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Extracellular Vimentin Modulation of Human Dendritic Cell Activation

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Extracellular Vimentin Modulation of Human Dendritic Cell Activation

By

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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PREFACE

My long-term goals include becoming a physician-scientist who collaborates with other physicians and scientists to improve patient care through clinical and translational research. My broad research interests involve understanding immune dysregulation in infectious and autoimmune diseases, and developing novel tests to assist with diagnostic and treatment decisions in these diseases. I have spent most of my time as a graduate student researching dendritic cells (DCs), which are antigen presenting cells crucial for initiating an adaptive immune response. The focus of my major, basic science project is how DCs are affected by extracellular vimentin. Vimentin is a protein which is traditionally considered to be inside the cell but has recently been reported to be outside in disease processes such as tissue damage, cancer, and autoimmunity. One of the most common autoimmune diseases is rheumatoid arthritis. My clinical project is about this disease and its most common first-line treatment, a small molecule called methotrexate.

This dissertation may be of interest to immunology and cancer researchers, as well as to health professionals such as rheumatologists and immunologists. The graduate school journey has been quite educational for me, and I believe that I have learned valuable skills that I will continue to use later in my career.

ABSTRACT OF THE DISSERTATION

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Dr. William Langridge, Chairperson

Vimentin is traditionally considered to be an intracellular protein with a primarily structural role. Evidence suggests that extracellular vimentin can be found in cancer, tissue injury, and autoimmunity. Extracellular vimentin has already been shown to alter innate immunity by increasing monocyte and macrophage ability to kill bacteria, but also decreasing neutrophil infiltration into inflamed tissue. How extracellular vimentin affects initiation of adaptive immunity has not been previously studied. To initiate adaptive immunity, antigen presenting cells prime naïve T cells. Since the most effective antigen-presenting cells are dendritic cells (DCs), the DCs are important in immune responses against cancer, self, and pathogens.

In this dissertation, I used primary human cell culture to demonstrate the effects of extracellular vimentin on DCs and T cells. In activated DCs, extracellular vimentin decreases secretion of pro-inflammatory cytokines IL-6 and IL-12 and increases secretion of the anti-inflammatory cytokine IL-10. As a result, there is less Th1 activation, resulting in an anti-inflammatory effect. This data supports the hypothesis that vimentin is a modulator of DC induced activation, resulting in a mild anti-inflammatory effect. By inducing suppression of the adaptive immune response, vimentin could be involved in cancer or trauma-complications.

I also had a clinically-focused side project focused on rheumatoid arthritis (RA), an autoimmune disease. The first line therapy for RA is methotrexate (MTX), but MTX is sometimes

ineffective or has side effects. I attempted to correlate in vitro MTX induced changes with MTX efficacy and MTX side effects in a small group of RA patients. The in vitro assay focused on MTX induced decreases in IL-17, a cytokine from pro-inflammatoryTh17 cells thought to be involved in RA pathogenesis. I found that there is greater variation in the effect of MTX on in vitro IL-17 secretion in those with side effects vs those without. This suggests that patients that had an unusually large or small reduction in IL-17 levels in response to MTX in vitro ultimately had side effects to the drug.

CHAPTER ONE

1. INTRODUCTION: DENDRITIC CELLS IN AUTOIMMUNITY, CANCER, AND TRAUMA

Note: This chapter contains components of my review article: M.B. Yu, W.H. Langridge, The function of myeloid dendritic cells in rheumatoid arthritis, Rheumatol Int, 37 (2017) 1043-1051. Specifically, the section "Types and Locations of Dendritic Cells" and the subsection on "Focus on Rheumatoid Arthritis" are from the review article.

Introduction

The immune system has both innate and adaptive segments. The innate immune system responds to established pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through pattern recognition receptors. Innate immune cells include granulocytes, monocytes, macrophages, dendritic cells (DCs), and natural killer cells. In contrast to the innate immune system, the adaptive immune system adapts over the course of a vertebrate's lifetime, developing the ability to recognize specific antigens as foreign and thus developing immunological memory. Adaptive immune cells include T cells and B cells. [1]

Antigen presenting cells (APCs) link the innate and adaptive immune systems. After APCs recognize PAMPs and DAMPs, they become activated. When APCs become activated, they present recently-ingested antigens bound to MHC (major histocompatibility complex) surface proteins, upregulate costimulatory signals such as cluster of differentiation 80 (CD80) or CD86, and secrete cytokines. The T cells only become activated if their T cell receptors are specific for that presented antigen on that particular MHC, bind to the costimulatory molecules, and detect immunostimulatory cytokines. The type of T cell differentiation that results is influenced by cytokines in the local environment, particularly those secreted from the APC. DCs have been

established as the most efficient APCs, and they are essential for activating naïve T cells, which are T cells that have never been activated before. These T cells can go on to activate B cells. Thus, DCs are crucial for initiating the adaptive immune response. [1-5].

The adaptive immune response is altered in many disease states, and it follows that DCs could potentially be involved in such disease states. In this chapter, we will discuss DCs and their role in the pathogenesis of autoimmunity, cancer, and trauma. Additionally, for each of these three general disease states, we will also focus on one subtype.

Types and Locations of Dendritic Cells

The major DC subsets are myeloid/classical DCs and plasmacytoid DCs. These DC subsets differ in morphology and gene expression, with myeloid DCs appearing more stellate in shape and more efficient at presenting exogenous antigens [5-8]. It was previously believed the two subsets had different origins. Myeloid DCs were thought to be derived from common myeloid progenitors (CMPs) whereas plasmacytoid DCs were considered to come from common lymphoid progenitors (CLPs). Recent research suggests that most DCs from both subsets are derived from CMPs, and more specifically arise from CMP-derivatives called common DC progenitors (CDPs). However, in instances of inflammation, monocytes are thought to give rise to a subtype of DCs called inflammatory DCs (infDCs) [6, 8, 9].

As myeloid DCs have been studied to a greater degree, the remainder of this chapter will focus on myeloid DCs. The immediate precursors of myeloid DCs differentiate into myeloid DCs after leaving the bone marrow [8]. The resulting myeloid DCs are found in the circulation and in tissues. After phagocytosing blood and tissue antigens, myeloid DCs migrate to secondary lymphoid organs such as lymph nodes. At these locations, the myeloid DCs present antigens to T cells [7, 8]. Myeloid DCs can also present antigens in ectopic lymph tissue, such as in synovial pannus in rheumatoid arthritis [10-12].

A convenient and commonly used myeloid DC model used for DC experiments is the monocyte-derived DC (moDC). These moDCs arise from peripheral blood monocytes that are differentiated into DCs in vitro by culture with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4. One potential weakness of this model is that moDCs may behave differently from myeloid DCs isolated in the absence of disease [7, 9, 13]. However, it was recently found that moDCs are strikingly similar to the infDCs, a new subtype of DCs found in vivo in inflammatory situations such as autoimmunity or infection. Thus, at this time, infDCs are the best candidates for an in vivo equivalent of moDCs [9].

For the remainder of this chapter, DCs refers to myeloid DCs, unless otherwise specified.

Autoimmunity

Discriminating between self and non-self is crucial for the immune system. In autoimmunity, there is aberrant activation of T and/or B cells that recognize epitopes from self-proteins, in their native form or altered by either post-translational or splicing modifications [2, 3, 14-17]. DCs are crucial to the pathogenesis and progression of autoimmune diseases, particularly T-cell mediated autoimmune diseases, due to their ability to guide differentiation of naive T cells.

For DCs to activate an autoreactive T cell that can then activate an autoreactive B cell, such autoreactive T cells and B cells must exist. T cells that recognize autoantigens are expected to be removed in the thymus during T cell development, and similarly B cells that recognize autoantigens are expected to be removed in the bone marrow during B cell development. One important aspect of both T cell and B cell development is negative selection, in which cells that bind too strongly to an autoantigen undergo apoptosis. In negative selection for developing T cells, the cells are screened in the thymus for binding to autoantigens expressed in the thymus, including some (such as insulin) that are otherwise specific to other tissues. Some of the thymic

cells express the transcription factor AIRE (autoimmune regulator), which induces production of these tissue specific antigens. In contrast, in negative selection for developing B cells, the cells are simply screened for binding to antigens present in the bone marrow, with no attempt to assess for binding to common proteins specific to other tissues [1, 18-20].

The second requirement to generate an activated, autoreactive T or B cell is that the antigen has to be available and appropriately presented. Naïve T cells usually require antigen presentation by DCs. DCs could be activated by PAMPs, DAMPs, or pro-inflammatory cytokines. These activation signals could be generated as part of a normal pro-inflammatory response. For example, in infection, PAMPs are present and can potentially activate DCs presenting self-antigen. Alternatively, the PAMPs can induce DCs to produce pro-inflammatory cytokines that activate nearby DCs presenting self-antigen. In fact, there is evidence that some infections can predispose to autoimmunity [20, 21]. A similar scenario may arise in the instance of tissue damage and the subsequent release of DAMPs. Accordingly, there is a recently discovered epidemiological connection between history of trauma and the development of the autoimmune disease lupus [22].

If self-presenting DCs are activated by activation signals or if there is a genetic defect resulting in spontaneously activated DCs, a DC-T cell interaction can lead to inappropriate T cell activation. This T cell activation results in a loss of tolerance, and autoimmunity [20, 23]. B cells often require T cell help, in that there often must first be an activated, autoreactive T cell [1].

Thus, if the autoantigen is in an area of activated DCs, the process of autoimmunity could begin. In such situations, the immune reaction could start as an immune system response to a small number of epitopes, and later develop into a response to a larger number of epitopes. This is called epitope spreading, and is a potential mechanism for both autoimmunity initiation and progression [24].

Focus on Rheumatoid Arthritis.

Rheumatoid arthritis (RA) is a systemic autoimmune disease that causes joint pain, inflammation, and loss of function [14]. Disease pathogenesis involves activation and proliferation of autoreactive pro-inflammatory effector T cells, and it is believed that DCs initially activate these autoreactive T cells [14-16]. There are elevated numbers of DCs in the synovium of RA patients, when compared to the synovium of osteoarthritis patients [4, 15, 25-28].. The DCs in RA synovium may very well be presenting antigens in the actual synovium, as synovial ectopic lymph tissue is a found in 40% of RA patients [12].

At a functional level, evidence from moDCs suggests that DCs from RA patients are more immunostimulatory, as they were shown to secrete increased amounts of pro-inflammatory cytokines and chemokines [29, 30]. In comparison with moDCs generated from healthy controls, RA moDCs secrete increased amounts of the proinflammatory cytokines IL-6 and IL-23 [30]. These cytokines are known to induce CD4+ T cell differentiation into Th17 cells, which are crucial pro-inflammatory cells in RA pathogenesis [14-16]. Indeed, RA moDCs have been shown to skew T cell differentiation towards Th17 at the expense of regulatory T cells (Tregs). The implication is that myeloid DCs have altered functions in RA, such as an enhanced ability to generate Th17 cells, and are partially responsible for the elevated Th17 numbers present in RA patients [30]. If these Th17 cells are autoreactive, the result would be chronic inflammation and tissue damage. In addition, RA moDCs have an increased capability to recruit macrophages, neutrophils, and monocytes due to their increased secretion of chemokines C-X-C motif ligand (CXCL) 8 and C-C motif ligand (CCL) 3 [29]. As a result, moDC activities could lead to increased leukocyte infiltration of the synovium, leading to exacerbated inflammation.

The importance of DCs in RA is further supported by the experimental finding that intra-articular injection with autoantigen-pulsed, pro-inflammatory mature DCs is sufficient to initiate

arthritis in mice [31]. This result suggests that DCs are able to drive disease onset, though it does not prove that pro-inflammatory mature DCs are the dominant driver of RA in human patients.

Additionally, synovial fluid in the joints of RA patients is enriched with pro-inflammatory cytokines, which may both stimulate DC activation and be sustained by DC activation. These cytokines include TNF-α, IL-1, IL-6, GM-CSF [32], and TSLP [33]. Except for GM-CSF, these cytokines have been shown to activate DCs in vitro. GM-CSF is important for differentiating monocytes into DCs. Lastly, there is another unexpected maturation factor present in the joints of RA patients: collagen, an extracellular protein that is usually physically isolated from immune cells in intact cartilage by collagen-associated proteins [34]. There is an increase in degraded collagen II in cartilage from RA patients in comparison with cartilage from non-arthritic controls [35]. This increase in degradation of collagen II is likely accompanied by increased exposure of collagen II to immune cells in the vicinity of synovial tissue [34]. Recently, it has been demonstrated that collagen II activates DCs, and in this way can help sustain the inflammation in RA [36].

Cancer

Tissue-resident DCs are found in most tissues [37]. If the DCs are in tissue that contains a tumor, the DCs take up antigens from dying tumor cells, travel to draining lymph nodes, and present the tumor-associated antigens to T cells. If the tumor proteins are not found in normal cells, T cells may recognize the tumor proteins as foreign antigens, become activated by the DCs, and mount an immune response against the tumor. This immune response involves T cell infiltration into tumors. Additionally, some DCs may remain in the tumor and help maintain activation of the T cell response against the tumor [38]. However, the tumor microenvironment is often immunosuppressive, such that effective T cell responses are not produced and the tumor can escape immune surveillance. Given the importance of DCs in T cell activation, DCs are also of interest in cancer immunology. [38-41]

Several studies have examined the relationship between immune infiltration of tumors and cancer prognosis. In general, patients with T cell infiltration into tumors have better prognosis, including greater survival rate. However, the story with infiltrating DCs is more complicated, with some studies finding DC presence in tumors to be associated with better prognosis while other studies finding the opposite. Part of the issue is that immature DCs are tolerogenic, while mature DCs are pro-inflammatory. The tolerogenic DCs may induce Tregs, which are anti-inflammatory T cells that promote immune tolerance and enable cancer to avoid immune detection. Tumor infiltrating DCs are often tolerogenic.

Tumor cells themselves often suppress DC activation through secretion of the anti-inflammatory cytokines IL-10 and TGF-β, as well as other factors such as prostaglandin E2 (PGE2) and Wnt proteins [39-41]. IL-10 and TGF-β inhibit DC expression of MHC, costimulatory molecules, and pro-inflammatory cytokines; as a result, the DCs are unable to induce pro-inflammatory T cell responses [41, 42]. PGE2 and Wnt induce DCs to produce IDO (indoleamine 2,3-dioxygenase), an enzyme which degrades tryptophan [41]. As a result of IDO expression, the DCs are less able to secrete pro-inflammatory cytokines and instead secrete anti-inflammatory cytokines such as IL-10. The IDO-induced change in cytokine expression and local depletion of tryptophan shifts DC-mediated T cell differentiation away from pro-inflammatory subtypes and towards anti-inflammatory Tregs, and can lead to apoptosis of activated T cells[40, 41, 43-45].

Focus on Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and approximately half of patients experience recurrence within three years. This recurrence is partially due to immune factors [46].

Anti-cancer adaptive immune responses likely depend on DCs traveling from a tumor to the tumor draining lymph nodes, and then presenting antigens [38]. DCs are less frequently

present in HCC tumors compared to in healthy liver tissue, perhaps leading to decreased tumor antigen presentation in the lymph nodes and contributing to a decrease in immune surveillance [47]. Additionally, the T cell compartment in the HCC tumor draining lymph node contains a relatively high percentage of anti-inflammatory Tregs and low percentage of pro-inflammatory Th1 and Th17 cells. The percentage of Tregs is higher in patients with more advanced tumors. It is possible that the tolerogenic status of the tumor draining lymph node in HCC involves tolerogenic DCs or a deficiency of activated DCs [48]. Indeed, there is a smaller number of activated DCs in the hepatic lymph nodes of HCC patients compared to in the hepatic lymph nodes of organ donors [49].

At a functional level, evidence suggests that DCs from HCC patients have impaired immunostimulatory capabilities. HCC patients but not healthy controls have an unusual subset of peripheral blood DCs that have been coined regulatory DCs. These regulatory DCs express CTLA4 [50] and PD-1 [50, 51], which are surface proteins with immunosuppressive abilities. CTLA4 is usually expressed on anti-inflammatory Treg cells and binds to co-stimulatory proteins on DCs. The CTLA4 on regulatory DCs binds to costimulatory proteins on other DCs, which enables the CTLA4-expressing DCs to secrete IL-10 and express IDO [50]. Most studies of PD-1 concern PD-1 expressed on the surface of T cells, and the functional details of PD-1 expression on DCs are not well understood. However, PD-1 expression on DCs has been shown to decrease the ability of DCs to induce T cell responses, such as proliferation [51]. Additionally, in comparison with moDCs generated from healthy controls, HCC moDCs express lower levels of MHC and secrete lower amounts of the pro-inflammatory cytokine IL-12 [46].

It is believed that this reduction in DC activation is due to the tumor microenvironment, which includes fibroblasts. Fibroblasts from HCC tumors attract DCs in vitro through secretion of CXCL12. Then, the HCC fibroblasts induce a tolerogenic phenotype in these DCs. These DCs are

prevented from upregulating surface expression of MHC and co-stimulatory factors, exhibit decreased secretion of IL-12, exhibit increased secretion of IL-10 and TGF-β, and express IDO. As would be predicted from such a tolerogenic phenotype, such DCs shift T cell differentiation towards anti-inflammatory Tregs and away from pro-inflammatory Th1 cells. Intriguingly, these HCC fibroblast-treated DCs are CTLA4+, suggesting that they have adopted the properties of the regulatory DCs found in the blood of HCC patients [52]. However, the in vitro evidence that HCC fibroblasts attract DCs is not in agreement with the finding from clinical samples that DCs are less frequently present in HCC tumors compared to in healthy liver tissue [47]. Perhaps other cell types in the tumor microenvironment secrete other factors to counter the ability of fibroblasts to attract DCs.

Tissue damage

Tissue damage is known to initially activate immunity but later suppress the immune response. Adaptive immunity is slower to initiate, and the effect of tissue damage on adaptive immunity is overall anti-inflammatory [53-55]. Tissue damage, at a cellular level, is cell death. Necrotic or damaged cells release specific molecules referred to as damage-associated molecular patterns (DAMPs) that initiate an inflammatory innate and adaptive immune response. DAMPs include intracellular proteins such as HMGB1 and various heat shock proteins (HSPs), as well as nucleic acid compounds such as genomic DNA or ATP itself [56, 57]. However, these same necrotic or damaged cells that release DAMPs may also release resolution-associated molecular patterns (RAMPs) that promote the reduction or resolution of acute inflammation [58]. Examples of RAMPs include members of the HSP family [58]. It has been proposed that the RAMPs act later and have longer-lasting effects compared to DAMPs, resulting in an overall suppressive effect on slow-to-start adaptive immunity. However, the mechanisms behind the temporal sequence of events are unclear, and the lists of RAMPs and DAMPs are incomplete and possibly overlapping [58]

As tissue-resident DCs are found in most tissues [37], they are likely to encounter RAMPs and DAMPs if the tissue becomes damaged. On DCs specifically, DAMPs can upregulate expression of MHC, co-stimulatory factors, and pro-inflammatory cytokines. Such changes enable DCs to initiate a pro-inflammatory adaptive immune response [56]. In contrast, with RAMPs, there is decreased differentiation of monocytes into DCs; and the DCs exhibit lower expression of MHC, co-stimulatory factors, and the maturation marker CD83, but higher expression of IDO [58, 59].

Focus on Post Traumatic Immunosuppression

Physical trauma involves tissue damage. After severe trauma, there is an initial systemic inflammatory response syndrome, followed by a compensatory anti-inflammatory response syndrome (CARS) [55]. Data from mouse models and from trauma patients suggest that DCs are involved in CARS.

In a mouse model, trauma increases DC apoptosis; decreases DC expression of MHC and CD83; and decreases lipopolysaccharide (LPS)-induced secretion of IL-12 but not IL-10. As would be expected from such phenotypic changes, these DCs have reduced ability to activate T cells [60, 61]. Similar findings have been documented in humans. In patients with trauma, there is a decrease in myeloid DC counts in the peripheral blood starting two days after the initiating trauma [62], and there is an increase in myeloid DC apoptosis [63]. Monocytes from a subset of trauma patients display inefficient in vitro (< 40%, compared to > 60%) differentiation into DCs in the presence of differentiation cytokines, and the resulting DCs have an altered phenotype. These altered DCs have reduced costimulatory factor expression and reduced ability to activate T cells. Interestingly, patients with dysfunctional monocyte to DC differentiation were more likely to develop infectious complications, suggesting that the observed in vitro differences are of clinical importance [64].

A few potential mechanisms of trauma-induced changes in DCs have been explored. For example, IL-10 is elevated post-trauma [60], and appears to play a role in inducing DC apoptosis in this setting [65]. Additionally, trauma decreases expression of pattern recognition receptors such as toll like receptors (TLRs) which explains the hypo-responsiveness to TLR ligands like LPS [61]. Thrombospondin, a protein involved in cell-to-matrix interactions, is also elevated post-trauma, and appears to be responsible for the dysfunctional monocyte to DC differentiation [64]. However, much is still unknown.

Conclusion

Based on their efficiency at presenting antigens and secreting cytokines, myeloid DCs may play an important role in stimulating pathogenesis of autoimmunity. Unfavorable environmental conditions and genetic defects may stimulate DC induction of autoreactive effector T cell differentiation, leading to development of autoimmunity [17, 20]. Conversely, dysfunction of the DCs may play a role in the immunosuppression sometimes observed in cancer, in that the tumor cells prevent DCs from initiating an effective anti-cancer immune response [38-41]. With tissue damage, the immune system can be initially activated but later become suppressed, which has implications for major surgery and severe trauma [55]. Given the importance of DCs in initiating adaptive immune responses, understanding how these cells function in these various types of disease conditions will establish a basis for development of more effective and safer therapies.

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CHAPTER TWO

2. EXTRACELLULAR VIMENTIN MODULATES HUMAN DENDRITIC CELL ACTIVATION

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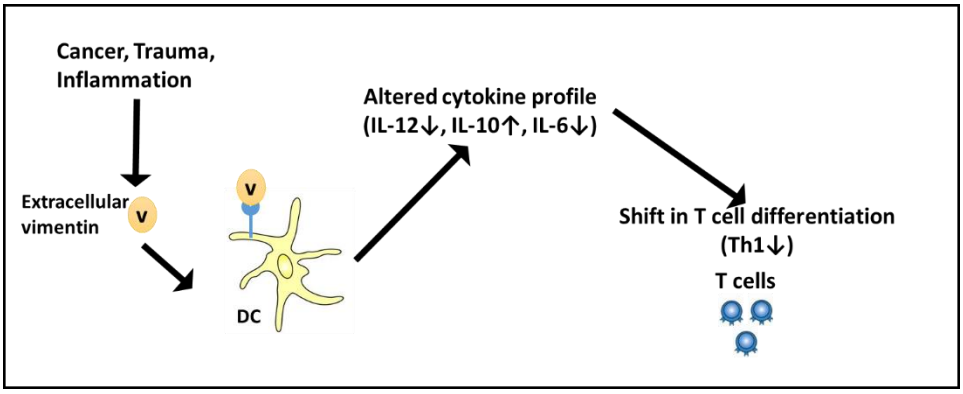
Note: This chapter is adapted from my article of the same title: M.B. Yu, J. Guerra, A. Firek, W.H. Langridge, Extracellular vimentin modulates human dendritic cell activation, Molecular immunology, 104 (2018) 37-46. An addendum has been added, containing information not in the original article.

Abstract

Vimentin is an intermediate filament protein traditionally considered to be an intracellular protein with a structural role. However, recent evidence suggests that vimentin can also be found outside the cell in disease conditions such as cancer, traumatic tissue injury, and inflammation. Extracellular vimentin was previously found to stimulate innate immunity by increasing monocyte and macrophage ability to kill bacteria. However, vimentin has also been previously found to decrease neutrophil infiltration into inflamed tissue. How extracellular vimentin affects the initiation of adaptive immune responses is unknown. Initiation of adaptive

immunity involves priming of naïve T cells by antigen-presenting cells, the most effective of which are dendritic cells (DCs). In this study, we demonstrate how extracellular vimentin modulates lipopolysaccharide (LPS) – induced activation of human DCs. Using cytometric bead arrays, we show that extracellular vimentin decreases LPS-activated DC secretion of pro-inflammatory cytokines IL-6 and IL-12 while increasing secretion of the anti-inflammatory cytokine IL-10. Using flow cytometry, we show that extracellular vimentin does not significantly affect LPS-induced DC surface expression of MHC I (HLA-ABC) or MHC II (HLA-DR) presentation molecules, costimulatory factors (CD80, CD86), or the DC maturation marker (CD83). Further, LPS-stimulated DCs co-cultured with allogeneic naïve CD4+ T cells (Th0) induced less secretion of the pro-inflammatory Th1 effector cytokine IFN-γ in the presence of vimentin than in the presence of LPS alone. This result suggests that vimentin reduces Th1 differentiation. Taken together, our data suggest that extracellular vimentin may inhibit pro-inflammatory adaptive immune responses, by blocking DC secretion of pro-inflammatory cytokines. Thus, extracellular vimentin may play an important role in cancer or trauma-complications by inducing suppression of the adaptive immune response. In a positive sense, the presence of extracellular vimentin may prevent tissue-damage from contributing to the development of autoimmunity. Consequently, extracellular vimentin may become a novel drug target for treatment of a variety of pro- and anti-inflammatory disease conditions.

Graphical Abstract



Keywords: vimentin; lipopolysaccharide; dendritic cells; cytokine; human; cell culture

1. Introduction

Vimentin is an intermediate filament protein normally found within the cells of many tissues, where it maintains cellular integrity, helps the cell resist mechanical stress [1, 2], transmits contractile forces [2], and positions cell organelles [3]. However, there is also evidence of extracellular or cell surface vimentin presence in a variety of diseases including: liver cancer [4], colon cancer [5], liver disease [6, 7], organ transplant [6, 8-10], sepsis [11], atherosclerosis [12], myonecrosis [13], pulmonary fibrosis[14], and systemic lupus erythematosus [15, 16].

Vimentin can be released to the extracellular environment in an unregulated way as a result of disrupted cell membranes related to traumatic cell injury or cell death [7, 10, 17], or as a result of overexpression [4]. Vimentin can also be secreted or trafficked to the cell surface in a regulated way [2].

Extracellular or surface vimentin has been documented to come from a variety of specific cell types. In pro-inflammatory environments, vimentin is released by macrophages [18], monocytes [12], neutrophils [19], and hepatocytes [7]. Vimentin is released in small amounts from

cultured endothelial cells and is found on the surface of endothelial cells specifically in capillaries and small veins [20]. The endothelial cells are induced to release more vimentin in the presence of TGF-β [14], a wound healing-associated cytokine with pro- and anti-inflammatory effects [21]. In apoptotic lymphocytes [22] and neutrophils [23], in injured skeletal muscle cells [13], and in activated platelets [24], vimentin is localized to the cell surface. Additional reports indicate that vimentin can be secreted from cultured astrocytes [25].

Extracellular vimentin's function is not well understood to-date, but recent literature indicates that extracellular vimentin could be involved in immune system modulation, wound healing, cancer progression, and pathogen entry into cells [2]. Of particular relevance to the present study, extracellular vimentin has been proposed to modulate the immune system by functioning as a damage associated molecular pattern (DAMP), as it has immunostimulatory properties. For example, extracellular vimentin induces superoxide production in macrophages [18] and monocytes [12], which increases the ability of these cells to kill bacteria [18]. Additionally, the presence of vimentin on the cell surface can induce NK cell-mediated lysis of the cell [26]. However, extracellular vimentin may also attenuate inflammation and promote tissue repair, as it decreases adhesion to platelets and activated endothelial cells in neutrophils [27], promotes axonal growth in neurons [28], and accelerates healing in a cataract surgery model [29]. However, very little is known about the effects of extracellular vimentin on adaptive immunity.

In the present study, we demonstrate the effects of extracellular vimentin on dendritic cells (DCs), the most effective antigen-presenting cells in the body. DCs play an important role in priming naïve T cells, and thus are key to initiating an adaptive immune response [30]. DC-mediated CD4+ T cell activation and differentiation is thought to be mediated by three signals: 1) peptide loading onto an MHC II molecule, 2) surface expression of co-stimulatory molecules, and 3) secretion of pro- or anti-inflammatory cytokines. The first two signals are required for T cell

activation, while the cytokines influence what type of CD4+ T cell (pro- or anti-inflammatory) arises from naïve CD4+ T cells. Because DCs are found at low frequencies in human peripheral blood (< 1% of peripheral blood leukocytes [31-33]), a common and convenient model for the human DC is the monocyte-derived DC (moDC), which is the specific DC model used in this study.

2. Materials and Methods

2.1 Isolation and culture of monocyte-derived dendritic cells from human peripheral blood

Monocyte-derived dendritic cells (moDCs) were prepared from freshly collected human peripheral blood cells from aphaeresis filter cones donated by the LifeStream blood bank (San Bernardino, CA). To deplete the red blood cells, the blood was incubated with a red blood cell lysis buffer (8.3 g/L ammonium chloride, 1.0 g/L potassium bicarbonate, 90 mg/L EDTA disodium, pH 7.1 – 7.4) for 15 min, centrifuged, and then the supernatant was removed [34]. This red blood cell depletion process was then repeated one more time to yield a leukocyte mixture. The CD14+ monocytes were isolated from the leukocyte mixture using magnetic CD14 microbeads human as described by the manufacturer (Miltenyi Biotech, CA; catalog #130-050-201). Briefly, CD14+ monocyte isolation involved: incubation of the leukocytes with anti-CD14 antibodies bound to magnetic beads for 15 min at 4°C in the dark, followed by separation of the CD14+ cells from all other leukocytes by binding the beads to a magnetic LS column (Miltenyi Biotech) in a magnet (MidiMacs separator, Miltenyi Biotech). The CD14+ monocytes were seeded (10^6 cells/mL) in 24-well (0.5 mL/well) or 96-well (0.2 mL/well) flat-bottom non-pyrogenic polystyrene tissue culture plates in moDC culture medium: RPMI 1640 medium (Mediatech Inc., VA) supplemented with 10% FBS (Atlanta Biologicals, GA), 100 U/mL penicillin-streptomycin (ThermoFisher, MA), 50 ng/ml human recombinant GM-CSF (Peprotech, NJ), and 10 ng/ml human recombinant IL-4 (Peprotech). The monocyte cell culture was fed at 2-day intervals by gentle replacement of 50% of the medium with pre-warmed fresh culture medium. The monocytes were cultured for 6 days

to allow their differentiation into moDCs, prior to treatment of the moDCs with vimentin, lipopolysaccharide (LPS), and polyinosinic-polycytidylic acid (poly I:C). For vimentin, LPS, and poly I:C concentrations, see section 2.3.

2.2 Isolation of naïve CD4+ T cells from human peripheral blood

Leukocytes were isolated from aphaeresis filter cones as described in section 2.1. The naïve CD4+ T cell fraction was isolated from the leukocyte mixture by negative selection using Naïve CD4+ T cell Isolation Kit II human as described by the manufacturer (Miltenyi Biotech, catalog #130-094-131). Briefly, isolation involved: initial incubation of the leukocytes with Biotin-Antibody Cocktail for 5 min at 4°C in the dark, followed by incubation with Microbead Cocktail for 10 min, and passage through a LS column in a magnet. The untouched, naïve CD4+ T cells were resuspended in R10 culture medium: RPMI 1640 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. See section 2.3 for final cell concentrations.

2.3 Cell treatment conditions

Vimentin protein (Sino Biological, China) was solubilized in PBS (phosphate buffered saline, pH 7.4) at 0.25 mg/mL.

For moDC monoculture, moDCs (10^6 cells/mL) were incubated in moDC culture medium (see section 2.1) for 48 hr at 37 °C in the presence of 5% CO_2. Treatments included no treatment, vehicle (PBS), vimentin (10 ug/mL unless otherwise stated), LPS (10 ng/mL) +/- vimentin, and poly I:C (10 ug/mL) +/- vimentin.

For moDC:T cell co-culture: moDCs (1 x 10^4 cells/well) were plated in R10 culture medium in 96-well tissue culture plates, and then treated. After the moDCs had been treated for 8 to 10 hours, naïve CD4+ T cells (0.8 – 1.0 x 10^5 cells/well) were added to the moDCs. The co-

culture treatment groups included: untreated, vimentin (10 ug/mL), and LPS (20 ng/mL) +/- vimentin. The naïve CD4+ T cell monoculture treatment groups were the same as the co-culture treatment groups. The co-cultures and naïve CD4+ T cell monocultures were incubated for an additional 4 days at 37 °C in the presence of 5% CO_2.

2.4 Surface staining and flow cytometry

For the purpose of staining cells in preparation for flow cytometry, washing is: addition of 200 uL of PBS, centrifugation (524 x g, 5 min, 4 °C), and removal of supernatant. Working solution of Fixable Viability Dye eFluor 450 (eBioscience, CA) is a 1:1000 dilution in PBS.

After treatment, the cells were incubated with 50 uL of working solution of Fixable Viability Dye eFluor 450 for 30 min at 4°C in the dark to distinguish between living (FVD450-) and dead (FVD450+) cells. The moDCs were subsequently washed once with PBS, resuspended in PBS (10 uL), and incubated with conjugated antibodies (indicated below) for 15 min at 4°C in the dark. The cells were washed with PBS and fixed with 1% paraformaldehyde solution before flow cytometric analysis using the MACSQuant Analyzer (Miltenyi Biotech).

The following anti-human antibodies were used to stain moDC monocultures: CD14 PerCP (clone HCD14), CD11c PE-Cy7 (clone 3.9), CD80 FITC (clone 2D10), CD86 PE (clone IT2.2), CD83 APC (clone HB15e) (all from Biolegend, CA); HLA-ABC FITC (clone G46-2.6) and HLA-DR FITC (clone L243) (both from BD Biosciences). The following isotype control antibodies were used: mouse IgG1-FITC, IgG2b-PE, IgG2-PerCP, IgG1-PE-Cy7, IgG1-APC (all from Biolegend).

The following anti-human antibodies were used to stain moDC:T cell co-cultures and T cell monocultures: CD3 FITC (clone HIT3a), CD25 APC (clone BC96, both from Biolegend); CD45RO PE (clone UCHL1, BD biosciences).

Singly-stained compensation beads (eBioscience) and a 1:1 mixture of live and heat-killed cells stained with Fixable Viability Dye eFluor 450 were used to establish compensation settings. Flow cytometry data analysis was performed using Flowjo data analysis software (FlowJo, OR).

2.5 Proliferation assay

Working solution of CellTrace Far Red is a 1:1000 dilution of CellTrace Far Red DMSO stock solution (1 mM) in pre-warmed PBS. Working solution was prepared immediately prior to use.

Isolated T cells were incubated with CellTrace Far Red working solution (10^6 cells / mL staining solution) for 20 min at 37°C in the dark. To remove free dye, R10 medium (5 x staining solution volume) was then added to the cells for an additional 5 min. The cell solution was centrifuged (524 x g, 5 min, 4 °C) and the supernatant was removed. The remaining pellet was resuspended in pre-warmed R10 medium.

The stained T cells were then cultured for 4 days as stated in section 2.3. As described in section 2.4, the cells were stained for viability and for surface markers, and analyzed via flow cytometry, with the following two modifications: The only antibodies used for surface staining were CD3 FITC (clone HIT3a) and CD4 PerCP-Cy5.5 (clone RPA-T4) (both from Biolegend). A 1:1 mixture of unstained T cells and CellTrace Far Red stained T cells was used to establish compensation settings.

2.6 Cytokine Cytometric Bead Array

MoDC culture supernatant was harvested after 48 hours of treatment. MoDC:T cell co-culture and T cell culture supernatant was harvested after 96 hours of treatment. Cytokine concentrations in the moDC culture supernatant were assessed with cytometric bead array (CBA) flex sets for IL-6, IL-10, and IL-12 as described by the manufacturer (BD Biosciences) on a

MacsQuant flow cytometer. Similarly, cytokine concentrations in the co-culture and T cell culture supernatant were assessed with CBA flex sets for IL-17 and IFN-γ. Data analysis was performed with FlowJo data analysis software. Protein concentrations were calculated from median fluorescence intensity. Relative expression is concentration of sample / concentration of sample treated with LPS alone.

2.7 ELISA

MoDC culture supernatant was harvested after 48 hours of treatment. Cytokine concentrations in the culture supernatant were assessed with Uncoated ELISA kits for IL-1β, IL-6, IL-10, IL-12, and TGF-β1 as described by the manufacturer (ThermoFisher). For TGF-β1, this procedure involves acid activation and neutralization. Relative expression is concentration of cytokine in the sample / concentration of cytokine in the sample treated with LPS alone.

2.8 Statistical Analysis

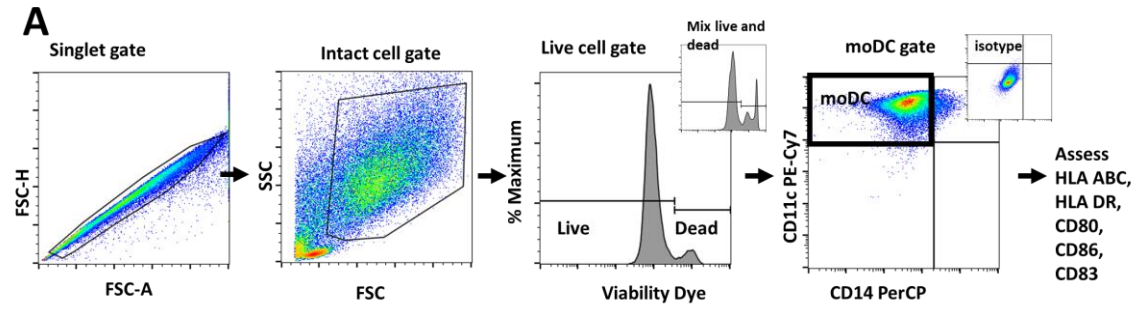
Unless otherwise specified, Student's paired t test was used to compare results from samples incubated with and without vimentin. In analysis comparing relative expression amounts (value of sample / value of sample treated with LPS alone) to the set value of 1, Student's single sample t test was used. Due to observed non-normality in cytokine expression levels in the DC monocultures, Wilcoxon signed rank test was used to compare cytokine expression levels from DC monocultures incubated with and without vimentin. P value less than 0.05 was considered significant.

3. Results

3.1 Immature moDCs

It is unknown whether vimentin alone can induce activation of DCs. We performed monoculture experiments to assess the effect of vimentin on immature moDCs. The moDCs were

treated with or without vimentin. No robust differences were found in the surface expression levels of the following activation-associated surface markers: MHC I protein HLA-ABC, MHC II protein HLA-DR, costimulatory factors CD80 and CD86, and maturation marker CD83 (Fig. 1A, B). In addition, no significant changes were found in the secretion of the cytokines IL-6, IL-10 or TGF-β1. IL-12 was undetectable or very low in all samples treated with nothing, vehicle, or vimentin alone (Fig. 1C, D, E). Similarly, IL-1β was undetectable in all samples treated with nothing, vehicle, or vimentin alone. The lack of significant differences suggests that vimentin alone does not activate DCs in most humans.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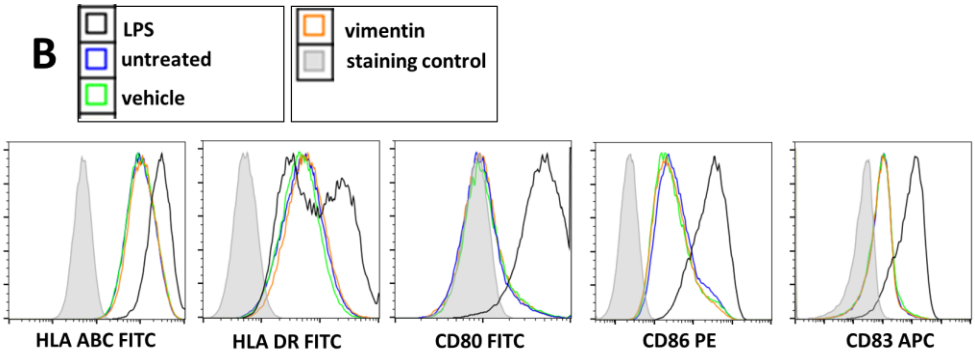
Assess HLA ABC, HLA DR, CD80, CD86, CD83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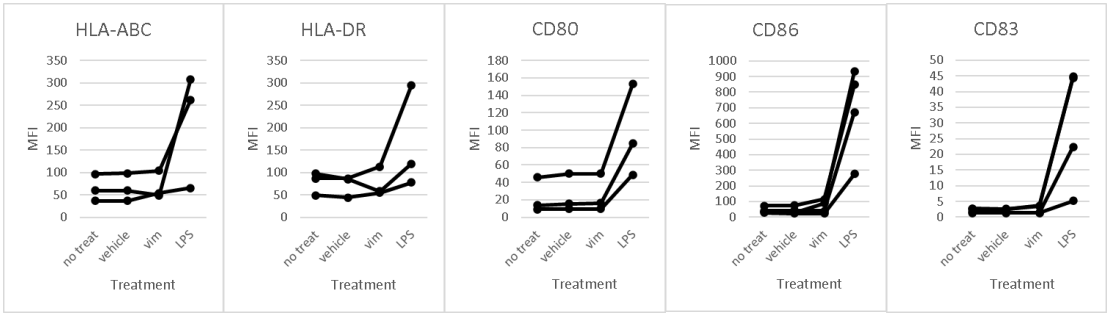
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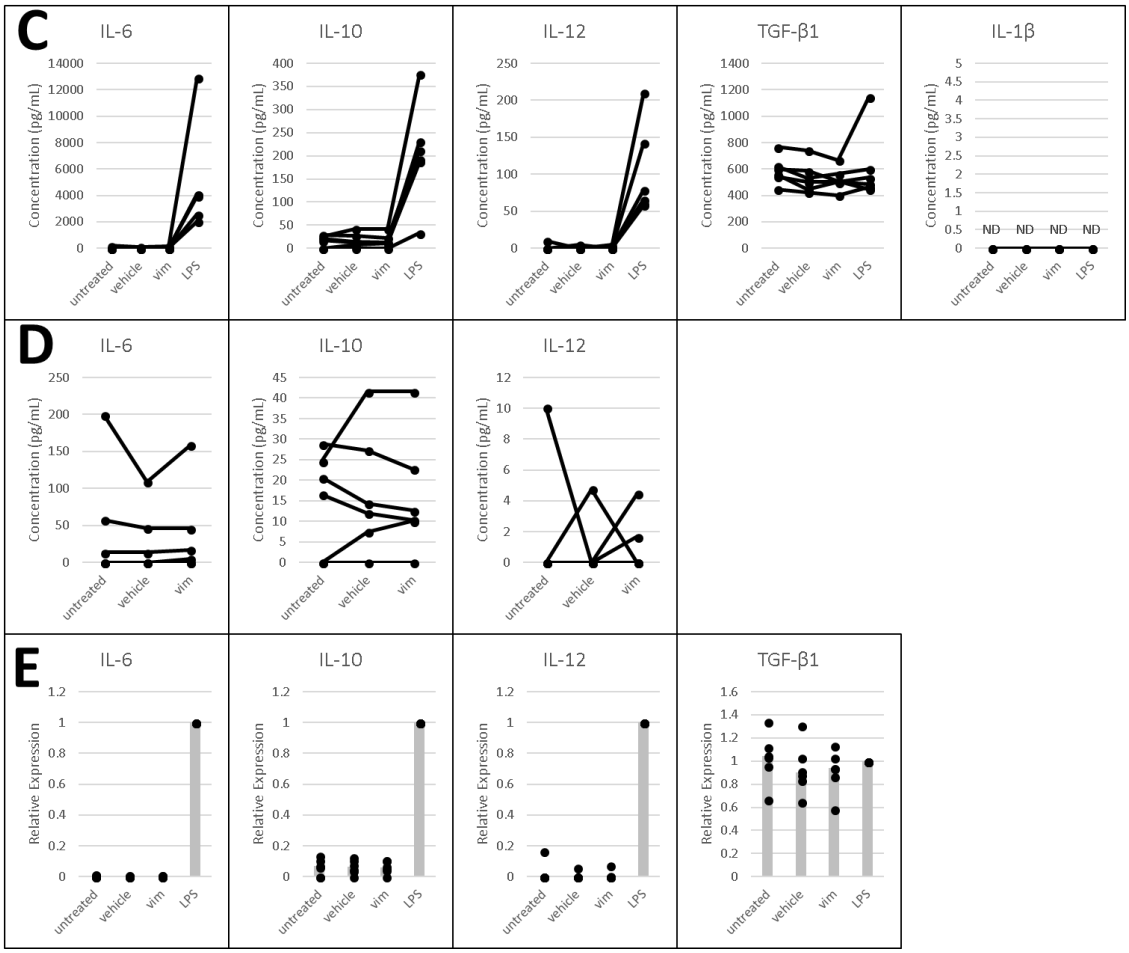
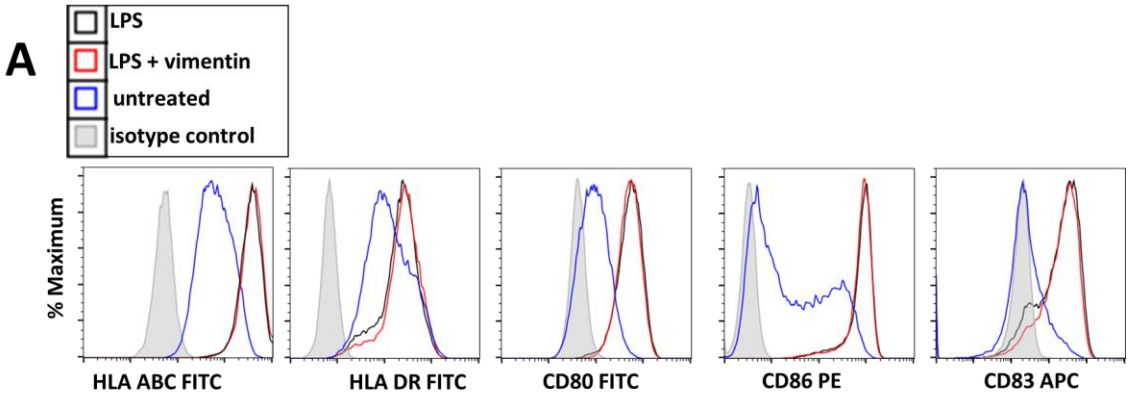


Figure 1. Vimentin does not activate moDCs.

The moDCs were treated with vehicle (PBS), vimentin, or LPS, or were untreated, for 48 hours. **A and B**. Expression of cell surface markers on moDCs analyzed by flow cytometry. **A.** Sequential gating strategy: single cells were selected based on forward scatter area and height, then intact cells were selected based on forward and side scatter, then living cells were selected based on detection of viability dye (FVD450), and then moDCs were selected based on detection of CD14 and CD11c **B.** Expression of cell surface markers on moDCs, analyzed by flow cytometry. The representative histograms show surface staining of HLA ABC, HLA DR, CD80, CD86, and CD83 in the gated moDCs. Histograms are shown from 1 representative donor. MFI (median fluorescence intensity) summary data is also shown, n = 3 to 4 healthy donors. Vim = vimentin. **C, D, and E.** Concentrations of IL-6, IL-10, IL-12, TGF-β, and IL-1β were detected by ELISA. n = 6 healthy blood donors. **C** is actual concentrations. ND: not detectable. **D** is actual concentrations of IL6, IL10, and IL12 but with the LPS group removed to facilitate comparisons between the other treatment groups. **E** is relative expression. Each black dot is one donor's cells in one treatment group. Gray bars are medians.

3.2 Addition of vimentin does not affect LPS-induced moDC expression of activation-associated surface markers

The effect of extracellular vimentin on DC activation is unknown. Therefore, we assessed whether extracellular vimentin could alter LPS-induced activation in moDCs. The moDCs were stimulated with LPS, in the absence or presence of extracellular vimentin. To determine whether vimentin alters LPS-induced moDC activation, we used flow cytometry to measure the surface expression of surface markers associated with moDC activation. These markers are MHC I protein HLA-ABC, MHC II protein HLA-DR, costimulatory factors CD80 and CD86, and the DC maturation marker CD83. All of these proteins were verified to be highly expressed in moDCs activated with LPS. The addition of vimentin had no significant effect on the LPS-induced high level of surface expression of any of these proteins (Fig. 2A).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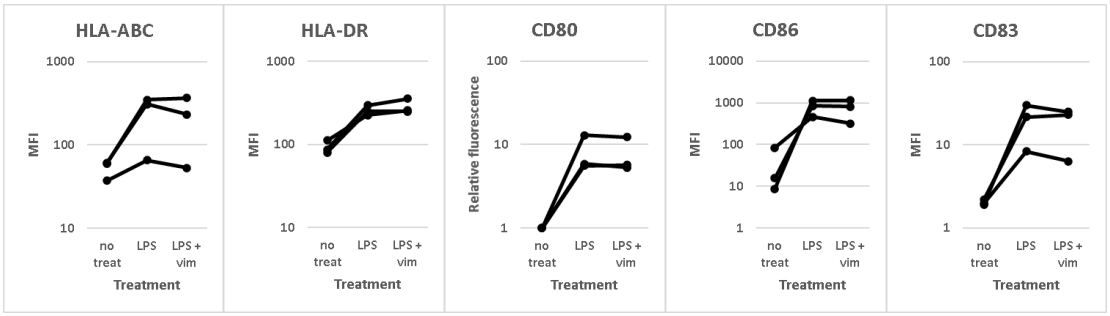
CD86 PE
CD83 APC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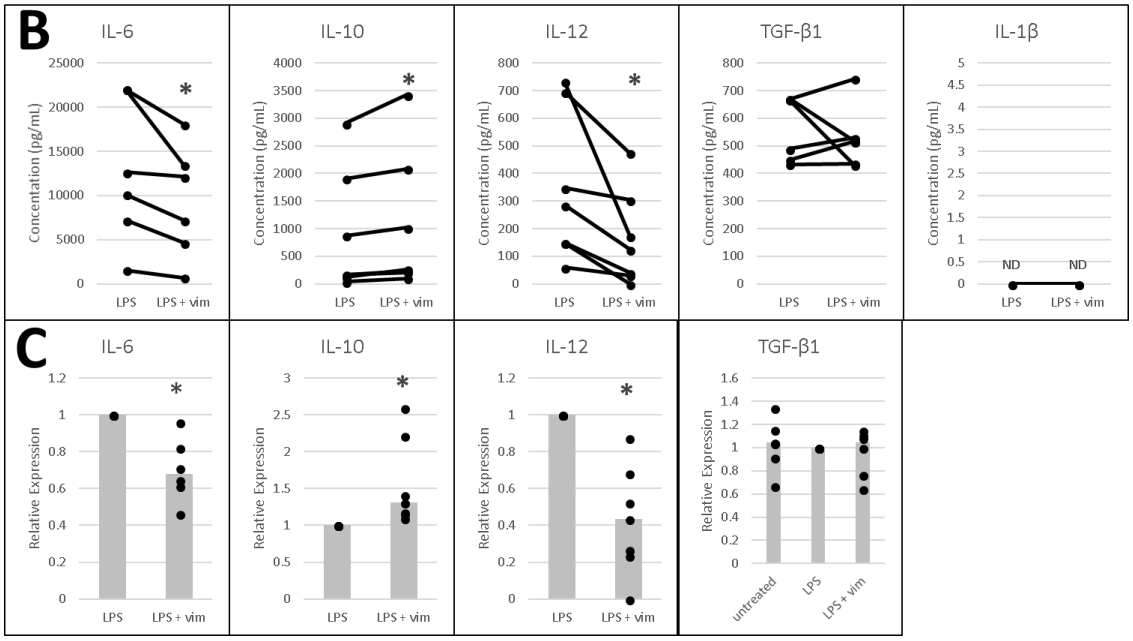
LPS + vim
Treatment


B
IL-6
IL-10
IL-12
TGF-β1
IL-1β
Concentration (pg/mL)
ND
C
Relative Expression
untreated
LPS
LPS + vim

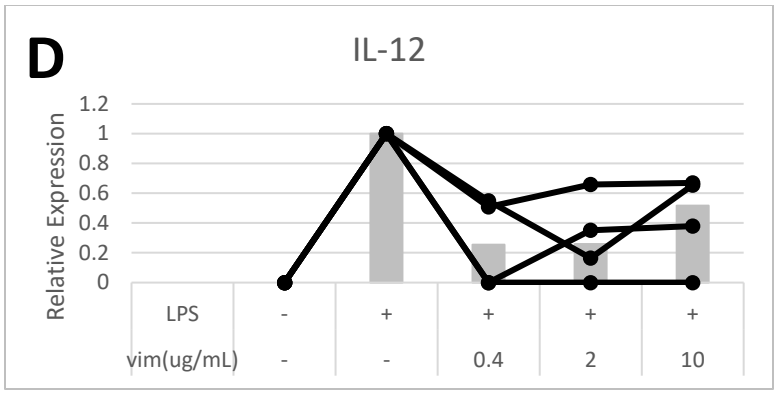


Figure 2. Effect of vimentin on LPS-induced moDC activation

The moDCs were treated with LPS +/- vimentin, or were untreated, for 48 hours. Unless otherwise stated, the vimentin concentration was 10 ug/mL. **A.** Expression of cell surface markers on moDCs analyzed by flow cytometry. The representative histograms show surface staining of HLA ABC, HLA DR, CD80, CD86, and CD83 in the gated live moDCs. Histograms are shown from one representative donor out of three analyzed. Summary data is also shown from n = 3 healthy donors. CD80 relative fluorescence is MFI relative to untreated, raw MFI data concerning CD80 in 2 experiments was lost (broken hard drive). **B and C.** Concentrations of IL-6, IL-10, and IL-12 in the cell culture supernatant were assessed by CBA. Concentrations of TGF-β1 and IL-1β were assessed by ELISA. n = 6 to 7 healthy blood donors. **B** is actual concentrations. ND: not detectable. **C** is relative expression. Each black dot is one donor's cells in one treatment group. Gray bars are medians. * $P < 0.05$ for comparison between LPS and LPS + vim. **D**. Effect of different concentrations (0, 0.4, 2, or 10 ug/mL) of extracellular vimentin on LPS-induced IL-12 secretion, as detected by CBA. n = 4 healthy blood donors. Each black dot is one donor's cells in one treatment group. Gray bars are medians.

3.3 Addition of vimentin changes LPS-induced moDC cytokine secretion

Another aspect of DC activation is cytokine secretion. Thus, we also assessed whether extracellular vimentin could alter LPS-induced cytokine secretion in moDCs. As these cytokine experiments were part of the first set of experiments, moDCs were stimulated with LPS, in the absence or presence of extracellular vimentin. CBA was used to assess the effects of vimentin on LPS-induced moDC production of IL-6, IL-10, and IL-12; ELISA was used to assess IL-1β and TGF-β1. In untreated moDCs, IL-10 and IL-12 concentrations were very low or undetectable by CBA, and IL-6 concentrations were < 5% of the value with LPS. LPS induced IL-6, IL-10, and IL-12 secretion. The moDCs also exposed to vimentin demonstrated a decrease in the LPS-induced secretion of IL-6 and IL-12 (Fig. 2B, C, D), cytokines known to be associated with the ability of moDCs to promote differentiation of Th17 cells [35, 36] and Th1 cells [36], respectively. Interestingly, moDCs treated with vimentin also demonstrated increased secretion of IL-10, a powerful anti-inflammatory cytokine that limits the differentiation, proliferation, and cytokine production of Th1, Th2, and Th17 cells [37, 38]. However, there was no change in the secretion of total TGF-β1, another anti-inflammatory cytokine. Additionally, IL-1β was undetectable in both moDCs treated with LPS and with LPS + vimentin. The inability of LPS alone to induce IL-1β secretion from DCs is consistent with literature [39].

3.4 MoDC:T cell co-culture

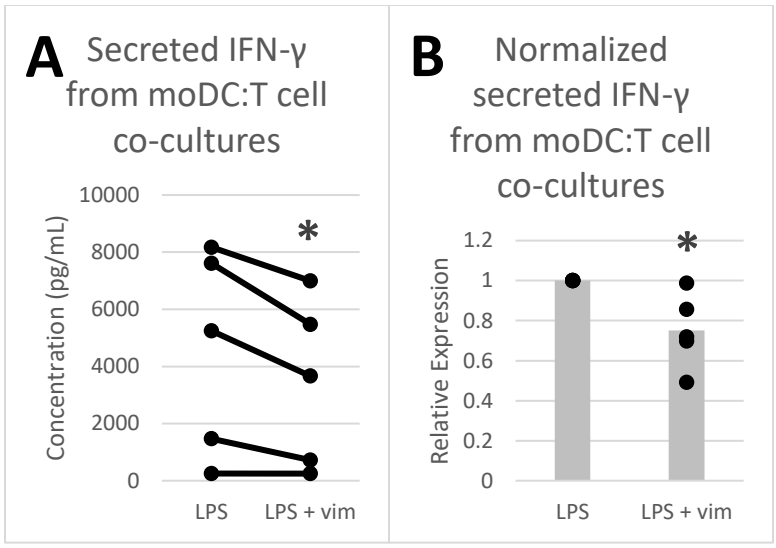
To address if extracellular vimentin alters moDC-mediated differentiation of T cells, moDCs were cultured with allogeneic naïve CD4+ T cells in the presence of LPS, with and without extracellular vimentin. The presence of LPS induces IFN-γ secretion, which is consistent with Th1 differentiation. However, the addition of vimentin decreases the amount of LPS-induced IFN-γ secretion, suggesting a decrease in Th1 differentiations (Fig. 3A, B). Neither LPS nor LPS + vimentin

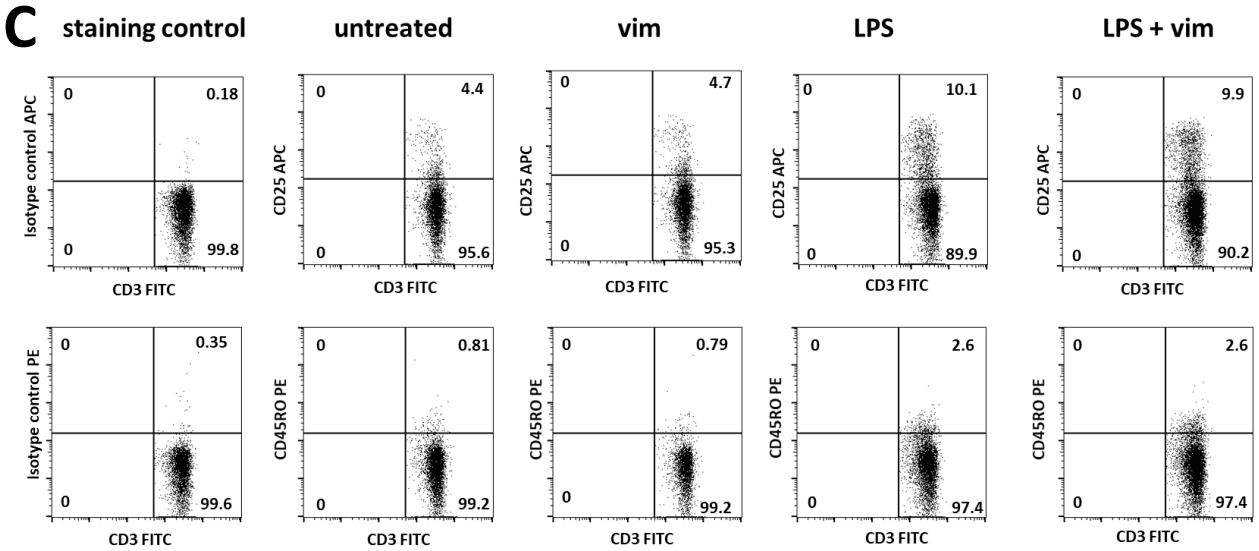
treatment resulted in significant IL-17 secretion, as IL-17 was undetectable by CBA in most co-cultures (4/5 independent experiments).

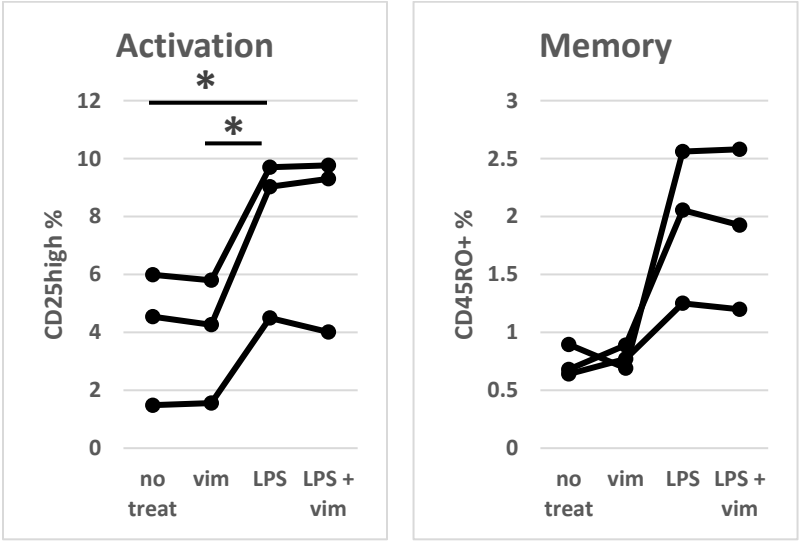
The addition of vimentin to LPS did not affect the proportion of T cells that became activated T cells (CD25+) or memory T cells (CD45RO+), as indicated by surface markers assessed by flow cytometry (Fig. 3C). As a control, co-cultures were also performed without treatment, or with the addition of vimentin alone. Vimentin alone did not alter the proportion of T cells that became effector T cells or memory T cells.

Another aspect of naïve T cell activation is proliferation, which was assessed by staining with a cell trace dye and detecting the dye with flow cytometry. LPS did increase moDC-mediated proliferation of allogeneic naïve CD4+ T cells. The addition of vimentin to LPS did not consistently impact this LPS-induced naïve CD4+ T cell proliferation (Fig. 3D, E).

We also assessed the effects of extracellular vimentin on naïve CD4+ T cells, independent of DCs. IFN-γ and IL-17 secretion were undetectable in naïve CD4+ T cells treated with nothing, vimentin, LPS, or LPS + vimentin. Additionally, activated T cells (CD25+) and memory T cells (CD45RO+) were rare or undetectable in naïve CD4+ T cells treated with nothing, vimentin, LPS, or LPS + vimentin (Fig. 4).


A
Secreted IFN-γ from moDC:T cell co-cultures
Concentration (pg/mL)
10000
8000
6000
4000
2000
0
*
LPS
LPS + vim
B
Normalized secreted IFN-γ from moDC:T cell co-cultures
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
LPS
LPS + vim


C
staining control
untreated
vim
LPS
LPS + vim
Isotype control APC
CD25 APC
CD3 FITC
0.18
99.8
4.4
95.6
4.7
95.3
10.1
89.9
9.9
90.2
Isotype control PE
CD45RO PE
0.35
99.6
0.81
99.2
0.79
99.2
2.6
97.4
2.6
97.4


Activation
CD25high %
12
10
8
6
4
2
0
*
*
no treat
vim
LPS
LPS + vim

Memory
CD45RO+ %
3
2.5
2
1.5
1
0.5
0
no treat
vim
LPS
LPS + vim

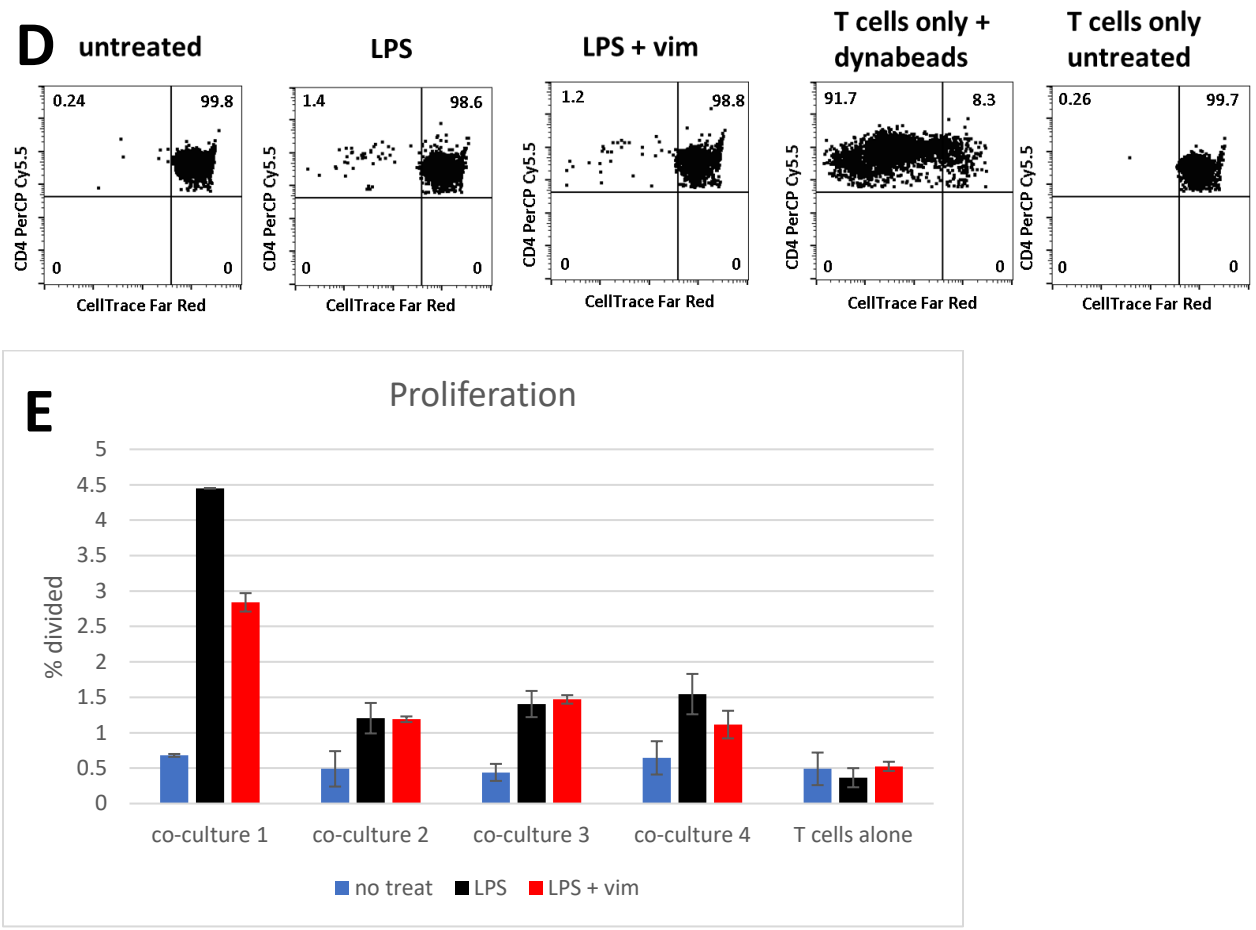


Figure 3. Effect of vimentin on naïve CD4+ T cell activation in moDC:T cell co-cultures

Co-cultures of moDCs and naïve CD4+ T cells were treated for 4 days. **A** and **B**. Supernatant was harvested. Cytokine concentrations were assessed by CBA. **A** is actual concentrations. **B** is relative expression. Each black dot is one donor's cells in one treatment group. Gray bars are means. * $P < 0.05$ for comparison between LPS and LPS + vim. **C**. We assessed expression of activation marker CD25 and memory marker CD45RO by flow cytometry. Plots are gated on live T cells (CD3+ FVD450- lymphocytes). Representative data are from 1 out of 3 independent experiments. Summary data is shown for the 3 experiments. * $P < 0.05$ for comparison between LPS and other treatment group. **D** and **E**. Naïve CD4+ T cells were stained with CellTrace Far Red. These naïve CD4+ T cells were co-cultured with treated moDCs for 4 days. **D.** Dot plots are gated on live CD4+ T cells (CD4+ FVD450- lymphocytes). Representative data are from 1 out of 4 independent experiments. **E.** Bar graph indicates frequency of CD4+ T cells that have proliferated at least once (CellTrace low). This contains data from 4 pairs of moDC:T cell donors, as well as a T cell monoculture control.

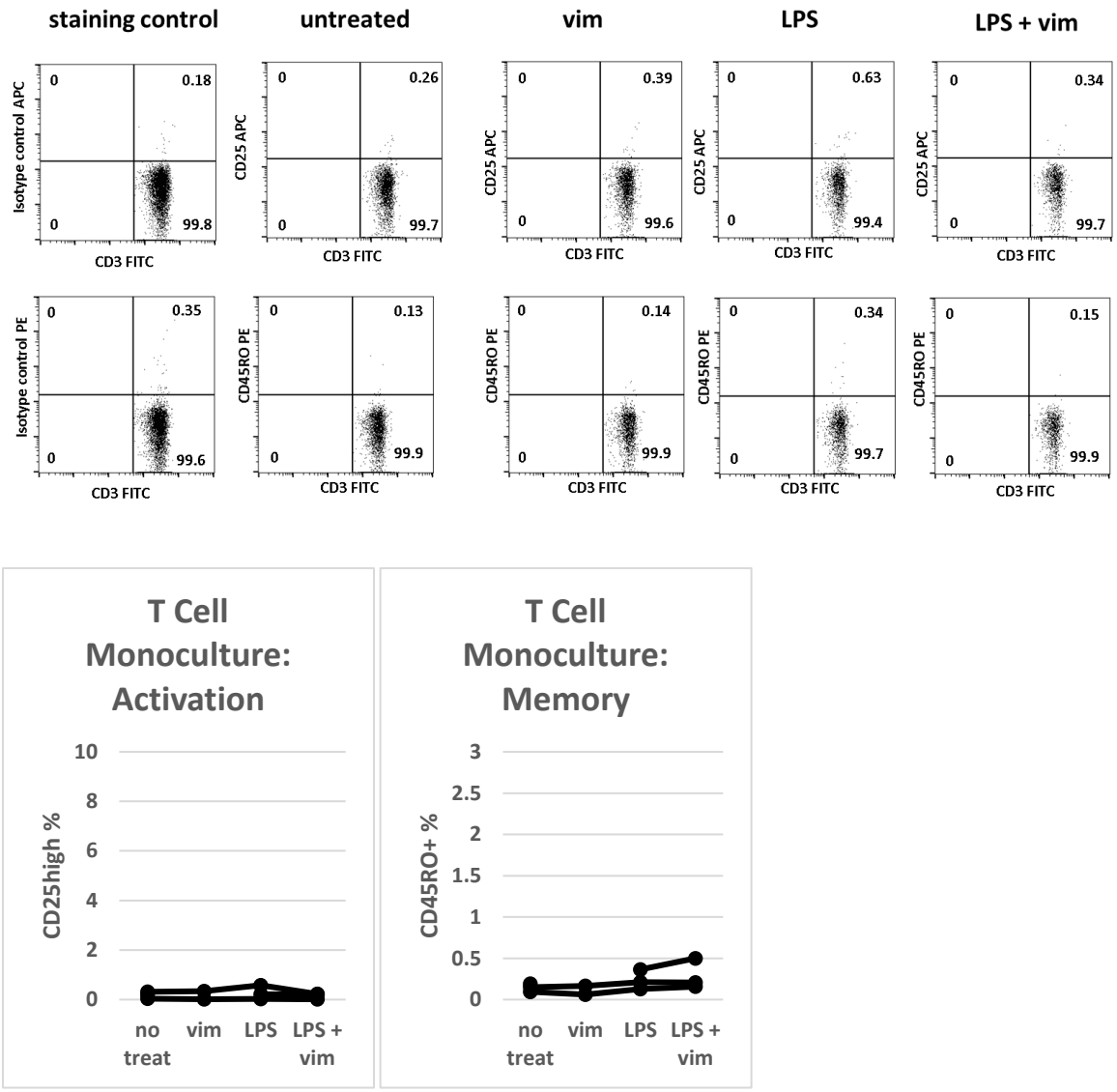
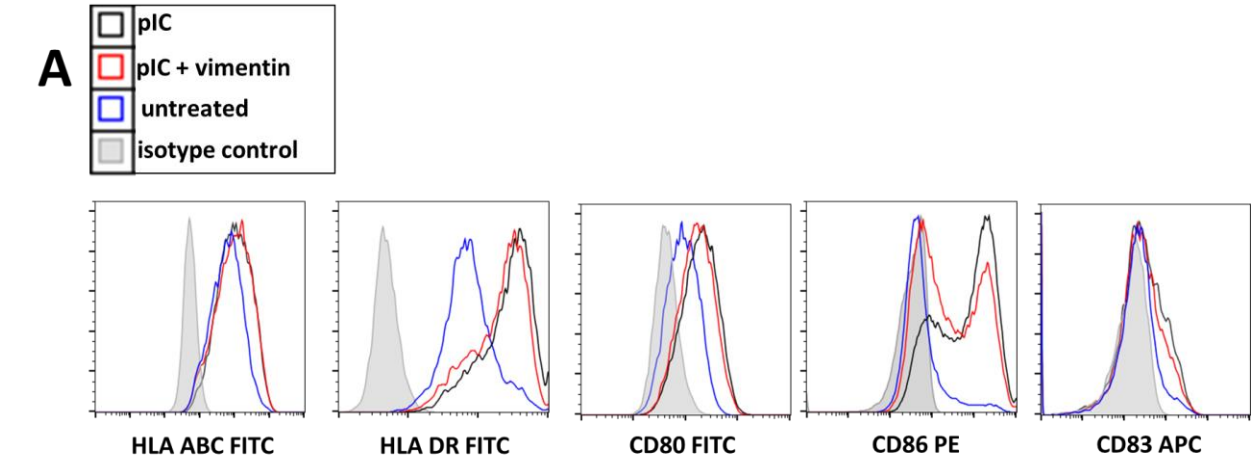


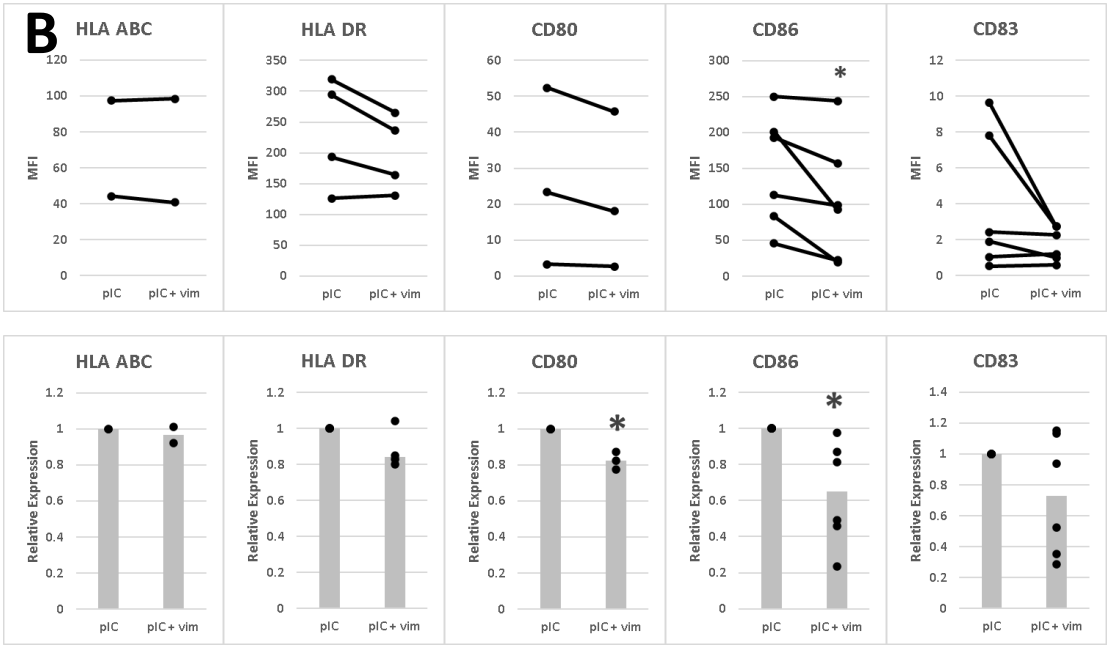
Figure 4. Direct effect of vimentin on naïve CD4+ T cells

Naïve CD4+ T cells were treated for 4 days. Expression of cell surface markers on T cells, analyzed by flow cytometry. Top: Plots are gated on live T cells (CD3+ FVD450- lymphocytes). Data are shown from one representative donor out of three analyzed. Bottom: summary graphs.

3.5 Addition of vimentin and poly I:C-induced moDC expression of activation-associated surface markers and cytokine secretion

In addition to activation by bacterial compounds, DCs are also subject to activation by viral compounds. One way to simulate an immune reaction to viral infection is with poly I:C, an analog of double-stranded RNA. Thus, we also assessed whether extracellular vimentin altered poly I:C-induced activation in moDCs. As expected based on literature [40], poly I:C increased surface expression of HLA ABC, HLA DR, CD80, and CD86. The addition of vimentin decreased poly I:C-induced surface expression of CD86 (Fig. 5A, B) and possibly also mildly decreased CD80. The addition of vimentin had no robust effect on HLA ABC, HLA DR, or CD83. Poly I:C induced secretion of IL-6 and IL-10, but the addition of vimentin had no consistent effect on poly I:C-induced secretion of IL-6 or IL-10 (Fig. 5C). Poly I:C did not induce secretion of IL-12 in most cases (5/6), and poly I:C + vimentin also did not induce secretion of IL-12 in most cases (5/6).


A
pIC
pIC + vimentin
untreated
isotype control
HLA ABC FITC
HLA DR FITC
CD80 FITC
CD86 PE
CD83 APC


B
HLA ABC
HLA DR
CD80
CD86
CD83
MFI
pIC
pIC+ vim
Relative Expression

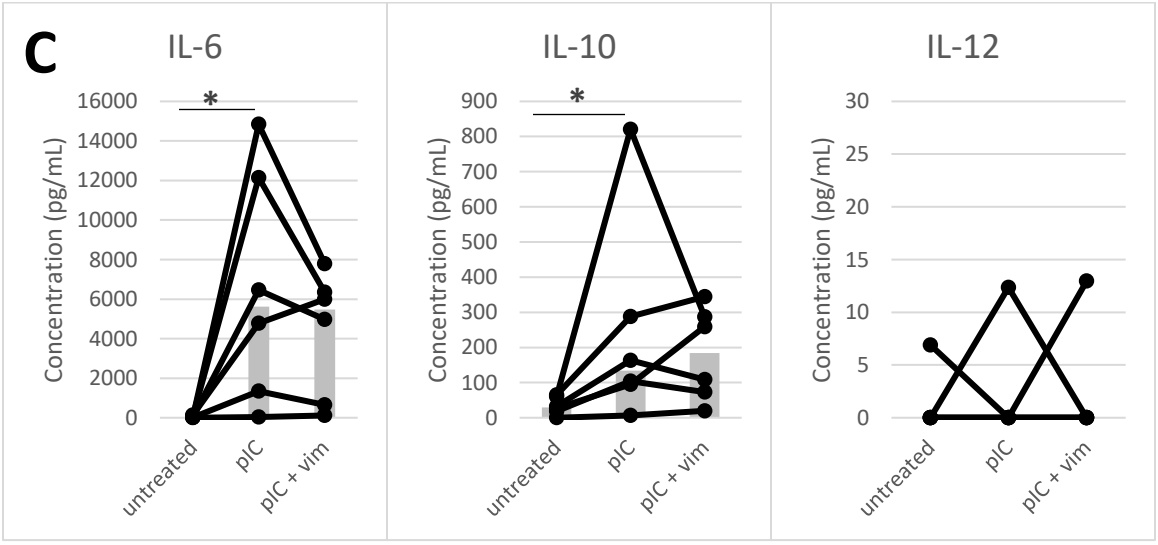

C
IL-6
IL-10
IL-12
Concentration (pg/mL)
untreated
pIC
pIC + vim

Figure 5. Vimentin modulates poly I:C-induced activation of moDCs.

The moDCs were treated with poly I:C +/- vimentin for 48 hours. **A** and **B.** Expression of cell surface markers on moDCs, analyzed by flow cytometry**. A**. The representative histograms show surface staining of HLA ABC, HLA DR, CD80, CD86, and CD83 in the gated moDCs. Data are shown from one representative donor. **B.** Summary data from multiple donors. Relative expression is normalized to the MFI in the poly I:C treated group. Each black dot is one donor's cells in one treatment group. Gray bars are means. * $P < 0.05$ for comparison between poly I:C and poly I:C + vim. n = 2 (HLA ABC), 3 (CD80), 4 (HLA DR), or 6 (CD86, CD83) healthy donors. **C.** Concentrations of IL6, IL10, and IL12 were detected by ELISA. Gray bars are medians. n = 6 healthy blood donors. * $P < 0.05$ for comparison between poly I:C and untreated.

4. Discussion

Extracellular vimentin has been previously shown to change the behavior of monocytes, macrophages [12, 18], and neutrophils [27]. Here, we have explored the effects of vimentin on DCs, the most effective antigen presenting cells. DCs recognize PAMPs and DAMPs, which enables them to direct activation of the adaptive immune system by inducing the differentiation of antigen-specific naïve T cells into effector T cells. The resulting effector T cells can be either pro- or anti-inflammatory [41]. In moDCs, vimentin does not appear to alter the LPS-induced surface expression of MHC I molecules, MHC II molecules, co-stimulatory molecules, or a marker of DC maturation (Fig. 2). However, exposure to vimentin does shift LPS-induced cytokine production away from pro-inflammatory cytokines, in ways suggestive of a decreased ability to differentiate naïve CD4+ T cells (Th0) into Th1 cells, which are pro-inflammatory (Fig. 2). We also observed that extracellular vimentin decreased LPS-induced moDC-mediated naïve CD4+ T cell differentiation into Th1 effector T cells (Fig. 3).

Our work could have broader implications for human health if vimentin also modulated DC activation induced by other stimuli from other pathogens. Thus, we assessed whether vimentin altered activation induced by poly I:C, known to simulate an immune response to viral infection through toll-like receptor 3 signaling [40, 42]. While there were no robust changes in DC cytokine secretion, vimentin did decrease the surface levels of costimulatory factor CD86, which suggests decreased activation of the DCs (Fig. 5).

A previous study by Carter et al. [6] demonstrated that exposure of phytohaemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) to vimentin decreases the secretion of pro-inflammatory cytokines secreted predominately by Th1 cells [6]. This observation suggests that exposure to extracellular vimentin decreases Th1 cell activation, a concept consistent with the data presented here. It is possible that the effects of vimentin identified in Carter et al.'s work

were partially mediated by mature DCs present in the PBMC sample. While Carter et al. used a stimulus specific for T cells, it is also known that stimulated T cells can induce DC maturation [43, 44]. Exposure of unstimulated PBMCs to vimentin had no effect on Th1 cytokines [6]. Similarly, in a study by Li et al. [14], in vitro exposure of unstimulated PBMCs to extracellular vimentin did not alter the proportion of Th1 cells in healthy volunteers. Our experimental protocol involving T cells is different from that of by Carter et al. [6] and Li et al. [14] in that we use moDCs and naïve CD4+ T cells only, and we stimulate the moDCs with LPS. As suggested by Carter et al.'s work [6], it is possible that extracellular vimentin has different effects depending on context.

Extracellular vimentin could result from tissue damage or immune activation, which can lead to tissue damage. Perhaps the availability of extracellular vimentin could be a sign to the immune system that there is or will likely be tissue damage. Based on our experimental results, we suggest that exposure of maturing DCs to extracellular vimentin could be a molecular mechanism that shifts naïve T cell differentiation away from Th1 cells. This alteration in the DCs could help to arrest tissue damage as well as helping to prevent autoimmunity by inhibiting the differentiation into Th1 cells of naïve T cells that recognize self-antigens released by damaged tissues (Fig. 6). Remaining pathogens could still be killed by monocytes or macrophages, as extracellular vimentin induces an oxidative burst in these cells, and the oxidative burst is known to kill phagocytosed bacteria [12, 18]. Additionally, there could be a transient decrease in monocytes, which may undergo apoptosis after an oxidative burst [45]. Such vimentin-induced pro- and anti-inflammatory effects could be beneficial in cases of mild injury or mild infection, by averting a major damaging pro-inflammatory immune response [46, 47].

However, during severe injury or severe infection, the immunosuppressive effects of extracellular vimentin could be harmful because extracellular vimentin might contribute to increased risk of prolonged infection unresolvable without DC-mediated Th1 responses. It has

been reported that severe injury or severe infection can cause systemic inflammatory response syndrome (SIRS), one effect of which is that the innate immune system becomes overactive while the adaptive immune system is suppressed [46-48]. Therefore, the possibility exists that vimentin could be one of the molecules responsible for this potentially dangerous imbalance in the immune system. If this hypothesis is correct, decreasing the effects of vimentin on the immune system may be an attractive therapeutic strategy for increasing trauma patient survival, as immune-system-related complications are a significant cause of death after trauma [49].

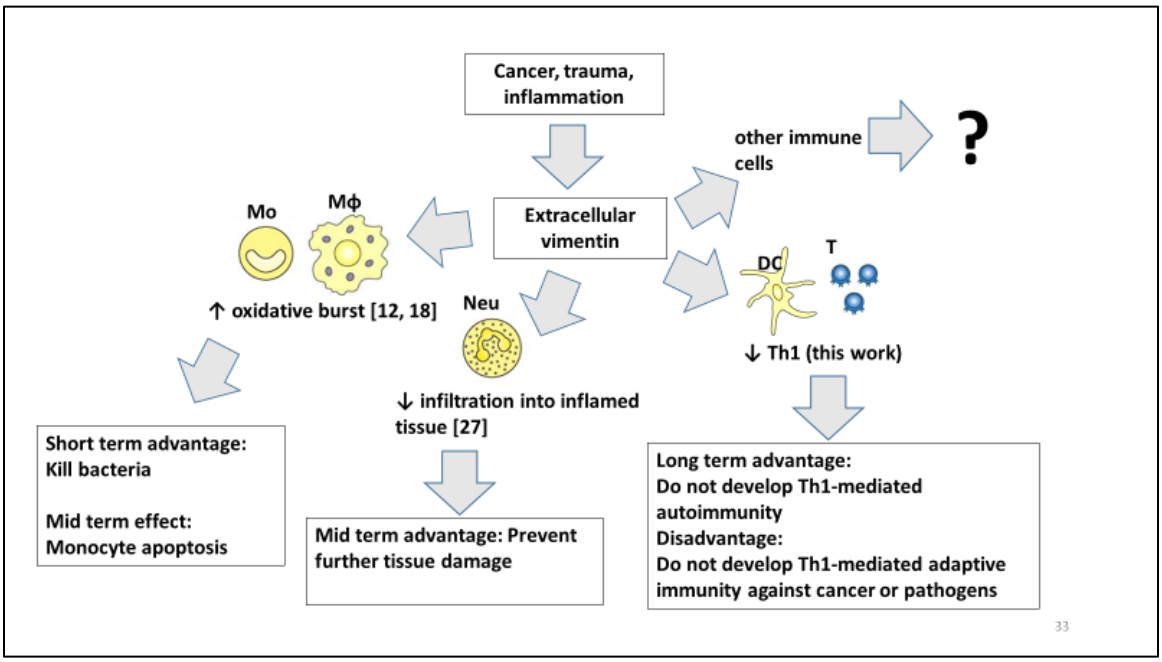


Figure 6. Proposed alteration of the immune response by extracellular vimentin.

Extracellular vimentin can result from cancer, trauma, or inflammation. Extracellular vimentin increases the oxidative burst in monocytes and macrophages, thus increasing bactericidal activity [12, 18] but possibly also inducing apoptosis in monocytes shortly afterwards [45]. Extracellular vimentin also decreases the infiltration of neutrophils into inflamed tissues [27]. In DCs, extracellular vimentin decreases the secretion of IL-12 and IL-6 while increasing IL-10 secretion. As a result, the DCs have decreased ability to stimulate the differentiation of naïve CD4+ T cells into Th1 cells. These opposing effects may have an advantageous effect as bacteria will be killed, further tissue damage will be prevented, and autoimmunity will be less likely. Potential disadvantages may include a decreased pro-inflammatory Th1 response against pathogens and cancer. However, there may be many other, unexplored effects of vimentin on immune cells.

In cancer, the tumor micro-environment is often immunosuppressive, which prevents the immune system from eliminating tumor cells [50, 51]. Vimentin was shown to be released constitutively by at least one cancer cell line [4], suggesting the immunosuppressive mechanism of extracellular vimentin could be used by these cancer cells to facilitate evasion of pro-inflammatory immune responses. Given the subtlety of our results, we think that vimentin alone is unlikely to have a major immunosuppressive effect. However, it may function in conjunction with other immunosuppressive molecules upregulated in cancer cells such as IDO (indoleamine 2,3 dioxygenase) [52] and TGF-β [21], to fine tune the immune response.

Future studies will explore the mechanism of extracellular vimentin's effect on moDCs, with a focus on how vimentin decreases LPS-induced IL-12 secretion. Literature reports indicate that vimentin interacts with the following cell surface receptors: dectin-1 [12], insulin-like growth factor 1 receptor (IGF1R) [28], NKp46 [26], and P-selectin [27]. Dectin-1 and IGF1R are known to be expressed in DCs, and there is transcriptional evidence that NKp46/NCR1 and P-selectin may also be expressed [53-58]. Thus, there are at least four receptors that could be responsible for the effects of extracellular vimentin on DC activation. Since dectin-1 surface expression was shown to be high in mature DCs [55], we believe that dectin-1 may be the most likely candidate. However, dectin-1 signaling is known to increase secretion of IL-6 and IL-12 in DCs [59], and to promote Th17 responses [60], which is not consistent with our data. Perhaps different dectin-1 ligands may generate different effects, as has been demonstrated for TLR4 [61]. Alternatively, IGF1R is also an attractive vimentin receptor candidate, as IGF1R signaling is known to increase IL-10 and decrease IL-6 secretion in DCs in the presence of LPS [62], which is consistent with our data on the effects of extracellular vimentin.

Another area of interest for further studies is how DC activation is modulated by modified vimentin, such as citrullinated vimentin, which was shown to be an important antigen in the pathogenesis of rheumatoid arthritis [63], or phosphorylated vimentin, which is thought to be the form of vimentin that is secreted from macrophages [2].

5. Conclusion

Our experimental data suggest a novel role for vimentin in immune regulation. Extracellular vimentin was shown to affect the stimulatory properties of moDCs. We propose that extracellular vimentin is involved in several clinical scenarios including organ damage, autoimmunity, and cancer. Augmenting the effect of extracellular vimentin on DC activation may prevent or ameliorate autoimmunity and organ damage. Conversely, blocking vimentin's immunosuppressive effects may help to prevent chronic infection and the progression of cancer.

6. Addendum

This addendum includes experiments that were not part of the published manuscript.

6.1 MoDC:T cell co-culture and intracellular cytokines

Materials and Methods

For these experiments, monocytes were isolated and differentiated into moDCs as described in section 2.1, and naïve CD4+ T cells were isolated as described in section 2.2. The moDCs (1 x 10^4 cells/well) and naïve CD4+ T cells (0.8 – 1.0 x 10^5 cells/well) were plated in R10 culture medium in 96-well tissue culture plates, and then treated. The co-culture treatment groups included: untreated, vimentin (10 ug/mL), and LPS (20 ng/mL) +/- vimentin.

The moDCs and T cells were co-cultured in R10 culture medium for 8 days. 6 hours before the end of the co-culture period, the culture medium was replaced with R10 culture medium containing PMA (phorbol 12-myristate 13-acetate, 50 ng/mL, ACROS Organics, Belgium), ionomycin (1 ug/mL, ALEXIS Biochemicals, NY), and monensin (2 uM, Biolegend) to allow

intracellular concentration of cytokines. After incubation, the cells were incubated with 50 uL of working solution of Fixable Viability Dye eFluor 450 for 30 min at 4°C in the dark to distinguish between living and dead cells. The cells were subsequently washed once with PBS, resuspended in PBS (20 uL), and incubated with CD3 FITC conjugated antibody (clone HIT3a; Biolegend) for 15 min at 4°C in the dark. The cells were subsequently washed with PBS, fixed with reagent A from the FIX & PERM cell permeabilization kit (ThermoFisher) for 15 min at room temperature in the dark. The cells were subsequently washed with PBS, resuspended in reagent B (10 uL) from the FIX & PERM cell permeabilization kit (ThermoFisher), and incubated with conjugated intracellular cytokine antibodies (indicated below) for 20 min at room temperature in the dark. The cells were subsequently washed with PBS and fixed with 1% paraformaldehyde solution before flow cytometric analysis using the MACSQuant Analyzer.

The following anti-human intracellular cytokine antibodies were used: IFN-γ PE (clone 25723.11; BD Biosciences) and IL-17 APC/Cy7 (clone BL168; Biolegend).

Fluorescent compensation and flow cytometry analysis were performed as described in section 2.4.

Results

Based on our moDC monoculture experiments involving cytokine detection (section 3.3), we hypothesized that extracellular vimentin would decrease moDC-induced Th1 and Th17 differentiation. So, we tested whether extracellular vimentin alters moDC-induced differentiation of T cells. MoDCs were cultured with allogeneic naïve CD4+ T cells in the presence of LPS, with and without extracellular vimentin. Vimentin exposure was shown to decrease differentiation of Th1 cells, as determined by measuring the percentage of T cells (CD3+ live lymphocytes) that are Th1 cells (IFN-γ+ CD3+ live lymphocytes) (Fig. 7). Neither LPS nor LPS + vimentin treatment

induced significant Th17 differentiation, as Th17 cells (IL-17+ CD3+ live lymphocytes) were undetectable by flow cytometry. These experimental results suggest that extracellular vimentin can decrease moDC-induced Th1 differentiation.

However, the amount of Th1 differentiation is lower than would be expected [64, 65], possibly due to the lack of delay between treatment of moDCs and the addition of T cells. Thus, in all other co-culture experiments in the chapter, moDC treatment preceded addition of T cells, and there was an 8 to 10 hour delay between treatment of moDCs and the addition of T cells. In these other co-culture experiments, results were again suggestive that extracellular vimentin can decrease moDC-induced Th1 differentiation (section 3.4).

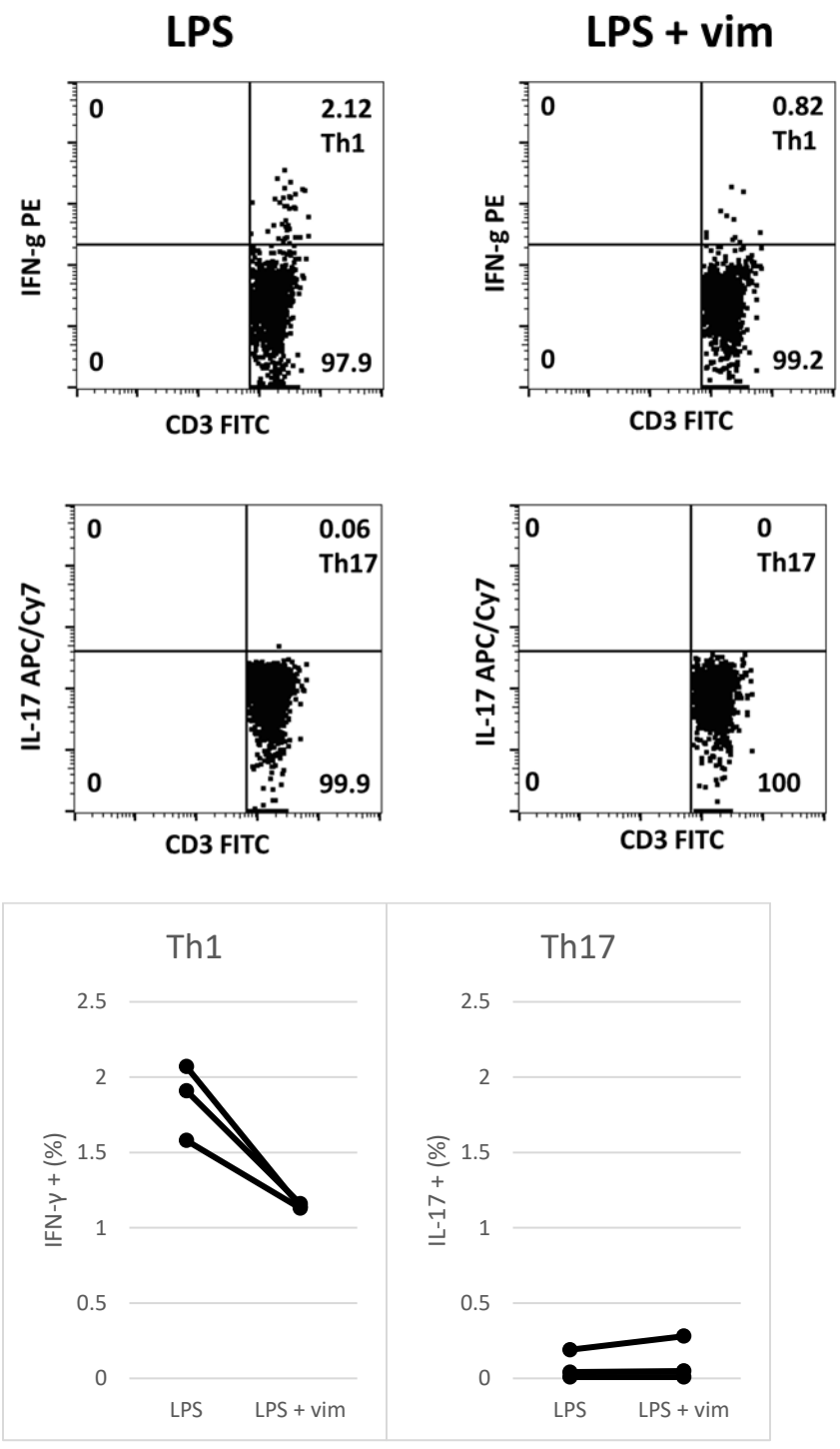


Figure 7. Effect of vimentin on naïve CD4+ T cell intracellular cytokines in moDC:T cell co-cultures

Expression of intracellular cytokines in CD4+ T cells analyzed by flow cytometry. Co-cultures of moDCs and naïve CD4+ T cells were treated with LPS or LPS + vimentin for 8 days. During the last 6 hours of incubation, the co-cultures were stimulated with PMA (50 ng/mL) and ionomycin (1

ug/mL) in the presence of monensin (2uM). Plots are gated on live T cells (CD3+ FVD450- lymphocytes). Top 2 rows: The representative dot plots indicate a lower percentage of Th1 (IFN-γ +) cells in co-cultures incubated with LPS + vimentin than in co-cultures with LPS alone. Th17 (IL17+) cells were undetectable. Representative data are from 1 out of 3 independent pairs of monocyte donor: naïve T cell donor. Bottom row: summary of data from the independent experiments with 3 pairs of blood cell donors. * $P < 0.05$ for comparison between LPS and LPS + vim.

6.2 Dectin-1 agonism is not the major mechanism for the effect of vimentin on LPS-induced moDC cytokine secretion

Materials and Methods

For these experiments, monocytes were isolated and differentiated into moDCs as described in section 2.1, and then moDCs were treated as described in section 2.3. Dectin was blocked with anti-dectin antibody (10 ug/mL, R&D, MAB1859) for 30 minutes prior to addition of other treatments. The positive control for dectin signaling is zymosan (2.5 ug/mL, Sigma Aldrich), a known agonist of dectin.

ELISA was performed as described in section 2.7.

For statistical analysis of representative data, the two-sample Student's t test was used.

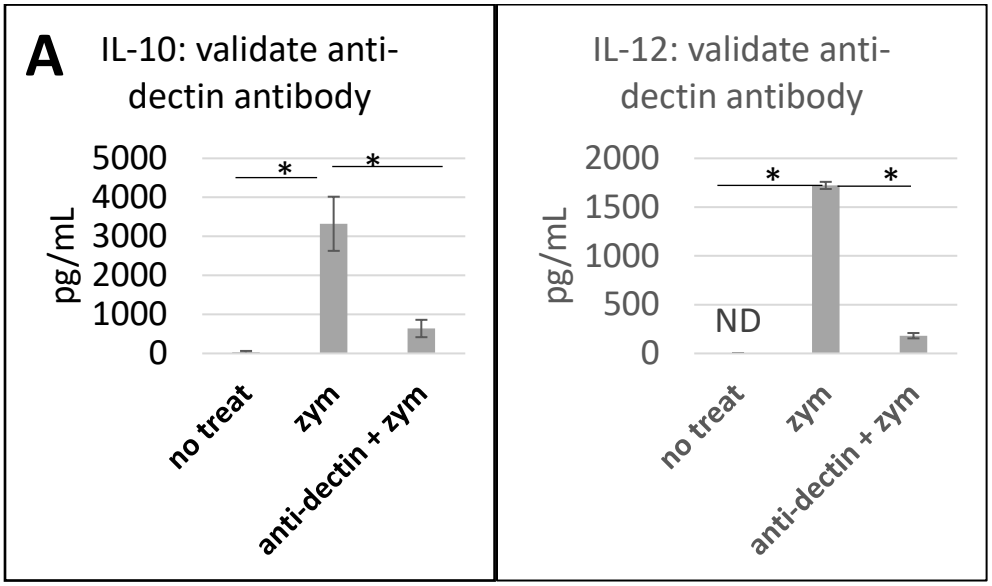
Results

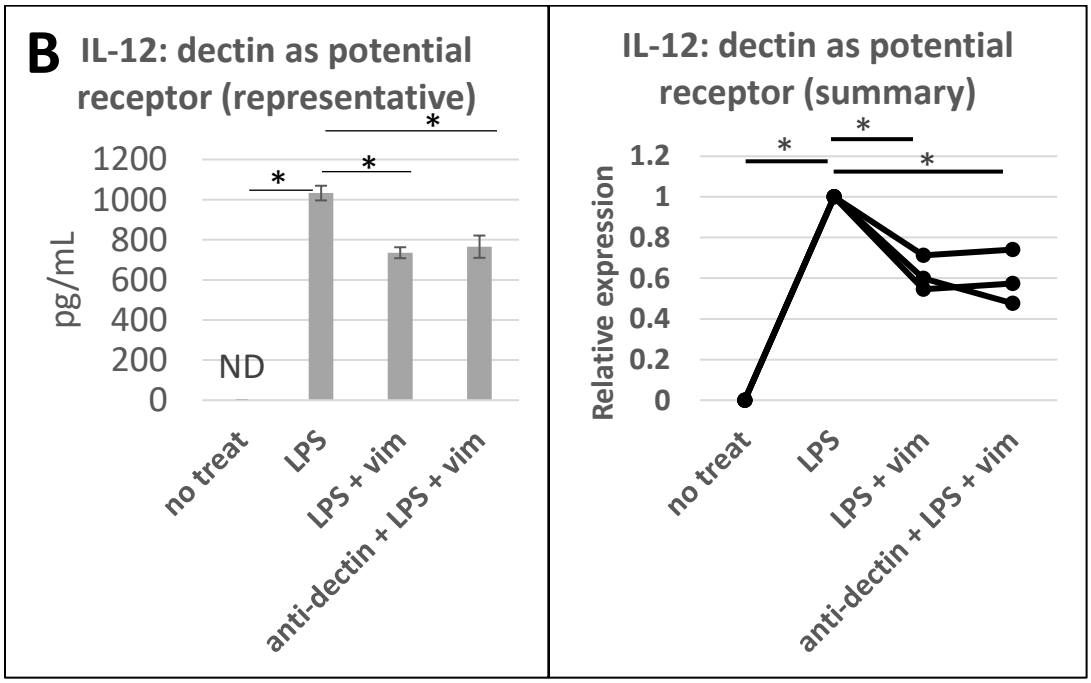
Literature reports indicate that vimentin interacts with dectin-1 in macrophages [12], which are closely related to dendritic cells [66, 67]. To assess potential involvement of dectin-1 in the effects of vimentin on LPS-induced cytokine secretion in moDCs, we used an anti-dectin antibody as a blocking reagent. This antibody was able to block IL10 and IL12 secretion induced by a known dectin agonist, zymosan, indicating that this antibody does block dectin-1 signaling (Fig. 8A). Use of the anti-dectin antibody did not reverse the effect of vimentin on LPS-induced IL12 secretion (Fig. 8B). However, the anti-dectin antibody may have partially reversed the effect of vimentin on IL10 (Fig. 8C), though this effect was not statistically significant.

Discussion

Dectin-1 agonism does not seem to be the sole major mechanism of vimentin's effects, though it could be partially responsible. Other receptors may be involved, and vimentin has

many binding partners as discussed in section 4 and [2, 68]. IGF1R is a particularly attractive candidate for mediating the effects of vimentin on DCs [28, 54, 57, 58, 62].





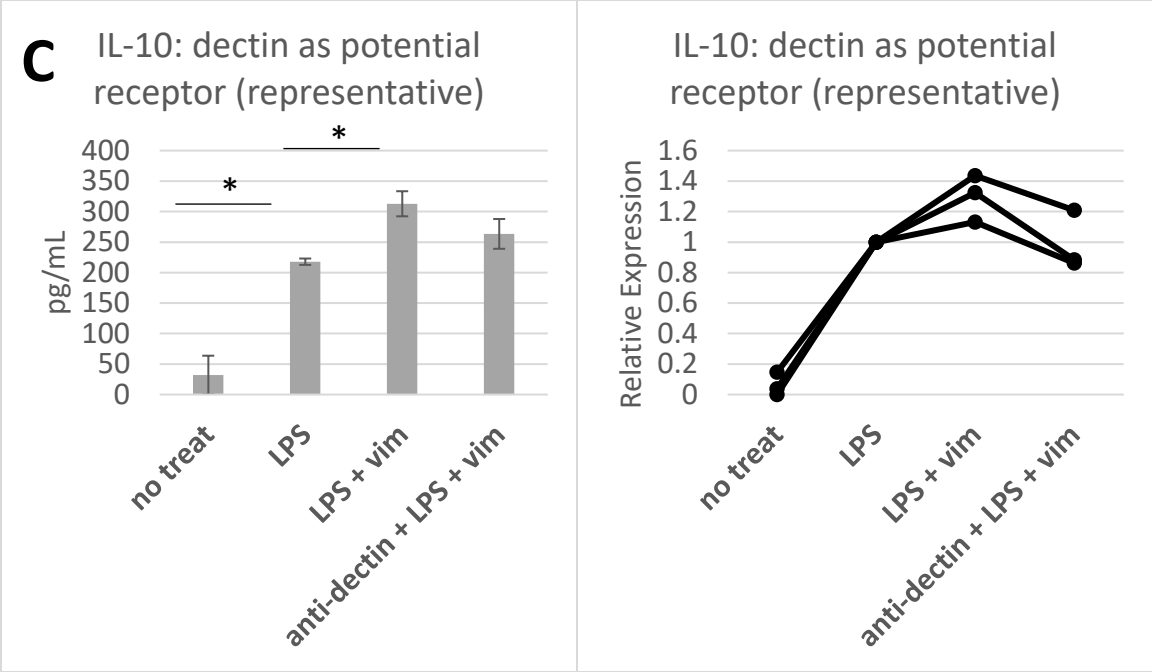


Figure 8. Evaluation of dectin as a potential receptor for vimentin.

The moDCs were pre-treated with blocking antibody for 30 minutes and then treated for 48 hours. Anti-dectin = anti-dectin antibody (10 ug/mL). zym = zymosan (2.5 ug/mL). vim = vimentin (2.5 ug/mL). Concentration of IL10 and IL12 were detected by ELISA. **A.** Anti dectin antibody is adequate to block dectin-1, as demonstrated by blocking IL-10 and IL-12 secretion induced by a known pro-inflammatory dectin-1 agonist, zymosan. **B.** Use of the anti-dectin antibody did not alter the modulating effects of vimentin on LPS-induced IL12 secretion. Left plot includes representative data using moDCs from one individual. This experiment was performed a total of 3 times with similar results, see summary data in right plot. * $P < 0.05$ for comparison between LPS and other treatment group. **C.** Use of the anti-dectin antibody may have partially reversed the effect of vimentin on LPS-induced IL10 secretion, though the effect is not statistically significant. Left plot includes representative data using moDCs from one individual. This experiment was performed a total of 3 times with similar results, see summary data in right plot. * $P < 0.05$ for comparison between LPS and other treatment group.

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CHAPTER THREE

3. REVIEW: PREDICTING METHOTREXATE RESISTANCE IN RHEUMATOID ARTHRITIS PATIENTS

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Note: This chapter is an updated version of my article of the same title: M.B. Yu, A. Firek, W.H. Langridge, Predicting methotrexate resistance in rheumatoid arthritis patients, Inflammopharmacology, 26 (2018) 699-708. The only significant change is the addition of the last paragraph in the methotrexate mechanism section, which briefly acknowledges a recent review article that explores more recently proposed, less studied mechanisms.

Abstract

Rheumatoid arthritis (RA) is an incurable, systemic autoimmune disease that decreases quality of life and can lead to severe disability. While there are many medications available to treat RA, the first-line of therapy is low-dose methotrexate (MTX), a small-molecule disease-modifying anti-rheumatic drug (DMARD). MTX is the recommended therapy due to its affordability and efficacy in reducing symptoms in most RA patients. Unfortunately, there is great person-to-person variability in the physiological response to MTX, with up to 50% of patients showing little response to the medication. Thus, many RA patients initially placed on MTX do not experience an adequate reduction of symptoms, and could have benefited more in both the short

and long terms if initially prescribed a different drug that was more effective for them. To combat this problem and better guide treatment decisions, many research groups have attempted to develop predictive tools for MTX response. Currently, there is no reliable, clinical-grade method to predict an individual's response to MTX treatment. In this review, we describe progress made in the area of MTX non-response/resistance in RA patients. We specifically focus on application of the following elements as predictive markers: proteins related to MTX transport and function, intracellular MTX concentration, immune cell frequencies, cytokines, and clinical factors.

Table 1. Abbreviations

ABC	ATP binding cassette
ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
ADA	adenosine deaminase
AICAR	aminoimidazole carboxamide ribonucleotide
AMPDA	AMP deaminase
ATIC	aminoimidazole carboxamide ribonucleotide transformylase
BCRP	breast cancer resistance protein
DC	dendritic cell
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DMARD	disease-modifying anti-rheumatic drug
EULAR	European League Against Rheumatism
IC50	half maximal inhibitory concentration
K_M	Michaelis constant
MDR	multidrug resistance protein
MRP	multidrug resistance-associated protein
MTX	methotrexate
MTX-PG	polyglutamated methotrexate
NSAID	nonsteroidal anti-inflammatory drug
PBMC	peripheral blood mononuclear cells
PCFT	proton-coupled folate transporter
PKA	protein kinase A
PMN	polymorphonuclear leukocytes
RA	rheumatoid arthritis
RF	rheumatoid factor
RFC	reduced folate carrier

SE	shared epitope
SLC	solute carrier
THF	tetrahydrofolate
TNFR	TNF receptor
TYMS	thymidylate synthetase

1. Introduction

Rheumatoid arthritis (RA) is a chronic disease that results in joint inflammation, pain, and swelling, and can often lead to irreversible deformity [1]. Most RA patients become unable to work full-time within 10 years of symptom onset [2]. Both the American College of Rheumatology (ACR) [3] and the European League Against Rheumatism (EULAR) [4] currently recommend low-dose methotrexate (MTX; Figure 9) monotherapy as the initial treatment for patients newly diagnosed with RA. The low-dose distinction is used to draw a contrast with the high-doses of MTX used in cancer treatment, which are 100 times larger [4, 5].The ACR's and the EULAR's reasons for choosing low-dose MTX are that MTX is inexpensive, and there is a lack of evidence that more expensive treatments have better long-term efficacy [3, 4].

However, there is significant variation in MTX efficacy between individual RA patients. Prevalence estimates of non-response/resistance to MTX therapy in RA patients range widely from 30 to 50% [6-10]. The mechanisms for this variable response are complex and unclear. Despite the obvious need, there is no validated clinical method available to predict MTX response in RA patients. In this review, non-response and resistance will be used interchangeably.

2. Methotrexate Mechanism

MTX (Figure 9) is classified as a small-molecule disease-modifying anti-rheumatic drug (DMARD). In broad terms, MTX is a folate derivative which acts as a competitive inhibitor for multiple folate dependent enzymes, leading ultimately to the inhibition of DNA synthesis and elevation of extracellular adenosine. The result is a decrease in both cell proliferation and

secretion of pro-inflammatory cytokines [5, 11-13]. In the immune system, MTX most dramatically affects T cells [14, 15], but is also reported to have anti-proliferative or anti-inflammatory effects on B cells [16], monocytes [15-17], and dendritic cells (DCs) [18].

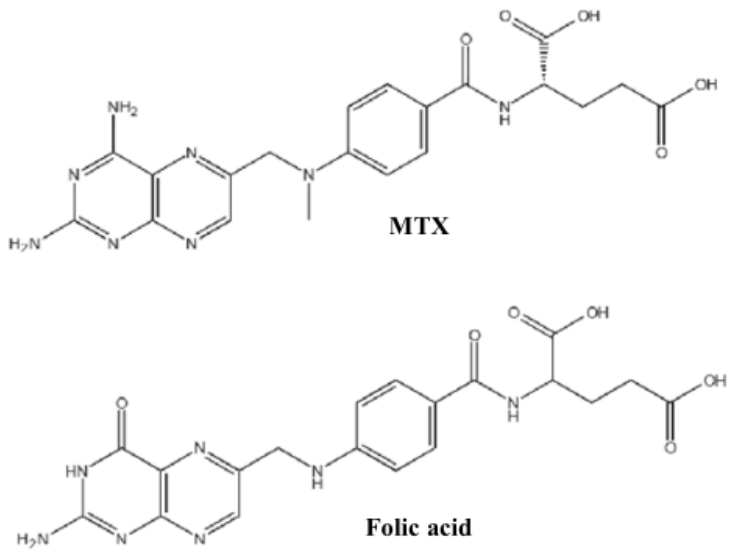

Figure 9. Structures of methotrexate and folic acid. MTX is a folate derivative

MTX is believed to prevent DNA synthesis and therefore normal cell division via inhibition of enzymes involved in de novo nucleotide synthesis (Figure 10). Specifically, MTX inhibits the enzymes thymidylate synthetase (TYMS), aminoimidazole carboxamide ribonucleotide transformylase (ATIC), and dihydrofolate reductase (DHFR). TYMS is involved in thymidine synthesis. ATIC is involved in de novo purine synthesis. DHFR reduces dihydrofolate (DHF) to tetrahydrofolate (THF), whose derivatives are cofactors for TYMS and ATIC. Thus, MTX decreases the activity of TYMS and ATIC by directly inhibiting these enzymes and by decreasing access to THF cofactors [11-13]. Inhibition of TYMS and ATIC decreases the amount of nucleotides available for DNA synthesis, particularly thymine [12, 19] and guanine [19, 20], and thus reduces DNA

synthesis [12, 13, 19, 20]. This molecular mechanism is particularly important for preventing T cell proliferation because activated T cells almost exclusively rely on de novo nucleotide synthesis as opposed to the nucleotide salvage pathway, and DNA replication is required prior to cell division [13, 15, 16, 19].

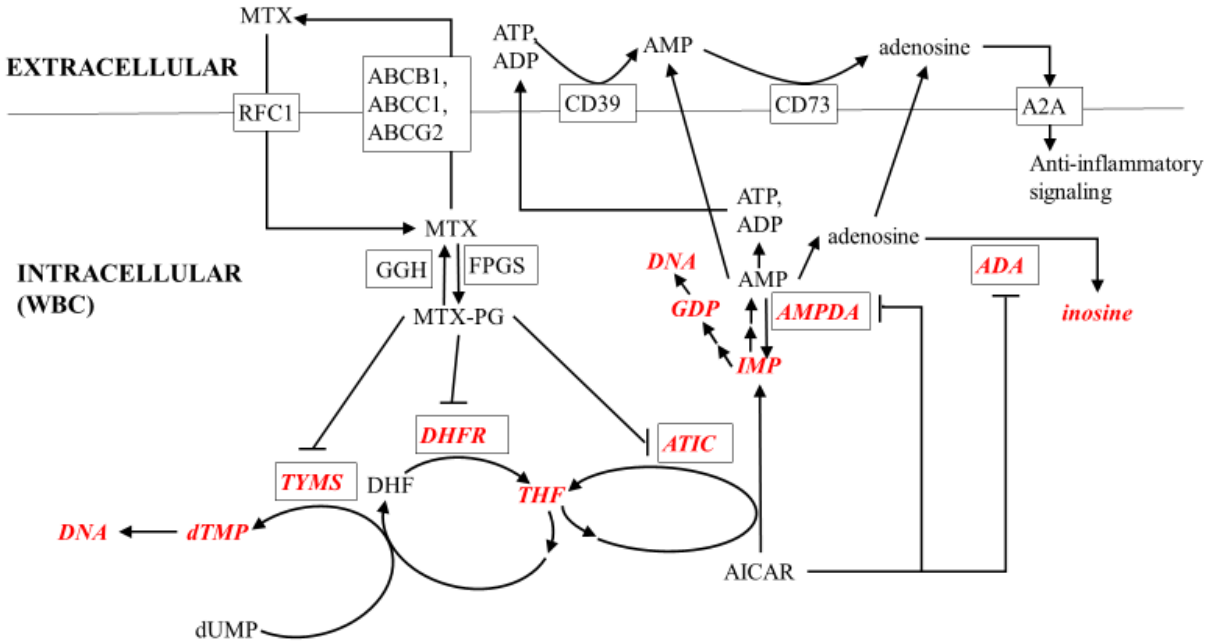

Figure 10 Mechanism of methotrexate. Boxes indicate proteins. Red, italicized type indicates those enzymes whose activity is reduced by MTX, or compounds whose levels are reduced by MTX. Transport of MTX into the cell occurs by RFC1, while transport out of the cell occurs by ABCB1, ABCC1, or ABCG2. MTX is polyglutamated by FPGS to become MTX-PG, and this process is reversed by GGH. MTX-PG inhibits TYMS, DHFR, and ATIC. Inhibition of DHFR decreases THF levels, leading to a decrease in the THF derivatives which are cofactors for TYMS and ATIC. This lack of access to cofactors contributes to the MTX-PG induced decrease in TYMS and ATIC activity. Decreases in TYMS and ATIC activity result in reduced synthesis of thymidine and purines, respectively. As a result, there are decreased amounts of thymine and purine nucleotides available for DNA synthesis, thus reducing DNA synthesis. The decrease in ATIC activity also leads to a build-up of AICAR. The increase in AICAR inhibits ADA and AMPDA, resulting in a build-up of adenosine and AMP. Adenosine, AMP, ADP, and ATP can be interconverted. The excess adenosine, AMP, ADP, and ATP are secreted into the extracellular space. Outside the cell, ATP, ADP, and AMP are hydrolyzed into adenosine by CD39 and CD73. Extracellular adenosine binds to A2A receptors, resulting in anti-inflammatory signaling

MTX promotes the increase of extracellular adenosine (Figure 10) by decreasing the activity of ATIC, resulting in an excess of ATIC's substrate aminoimidazole carboxamide ribonucleotide (AICAR). AICAR in turn inhibits adenosine deaminase (ADA) and AMP deaminase (AMPDA), resulting in a buildup of their respective substrates, adenosine and AMP. Adenosine, AMP, ADP, and ATP can interconvert into each other by phosphorylation and hydrolysis [5, 11-13]. Excess adenosine, AMP, ADP, and ATP are released into the extracellular space. The mechanism of this release is unclear, but the proposed mechanisms include vesicle exocytosis and facilitated diffusion [21]. Extracellular AMP, ADP, and ATP are converted into adenosine by cell-surface enzymes CD39 and CD73. Then, adenosine binds to adenosine receptors [12, 13, 21].

Adenosine receptors are expressed on immune cells including neutrophils, macrophages, DCs, T cells, and B cells. These receptors are G protein coupled receptors. The adenosine receptor subtypes A_{2A} and A_{2B} activate G_s proteins, which activate the adenylate cyclase-cAMP-protein kinase A (PKA) pathway. In contrast, adenosine receptor subtypes A_1 and A_3 activate G_i proteins to inhibit the adenylate cyclase-cAMP-PKA pathway [22]. The receptor of greatest relevance to MTX induced effects is believed to be the A_{2A} receptor [13].

Through the A_{2A} receptors, extracellular adenosine induces anti-inflammatory effects in several types of immune cells. In neutrophils, A_{2A} receptor signaling decreases expression of pro-inflammatory cytokines and prevents apoptosis. In macrophages and mature DCs, A_{2A} receptor signaling decreases expression of pro-inflammatory cytokines and increases expression of anti-inflammatory cytokines. In CD4+ T cells, A_{2A} receptor signaling reduces both proliferation and expression of pro-inflammatory cytokines [22]. In activated B cells, A_{2A} receptor signaling decreases antibody production, but this is usually balanced by A_1 and A_3 receptor signaling which promotes antibody production [23].

More recent work has indicated that MTX also uses other mechanisms such as altered long non-coding RNA expression, decreased nitric oxide production, and inhibition of JAK-STAT signaling. However, these mechanisms are not well understood [24].

3. Alterations in gene sequences

Variations in gene sequences are attractive as potential predictive markers for MTX response because DNA sequences are static over the course of a lifetime. As a result, it does not matter if the patient samples were acquired before or after beginning MTX treatment. Much work has been done evaluating correlations between MTX efficacy and variations in the DNA sequences of genes involved in MTX pharmacokinetics and pharmacodynamics. Progress in the pharmacogenetics of MTX response in RA has been recently reviewed elsewhere [25], and will not be extensively discussed here. Several studies report associations between MTX response and single gene polymorphisms. However, most of these findings have either been contradicted by other studies, or have not yet been independently confirmed [25].

For example, ATP binding cassette B1 (ABCB1) codes for a protein that removes MTX from cells, and one of its polymorphisms is rs1045642, 3435C>T. The TT genotype was reported to be associated with increased MTX efficacy in a Polish [26] and a Japanese [27] cohort of RA patients. However, this same genotype was associated with decreased MTX efficacy in another Japanese cohort [28], and it was the CC genotype that was associated with increased efficacy in an Indian cohort [29]. No association between this SNP and MTX efficacy was found in studies with other populations of RA patients [8, 30-33]. Unfortunately, in the field of the pharmacogenetics of MTX, such direct contradictions are common [25].

Many reasons have been suggested for the lack of consistency. Many of these studies could be considered small for genetics studies (well under 1000 people), and thus the results may not be replicable [25]. It is possible that some of the positive findings may be false positives, with

the positive result due to random chance [34]. MTX response is affected by many genes, and the effect of any one gene is likely to be small. Small effects are difficult to consistently detect, but more importantly, a gene with a small effect is unlikely to be a helpful biomarker in personalized medicine. Additionally, the genes may interact with each other, and linkage disequilibrium may play a role [25]. So, models utilizing multiple polymorphisms and haplotype blocks have also been developed [25, 35, 36].

4. Alterations in protein expression and activity

Expression levels and activity of some MTX-associated proteins have been evaluated as potential predictors of the MTX response. These proteins described below are grouped by function.

4.1Absorption: Transport into the bloodstream

After oral delivery, MTX is absorbed into enterocytes of the small intestine by proton-coupled folate transporter (PCFT). PCFT transports physiological folates with Michaelis constant K_M 1 – 3 uM, and it can also transport MTX with K_M 3 – 6 uM because MTX is a folate derivative. It is conceivable that differences in the expression level or activity of PCFT could contribute to differences in response to MTX [11].

A study in RA patients indicated there is no association between PCFT expression and MTX efficacy [37]. But PCFT has been shown to be inhibited in vitro by various compounds such as the DMARD sulfasalazine, nonsteroidal anti-inflammatory drugs (NSAIDs) diclofenac and indomethacin, tea flavonoid epigallocatechin-3-gallate, and wine flavonoid myricetin [11, 38, 39]. The inhibitory effects of sulfasalazine on PCFT may explain why the combined therapeutic effects of MTX and sulfasalazine are less than additive [11, 39].

4.2 Distribution: Transport into cells

Figure 10 illustrates the pathway of MTX transport into the cell.

MTX is transported into cells by reduced folate carrier 1 (RFC1)/solute carrier 19A1 (SLC19A1), which physiologically transports folates [11, 13, 40]. RFC1 is expressed in a variety of tissues, with the highest expression in the placenta, liver, and leukocytes [11]. The high expression of RFC1 in the placenta may contribute to the efficacy of high-dose MTX to induce abortion [41]. Additionally, while it has not been shown that low-dose MTX leads to miscarriage, the RFC1 expression in the placenta could be used as a biochemical rationale to support the current recommendation that low-dose MTX not be used to treat RA during pregnancy [42].

One study in RA patients indicated that there is a positive correlation between RFC1 expression in peripheral blood mononuclear cells (PBMCs) and MTX efficacy. Subjects with higher expression of RFC1 were more likely to experience lower disease activity while on MTX, which is consistent with the concept that MTX enters target cells through RFC1 [37]. However, another study found that low RFC1 expression in PBMCs is associated with MTX efficacy in RA patients with low activity from ABCB1, a transporter that removes MTX from cells [43]. These studies only measured RFC1 expression after MTX treatment and not before [37, 43]. For RFC1 expression to be of benefit to patients and truly predictive, the markers must be observed before the patient is on MTX, as MTX treatment has been shown to increase RFC1 expression [44]. It is unknown if this difference in RFC1 expression was present prior to MTX administration. The observed differences in RFC1 expression in MTX responsive compared to MTX resistant patients could be a result of MTX's effects, and it is possible that the non-response could be due to the lack of MTX's effect to increase RFC1.

4.3 Distribution: Transport out of cells

Figure 10 illustrates the pathway of MTX transport out of the cell.

There are several ABC transporters involved in the efflux of MTX from cells. These include ABCB1/multidrug resistant protein 1 (MDR1)/ P-glycoprotein (P-gp), ABCC1/multidrug resistance

associated protein 1 (MRP1), and ABCG2/breast cancer resistance protein (BCRP) [11, 40]. Theoretically, increased expression or function of these transporters should decrease MTX concentrations in target cells, resulting in lack of therapeutic response to MTX.

ABCB1/MDR1/P-gp

ABCB1 is present on the surface of peripheral blood leukocytes in RA patients and healthy controls [32, 43, 45], but absent in RA synovial tissue [46]. Studies have been performed to assess the association between ABCB1 expression and function with MTX resistance.

Prior to treatment, there is no difference in lymphocyte ABCB1 expression between RA patients who will respond to MTX and those will not. After four months of MTX treatment, lymphocyte ABCB1 expression decreased in RA patients whose symptoms had also significantly improved, while ABCB1 expression levels were unchanged for those whose symptoms had not improved with MTX [32]. Although changes in ABCB1 expression may be indicative of MTX response, these findings are not practical for prediction as the patient has already been exposed to MTX.

It has been proposed that the functional activity of ABCB1 may be more meaningful than its expression. The functional activity is measured by fluorescence of cells incubated with calcein, a fluorescent substrate for ABCB1, with and without an inhibitor of ABCB1 [45]. There are conflicting results concerning associations between ABCB1 activity and response to MTX. One study found that lymphocyte ABCB1 activity between responders and non-responders is the same at baseline. After MTX treatment, lymphocyte ABCB1 activity decreased relative to baseline in RA patients whose symptoms had also significantly improved, while ABCB1 activity was unchanged for those whose symptoms resisted MTX [32]. The results of this study are in agreement with the accepted role of ABCB1 in MTX pharmacokinetics.

However, in a study utilizing blood samples after MTX treatment, it was found that there was no association between ABCB1 activity in lymphocytes and MTX resistance, and that high ABCB1 activity in granulocytes is associated with MTX efficacy [45]. More specifically, in RA patients whose PBMCs express high levels of RFC1, higher ABCB1 activity in PBMCs is associated with increased MTX efficacy [43]. The results from these two latter studies conflict with the accepted role of ABCB1 in MTX pharmacokinetics since high ABCB1 should remove MTX and thus decrease the ability of MTX to influence target cells. It is clear that the role of ABCB1 in the overall action of MTX needs additional clarification.

ABCC1/MRP1

ABCC1 is expressed on the surface of peripheral blood leukocytes in RA patients and healthy controls [43, 45]. Additionally, this protein is present in low levels on synovial T cells and synovial macrophages in a subset of RA patients, and completely absent from synovial tissue in other RA patients [46]. An ovarian cancer cell line [47] study suggests that increased ABCC1 leads to MTX resistance. However, one study utilizing blood samples from RA patients after MTX treatment found no association between ABCC1 activity and MTX resistance. The functional activity was measured by fluorescence of cells incubated with calcein, a fluorescent substrate for ABCC1, with and without an inhibitor of ABCC1 [45].

ABCG2/BCRP

ABCG2 is present in synovial macrophages, T cells, and endothelial cells in RA patients, but present in very few synovial cells in patients without RA [46]. A study has been performed to assess the association between ABCG2 expression and DMARD resistance. In this study, the RA patients were treated with either MTX or leflunomide, another small molecule that also inhibits nucleotide synthesis. High expression of ABCG2 in synovial tissue after DMARD treatment is associated with DMARD resistance. Correlation between ABCG2 expression before and after

treatment suggested that differences in ABCG2 expression between responders and non-responders may have been present before treatment, and thus could be used to predict response to MTX [46].

4.4 Adenosine pathway

Figure 10 illustrates how MTX increases extracellular adenosine.

One of the downstream effects of MTX is release of ATP and ADP into the extracellular environment. Extracellular ATP and ADP are hydrolyzed into AMP by cell surface bound CD39, also known as ectonucleoside triphosphate diphosphohydrolase-1. AMP is then hydrolyzed into adenosine by cell surface bound CD73, also known as ecto-5'-nucleotidase. As described earlier, this extracellular adenosine then causes anti-inflammatory signaling. Both CD39 and CD73 are highly expressed on Tregs, and the CD39 and CD73 mediated production of adenosine contributes to the anti-inflammatory effect of this cell type [21]. A recent study found that low expression of CD39 in Tregs prior to MTX treatment is associated with lack of response to MTX [48]. CD39 expression in Tregs could potentially be used as a powerful biomarker to predict response to MTX.

5. Polyglutamated methotrexate as a marker

The MTX concentration in blood or in cells could be used as a pharmacokinetic marker. However, plasma MTX is not used because plasma MTX concentrations are insignificant 24 hours after a dose [49]. Since plasma MTX is transported into a cell, polyglutamated, and then able to act on its target enzymes, intracellular polyglutamated MTX (MTX-PG) is a better choice. Even though MTX's therapeutic effects are thought to be due to its interactions with enzymes in leukocytes and synovial cells, the MTX-PG level in the RBCs is more frequently measured. This is due to the greater number of RBCs in the blood and due to the longer lifetimes of RBCs relative to some of the leukocytes, such as neutrophils. It was reported that there is an association between MTX efficacy and higher MTX-PG in RBCs [50]. However, in a recent paper utilizing lower

doses of MTX and shorter time between MTX initiation and sample collection, no association was found with RBC MTX-PG and disease activity [27].

MTX-PG is a promising tool to study the pharmacokinetics of MTX but is limited as a predictor for MTX response, since the patient must take MTX for several weeks before RBC MTX-PG levels stabilize.

6. Immune cell frequencies as markers

Given the importance of immune cells in RA, the relative number of immune cell subtypes have been examined as possible markers of MTX response. One study found that a higher naïve T cell frequency before treatment is associated with remission induced by MTX monotherapy. This observation was found in both a pilot cohort and a validation cohort, though the pilot cohort was frozen blood samples from RA patients on either MTX monotherapy or MTX and TNF-inhibitors. The association between higher naïve T cell frequency and remission on MTX is consistent with the idea that the T cell balance may be dysregulated in MTX-resistant RA. [51]

Another study found that a lower Th17 cell frequency before MTX treatment is associated with greater decrease in inflammation induced by MTX monotherapy [52]. This was a very small study and their observations have yet to be repeated by other larger studies, but this observed association between Th17 cells and MTX response is consistent with the purported importance of Th17 in sustaining RA-associated inflammation.

A recent study found that higher number of circulating monocytes is associated with a lack of response to MTX [53]. This is consistent with the finding that MTX can activate DCs, which are closely related to and can be derived from monocytes [54]. However, there was another study that found no significant differences in circulating monocyte counts between MTX responsive and non-responsive RA patients [55].

Additionally, in a study utilizing blood taken after at least two months of MTX monotherapy, it was found that RA patients who are responsive to MTX have lower number of circulating polymorphonuclear leukocytes (PMN) than those who have a partial or no response to MTX [50]. However, this information cannot be used to predict MTX response as this difference in PMN numbers was not determined prior to MTX treatment.

7. Cytokines and chemokines as markers

Given the importance of cytokines and chemokines in RA pathogenesis, their baseline concentrations have been explored as possible biomarkers to predict MTX response. Favorable MTX response is mildly associated with increased baseline serum concentration of soluble TNF receptor (TNFR), which decreases TNF-α signaling by competing with membrane-bound TNFR for binding to TNF-α [55]. Conversely, decreased baseline serum concentration of TNF-α has been reported as predictive of MTX response [56]. However, in another study focusing on early RA patients, serum TNF-α was not predictive of MTX response [57]. Additionally, it was reported that there is an association between MTX resistance and increased baseline serum concentration of MPIF-1, a chemokine which attracts monocytes and naïve T cells [58]. Multiple studies found that serum concentrations of the following cytokines are not correlated with MTX efficacy: IL-1 receptor antagonist [55, 57], IL-1β, IL-6, IL-8, IL-10, and IL-12 [56, 57]. These studies indicate that baseline levels of cytokines are not consistently predictive of a clinical response to MTX treatment.

A similar approach is to culture PBMCs and then measure the concentration of secreted cytokines. Through this approach, it was found that a favorable MTX response was associated with increased IL-1β and soluble TNFR secretion from untreated PBMC, prior to in vivo MTX treatment [55]. Another group cultured PBMCs in the presence of varying concentrations of MTX, and then calculated half maximal inhibitory concentration (IC50) values for the concentration of MTX required to suppress cytokine production. This group found a negative correlation between

the clinical response and IC50 values for TNF-α and IFN-γ. Patients who experienced a larger clinical response to MTX tended to require a lower concentration of in vitro MTX to decrease secretion of TNF-α and IFN-γ [59].

8. Other proteins as markers

Autoantibodies are a common occurrence in RA. Two of the best-studied, common RA autoantibody types are anti-citrullinated protein antibody (ACPA), against citrullinated peptides, and rheumatoid factor (RF), against the constant region of IgG antibodies. In most studies exclusively with patients on MTX monotherapy, it has been found that ACPA status is not predictive of response to MTX [51, 57, 60]. However, one study found that ACPA positive RA patients were more likely than ACPA negative RA patients to achieve remission with MTX and prednisone [61]. Furthermore, RF status was not shown to be predictive of response to MTX [51, 56, 57, 60-62].

Myeloid related proteins 8 and 14 are endogenous proteins that activate TLR4 signaling and are found in increased levels in the serum of RA patients. They are secreted by monocytes, macrophages, and neutrophils in pro-inflammatory environments. Recently, it was found that a higher concentration of myeloid related proteins 8 and 14 in the serum of RA patients before MTX monotherapy is associated with larger therapeutic response to MTX. Thus, serum myeloid related proteins 8 and 14 are promising biomarkers that could be used to predict MTX response. [63]

9. Clinical predictors of methotrexate resistance

9.1 Disease-related

Disease duration has also been examined as a possible factor for MTX resistance. Some [56, 60] but not all studies found that MTX is slightly less efficacious if the disease symptoms were of longer duration before treatment. For example, in a cohort with a wide range of symptom durations, MTX responders had symptoms for 3.1 ± 5.4 years prior to treatment while MTX non-

responders had symptoms for 10.2 ± 10 years prior to treatment, and this was statistically significant [56]. In cohorts with symptom onset less than one year prior to MTX treatment initiation, Saevarsdottir et al. [60] reported that the odds ratio for response was 0.88 – 0.99 per month increase in disease duration, but Ponchel et al. [51] reported this odds ratio to be 0.89 - 1.2, which is not significant. Additionally, in a cohort with symptom onset less than two years prior to MTX treatment initiation, the median symptom duration was 24 weeks for responders and 25 weeks for non-responders, and this was not statistically significant [36].

9.2 Patient-related

A number of studies have examined the impact of patient-related clinical factors on MTX efficacy. Most studies found that MTX is more likely to be effective in male RA patients compared to female RA patients. The odds of effective response to MTX are approximately double for male compared to female RA patients [36, 55, 60, 61].

Age of the patient has not shown to be consistently predictive. Some studies indicate increased MTX efficacy with increasing age [60, 64]. For example, Saevarsdottir et al. [60] reported that the odds ratio for response was 1.3 per 10 years increase in age, and Sharma et al. [64] reported that the average age was 45 years old for MTX responders and 41 years old for MTX non-responders. However, other studies indicate that age is not associated with significant changes in likelihood of response to MTX [36, 55, 61].

People with the shared epitope (SE), which are HLA-DR alleles sharing a specific motif, are more susceptible to RA. According to a study focusing on RA patients taking MTX and sulfasalazine, SE+ RA patients are more likely than SE- RA patients to be resistant to these DMARDs [9].

Lifestyle factors have also been examined, as they can be altered in addition to being theoretically used in prediction of MTX efficacy. Smoking is the best established environmental

risk factor for RA onset [1, 65]. There have been studies indicating that smoking does not predict MTX response [9, 36]. However, in a recent, large study on patients with early RA, it was found that current smoking may predict MTX resistance, but history of smoking is not associated with MTX resistance [66]. While smoking cessation is advisable to increase the probability of MTX efficacy, it is apparently not a large enough effect by itself to reliably predict who will respond to MTX and who will not.

Since caffeine is an adenosine receptor antagonist and increasing adenosine receptor signaling is part of MTX's mechanism, there has been some concern that caffeine could reduce MTX's efficacy. While a small study initially indicated that high caffeine intake decreases the efficacy of MTX [67], a larger study later found that caffeine did not significantly impact MTX efficacy [68].

MTX is a folate derivative, and it interferes with folate metabolism [5, 11-13, 69]. Folic acid supplementation prevents MTX-induced liver toxicity and gastrointestinal side effects. While there are concerns that this folic acid supplementation might reduce the efficacy of MTX, meta analysis indicates that folic acid supplementation does not significantly change the efficacy of MTX [69]. As a result, EULAR recommends folic acid supplementation in conjunction with MTX [4]. In the United States, roughly half of all MTX-treated patients have also been prescribed folic acid [70].

There is a lack of studies examining association of other dietary components with MTX efficacy in RA patients.

10. Conclusion

MTX is an effective, well-tolerated drug for many RA patients. However, there remains a lack of efficacy in up to 50% of patients [6-10]. In addition, this drug can cause significant side effects in a small minority of RA patients [10]. A reliable clinical test to determine MTX response

in RA patients would be a major clinical advance. Such a test would promote earlier use of non-MTX based therapies and avoid the possibility of MTX-induced adverse effects in patients who would have poor response to MTX. Unfortunately, despite many studies in this research area, there is still no reliable test that can be applied clinically to predict MTX response. Given the complexity of MTX transport, cellular delivery, and action, it is likely that MTX response is dependent on multiple variables, including clinical characteristics. There is clearly an unmet need to develop a clinical predictive method for determining MTX response in this devastating disease.

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CHAPTER FOUR

4. Effect of Methotrexate on IL-17 Secretion from Resting and Stimulated Leukocytes: Implications for Rheumatoid Arthritis Treatment

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Keywords: rheumatoid arthritis, methotrexate, resistance, response, predict, cytokine, IL-17

Abstract

Background: Rheumatoid arthritis (RA) is a systemic autoimmune disease that is often treated with methotrexate (MTX), which targets activated T cells. Unfortunately, 30 to 50% of RA patients do not experience adequate reduction of symptoms while on MTX. Predicting MTX response would be helpful for guiding treatment decisions and improving the lives of RA patients. The purpose of this pilot study was to explore a possible association between clinical response to MTX and in vitro effects of MTX on leukocyte secretion of IL-17 and IFN-γ, pro-inflammatory cytokines associated with the activated Th17 and Th1 cells.

Methods: Peripheral blood samples were collected from 18 RA patients prescribed MTX but prior to initiation of MTX treatment. Leukocytes were isolated from the blood samples, activated with anti-CD3 and anti-CD28 antibodies, and treated with several concentrations of MTX (0 to 1 uM). Supernatant was harvested at 72 hours, and concentrations of IL-17 and IFN-γ were assessed by ELISA. The RA patients' charts were reviewed to assess the clinical effects of MTX after 3 months of MTX treatment, as described by changes in clinical disease activity index (CDAI) and any side effects.

Results: 1 uM of MTX decreased secretion of IL-17 in 15/18 blood samples, and secretion of IFN-γ in 16/18 samples. Maximal effect on IL-17 ranged from 87% decrease, to 30% increase. Maximal effect on IFN-γ ranged from 92% decrease, to 88% increase. The concentration of MTX

required to achieve half-maximal decrease (IC50) also varied for both IL-17 (0.03 – 0.3 uM) and IFN-γ (0.01 – 0.3 uM). Based on samples from 14 patients with at least partial follow-up, there is greater variation in the maximal effect of MTX on in vitro IL-17 secretion in those with side effects vs those without ($p < 0.05$). Absence of side effects was associated with MTX-induced decrease in IL-17 from 20 to 70%. In contrast, out of 6 patients with side effects, 2 patients experienced MTX-induced increase in IL-17, and 2 other patients experienced MTX-induced decrease in IL-17 of greater than 70%. Only 11 RA patients completed the MTX trial of 3 months. No statistically significant correlations were found between clinical efficacy and in vitro results.

Conclusions: There is large person-to-person variation in the in vitro effects of MTX on IL-17 and IFN-γ. This variation could potentially be used to predict the occurrence of side effects, as immune cells from patients with side effects may be more likely to be unusually sensitive or unusually resistant to the effects of methotrexate.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that decreases quality of life and can lead to severe disability by causing joint inflammation and irreversible deformity. The first-line of therapy for RA is methotrexate (MTX), a small-molecule disease-modifying anti-rheumatic drug (DMARD) [1]. Unfortunately, 30 to 50% of RA patients do not experience adequate reduction of symptoms while on MTX [2-6]. Additionally, MTX resistance is only apparent after several weeks of treatment. As a result, RA patients who are initially placed on MTX but do not respond may not experience relief until placed on a different drug, by which time their disease has further progressed. A tool that could accurately predict MTX response and resistance would be helpful for guiding treatment decisions and improving the lives of RA patients.

Several studies have reported potential biomarkers for MTX resistance including genetic polymorphisms and expression levels of proteins involved in MTX transport and function. But at

this time, none of these biomarkers has been independently shown to be robust, with findings from one study often directly contradicted by findings from other studies [7]. The lack of agreement between studies may be due to the possibility there could be different underlying causes of MTX resistance in different people who exhibit MTX resistance.

MTX is a folate derivative with two major mechanisms of action. This drug inhibits de novo nucleotide synthesis, thus hindering DNA replication and cell proliferation. Additionally, MTX induces release of adenosine into the extracellular environment, and adenosine induces anti-inflammatory effects through binding to the adenosine 2A (A2A) receptor [8]. MTX most dramatically affects activated T cells, resulting in decreased proliferation and decreased pro-inflammatory cytokine secretion from these cells [9].

The pathogenesis of RA involves aberrant activation of T cells. Historically, RA was believed to be a T helper cell (Th) 1-mediated disease. However, recent research suggests that Th17 cells also play a major role [10]. Th17 cells are characterized as T helper cells that produce the pro-inflammatory cytokine interleukin (IL) -17 [11]. Through secretion of IL-17, Th17 cells promote bone resorption, cessation of cartilage repair, and secretion of other pro-inflammatory cytokines [10, 11]. Additionally, MTX treatment in RA patients reduces the ratio of Th17 cells to total Th cells [12], and reduces the ratio of Th17 cells to anti-inflammatory Treg cells [13].

Instead of examining the many potential upstream predictors of MTX resistance, our strategy is to directly assess the downstream effects of MTX on a sample of peripheral blood leukocytes, which could be thought of as a microcosm of an individual's immune system. Given the mechanism of MTX and the importance of Th17 cells in RA, we investigated the possibility that clinical response to MTX in RA patients is associated with magnitude of MTX-induced decrease in secretion of IL-17 in vitro.

2. Methods

Samples from healthy donors

Apheresis filter cones were donated by LifeStream blood bank (San Bernardino, CA).

Samples from RA patients

The study subjects are 18 RA patients who were prescribed MTX monotherapy but had never been treated before with MTX or any other DMARD (disease-modifying antirheumatic drug). Blood samples were collected from RA patients, using EDTA (ethylenediaminetetraacetic acid) tubes, before initiation of MTX therapy. The patients were instructed to also take folic acid supplements to decrease MTX's side effects, which is in-line with standard practice when using MTX for RA.

Blood sample processing

Human peripheral blood from the filter cones or EDTA tubes was incubated with a red blood cell lysis buffer (8.3 g/L ammonium chloride, 1.0 g/L potassium bicarbonate, 90 mg/L EDTA disodium, pH 7.1 – 7.4) to deplete the red blood cells. The leukocytes were seeded (2 x 10^6 cells/mL; 200 uL/well) in 96-well tissue culture plates.

All cell cultures were carried out in R10 medium: RPMI 1640 medium (Mediatech Inc.) supplemented with 10% FBS (Atlanta Biologicals), and 100 U/mL penicillin-streptomycin (ThermoFisher). In all cell cultures, supernatant was collected after 72 hours. Supernatant was stored at -80 C until use.

In the stimulated cell cultures, the cells were stimulated with a combination of low-endotoxin azide-free anti-CD3 (OKT3, 1 ug/mL, Biolegend) and anti-CD28 (CD28.2, 1 ug/mL, Biolegend). In the resting cell cultures, no stimuli were added.

MTX (ThermoFisher) stock solution (10 mg/mL; 22 mM) was prepared in DMF, diluted in PBS (20 uM), serially diluted in R10 medium, and then added at the start of cell culture. Folic acid

(ThermoFisher) stock solution (40 mg/mL) was prepared in 1M NaOH, diluted in R10 medium, and then added at the start of cell culture (final concentration 40 ug/mL). Folinic acid (ThermoFisher) stock solution (4 mg/mL) was prepared in water, diluted in R10 medium, and then added at the start of cell culture (final concentration 40 ug/mL). 8-(3-chlorostyryl)caffeine is a specific antagonist for the A2A adenosine receptor, while alloxazine is a specific antagonist for the A2B adenosine receptor. 8-(3-chlorostyryl)caffeine (ThermoFisher) and alloxazine (Santa Cruz Biotechnology) stock solutions (10 mM) were prepared in DMSO, diluted in R10 medium, and then added at the start of cell culture (final concentration 10 uM).

Cytokine ELISA

Supernatant was tested by sandwich enzyme-linked immunosorbent assay (ELISA). IL-17 and IFN-γ were measured with the uncoated ELISA kit with plates (ThermoFisher) according to the manufacturer's protocol.

Clinical assessment

Clinical RA disease activity was quantified with the CDAI (clinical disease activity index), a validated way to measure disease activity [14]. The CDAI is the sum of: tender joint counts (out of 28 commonly affected joints), swollen joint counts (out of 28 commonly affected joints), patient global assessment (0 to10), and provider global assessment (0 to 10). The CDAI was measured using data acquired about the patient's state before MTX treatment and after 3 months of MTX treatment. Additionally, any side effects were also recorded. The data was taken from the subject's medical records.

Statistical Analysis

In experiments with multiple doses of MTX, dose-response curves were fitted with a four parameter log logistic model, performed with the drc package in R. Correlation was calculated by the Pearson's correlation coefficient. Comparison between groups with and without side effects

was performed with the t test and the F test. In experiments related to the mechanism of MTX, ANOVA was used to evaluate potential differences between treatment groups. P value less than 0.05 was considered significant.

3. Results

Effect of MTX on IL-17 secretion from unstimulated leukocyte cultures

The eventual goal is to develop a clinical grade test. We chose to use leukocyte cultures to have a comprehensive representation of the immune system, and to simplify sample preparation. We chose to use ELISA to detect cytokine secretion because of this assay's high sensitivity, high specificity, safety, simplicity, and the high availability / relatively low cost of microplate readers.

Baseline IL-17 secretion from the unstimulated leukocytes of our healthy donors (average 160 pg/mL) was comparable to what has been previously reported (average 145 pg/mL) under similar conditions [15]. MTX decreased IL-17 secretion in unstimulated leukocytes from some healthy donors, but increased IL-17 secretion in unstimulated leukocytes from others (Fig. 11).

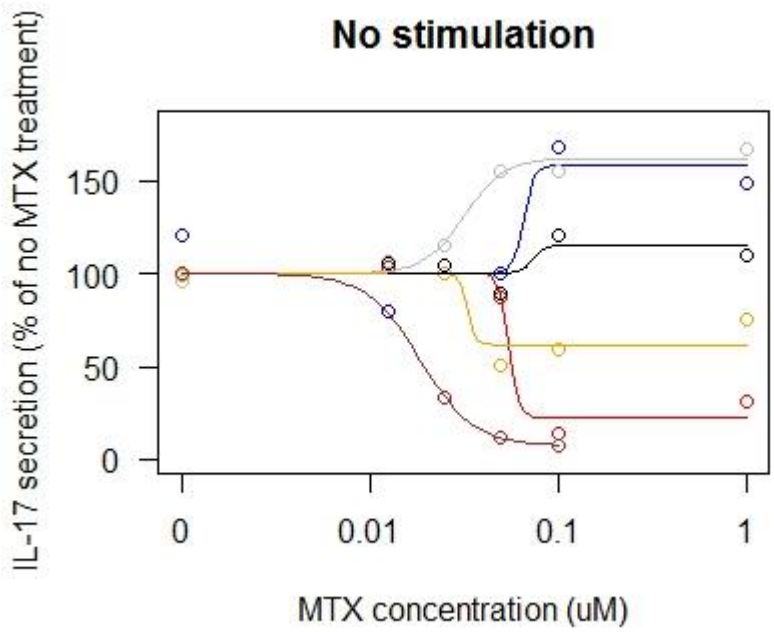

Figure 11: Effect of methotrexate on resting leukocyte cultures from healthy blood donors. Samples from 6 different healthy blood donors were exposed to a range of MTX concentrations. Supernatants were harvested after 72 hours, and then IL-17 secretion was assessed by ELISA.

Effect of MTX on IL-17 secretion from anti-CD3/anti-CD28 stimulated leukocyte cultures

We also assessed IL-17 secretion from leukocytes incubated with anti-CD3 and anti-CD28 antibodies, which specifically stimulates T cells. In these stimulated leukocyte cultures derived from healthy donors, MTX decreased IL-17 secretion. However, there was great variation in the degree of decrease and the concentration of MTX required to decrease IL-17 secretion. (Fig. 12)

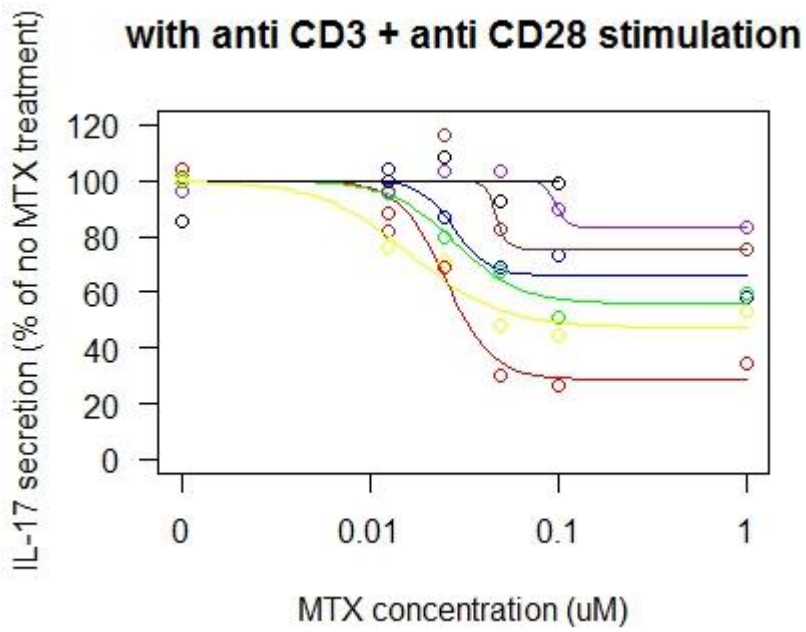

Figure 12. Effect of methotrexate on stimulated leukocyte cultures from healthy blood donors. Leukocyte cultures from 6 different healthy blood donors were stimulated with anti CD3 and CD28, and exposed to a range of MTX concentrations. Supernatants were harvested after 72 hours, and then IL-17 secretion was assessed by ELISA.

Correlation with clinical response to MTX

Because MTX's cellular target of interest in RA is activated T cells, we focused on anti-CD3/anti-CD28 (T cell specific stimulant) stimulated leukocyte cultures when using samples from RA patients.

In 15/18 blood samples, MTX decreased secretion of IL-17, and the greatest decrease was an 87% reduction. In 2/18 blood samples, MTX increased secretion of IL-17, and the greatest increase was by 30%. For the samples in which there was a MTX-induced decrease in IL-17 secretion, there was variation in the concentration of MTX required to achieve half-maximal decrease (IC50), ranging from 0.03 – 0.3 uM.

In 16/18 blood samples, MTX decreased secretion of IFN-γ. The greatest decrease was a 92% reduction. The IC50 ranged from 0.01 – 0.3 uM.

There is a weak correlation between the IC50s from IFN-γ and IL-17 ($p < 0.05$, $R2 = 0.50$), and the amount of MTX-induced decrease in IFN-γ and IL-17 ($p < 0.05$, $R2 = 0.59$)

Based on samples from 14 patients with at least partial follow-up, there is greater variation in the maximal effect of MTX on in vitro IL-17 secretion in those with side effects vs those without ($p < 0.05$) (Figure 13). Absence of side effects was associated with MTX-induced decrease in IL-17 from 20 to 70%. In contrast, out of 6 patients with side effects, 2 did not experience a MTX-induced decrease in IL-17, and 2 others experienced a MTX-induced decrease in IL-17 of greater than 70%.

Only 11 RA patients completed the MTX trial of 3 months. No statistically significant correlations were found between clinical efficacy and in vitro results. (Figure 14)

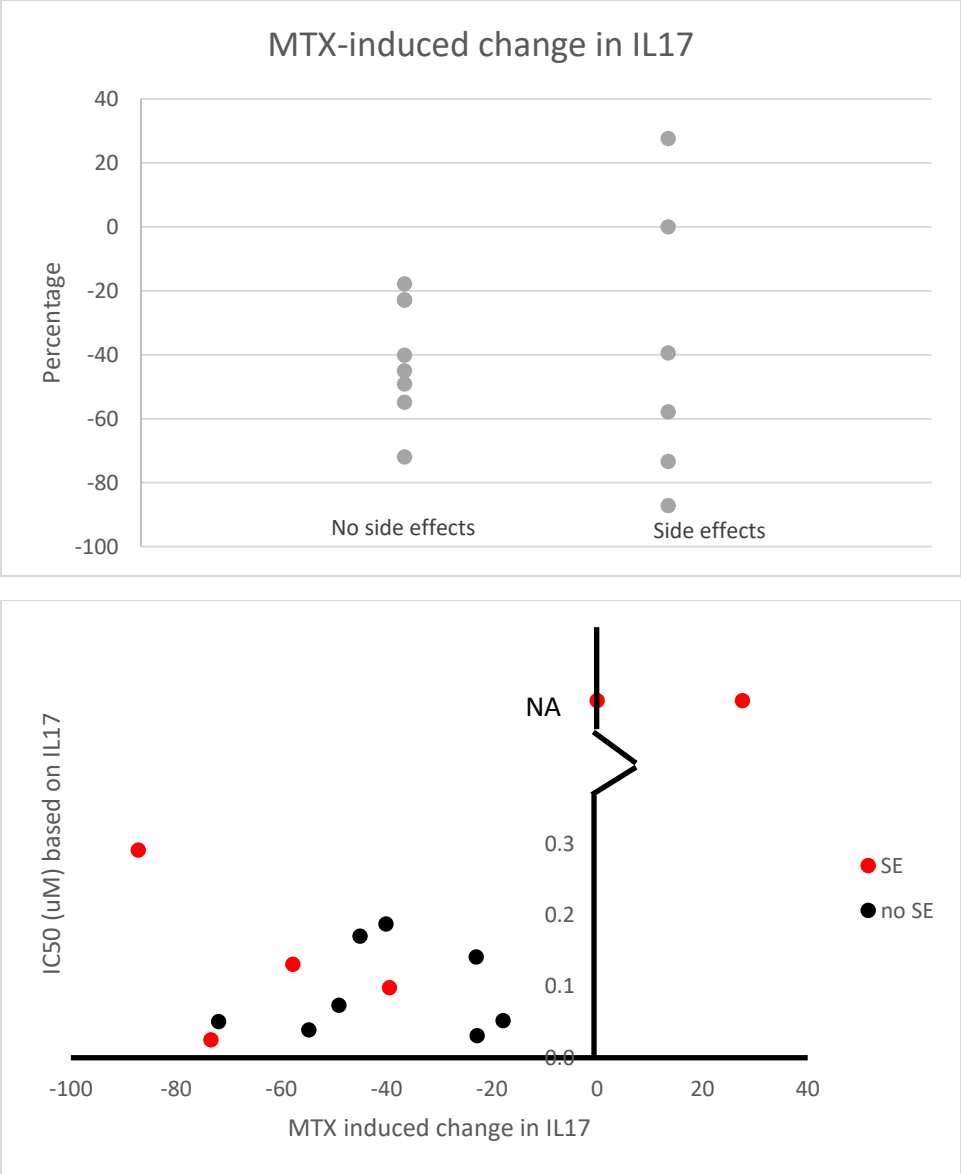

Figure 13. Results from stimulated leukocyte cultures from RA patients, and relationship to side effects. Leukocyte cultures from RA patients were stimulated with anti CD3 and anti CD28, and exposed to a range of MTX concentrations. ELISA was used to assess the maximum MTX-induced change in IL-17 secretion (top and bottom graph) as well as IC50 (bottom graph). Side effect occurrence was gauged from chart review. There was greater variation in the maximum effect of MTX on the group with side effects than the group without side effects (top). The IC50 could not be calculated (NA) in samples from 2 patients in which MTX did not have an inhibitory effect on IL17 secretion. SE = side effects.

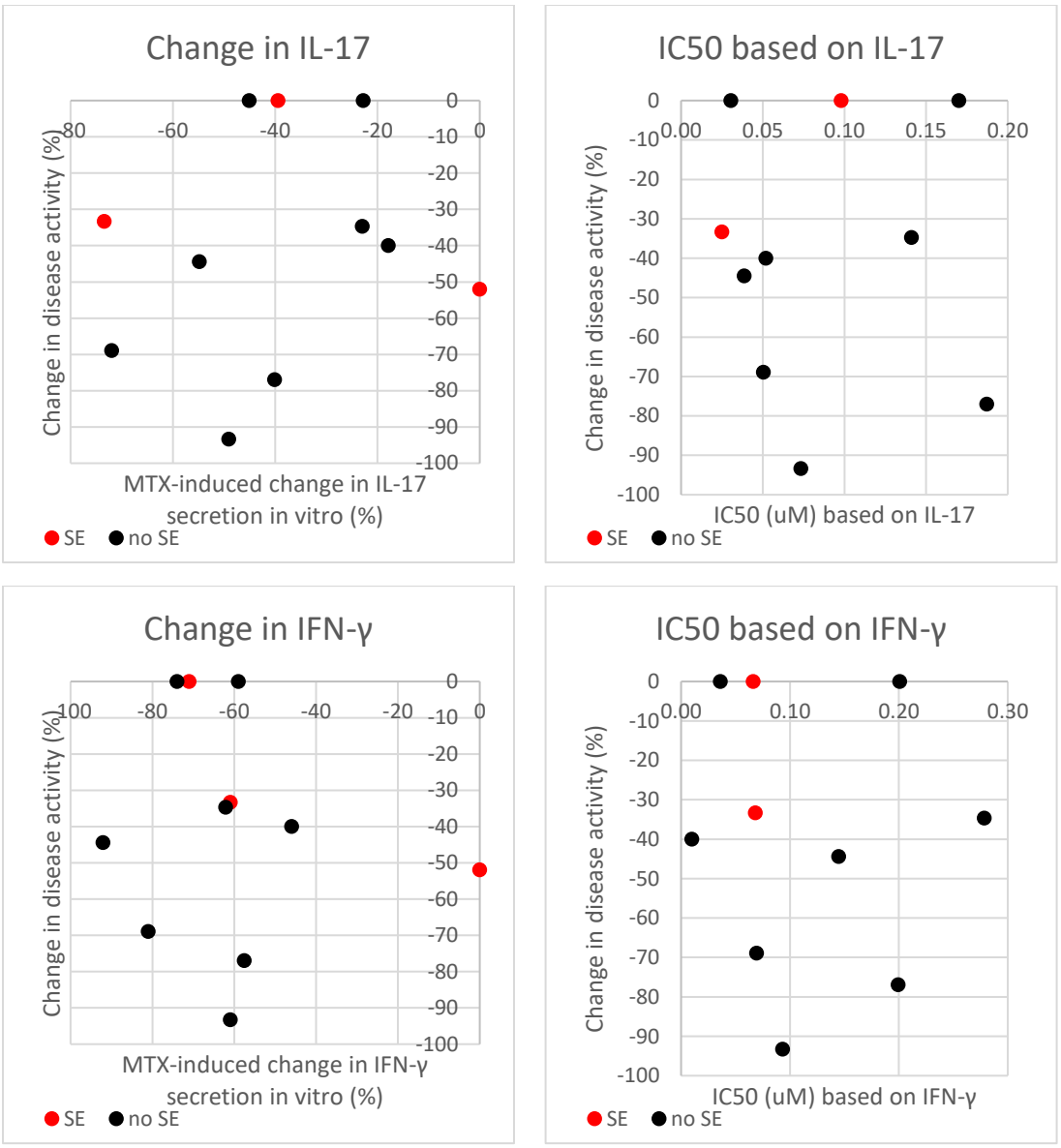

Figure 14. Results from stimulated leukocyte cultures from RA patients, and relationship to efficacy. Leukocyte cultures from RA patients were stimulated with anti CD3 and anti CD28, and exposed to a range of MTX concentrations. ELISA was used to assess the maximum MTX-induced change in cytokine secretion and the IC50 based on cytokines. Clinical efficacy is based on CDAI (change in disease activity) score. No significant correlation found between clinical efficacy and in vitro results.

Mechanism

The proposed mechanisms of MTX involve inhibition of folate-dependent pathways and release of adenosine into the extracellular milieu. We assessed whether the MTX induced effects

on IL-17 secretion are dependent upon folate depletion or extracellular adenosine. Folic acid had no impact on MTX induced changes in IL-17 secretion (data not shown). However, folinic acid, a biologically active derivative of folic acid, does reverse the MTX induced decrease in IL-17 secretion in stimulated cultures (Fig. 15). This result suggests that MTX's interference with folate metabolism is responsible for its effects on IL-17 secretion. In both resting and stimulated cultures, adenosine receptor antagonists did not reverse MTX induced decreases in IL-17 secretion (data not shown), suggesting that MTX's impact on IL-17 secretion is independent of MTX-induced adenosine signaling.

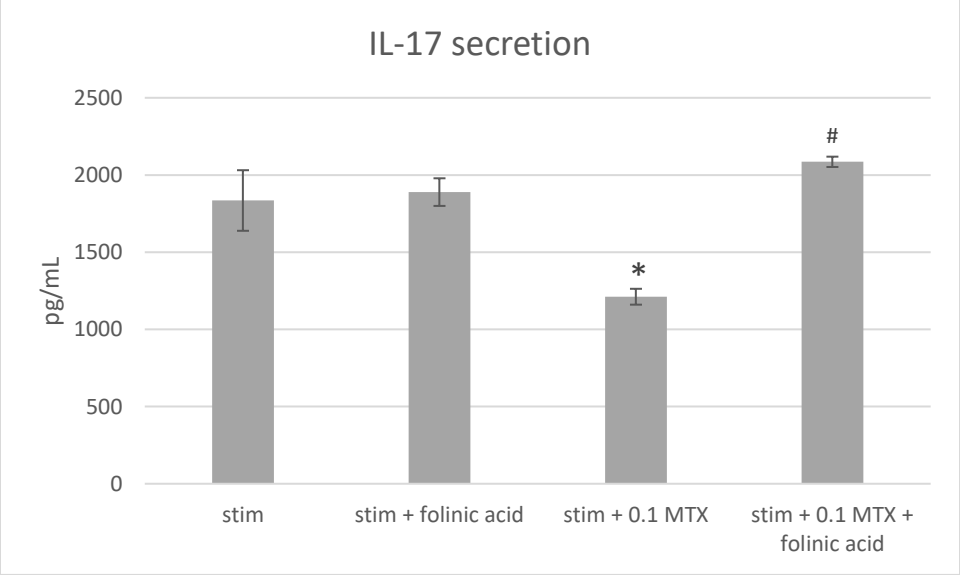

Figure 15. Possible mechanism of methotrexate effects on IL-17. Leukocyte cultures were stimulated with CD3 and CD28 (stim), and exposed to folinic acid (40 ug/mL) and MTX (0.1 uM) alone and in combination. Supernatants were harvested after 72 hours, and then IL-17 secretion was assessed by ELISA. * $p < 0.05$ compared with stimulation alone. # $p < 0.05$ compared with stimulation + MTX (0.1 uM). This is representative data using leukocytes from 1 individual.

4. Discussion

We demonstrate here that there is significant person-to-person variability in MTX-induced suppression of IL-17 secretion from leukocytes. In this study, we examined both resting

and activated leukocytes, as MTX can have differing effects on activated and resting leukocytes. For example, activated T cells have been reported to be more sensitive than resting T cells to MTX-induced apoptosis [16].

A previous in vitro study [17] demonstrated that MTX decreases secretion of IL-17 from anti-CD3/anti-CD28 stimulated PBMCs, which we confirm here. The previous study found that the MTX-induced effects on IL-17 secretion were not dose dependent, but we found that it is dose dependent. This discrepancy is due to the concentrations of MTX used. They used concentrations from 0.22 to 55 uM (0.1 to 25 ug/mL) [17], whereas we used 0.0125 to 1 uM. Since our lowest MTX concentration was lower, we could detect dose dependence. Additionally, the IC50s calculated here range from 0.03 to 0.3 uM MTX, concentrations which are easily achievable in the plasma after ingestion of a typical dose of MTX for treatment of RA [18].

Further experiments suggested that the mechanism of MTX-induced suppression of IL-17 secretion is dependent on folate pathway antagonism, but not adenosine signaling. Blocking adenosine receptors was unable to reverse the effects of MTX in both resting and stimulated cells. Folinic acid was able to reverse MTX-induced suppression of IL-17 in stimulated cells, suggesting dependence on folate pathway disruption. However, folic acid had no effect, perhaps because folic acid is upstream of the enzyme dihydrofolate reductase (DHFR), which is directly inhibited by MTX [8, 19-21]. Folinic acid, in contrast, is downstream of DHFR [21].

We found that in vitro MTX decreased IL-17 secretion from leukocytes from most individuals. This decrease in IL-17 secretion could be a result of a decreased number of Th17 cells, and could potentially decrease further Th17 differentiation [22]. Guggino et al. [23] reported that in vitro MTX treatment of unstimulated PBMCs decreases the proportion of Th17 cells in early RA patients, but does not significantly change the Th17 proportion in PBMCs from healthy donors.

We found that in vitro MTX increased IL-17 secretion from resting leukocytes from some healthy donors and from stimulated leukocytes from some RA patients, which has not been previously reported. Currently, the mechanism behind a MTX-induced increase in IL-17 secretion in resting leukocytes is unclear. We speculate that this could be due to the activation of the adenosine 2B (A2B) receptor. Unlike signaling through the A2A receptor which has anti-inflammatory effects, signaling through the A2B receptor has pro-inflammatory effects [24]. However, neither A2A receptor antagonists nor A2B receptor antagonists were able to reverse the effects of MTX on resting leukocytes. At this time, the mechanism remains unknown. We also speculate that the unknown mechanism of MTX-induced IL-17 secretion could be responsible for MTX resistance in some RA patients.

Similar to our work, Haroon et al. [25] correlated clinical response to MTX with the effect of MTX on anti-CD3/anti-CD28 stimulated whole blood cultures, but chose to examine secretion of different cytokines: TNF-α and IFN-γ. The IC50s for these cytokines were negatively correlated with clinical response to MTX, with stronger association for TNF-α. However, 50% of RA patients with low IC50 for TNF-α had less than 50% reduction in symptoms [25]. Since TNF-α and IFN-γ are associated with Th1 cells, and there has been a shift in the understanding of RA pathogenesis to include a major role for Th17 cells [10], we sought to improve on Haroon et al.'s work by examining secretion of a cytokine specifically from Th17 cells, IL-17. Unfortunately, we were unable to find a significant correlation between clinical efficacy and the in vitro effects of MTX on IL-17 secretion. However, there may be a relationship between side effects and the in vitro effects we observed, with side effects more likely if MTX induced a large decrease in IL-17 or did not decrease IL-17. Perhaps a large decrease in IL-17 is representative of general sensitivity of the individual's cells to MTX, which clinically could manifest as side effects. Perhaps a failure of MTX

to induce suppression of WBC secretion of IL-17 could reflect an abnormality in MTX transport, such that less MTX is inside target cells and more is outside where it could affect non-target cells.

5. Conclusion

There is large person-to-person variation in the in vitro effects of MTX on IL-17 and IFN-γ in blood samples from RA patients. This information could possibly reflect the effects of MTX in vivo in the RA patients, and be harnessed to predict side effects. Increased sample size is needed to assess whether this variation is of clinical significance.

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CHAPTER FIVE

5. CONCLUSIONS

Summary of Previous Chapters

This dissertation explored the modulation of the adaptive immune response by two very different stimuli, an intracellular human protein and a commonly used folic-acid based medication. Both stimuli have anti-inflammatory effects. This dissertation has broad implications for basic immunology and human disease.

Chapter 1 broadly summarizes the role of dendritic cells in human diseases including autoimmunity, cancer, and tissue damage. This review indicates that dendritic cells are crucial for initiation of the adaptive immune response by activating naïve T cells, and thus dendritic cells may be involved in the many disease processes involving modulation of the adaptive immune response.

Chapter 2 shows that dendritic cell activation can be modulated by extracellular vimentin, an intracellular protein that can be found outside the cell in cancer, traumatic tissue injury, and inflammation. Extracellular vimentin seems to have a mild anti-inflammatory effect on dendritic cell activation, resulting in less pro-inflammatory T cell activation. Interestingly, others have found extracellular vimentin to be anti-inflammatory in neutrophils[1], but pro-inflammatory in monocytes and macrophages [2, 3]. Perhaps such a system, where the same stimulus has different effects, could allow the body to fight a potential infection with monocytes and macrophages while also preventing dendritic cells from initiating autoimmunity.

One of the most common autoimmune diseases is rheumatoid arthritis, and one of the autoantigens in this disease is citrullinated vimentin [4].

Chapters 3 and 4 are about a clinical problem in rheumatoid arthritis treatment: predicting clinical response to methotrexate, a commonly used immunomodulatory drug. We used patient samples to attempt to correlate clinical response to methotrexate with in vitro T cell response to methotrexate. In particular, we were examining the Th17 subset of T cells, and found that there was a large amount of person-to-person variability in the in vitro effect of methotrexate. However, we were unsuccessful in finding any statistically significant correlations between efficacy and the in vitro results, due in part to small sample size.

Future Directions

The future directions for these projects are potentially diverse. In regards to the vimentin project, future directions include further exploring the mechanism of vimentin-induced dendritic cell modulation. From a drug development perspective, if vimentin-induced effects were more dramatic, then the involved receptor would be a potential drug target. For the methotrexate in rheumatoid arthritis project, there is the possibility of increasing the sample size, or of changing the methodology (e.g. use unstimulated samples instead of stimulated ones). However, given the lack of promising data so far, it may be more worthwhile to use this approach in other rheumatologic diseases. For example, this type of study could also be performed with samples from psoriatic arthritis patients, as methotrexate is also used to treat this disease and there is strong evidence of Th17 involvement in the disease pathogenesis [5, 6].

Conclusion

Overall, this dissertation has enhanced knowledge of adaptive immune response activation, by adding information about extracellular vimentin-induced effects on dendritic cells and then the downstream effect on T cells. Based on our work presented here and the work of others[1-3], we propose that extracellular vimentin can have dual pro-inflammatory and anti-

inflammatory effects depending on context. This has implications for basic immunology and may have clinical significance. Additionally, as shown in my clinical project, there is a large amount of person-to-person variation present in in vitro response to methotrexate, suggesting underlying variation in immune cell function. This underlying variation could potentially be used for personalized medicine.

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