CELLULAR INTERACTIONS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS: CROSS-TALK BETWEEN T CELLS AND FIBROBLAST-LIKE SYNOVIOCYTES

by

Chinh Nguyen Tran

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Doctoral Committee:

Professor David A. Fox, Chair
Professor Deborah L. Gumucio
Professor Steven L. Kunkel
Professor Lloyd M. Stoolman
Associate Professor Lilli M. Petruzzelli
Dedication

To Mom, Dad, and Mike
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1-1. Clinical Overview of Rheumatoid Arthritis

The characteristic feature of rheumatoid arthritis (RA) is the development of joint
damage and deformity due to chronic inflammation of the synovium, most notably swan-
neck deformities of the distal phalanges. Histologically this is manifested by a
hyperproliferation of resident joint structural cells, which are intermixed with invading
immune cells, forming a tissue termed the pannus. The RA pannus is thought to
responsible for the cartilage and bone erosion leading to the eventual remodeling and
disfigurement of the joint. However, RA is also a systemic inflammatory disease and can
affect many organ systems outside the joint [1].

Despite RA’s profound impact on the body, it is a disease that defies absolute
characterization, as there are no definitive features that are known to be entirely unique to
it, and must be defined by clinical diagnostic criteria. The 1987 revised criteria for the
classification of RA are typically used to diagnosis this disease [2]. These criteria are efficient in separating RA from other inflammatory arthritides and have a sensitivity and specificity of 90% [3]. Based on these criteria, the female to male ratio of RA is approximately 2 to 1, and it has a global prevalence of approximately 1% [4]. Beyond the biologic impacts of RA, it has been shown to have profound negative effects on quality of life as the disease progresses: The rate of disability by 10 years after the onset of disease is at least 50%, RA patients are hospitalized twice as frequently, and median life expectancy is reduced 7 years for men and 3 years for women [4].

1-2. T cell influences in RA

Currently, the etiology of RA remains elusive. Several mechanisms have been proposed (T cell mediated, humoral/B cell mediated, cytokine networks, microbial pathogens), but no consistent factor documented in all cases of RA [5].

T cells in RA etiology

Several lines of evidence suggest a T cell mechanism may be involved in RA pathogenesis [6]. Strong circumstantial evidence comes from the observation that the T cell is the most prominent infiltrating lymphocyte within the pannus. Enumeration of the invading lymphocyte population from digested synovial tissues showed that approximately 80% of the cells were T cells [7-9].

Another observation supporting the role of the T cell in RA pathogenesis, comes from the MHC II allele, which CD4+ T cells recognize. HLA-DR4 (*0401) is an MHC II allele
that has been closely associated with RA incidence within the Caucasian population [10]. Several other MHC alleles, were also found to be associated with RA, and the “shared epitope” hypothesis of RA etiology was proposed based upon the amino acid residues from 70–74 of the DR β chain [11]. HLA-DR alleles associated with RA were found to possess QKRAA or QRRAA as a motif within this region. Possession of an allele containing the shared epitope also correlated with increased disease severity [12-14]. It has been hypothesized that the shared epitope can present a specific pattern of autoantigenic peptides distinct from other MHC II alleles [12]. Consequently, it has also been proposed that the T cell repertoire is shaped by education during thymic development against the shared epitope and that this skewed T cell population could contribute to RA pathology [15, 16]. It has even been suggested that the shared epitope itself could be presented by other MHC II alleles, thus creating a population of autoreactive T cells that would be activated by re-encountering the QKRAA motif within proteins of microbial pathogens [17, 18].

Unfortunately, no autoantigen has been consistently reported in RA nor has a pathogenic T cell clone been positively identified. Additionally, clinical trials testing T cell depletion by antibody directed at CD4 did not produce robust therapeutic effects [19, 20].

However, recent trials have revived the concept of targeting the T cell as a therapeutic mechanism. The CD28 receptor on T cells receives co-stimulatory activation signals when bound by its ligand B7-1/CD80 [21-23]. To limit uncontrolled T cell activation, stimulated T cells upregulate expression of a second receptor for B7-1, CTLA-4. CTLA-4
is an inhibitory receptor and has approximately 1000 fold greater affinity for B7-1 relative to CD28, thus enabling it to out-compete activating signals [24]. By combining the human extracellular domain of CTLA-4 with the Fc portion of human IgG1, CTLA-4Ig was created. In clinical trials, CTLA-4Ig (Abatacept) significantly improved RA patient outcomes as a monotherapy [25, 26], in combination with methotrexate [27], and in patients refractory to anti-TNF therapy [28]. These findings suggest that despite the negative results from T cell depletion trials, T cell modulation by inhibition of activation signals is a viable mechanism to treat RA, and that T cell activation contributes to RA clinical manifestations. The rapidity of the onset of clinical benefit in controlled trials of CTLA-4-Ig indicates that T cells contribute actively to the inflammatory manifestations of this disease throughout its course, not just at disease initiation.

With these considerations in mind, better characterization of the nature of T cell function in RA is an important research goal.

*T cell characterization in RA*

Two ways to better understand the T cells in RA is to characterize them by surface marker expression and by cytokine production. The CD4 marker characterizes the helper subset of T cells normally responsible for full adaptive activation of the immune system. The CD4 T cell subset in RA displays markers indicative of activation and in RA cytokines skew towards a Type 1 immune profile [29, 30]. Compared to CD4 T cells from peripheral blood, synovial CD4 T cells produced more IFNγ, a prototypic type I cytokine, and paradoxically IL-10, a normally inhibitory cytokine [31]. Characterization of CD27 surface expression (who’s loss is a marker for prolonged T cell activation [32,

4
33]) on CD4 naïve and memory T cells, found that early activated CD4 T cells were found in perivascular aggregates of synovium and that the diffuse synovial T cells displayed the late activation loss of CD27 [34], suggesting a pattern of naïve CD4 T cells from peripheral blood progressing to late activation as cells migrate into the synovium, similar to what would be expected from T cells migrating from peripheral blood through a lymph node.

Another peculiar characteristic of RA T lymphocytes, is the expansion of CD4+CD28null population in some patients. These cells display markers of autoreactivity [35], are not anergic [35], are resistant to apoptosis [36], and show signs of oligoclonality [37]. However the CD28 pathway has been shown to be intact in T cells hyporesponsive to CD3 activation [38] and CTLA-4Ig has been found to be therapeutic in clinical trials, reinforcing the concept that no one T cell population can easily account for all of the roles of T cells in human RA.

Looking at another T cell subset, the CD8 T cells are primarily associated with cell-mediated cytotoxic responses towards virally infected cells and malignancies. In RA, CD8 T cells have been shown to be crucial for maintenance of ectopic germinal centers in synovial pannus lesions [39]. Using human synovial tissue transplanted into SCID mice, it was demonstrated CD8 T cells were crucial for the maintenance of germinal centers and antibody production from human explants [40]. Not surprisingly the CD8 T cells expressed CD40L and were associated with lymphotoxin α/β production (a cytokine associated with lymph node maintenance).
While the characterization of RA T cells continues, the exact type of pathologic T cell which could be responsible for disease initiation and maintenance is not adequately understood.

1-3. B cell influence in RA

Given the equivocal evidence for the primacy of T cells in pathogenesis and maintenance of RA, other cell types deserve evaluation. Though the B cell is not as numerous as the T cell in pannus lesions, it is found within those tissues. The initial suggestion that RA might be caused by a B cell abnormality was based on the observation of high levels of circulating autoantibodies in RA patients. Rheumatoid factors (RF) are autoantibodies of any isotype with specificity for the Fc portion of IgG. RF is generated during most immune responses, but RF persistence is associated with autoimmune disease [41]. RF has been shown to fix and activate complement and could potentially contribute to local inflammation [42, 43]. RF has not been shown to be a causative agent in arthritis when transferred into healthy individuals [44]. But, RF is a positive predictor of disease severity and progression in RA [45]. When combined with autoantibodies directed against cyclic citrullinated peptides (anti-CCP), RF and anti-CCP become highly specific for rheumatoid arthritis and negative clinical outcomes [3, 46-48]. Citrullinated proteins are proteins that have had lysine residues converted to citrulline residues by a deiminase reaction catalyzed by peptidylarginine deiminase [48].

Despite little evidence that autoantibodies are pathogenic in RA, B cell depletion trials have shown positive clinical results without the development of hypogammaglobulinemia
[49, 50]. This suggests that in responsive patients, B cell antibody production might not be the only way that B cells contribute to RA pathogenesis, and that perhaps B cells may also participate in disease progress through antigen presentation, cytokine production, or cell-cell contact interactions.

It should be noted that there are rare case reports of patients suffering from agammaglobulinaemia or hypogammaglobinaemia who meet the ACR criteria for RA [51, 52]. These patients lack mature B cells and circulating immunoglobulins, yet still develop an inflammatory synovitis characterized by a T cell infiltrate as is seen in RA.

1-4. Microbial infections – do they have a role in RA?

Taking a step back, since a precise autoimmune, T cell or B cell mediated mechanism of RA etiology has been not proven, it is possibly that RA is a manifestation of an aberrant immune response to an invading or local pathogen. The evidence for microbial participation in RA pathogenesis is suggestive, but not conclusive. A variety of microorganisms or components of microorganisms can be detected in arthritic joints. It is suggested that perhaps, following an initial microbial infection in the joint or elsewhere in the body, immune responses might be directed into an aberrant reaction in the joint. Some viruses know to cause an inflammatory arthritis, and potentially affecting RA are parvovirus B19 [53, 54], Epstein-Barr virus (EBV) [55-57], and herpes simplex [58]. But there is also evidence against an RA association with viral infection as measured by viral load[58-60], and no evidence that RA is ameliorated by anti-viral agents.
Some bacterial pathogens implicated in RA are E. coli and mycoplasma. Mycoplasma DNA has been detected in synovial fluid and tissue [61, 62]. Several clinical trials have documented positive clinical outcomes using antibiotics alone [63, 64] or in combination with methotrexate [65]. However, these reports do not confirm a mechanism of action for the antibiotics used, typically tetracyclines, which can also inhibit collagenases.

E. coli heat-shock protein dnaJ contains the shared epitope sequence and synovial cells have been reported to respond to dnaJ [17]. Similarly EBV protein gp110 was also found to contain the shared epitope [66]. But again, the evidence for a microbial association with RA is inconsistent through all disease incidences.

1-5. Cytokine networks in RA

In contrast to the etiology of RA, the importance of the cytokine milieu in the joint has been better described clinically. Cytokine production in RA is robust and varied. Proinflammatory, immunoregulatory, chemotactic, and mitogenic cytokines have all been documented at the mRNA and/or protein level from RA tissues [67]. The pathogenicity of RA cytokine production was convincingly demonstrated by dramatic reduction in disease severity after cytokine neutralization in clinical trials. The first dramatic responses were seen with TNFα blockade with monoclonal antibodies [68, 69]. From these first trials new cytokine targets have emerged. Inhibition of IL-1 signaling pathways utilizing recombinant IL-1 receptor antagonist slowed radiographic progression of RA [70, 71]. Blockade of IL-6 by neutralizing antibody resulted in reduced clinical severity of RA symptoms [72, 73]. Unfortunately, cytokine neutralization is not curative and treatment cessation may lead to recurrence of symptoms. This indicates that the
underlying inflammatory process is still active despite the blockade of soluble inflammatory mediators.

1.6 Fibroblast-like synoviocytes: active participants in joint destruction

Normal synovial fibroblast function

Looking the joint inflammation it is important not to solely focus on the invading lymphocytes, leukocytes, and pathogens. The resident cells native to the joint are also participants in the complex cell-cell interactions of the pannus. The normal synovium morphology is composed of an intimal layer 1-3 cells thick atop a subintima [74]. The intima does not contain a basement membrane, is exposed to the joint space, and is populated by two cell types [75]. They have been termed type A (synovial macrophage, macrophage-like synoviocyte, MLS) and type B (synovial fibroblast, fibroblast-like synoviocytes, FLS) synoviocytes. FLS help maintain joint homeostasis by synthesis of protein products that protect the joint space and control synovial fluid volume [75].

FLS are active participants in RA joint destruction

Far from being passive structural cells responding to an inflammatory environment of cells and cytokines, the evidence indicates that FLS are active participants in joint destruction and maintenance of inflammation [75-77]. The most convincing evidence of the innate aggressive nature of RA FLS comes from transplant of human cartilage with pure populations of RA FLS into SCID mice [78]. RA FLS were observed invading cartilage in the absence of lymphocyte or cytokine stimulation, showing an innate trait of
cartilage degradation. Osteoarthritis (OA) FLS and dermal fibroblasts do not share this phenotype.

**FLS degradative enzymes**

FLS elaborate many degradative enzymes which can breakdown the extracellular matrix and connective tissue. Cultured FLS produce collagenase (MMP-1), stromelysin (MMP-3), and MMP-10 de novo [79, 80], and secretion of these enzymes is correlated with invasive ability through artificial matrices. IL-1 and TNFα stimulation enhanced the production of stromelysin and collagenase from FLS, but did not enhance production of TIMP, implying conditions within the RA synovium favor production of factors leading to destruction of tissues versus tissue protection. Other pro-inflammatory factors are also present within the synovium apart from IL-1 and TNFα, including IL-6, TGFβ, and other mediators. The multiplicity of factors which promote FLS destruction of cartilage is seen by retention of cartilage invasive properties after stimulation of FLS by synovial fluid incubated with neutralizing antibodies to the aforementioned cytokines [81]. FLS interaction with the extracellular matrix has also been shown to enhance MMP production [82]. A recent study using stable knockdown of MT1-MMP in FLS by retrovirus, has shown a reduced but not eliminated capacity of transfectants to invade cartilage in the SCID transplant model [83], suggesting that MT1-MMP is a major contributor to FLS cartilage destruction.
An attractive mechanism for synovial hyperplasia seen in RA would be simply the increased mitotic rate of FLS. FLS certainly proliferate when cultured ex vivo and cultures can be maintained for months [76]. The lining layer cells in RA synovium tissues also showed greater thymidine incorporation relative to OA tissues [84]. Within the RA joint there are also elevated levels of growth stimulating cytokines [75]. However, phenotypic characterization of the thymidine incorporating cells from synovial tissues revealed that thymidine was mostly incorporated by CD8 cells [85]. Similarly while 5% of cultured FLS showed positive DNA synthesis as an indicator of proliferation, only 1% of synovial lining cells in vivo displayed active DNA synthesis, suggesting mechanisms are present in the synovium to retard FLS growth [86].

An alternative method to explain increased FLS numbers in synovial lesions is defective apoptosis. The role of Fas (CD95) expression on RA FLS remains unclear. Initial studies indicated that stimulation by TNFα sensitized FLS to apoptosis through Fas ligation through the downregulation of FLIP [87, 88]. More recent studies reported results directly contradictory to the previous findings. These studies showed that FLIP levels are enhanced after FLS are stimulated by TNFα [89], and that FAS ligation on TNFα stimulated FLS lead to proinflammatory transcription factor activation, not apoptosis [89]. The studies indicating TNFα sensitization of FLS to apoptotic signals [87, 88], possibly could be due to FLS line variability in sensitivity to apoptosis [89]. Apoptotic resistance leading to increased inflammatory capability could explain increased FLS numbers in the RA pannus.
FLS cytokines and chemokines

FLS release a plethora of soluble mediators involved in local inflammation, lymphocyte recruitment, angiogenesis, cellular activation and differentiation [75-77, 90-92]. The mediators released from FLS are indicative of their inflammatory state. Culture supernatants from FLS stimulate cellular replication in bioassays [93]. Comparing cDNA from RA FLS and OA FLS revealed a pattern of inflammatory cytokine production. A potent cytokine produced spontaneously by RA FLS is IL-15 [94, 95]. Synovial T cells migrate and proliferate in response to stimulation with IL-15 [96]. IL-15 enhances effector functions of T cells in the induction of TNFα by monocytes [97]. FLS also produce the osteoclast differentiation factor RANKL, and can induce osteoclast development from peripheral blood monocytes [98].

1.7 T cell-FLS interactions

FLS activation by T cells

Given the extensive infiltration of the synovial pannus by T cells, interactions between invading T cells and FLS could be important in RA pathogenesis. Based upon the heavy lymphocytic infiltration of synovial membranes, early experiments recapitulated T cell adherence to FLS via antigen independent mechanisms in vitro. Using unstimulated FLS, it was noted that activated T cells bound more vigorously to FLS than did resting T cells, and that this adhesive interaction could be partially inhibited by blocking the CD2/LFA-3 interaction [99]. When FLS are stimulated with inflammatory cytokines (such as IFNγ,
TNFα, or IL-1β) the percentage of adherent T cells increased [100, 101]. These cytokines induced much greater expression of ICAM-1 (intercellular adhesion molecule-1, CD54) [101] and the adherent T cell subset displayed a higher expression of the ICAM-1 counter-receptor LFA-1 (leukocyte functional antigen-1, CD11a/CD18) [100]. These studies note the decreased capacity of resting T cells to bind to FLS, which is probably due to the short time course of the adhesion assay.

Expanding beyond adhesion assays, experiments on cocultures of T cells and FLS demonstrated that interaction between these cell types could activate FLS. Phorbol myristate acetate (PMA) activated T cells triggered IL-1β transcription in FLS and release of IL-1 into culture supernatants, dependent on interactions between LFA-1 and ICAM-1 [102]. LFA-1/ICAM-1 interactions are important in T cell adhesion and immunologic synapse formation between T cells and conventional antigen-presenting cells (APC). It was noted that resting T cells did not adhere within a time course of 30 minutes, while PMA-treated T cells had firm LFA-1/ICAM-1 mediated adhesion.

Potential limitations of this study involve its use of SV-40 transformed FLS lines and PMA treatment of T cells. The transformed FLS constitutively expressed ICAM-1 at a high level that was uninfluenced by IL-1β, suggesting a preactivated state, while PMA activation of T cells might not be physiologically relevant. Nonetheless, this study demonstrates the potential for antigen-independent interaction of T cells with FLS, leading to inflammatory mediator production. More physiologic activated T cells have also been shown to activate FLS. Comparing IL-2 expanded T cells from both RA synovial fluid and normal peripheral blood as a stimulus for RA FLS, it was found that
both T cell types produced factors that enhanced FLS growth, but that clones derived from synovial fluid produced more [103]. The growth effect of these factors was not attributed to IFN$\gamma$ or IL-2.

Work in our laboratory has also focused on the interaction between T cells and FLS. It was found that even resting T cells could stimulate FLS. Using resting T cells as a stimulus, we have documented activation of FLS and release of proinflammatory mediators [104]. We found that autologous or allogeneic resting T cells have similar activating potential on FLS. This effector function of resting T cells is not restricted to a particular T cell population. Various subsets of T cells, CD4+, CD8+, CD45RO+, and CD45RA+ all had comparable ability to induce synovial fibroblast activation. Activated FLS showed induction or augmentation of mRNA for stromelysin, IL-6, and IL-8, gene products important in joint inflammation and joint destruction. Furthermore, increased production of IL-6 and IL-8 was quantitated both by ELISA and by intracellular cytokine staining. Another striking observation was that the T cell specific cytokine IL-17 synergized with T cells to active FLS. These coculture systems spanned up to 24 hours and no T cell activation was noted, by assessing upregulation of CD154/CD40L or CD69. Thus, using resting T cells as a stimulus on untransformed FLS lines, we documented further evidence of T cell-FLS antigen independent interactions resulting in induction of an inflammatory profile of FLS.

Two studies have recently provided further support for a role of T cell activation of FLS in antigen independent systems. One study used unstimulated purified T cells [105] and
the other used collagen type II (CII) responsive T cells [106]. Both studies cite the importance of IL-15 expression by FLS and its upregulation after stimulation by T cells [105, 106]. The first study utilized unstimulated T cells purified from peripheral blood or RA synovial fluid. After 96 hours of coculture with these T cells, induction of ICAM-1, IL-8, IL-6, and IL-15 was noted on the FLS [105]. This induction was dependent on cell-cell contact, as evident when transwell inserts separating T cells from FLS blocked activation. Similarly blockade of CD69, CD11a, IL-17, TNFα, and IFNγ also inhibited activation of FLS. Another interesting observation was that T cells showed signs of activation by induction of CD69, CD25, IL-17, TNFα and IFNγ after coculture with FLS [105]. This is contrary to our findings described above [104], and the discrepancy is explained by the duration of coculture, 96 versus 24 hours. T cell responses were blunted by blocking antibodies towards ICAM-1 and IL-15, demonstrating the importance of FLS factors for T cell stimulation. When CII activated T cells were used as the stimulus, FLS displayed production of TNFα, IL-15, and IL18 [106]. These CII activated T cells were generated by culture of T cells with bovine CII and autologous irradiated APC for extended periods. An intriguing observation was that the increased length of stimulation of CII before coculture with FLS resulted in increased IL-17 and IFNγ production by T cells, which implies the level of T cell activation is proportional to the effector function.

**Cross-talk between T cells and FLS**

FLS are not the only “beneficiaries” in cocultures with T cells. IL-2 expanded T cells rapidly die after withdrawal of cytokine stimulation. FLS were able to rescue IL-2 expanded T cells from cytokine deprivation [107]. This rescue could not be explained by
FLS cytokine production and was thought to be mediated by an unknown stromal factor. RA synovial T cells display a phenotype indicative of susceptibility to apoptosis: low Bcl-2, high Bax, and high Fas [108]. However, these T cells are resistant to apoptosis relative to T cells from crystal arthritis, but die when removed from the joint. Rescue of both RA synovial T cells and gout T cells occurred when they were cultured with FLS. Integrin interactions are implicated in the survival signals, as RGD peptide stimulation enhanced T cell longevity [108].

Long term cultures of RA FLS with autologous T cells results not only in long term T cell survival, but also T cell expansion [109]. This mechanism behind this observation was attributed to TSP1 on FLS stimulating CD47 on T cells in a costimulatory fashion distinct from CD28 [109]. However, a role for other receptor-ligand interactions is not excluded.

As previously stated, T cell coculture with FLS results in the upregulation of the activation markers CD69 [105, 110] and CD25 [105], and this process is dependent on FLS IL-15 and ICAM-1.

**FLS as APC**

The above observations describing FLS and T cell cross signaling were conducted using antigen free systems. Studies have also explored the role of FLS as APC for T cells. Initial experiments using human dermal fibroblasts showed that fibroblasts were poor generators of allogeneic responses [111]. This defect was not due to inadequate
expression of MHC II, but was due to lack of an accessory molecule that could be provided by conventional APC. However, it was noted that dermal fibroblasts could stimulate previously activated allogeneic T cells [111]. Expanding on this work, the capacity of dermal fibroblasts to function in antigen presentation was evaluated. Dermal fibroblasts were able to process antigen, but did not function well as APC without accessory cell help [112]. In both these studies INFγ was used to induce MHC II, and antigens relevant to RA were not evaluated. These studies do document fibroblast expression of functional MHC II. Another report documents costimulatory properties of FLS dependent on IFNγ or IL-1 stimulation [113].

FLS of RA synovium express high levels of MHC II ex vivo [114], indicating the potential for antigen presentation by FLS in RA. Early studies suggest that FLS can process antigen similarly to professional APC [115]. In those experiments, FLS were able to take up and process various antigens, and present them to T cell clones via an MHC II restricted mechanism [115]. This gives support to potential antigen specific interaction between T cells and FLS (as APC). However, antigens relevant to RA were not assayed nor were observed responses robust.

Our laboratory also observed MHC II dependent signaling between FLS and T cells. Superantigens activate FLS to secrete inflammatory mediators and potentially participate in RA pathology. Thus, we assessed the ability of IFNγ treated FLS to present superantigens to T cells [116]. FLS can indeed present superantigens, inducing resting T cells to proliferate. T cell proliferation to superantigens was dependent on MHC II, CD2,
LFA-1, and the cytokine IL-2. This study provides a mechanism for FLS to activate naïve T cells, but does not demonstrate an “antigen specific” response to autoantigens or to peptide antigens that are presented in the cleft of the MHC molecule.

There is also evidence that FLS might not activate T cells, but instead induce anergy [110]. These experiments assessed the APC and allostimulatory functions of FLS. Similar to previous studies, FLS were able to load antigen onto MHC II. However, allogeneic responses depended upon the addition of accessory cells expressing CD80, and blockade of CD80 abolished the response. When FLS without accessory cells were cultured with T cells, they adopted a phenotype resembling anergy: upregulation of CD25, reduced proliferation, and reconstitution of proliferation by exogenous IL-2 (21). Similar to other studies, CD69 on T cells was also upregulated after T cell culture with FLS. This study implies that FLS cause anergy due to a lack of costimulatory molecules, but that bystander cells expressing costimulatory molecules could overcome this. The potential for accessory costimulation exists abundantly within RA synovium due to the close proximity of FLS with B cells, macrophages, and dendritic cells.

1-8. Thesis objective

Given the unknown etiology of RA, the data supporting active FLS participation in pathology, and the unusual interactions between T cell and FLS, this thesis sought to explore the mechanisms involved in T cell-FLS bidirectional signaling as a pathologic process in RA.
References


70. Jiang, Y., et al., *A multicenter, double-blind, dose-ranging, randomized, placebo-controlled study of recombinant human interleukin-1 receptor antagonist in*


2. Antigen Presenting Cell Function of FLS for Autoantigens

2-1. Summary

The abundance of both T lymphocytes and fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA) synovium suggests that interactions between these two cell types could be important in the pathogenesis of RA. FLS express high levels of MHC II in vivo, suggesting that these cells might present peptide antigens to T cells. We found that human FLS could present peptides from the autoantigens human cartilage gp39 (HCgp39) and human collagen II (hCII) to antigen-specific MHC-restricted T cell hybridomas derived from a human HLA-DR4 transgenic mouse. This response required pretreatment of FLS with IFNγ, showed MHC restriction, and was dependent on human MHC II and murine CD4 for effective antigen presentation. Furthermore, FLS were able to extract and present antigens found within human synovial fluid to both the HCgp39 and hCII T cell hybridomas, in an IFNγ-dependent and MHC-restricted manner. In the
context of inflamed synovial tissues, FLS may be an important and hitherto overlooked subset of APCs that contribute to joint pathology.

2-2. Introduction

In contrast to the usual functions of fibroblasts, FLS have been hypothesized to be important in joint inflammation and destruction [1-4]. In RA, FLS adopt an inflammatory phenotype, and secrete cytokines, proteases and other mediators. FLS secrete chemokines that enhance leukocyte recruitment through synovial endothelium [5]. The most abundant infiltrating leukocyte is the T lymphocyte, which may come into close proximity to FLS, and interaction between these cells could contribute to RA pathology. When FLS and T cells are co-cultured in vitro, activation of both cell types is observed. FLS produce inflammatory mediators such as IL-6, IL-8, and prostaglandin E2 [6], and T cells upregulate the activation markers CD69 and CD25 [7]. The functional consequences of these co-cultures mirror the molecular characteristics of RA synovium, which suggests that similar interactions could be occurring within the inflamed RA joint. These experiments were performed without addition of antigen; thus, the bidirectional activation observed was by mechanisms independent of exogenous antigen presentation.

The etiology of RA is still unknown, although several hypotheses have been proposed; however, a strong genetic contribution to RA is well established. Within the Caucasian population, the most important genetic association is with the MHC locus -- specifically with HLA-DR4 and other closely related DR alleles [8]. One of the RA associated DR4 alleles, HLA-DRB1 (*0401), may also be predictor of disease severity, with homozygous individuals showing worse clinical outcomes [9-12]. The RA associated DR4 alleles all
share a common motif near the peptide binding groove at residues 70-74 of the β chain [13-15]. When human HLA-DRB1 (*0401) is expressed as a transgene in mice, it confers susceptibility to induction of arthritis by immunization with certain autoantigens, even on otherwise genetically resistant backgrounds [16].

Prior work has yielded mixed results regarding the capacity of fibroblasts to function as true APC for MHC-restricted responses to exogenous antigens from pathogens [17-20]. The present study sought to evaluate the ability of FLS to function as an antigen presenting cell (APC) for specific autoantigens present within the joint and relevant to RA. Mouse T cell hybridomas were employed that are specifically responsive to arthritogenic peptides of human cartilage gp39 (HCgp39, YKL-40) or human collagen II (hCII) presented by the human MHC II allele HLA-DRB1 (*0401), as measured by production of murine IL-2. These hybridomas were developed from a mouse transgenic for the human class II allele HLA-DR4 (*0401). Since these T cell hybridomas have been shown to recognize their respective antigens when presented by human dendritic cells or human monocytes which express *0401 [21], they were used to evaluate the APC potential of FLS for arthritogenic autoantigens.

2-3. Methods

Fibroblast isolation and culture

All procedures involving specimens obtained from human subjects were performed under protocols approved by the University of Michigan Institutional Review Board. FLS were obtained by collagenase (Worthington Biochemical) digestion of human synovial tissue obtained at arthroplasty or synovectomy from RA or osteoarthritis (OA) joints. RA
diagnosis was based upon the presence of at least 4 out of the 7 ACR criteria for RA [22]. The diagnosis of OA was based upon characteristic clinical and radiographic features, and confirmed by pathological findings at joint surgery. Cells were maintained in CMRL medium (Invitrogen) supplemented with 10% FCS (Atlanta Biological), 2mM glutamine (Cambrex), 50 U/mL penicillin (Cambrex), and 50µg/mL streptomycin (Cambrex). FLS were used after passage 4 from primary cultures.

*T cell hybridoma generation and culture*

T cell hybridomas specific for a 13 amino acid peptide of the arthritogenic human cartilage gp39 (HCgp39) and a 15 amino acid peptide from human collagen II (hCII) proteins (HCgp39 263-275 and hCII 259-273) were developed and characterized as previously described [21]. T cell hybridomas were cultured in RPMI medium (Invitrogen) supplemented with 10% FCS (Atlanta Biological), 2mM glutamine (Cambrex), 50 U/mL penicillin (Cambrex), 50µg/mL streptomycin (Cambrex), 0.6mM sodium pyruvate (Cambrex), 1mM HEPES (Cambrex), and 0.055mM β-mercaptoethanol (Invitrogen).

*Antigens*

Peptide antigens RSFTLASSETGVG and GIAGFKGEQGPKGEP, corresponding to HCgp39 263-275 and hCII 259-273 respectively, were synthesized by the University of Michigan Protein Core. Synovial fluid was obtained at therapeutic arthrocentesis, centrifuged to remove cells, and stored at -80 C for subsequent use. For testing of responses to synovial fluid antigens, FLS were loaded with 50%/50% synovial
fluid/medium, for the HCgp39, or 20%/80% fluid/medium, for the hCII hybridoma, as an antigen source. FLS were incubated with antigens for between 3 to 7 days before addition of T cell hybridomas. Antigen incubation times less than 3 days resulted in suboptimal antigen presentation.

**FLS and T cell hybridoma co-culture.**

FLS were cultured with CMRL in 24 well plates at 10,000 cells per well and were allowed to adhere for 48 hours. FLS were then stimulated with 1000 U/mL IFNγ for an additional 48 hrs to re-induce MHC II, which is expressed in vivo by FLS. Four days after initial plating, the medium was changed and new medium containing 1000 U/mL IFNγ and 10ug/mL of peptide antigen (HCgp39 or hCII) was added for another 3 days. Seven days after initial plating, the medium was changed to T cell hybridoma medium, containing HCgp39 or hCII peptide at 10ug/mL. 200,000 T hybridoma cells were added while maintaining antigen concentration at 10ug/mL. T cells and FLS were allowed to interact for 3 days before plates were frozen and thawed. Plates were spun down and supernatants harvested.

**Mouse IL-2 ELISA**

ELISAs were performed using OptEIA Mouse IL-2 kits (BD Biosciences) and the manufacturer’s protocols were followed.

**MHC Typing**
DNA was isolated with the DNeasy Tissue Kit (Qiagen). MHC analysis used the HLA-DR Typing Tray and DR 4T SSP Unitray (both Pel-Freez) to identify the presence of a DR4 allele and the subtype of DR4, respectively. Manufacturer’s protocols were observed.

**Blocking Antibodies**

For blocking assays, T cell hybridomas were incubated, before being co-cultured with FLS, in medium containing 10ug/mL anti-CD4, anti-CD11a, anti-CD2, or rat IgG. FLS were exposed to medium containing 10ug/mL anti-MHC II, anti-CD54, anti-CD58 or mouse IgG before the addition of T cell hybridomas. When cells were co-cultured, additional antibodies were added to maintain a final antibody concentration of 10ug/mL.

**HCgp39 ELISA**

ELISAs were performed using YKL-40 ELISA Kit (Quidel) and the manufacturer’s protocols were followed.

**2-4. Results**

*FLS can present HCgp39 and hCII peptides*

To assess APC function of FLS, these cells were treated with IFNγ, loaded with immunodominant peptides from HCgp39 or hCII, and co-cultured with the HCgp39 or hCII specific T cell hybridoma. As a control the peptide and T cell hybridoma pairs were mismatched (FLS+ HCgp39 peptide+hCII hybridoma, or FLS+hCII peptide+HCgp39
hybridoma). These mismatched peptide pairs are subsequently referred to as irrelevant peptide controls. The supernatants from these co-cultures were evaluated for murine IL-2 as a measure of T cell stimulation due to FLS presentation of peptide antigen. FLS co-cultured with the HCgp39 or hCII specific T cell hybridoma cells along with the cognate peptide induced significant release of IL-2 from the hybridomas (Figure 2-1A). When irrelevant peptide was presented, IL-2 production was greatly reduced. Hybridomas cultured with cognate peptide in the absence of FLS produced very little or no IL-2, indicating the requirement for an APC.

To explore the effects of antigen concentration on the ability of FLS to activate T cell hybridomas, FLS were loaded with increasing concentrations of peptide antigen, ranging from 0.1ug/mL to 10ug/mL, and used as APC for T cell hybridomas. IL-2 production by the T cell hybridomas increased with the antigen concentration (Figure 2-1B).

**FLS APC function is dependent on IFNγ**

The requirement of IFNγ pretreatment for effective APC function by FLS was assessed. FLS, with or without IFNγ pretreatment, were cultured in medium containing HCgp39 peptide antigen, and used as APC for the HCgp39 T cell hybridoma (Figure 2-1C). IFNγ pretreatment was necessary to reinduce expression of MHC II found in vivo [23]. IFNγ-treated FLS were able to present antigen to T cell hybridomas, but FLS cultured without IFNγ did not function as APC. The IFNγ dependence of FLS APC function is consistent with MHC II dependent antigen presentation and recognition.
Fibroblast APC function is MHC II restricted

Not all FLS lines were able to function as APC, even with IFNγ stimulation. It was hypothesized that lack of APC function by FLS was due to the MHC restriction of the T cell hybridoma response. Chromosomal DNA from various FLS lines, as well as from fibroblast lines from other tissue sources, were harvested and screened for HLA type by PCR. Using HLA-DRB1 (*0401) positive and DR4 negative FLS lines as APC for peptide antigens, we found that only *0401 positive cell lines could present antigen to the HCgp39 T cell hybridoma (Figure 2-2A). When a DR4 negative cell line was used as APC, no IL-2 was produced by the T cell hybridoma.

We next sought to assess the DR4 subtype specificity of antigen presentation and also the antigen presentation potential of fibroblasts other than FLS. To address these issues, MHC typed lung fibroblasts cultured from interstitial pneumonia biopsies were treated with IFNγ, loaded with cognate antigen or irrelevant antigen, and used as APC. Similar to FLS, lung fibroblasts were able to load peptide antigen and present it to the HCgp39 T cell hybridoma (Figure 2-2B). Comparing *0401 positive, *0404 positive, and DR4 negative lung fibroblast lines, this system showed stringent specificity for antigen recognition only in the context of *0401. Even the *0404 allele, which also contains the shared epitope and is associated with RA, did not function as an effective activation signal for the T cell hybridoma. The ability of fibroblasts to function as APC is therefore not unique to fibroblasts from a synovial source, and antigen presentation by fibroblasts of any tissue follows strict MHC restriction. Table 1 summarizes the results of HLA-DR typing of fibroblasts from various tissue sources and assessment of APC function of the
fibroblasts used as APC. The ability of fibroblasts to act as APC corresponds strictly to possession of the correct MHC II subtype, specifically HLA-DRB1 (*0401), irrespective of the tissue source. Notably, DRB1 *0403 and *0404 expressing fibroblasts did not function as APC for the HCgp39 T cell hybridoma. Similar to professional APC, fibroblast APCs must express the MHC II allele with which T cells were educated.

*APC function is dependent upon MHC II and CD4*

Given that IFNγ is required to achieve effective APC function of FLS and MHC II restriction is displayed by T cell hybridomas and since IFNγ stimulation upregulates many proteins important for antigen presentation, the roles of Class II MHC and other structures important to T cell activation were further evaluated. We sought to isolate those critical to FLS/T cell interactions. Blocking antibodies were added to co-cultures of antigen loaded FLS and T cell hybridomas, including human MHC II, CD54, and CD58 on the FLS surface and murine CD4, CD2, and CD11a on the T cell surface. Blockade of human MHC II or murine CD4 by antibody abolished the FLS activation of the HCgp39 T cell hybridoma (Figure 2-3), and thus further demonstrated the importance of a functional peptide-MHC complex. Interference with human CD54 adhesion yielded less robust effects, and blockade of murine CD11a did not cause a similar reduction in IL-2 production. Thus, it is unclear at this time whether a CD54-CD11a interaction is required for FLS to present antigen to T cell hybridomas. A CD58-CD2 interaction also does not seem to be required for FLS APC function in this system.
FLS are also able to present autoantigens from synovial fluids

To use a source of antigen more biologically relevant than synthetic peptides, human synovial fluids (SF) from RA and osteoarthritis (OA) patients were added to cultures of FLS. SF was collected from patients undergoing therapeutic arthrocentesis. Collected SF was centrifuged at 2000 rpm for 30 minutes to pellet synovial cells. The supernatant was used as an antigen source after being diluted to 20% in medium. FLS were able to extract hCII antigens from some SFs and activate the hCII T cell hybridoma (Figure 2-4A). One SF was able to elicit IL-2 responses that were comparable to 10ug/mL of peptide antigen (RA SF8). Other SFs (RA SF6 and OA SF3) were presented by FLS, but were not as potent as 10ug/mL of peptide antigen. FLS were unable to present antigen from some fluids. None of the fluids tested activated the hCII hybridoma in the absence of FLS; however, one fluid did activate the HCgp39 hybridoma minimally (data not shown). FLS presentation of antigens from SF was dependent on IFNγ pre-treatment, and antibody blocking studies revealed that APC function of FLS for SF antigens was MHC II dependent (data not shown).

To assess whether the variability in the ability of different SFs to function as a source of antigen reflected the antigen concentration present within the fluid, various SFs were presented to the HCgp39 T cell hybridoma by FLS. The HCgp39 content within these fluids was measured by ELISA, and these SFs were diluted 50% in media and then loaded onto FLS for antigen presentation. The HCgp39 concentration was lower in SF than the amount of peptide used for antigen presentation. SF HCgp39 concentrations ranged from 1.19 - 3.10 ug/mL (Figure 2-4B). FLS were able to extract HCgp39 from
some SFs and activate T cell hybridomas (Figure 2-4B). Functional HCgp39 antigen was present in both RA and OA SF, similar to hCII antigen, as indicated by induction of IL-2. However, not all SF samples allowed FLS to activate hybridomas, even when HCgp39 antigen was detectable by ELISA at concentrations similar to functionally presentable fluids. One fluid activated the HCgp39 hybridoma in the absence of FLS and was excluded (data not shown).

The lack of correlation between antigen concentration in SF and the level of IL-2 produced by the HCgp39 T cell hybridoma contrasted with previous results (Figure 2-1B), which showed that the level of IL-2 produced by T cell hybridomas correlated with the concentration of peptide antigen presented. To explore this discrepancy we pooled the data from several experiments that measured the antigen concentration of SF and the ability of FLS to present HCgp39 from SF to the HCgp39 hybridoma. As an internal control, the resultant IL-2 response from these experiments was normalized as a percentage of the IL-2 response elicited by loading the same FLS lines with 10 ug/mL of synthetic peptide. This normalized response was plotted against the HCgp39 concentration in the SF as measured by ELISA. A close correlation between the HCgp39 concentration in SF and the amount of IL-2 produced by the HCgp39 specific T cell hybridoma was not observed (Figure 2-4C). Curve fitting analysis yielded a very low r² value of 0.0677. This result implies that other factors in SF regulate the capacity of FLS to function as APCs.
2-5. Discussion

Presentation of autoantigens to T cells in synovial tissue could be very important in the initiation and perpetuation of inflammatory arthritis. The RA-associated MHC alleles possess a common sequence motif referred to as the “shared epitope” [13]. Structural analysis of the shared epitope region shows that it is positioned near the MHC peptide binding groove [15]; thus, influencing bound peptides or affecting interactions with T cell TCRs [14]. This suggests that antigen presentation may be an important pathogenic mechanism in RA.

Previous data regarding fibroblasts as APC have been mixed. Early studies using dermal fibroblasts observed that they were poor generators of allogeneic responses [17]. This defect was not due to inadequate expression of MHC II, but was due to lack of an accessory molecule that could be provided by conventional APC. However, it was noted that dermal fibroblasts could stimulate previously activated alloreactive T cells. Expanding on this work, the capacity of dermal fibroblasts to function in antigen presentation was evaluated. Dermal fibroblasts were able to process antigen, but did not function well as APC without accessory cell help [18, 24]. In both of these studies IFNγ was used to induce MHC II, but autoantigens were not evaluated. These previous studies do document fibroblast expression of functional MHC II.

FLS of RA synovium express high levels of MHC II in vivo and ex vivo [23], suggesting that the potential for antigen presentation by FLS in RA exists. Previous work indicated that FLS can process and present bacterial antigens to T cell clones via an MHC II
restricted mechanism [19]. Our current study expanded on these observations by examining the APC function of FLS for arthritogenic autoantigens, including endogenous proteins present in SF.

There is also evidence that under some conditions FLS might not activate T cells, but instead induce anergy, in experiments that assessed the APC and allostimulatory functions of FLS [25]. In these studies, FLS were able to load antigen onto MHC II, but allogeneic responses depended upon the addition of accessory cells expressing CD80, and blockade of CD80 abolished the response (i.e. FLS are poor allogeneic stimulators similar to dermal fibroblasts [17]). When FLS without accessory cells were cultured with T cells, the T cells adopted a phenotype resembling anergy: upregulation of CD25, reduced proliferation, and reconstitution of proliferation by exogenous IL-2. This study implies that FLS cause anergy due to a lack of costimulatory molecules, but that bystander cells expressing co-stimulatory molecules could overcome this. The potential for accessory co-stimulation exists abundantly within RA synovium due to the close proximity of FLS to B cells, macrophages, and dendritic cells, as well as other T cells.

The evidence that FLS can express functional MHC II is strong, but the function of FLS MHC expression has not been thoroughly explored. Work in transgenic mice suggests that the specific MHC II allele subtype has a striking effect on T cell polarization. Evaluation of the T cells from DR4 transgenic mice reveals functional differences between RA associated and non-associated alleles. T cells from *0401 transgenic mice, possessing the RA associated allele, differ from T cells from *0402 mice, which carry a
non-RA associated allele, by their cytokine profile after antigen stimulation. *0401 mice show a skew towards a Type I response and make greater levels of IFNγ and TNFα after stimulation with antigen compared to *0402 mice [20]. RA has been classified as a Type I disease and both of these cytokines are found within RA synovium.

A possible reason for differing T cell responses to antigens presented by different DR4 alleles is that the peptide binding repertoire of RA associated DR4 alleles is distinct from other DR4 alleles. HLA-DRB1 (*0401) presents immunodominant peptides from the autoantigens HCgp39 and hCII, which are peptides distinct from those presented by non-RA DR4 alleles [20]. Perhaps, presentation of a unique panel of peptides by RA associated MHC activates a distinct set of T cells in the periphery and educates a corresponding set of T cells in the thymus, thus predisposing to autoimmunity [26, 27].

Even though a definitive autoantigen (or autoantigens) has yet to be consistently identified in this process of T cell development and activation in RA, HCgp39 and hCII are plausible candidates. These autoantigens are found within cartilage and synovial tissues, and are arthritogenic, able to induce inflammatory arthritis in susceptible strains of rodents [28]. HCgp39 is made by chondrocytes and macrophages, two cell types that are found in RA joints. Elevated serum HCgp39 appears to correlate to increased RA disease activity [29-31]. Peripheral blood mononuclear cells from RA patients show an increased proliferative response to HCgp39 antigen when compared to healthy controls [32]. The prevalence of HCgp39 responsive T cells in peripheral blood of RA patients was similar to that seen in controls in one study [33], but RA T cells produced increased
IFNγ in response to HCgp39, whereas controls showed an IL-10 response, indicating that there is a skew towards inflammation in RA patients while controls show a regulatory response [34]. Histologic studies have even identified HCgp39 complexed with HLA-DRB1 (*0401) on APC within human RA synovial tissue sections [35]. These observations support the idea that HCgp39 responsive T cell clones might contribute to RA pathogenesis or joint inflammation.

Like HCgp39, hCII is a human autoantigen, also used to induce arthritis in mice, that serves as a model of RA [36]. It is only found within cartilage and synovial fluid or tissue. Although, autoantibodies towards hCII and hCII responsive T cell clones are not specific for RA [37], T cell clones in RA patients have been identified that have a TCR repertoire similar to hCII expanded T cells [38]. There is also evidence that T cell responses to altered forms of CII are important to RA [39]. Furthermore, co-cultures of hCII reactive T cells with FLS increased the production of IFNγ, IL-17, TNFα, IL-15 and IL18 [40]. These studies suggest that T cell responses are altered in RA patients such that responses to antigens might be skewed towards a pro-inflammatory response centered around HCgp39 and/or hCII antigens, even when measurable T cell proliferative responses are not remarkably elevated.

In this current study HLA-DRB1 (*0401) positive FLS from different tissue sources were able to present immunodominant peptides from the arthritogenic proteins HCgp39 and hCII to antigen-specific T cell hybridomas. The key requirement appears to be the expression of the correct MHC II allele. Since T cell hybridomas represent previously-
activated T cells, minimal co-stimulation may be needed for reactivation. Nonetheless, any activation of the T cell hybridoma indicates that a functional peptide-MHC II complex is present on the FLS. If irrelevant peptide or an MHC II mismatch is present, no T cell stimulation occurs. The significance of other structural interactions outside of MHC-TCR is not yet clear. The CD54-CD11a and CD58-CD2 interactions are often important for professional APC interaction with T cells. Inhibiting these interactions did not substantially disrupt FLS-APC function. Perhaps, cross-species limitations of some co-stimulatory receptor/ligand interactions and the relative lack of dependence of hybridomas on co-stimulatory signals minimize the roles of these molecules in the system that we used.

FLS are also able to extract antigens from SF and present the antigens to both HCgp39 and hCII T cell hybridomas. The exact nature of the antigenic material in SF is still unknown. It may include predigested peptide fragments and/or intact proteins, such as HCgp39. Whether these antigens are passively loaded onto FLS or externally taken up and processed remains to be discovered, and it is possible that both processes can occur.

SF also contains many factors that could affect antigen presentation, enhancing or inhibiting it. This study did not find a correlation between the concentration of HCgp39 in SF and the ability of FLS to present antigens to the HCgp39 T cell hybridoma. Inflammatory and inhibitory cytokines (e.g. IL-1 and IL-10) are present in SF, and may affect immune cell function. It is also possible that HCgp39 degradation products are present in SF which were not recognizable by the HCgp39 ELISA used. These
fragmented portions of HCgp39 could result in a “functionally” high concentration of HCgp39, while leaving the measurable HCgp39 low. These issues will require further analysis in order to identify additional factors that govern FLS APC function in vitro and in vivo.

FLS exist under unique conditions that provide mechanisms for activation or reactivation of T cells specific to arthritogenic autoantigens. FLS express high levels of MHC II in RA synovium and are chronically exposed to autoantigens present within SF. In the context of an inflamed RA joint, FLS could take up antigen, display antigenic peptides on MHC, and activate T cells in an antigen dependent mechanism as proposed by our in vitro studies. These T cells would either have been activated de novo within the synovium or previously activated in lymphoid tissues. The fact that RA FLS are surrounded by activated immune cells which express high levels of co-stimulatory molecules would further enhance T cell-FLS interactions through MHC II and TCR by cross-costimulation. APC function of FLS may be a hitherto overlooked mechanism of T cell activation in RA synovium. Moreover, the role of non-classical APCs, such as fibroblasts, as participants in autoimmunity deserves further consideration.
Figure 2-1. *FLS present arthritogenic peptides to T cell hybridomas.*
A) FLS were plated and treated with IFNγ. HCgp39 or hCII peptides were pulsed onto FLS before they were co-cultured with T cell hybridomas. Mouse IL-2 was measured from culture supernatants from HCgp39 or hCII specific T cell hybridomas by ELISA. Error bars represent the range of the 95th % confidence intervals. B) FLS were pulsed with decreasing concentrations of HCgp39 peptide before coculture with the HCgp39 specific hybridoma. C) FLS either received medium containing or devoid of IFNγ, before being pulsed with peptide and cultured with the HCgp39 specific hybridoma.
Figure 2-2. MHC Restriction of antigen presentation by FLS
A) HLA-DRB1 (*0401) positive and negative FLS lines were used to present peptide antigen to an HCgp39 T cell hybridoma. B) Arthritogenic peptide antigen presentation by FLS is restricted to HLA-DRB1 (*0401). Lung fibroblasts expressing the RA associated DR4 subtype alleles 0401 or 0404 were pulsed with HCgp39 peptide and co-cultured with the HCgp39 T cell hybridoma. IL-2 was measured from supernatants by ELISA. Error bars represent the range of the 95th % confidence interval.
A

HLA-DR Phenotype of FLS

mIL-2 pg/mL

-DR4 (*'0401)  -DR4 (*'0401)  No FLS

- DR4 (*'0401)
- DR4 ('0401)
No FLS

cognate peptide
irrelevant peptide
no hybridoma

B

Lung Fibroblast HLA Type

mIL-2 pg/mL

-DR4  *0404  *0401

-DR4  *0404  *0401
Figure 2-3. APC function of FLS for peptide antigen responses of T cell hybridomas is MHC and CD4 dependent

Blocking antibodies towards cell surface structures were added to co-cultures of FLS and the HCgp39 T cell hybridoma. Mouse IL-2 was measured by ELISA. Error bars represent the range of the 95th % confidence interval.
No antibody
No FLS
MHC II
CD54
CD58
MsIgG
CD4
CD11a
CD2
RlgG

Anti-human blocking antibodies
Anti-murine blocking antibodies

mIL-2 pg/mL
**Figure 2-4.** *FLS can extract antigen from SF and present it to HCgp39 and hCII T cell hybridomas.*

A) SF from RA and OA patients were diluted to 20% in medium and loaded onto FLS, before co-culture with the hCII specific T cell hybridoma. B) FLS were loaded with RA or OA SF diluted to 50% in medium before culture with the HCgp39 specific T cell hybridoma. The HCgp39 concentration within the SF was measured by ELISA and is indicated in the lower row beneath the graph. Error bars represent the 95th percentile confidence interval. C) OA or RA SF was loaded onto FLS and presented to the HCgp39 T cell hybridoma. The resultant IL-2 production was represented as a percentage of the IL-2 produced from 10ug/mL of HCgp39 peptide and plotted on the y-axis. The x-axis displays the concentration of the HCgp39 antigen contained within SF. Linear regression analysis yielded an \( r^2 \) value of 0.0677.
A

![Bar chart showing cytokine production in response to different peptides.]

- **Cognate Peptide**: RA SF5, RA SF6, RA SF7, RA SF8, OA SF3, RA SF9
- **Irrelevant Peptide**:

**Antigen Source**
- **mIL-2 pg/mL**
  - FLS + T cell Hybridoma
  - T cell Hybridoma Alone

B

![Bar chart showing cytokine production in response to different peptide sources.]

- **HCgp39 peptide**: RA1 SF, RA2 SF, RA3 SF, RA4 SF, OA2 SF

**Antigen Source**
- **mIL-2 pg/mL**
  - HCgp39 peptide
  - RA1 SF
  - RA2 SF
  - RA3 SF
  - RA4 SF
  - OA2 SF

**HCgp39 protein [μg/mL]**
- *: 1.38
- : 2.24
- : 1.19
- : 3.10
- : 2.46

C

![Scatter plot showing cytokine production in response to varying HCgp39 concentration.]

- **R² = 0.067**
- **Antigen Sources**
  - Rheumatoid Fluid
  - Osteoarthritis Fluid

**x-axis**: HCgp39 ug/mL
**y-axis**: mIL-2 pg/mL

50
Table 1 MHC Typing of fibroblasts compared to antigen presenting activity.

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3. Activation of Synovial Fibroblasts by Cytokine Activated T cells

3-1. Summary

Two striking features of RA synovium are cartilage-invading fibroblast-like synoviocytes (FLS) and a prominent T cell infiltrate. It is difficult to observe the in vivo interactions of these cells, but in vitro systems have documented bidirectional activation of both cell types. The current experiments were performed to more definitively analyze interactions between T cells and FLS at a molecular level, using imaging techniques and functional assays of FLS activation. Fluorescence confocal microscopy showed that the integrin LFA-1 localized to the contact point or “synapse” between T cells and FLS during the presentation of superantigen to T cells by FLS. When cytokine-activated T cells (Tck) are cultured with FLS (which have been shown to functionally mimic T cells isolated from RA synovial fluid), the synapse between Tck and FLS showed colocalization of LFA-1 and its counter-receptor, CD54/ICAM-1. Cocultures of Tck with FLS resulted in contact-dependent FLS production of IL-6 and IL-8, an effect that was synergistically enhanced by the T cell cytokine IL-17. TNFα blockade inhibited Tck activation of FLS and eliminated Tck synergy with IL-17, even though secreted TNF was not detectable in the
culture supernatant. Surface blocking of TNFα inhibited similarly inhibited effector function of Tck and synergy with IL-17. These results implicate membrane-bound TNF in the Tck/FLS interaction, and provide an additional mechanism for the therapeutic utility of TNF blockade in RA.

3-2. Introduction

The normal synovial lining is one or two cell layers thick, contains both synovial macrophages and FLS, and is responsible for maintaining synovial fluid homeostasis. In RA, the synovial lining transforms into the synovial pannus, a multicellular tissue composed of FLS closely intertwined with invading lymphocytes and antigen presenting cells. The current consensus is that FLS are not merely bystanders in the inflammatory process, but are active participants in joint destruction [1-5]. However, the mechanism underlying this inflammatory transformation remains unknown.

The most prominent invading lymphocyte in the RA synovium is the T cell [6-8]. The in vivo contribution of synovial T cells to RA etiology and pathology remains elusive, but in vitro studies have shown that T cells can adhere to and activate FLS. Functional studies have shown that FLS can present superantigen to naïve T cells, inducing a proliferative response [9], and peptide antigens to T cell hybridomas, inducing cytokine secretion (Chapter 2). Moreover, T cells can activate FLS to produce inflammatory mediators [10-12], and this is synergistically augmented by the T cell specific cytokine IL-17 [10], which is present in RA synovium [13].
The current studies were undertaken to better define the molecular basis of T cell/FLS functional interactions.

3-2. Methods

*FLS isolation and culture:*

All procedures involving specimens obtained from human subjects were performed under protocols approved by the University of Michigan Institutional Review Board. FLS were obtained by collagenase (Worthington Biochemical) digestion of human synovial tissue obtained at arthroplasty or synovectomy from RA or osteoarthritis (OA) joints. RA diagnosis was based upon the presence of at least 4 out of the 7 ACR criteria for RA [14]. The diagnosis of OA was based upon characteristic clinical and radiographic features, and confirmed by pathological findings at joint surgery. Cells were maintained in CMRL medium (Invitrogen) supplemented with 10% FCS (Atlanta Biological), 2mM glutamine (Cambrex), 50 U/mL penicillin (Cambrex), and 50µg/mL streptomycin (Cambrex). FLS were used after passage 4 from primary cultures.

*T cell generation:*

T cells were obtained from peripheral blood by negative selection using RosetteSep Human T cell Enrichment Cocktail (Stemcell Technologies) and separation on a Ficoll gradient. Briefly, freshly isolated blood from volunteers was incubated with kit antibody cocktail for 20 minutes at room temperature. Blood was diluted 1:1 with 2% NCS/PBS before overlay onto room temperature Ficoll, and spun at 1200 RCF with the brake off. The buffy coat containing only T cells was recovered and used for subsequent experiments. Trest were unstimulated T cells. Tck were cytokine activated T cells
stimulated by a cocktail of 25 ng/ml TNFα, 100 ng/ml IL-6, and 25 ng/ml IL-2 for 8 days [15]. Tsea were T cells activated with the superantigen SEA (staphylococcal enterotoxin A) by coculture with IFNγ stimulated FLS [9].

**Tck and FLS coculture.**

FLS were seeded into 12 well plates at a density of 10,000 per well and allowed to adhere for 48 hours before the addition of 250,000 Tck. IL-17 (R&D Systems) was added to cocultures to yield a final concentration of 20 ng/mL. Final culture volumes were 1.5mL/well. Coculture times were between 24 and 72 hours depending on the assay. Coculture supernatants were harvest and assayed by ELISA.

**Confocal microscopy.**

20,000 – 50,000 FLS were grown on glass cover slips in 6 well plates for 48 hours. Media was changed and 500,000 – 1 million T cells (Trest, Tck, or Tsea) were added to the cultures. Cocultures were allowed to interact for 1 to 24 hours before cover slips were washed with PBS (Invitrogen) and fixed in 4% paraformaldehyde/PBS (Electron Microscopy Sciences) for 1 hour at room temperature or overnight at 4°C. For cocultures utilizing Tsea, after FLS were allowed to adhere for the initial 48 hours, they were stimulated with 1000 U/mL of IFNγ for an additional 48 hours. Subsequently, the IFNγ medium was changed to medium containing 1ng/mL of SEA (final concentration) before the addition of T cells. After fixation, cover slips were washed in 2%NCS/PBS to remove residual paraformaldehyde, and blocked in 5% nonfat milk/PBS for 30 minutes. Coverslips were again washed with 2%NCS/PBS. Primary antibodies used for staining
were mouse anti-human CD11a [9] and mouse anti-human ICAM-1 (Clone R6.5.D6.E9.B2). Secondary antibodies used were goat anti-mouse IgG Alexa 594 (Molecular Probes) and rabbit anti-goat IgG Alexa 488 (Molecular Probes). For coculture studies comparing CD11a and ICAM-1 localization, before FLS were plated on glass coverslips they were transfected with an expression vector for a fusion protein of ICAM-1 and eGFP. This expression vector was termed CD54-eGFP and was created as follows. Primers were constructed against the sequence described by accession BC015969 to PCR amplify the ORF (without the stop codon) of human ICAM-1. A HindIII restriction site at added to the beginning of 5’ primer and an AgeI site added to the tail of the 3’ primer facilitated ligation of the amplified fragment into the pEGFP-N1 expression vector (Clonetech). After ligation the insert sequence was verified to be free of mutation. This resulted in a CD54-eGFP fusion protein expression vector, with ICAM-1 at the N terminus and eGFP at the C terminus (intracellular) and a short peptide linker (GSTPVAT) in between. Transfection was carried out using the Adult Human Dermal Fibroblast Kit (Amaxa), and manufacturer’s protocols were observed.

Transwell Assays.

FLS were seeded into 12 well plates at a density of 10,000 per well and allowed to adhere for 48 hours. FLS location will subsequently be referred to as the bottom chamber. Transwell inserts with a 0.4 uM membrane pore size were placed on top of the wells containing FLS. These transwell inserts had been placed in medium for 24 hours before adding to plates containing FLS, to saturate the membrane. 500,000 Tck were added to the bottom chamber or into the transwell insert. Total volume in the bottom chamber was
1.5 mL. Total volume in the transwell was 0.5 mL. IL-17 was added to the transwell insert at 0.04, 0.4, and 4ng/mL concentration in 0.5mL, but would diffuse through the transwell to give a final concentration of 0.01, 0.1, and 1.0 ng/mL.

**Blocking studies**

FLS were seeded into 12 well plates at a density of 10,000 cells per well and allowed to adhere for 48 hours. Tck were incubated with one of two TNFα blocking antibodies from different clones (eBiosciences and R&D Systems) or control mouse IgG at 1 ug/mL for 30 minutes at room temperature. 250,000 Tck were added to FLS containing wells with additional antibody to maintain a concentration of 1 ug/mL. IL-17 was titrated into cocultures in following concentrations: 0.02, 0.2, and 2.0 ng/mL (we found that 2 and 20 ng/mL IL-17 synergized similarly with Tck, data not shown). Cocultures were maintained for 24 to 72 hours.

**3-4. Results**

*Cytokine activated T cells induce FLS production of inflammatory cytokines*

Previous work in our laboratory utilized two types of T cells [9, 10]. Trest and Tsea could be viewed as representing polar states of T cell stimulation from naïve to full activation. An alternative state of T cell activation state has been described, termed cytokine activated T cells (Tck). Tck are generated through stimulation independent of the TCR with a cocktail of cytokines: TNFα, IL-6, and IL-2. These cytokines, in particular TNFα and IL-6, are found abundantly within synovial tissues. Tck have been documented to possess effector function identical to T cells purified from RA synovial fluid, most
notably in the induction of TNFα from monocytes. The stimulatory mechanism of these cells is clearly distinct from T cells activated through CD3/CD28 cross-linking [15]. We sought to explore Tck effector function in T cell cocultures with FLS.

FLS cultures were stimulated by the addition of Tck and allowed to interact for 36 hours. The cytokine IL-17, produced by RA synovial tissues [13], has been shown to synergize with Trest in the activation of FLS [10], and was also included in some wells. Three FLS lines were assayed for stimulation by Tck (Figure 3-1A and 3-1B). Tck stimulated FLS to produce the inflammatory cytokines IL-6 and IL-8, and this effector function synergized significantly with IL-17. No cytokine production was detected in cultures of Tck and IL-17 in the absence of FLS (data not shown). Imaging of Tck/FLS interactions revealed strong adhesion of Tck to monolayers of FLS (Figure 3-1C). Tck were found to adhere strongly to FLS by 1 hour of coculture, faster than Tsea or Trest (data not shown).

**Tck activation of FLS requires cell-cell contact**

The observed Tck effector functions on FLS were thought to require cell-cell contact, given that Tck-induced monocyte production of TNFα also required cellular interaction [15]. To explore the role of cell-cell contact, cocultures of Tck and FLS were set up using transwells. FLS were cocultured with Tck, and were either allowed to interact or Tck were separated from FLS by a transwell insert. The transwell insert allowed the free flow of cytokine, but prevented intercellular adhesive interactions to occur between FLS and Tck. IL-17 was added in increasing titrations directly into the transwell insert and allowed to diffuse through the entire culture. Only when Tck were allowed to contact FLS was significant IL-8 production and synergy with IL-17 observed (Figure 3-2).
When Tck were separated from FLS by the transwell insert, IL-8 production was similar to IL-17 stimulation, best observed in the 1ng IL-17 column. This suggests that IL-17 alone did cause Tck to produce soluble inflammatory mediators, which in turn could activate FLS.

LFA-1 localizes to the contact between point FLS and Tsea and Tck

Given that cell-cell contact is important for T cell interaction with FLS, we sought to explore the structures crucial to these interactions. There is evidence that the FLS-adherent population of T cells expresses greater levels of the integrin LFA-1 [16]. It has also been observed that adhesion of T cells to FLS in static assays could be inhibited with blocking antibodies against LFA-1 (CD11a/CD18) and its counter-receptor ICAM-1/CD54 [17, 18]. Beyond adhesion, LFA-1 has been implicated in supplying a costimulatory signal to T cells [19]. The adhesive and/or costimulatory properties of LFA-1 are important in the presentation of superantigens to T cells [20-22], and in particular superantigen presentation by FLS [9]. We therefore sought to define the contact point, or synapse, between FLS and T cells, and whether LFA-1 and ICAM-1 colocalized in this region. First we sought to define the localization of LFA-1 in an antigen dependent system. Cultures of FLS were treated with IFNγ and used as APC for the superantigen SEA to activate T cells. These cultures were fixed, stained for LFA-1, and examined by confocal microscopy. 3-D reconstruction of adherent Tsea, which had been stained with anti-CD11a antibody, showed that LFA-1 is arrayed in an asymmetric distribution and suggests LFA-1 intensity is greatest at the contact point between Tsea and FLS (Figure 3-3).
T cells can also interact with FLS independent of antigen presentation [10-12]. ICAM-1 (CD54), the counter receptor to LFA-1, is an adhesion molecule expressed abundantly on the FLS surface [23]. In addition to adhesion, ICAM-1 ligation induces stimulatory signals in the cell that expresses this molecule [22, 24-26]. To observe the localization of ICAM-1 in antigen independent activation of FLS by Tck, FLS were transfected with an ICAM-1/eGFP fusion protein expression vector, termed CD54-eGFP, before coculture with Tck. Cocultured cells were fixed and Tck were indirectly stained for CD11a by Alexa594. At the synapse of Tck and FLS, confocal imaging revealed merged signals between ICAM-1 and LFA-1 (Figure 3-4). Given the strong colocalization of ICAM-1 and LFA-1 to the contact point of Tck and FLS, we attempted to disrupt the effector function of Tck on FLS using blocking antibodies to these adhesive proteins. Unexpectedly, blocking antibodies directed towards LFA-1 and ICAM-1 showed only modest inhibition of Tck effector function on FLS cytokine production (data not shown).

**TNFα is required for optimal Tck activation of FLS and synergy with IL-17**

A potent stimulatory molecule in RA is TNFα. Given the abundance of TNFα in RA synovial tissue and fluid [27, 28], and the use of TNFα blockade as an effective therapy for RA [29], the role of TNFα in Tck/FLS interactions was examined. Although, macrophages are considered to be the primary source of TNFα in RA, T cells are also known to produce TNFα [30]. Two different TNFα blocking antibodies were used to inhibit Tck/FLS interaction and Tck synergy with IL-17. TNFα blockade substantially inhibited Tck activation of FLS and Tck synergy with IL-17, as assayed by IL-8.
production (Figure 3-5). However, TNFα inhibition did not affect IL-17 stimulation of FLS, indicating that IL-17 was not causing FLS to produce autocrine TNF.

Based upon these findings, the TNFα concentration of coculture supernatants were evaluated by ELISA. No TNFα was detected from any of these cultures (data not shown). Since TNFα is initially expressed as a membrane bound protein and assuming membrane bound TNFα was responsible for TNFα effector function, Tck were preincubated with blocking antibody against TNFα for 30 minute and washed to remove excess antibody. These antibody-coated Tck were used to activate FLS. Preincubation of Tck with blocking antibody to TNFα significantly reduced Tck activation of FLS and synergy with IL-17, while Tck preincubated with control antibody did not inhibit activation of FLS (Figure 3-6A). To determine the expression of membrane TNFα, Tck were stained with the same antibody as was used to block TNFα function and examined by flow cytometry. Unexpectedly, the levels of membrane TNFα were not distinguishable above control antibody (Figure 3-6B).

3-4. Discussion

Accumulating evidence indicates that bidirectional signaling between T cells and FLS is robust and functionally significant. Several reports have documented T cell effector function towards FLS, resulting in augmentation of pro-inflammatory properties of FLS. Resting, unstimulated T cells have been shown to alter gene expression in FLS, and cause release of IL-6, IL-8, and PGE₂ [10]. This effector function of resting T cells was not confined to a unique T cell subset, as CD4+, CD8+, CD45RA+, and CD45RO+ T cells
all had a similar capacity to activate FLS. T cells recovered from RA synovial fluid likewise elicit strong inflammatory responses when cultured with FLS, in particular strongly upregulating the immune modulating cytokine IL-15 [11]. As a prototype for autoreactive T cells that are potentially arthritogenic, collagen II stimulated T cells enhanced FLS production of many inflammatory mediators, and this effector function appeared to be proportional to the length of T cell activation with collagen [12]. Conversely, FLS have also been shown to positively stimulate T cells. Simple coculture of T cells with FLS enhances T cell survival and prevents apoptosis [31, 32]. More robust responses have also been reported. FLS can induce naive T cell proliferation by presentation of superantigen [9, 33]. When cocultured with FLS, T cells upregulate the activation markers CD69 and CD25 [11, 34], as well as many cytokines, such as IL-17, TNFα, and IFNγ [11, 12].

Our studies and work by others [11, 15] have demonstrated the importance of cell-cell contact for the bidirectional signaling of T cells for FLS and other cell types. Early experiments on T cell/FLS interaction noted that these cells readily adhered in coculture [16-18, 35]. The CD2/LFA-3 [35] and LFA-1/ICAM-1 [16-18] adhesive interactions have been implicated in T cell/FLS association. The relative requirements of these protein-protein interactions for T cell adhesion to FLS remains ill defined, as different studies report differing effects when these interactions were inhibited using blocking antibodies [17, 18, 35]. These differences were most likely due to the varying activation states of the T cells used, as well as differing methodologies used to assess adhesion. However, two studies characterizing the phenotype of adherent T cells and FLS reported
increased expression of LFA-1 and ICAM-1, respectively, on the adhesive population [16, 18]. Also, cell-cell interactions have been documented to positively signal in T cell-FLS cocultures, leading to FLS upregulation of VCAM-1 and ICAM-1 [36]. We have been able to visualize LFA-1/ICAM-1 interactions between T cells and FLS by confocal microscopy and localize it to the contact point of the two cells. Specifically, we imaged the contact of point between FLS and T cells representative of two activation states: Tck and Tsea. Consistent with studies demonstrating the importance of LFA-1 for adhesion of T cells to FLS [16-18], LFA-1 localized to the contact point of Tsea and FLS during T cell stimulation by SEA. Also in systems devoid of exogenous antigens, using Tck cells that are activated independent of the TCR, LFA-1 could again be seen localized to the contact between Tck and FLS in conjunction with the counter-receptor ICAM-1 expressed on the FLS surface.

The signaling mechanisms between T cells and FLS involve a variety of mechanisms. The LFA-1/ICAM-1 interaction may play a role in this signaling beyond its role in adhesion. ICAM-1 [22, 24-26] and LFA-1 [19-21, 37, 38] have also been implicated as costimulatory structures that can enhance cellular activation. Clustering of LFA-1 to the contact sight between FLS and Tsea during presentation of SEA and colocalization of LFA-1/ICAM-1 at the synapse between Tck and FLS implied that activating signals could be conveyed by these proteins. A previous study reported that interference with this interaction could greatly blunt the outcome of T cell/FLS interactions [11]. Unexpectedly, in our system inhibition of LFA-1/ICAM-1 by blocking antibody only resulted in modest inhibition of Tck effector function. This implies that similar to the
contribution of the LFA-1/ICAM-1 interaction in T cell adhesion assays to FLS, the overall contribution of this interaction to effector function of T cells in FLS activation depends on assay methodologies and T cell activation state.

Beyond costimulation through adhesive molecules, many other pathways have been identified as activation mechanisms between T cells and FLS. The cytokine IL-15 has been identified as a mediator of T cell and FLS interactions [11]. Engagement of the costimulatory receptor CD40 on FLS by CD40L on activated T cells results in enhanced VEGF production [39]. The CD47/TSP-1 pathway has been shown to be involved in T cell adhesion and survival in cocultures with FLS [40].

Our study extends the repertoire of T cell/FLS interactions by focusing on the Tck, which are T cells that have been activated not through the TCR, but instead by a pool of cytokines similar to those found within RA synovium. Tck have been shown induce TNFα production from monocytes through a mechanism distinct from pathways used by T cells stimulated through CD3/CD28 engagement, requiring NF-κB and inhibited by PI 3-kinase [15]. Consequently, Tck provide a convenient in vitro system to generate T cells that function in a manner similar to RA synovial T cells.

Tck proved to be potent activators of FLS, increasing production of IL-6 and IL-8 during cocultures. Similar to earlier experiments with resting T cells [10] and consistent with one of its proposed functions [41], IL-17 synergized with Tck. Based on previous reports [11, 18], and our imaging studies, it was expected that blocking antibodies towards LFA-
1 and ICAM-1 would have more robust effects on Tck activation of FLS, but only marginal inhibition was observed. However, this effector function of Tck and synergy with IL-17 was dependent on cell-cell contact, and not due to basal cytokine production, as separation of Tck from FLS by transwell inserts abolished FLS stimulation.

We explored TNFα as a potential signaling mechanism between T cell and FLS. TNFα is found abundantly within rheumatoid synovial tissues [27, 28, 42]. The binding of TNFα to one of its two distinct receptors [43, 44] results in many varied biological effects [45]. TNFα is initially synthesized as a transmembrane protein. Soluble TNFα is produced by shedding of the membrane bound form by the membrane-associated metalloproteinase TACE [46]. Membrane-bound TNFα is not simply a precursor of the fully functional cytokine. It has been reported to function in cytotoxic killing [47, 48]. It has been suggested that these two forms TNFα can mediate different biological effects [49, 50].

TNFα has been shown to be a potent proinflammatory signal for human FLS [51]. The first suggestion that TNFα might be contributing to the joint pathology seen in RA was with the creation of the mouse transgenic for human TNFα. This mouse developed a spontaneous inflammatory arthritis similar to RA, and was protected from this arthritis by TNFα neutralization [52]. In humans, in vitro cultures showed that synovial cell culture IL-1 production was inhibited by using TNFα blocking antibodies [53]. Ultimately, TNFα neutralization was shown to be an effective therapeutic in human RA [54, 55].
In view of the effective use of TNFα blockade in RA, and reports of IL-17 synergy with TNFα [41, 56], we explored TNFα blockade as an approach to inhibit Tck function. Anti-TNFα proved to be an effective inhibitor of Tck function and synergy with IL-17. In these experiments there was no evidence that IL-17 induced production of TNFα by FLS, as TNFα blocking antibody had no effect on IL-17 stimulation of FLS. We also found that, on Tck, TNFα is membrane bound, as indicated by the blocking effect on FLS activation seen with pre-coating of Tck with anti-TNFα, and by the lack of detectable soluble TNFα in the culture medium. The membrane bound TNFα on Tck could not be detected above background on Tck. This implies that even small amounts of membrane bound TNFα are effective inflammatory signals. It should also be noted that TNFα blockade greatly diminished Tck effector function and synergy with IL-17, effector function was not completely abrogated. This could be due to incomplete neutralization of all TNFα molecules, but is most likely due to the diverse signaling pathways between T cells and FLS that were not disrupted.

Although the role of a T cell population expressing membrane bound TNFα has not been well studied in RA pathophysiology, research in Crohn’s Disease (CD) has documented the pathologic functions of these cells. Similar to the dramatic results for RA, anti-TNFα therapy has proven to be an effective treatment for CD [57, 58]. In determining the mechanism of this therapeutic effect, it was found that neutralization of TNFα resulted in lowered IFNγ and TNFα production from CD patient T cells [59-61]. Further study revealed that therapeutic anti-TNFα monoclonal antibodies exerted their effects by specifically binding to T cells surfaces. Antibody binding to T cell membranes leads to
caspase-3 activation and apoptosis in cell cultures, with intestinal lamina propria T cells showing increased susceptibility compared to peripheral blood T cells [62]. This apoptotic effect was further verified by endoscopic tissue biopsy of CD patients receiving anti-TNFα therapy for T cell death [63].

In our studies, we have used a population of T cells which resemble T cells isolated from RA SF to activate FLS. Similar to T cells from CD these Tck utilize membrane bound TNFα as a mechanism to convey inflammatory signals. This suggests that along with soluble TNFα, TNF on T cell surfaces could be an important signaling interaction in RA. These results provide another mechanism for the utility of TNF blockade in the treatment of RA, and imply that membrane bound TNF on T cells may be important in RA pathogenesis.
Figure 3-1. *Tck adhere to and activate FLS.*
Tck were cocultured with three different FLS lines and the resultant IL-8 A) and IL-6 B) in culture supernatants were measured by ELISA. Error bars represent the 95% confidence interval. C) FLS were grown on glass coverslips and cocultured with Tck. Coverslips were fixed with 4% paraformaldehyde and imaged by light microscopy.
Figure 3-2. Tck stimulation of FLS requires cell-cell contact.
FLS were grown in the bottom chamber of a transwell assay plate. Tck were added directly into the bottom chamber with FLS (+Tck) or above FLS separated by transwell inserts ([+Tck]). IL-17 was titrated into the transwell inserts to diffuse throughout the cocultures. The resultant IL-8 production in supernatants was measured by ELSIA. Brakets ([]) denote additions inside the transwell and not directly to FLS. Error bars represent the 95% confidence interval.
Figure 3-3. LFA-1 localizes to the contact point between Tsea and FLS.
IFNγ stimulated FLS were grown on glass coverslips and used to present superantigen to
Tsea. Confocal slices of a Tsea adhering to an FLS were reconstructed to form a 3-D
image of LFA-1 distribution on the Tsea. This 3-D reconstruction was rotated and 3
different images of the same Tsea upon an FLS were captured from different viewpoints.
Figure 3-4. LFA-1 and ICAM-1 colocalize to the contact point between Tck and FLS. FLS were transfected with an expression vector for CD54-eGFP fusion protein before being cocultured with Tck. Localization of LFA-1 on Tck surfaces was stained (red) and compared to ICAM-1 (green) on the FLS. Confocal imaging of stained cocultured cells was performed to examine cell-cell interactions. Merged signals where LFA-1 and ICAM-1 colocalize appear yellow. Top row: 60X magnification of a CD54-eGFP transfected FLS with many Tck adhered. Middle and bottom rows: Digitally zoomed images of different Tck adhered to different FLS.
**Figure 3-5.** *Tck activation of FLS requires TNFα.*

IL-8 production from three different cellular cultures was compared: Tck cultured alone (Tck alone), FLS cultured alone (FLS alone), or FLS cultured with Tck (FLS+Tck). Before coculture with FLS, Tck were incubated with either one of two different TNFα blocking antibodies (anti-TNFα #1 or anti-TNFα #2), control antibody (MsIg), or no antibody (Media). The concentration of antibody in cocultures was kept constant. The resultant IL-8 production from different conditions is represented by a 3-D chart. The x-axis displays culture conditions with blocking antibodies used. The y-axis shows IL-8 production. The z-axis shows the concentration of IL-17 titrated into the cultures and is also represented by increasing color density of blue for the graphed columns, ranging from white for no IL-17 to navy blue for 2.0 ng/mL of IL-17.
Figure 3-6. *Tck activation of FLS requires TNFα that is membrane bound.*
A) Tck were incubated with TNFα blocking antibody for 30 minutes to bind all surface TNFα. Excess blocking antibody was removed by pelleting and sequential washes of Tck with PBS before coculture with FLS (pre anti-TNFα). As a controls, Tck were also similarly pre-blocked with mouse IgG (preMsIg) or TNFα neutralizing antibody was directly added to cocultures with FLS and Tck without removal of excess antibody (MsIg or anti-TNF respectively). The effects of these antibody regimes were compared using stimulation by Tck and Tck+IL-17. IL-8 production was measured by ELISA. Error bars represent the 95% confidence interval. B) Tck were generated by culture with cytokines and stained with control antibody (MsIg), anti-human CD3, or with anti-human TNFα. Surface staining of these antibodies was visualized by flow cytometry.
References


Chapter 4

4. Fibroblast-Like Synoviocytes Express the B7 Family Costimulatory Molecule B7-H3

4-1. Summary

FLS and T cells can activate each other in vitro, and in vivo interactions between these cells may be important in the pathogenesis of RA. However, FLS lack significant expression of the ligands for CD28, the prototypic T cell costimulatory receptor. We sought to identify other molecules homologous to CD28 ligands that would be more strongly expressed by FLS, such as the molecule termed B7-H3. By flow cytometry we documented strong B7-H3 expression on FLS, which was unaffected by the cytokines IFNγ, IL-4, IL-10, IL-17, IL-1β, or TNFα. Western blot analysis was employed to identify the specific isoforms of the B7-H3 expressed by FLS, and showed predominant expression of the larger, 4-Ig-domain isoform of B7-H3. Immunohistochemical staining of RA synovial tissue revealed substantial expression of B7-H3 in vivo, and B7-H3 expression patterns were similar to fibroblast specific markers Cadherin-11 and CD90. These sections also showed that B7-H3 was distinct from but in close proximity to cells that expressed CD45, CD20, and CD3. Confocal microscopy of FLS and T cell cocultures showed localization of B7-H3 in the region of the T cell-FLS contact point,
but distinct from the localization of T cell CD11a (LFA-1) and FLS CD54 (ICAM-1).
RNA interference was used to knock down B7-H3 expression in FLS and these FLS were cocultured with T cells to observe the effect on T cell cytokine production. Some T cells that were cultured with B7-H3 knock down FLS had reduced or enhanced cytokine production depending upon the activation state of the T cell. These observations suggest that B7-H3 may be important in the interactions between FLS and T cells in RA, and potentially other diseases.

4-2. Introduction

The RA synovial environment comprises a complex mix of cell types. Invading lymphocytes are in close proximity to resident structural cells. In this milieu, particular focus has turned to the interaction between the most numerous lymphocyte, the T cell, and the structural cell which invades cartilage, the FLS [1-4].

In vitro assays have documented strong association and bidirectional signaling between these two cell types. Similar to professional APC-T cell interactions, FLS and T cells in co-cultures have been shown to interact in antigen dependent systems. FLS can present superantigen to T cells inducing a proliferative response [2]. FLS are also able to take up and present arthritogenic peptide autoantigens to HLA-DR4 restricted T cell hybridomas [5]. Moreover, T cells previously stimulated by antigen presented by professional APC have augmented ability to interact with FLS, leading to increased release of IL-15, TNFα, IL-18, IL-17 and IFNγ [6].
T cells and FLS can also interact and signal in the absence of exogenous antigen. Naïve T cells cultured with FLS cause FLS to increase production of inflammatory mediators such as IL-6, IL-8, and PGE2 [4]. Conversely, T cells have been reported to express the activation marker CD69 after co-culture with FLS [1].

FLS share features in common with professional APC, and they express many surface glycoproteins related to lymphocyte trafficking and signaling, such as VCAM-1 and CD54 [7]. The integrin CD11a/CD18 (LFA-1) clusters at the contact point between T cell and FLS [8], and T cell adherence to FLS is inhibited by blocking antibody to LFA-1 [9]. FLS also express significant MHC II in vivo [10], which can be reinduced in vitro [11, 12]. However, unlike traditional APC, FLS do not express the classic costimulatory molecules B7-1 or B7-2 (CD80, CD86) [13, 14], which typically provide the second signal for full T cell activation.

A family of B7 related proteins has been discovered that have variable homology to CD80 or CD86. One of these proteins is termed B7-H3, a type I transmembrane protein with approximately 20% sequence homology to B7-1, B7-2, PD-L1, and ICOS-L [15]. Human B7-H3 was initially described as a costimulatory molecule expressed only on some activated APC and lymphocytes. Ligation of B7-H3 to a putative receptor on activated T cells enhanced proliferation and IFNγ production. Further genomic analysis of B7-H3, in human and mouse, revealed that the human form can be expressed as two distinct isoforms while the mouse expresses only one isoform [16-18]. The human B7-H3 gene contains four Ig-like repeats which can be alternatively spliced to express a protein
containing either four Ig-like (4IgB7-H3, B7-H3b) or two Ig-like (2IgB7-H3, B7-H3) domains. The mouse gene only contains two Ig-like repeats, and thus only one form is expressed.

Similar to the other B7 family members, subsequent studies have shown that B7-H3 can mediate inhibitory effects [17, 19, 20] on T cell function as well as costimulatory effects [15, 21-23]. Unlike B7-1 and B7-2, B7-H3 has been shown to also be expressed on non-hematopoietic cells [17]. Although FLS lack expression of the classic costimulatory molecules B7-1 and B7-2, we hypothesized that FLS might express other costimulatory molecules, such as B7-H3, and that these ligands might participate in T cell-FLS interactions.

4-3. Methods

**FLS isolation and culture**

All procedures involving specimens obtained from human subjects were performed under a protocol approved by the University of Michigan Institutional Review Board. FLS were obtained by collagenase (Worthington Biochemical) digestion of human synovial tissue obtained at arthroplasty or synovectomy from RA or osteoarthritis (OA) joints. RA diagnosis was based upon the presence of at least 4 out of the 7 American College of Rheumatology criteria for RA [24]. The diagnosis of OA was based upon characteristic clinical and radiographic features, and confirmed by pathological findings at joint surgery. Cells were maintained in CMRL medium (Invitrogen) supplemented with 10% FCS (Atlanta Biological), 2mM glutamine (Cambrex), 50 U/mL penicillin (Cambrex),
and 50µg/mL streptomycin (Cambrex). FLS were used after passage 4 from primary cultures.

**T cell generation**

T cells were obtained from peripheral blood by negative selection using RosetteSep Human T cell Enrichment Cocktail (Stemcell Technologies) and separation on a Ficoll gradient. Briefly, freshly isolated blood from volunteers was incubated with kit antibody cocktail for 20 minutes at room temperature. Blood was diluted 1:1 with 2% NCS/PBS before overlay onto room temperature Ficoll, and spun at 1200 RCF with the brake off. The buffy coat containing only T cells was recovered and used for subsequent experiments. T cells were maintained in RPMI (Cambrex) supplemented with 10% FCS (Atlanta Biological), 2mM glutamine (Cambrex), 50 U/mL penicillin (Cambrex), and 50 ug/mL streptomycin (Cambrex). Trest were resting T cells that had been freshly isolated. Tck were cytokine activated T cells stimulated by a cocktail of 25 ng/ml TNFα, 100 ng/ml IL-6, and 25 ng/ml IL-2 for 8 days [25]. Tsea were T cells activated with superantigen SEA (staphylococcal enterotoxin A) 10 ng/mL by co-culture with IFNγ stimulated FLS [2]. TCd3 were T cells stimulated by plates coated with a combination of 10 ug/mL mouse anti-human CD3 and 10 ug/mL mouse anti-human CD28 for 8 days.

**Flow cytometry**

Fibroblasts were grown for 48 hours in plates before stimulation with cytokines: IFNγ 1000U/mL, TNFα 10ng/mL, IL-1β 1ng/mL, IL-17 20ng/mL, IL-10 10ng/mL, or IL-4 10ng/mL. Cytokine stimulation proceeded for 72 hours. Fibroblasts were removed from
plates for staining by incubation with 3mM EDTA in PBS for 20 minutes at room
temperature. Cells were stained with mouse anti-human antibodies towards CD98, CD54,
or B7-H3 (R&D Systems), or with a mouse IgG control. A secondary goat anti-mouse
IgG Alexa488 (Molecular Probes, Invitrogen) conjugate was used to visualize
fluorescence. Cytometry was performed on a Coulter EPICS XL.

*Western Blot*

Western blot analysis used standard protocols. Fibroblasts were grown in 175 cm² flasks
to confluence and lysed with a buffer containing 1mM PMSF, 5mM iodoacetamide, 8mM
Tris-HCl, 100mM NaCl, 0.02% NaN₃, and 1% NP-40 or 1% saponin as the primary
detergent. Preclearing steps were: 1) 25 uL protein-G agarose beads (Invitrogen), 2) 1 ug
mouse IgG (Sigma) followed by 25 uL protein-G agarose, and 3) 1 ug goat IgG
(Antibodies Inc) followed by two rounds of 25 uL protein-G agarose. Lysates were run
in reducing buffer on Tris-Glycine 4-20% acrylamide gradient gels (Novex, Invitrogen)
and transferred onto an Immobilon-P membrane (Millipore). Primary antibody, goat anti-
human B7-H3 (R&D Systems), was used at a concentration of 1 ug/mL for 1 hour at 4
°C. Control goat IgG antibody (Sigma) was also used at 1ug/mL. Membranes were
washed 3 times with wash buffer before development with rabbit-anti-goat-HRP
conjugate (Molecular Probes, Invitrogen) at 0.2ug/mL for 1 hour at 4 °C. Membranes
were developed by ECL (Amersham).

*Immunohistochemistry*

RA synovial tissue was fixed in 10% neutral-buffered formalin or frozen in OCT, and
sections were mounted onto glass slides. Immunohistochemistry was performed using standard protocols for dichromatic staining of frozen and paraffin embedded sections [26]. Goat anti-human antibodies were anti-B7-H3 (R & D systems), biotinylated anti-B7-H3 (R & D systems), anti-CD90 (insert manufacturer), and anti-cadherin-11 (R & D systems). Mouse anti-human antibodies were anti-CD3 (R & D Systems), anti-CD68 (Dako Cytomation), anti-CD20 (Signet Laboratories), anti-CD45 (Signet Laboratories), and anti-cadherin-11 (Zymed). Detection antibodies and secondary detection reagents used were biotinylated horse anti-goat IgG (Vector Laboratories), alkaline phosphatase horse anti-mouse IgG (Vector Laboratories), R.T.U. VECTASTAIN Elite ABC Reagent (Vector Laboratories), Vector Blue Substrate (Vector Laboratories), Vector Red Substrate (Vector Laboratories), and AEC substrate (Vector Laboratories). After staining sections were sealed using Faramount.

Confocal Microscopy

FLS were grown on glass coverslips for 48 hours. Trest, Tck, or Tsea were co-cultured with FLS for various time points. Coverslips were then washed with PBS and fixed in 4% paraformaldehyde (Molecular Probes) for 1 hour at room temperature or overnight at 4°C. Coverslips were stained using goat anti-human B7-H3 (R & D Systems), mouse anti-human CD11a, mouse anti-human CD54. Secondary antibodies used were goat anti-mouse IgG Alexa594 and rabbit anti-goat IgG Alexa488. For co-culture studies comparing LFA-1 and ICAM-1 localization, before FLS were plated on glass coverslips they were transfected with an expression vector for a fusion protein of ICAM-1 and eGFP. The methodology and vector were identical to those in Chapter 3.
B7-H3 Knockdown and Co-culture of T cells and FLS

Three stealth RNAi and two control RNAi were obtained from Invitrogen algorithms based against the sequence accession AJ583695 from GenBank. Stealth RNAi’s were pooled in equimolar amounts and used at a working concentration of 10 uM, to knock down B7-H3 expression. Control RNAi was also pooled in equimolar amounts and used at a working concentration of 10 uM. FLS were transfected with RNAi using the adult human dermal fibroblast kit (Amaxa Systems). For transfection, FLS were removed from plates by trypsinization and pelleted. FLS were then resuspended in transfection buffer with 1-5 uM final concentration of RNAi and placed in cuvettes. FLS were electroporated and replated for 48 hours before use. As an additional control, FLS were mock transfected without the use of RNAi. Transfected FLS were allowed to rest for 48 hours before being seeded onto plates. FLS were left to adhere for an additional 48 hours after seeding. To evaluate the effect of FLS on T cell cytokine production, Trest, Tck, or T\textsubscript{CD3} were then added to FLS at a ratio of 1:6.66 FLS to T cells. After 8 days, T cells were collected from co-cultures by gentle washing with media. Harvested T cells were washed twice in media and then replated at 500,000 cells in 1.5 mL of medium. T cells were then stimulated for 12 hours with 10 ng/mL PMA and 0.5 uM ionomycin. Supernatants were harvested and TNF\textalpha, IFN\gamma, and IL-2 levels were measured by ELISA (BD Biosciences).

4-4. Results

B7-H3 is expressed by FLS and other fibroblasts

Surface staining of FLS revealed strong expression of B7-H3 by the resting and IFN\gamma
stimulated cells (Figure 4-1A). In contrast, the B7 family member ICOS-L (B7-H2) was not detected (Figure 4-1A). Expression of B7-1 or B7-2 (CD80 and CD86 respectively) by FLS was previously reported to be negligible [13, 14]. Similar to ICOS-L expression, B7-H1 and PD-L2 expression was negligible on FLS and did not increase with IFNγ stimulation (Figure 4-1B). To assess whether other fibroblasts also selectively expressed B7-H3 and whether immunoregulatory cytokines affected B7-H3 expression, we compared B7-H3 expression on RA FLS, OA FLS, skin fibroblasts, and lung fibroblasts. These various fibroblasts were stimulated, before surface staining, with various immunoregulatory cytokines for 72 hours, including IFNγ, TNFα, IL-1β, and IL-17. As a control, CD54 (ICAM-1) expression was also measured. All four types of fibroblasts (RA FLS, OA FLS, skin, and lung) expressed high basal levels of B7-H3, which was unaffected by cytokine stimulation (Figure 4-2). In contrast, CD54 expression increased or decreased depending on the cell type and cytokine (Figure 4-2).

**B7-H3 expressed by FLS and other fibroblasts is predominantly 4Ig**

Since human B7-H3 can be alternatively spliced to yield 4IgB7-H3 (B7-H3b) or 2IgB7-H3 (B7-H3), western blot analysis was used to determine the molecular weight of B7-H3 expressed by fibroblasts. We first established that the antibody employed for western blot analysis recognized both isoforms of B7-H3, detecting a band at slightly less than 98 kDa for recombinant 4IgB7-H3 and slightly less than 50 kDa for 2IgB7-H3 (Figure 4-3A).

Whole cell lysate, from RA FLS lysed in NP-40 was precleared, separated by SDS-PAGE, blot transferred and probed with B7-H3 antibody. This revealed only one band at
approximately 110 kDa corresponding to glycosylated 4IgB7-H3. Extending the blot development time to 3 hours did not reveal the presence of a 2IgB7-H3 band near 50 kDa, but nonspecific background staining was visible (Figure 4-3B).

To determine whether OA FLS or fibroblasts from skin and lung also predominantly express 4IgB7-H3, western blot analysis was again employed. Whole cell lysates from RA FLS, OA FLS, skin fibroblasts, or lung fibroblasts were created using saponin as the detergent, (which resulted in better recovery of B7-H3 compared to NP-40, data not shown). To minimize any potential loss of 2IgB7-H3 signal, this lysate was not precleared before separation on SDS-PAGE. Blot development yielded a strong band at 110 kDa for all fibroblast types and a much weaker band at approximately 50 kDa (Figure 4-3C). This suggests that the 4Ig isoform is the major and almost exclusive isoform of B7-H3 expressed on fibroblasts.

**B7-H3 is broadly expressed in RA synovium.**

To evaluate the expression of B7-H3 in vivo, synovium was obtained from arthroplasty or synovectomy of RA joints, and immediately fixed in formalin for embedding in paraffin, or frozen in OCT. To locate regions rich in FLS, RA synovial sections were stained with the FLS specific anti-cadherin-11 (Cad-11), which was compared to B7-H3 staining (Figure 4-4A). Cadherin-11 is used by FLS in homotypic adhesive interactions, is expressed by synovial lining FLS, and is important for the overall morphology and development of the synovial lining [27]. Monochromatic staining of B7-H3 or Cad-11 showed almost identical patterns of expression. The strongest localization of Cad-11/B7-
H3 was to the lining layer of RA synovium, however, Cad-11/B7-H3 was also found diffusely throughout synovial tissue (Figure 4-4A). CD90, another fibroblast marker, was used to show the diffuse expansion of FLS in RA synovium, but unlike Cad-11/B7-H3, CD90 localized strongest to blood vessel rich reasons (Figure 4-4A). This is strong indication that, similar to cultured FLS, the FLS in RA synovium express B7-H3.

Dichromatic immunohistochemical staining was performed, to compare the expression of B7-H3 to immune cell markers. Staining of paraffin sections for B7-H3 and CD20 (B cell), CD45 (hematopoietic), or CD68 (macrophage) surface structures revealed that B7-H3 staining was distinct from CD20 and CD45 (Figure 4-4B). There was diffuse B7-H3 expression throughout the pannus with slightly stronger localization towards the lining layer and around blood vessels. Patches of B cells and hematopoietic cells appeared distinct from the B7-H3+ cells. The macrophage marker CD68 showed broad staining that overlapped partially with B7-H3 expression. This most likely represents resident macrophage-like synoviocytes and invading macrophages. The control antibody for B7-H3 (goat IgG) displayed only a background staining pattern (Figure 4-4B). Frozen sections were stained to compare B7-H3 staining to CD3 (T cell) or CD80 (APC) staining (Figure 4-4C). CD3+ T cells were abundant and in close proximity to B7-H3+ cells. CD80 staining showed a diffuse pattern, indicating the broad distribution of professional APC within the synovium. However, at the synovial lining, B7-H3 staining was strong and there was little CD80 signal.

By the staining patterns of B7-H3 and CD3, T cells appear to be juxtaposed to FLS
throughout RA synovial lesions. However, since B7-H3 is not unique to FLS, CD3 staining was compared to Cad-11 and CD90 (Figure 4-4D). Similar to the pattern seen with B7-H3/CD3 (Figure 4-4C), Cad-11/CD3 and CD90/CD3 staining showed both well-organized and diffuse T cell regions, with many T cells in close proximity to Cad-11+ FLS (Figure 4-4D).

**B7-H3 localizes to the contact point between FLS and T cells.**

T cells representing three different T cell activation states were co-cultured with FLS. Tsea were T cells that had been stimulated through their TCR by superantigen presented by FLS. Cytokine activated T cells (Tck) were activated with a cytokine cocktail, (and not by the TCR), consisting of IL-6, TNFα, and IL-2, and represent a population that share characteristics similar to T cells isolated from RA synovial fluid [25]. Trest were T cells that were purified from peripheral blood and not otherwise stimulated. After 2 or 24 hours, the co-cultures were fixed with paraformaldehyde and stained for B7-H3 and CD3. By 24 hours, B7-H3 signal could be seen outlining the contact point of both Tck and Tsea on FLS (Figure 4-5A&B). At 24 hours, Trest adherence to FLS was marked, but strong B7-H3 signal at the contact site was not clearly visible (Figure 4-5A). This localization of B7-H3 to the contact region with Tck and Tsea on FLS is clearly distinct as early as 2 hours of co-culture (Figure 4-5B).

**B7H3 localization at the FLS/Tck point contact is distinct from the zone of CD54/LFA-1 engagement.**

B7-H3 localization was compared to the localization of CD54 (ICAM-1) on FLS and its
counter receptor, LFA-1 (CD11a/CD18), on the T cell. FLS were transfected with a
CD54-eGFP (green) expression vector before co-culture with Tck. Co-cultured cells
were fixed and Tck were indirectly stained for CD11a which was visualized by means of
the fluorochrome Alexa594 (red). At the synapse of Tck and FLS, confocal imaging
revealed merged signals (yellow) between CD54 and LFA-1 (Figure 4-6A). In contrast,
B7-H3 at the FLS/Tck contact point showed a staining pattern that was distinct from
CD54 (Figure 4-6B), as seen by the separation of green and red signals. Similarly no
merging of B7-H3 and LFA-1 fluorescence was detected (Figure 4-6C). The separation
of B7-H3 from regions dense in LFA-1/CD54 at the contact point between FLS and Tck
could be a temporal artifact. It is possible that B7-H3 might migrate to and from
LFA/CD54 dense areas in a rapid sequence or at extremely slow rate not detected by the
time points of confocal image. However, we have not observed any merged signal of B7-
H3 with LFA-1 or CD54 at co-culture times up to 24 hours (data not shown).

Distinct functions of B7-H3 in interactions with resting versus cytokine activated T cells

To assess the role of B7-H3 in co-cultures of T cells and FLS, RNA interference (RNAi)
technology was used to knock down B7-H3 expression in FLS before co-culture with T
cells. Pools of specific or control RNAi's were transfected into FLS and screened for
effective inhibition of B7-H3. Effective and specific knockdown of B7-H3 persisted for
at least 12 days post-transfection (Figure 4-7). RNAi transfection did not adversely affect
CD98 expression in mock transfected or RNAi transfected FLS (Figure 4-7).

T cells representative of three activation states were co-cultured with FLS: Trest were
resting T cells freshly isolated from peripheral blood; Tck were cytokine activated T cells
similar to RA synovial T cells [25]; \( T_{\text{CD3}} \) were T cells that had been previously activated through plate bound anti-CD3/anti-CD28. \( T_{\text{CD3}} \) were used in place of Tsea to represent TCR stimulated T cells in these experiments, to avoid the need for exposure of T cells to APC prior to incubation with B7-H3 knockdown FLS, which could potentially confound interpretation of the results. These various types of T cells were co-cultured with FLS that had been transfected with pooled B7-H3 RNAi, pooled control RNAi, or mock transfected FLS (no RNAi). After 8 days of co-culture with B7-H3 knockdown FLS, T cells were harvested and restimulated with PMA and ionomycin for 12 hours. Production of TNF\( \alpha \), IFN\( \gamma \), and IL-2 by the T cells was measured by ELISA. While cytokine production by \( T_{\text{CD3}} \) following co-culture with FLS was not substantially affected by B7-H3 knockdown, striking and opposite effects on cytokine production of Tck and Trest were observed. Trest co-cultured with B7-H3 negative FLS showed increased levels of cytokine production compared to FLS transfected with control RNAi (Figure 4-8A to C). In contrast, Tck co-cultured with B7-H3 negative FLS showed reduced cytokine production compared to co-culture with control RNAi FLS (Figure 4-8A to C). This suggests that B7-H3 has an inhibitory effect on Trest and a stimulatory effect on Tck.

4.5 Discussion

B7-H3: inhibitory or costimulatory?

To achieve full activation during the response to peptide antigens, T cells require two stimulatory signals. TCR engagement of MHC/antigen triggers complex signaling cascades in T cells, but alone this is insufficient for activation [28-31]. A costimulatory signal is required in conjunction with TCR signaling to induce clonal proliferation and
prevent anergy. This additional signal to the TCR is typically provided by a surface structure present on accessory cells, such as B7-1 (CD80), which can engage the CD28 molecule on the T cell surface [32-36]. The discovery of a second receptor for B7, CTLA-4, which is important in limiting T cell responses, established a molecular basis for the bifunctional role of B7 as both an activating and inhibitory ligand [37].

Starting from the initial B7-1 costimulatory ligand, a family of B7 molecules has been discovered. B7-2 (B70, CD86) is a second ligand for both CD28 and CTLA-4. New ligand/receptor pairs include PD-L1 (B7-H1)/PD-1, PD-L2 (B7-DC)/PD-1, ICOS-L (B7-H2, B7h, B7RP-1)/ICOS, and B7-H4/BTLA [38-40]. These novel B7 family molecules have documented effects in enhancing T cell functions, inhibiting T cell functions, or both.

This duality is further reinforced by the more recent characterization of the B7 related protein VSIG4. Initially, VSIG4 (CRIg) was described as a complement receptor necessary for binding enzymatic products of C3 and for effective phagocytosis by Kupffer cells [41]. In addition, VSIG4 has been also been found to reduce T cell proliferation by CD3/CD28 stimulation in vitro, and to blunt CD8 T cell responses and IFNγ production in vivo [42]. This complex pattern of inhibition and/or activation is an important characteristic of the B7 family. Thus beyond the their role in immune activation, the B7 family functions in the complex realm of immune modulation [38-40]. This is exemplified by the discoveries that CD28 and CTLA-4 not only transduce signals into the T cell by ligation of B7 family members, but that the signaling is bidirectional.
with functional signals transmitted into the APC as well [43, 44]. Further complicating immune modulation by B7 family members is the more recent discovery that PD-L1 and B7-1 can indeed bind, and that is interaction is inhibitory and signals bidirectionally [45].

The current study has documented expression of a B7 family costimulatory molecule, B7-H3, on FLS and other fibroblasts, which are not typically viewed as professional APC. Unlike professional APC, which only express B7-H3 after activation [15], FLS expression is constitutive and uninfluenced by immunoregulatory cytokines. This constitutive B7-H3 expression is overwhelmingly of the 4Ig isoform and is robust both in vivo and in vitro.

The literature on the effects of B7-H3 on T cells has not hitherto provided consensus as to its function. B7-H3 was originally described in human systems as a 2Ig costimulatory molecule that increased T cell proliferation, enhanced cytotoxicity, and increased IFNγ production [15]. However, a subsequent study indicated that the predominant isoform of B7-H3 is the 4Ig form [17]. This report did not recapitulate the costimulatory effects originally documented. In yet another study, NK lysis of neuroblastoma lines was enhanced by blocking antibodies against 4Ig B7-H3 [46].

Studies of B7-H3 in mice, which only contain one isoform, 2Ig [16, 18], do not resolve this issue. B7-H3 knockout mice showed increased airway hypersensitivity and earlier onset of EAE [20]. Similar results were obtained using B7-H3 blocking antibodies, notably that T cells increased cytokine production and EAE symptoms were exacerbated.
by B7-H3 blockade [19]. In contrast to reports in humans, tumor expression of B7-H3 enhances cytotoxicity [21, 22]. And when B7-H3 knockout mice are used in allograft survival studies, B7-H3 deficient transplants fare better when combined with immunosuppression [23]. Thus far, there appears to be no specific and consistent association of 2Ig or 4Ig B7-H3 with activating or inhibitory effects.

We found B7-H3 expression was associated with FLS rich areas and was in close proximity to T cells in the RA pannus. When the T cell-FLS interaction was explored by confocal microscopy, B7-H3 was found at the contact point, but was differentially localized from CD54 and LFA-1. This suggested that B7-H3 could be an important signaling molecule between FLS and T cells. However, knockdown of B7-H3 in FLS did not affect the ability of FLS to present superantigen to T cells or to respond to contact with T cells by the secretion of IL-6 and IL-8 (data not shown). Nevertheless, interesting B7-H3 dependent effects on cytokine secretion by T cells following co-culture with FLS were documented, including the differential effects of B7-H3 on cytokine production by resting or cytokine activated T cells. This suggests that the activation state of the T cell has significant influence over the outcome of T cell ligation of B7-H3, and also suggests that there may be two receptors for B7-H3 (which is a pattern that would be similar to other B7 family ligands). Furthermore, cytokine production by $T_{CD3}$ did not greatly differ after co-culture with B7-H3 expressing or knockdown FLS.

A simplistic model to explain our observations would be that resting T cells predominately express an inhibitory receptor for B7-H3, while cytokine activated T cells
predominantly express an activating receptor for B7-H3. T cells activated through stimulation by CD3 and CD28 did not have significant differences in cytokine production, suggesting that this type of activated T cells does not express a functionally significant level of B7-H3 receptor, or that, perhaps, opposing effects of engagement of both positive and negative receptors are offsetting. So far we have not observed an effect on FLS, by engagement or interference with B7-H3 signaling, implying that perhaps B7-H3 signaling is not bidirectional in FLS-T cell interactions as compared to interactions of T cells with professional APC. However, an alternative possibility is that other assays need to be employed to measure the functional consequences of B7-H3 engagement on the B7-H3 expressing cells.

Costimulation in RA

The strong expression of B7-H3 by FLS of the RA pannus raises interesting issues regarding costimulation of T cells in RA. It has been suggested that an unusual subset of CD4+CD28null T cells is expanded in RA [47, 48]. These T cells are likely to require costimulatory signals distinct from CD80/CD86. There is evidence that constant exposure to TNFα (which is abundant in RA) blocks the transcription of CD28 [49]. These CD4+CD28null T cells have been shown to be potent producers of cytokines (including TNFα and IFNγ) [50], and to also express costimulatory molecules normally associated with NK cells [51]. It will be interesting to determine whether this CD4+CD28null T cell subset interacts with FLS through the B7-H3 molecule.

CD4+CD28null T cells are, of course, not the only inflammatory T cell subset in RA.
Recent trials indicate that the drug Abatacept (CTLA-4-IgG fusion protein) has clinical benefit in RA [52]. Unfortunately, as with all current therapy for RA, complete remission of disease is unlikely. Nonetheless the clinical data proves that interference with costimulatory molecules may be an effective approach for treatment of RA, and it is likely that targets other than CD28 will also be studied. The robust, constitutive and selective expression of B7-H3 by FLS in vivo and in vitro, and its involvement in FLS/T cell interactions implies a unique and significant role for this molecule in the pathogenesis of synovitis. Definition of its receptor(s) on the T cell, and further insights into their costimulatory or inhibitory signaling functions will be required in order to determine whether B7-H3 or its receptor(s) are, like CD28, potential targets for novel molecular therapeutic agents in RA and other diseases.
Figure 4-1. FLS express the B7 family molecule B7-H3.

A) FLS were stimulated with IFNγ for 72 hours. FLS were removed from culture wells with EDTA and stained with mouse anti-human B7-H3 antibody (solid black line), mouse anti-human ICOS-L (dashed black line), or control mouse IgG (solid grey line). B) The panel of antibodies was expanded in a separate experiment to include B7-H3 (solid black line), PD-L2 (dashed black line), B7-H1 (solid dark grey line), or mouse IgG control (light grey fill). Staining is representative of two experiments.
**Figure 4-2.** *Fibroblasts from various tissue sources express B7-H3.* RA FLS, OA FLS, lung fibroblasts, and skin fibroblasts were stimulated with TNFα, IFNγ, IL-1β, IL-17, IL-4, or IL-10 for 72 hours. Fibroblasts were removed from culture wells with EDTA and stained with mouse anti-human B7-H3, CD54, or control antibody. Staining is representative of two experiments.
Figure 4-3. *Fibroblasts express the 4Ig isoform of B7-H3.*

Numbers shown to the left of each panel represent molecular mass markers (kDa). A) Recombinant 4Ig and 2Ig B7-H3 was separated on SDS PAGE and stained with goat anti-human B7-H3 antibody. After transfer to Immobilon-P, blots were developed by rabbit anti-goat HRP and ECL. B) RA FLS whole cell lysates were precleared, and then separated on SDS PAGE, blot transferred, and developed with goat anti-human B7-H3 antibody or goat IgG. Lysates from the same RA FLS line were run in all lanes. These results representative of two experiments. C) Whole cell lysates from RA FLS, OA FLS, lung fibroblasts, and skin fibroblasts were run on SDS-PAGE without preclearing, blot transferred, and developed with goat anti-human B7-H3 antibody or goat IgG. The film exposure duration was 5 seconds. Staining is representative of two experiments.
Figure 4-4A&B. B7-H3 expression within the RA synovium
Sections RA synovial tissue were stained. Sections are representative of these experiments and come from RA tissue. A) B7-H3 staining was compared to cadherin-11 and CD90 staining on frozen sections. To control for dichromatic staining, control antibody was developed in blue for B7-H3/Cadherin-11 and red for CD90. Images were taken at 20X magnification. B) Paraffin embedded sections of RA synovial tissue were dually stained with goat anti-human B7-H3 (red) and mouse anti-human CD20, CD45, or CD68 (all blue). As a control for B7-H3, goat IgG was used. Images were taken at 40X magnification.
Figure 4-4C&D
C) Frozen sections were stained dually stained with goat anti-human B7-H3 (red) and mouse anti-human CD3 or CD80 (both blue). Images taken at 40X magnification. D) CD3 (blue) staining was compared to cadherin-11 (red) staining on frozen sections, with goat-IgG control for cadherin-11 (red) (top). In addition, CD3 (red) was compared to CD90 (blue), with mouse IgG (blue) control for CD90 (bottom). Images taken at 40X magnification.
Figure 4-5. B7-H3 localization at the T cell/FLS synapse.
A) FLS were grown on glass coverslips and co-cultured with Tsea, Tck, or Trest for 24 hours. Coverslips were fixed in 4% paraformaldehyde and stained with goat anti-human B7-H3 (green) and CD3 (red). B) Staining of B7-H3 (green) and CD3 (red) after 2 hours of co-culture. Images are representative of three experiments.
**Figure 4-6** B7-H3 localization compared to CD54 and LFA-1 localization at the T cell/FLS synapse.

Images are representative of three experiments and digitally zoomed before capture. A) FLS were transfected with an expression vector for CD54-eGFP fusion protein (green) before plating on glass coverslips and co-culture with Tck. Tck were stained with anti-LFA-1 (red). This image is the bottom row of Figure 3-4. B) Tck were co-cultured with FLS and stained for B7-H3 (green) and CD54 (red). C) B7-H3 (green) dually stained with anti-LFA-1 (red) in co-cultures of FLS and Tck.
Figure 4-7. *B7-H3 specific RNAi knockdown B7-H3 long term cultures.*
FLS were transfected with pooled B7-H3 specific RNAi, pooled control RNAi, or mock transfected (with no RNAi) before staining with B7-H3 (black line), CD98 (dashed line), or control mouse IgG (gray fill) at 12 days post transfection. Histograms are representative of 5 experiments.
Figure 4-8 T cell cytokine production after co-culture with B7-H3 RNAi transfected FLS. FLS were transfected with pooled B7-H3 RNAi (B7-H3), pooled control RNAi (Control), or mock transfected (No RNA) and co-cultured with Trest, Tck or Tcd3/cd28. T cells were harvested after 8 days of co-culture and restimulated with PMA and ionomycin. Supernatants were measured for cytokines by ELISA. Error bars represent 95% confidence intervals. Figures are representative of three experiments. A) TNFα. Using two tailed t-Test analysis comparing cytokine production from Trest, cultured with B7-H3 negative FLS (B7-H3) to B7-H3 positive FLS (Control) and with B7-H3 negative FLS (B7-H3) to mock transfected (No RNA), yielded p-values <0.0021 and <2.2x10^-5 respectively. Similarly t-Test analysis for Tck yielded p-values <9.1x10^-8 and <5.6x10^-7 respectively. B) IFNγ. p-values for Trest cytokine production are <1.8x10^-5 and <0.0013 respectively, and for Tck are <2.8x10^-9 and <1.2x10^-7 respectively. C) IL-2. p-values for Trest cytokine production are <2.5x10^-5 and <0.0005 respectively, and for Tck are <2.4x10^-5 and <2.7x10^-6 respectively.
References


5-1. Summary of findings

*FLS presentation of arthritogenic peptides*

Extending the earlier findings of FLS presentation of superantigens to T cells, we focused on whether FLS could serve as APCs for peptide specific responses. When FLS were used as APCs for immunodominant peptides from the arthritogenic antigens HCgp39 and hCII, we found that T cell hybridomas recognized antigenic peptides presented by FLS and released IL-2. Demonstrating that true antigen presentation was occurring between FLS and T cell was important, because of the various antigen independent signaling pathways possible between these cell types. Similar to professional APCs, antigen presentation by FLS was dependent on functional MHC II, which was induced by IFNγ. The HLA-DR4 alleles of the FLS and the corresponding TCR on T cells had to be precisely matched to engender a T cell response, illustrating MHC restriction as well as strict peptide specificity. SF from RA and OA patients contained both HCgp39 and hCII
antigens that could be presented to the HCgp39 and hCII specific T cell hybridomas, respectively. However, there are as yet unidentified factors present in SF that may inhibit or augment FLS antigen presentation in the presence of adequate levels of antigen. Given the expression of MHC II by FLS in vivo, coupled with FLS-T cell proximity in pannus lesions and chronic exposure to autoantigens, these results suggest that FLS might be an important APC in the inflamed synovium.

Further work must be done to support the hypothesis that in vivo FLS antigen presentation is of importance in RA. Although, T cell hybridomas are a good measure of antigen presentation they are not ideal representations of human RA T cells. Recapitulation of our findings using human T cell lines or clones developed against hCII or HCgp39 would be strong support for FLS APC function in RA. The exact mechanism of antigen presentation used by FLS also remains to be discovered. Is this mechanism identical to professional APC such as dendritic cells and macrophages? Do FLS possess the intracellular machinery necessary to degrade proteins to their antigenic peptides or can they only process and present small peptides?

Along similar lines, do all fibroblasts possess innate APC capabilities and how does this relate to our understanding of autoimmune disease? We have shown that some lung fibroblast lines can present antigen similar to FLS. Is this APC potential of FLS and other fibroblasts only manifested in settings of chronic inflammatory cytokine exposure (i.e. an autoimmune setting)? And how does the initiation and continuation of antigen presentation differ in a normal immune response (say in septic arthritis) from an
autoimmune response (RA)? If all fibroblasts possess innate APC potential, are fibroblasts important in extra-articular manifestations of RA, or in diseases in which fibrosis of tissue rather than tissue destruction by fibroblasts is the pathologic hallmark? To answer these questions, it will be necessary to further dissect out the components of antigen presentation in RA by professional APC and FLS.

*Tck interactions with FLS*

Earlier imaging analysis in our laboratory of FLS and T cell interactions utilized opposite states of T cell activation: resting and superantigen activated. We turned our attention towards a distinct type of T cell, the Tck, which is activated not by TCR engagement, but instead by a cytokine cocktail composed of mediators relevant to RA. Even more than Trest, Tck could potently activated FLS in cocultures and this effector function synergized with IL-17. Imaging of Tck/FLS cocultures documented extensive cellular interactions, and confocal imaging found colocalization of the ICAM-1 and LFA-1 to the Tck/FLS synapse. However, blocking antibodies against LFA-1 or ICAM-1 could not significantly inhibit Tck activation of FLS. Significant reduction in Tck activation of FLS was, however, observed with TNFα blockade, but soluble TNFα could not be detected in coculture supernatants. Effective inhibition of Tck effector function on FLS was obtained by blockade of membrane-bound TNF on Tck. These findings suggest that in addition to the soluble TNFα produced from synovial macrophages, membrane bound TNFα on Tck may also contribute to FLS transformation and activation.
Given the lack of response to initial T cell targeted therapies and the robust therapeutic effects seen after TNFα blockade, it appeared that cytokine networks were key to RA treatment. However, recent trials demonstrate that T cell targeted therapies (e.g. CTLA4-Ig) also improve clinical and radiographic endpoints in RA. In theory, CTLA4-Ig would inhibit T cells indirectly by costimulation deprivation or directly inhibit APC. So, in addition to cytokine networks, the T cell once again is an attractive target for RA therapy and by extension a likely culprit in RA pathology. The Tck provides a novel in vitro model to mimic RA T cells. It would be of great interest if RA T cells from patients also expressed biologically active, membrane bound TNFα. Along similar lines and given the promiscuity of B7 receptor-ligand interactions, would CTLA4-Ig inhibit Tck ability to active FLS and would this effect synergize with TNFα blockade? No RA therapy has produced complete remission of symptoms in a majority of patients with RA, and this is paralleled by our in vitro studies of incomplete inhibition of FLS activation by TNF blockade. It is distinctly possible (and rather likely) that other cognate receptor-ligand interactions, as yet unidentified, contribute to FLS-T cell signaling.

The Role of B7-H3 on FLS

Extending the identification of further mechanisms of FLS-T cell interactions, we found that FLS constitutively express the B7 family member, B7-H3, but not other B7 family ligands. In contrast to observations with immune cells, immunoregulatory cytokines did not modulate FLS B7-H3 expression. Western blot analysis confirmed that the 4Ig isoform of B7-H3 was the predominant form expressed by FLS. Immunohistochemical
staining of RA synovial sections showed that B7-H3 expression was robust in vivo and mirrored the expression of the FLS marker cadherin-11, and the fibroblast marker CD90. B7-H3 expression in synovial lesions was distinct from B cells, but did show some overlap with macrophage regions. T cell markers in synovial sections were also distinct from B7-H3, but were in close proximity to FLS. Confocal fluorescence imaging of Tck/FLS cocultures showed B7-H3 localization near the contact point between the two cell types. Based upon our data of ICAM-1/LFA-1 colocalization to the contact site between FLS and Tck, we compared B7-H3 localization to LFA-1 and ICAM-1. B7-H3 localized near the contact point of FLS and Tck, but in regions distinct from LFA-1 and ICAM-1. When B7-H3 was knocked down in FLS by RNAi and these FLS were used to stimulate different types of T cells, it was found that Tck cytokine production was inhibited and Trest cytokine production was enhanced relative to controls, indicating that putative receptors for B7-H3 are activating in Tck while inhibitory in Trest.

These results raise the question of what role B7-H3 plays in the immune system and, given FLS strong constitutive expression of B7-H3, what part it plays in RA pathology. The different isoforms of human B7-H3 need to be more fully differentiated in terms of effector function and tissue/cellular expression profile. In this regard, do the two isoforms signal differently, are they differentially expressed temporally, and what influences expression of one over the other? All of these questions also beg the discovery of the counter ligand(s) to B7-H3. After this ligand is discovered, its expression profile and tissue distribution would be quite informative. Based upon our data and likely similar to other B7 family ligands, the counter ligand would likely be expressed as two distinct
receptors, one inhibitory and one activating. Would the different isoforms of B7-H3 have distinct preference for activating or inhibitory receptors? Do CD28-negative T cells, a subset that is expanded in RA, preferentially express ligands for B7-H3? It is exciting to think that similar to CTLA4-Ig, one of these receptors could become a potent therapy in autoimmune disease.

Much thought has been given to B7 family members as costimulatory molecules, but they also signal into the cells on which they are expressed. On this front much work still needs be accomplished to discern what effect (if any) ligation of B7-H3 has upon the cells which express it. Similarly, does ligation of different isoforms result in different signaling? Thus far we have not seen a functionally different response from FLS that have had B7-H3 knocked down by RNAi.

Once these questions have been answered, the results will hopefully provide another therapeutic target for RA and possibly other autoimmune diseases. At the least, it will further understanding of the immune system’s complexity.

5-2. In closing

T cell/FLS cross-talk is an important pathologic mechanism in RA. These two cell types share potent interactions via antigen dependent and antigen independent mechanisms – following some known paradigms of immune system function but also interacting in completely novel mechanisms. These interactions may ultimately prove to be effective targets for new treatments for RA. Further work that utilizes a combination of
approaches, ranging from ex vivo studies of RA tissues to genetic and immunologic interventions in animal models, will further establish the conceptual basis for new treatment strategies.