RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS SYNOVIAL FIBROBLASTS EXPRESS FcαR (CD89) *in vitro* AND in *vivo*: IMPLICATIONS FOR RHEUMATOID ARTHRITIS

BY

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Master of Science

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" Rheumatoid Arthritis and Osteoarthritis Synovial Fibroblasts Express FcαR (CD89) *in vitro* and in *vivo*: Implications for Rheumatoid Arthritis"

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ABSTRACT

Rheumatoid arthritis (RA) is a common chronic inflammatory and autoimmune disorder of unknown etiology that to date has no known cause or cure. RA is characterized by synovial inflammation, proliferation and progressive joint destruction, and patients with high titers of IgA-type rheumatoid factor typically follow a more aggressive and destructive disease course. In the present study, the role of IgA in RA was evaluated by investigating whether RA fibroblast-like synoviocytes (FLS) expressed receptors for IgA. Immunofluorescent staining of RA and osteoarthritis (OA) FLS cell cultures showed that the IgA receptor FcαR (CD89) previously described in granulocytes was expressed on both types of FLS, but not in fibroblasts from uninflamed non-synovial tissue. All RA and OA FLS were tested for their ability to express mRNA for IgA receptors and FcγRIII via RT-PCR. DNA sequencing of the RT-PCR products confirmed FcαR and FcγRIII identity. While every RA and OA patient tested positive for both FcαR and FcγRIII, none expressed mRNA for the polymeric immunoglobulin receptor described in epithelial cells or the Fcα/μR in white blood cells. Immunohistochemistry on fresh frozen RA and OA tissue showed specific staining for FcαR predominantly in the intimal lining layer, confirming expression *in vivo*.

In conclusion, RA and OA FLS constitutively express the IgA receptor FcαR both *in vitro* and *in vivo*. While this work confirms the presence of the receptor on FLS cells, which have the ability to contribute to the powerful pro-inflammatory process that characterizes RA, further studies are required to define the role of FcαR in arthritis.

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Abbreviations

α	alpha
γ	gamma
κ	kappa
µg	microgram
µl	microliter
µm	micrometer
bp	base pair
C. trachomatis	*Chlamydia trachomatis*
CO2	carbon dioxide
COX-2	cyclooxygenase 2
DAB	diaminobenzidine hydrochloride
DAF	Decay Accelerating Factor (CD55)
ddH_2O	distilled deionized water
dH_2O	distilled water
DMARDS	disease modifying anti-rheumatic drugs
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ERA	Early Rheumatoid arthritis
EtBr	ethidium bromide
FcαR	Fc alpha receptor (CD89)
FcγRIII	Fc gamma receptor III (CD16)
FcRγ	FcR γ-chain subunit
Fas-L	Fas ligand
FBS	fetal bovine serum
FcαRI	CD89
FMLP	formyl-methiony-leucyl-phenyalanine
FLS	Fibroblast-like synoviocytes
GM-CSF	granulocyte-macrophage colony stimulating factor
H_2O_2	hydrogen peroxide
HDF	human dermal fibroblasts
HLF	human lung fibroblasts
IgA	immunoglobulin A
IgAN	IgA nephropathy
IgG	immunoglobulin G

IgM	immunoglobulin M
IC	Immune complexes
ICAM-1	intracellular adhesion molecule 1
IL-1	interleukin 1
IL-6	interleukin 6
IL-8	interleukin 8
JPEG	Joint Photographic Experts Group
LPS	lipopolysaccharide
mIgA	monomeric IgA
mg	milligrams
ml	mililiter
mm	millimeter
mM	milimolar
NFκB	Nuclear Factor kB
nm	nanometer
OA	Osteoarthritis
P. mirabilis	*Proteus mirabilis*
PBS	phosphate buffered saline
PBS – T	phosphate buffered saline with Tween™
pIgA	polymeric IgA
pIgR	polymeric immunoglobulin receptor
PFA	paraformaldehyde
PFS	phosphate buffered fish skin gelatin solution
RA	Rheumatoid arthritis
RF	Rheumatoid Factor
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse-transcriptase polymerase chain reaction
SC	secretory component
sIgA	secretory IgA
SS	Sjögren's Syndrome
TNF-α	tumor necrosis factor alpha
UDPGD	uridine-diphosphoglucose dehydrogenase
VCAM-1	vascular cell adheshion molecule 1
VEGF	vascular endothelial growth factor
v/v	volume/volume
Absorbance $_{260}$	absorbance at 260 nm
Absorbance$_{280}$	absorbance at 280 nm

1. INTRODUCTION

1.1 FOCUS OF STUDY

The focus of this work is to investigate the possibility that human fibroblast-like synoviocytes (FLS) express IgA receptors, determine how this compares to fibroblasts derived from other tissues and whether FLS expression of IgA receptors occurs in vivo. Based on the clinical evidence that patients with elevated levels of IgA-RF are subject to a more rapid disease course, we investigated the possibility of a connection between IgA and the intimal fibroblast-like synoviocytes. The discovery of FcαRI receptor on FLS might offer not only insight into disease mechanism, but also the possibility of an unexplored therapeutic target.

This work is entirely novel as FcαRI has never been described previously on FLS. Cells in the synovium are active participants in the inflammatory process of rheumatoid arthritis (RA) express FcαRI. FcαRI expression on FLS may play a part in the inflammation that accompanies RA. Furthermore, the FLS FcαRI is not present on normal dermal or lung fibroblasts, suggesting FcαRI is intrinsic to synovial cell biology in arthritis.

First, rheumatoid arthritis will be described, including the role of TNF-α in RA inflammation. RA FLS will be presented from a physiological perspective and their role in the cytokine network of RA as aggressive contributors to the perpetuation of inflammation will be discussed.

Focus will shift to an overview of IgA that addresses various molecular forms of IgA and IgA function. The structure and genetics of the IgA receptor FcαRI is detailed and biological functions that result from IgA/ FcαRI association illustrated.

The involvement of IgA type rheumatoid factor and associated immune complexes containing IgA in the overall prognosis of RA will be outlined along with the possible mechanisms which cause RF-immune complex formation. The final section of the introduction will bring together various diseases characterized by IgA-containing immune complex formation, creating the foundation for the discussion of both presence and potential function of FcαRI on FLS.

1.2 RHEUMATOID ARTHRITIS

1.2.1 INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic inflammatory destructive arthropathy that affects one out of every hundred Canadian adults (www.arthritis.ca) and is diagnosed clinically by typical symptoms and characteristic patterns of articular and extra-articular disease. No one particular diagnostic study or autoimmune biological marker exists for RA as some patients with RA do not produce rheumatoid factor, however typical abnormalities include high titers of antibodies against IgG, polyclonal hypergammaglobulinemia and anemia of chronic disease. Recently the fluctuation in serum levels of anti-cyclic citrullinated peptide antibody (anti-CCP), has been found to be useful for not only the diagnosis but also the management of RA [1]. To date, it has not been possible to prevent the disease or to completely arrest the disease process through medical therapy. While several recent advances in anti-TNF-α monoclonal antibodies, which are often prescribed in combination with various disease modifying anti-rheumatic drugs (DMARD), have substantially decreased RA morbidity, long term disability remains an issue [2, 3]. Once diagnosed, long term prognosis is poor; 80% of patients are disabled after 20 years and overall life expectancy is reduced by 3-18 years on average [4-8].

RA associated morbidity has substantial personal, social and economic costs. It is impossible at the time of diagnosis to predict the pattern of disease course in an individual patient and is therefore a topic of hot research. However, a more destructive disease course has been associated with patients that have many involved joints, extra-articular manifestations, in those patients suffering psychosocial problems and in those with high titers of rheumatoid factor or serum immune complexes [9, 10]. RA is three times more prevalent in women than men and occurrence increases with age in both sexes, resulting in increased RA incidence in the elderly[11]. For unknown reasons, RA typically follows a more severe disease course in men and the elderly [5, 12].

RA is an inflammatory autoimmune process affecting the small diarthrodial joints of the hands and feet and a myriad of other tissues. Currently available DMARDS, including inhibitors of TNF-α, particularly when used in combinations, have dramatically changed the outcome of RA in most patients, with marked improvements in long term prognosis. Normally the tightly regulated inflammatory process is a balance of mediators that act to both initiate and maintain inflammation and mediators that shut the process down. In a state of chronic inflammation, levels of the mediators that initiate and maintain

inflammation increase, resulting in cellular damage. Current therapy is directed toward diminishing the inflammatory response and treating the sequelae of uncontrolled inflammation manifested by the destruction of cartilage and bone via early diagnosis and treatment [12].

The discovery of what initiates and perpetuates RA lies in the continued progression of knowledge of the molecular basis of immune system mediator function. Further elucidation will provide a better understanding of the mechanisms of autoimmune pathogenesis in RA.

1.2.2 PATHOLOGY

1.2.2.1 Normal Synovial Tissue

The synovium lines the non-cartilaginous surfaces of the diarthrodial joints and provides nutrients to avascular structures such as cartilage. Normal synovial tissue surrounds articular spaces as well as tendon sheaths. In the joint space, the intimal lining layer is comprised of synoviocytes one to two cells deep without an underlying basement membrane, and the synovial sublining which merges with the joint capsule[13]. The intimal lining layer consists mostly of intimal macrophages and fibroblast-like synoviocytes (FLS) [14]. The intimal macrophages are commonly referred to as Type A synoviocytes which are

derived from the bone marrow and make up 25% of the synovial cell lining layer [15] [16]. Type B synoviocytes express high levels of uridine-diphosphoglucose dehydrogenase (UDPGD)[17]. The synovial sublining is relatively acellular, with scattered blood vessels, fat cells and fibroblasts.

1.2.2 .2 Rheumatoid Synovial Tissue.

In RA, the synovium becomes hypertrophic and edematous due to microvascular injury and increased vascular permeability, accompanied by an influx of inflammatory cells in the perivascular space [14]. With continued inflammation, Type B synoviocyte proliferation rates increase, creating villous projections of synovial tissue which protrude into the joint cavity, where it overgrows and invades the underlying cartilage and bone [18]. Proliferating synovial tissue at the cartilage-synovium junction is known as pannus.

1.2.2.3 Synovial Tissue in Early Rheumatoid Arthritis (ERA)

Synovial inflammation varies largely between individual patients in all phases of RA therefore study of the pathological changes in synovial tissue of patients with long standing RA compared to those in early RA is crucial for the improved understanding of RA[19] [20]. It is known that asymptomatic synovial inflammation precedes clinically-overt arthritis [21]. The average expression of

adhesion molecules and cytokines is similar when synovial tissue from ERA patients is compared with that from patients with long standing RA [19, 22]. Furthermore, the major cell types that infiltrate the synovial tissue in RA are independent of disease duration [23]. Although there appears to be no clear differences between the cell populations seen in ERA tissues and those of patients with advanced disease, this may be a result of ERA actually being a well established disease. ERA in actuality represents a state of persistent inflammation. Persistent inflammation is a key feature of RA at all stages, although the nature of this inflammation varies at different stages of the disease: chronic inflammation tends to be mediated by mononuclear cells while acute inflammation tends to be exudative. Both can co-exist during disease "flares". While it is can be challenging to distinguish between ERA patients who will progress into RA or remit, individuals with synovitis of recent onset who have symmetrical polyarthritis that is associated with the presence of one or more RA autoantibodies in the blood, will almost invariably have persistent, progressive RA.

1.2.3 Inflammation

A gigantic influx of leukocytes precedes vascular proliferation. Most of the infiltrating cells are helper T lymphocytes with a mature memory phenotype, but

plasma cells, macrophages, B cells, mast cells, natural killer cells and dendritic cells are also found in the synovial sublining [16]. Cytokines TNFα, IL-1 and IL-6 are released and drive inflammation in RA. A high proportion of the lymphocytes and monocytes appear to be retained in the synovial stroma. Neutrophils on the other hand accumulate in the synovial fluid. B lymphocytes mature into plasma cells which locally produce rheumatoid factor and other antibodies that further aggravate the inflammation. Immune complexes activate the complement system, releasing chemokines and increasing vascular permeability. The recruitment of inflammatory cells accompanied by local retention and exuberant cell proliferation increasingly adds to the cellularity of the rheumatoid synovium. Very few apoptotic cells exist in RA synovium [14], and impaired programmed cell death was found to enhance hyperplasia in RA synovium [24, 25]. Therefore the increased cellularity is due to a combination of factors that reduce apoptosis and enhance the influx of inflammatory cells into the synovium [26-28]. Patients develop swelling, pain, and joint stiffness with the onset of vascular injury and angiogenesis in the synovial membrane.

1.2.3.1 The Role of TNF-α in RA

TNF-α is a cytokine that elicits diverse pro-inflammatory effects by stimulating a wide variety of cells. Produced primarily by macrophages and monocytes, B

cells, T cells and fibroblasts also produce TNF-α. TNFα is best known for its ability to promote inflammation via autocrine stimulation and paracrine induction of other inflammatory cytokines, such as IL-1, IL-6, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF) [29]. Fibroblasts stimulated with TNF-α express adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1), which interact with their respective ligands on leukocytes, thereby increasing the recruitment of leukocytes into inflammatory sites such as the RA joint [30].

Understanding the intracellular targets that regulate TNF-α in RA can potentially lead to new therapeutic interventions. Nuclear factor-κB (NF-κB) is expressed in all cells and plays a broad role in gene transcription. NF-κB plays a key role in the expression of as many as 70 genes central to the inflammatory process, including TNF-α, and has been detected in vivo in rheumatoid synovium [31, 32]. Activated NF-κB in the RA synovium regulates pro-inflammatory genes including, TNF-α IL-6, IL-8, inducible nitric oxidase synthase and cyclooxygenase-2 (COX-2) [33]. Targeting TNF-α is an effective therapeutic strategy that inhibits NFκB, and has proven successful in many animal models of arthritis [34] [35]. Directly inhibiting TNF-α has also proven effective in humans, as TNF-α production may serve as an autologous stimulus

for other cytokines in the RA synovium. Recent studies have found that RA patients who respond well to TNF-α blockade have inactive disease and have lower levels of serum TNF-α. RA patients who are non-responsive to TNF-α inhibitors have active disease and higher serum levels of TNF-α when compared to patients with inactive RA [32]. While many patients experience a dramatic response to TNF-α inhibitors, the complexities of the RA cytokine network in conjunction with other pathophysiological mediators continue to perpetuate the disease.

1.2.4 Joint Destruction

Immune complexes promote phagocytosis, leading to greater lysosomal enzyme release and the digestion of collagen, cartilage matrix and elastic tissues. The release of oxygen free radicals injures cells, which in turn release phospholipids that fuel the arachidonic acid cascade and exacerbate the local inflammatory response. In response to the inflammatory injury in RA, mesenchymal elements of the synovium proliferate, forming an invasive pannus, and eroding through cartilage and subchondral bone. Pannus is an expanding inflammatory mass of synovial lining cells that extends into the articular cartilage and bone and is the destructive element in rheumatoid arthritis [6]. Cells in the RA pannus tissue assume many characteristics of transformed malignant cells, exhibiting the

properties of invasion of cartilage and bone, neovascularizatoin and oncogene expression [6]. At the cartilage-pannus junction, "pannocytes" have been identified which exhibit phenotypic and functional features of both FLS and chondrocytes. Cytokines from activated synovial macrophages are believed to directly stimulate cells within the pannus, causing them to pseudotransform and invade both cartilage and bone [13]. At this time, it is unclear whether pannocytes are of a separate cellular lineage, but it is apparent these cells are derived from the monocyte/macrophage lineage[36].

1.3 FIBROBLAST-LIKE SYNOVIOCYTES (FLS)

The synovial intimal lining is the interface between the synovium and intra-articular space. The lining is normally one to two layers deep and supported by a relatively disorganized accumulation of matrix proteins rather than a proper basement membrane. It lacks blood vessels, receiving oxygen via a sublining vascular network. Two types of synoviocyte are present in the intimal lining layer: fibroblast-like and macrophage-like. These cells do not form tight junctions with each other, but rather associate via homotypic cell-cell interactions between FLS with each other or heterotypic interaction of the FLS with macrophage-like synoviocytes. These two types of lining cells were originally designated "type B" and "type A" respectively according to their appearance in

electron microscopy however, it is more descriptive to refer to them by their lineage derivation [37].

1.3.1 Fibroblast-Like Intimal Synoviocytes

Unique in structure and function, the joint is primarily formed from mesenchymal cells. The cavity of the joint is lined primarily by fibroblast-like intimal synoviocytes . This cell population comprises approximately two-thirds of the lining cells in an uninflamed joint [38]. Differentiation of fibroblast-like intimal synoviocytes follows a defined pattern of gene expression, resulting in a series of functions critical to the biological function of the normal joint. The primary function of the joint is to permit weight-bearing motion on avascular cartilage thus the specialization of the lining cells is aimed at maintaining the integrity of the joint. Key functions of fibroblast-like intimal synoviocytes include maintaining cartilage viability and function, removal of cartilage debris resulting form impact and weight bearing stresses, and coordinating the surveillance of the synovial fluid space. These functions require polarization and organization of the fibroblast-like intimal synoviocytes. Polarization is an unusual characteristic for typical connective tissue fibroblasts and is more commonly encountered in epithelial cells.

Distinct from other fibroblasts derived from the same mesenchymal progenitor, such as osteocytes, fibroblast-like intimal synoviocytes are specialized in a variety of functions unique from regular fibroblasts. Fibroblast-like intimal synoviocytes appear early in joint development and their subsequent functions suggest that they are responsible for attracting circulating monocytes lineage cells to become resident phagocytic cells in the intimal lining. Interactions between monocytes and synoviocytes are responsible for patterning the histogenesis of the normal joint [39] and it is possible that fibroblast-like intimal synoviocytes enhance the recruitment of T and B cells into the joint cavity, thereby enhancing the performance of parallel immune surveillance functions of the joint cavity's extracellular space [40].

Fibroblast-like intimal synoviocytes express a variety of surface adhesion receptors that anchor them to the extracellular matrix and regulate the flux of cells that pass into the synovial space. The surface expression of vascular cell adhesion molecule 1 (VCAM-1) and the intracellular localization of the enzyme UDPGD are also unique to fibroblast-like intimal synoviocytes [41-43]. VCAM-1 is expressed in the RA intimal lining in amounts that are higher than those found in normal tissue [43]. Frozen sections of both normal and RA synovial tissue show prominent VCAM-1 expression by both immunohistochemisty and in situ

hybridization [42, 44]. In FLS cell culture, VCAM-1 is constitutively expressed however, subintimal fibroblasts and fibroblasts from other sources do not express VCAM-1 [39]. UDPDG is involved in hyaluronan synthesis and increased UDPDG activity is increased in a narrow band of cells at the presumptive joint line prior to cavitation during development. Edwards proposes that joint cavitation is facilitated by a rise in hyaluronan concentrations in an area of tissue where cohesion is dependent on macrophages expressing CD44 and extracellular hyaluronan [38]. Based on this proposal, it is possible that the early role of macrophages in the joint histogenesis is the removal of cells that may undergo apoptosis during the formation of the joint cavity, and a dysfunction of apoptosis could contribute to synovial hyperplasia [45]. How UDPDG production by fibroblast-like intimal synoviocytes affects synovial hyperplasia is unknown.

1.3.2 Macrophage-Like Intimal Synoviocytes

The intima of the joint contains a second intercalated cell type derived from the CD14+ branch of the monocytes lineage designated as macrophage-like intimal synoviocytes. These cells exhibit a specialized phenotype that distinguishes them from the typical monocytes and feature characteristics found in certain dendritic cells [46, 47]. The monocyte progenitors of the macrophage-like intimal

synoviocytes enter the intima after leaving blood vessels and differentiate into their mature form in response to interactions with the fibroblast-like intimal synoviocytes [47]. Understanding the function and interaction between these two cell types as well as the molecules involved in initiating joint histogenesis is of special importance both in the normal and inflamed joint.

1.3.3 Limitations of in vitro Models in the Study of Snyoviocytes

Two major problems currently present themselves to researchers studying fibroblast-like intimal synoviocytes. The first is the uncertainty as to whether a given fibroblast-like cell propagated in tissue culture originated from the intima or subintima and therefore, cells cultured from the joint are referred to as fibroblast-like synoviocytes (FLS). In spite of the increased number of fibroblast-like intimal synoviocytes in the hyperplastic synovial intimal lining in RA, the problem of being able to reliably distinguish the origin of cells from the intima or subintima remains. The second problem is that the inflammatory state has induced alterations in phenotype by cytokines and growth factors from lymphocytes or additional activation pathways involving monocytes and/or FLS. It has been proposed that due to prolonged exposure to these paracrine effects FLS undergo "phenotypic immunological imprinting" which may explain the behavior of these cells in culture [48]. Cultured FLS are a starting point,

especially since macrophage-like intimal fibroblasts are short lived in culture. A fundamental, although unproven, premise is that findings made on cultured FLS may be directly extrapolated to provide insight into the details of the intimal synoviocyte as it behaves in the inflamed joint. This is why all data collected *in vitro* must be followed up by *in vivo* models.

1.3.4 The Role of FLS in Synovial Hyperplasia

One of the most striking features of RA is the marked hyperplasia of the synovial lining layer and the invasion and destruction of cartilage and other joint structures [49]. The synovial membrane becomes edematous, and infiltrated with an profusion of inflammatory cells, primarily CD4+T cells, which are the main orchestrator of cell-mediated immune responses. Changes in the intima include a massive increase in the number of fibroblast-like intimal synovicocytes and an altered cell-cell relationship with the macrophage-like synoviocytes resulting in the formation of villous projections. It is unknown what mechanisms precisely direct the immune response to the joint and whether the autoimmune response of RA occurs entirely in the setting of the normal intima, or if minor degrees of non-specific hyperplasia direct the immune response to the joint through the repertoire of immunologically relevant molecules expressed by FLS. Hyperplasia could be initiated by events such as infection or traumatic

insult and be driven by a local immune response to a common pathogen. The constitutive production of the resulting chemokines might provide a non-antigen-specific mechanism for the localization of the immune response to the joint. The local inflammatory response would include the production of chemoattractant molecules, activating the fibroblast-like intimal synoviocytes causing an increase in transcription and thus proliferation. Hyperplasia may be an intrinsic response of intimal synoviocytes to injury and healing. However, in RA the synovial membrane undergoes striking changes and is transformed from a nutritive tissue into the primary instrument of joint destruction, demolishing cartilage with enhanced levels of degradative enzymes. Why activated RA fibroblast-like intimal synoviocytes have increased proliferation rates [50] is unknown but cellular accumulation through decreased apoptosis in fibroblast-like intimal synoviocytes has been demonstrated [49]. The resulting cellular accumulation may in part explain hyperplasia of the synovium in RA. Furthermore, fibroblast-like intimal synoviocytes overexpress several genes which are involved in increased cytokine expression and the regulation of cell proliferation in the rheumatoid synovium [51]. Recent studies suggest NF-κB plays a key role in the regulation of synovial fibroblast apoptosis and gene expression due to its high levels of expression both in the rheumatoid synovium

and FLS [33]. NFκB inhibition predisposes these cells to TNF-α and Fas-L mediated apoptosis [52].

1.3.5 FLS Produce Cytokine Networks that Perpetuate Synovitis

Major insight has been gained by defining the cytokine networks that contribute to the perpetuation of rheumatoid synovitis. Consistently, results indicate an abundance of fibroblast-derived and macrophage-derived factors compared to T cell cytokines in synovial fluid and serum [53]. Antigen-activated CD4+T cells stimulate monocytes, macrophages and FLS to produce the cytokines, interleukin-6 (IL-6), interleukin-1 (IL-1) and TNFα and to secrete matrix metalloproteinases along with soluble mediators interferon-γ and interleukin-17 [54, 55]. Synovial macrophages are responsible for the majority of IL-1, TNF-α, GM-CSF and chemokine production [56]. FLS are also responsible for the production of IL-6 and angiogenic factors like vascular endothelial growth factor (VEGF). FLS are further activated by the macrophage derived IL-1 and TNF-α leading to additional GM-CSF and IL-8 gene expression [57]. Synovitis is further perpetuated through the complex autocrine and paracine intereactions between macrophage-like intimal synoviocytes and fibroblast-like intimal synoviocytes. As discussed in *1.2.2.3*, IL-1, IL-6 and TNF–α are the key cytokines that drive inflammation in RA by stimulating FLS which increases the production of

fibroblast growth factor (FGF). Consequently, FLS experience enhanced autocrine activation [58]. While the inflammatory cytokine networks in RA account in part, for aberrant rheumatoid FLS function, there is no doubt that the presence of IL-1 and TNF-α creates an environment conducive to FLS activation.

1.3.6 FLS Transformation to a Hyperproliferative and Invasive State

Based on the argument that the cytokine networks in RA require occasional exogenous stimulation from T cells, superantigens or other pathogens, FLS have been viewed as passive participants in arthritis, merely responding to the pro-inflammatory environment. However, the concept that the rheumatoid synovium behaves like a locally invasive tumor suggests FLS exhibit characteristics of transformed malignant cells that contribute to the pathogenesis of RA [14]. Mounting evidence for this premise exists from a variety of experiments in which both the phenotypic and genetic properties of RA FLS are altered from their normal states. While FLS typically thrive and proliferate under conditions which permit adherence, they can under some circumstances, grow in an anchorage-independent manner, which is a characteristic of transformed cells [59]. Cultured FLS also express several oncogenes, most importantly *c-myc,* which is responsible for initiating cell proliferation and characteristic of cells that have escaped normal growth-regulatory mechanisms

[60]. Synoviocyte transformation is also marked by a loss of contact inhibition. Normal FLS proliferate in culture until confluent, at which point DNA synthesis stops and the cells become quiescent until harvested. Transformed fibroblasts proliferate after contact has been made, causing them to cluster and pile on top of each other [61]. The most aggressive potential of these transformed cells in vivo was demonstrated when serially cultured FLS, looking very much like destructive pannus, invaded co-implanted human cartilage engrafted into severe combined immunodeficient (SCID) mice[61]. Control synoviocytes from OA patients and normal dermal fibroblasts do not invade the cartilage [14]. The accumulating evidence proves that FLS are irreversibly altered in RA and that an autonomous process allows them to remain activated even after they are removed from the articular inflammatory milieu [62].

1.4 IMMUNOGLOBULIN A (IgA)

Immunoglobulin A (IgA) abundantly coats the enormous surface area of the mucosal epithelium, which measures about 400 m^2 in adult humans. IgA makes up only 10 to 15% of total serum immunoglobulin, but is the richest immunoglobulin in extravascular secretions [63]. The majority of adult human plasma cells are committed to IgA production and more IgA is produced per day (66mg/kg/day) than all other classes combined [64]. In spite of its abundance,

relatively little is known about the mechanism of action of IgA in host immune defense and immune tolerance. The mucosal epithelium of the upper airway and gastrointestinal tract functions as a critical barrier that must protect the internal environment from potential pathogens and toxins in the external environment. The mucosal epithelium is physically vulnerable to continuous exposure to potentially infectious agents, such as bacteria and viruses, as well as to substances in the environment or diet. IgA represents a key first line of defense against invasion by inhaled and ingested pathogens at the vulnerable mucosal surface.

1.4.1 Comparison of IgA Amongst Different Species

Interestingly, IgA appears to share a common protective function across the different species where it is found, but variation in gene number, allotype and molecular forms have been distinguished. Rabbits remarkably exhibit 13 distinct heavy constant region (Cα) genes [65], of which 11 appear to be expressed [66]. In contrast, the majority of other species studied such as pigs, dogs, orangutans, rat and mouse, have just one Cα gene [67, 68]. IgA sequencing of several primate species exposed interesting evolutionary developments [69]. Humans, along with chimpanzees, gibbons and gorillas have two IgA Cα genes giving rise to two subclasses, IgA1 and IgA2 which are present in every normal individual.

The two subclasses have arisen through gene duplication and share considerable sequence similarity. The main difference between IgA1 and IgA2 is that the former possesses an elongated hinge region that is rich in proline residues lacking in IgA2. It has been postulated that the longer hinge region of IgA1 confers an evolutionary advantage in antigen recognition by allowing higher affinity bivalent interactions with distantly spaced antigens [70]. However the extended reach increases vulnerability to proteolytic attack from pathogenic bacteria such as *Haemophilus influenzae* that have evolved enzymes that specifically cleave the IgA1 hinge region [71].

1.4.2 Molecular Forms of IgA

While IgA has the ability to exist in many molecular forms in the body, it is interesting to note that various molecular forms of IgA play a wide range of roles in immune defense. In humans, the ability of IgA and its receptors to perform a wide range of specific tasks exemplifies not only the importance of IgA, but the evolutionary development of the body to utilize it in regulatory systems.

IgA bearing B cells appear during the eleventh week after birth, in contrast to IgM and IgG bearing B cells that appear earlier in development [72]. IgA is synthesized in different molecular sizes which includes monomers, dimers,

tetramers and higher polymeric forms, and it has been suggested that IgA-secreting cells in different organs can produce IgA with different size profiles [73, 74]. The dimers, composed of both the heavy and light chains of two monomers, are arranged in an end to end configuration stabilized by disulphide bridges and the incorporation of J (joining) chain [75]. The serum distribution of monomeric IgA (mIgA) and polymeric IgA (pIgA) vary significantly from species to species. For reasons that remain unclear, the predominant form in human serum is mIgA, but mainly pIgA in the serum of other animals. Monomeric IgA is produced by B lymphocytes in the bone marrow and in some peripheral lymphoid organs [76] [62]. The IgA concentration in human plasma is 2 to 3 mg/ml and is 85-95% mIgA [77]. Plasma cells at mucosal sites produce pIgA which is transported across the epithelial cell boundary and out into secretions (saliva, tears, colostrum, gastrointestinal fluids, nasal secretions, bronchial secretions and urine) by interaction with the polymeric immunoglobulin receptor (pIgR). During this process pIgR is cleaved and secretory component (SC), the major extracelluar fragment, remains covalently attached to the IgA dimer. Due to its incorporation, SC becomes a central part of secretory IgA (sIgA), the primary form of IgA released into secretions, and plays a pivotal role in protecting sIgA from degradation in a proteolytic rich

environment. sIgA is the most abundant form of IgA found in the secretions of mammals, although sIgA levels in colostrum and milk vary between species.

1.4.2.1 J Chain

Discovered as a component polypeptide, J chain associates with both IgA and IgM, although its exact role in immunoglobulin polymerization has never been clarified. Nevertheless, J chain appears to be mandatory for the initiation of polymerization of both IgA and IgM and appears to be important in maintaining the correct conformation of assembled chains. It is also thought to be important in its interaction with SC to form sIgA or sIgM, as J chain knock-out mice have increased plasma and decreased bile and fecal levels of IgA while serum levels of IgM remain normal [78]. J chain is not homologous to immunoglobulins and has been mapped to a chromosome separate from those containing immunoglobulin genes [79]. The same J chain is found in both IgM and IgA polymers with one J chain per polymer attached to the C-terminal cysteine in an alpha or mu chain. There is a high degree of serological cross-reactivity between J chains of different species which implies a high rate of conservation over extended evolutionary periods [80]. J chain is expressed early in human development (the sixth gestational week) [81], and synthesized early in B cell development prior to expression of immunoglobulin chains [62]. These findings suggest that J chain

may have an alternative immune defence function, preceding immunoglobulins in both phylogeny as well as in ontogeny[82].

1.4.2.2 Secretory Component

Providing the first line of immunological defense, SC plays a pivotal role by ensuring the integrity of sIgA, opsonizing microorganisims thereby limiting adhesion and colonization on the lining of the mucosal surface. SC bound to pIgA undergoes proteolytic cleavage from pIgR before it is released into mucosal secretions. SC is a normal component of sIgA in mucous fluids and may or may not be disulfide bonded to the IgA dimer depending on the species. SC also acts to stabilize sIgA from proteolytic degradation by bacterial enzymes while neutralizing pathogens, especially viruses. The most predominant type of antibody in external secretions, 3 g of sIgA are transported daily into the average adult intestine [83].

1.4.3 Functions of IgA

More than 70% of immune cells, dedicated daily to resisting systemic infections, comprise the antibody producing cells that produce IgA. It has been proposed that sIgA acts via three mechanisms in mucosal immune defense, thus playing a major role in the innate immune system preventing microorganisms and foreign

proteins from penetrating the mucosal surface [84, 85]. The first mechanism, immune exclusion, occurs on the lumenal side where sIgA inhibits adherence of microorganisms by surrounding pathogens with a hydrophilic shell that is repelled by the mucin gycocalix at mucosal surfaces [86]. The second mechanism by which sIgA protects the mucosal surfaces has two levels: one is the intracellular interception of viral antigens during transepithelial IgA transport; the second is the transport of IgA complexed with antigens from the submucosal side to the lumenal side [87]. The third mechanism involves large amounts of uncomplexed SC, which acts as a non-specific microbial scavenger within mucosal secretions, limiting infection by enterotoxigenic bacteria such as *E. coli* [88].

1.5 RECEPTORS FOR IgA

Fc receptors are defined by their specificity for the Fc fragment of immunoglobulin isotypes, and Fc receptors for IgA are referred to as FcαR [89]. Receptors to IgA were first described 30 years ago by Lawrence et al. which showed binding of an IgA1 myeloma protein and sIgA to blood neutrophils [90]. A key link between the humoral and cellular branches of the immune system, Fc-receptors (FcR) are present on a variety of cells. Members of the immunoreceptor family, FcR are classified along with T-cell receptors, NK

receptors and B cell receptors who, when functioning properly, recognize foreign antigens. Interaction between antibodies and FcR affords antigen specific recognition to cells that express FcR. The resulting effects of this interaction include a variety of responses including endocytosis, phagocytosis, transcytosis, exocytosis, superoxide generation, antibody-dependant cell mediated cytotoxiciy (ADCC) and release of cytokine inflammatory mediators involved in the modulation of cell survival.

Though structurally unrelated, there are five recognized types of FcαR, of which three are considered legitimate FcαR. CD89 (FcαRI) binds both human IgA1 and IgA2 subclasses (38). The polymeric immunoglobulin receptor (pIgR) is involved in the transport of IgM and IgA across the epithelial barrier by binding principally to the J chain [91]. Fcα/μR is expressed on the majority of B lymphocytes and macrophages, and can bind to the Fc portion of either IgA or IgM [92]. The two alternative receptors are the hepatic asialoglycoprotein receptor (ASPGR) and the transferrin receptor [93] [94]. In addition, poorly characterized IgA receptors have been reported on a range of cell types [95-99].

1.5.1 FcαRI Cellular Distribution

Expression of the FcαRI transmembrane receptor is restricted to cells of the myeloid lineage including neutrophils, eosinophils, most monocytes/macrophages, a subpopulation of dendritic cells, Kuppfer cells and cell lines that are related to these cell types [100]. FcαRI was first purified from neutrophils by affinity chromatography of radiolabeled solubilized cell membranes on IgA resins [101] and later from monocytes [98] and eosinophils [98] using IgA resins or anti-CD89 monoclonal antibodies. The level of FcαRI expression on cells is estimated to be 57, 000 per monocyte and 66,000 per neutrophil [102]. This estimate is supported by a recent study that documented high FcαR expression on neutrophils, but lower FcαRI expression on recently migrated monocytes [64]. Receptor size varies between 55-75 kDa on monocytes and neutrophils and 70-100 kDa on eosinophils due to an increase in glycosylation [103]. A variety of cytokines and other agents modulate FcαRI expression. On neutrophils FcαRI expression was upregulated by formyl-methionyl-leucyl-phenylalanine (FMLP), TNF-α and IL-8 [98]. The rapid upregulation of FcαRI on neutrophils is a result of recruitment from intracellular pools and occurs via a Ca^2 -dependent signaling pathway [101, 104-107]. Expression of FcαRI on monocytes is induced by lipopolysaccharide (LPS), TNF-

α and GM-CSF and IL-1 [103]. FcαRI is downregulated by transforming growth factor-β, interferon-γ, suramin and its ligand, pIgA [108] [109].

1.5.2 FcαRI Genetics

The gene encoding the FcαRI singe gene is located in the distal part of the q-arm on chromosome 19 at 19q13.4. [109, 110] Interestingly, it shares ~20% homology with other FcR, such as FcγR and FcεRI genes but is more homologous (~35%) to another family of receptors known as the leukocyte receptor cluster, that includes natural killer cell inhibitory receptors (KIR/KAR) and leukocyte Ig-like receptors (LIRs, MIRs) [111]. A bovine FcαRI that shares homology with human FcαRI and is able to bind both human and bovine IgA has been discovered [112] but to date, no murine homologue for FcαRI has been identified. Human FcαRI is also closely related to the bovine Fcγ2R as well as human and mouse platelet-specific collagen receptor (GVPI) [113] suggesting that FcαRI and Fcγ2R are a separate group of FcR, evolving from a common ancestral gene. The FcαRI gene consists of five exons that span approximately 12kb [114]. The first exon (S1) includes the 5′ untranslated region, an ATG translational initiation codon and most of the signal peptide. S2 is a mini-exon only 36 bp long, which encodes the second part of the signal peptide including the predicted signal peptidase cleavage site, whereas the extracellular domains are encoded by EC1 and EC2.

The last exon, called TM/C encodes the transmembrane domain and the short cytoplasmic tail.

1.5.3 Transcripts and Structure of FcαRI

While a number of alternatively spliced FcαRI transcripts have been identified by using RT-PCR, FcαRI exists *in vivo* as two different isoforms, a.1 and a.2, differing by a deletion in the extracellular domain. Isoform a.1 is expressed on blood monocytes, neutrophils, eosinophils, cultured blood monocytes and peritoneal macrophages while the a.2 isoform is found exclusively on alveolar macrophages [115, 116]. FcαRI is composed of two extracellular Ig-like domains (206 amino acids) that are oriented approximately 90^0 apart, a predicted transmembrane region (19 amino acids) and a cytoplasmic tail (41 amino acids) which is devoid of recognized signaling motifs [103, 117]. While sharing many structural similarities with FcγRIII and FcεRI, FcαRI is radically different in the way it binds it ligand compared to other similar FcRs. FcγRIII and FcεRI bind their ligands via the membrane proximal EC2 domain, in contrast to FcαRI which binds its ligand on the membrane distal EC1 domain [115, 118, 119]. Binding of IgA to FcαRI is also unique compared to FcγRIII and FcεRI, as IgA binds to the CH2-CH3 interface on the FcαRI, rather than to the top of the C_H2 or C_H3 regions.

The hydrophobic protein core of the FcαRI:IgA interface is flanked by charged residues [117]. Mutation analysis of the charged residues on IgA revealed that, in spite of the basic nature of FcαRI residues and the acidic nature of IgA residues, electrostatic interactions are unlikely to play a role in receptor-ligand binding [120].

1.5.4 IgA binding to FcαRI

The FcαRI has a low/medium affinity for IgA, (Ka ~ $10^6 M^{-1}$) [120]. The rapid dissociation of mIgA and FcαRI ($t^{1/2}$ ~ 25 s) suggests that mIgA interacts only transiently, while pIgA and IgA complexes bind avidly [121]. Due to the rigid conformation of IgA1 caused by heavily linked O-glycosylation in the hinge region, IgA binds FcαRI in an upright conformation unlike FcγRIII and FcεRI which bind their respective ligands in a downwards orientation [115]. Recent X-ray crystallography findings confirmed the stoichiometric configuration of FcαRI:IgA = 2:1 [117]. IgA can bind two FcαRI simultaneously however, the binding of one IgA molecule to two FcαRI is not sufficient to induce the receptor crosslinking necessary for signal transduction [122].

1.5.5 FcαRI Signal Transduction

A member of the multichain immune recognition receptor (MIRR) family, FcαRI signaling is dependant on association of FcαRI with the FcR γ-chain subunit (FcRγ) [123]. Association is necessary to induce signal transduction as the cytoplasmic tail of FcαRI bears no known signaling motifs and the FcRγ bears an immunoreceptor tyrosine (TyR)-based activation motif (ITAM) in its cytoplasmic region [124]. Cross-linking of IgA bound to FcαRI induces relocation into the glycosphingolipid and cholesterol rich domains known as "rafts" in the cell membrane that are abundant in signaling effector molecules. Tyrosine phosphorylation is critical in the initiation of activatory signals. Upon cross-linking, FcRγ – ITAM's are phosphorylated by p56 lyn that leads to the recruitment of p72syk. FcαRI crosslinking in neutrophils triggers calcium release from intracellular stores and induction of NADPH oxidase activity that is sensitive to inhibition by PI 3 kinase inhibitors [125].

Although the cytoplasmic tail has no known signaling motif, FcαRI can be expressed both associated and unassociated with the FcRγ (γ-less FcαRI) on neutrophils and monocytes, suggesting that the positively charged arginine at position 209 may associate with another yet unknown complex [121, 125]. IgA binding to FcαRI and the subsequent trafficking route taken by the

FcαRI/FcRγ complex is pH dependant. Under alkaline conditions (pH=5) the FcαRI/FcRγ associated complex traffics to the late endosome and results in IgA degradation. FcRγ-less FcαRI ligand binding occurs under more acidic conditions (pH = 6) and traffics bound IgA towards the early sorting endosomes, where a high portion of IgA remains bound and is subsequently recycled back to the cell surface [125]. This suggests a regulatory role for γ-less FcαRI in maintaining IgA serum homeostasis [126].

1.5.5. FcαRI Shedding and the Creation of Ig- Associated Immune Complexes

IgA ligand binding and the subsequent crosslinking of FcαRI on monocytes can result in the shedding of FcαRI. Two soluble forms (sFcαRI) have been identified in humans. A 30 kDa soluble product is generated by FcRγ chain dependant proteolytic cleavage [127]. The second sFcαRI was identified in patients with IgA nephropathy (IgAN), in which sFcαRI was shown to associate with pIgA forming 50-70 kDa complexes [125, 128].

1.5.6 Biological Functions

Accumulating evidence supports a pro-inflammatory role for FcαRI based on its ability to trigger a wide array of inflammatory functions. Association with FcRγ chain is essential for the initiation of cellular functions, such as the release of Ca^{2+}

and IL-2, degranulation and the degradation of IgA [125]. Interestingly, both FcαRI and γ-less FcαRI are endocytosed at similar rates [129]. FcαRI activation depends on receptor clustering at the cell surface. In monocytes, receptor cross linking results in the induction of IL-6 and TNF-α release [130] and FcαRI triggering on monocytes by CD89 mAb or IgA immune complexes induces the release of IL-1β, IL-8, leukotrienes C4 and B4, and prostaglandin E2 as well as superoxide release [131]. Neutrophils undergo respiratory burst when triggered with aggregated serum IgA and crosslinked mIgA, pIgA, sIgA or CD89 mAb My43, inducing superoxide release[132]. sIgA is also a potent stimulus of eosinophil degranulation [133].

FcαRI mediates phagocytosis of IgA-opsonized bacteria and yeast particles. Priming of monocytes and neutrophils increases IgA-mediated phagocytosis. Phagocytotic capacity of these cells increases when neutrophils are primed by cytokines GM-CSF and IL-8, and when monocytes are exposed to IL-1, TNF-α, GM-CSF and LPS. Eosinophils must be primed with IL-4, IL-5 and GM-CSF in order for IgA coated beads to be phagocytosed [103]. Priming is thought to increase the number of FcαRI molecules on the cell surface or increase FcαRI avidity for its ligand [134]. It is important to note that pathogenic cells, such as bacteria and tumor cells that are primed with IgA antibody are targeted for

antibody-dependant cell-mediated cytotoxicity (ADCC) by FcαRI expressing cells [135].

1.6 RHEUMATOID FACTOR

The term rheumatoid factor (RF) represents a group of autoantibodies specific for antigenic epitopes on the Fc portion of IgG antibodies. Most RF are of the IgM isotype, however RF is also able to bind IgG, IgA, IgD, and IgE. Generally associated with arthritis, rheumatoid factors have also been detected in a variety of other autoimmune and lymphoproliferative disorders. RF is also detected in a proportion of normal individuals [136]. Even in normal individuals, a variety of environmental stimuli can trigger RF production. The resulting polyclonal B cell activation produces exogenous antigen-bearing cross-reactive determinants to human IgG [137] [138]. An additional mechanism by which RF is thought to be stimulated is by cross-reactive epitopes on foreign antigens or by components of antigen-antibody complexes [139, 140]. Polyclonal activators of B lymphocytes, such as EBV, can induce RF production [141, 142]. RF develops over the course of many acute and chronic inflammatory diseases. The RF found in RA has a higher affinity and specificity for human IgG in comparison to RF found in other diseases.

1.6.1 *Physiological Role of RF in Autoimmunity*

RF B cells capture immune complexes and present antigen to T cells [143]. It is thought that early in the secondary immune response, IgM-expressing B cells might act as antigen presenting cells (APC) for immune complexed antigen. To complicate matters, IgG-RF, in contrast to other autoantibodies, has the unique ability to self-associate to form immune complexes in the absence of exogenous antigen [144]. The elevated levels of autoimmune complexes in the localized area of the joint would augment antigen-specific T cell expansion. Often IgM-RF is detected during acute infections, implying an underlying physiological function. IgM-RF is thought to amplify the humoral immune system by contributing to the formation of multivalent and multispecific complexes. RF's of the IgM class activate complement upon binding to IgG which results in the lysis of IgG-coated invading microorganisms. Secreted RF enhances immune complex clearance and RF B cells contribute to accessory cell function by activating complement through presentation of IgG-complexed antigen.

1.6.2 *RF Role in RA Associated Inflammation*

The chronic inflammatory process in the synovium of RA patients is characterized by the infiltration of macrophages, T and B lymphocytes which form ectopic lymphoid tissue [145]. Recruited B lymphocytes mature under pro-

inflammatory conditions and produce a variety of antibodies, including RF [146]. Locally produced RF contributes to the pathogenesis of RA by virtue of its ability to form immune complexes and activate complement. Interestingly, it has been demonstrated that synovial fluid from RA patients has increased complement activation and consumption when compared to synovial fluid of non-RA individuals [147]. The immune complexes which collect in the diseased joint promote the inflammatory process by recruiting neutrophils. As a result, lysosomal enzymes and inflammatory mediators are released into the arthritic joint, thereby exacerbating joint inflammation and contributing to joint destruction [148] [149].

Although there is no direct linear correlation between RF level and RA prognosis, patients with high titer of RF tend to have more aggressive disease. High titers of RF, in general are associated with an increased risk of vasculitis [150], and IgA RF titers may correlate with bone erosion [151]. However, it has been shown that elevated RF titers do occur long before the clinical onset of RA, suggesting that high affinity RF production is not a secondary phenomenon of the disease, but rather an active element in RA inflammation and the ensuing tissue destruction.

1.6.3 IgA-RF Association with Aggressive Disease in RA

Elevated serum titers of IgM-RF are one of the several diagnostic criteria for establishing the diagnosis of RA. Yet, the combined elevation of IgM-RF and IgA-RF is highly specific for RA and this combination is very rarely found in rheumatic diseases other than RA [152]. In a cross-sectional study, the majority (74%) of RA patients had elevations of 2-3 RF isotypes, and 67% had the combined elevation of IgA and IgM [153]. Of those patients with RA, 65% were positive for IgA-RF and 92% were positive for IgM-RF [154, 155].

IgA-RF can occur in serum and synovial fluid, and is predominantly polymeric [156]. Several studies have reported significant clinical implications to IgA-RF in RA. RA patients with a predominant increase in IgA-RF have more erosive disease [157, 158]. IgA-RF is associated with extra-articular manifestations of RA [158-160]. Detection of IgA-RF early in disease predicts poorer prognosis with a more rapidly progressive course [161, 162]. To date, it is unknown why IgA-RF is associated with a more aggressive RA.

1.6.4 IgA -RF and IgA Associated Immune Complexes in Other Autoimmune Diseases

RF is not exclusive to RA and has been associated with a variety of other autoimmune diseases. The production of RF associated immune complexes is thought to contribute to the perpetuation of the autoimmune response in diseased individuals. RF immune complexes are thought to incite inflammatory responses locally, as in RA, or form large complexes that can not be readily cleared by the body.

1.6.4.1 Sjögren's Syndrome

Sjögren's Syndrome (SS) is a chronic autoimmune disorder characterized by dysfunction and destruction of the exocrine glands. It affects predominantly, but not exclusively, the salivary and lacrimal glands, leading to dryness of the mouth and eyes. Exocrinopathy is associated with dense lymphocytic infiltrates of glandular tissues and B-cell hyperreactivity. SS has a broad clinical presentation, ranging from disease confined to the exocrine glands, to extraglandular systemic involvement affecting multiple organs. It may occur alone (primary Sjögren's Syndrome) or in association with several other autoimmune diseases (secondary Sjögren's Syndrome) such as RA.

Secondary Sjögren's Syndrome differs from the primary syndrome in terms of clinical presentation, serology and immunogenetic background [163]. Approximately 5% of patients with RA have a clinically overt Sjögren's syndrome, while subclinical complaints can be confirmed in as many as 20% of patients [164]. Destruction of the corneal and bulbar conjunctival epithelium (karatoconjuctivitis sicca, KCS) is the predominant sicca manifestation of patients with RA and SS, whereas xerostomia and enlargement of the major salivary glands are significantly less common than in primary Sjögren's syndrome [165].

Sjögren's syndrome continues to be associated with polyclonal B-cell hyperreactivity. Specific serological studies usually reveal autoantibodies directed against both organ and nonorgan specific autoantigens, as well as the occurrence of high amounts of circulating immune complexes and cryoglobulins [166]. Elevated plasma levels of IgA have been detected in patients with primary SS [167]. An excess of pIgA, together with mIgA immune complexes [168] and IgA-RF have been described in this condition. It has been proposed that elevated IgA in SS is caused by defective clearance, rather than by polyclonal overproduction of the antibody [169]. This may involve intrinsic abnormalities in IgA structure which would prevent IgA binding to the receptors, thereby lowering their catabolism [170-172].

1.6.4.2 IgA Nephropathy

Presenting with a very wide array of clinical presentations, primary IgA nephropathy (IgAN) is the most frequent form of primary glomerulonephritis. Progressive renal failure occurs in one third of all patients. The disease is characterized by mesangial co-deposits of IgA binding to either C3, IgG or IgM . A putative pathogenic role credited to the presence of large amounts of pIgA and pIgA-IC in the serum of IgAN patients [173]. While levels of plasma IgA1 and plasma IgA1 containing immune complexes (IgA-IC) are elevated in about half the patients with IgAN [174], they are not sufficient to cause mesangial deposition of IgA1 [175-178]. Rather, a pathogenic role has been attributed to the circulating IgA-IC [179, 180]. Schena et al, found high levels of pIgA and IgA-RF in the blood of patients with IgAN and a subsequent reduction in the capacity of serum to solubilize IC in a high percentage of patients [181]. Increasing amounts of pIgA were found to inhibit the complement-mediated solubilization (CMS), while inhibitory activity is also exhibited by RF. These results suggest that pIgA and IgA-RF are responsible for the presence of insoluble nephritogenic IC in the serum of patients with primary IgAN. Fcα/μ on mesangial cells is another conjectured player in IgAN.

2. Project Rational

FLS act as transformed aggressive cells in RA, contributing to cytokine production by both autocrine and paracrine mechanisms. The functional specialization of the joint results in a privileged immune site, leaving it susceptible to autoimmune disease. It is unknown why IgA-RF is associated with a more aggressive RA, however it has been proven that IgA-associated immune complexes bind avidly to FcαR and trigger many pro-inflammatory responses in myeloid cells.

The relationship between elevated IgA–RF levels and a more destructive disease course in RA remains to be determined. While there are abundant amounts of IgA and IgA immune complexes associated with RA and IgA receptors on neutrophils, monocytes/macrophages recruited to the inflamed joint, to date there has been no description of IgA receptors on FLS.

3. PROJECT HYPOTHESIS AND AIMS

Hypothesis

FLS cells of the RA joint express a receptor for IgA.

Aims

1.) To determine if RA, OA and ERA FLS in cell culture express a receptor for IgA *in vitro*, and if so, to identify which one.

2.) To investigate the potential expression of IgA receptors on fibroblasts derived from tissue other than that of the joint.

3.) To determine whether synovial tissue from patients with RA, OA and ERA express IgA receptors *in vivo*.

4. MATERIALS AND METHODS

4.1 REAGENTS

All laboratory chemicals were purchased from Sigma-Aldrich Chemicals (Sigma-Aldrich Ltd., Oakville, Ontario, Canada) unless otherwise indicated. All solutions and buffers were prepared using distilled deionized water (ddH_2O) and sterilized by autoclaving prior to use.

4.2 TISSUE CULTURE MEDIA

All media was purchased from Gibco BRL Life Technologies (Gaithersburg, MD, USA). Commercially prepared Dulbecco's Minimal Essential Media (DMEM) was supplemented with 2 millimolar (mM) L-glutamine, 1.0 mM sodium pyruvate, 100 units/ml of penicillin-G and 50 units/ml of streptomycin sulfate. Pre-mixed powder for RPMI 1640 and Minimal Essential Medium (MEM) containing L-glutamine were made according to the manufacture's instructions, supplemented with 1.0 mM sodium pyruvate 100units/ml of penicillin-G and 50 units/ml of streptomycin sulfate. The pH was adjusted between 7.0 and 7.5 using concentrated HCl. All cell culture media was prepared using ddH_2O and filter sterilized using a 0.2 micrometer (μm) VacucapR filter unit (Sarstedt, Missassauga, Ontario, Canada) into autoclaved sterile glass bottles. Media was stored in the dark at 4°C until required.

4.3 CONTROL CELL LINES

For use as comparison fibroblast cell lines - human dermal fibroblasts (HDF) were a gift from Dr. J. Wilkins (University of Manitoba, Winnipeg, Manitoba, Canada). Human lung fibroblasts (HLF) were purchased from Cambrex (Walkersville, MD). The myelomonocytic cell line, U937 (ATCC), was used as a positive control cell line for FcαR, FcγRIII and Fcα/μR expression. CALU-3 airway epithelial cells (ATCC) were used as a positive control cell line for pIgR expression.

4.4 RA, OA AND ERA TISSUE COLLECTION

All synovial tissue was generously donated from the lab of Dr. Hani El-Gabalawy (Arthritis Research Center, University of Manitoba, Canada). Synovial tissue was obtained from patients with RA and OA at the time of joint replacement surgery, or synovial biopsy for the one patient with early RA, according to a protocol approved by the Human Research Ethics Board at the University of Manitoba. All RA patients met the American College of Rheumatology criteria for the disease [182]. Tissue was either snap-frozen in OCT mounting media (Canlab, Mississauga, Ontario, Canada) or immediately processed for primary cell culture of FLS.

4.5 ISOLATION OF FLS

All primary cell cultures of FLS were prepared by Keng Wong in the laboratory of Dr. Hani El-Gabalawy. Synovial tissue was digested with collagenase (1mg/ml) and hyaluronidase (0.05 mg/ml) in Hank's balanced salt solution (Gibco, Burlington, Ontario, Canada) for 1-2 hours at 37°C. Cells were washed with 10% Fetal Bovine Serum (FBS) in DMEM (both from Gibco), centrifuged at 1,000 revolutions per minute for 10 minutes, resuspended in FBS/DMEM and cultured overnight at 37°C in 5% carbon dioxide (CO_2).

4.6 CELL CULTURE OF FLS AND CONTROL LINES

Primary RA and OA cells with a doubling time of 5 days were grown in DMEM with 10% FBS. Both human HDF and HLF were cultured under identical conditions. Both cell lines double in 3 days. U937 cells double in 24 hours and were grown in 1640 RPMI media with 10% FBS. CALU-3 cells were cultured in MEM with 5% FBS and double every 48 hours. Cells were cultured in a humidified 5% CO_2 incubator (Forma Scientific Inc. Marietta, OH USA) at 37°C. FLS cells were split every seventh day, HDF and HLF every fourth day, and U937 and CALU-3 cells were split every two days.

4.7 IMMUNOFLUORESCNCE

4..7.1 FcαR staining of FLS, HDF and HLF cell lines

Cells were grown on 1 cm diameter coverslips (Fisher Scientific Company, Nepean, Ontario, Canada) at the bottom of a 6 well polystyrene plate (Starstat) until 50-70% confluent before transferring individual coverslips to a 24-well tissue culture plate. Cells were then washed with 3 ml of Phosphate-Buffered Saline (PBS), fixed with 2 ml 4% paraformaldehyde (PFA) for 30 minutes rocking on ice. The PFA was washed off twice with PBS then quenched in a solution of 75mM ammonium chloride (NH_4Cl), 20mM glycine in PBS for 10 minutes. Following quenching, cells were rinsed twice with PBS and blocked with permeablizing solution (PFS) comprised of fish skin gelatin (0.07%) (Fisher) and 0.025% Saponin in PBS for 1 hour at 37°C. After blocking, cells were incubated with antibodies to FcαR (A59 1: 50, BD Pharmingen, San Diego, California USA; N20 or T20 1:50, Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted in PFS for 1 hour at 37°C or overnight at 4°C. After primary incubation, the cells were washed 3 times for 5 minutes with 1XPBS and the respective FITC-conjugated secondary antibodies of donkey anti-goat FITC 1:200 or rabbit anti-mouse FITC 1:200 (Jackson Immunoresearch, West Grove, PA) diluted in PFS were added for 30 minutes at 37°C. The polystyrene plate was wrapped in aluminum foil to prevent bleaching of the FITC conjugated antibody. Cells were

washed 3 times for 5 minutes in 1X PBS, then mounted with Antifade containing DAPI (Molecular Probes, Eugene, Oregon) on Premium Cover Glass™ slides (Fisher). Control conditions included staining with the secondary antibody alone and in the case of FLS, blocking solution alone to confirm complete blockade of continually observed autofluorescence. Data was examined under an Olympus BX51 microscope. Images were digitally captured using Spot Version 4.0.4 software (Diagnostic Instruments Inc, Sterling Heights, MI USA) on a Spot PCT-CE colour camera at both 20X and 40X magnification. Images were saved in tagged image file (TIF) formatted and converted to JPEG format using Spot Version 4.0.4 software (Diagnostic Instruments Inc.)

4.7.2 Preparation and Addition of N20 Antibody Blocking Peptide.

In a 1.5 mL Eppendorf™ tube the N20 antibody was diluted at a 20% volume/volume (v/v) in commercially prepared blocking peptide for N20 (Santa Cruz) for a 2h incubation at room temperature as per manufacture's instructions. The antibody/peptide mixture was diluted into PFS accommodating the 20% v/v antibody dilution and processed in parallel as outlined in Section 3.7.1.

4.8 RT-PCR OF FLS AND OTHER PRIMARY CELL CULTURES

Note: RNase and DNase free barrier pipette tips (Sarstedt) were used in all steps of RNA isolation and manipulation to help decrease the probability of aerosol cross-contamination of RNA and/or RNases.

4.8.1 RNA Isolation From FLS via modified TRIzol® Method

Total RNA was extracted from confluent RA and OA FLS (2x T75 culture flasks (Sarstedt), by trypsinizing the cells with 5ml 0.25% trypsin solution (Gibco) for 5 minutes at 37^{0}C. Cells were then transferred into a 10ml conical tube (Sarstedt) and 5 ml of media was added to neutralize the trypsin before spinning down the samples for 5 minutes at 1000 rpm in an Eppendorf™ R810R (Brinkman Instruments Inc. Westbury NY, USA) centrifuge. The media was aspirated off and the cells resuspended in 1ml TRIzol® reagent, transferred into a sterile 1.5 mL Eppendorf™ tube and incubated at room temperature for 5 minutes. To extract the RNA, 0.2ml of chloroform was added to each sample, and the sample immediately shaken by hand for 15 seconds then allowed to sit 3 minutes at room temperature before being centrifuged at 12000 x g in a Biofugepico™ centrifuge (Heralus Instruments, Germany) for 15 min at 4^{0}C. The aqueous top layer was carefully transferred to a sterile 1.5 mL Eppendorf™ tube avoiding the white protein interphase layer. An equal volume (normally 0.5ml) of 2-butanol

(isopropanol) was added to each tube, vortexed and incubated for 10 minutes at room temperature to precipitate the RNA. The samples were centrifuged at 12,000 x g for 10 minutes at 4°C and the supernatant discarded. One ml of 75% ethanol was added to each tube to wash the pellet and ensure the removal of residual salts. The tubes were centrifuged at 7,500 x g for 5 minutes at 4°C and the 75% ethanol discarded. The pellets were allowed to air dry for 20 minutes and the pellets dissolved in 50 µl of RNase-free ddH2O. RNA samples were heated at 65°C for 10 minutes if required to dissolve the RNA and 5µl aliquots from each sample were diluted 1/25 in RNAse-free H_2O. RNA concentration was determined by measuring the absorbance at 260 nanometers [173] using a 1.0mL quartz cuvette in a Cary 1 spectrophotometer (Varian, Mississauga, Ontario, Canada) where an absorbance reading of 1.0 at 260 nm equals a RNA concentration of 40 µl/ml. The yield was calculated as follows: $Absorbance_{260}$x 40 ml/mL x dilution factor. Purity of RNA preparations was estimated by measuring the ratio of $Absorbance_{260}$ to Absorbance at 280 nm ($Absorbance_{280}$). RNA preparations having a ratio of 1.5 to 2.0 are considered to be suitable for further use. RNA samples were stored in RNase-free ddH_2O at –80°C until required.

4.8.2 RNA Isolation from Control Cell Lines via TRIzol® Method

Confluent T25 culture flasks (Sarstedt) of dermal fibroblasts, U937 and CALU-3 cells underwent RNA isolated by TRIzol® reagent (Invitrogen Life Technologies) as described by the manufacturer. RNA samples were stored in RNase-free ddH_2O at –80^{0}C until required.

4.8.3 RT-PCR of FLS CELLS AND CONTROLS

4.8.3.1 Reverse Transcriptase of FLS and Control Cell RNA

FLS, HDF, HLF, U937 and Calu-3 RNA was reverse transcribed to cDNA by mixing 3 µl RNA with 1µl oligo dT, 1µl of 10mM dNTP stock (composed of 10 mM each cATP, dGTP, dCTP and dTTP at neutral pH), 4 µl of 5x RT buffer, and 2µl of 0.1M dithiothreitol (Gibco BRL Life Technologies) in a sterile 200 µl Eppendorf™ tube followed by incubation at 37^{0}C for 2 minutes in Forma Scientific EL745 water bath (Forma Scientific Inc. Marietta, OH USA). One µl of murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies) was added to the RNA mixture followed by incubation at 37^{0}C for 50 minutes. The first strand cDNA synthesis reaction was inactivated by heating at 70^{0}C for 15 minutes. Samples were stored at -80^{0}C until required.

4.8.3.2 PCR of FLS and Control Cell Lines

Thermal cycling involved an equal 5 μl volume of cDNA from each RT reaction amplified with 1 μl of each upper and lower primer (10 mM stock), 5μl of 10X PCR buffer, 1.5μl of 50 mM $MgCl_2$, 1 μl of 10mM dNTP mix and 0.4 μl of *Taq*™ polymerase (Promega, Madison, WI) for a final 50μl volume in a sterile 200 μl Eppendorf™ tube. Table 1 lists the primer sequences used for FcαR, Fcα/μR, pIgR, FcγRIII and GAPDH. Edwards et al found that in normal adult synovium FcγRIII was restricted to intimal cells and thus FcγRIII serves as an accurate marker of cells derived from the synovial intimal lining [183]. All PCR reactions consisted of initial denaturing at 94^{0}C for 3 minutes followed by 30-40 cycles at 94^{0}C for 45 s, 55^{0}C for 30 s, and 72^{0}C for 90 s in an Eppendorf™ Mastercycler Gradient thermocycler (5331-1198 Eppendorf Scientific, Westbury NY, USA).

TABLE 1: PRIMER SEQUENCES FOR FcαR, Fcα/μR, pIgR, FcγRIII AS USED IN PCR OF FLS AND CONTROL CELL LINES.

Receptor	Upper Primer	Lower Primer
FcαR	CCT CAG TCT GGG GCT TTC TTT	CTT GTT TGC GTC CAT GTG GTC
Fcα/μR	GAC AAC TAC CAA GCC TGA TAG G	TCT GTC CCT CAG GGT CCT GGA T
pIgR (cytoplasmic)	GAC CCC ACT CCC TGC TCT AAC	AGA AGA GGG GAA GGA CGG GAG
FcgRIII	AAG ATC TCC CAA AGG CTG TG	ATG GAC TTC TAG CTG CAC CG
GAPDH	CCA TGG AGA AGG CTG GG	CAA AGT TGT CAT GGA TGA CC

4.8.3.3 Identification of FcαR and FcγRIII via RT-PCR Products

15 µl of FLS, dermal fibroblast, U937 and Calu-3 cDNA was combined with 2 µl of DNA running buffer in a 200 µl Eppendorf™ tube, vortexed and quick spun on a centrifuge to ensure mixing of the samples with the loading buffer. The samples were loaded in a 1.2% agarose gel containing 0.05 mg/ml ethidium bromide (EtBr). In addition to FLS DNA and control DNA, 1 µg of 500bp DNA ladder (Invitrogen) was loaded flanking the FLS DNA to allow evaluation of electrophoresis. The agarose gel was electrophoresed in a Sub-Cell GT (Biorad) at 60 volts/centimeter in 1x TBE running buffer until the 500bp ladder band had migrated approximately 7 cm from the well. The agarose gel was viewed using a Syngene (Genegentics) transilluminator and documented using Polapan 667, ISO $3000/36^0$ instant film. The elecrophoresed RT-PCR products of each FLS sample were cut from the agarose gel at 241 bp (FcαR) and 254 bp (FcγPIII) and purified for DNA analysis with QIAquick Gel Extraction kit (Qiagen, Mississauga, Ontario, Canada) as per manufacturers instruction. The purified DNA was sequenced to confirm band identity at the Manitoba Institute of Cell Biology (MICB). The resulting DNA sequences were compared to those in the Genebank of NCBI human genome database for identification of FcαR and FcγRIII.

4.9 IMMUNOHISTOCHEMISTRY

4.9.1 Preparation of Frozen Synovial Tissue Blocks

Synovial tissue sections embedded in OCT were prepared by Keng Wong in Dr. El-Gabalawy's lab and provided as required. Sections were transported to MICB in Styrofoam coolers on dry ice and stored at –80°C until required. Prior to cutting, sections were warmed to cryostat cutting temperature (-10°C) for 30 minutes. Tissue was sliced on a Thermoshandon cryostat (77200227, Thermoshandon Ltd, Cheshire UK), into 8 μm sections of RA, OA and ERA tissue. The tissue was mounted on ProbeON Plus™ polarized coated slides (Fisherbiotech 15-188-52) developed for immunohistochemistry. Once the tissue section adhered to the slide, tissue sections were dried at room temperature for 30 minutes to ensure all water had evaporated from the samples. Slides were cooled in a slide box (Fisher) for 15 minutes on dry ice before being transferred to storage at –80°C

4.9.2 Staining for FcαR in RA, OA and ERA Synovial Tissue

Slides were removed from –80°C and allowed to warm to room temperature on filter paper (Fisher) for 10-15 minutes. Slides were immersed in 70 ml ice cold acetone (4°C) for 10 minutes in an 8 slide glass developer (Fisher), air-dried, then rehydrated with 70 ml PBS, and pretreated with 100 μl of 0.03% H_2O_2 in 1X PBS-T

for 20 minutes to block endogenous peroxidase activity. All tissue sections were blocked with PFS (described in section 3.7.1) at room temperature for 1h in a humidity chamber (Fisher). The PFS was carefully removed, and the tissue was incubated for 1 hour at room temperature with 100 µl of: (a) 1-3 different antibodies to the FcαR, all at 1:100 dilutions (N20 and T20 goat polyclonal, and A3 mouse monoclonal antibodies; Santa Cruz); (b) a mouse monoclonal antibody to decay accelerating factor (DAF) diluted 1:500 (CD55; DAKO, Carpinteria, CA); or (c) a mouse monoclonal antibody to the macrophage marker CD68 (DAKO) 1:200. Control conditions included the use of isotype matched irrelevant mouse and goat IgG at 1:100 dilutions (IgG1 DAKO, IgG1 Sigma) as well as secondary antibodies alone. Slides were washed 3 times in PBS with 0.05% Tween to remove the primary antibody. One hundred µl HRP-conjugated secondary antibodies to mouse 1:200 (DAKO) and goat IgG 1:200 were diluted in PFS then added to each tissue sample for 1 hour at room temperature. Slides were washed 3 times in PBS-T to ensure removal of excess secondary antibody. Tissue samples were developed with diaminobenzidine hydrochloride (DAB) (DAKO) on average for 5 minutes and immersed in tap water for 1 minute to stop the DAB reaction. Slides were counterstained with Meyers' hematoxylin (Fisher) for an average of 3 minutes, then immersed in running tap water for 1 minute to stop the reaction. Tissue was dehydrated in 50-100% ethanol gradients; followed

by 100% xylene to ensure all water was removed and then mounted in Permount (Fisher).

4.9.3 Confirmation of FcαR Expression on Synovial Tissue by N-20 Blocking Peptide

To confirm specificity of the staining for FcαR, the blocking peptide for N20 was pre-incubated with the antibody at a ratio of 1µl of N20: 5ul of blocking peptide for 2h at room temperature. The resulting solution was applied at a 1:100 dilution (antibody:blocking peptide vol/vol) in PFS. Tissue sections were visualized using an Olympus BX51 camera.

5. RESULTS

5.1 RA and OA FLS EXPRESS FcαR PROTEIN BY INDIRECT IMMUNOFLUORESCENCE

Expression of FcαR on FLS was studied by indirect immunofluorescence using cell cultures prepared from at least 8 RA subjects, 4 OA subjects and one patient with untreated early RA. Three different antibodies to FcαR were used: N-20 binds between amino acids 23-227 of the extracellular domain; T20 goat antibody bind amino acids 150-227 of a narrower extracellular region than N20 (Figure 1c). The N-20 antibody that recognizes the N-terminus site stained all RA, OA and early RA FLS tested (Figure 1a). Each subjects' FLS cells examined in all fields showed diffuse staining throughout the cell. This staining was inhibited by pre-incubating the N20 antibody with the blocking peptide, confirming that the positive staining was specific for the FcαR (Figure 1b). Staining with the two other anti-FcαR antibodies (T-20 and A59) yielded identical patterns to that obtained with the N20 antibody and FcαR expression was present in FLS from passage 4 through passage 9 (data not shown). In contrast to FLS, FcαR staining was absent in normal human dermal fibroblasts and lung fibroblasts (Figure 1a). Therefore, while FcαR was expressed by all RA and OA FLS tested, it was undetectable on fibroblasts of non-synovial origin.

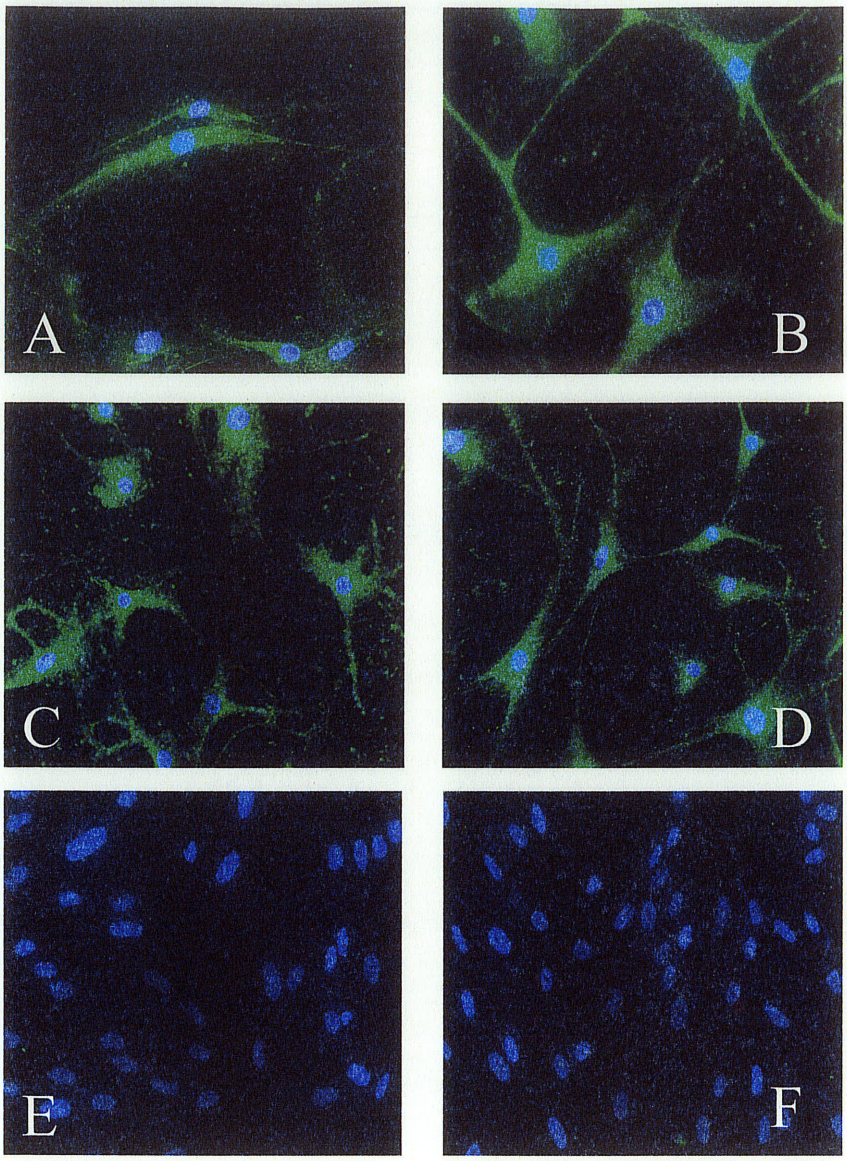

FIGURE 1(a) FcαR IS EXPRESSED BY ERA, RA AND OA FLS BUT NOT BY DERMAL OR LUNG FIBROBLASTS.
Indirect immuofluorescence of early RA(**A**), RA(**B,C**) and OA(**D**) FLS cells showing staining for FcαR. Cells above were fixed with 4% PFA, blocked with fish skin gelatin and stained for FcαR with N20 and FITC-conjugated donkey anti-goat IgG. Both dermal (**E**) and lung fibroblasts (**F**) are negative for FcαR expression. DAPI staining shows blue nuclei. (Original magnification x 400 for **A-F.**)

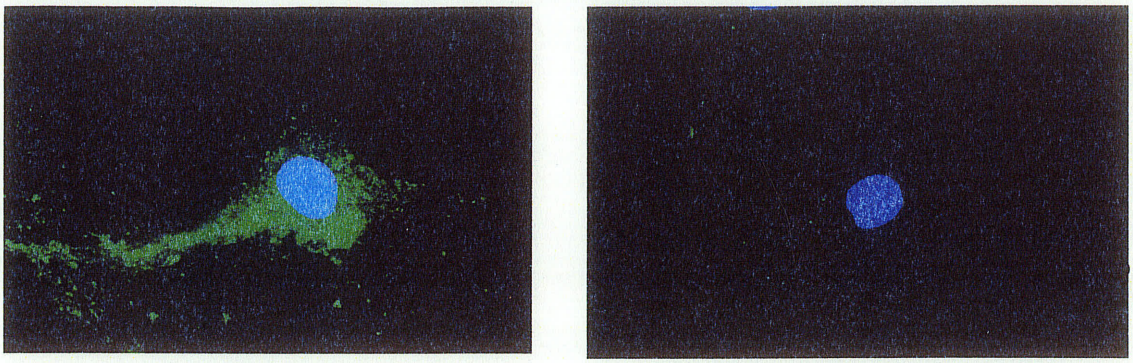

FIGURE 1(b): STAINING OF FLS IS BLOCKED BY INHIBITORY PEPTIDE TO N20 GOAT ANTIBODY TO FcαR.
Indirect immunofluorescence of RA FLS staining for FcαR is blocked by the inhibitory peptide to the N20 goat antibody. The antibody to blocking peptide was incubated at room temperature for 2 hours at a 1:5 volume ratio to sequester the N20 binding site, and then applied to the FLS to confirm FcαR staining specificity. Similar results were obtained with OA FLS (data not shown). (Original magnification X 400)

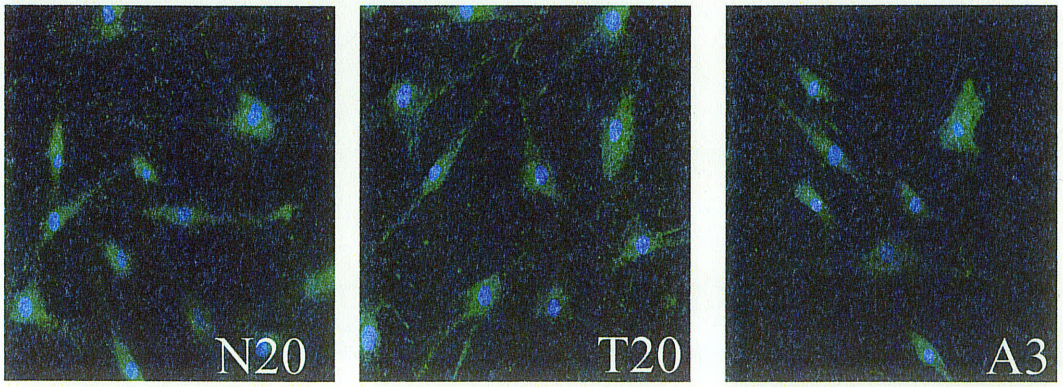

FIGURE 1(c): FcαR IS DETECTED BY THREE DIFFERENT ANTIBODIES TO CD89. RA and OA FLS cells were stained with three different antibodies to FcαR: N20 goat antibody to the N-terminus, between aa 23-227 of the extracellular domain; T20 goat antibody to aa 150-227 but a different extracellular region than N20; and A3 mouse monoclonal antibody to an unmapped domain. FITC-conjugated secondary antibodies and DAPI for blue nuclear staining were used. (Original magnification X 400)

5.2 RA AND OA FLS EXPRESS FcαR AND FcγRIII mRNA BY RT-PCR

To confirm FcαR mRNA expression by FLS, RT-PCR was carried out with RNA obtained from FLS prepared from at least 9 different patients with RA, 1 with ERA and 7 with OA. RT-PCR primers specific for FcγRIII receptors were chosen to ensure that primary FLS cultures were comprised of FLS from the intimal synovial lining. All patients' FLS obtained for the study were analyzed between passages 4 and 6, and were found to express FcαR, which was confirmed by DNA sequencing of the RT-PCR products (Figure 2). Every sample of FLS tested for the FcγRIII (n=5) was also positive.

In addition to FcαR and FcγRIII receptors, the potential expression of other IgA receptors on FLS was investigated. RT-PCR primers to both the Fcα/μR (endogenously expressed in U937) and the polymeric immunoglobulin receptor (pIgR), the epithelial IgA receptor (endogenously expressed in CALU-3 airway epithelial cells) were analyzed with mRNA from both RA and OA FLS, U937, and CALU-3 cells. As expected, U937 cells expressed mRNA Fcα/μR and the CALU-3 cells expressed mRNA for pIgR however, none of the FLS cells expressed mRNA for Fcα/μR or pIgR. Dermal and lung fibroblasts did not express mRNA for any of the IgA receptors tested.

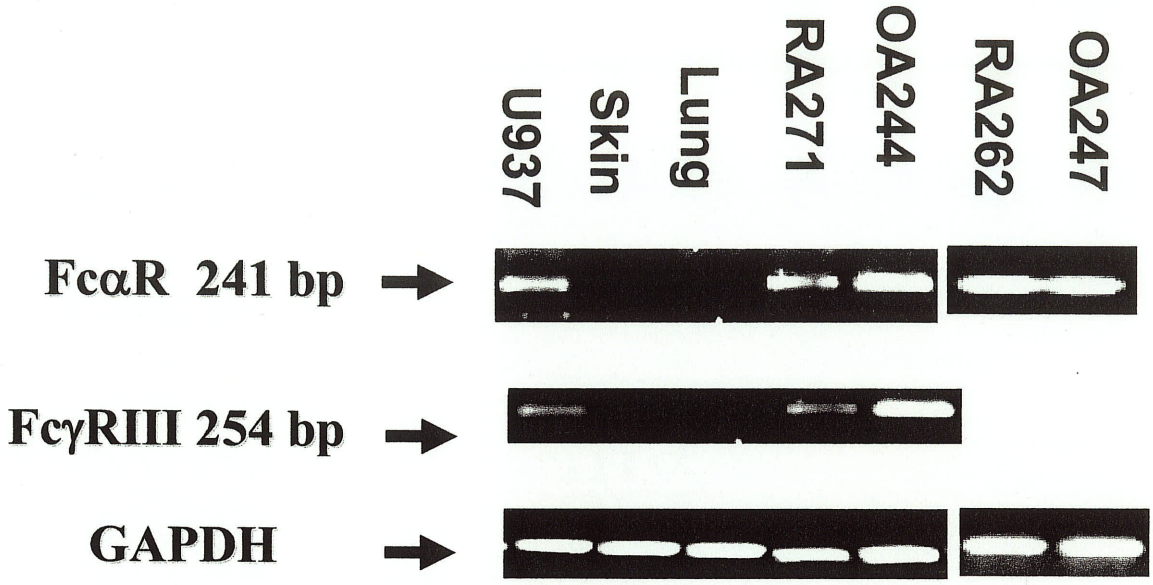

FIGURE 2: FcαR mRNA IS EXPRESSED BY FLS BUT NOT LUNG OR DERMAL FIBROBLASTS. RT-PCR products obtained with mRNA from RA and OA FLS, human dermal and lung fibroblasts for FcαR, FcγRIII, and GAPDH. U937 myelomonocytic cells were used as a positive control cell line for the FcαR. All RA and OA patients confirmed the presence of receptor mRNA, showing bands at 241bp for FcαR and 254bp for FcγRIII. DNA sequencing of RT-PCR products confirmed FcαR identity in FLS.

5.3 FcαR EXPRESSION IN RA AND OA SYNOVIAL TISSUE

Expression of FcαR in RA and OA synovium was investigated in frozen synovial tissue obtained from a total of 7 patients with RA, 1 with early RA and 4 with OA was stained for FcαR (N-20), CD68 and DAF. DAF is expressed at high levels on cells lining body cavities that make it an ideal marker for synovial lining identification [184]. CD68 belongs to a family of highly glycosylated lysosomal glycoproteins that is found on the surface of macrophages as well as the synovial intimal lining, making it another ideal marker for synovial tissue [185]. Synovial tissue from all subjects investigated stained intensely for all of FcαR, DAF and CD68 in the synovial lining (Figure 3a and b). All patients stained positive for DAF in the synovial lining. CD68 staining was predominantly observed in the synovial lining with diffuse staining throughout the subintimal region. The staining for FcαR in the subintimal layer was diffuse, less intense, and appeared to be predominantly in macrophage-like cells while some positively stained cells had a fibroblast-like appearance. Seven of the 10 patients' tissue was stained with 3 different antibodies to FcαR, (N-20, A3 and T-20) all of which showed the same consistent pattern of intense staining in the synovial lining layer. Staining with an irrelevant IgG or with the secondary antibody alone was negative. Staining for FcαR was inhibited by the blocking peptide for N20 in n=3 subjects, confirming the specificity of the observed FcαR staining with N20 (Figure 3c).

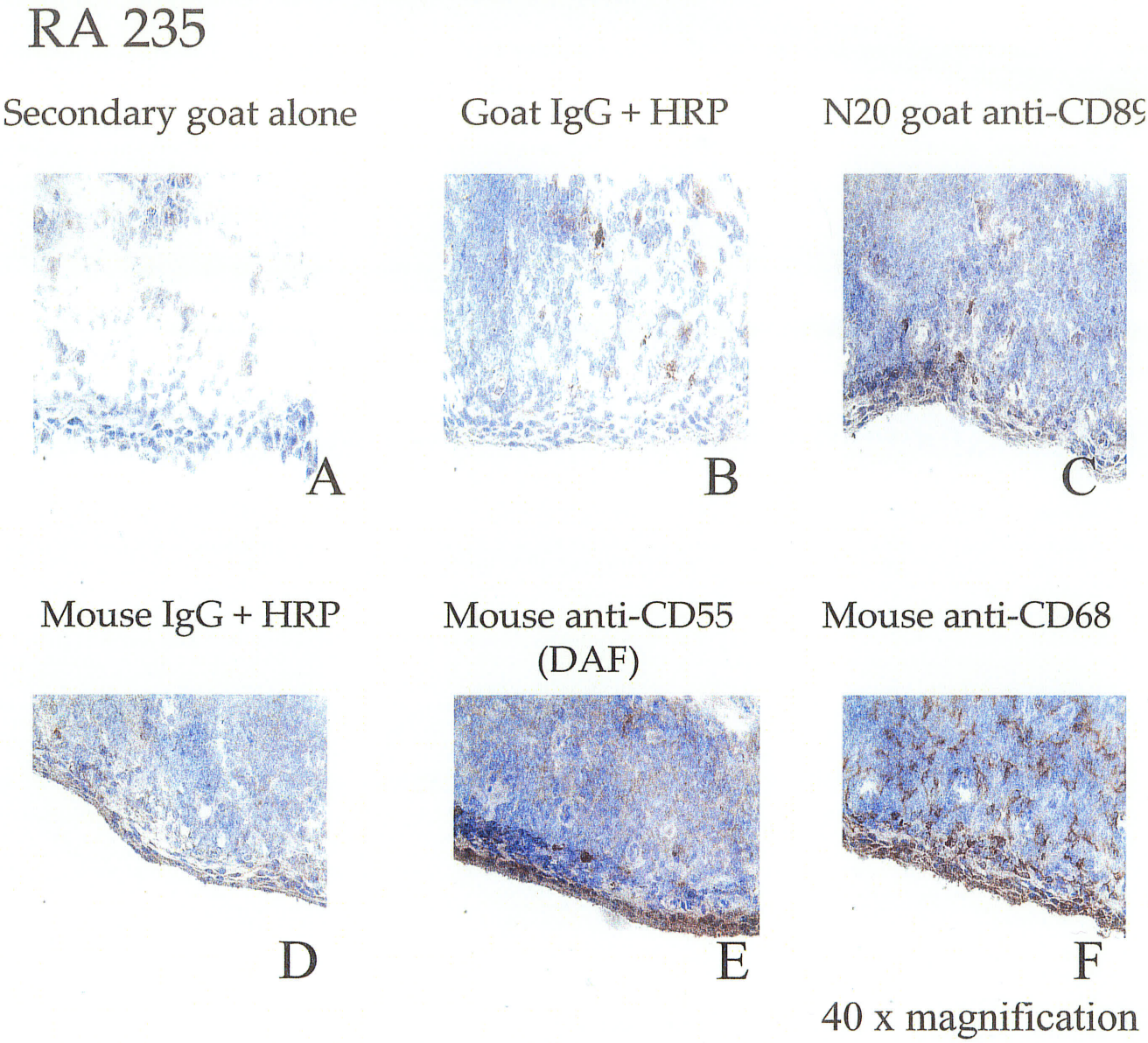

FIGURE 3 (a); FcαR, DAF AND CD68 STAINING APPEARS PREDOMINANTLY IN THE SYNOVIAL LINING OF RA SYNOVIAL TISSUE The intitmal lining stains positive for FcαR (C), DAF (E) and CD68 (F), with some diffuse staining of CD68 throughout the subintimal region (F). Irrelevant secondary antibodies goat IgG (B) tested negative and mouse IgG (D) weakly positive due to prolonged DAB exposure.

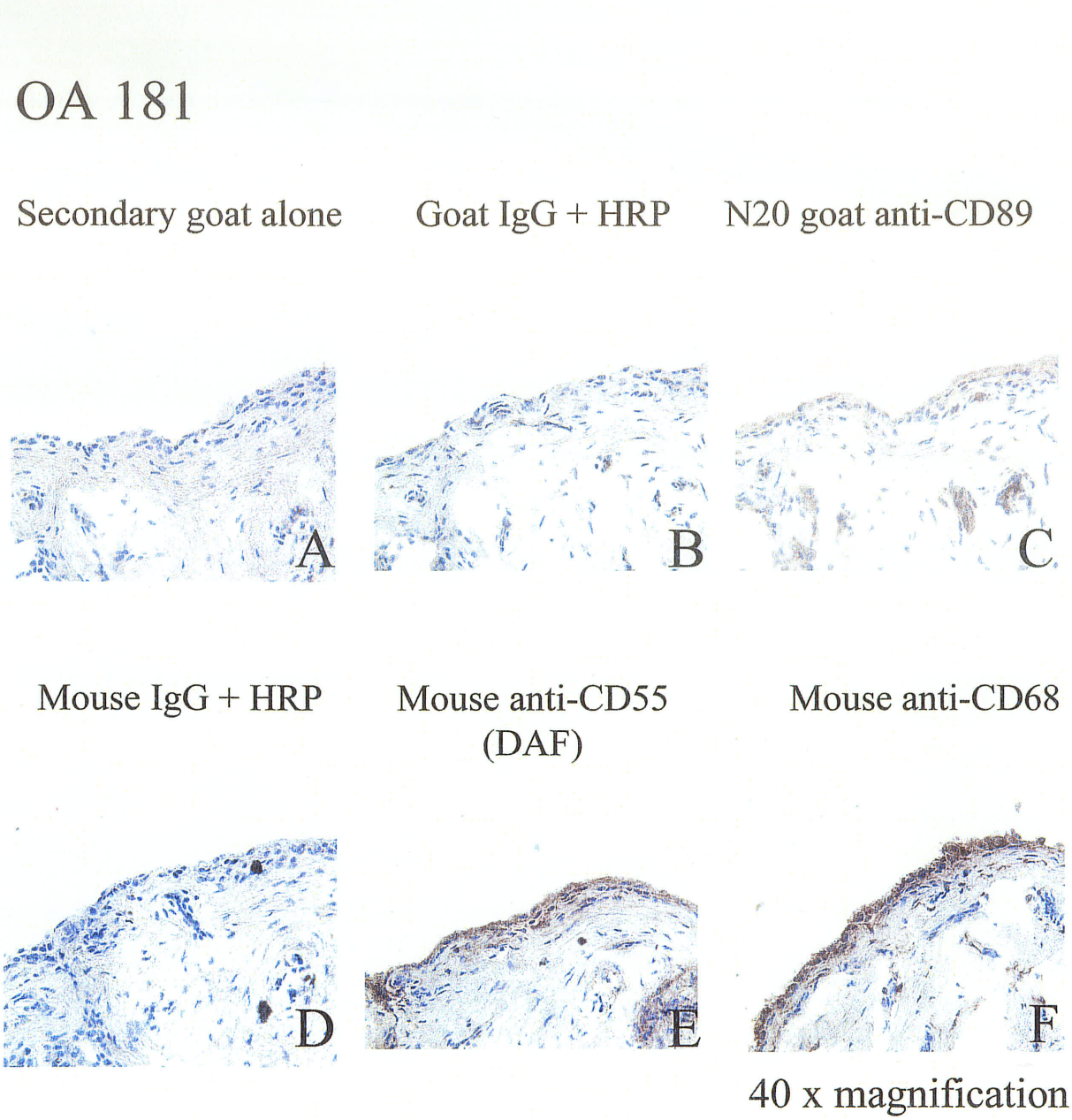

FIGURE 3(b): FcαR, DAF AND CD68 STAINING APPEARS PREDOMINANTLY IN THE SYNOVIAL LINING OF OA SYNOVIAL TISSUE. The initmal lining stains positive for FcαR (C), DAF (E) and CD68 (E), with some diffuse staining of CD68 throughout the subintimal region (F). Irrelevant secondary antibodies to both mouse and goat IgG (B,D) tested negative.

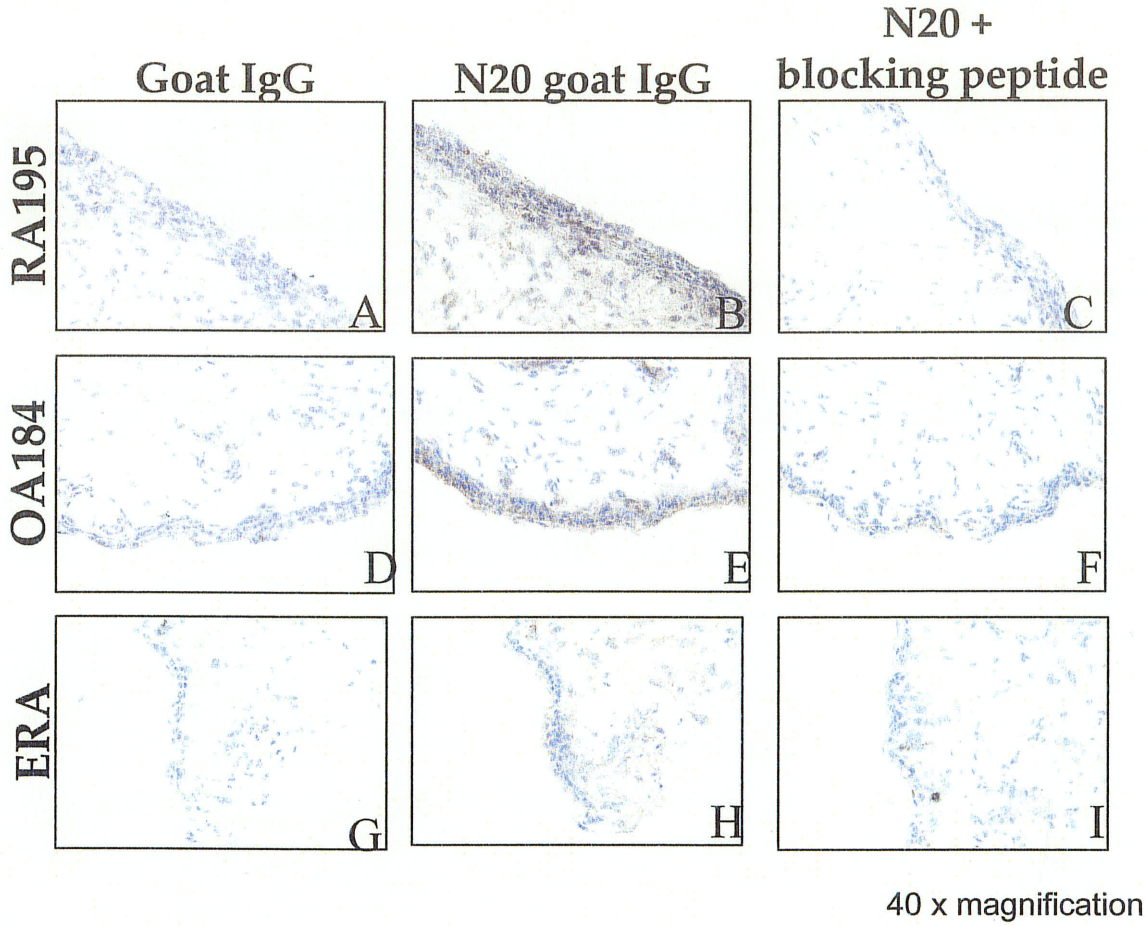

FIGURE 3(c): FcαR APPEARS PREDOMINANTLY IN THE INTIMAL LINING LAYER OF RA, ERA AND OA TISSUE AND IS SPECIFICALLY BLOCKED BY N20 INHIBITORY PEPTIDE. FcαR is detectable on RA, ERA and OA synovial tissue. FcαR staining predominates in the intimal lining layer of RA(B), ERA(E) and OA(H) synovial tissue. Serial tissue sections from each patient were stained for DAF to confirm that FLS staining for FcαR were indeed part of the synovial intimal lining (data not shown). Serial tissue sections from each patient were also stained with N20-blocking peptide complex which specifically inhibited antibody binding to the FcαR (C,F,I). Staining was absent with an irrelevant goat IgG (A,D,G)

6. DISCUSSION

Fibroblast-like synoviocytes in culture and synovial tissue from patients with RA and OA express the FcαR. This work is the first to demonstrate the presence of any IgA receptor and specifically, FcαR on synovial fibroblasts. FcαR expression by FLS is not unique to RA because OA patients also express FcαR on their FLS. Human fibroblast cell culture results confirm that FcαR mRNA expression is unique only to FLS, and not fibroblasts of non-synovial origin from uninflammed tissue. This suggests that the presence of FcαR is intrinsic to synovial cell biology, and not unique to a particular arthropathy. The observation of FcαR expression on FLS in synovial tissue supports the idea that FcαR expression is not an artifact of primary cell culture conditions. The predominantly intimal location of this staining in RA and OA tissue is particularly interesting, because it is these cells that, when cultured, demonstrate both macrophage-like and fibroblast-like phenotypes. However, only passages 4 through 9 were used for FcαR investigation, as the macrophage-like fibroblasts are known to disappear from culture after passage 3 [96]. This suggests that the observed staining of FcαR is predominantly, if not exclusively due to FLS, not the macrophage-like lineage. Furthermore, data obtained by Erika Gibon and Liz Henson (JRE lab, Appendix 1, unpublished data) supports the idea FLS can bind and internalize IgA crosslinked by F(ab')2 pseudo immune complexes, but not mIgA suggesting

that receptor aggregation is necessary for receptor internalization like the FcαR found on neutrophils and monocytes. This observation suggests that FcαR on FLS has the ability to bind and internalize its ligand. The ability to bind and internalize IgA implies a novel mechanism by which FcαR on FLS may contribute to RA inflammation. The presence of FcαR on FLS may contribute to the overall maintenance of the joint microenvironment through the uptake of IgA complexes. The role in which FcαR plays in the development of various arthropathies remains to be elucidated

The role of the FcαR in the synovium remains to be determined. In phagocytes, this receptor plays an important role in clearance of immune complexes and enhances phagocytosis of IgA-coated particles or bacteria by neutrophils and macrophages [104, 106]. Priming of these cells increases the level of IgA-mediated phagocytosis of IgA coated particles suggesting the importance of FcαR in clearance of immune complexes. Activation and subsequent internalization of the FcαR is dependant upon cell surface receptor crosslinking [186]. Using the murine B cell line IIA1.6 transfected with FcαR Reterink et al, demonstrated no significant binding when incubated with mIgA, however incubation with aggregated IgA resulted in receptor binding greater than 90% [63]. Depending upon which isoform of IgA the immune complex is associated

with, phagocytosis will result in a pro-inflammatory or anti-inflammatory response. The receptor is reported to have anti-inflammatory effects because serum IgA immune complexes may be phagocytosed with little resulting inflammation and mIgA inhibits functions such as oxidative burst and cytokine release [187]. mIgA is extremely abundant and there is sufficient concentrations in the blood to completely bind to all FcαR sites on circulating human neutrophils however, such binding does not induce an inflammatory response until the receptor is cross linked [120, 188]. In contrast, pIgA and IgA immune complexes effectively activate the FcαR on blood leukocytes, resulting in an array of immune effector functions. The presence of pIgA in the non-mucosal site of the joint binding to FLS FcαR may be responsible, in part for the array of cytokines released in RA.

FcαR is a low affinity receptor that only binds mIgA transiently on monocytes, whereas pIgA and IgA immune complexes bind avidly [154] The live cell uptake confocal microscopy experiment (Appendix 1) suggests that binding by FLS occurs in the same manner as white blood cells, because crosslinking of the FLS FcαR is necessary for internalization. In RA, resident plasma cells of the synovium produce immunoglobulins such as IgA-RF that form immune complexes. These complexes may trigger FcαR aggregation on FLS allowing

them to be internalized, suggesting a role for immune complex clearance by FLS. Although FLS FcαR may bind crosslinked IgA, the downstream consequences of IgA binding via FcαR-mediated endocytosis in FLS, remains to be determined.

Different splice variants of FcαR have been reported, as IgA receptor biology differs among macrophages of several different organs. The FcαR a.1 isoform is expressed on blood monocytes, cultured blood macrophages and peritoneal macrophages, the a.2 isofom is found exclusively on alveolar macrophages [189]. Human alveolar macrophages stimulated with PMA undergo respiratory burst when treated with pIgA and sIgA and a subsequent increase in TNF-α production [117]. Conversely, macrophages from the gastrointestinal tract, which produces considerable amounts of pIgA (66 mg/kg body weight per day [190]) , fail to express FcαR suggesting a programmed anti-inflammatory system for protection of mucosal integrity depending on which IgA isoform is presented [173]. The gastrointestinal tract is an immune tolerant organ; a gastrointestinal tract (GI) derived macrophage response would result in unnecessary immune response and subsequent inflammation if GI macrophages expressed FcαR. This work demonstrates that FLS express FcαR and therefore possess the machinery necessary to participate in clearance of IgA type immune complexes such as IgA-RF.

Mucosal and synovial inflammation is associated with a variety of diseases in which production of IgA increases the formation of IgA immune complexes. Clinical studies describe the development of reactive arthritis in the setting of mucosal infection. For example, patients infected with enteric pathogens such as *Yersinia, Campylobacter,* and *Salmonella* or with genitourinary pathogens such as *C. trachomatis* may develop reactive arthritis [191]. It is interesting that over 90% of patients who develop chronic forms of reactive arthritis are positive for HLA-B_{27} positivity. IgA-mediated mucosal immunity has been proposed to play a role in precipitating reactive arthritis in genetically susceptible individuals [192, 193]. Mucosal infection by one of these arthritogenic organisms can result in dissemination of bacterial material to synovial tissues [194-196]. Live *Chlamydia* in synovial tissue and *C. trachomatis* nucleic acid in the synovial fluid has been detected in patients with reactive arthritis [197], suggesting that the cells of the synovium may serve as a reservoir for microorganisms. While the provocative agent may have disappeared from the gut by the time joint symptoms arise, patients develop oligoarthritis, most commonly of the lower extremities and involve the joints where FLS are present. Without effective clearance, the resident pathogens may alter host immune response to perpetuate synovitis. It has been suggested by Schoen et al. that IgA complexes that arrive in the joint via

the blood stream would also activate synovial macrophages, resulting in the release of TNF-α and IL-1, further contributing to inflammation [198].

Comparison of reactive arthritis with inflammatory bowel disease (IBD) associated arthritis suggests that a common antigen is shared by reactive arthritis associated bacteria and bacteria of the gut flora. In IBD, inflammation of the intestine compromises the integrity of tight junctions of the mucosal epithelium cell membrane, resulting in a leaky epithelium that allows for free passage of lumenal contents into the lamina propria [199]. Salmi et al demonstrated that different leukocyte populations derived from inflamed gut avidly bind to synovial vessels using a distinct repertoire of adhesion molecules. The direct cellular interactions between inflammatory cells of patients with ulcerative colitis or Crohn's disease and cells of the synovium comprises a significant pathogenic element in the development of enteropathic arthritis [199]. Thus mucosal responses appear to play a role in the pathogenesis of a spectrum of inflammatory arthropathies. FcαR expression by FLS may be another missing link that connects breaches in the mucosal epithelial barrier with subsequent joint inflammation.

Given the close clinical associations between reactive arthritis and RA, a pathogenic role for bacteria has been proposed in RA. In patients with active RA, an increase in antibodies to *P. mirabilis* has been demonstrated, suggesting that the presence of these infectious agents would elicit an immune response resulting in the formation of IgA complexes via bacterial opsonization. FLS in RA could play a major role trying to clear this chronic bacterial infection. Abundant pIgA production occurs at the mucosal interface, binding invading pathogens and forming immune complexes. An arthritogenic gastrointestinal or genitourinary microorganism infection would trigger an immune response, generating peptides within host antigen presenting cells, resulting in dissemination of the microbial antigens to the synovium. In addition, breaches of the gastrointestinal epithelial lining could result in IgA-complexed pathogens or immune complexes entering the systemic circulation and theoretically depositing in the synovium via the FcαR. Thus, in some cases an IgA-associated immune complex binds to the FcαR on FLS, and in others the complex containing a pathogen binds to the FLS FcαR. The microenvironment of the gut depends heavily on IgA to protect against invading microogranisms and to neutralize toxins and infectious organisms. By whatever mechanism, invading pathogens are able to leave the digestive system and make their way throughout the body. In RA, FcαR on FLS might act as one of the synovial tissue's first line of defense

for clearance of bacteria or immune complexes that perpetuate the chronic inflammation.

This work demonstrates that RA and OA FLS express the FcαR both in cell culture and in tissue. We propose that FcαR by FLS is intrinsic to the biology of these cells because all RA and OA patients tested were positive for the receptor, but not fibroblasts from uninflamed lung or skin. To further confirm this finding and address the issue of whether FcαR expression is common to all fibroblasts requires more investigation in FcαR expression. Studies in which dermal and lung fibroblasts are challenged with a variety of cytokines from the RA inflammatory milieu, such as IL-4, IL-6 and TNF-α, then checked for FcαR expression would fully answer if FcαR is unique to synovial cell biology or if FcαR is transiently expressed in the context of an inflammatory environment.

The worse prognosis associated with IgA-RF may somehow be the result of FcαR expression on FLS. It is possible that during mucosal infection, increasing levels of IgA and the resulting immune complexes access the systemic circulation and bind to the FcαR in the joint, contributing to the initiation and perpetuation of RA. The role of FcαR in clearance of immune complexes in RA pathology remains to be determined and the discovery of IgA receptors on FLS may open

the way for new understandings of the mechanisms involved in a variety of arthropathies associated with mucosal inflammation. It is possible that FLS FcαR plays an auxiliary role in joint maintenance by clearing IgA-associated complexes. However, when these complexes increase in size and affinity for the FLS FcαR, the resulting effector functions could play a role in perpetuating the inflammatory process of RA. FcαR expression in the joint might also account for the onset of arthritis after infection in the gastrointestinal or genitourinary tract. While this work clearly establishes that FLS express FcαR further studies are required to define its role in arthritis.

In conclusion, this work clearly demonstrates the presence of FcαR on RA and OA synovial fibroblasts both *in vitro* and *in vivo,* but not on fibroblasts of non-synovial origin. This conclusion is supported by specific immunofluorescent staining in both RA and OA FLS cells. This expression is not transient because it persists in cultured cells through passage 6 and occurs in every patients' FLS cells. Immunohistochemistry of fresh frozen tissue confirmed FcαR expression of the synovial lining *in vivo* in both RA and OA patients. Additional studies are required to further characterize FcαR expression in FLS and determine how IgA binding to FcαR on FLS contributes to the RA inflammatory process.

Appendix

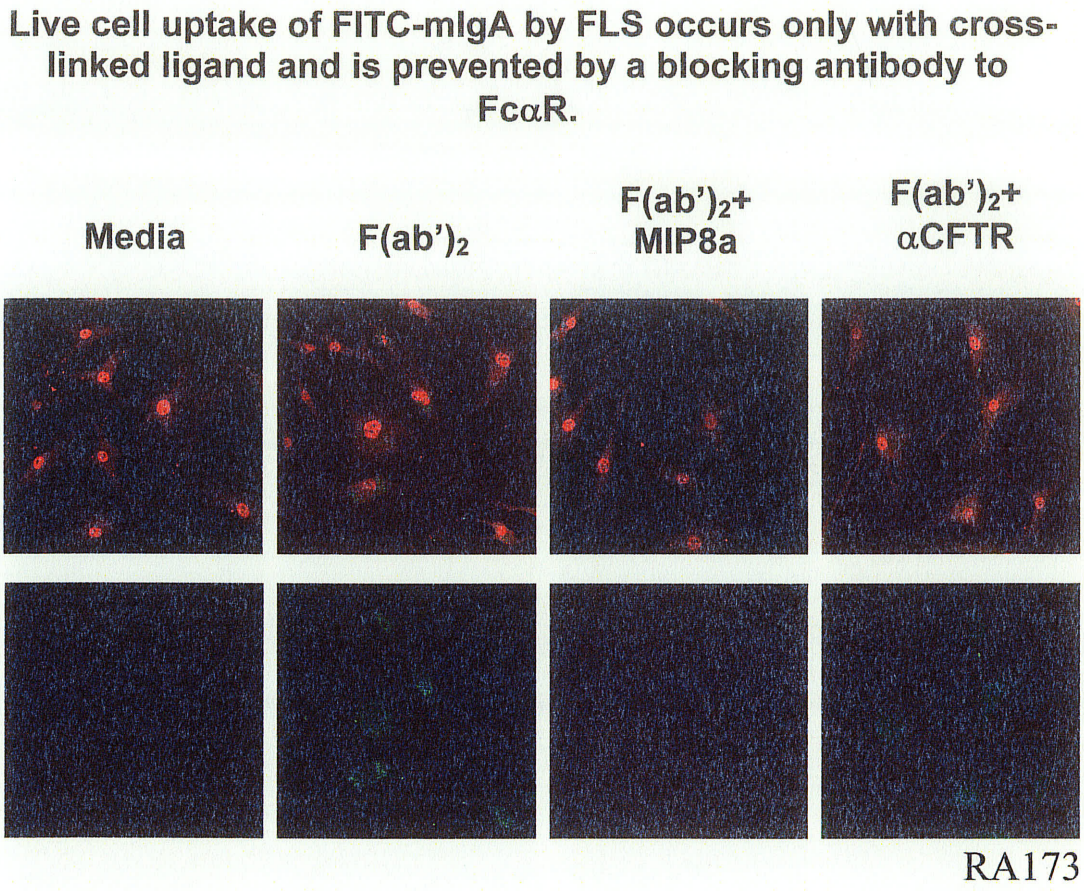

Figure 1 - Live cell uptake of FITC-mIgA by FLS occurs only with cross-linked ligand and is prevented by a blocking antibody to FcαR. RA FLS were incubated overnight with FITC-mIgA in media alone, with anti-IgA F(ab')2, with anti-IgA F(ab')2 and MIP8a blocking antibody to FcαR, or with anti-IgA F(ab')2 and an isotype control antibody (to CFTR). Cells were washed, fixed, stained for actin (red), and mounted with DAPI for imaging with confocal microscopy. Top row shows red and green images merged while the bottom row shows the green image alone. (Red nucleus is bleed-through from DAPI on this microscope.) Image courtesy of Erika Gibson and Liz Henson on behalf of the Richman-Eisenstat lab.

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