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Integrated Immune Cell-Cell Communication Networks Underpinning Rheumatoid Arthritis Pathogenesis

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ABSTRACT

Rheumatoid Arthritis (RA) is a chronic, systemic auto-inflammatory disease that targets peripheral joints causing bone erosion, worsened mobility, and lower quality of life. RA is a complex autoimmune disease that develops through the combined actions of many cell types. In particular, monocytes, fibroblasts, and T cells play critical roles within the arthritic joint driving disease pathogenesis. These cells communicate with each other via ligand-receptor interactions orchestrating inflammatory responses ultimately leading to joint destruction. However, it remains unclear how the combined efforts of these different cells form an integrated network of immune responses which ultimately lead to disease development. Therefore, we hypothesized that an integrated communication network between the different immune cells found within RA joints functions to promote inflammation and drive RA disease pathogenesis. I utilized predictive assessment of cell-cell communication networks through single-cell RNA-sequencing to determine how immune cell interactions differ between autoimmune and non-autoimmune joint destruction. These studies identified a novel cell-cell communication network in which monocytes, fibroblasts and T cells cooperate to promote cell-cell interactions, immune activation, and induction of pro-inflammatory gene networks. Together, these results identify numerous potential therapeutic targets for the intervention of RA disease.

Introduction

Rheumatoid Arthritis (RA) is a chronic, systemic autoinflammatory disease that targets peripheral joints causing bone erosion and negatively affects mobility.[1] RA does not directly cause death, but it significantly reduces quality of life and the life expectancy of those affected by it if left untreated.[2] Globally, RA affects approximately 0.5-1.0% of the population with two to three times greater incidence in women.[3] The development of RA joint inflammatory response usually appears as a result of microvascular changes and an increase in the number of cells or synovial lining hyperplasia.[4] During the development of the disease, it is also possible for other organs to be affected as well. This systemic inflammation can cause cardiovascular, pulmonary, and skeletal complications.[3] The control group for the analysis performed was patients with osteoarthritis (OA), which is a debilitating chronic condition requiring long-term treatment of pain and functional impairment.[5] The symptoms of OA are like that of RA, consisting of joint pain and stiffness, with an emphasis on pain in the knee. A major difference between these two diseases is that RA is an autoimmune disease while OA is not, so differences between the two diseases are likely to be attributed to the autoimmune reaction underpinning RA.

The pathogenesis of RA generally takes place in the synovial tissue and is characterized by recruitment and accumulation of T cells, B cells, macrophages, and dendritic cells in the synovium.[1] As all these types of cells are interconnected with one another, cytokines and chemokines signal from one cell to another. One such chemokine is CCL2, which is also known as monocyte chemoattractant protein-1 (MCP-1), induces the locomotion and recruitment of monocytes to the site.[6] Another cytokine is IL1B, which is associated with the expression of different characteristic

features of RA.[7] IFNγ is another important cytokine, and it mediates both immunostimulatory and immunosuppressive effects of the disease.[8]

Memory CD4+ T cells are spread throughout the affected joints of RA patients, and highly expanded CD4+ T cells are found in the synovial tissue during the early stages of disease. Clonally expanded T cells is a key characteristic of several autoimmune syndromes, including RA.[1] In 1986, immunologists Mosmann and Coffman proposed the idea that these CD4+ T cells could be divided into two different subsets, Th1 or Th2, based on differential ability to secrete various cytokines, including IFNγ, IL-2, TNF, IL-4, and IL-5.[1] Th1 cells are characterized by an ability to produce IFNγ, IL-2, and TNF, while Th2 cells secrete IL-4 and IL-5. RA has been determined to be a Th1 CD4+ T cell driven disease based on the heightened abundance of IFNγ-secreting T cells in the synovial fluid of RA patients.[1] Th1 cells induce macrophage activation, leading to an increased capacity to secrete pro-inflammatory cytokines, such as TNF.[1] However, more recent research suggests that other types of T cells exist, such as Th17 and Th22, and they have a clear involvement in the disease as well.[2]

The HLA-DR class of Major Histocompatibility Complex molecules are critical to driving T cell-mediated responses and play a key role in the pathogenesis of RA.[9] The primary role of HLA-DR is to present processed antigenic peptides to T cells driving T cell receptor (TCR)-mediated activation. Within the arthritic joint, HLA-DR is expressed on the surface of antigen presenting cells such as macrophage and B cells as well as by activated CD4+ T cells.[10] Specifically, the association between the HLA-DRB1 molecule and RA disease development encodes the polymorphic HLA class II DRβ chain, and its greatest variation is confined to a stretch of the DRβ chain alpha helix known as the shared epitope; this is the strongest genetic evidence of CD4+ T cells and its role in the pathogenesis of human disease.[9]

B cells are located in the synovial fluid, and play a major role in the pathogenesis of RA.[11] Prior to entering the joint, these cells undergo a process called B cell checkpoints, which are a series of quality-control steps that influence the proliferation, differentiation, apoptosis, and other aspects of B cell development. Some of these checkpoints, known as tolerance checkpoints, function to promote B cell tolerance and limit the amount of self-reactive B cells in the body. During the first stage of B cell development, the cells are checked by the first tolerance checkpoint in the bone marrow.[11] After the now immature B cells leave the bone marrow, they are checked by a second tolerance checkpoint, and soon become mature B cells. Several functions of B cells, including antigen presentation, cytokine secretion, and autoantibody production, are major contributors to the pathogenesis of RA.[11] The abnormal generation and persistence of self-reactive B cells in RA suggests that defects in these checkpoints also contribute to disease development.[11]

B cells are one of three types of professional antigen presenting cells (APC). The primary function of these cells is to present antigens to the CD4+ T cells driving their activation. B cells secrete a variety of cytokines, including TNF-a, IFNγ, IL-6, IL-1b, IL-17 and IL-10. B cells also produce and secrete autoantibodies after differentiating into plasma cells. Autoantibodies contribute to the pathogenesis of RA through immune complex formation that are deposited in inflamed joints activating the complement pathways. Complement activation drives the production of C5a and membrane attack complexes which further promote tissue damage. Interestingly, some B cells function to prevent RA through production of natural antibodies and anti-inflammatory cytokines such as IL-10.[11]

Monocytes are a type of white blood cell from the myeloid lineage. These cells have plastic characteristics, allowing them to differentiate into their own subsets or into other cells, such as macrophages. Monocytes develop in the bone marrow from hematopoietic stem cells, just like other cells of the myeloid lineage. Monocytes are identified by their characteristic expression of CD14 and CD16. A classification system created by Ziegler-Heitbrock et al. categorized monocytes into three groups: classical monocyte (CD14++CD16−), intermediate monocyte (CD14++CD16+), and non-classical monocyte (CD14+CD16++) (12). Intermediate monocytes produce proinflammatory cytokines, classical monocytes secrete high levels of IL-1β, IL-10, IL-6, and TNF-α, and non-classical monocytes are responsible for an early inflammatory response. Monocytes express cell surface HLA-DR, contributing to a higher production of TNF and promoting T cell activation.[12] A key characteristic of monocytes is that they differentiate into various macrophages, which can further differentiate into osteoclasts. Osteoclasts are a major hallmark of RA

pathogenesis, as they are responsible for the dissolution of bones. Specifically in RA, osteoclasts populate the synovial membrane and play a role in the structural damage of chronic inflammatory joint disease.[13]

Within tissues, fibroblasts function to maintain tissue homeostasis, coordinate inflammatory responses, and mediate tissue damage. Chronically activated fibroblasts can differentiate into myofibroblasts, which are responsible for producing collagen and are important for the process of fibrosis in lung, liver, gut, skin, and other tissues. In RA, chronically activated fibroblasts drive excessive matrix degradation that destroys cartilage and causes permanent joint damage. Furthermore, these fibroblasts in the synovial tissue have distinct surface markers, their subpopulations have distinct mRNA signatures, and there are three major fibroblast subsets: CD34– THY1– , CD34– THY1+, and CD34+.[14]

RA is a disease that greatly affects the lives of those who are diagnosed with it. RA is ranked as one of the highest diseases for the negative effects it has on the quality of life of patients. RA causes severe limitations in patients' ability to perform physical work, increased pain and fatigue, and reduced participation in leisure activities.[7] As a result, many patients with RA develop mental/emotional limitations, which further decrease quality of life.[7] Additionally, as previously mentioned, RA affects around 0.5-1.0% of the world population, which is a considerable amount.[1] There are several therapeutic approaches to limit the negative impacts of RA; however, to date treatment is unable to cure the disease and often leads to severe side effects. Therefore, the generation of more effective therapeutic approaches will require a more comprehensive understanding of disease pathogenesis and its molecular underpinnings.

Here, I report an assessment of the cell-cell communication networks characterizing the autoimmune landscape of RA patients' joints. Novel analysis of previously published single cell RNA sequencing (scRNA-seq) data revealed a unique cellular communication network distinguishing non-autoimmune (osteoarthritis) and autoimmune (RA) joint inflammation. Specifically, I found a communication network in which joint-infiltrating monocytes produce increased amounts of IL-1b that act upon synovial fibroblasts, driving the production of chemotactic agents such as CCL2 and the pro-inflammatory cytokine IL-6. These chemoattractants are, in turn, sensed by immune cells such as monocytes and T cells that likely promote physical interactions, ultimately enhancing T cell activation. Activated T cells then express pro-inflammatory cytokines such as IFNγ and TNF, further activating monocytes and fibroblasts. In all, these analyses have unearthed a previously unappreciated interconnected signaling network that forms a feedback loop, likely underpinning the progressive nature of RA inflammation.

Methods

Dataset

The dataset used for this study was downloaded from Immport, accession #SDY998.

NicheNet Analysis

The NicheNet analysis took place by using R Studio, and by starting with creating a Seurat object, which is an R package that is designed for single cell RNA sequencing. In order to create the Seurat object, the dataset needed to be put into different functions that turned the data into a Seurat object. From here, the NicheNet object could be created for each cell type by setting the receiver celltype as the one the object is being created for, and the sender cell types as each of the other cells. Finally, for each NicheNet object, the aforementioned ligand heat map and DotPlots were created. All code used can be seen here: https://github.com/amaanb/RA-Nichenet-Analysis.

Graphical Abstract

Graphical representation of the findings of this study were generated using Biorender (www.biorender.com).

Results and Discussion

Single cell transcriptional landscape of RA

To gain a deeper understanding of the cellular communication networks underpinning RA pathogenesis, I performed predictive analyses of cell-cell communication using previously published single-cell RNA-sequencing data.[15] The data used throughout my analysis was taken from 36 patients with RA who met the 1987 American College of Rheumatology (ACR) classification criteria and 15 patients with OA from ten clinical sites over 16 months. After obtaining tissue samples from both the RA and OA patients, a variety of strategies were used to separate the main cells from one another: B cells, T cells, monocytes, and fibroblasts.[15] As such, the analysis performed determines ligand signaling enriched in the cells from RA patients, while using the data from OA patients as a reference. Using OA patients as the control rather than healthy patients is to ensure all differences between the control is due to the autoimmunity of RA.

In the dataset for the analysis, patients with OA had 104 B cells, 248 fibroblasts, 200 monocytes, and 204 T cells. Patients with RA had 858 B cells, 2017 fibroblasts, 784 monocytes, and 1574 T cells (Fig 1A,B). RA has a greater sample size than OA (Fig 1C) because the data came from 36 RA patients, but only 15 OA patients. To accommodate for the difference in the quantity of samples for both diseases, the percentages of each cell type in each disease gives an accurate representation of the relative abundance of each cell type (Fig 1C). The samples from RA had a slightly higher percentage of B cells, fibroblasts, and T cells in the sample, but a significantly less percentage of monocytes when compared with the samples from OA.

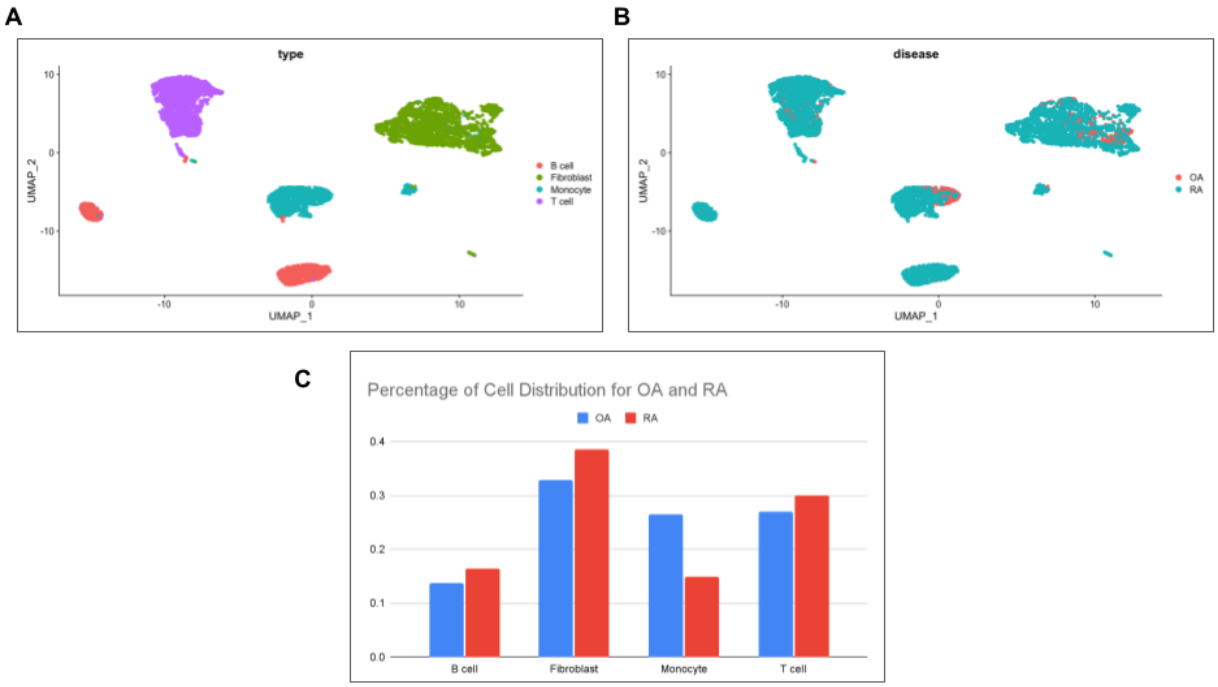

Figure 1. Cell type composition of arthritis patient joints. (**1A**) Uniform manifold approximation and projection (UMAP) dimensionality reduction of cell types identified in scRNA-seq dataset. (**1B**) UMAP projection of scRNA-seq dataset colored by disease. (**1C**) Frequency distribution of B cells, fibroblasts, monocytes, and T cells in scRNA-seq dataset.

Monocyte-derived IL-1B drives chemokine expression by synovial fibroblasts in RA

Fibroblasts are a key tissue-resident cell type involved in the pathogenesis of RA. Therefore, I began by assessing the cell-cell communication networks of RA and OA joints from the perspective of fibroblasts as the receiver cell type. NicheNet analysis identified TGFB1 and IL1B as the strongest signaling ligands in RA fibroblasts (Fig 2A). Examination of ligand expression demonstrated T cells or monocytes are the major source of TGFB1 or IL1B, respectively (Fig 2B). IL1B signaling in fibroblasts induced expression of several genes, including the chemokines CCL2, CXCL12, and CXCL2. TGFB1 signaling promotes fibroblast activation as evidenced by induction of FOS, JUN, ID2 and ID3 expression. IL1B and TGFB1 synergize to drive the production of collagen genes (COL1A1 and COL3A1) and the pro-inflammatory cytokine IL6 (Fig 2A). Additionally, T cell-derived IFNG similarly promotes expression of the chemokines CCL2 and CXCL2, as well as several genes associated with fibroblast activation (Fig 2A-B). Together, these data suggest that the T cell-derived TGFB1 and IFNG, and the monocyte-derived IL1B promote the activation of synovial fibroblasts in RA joints driving the production of chemoattractants, pro-inflammatory cytokines, and possibly collagen deposition.

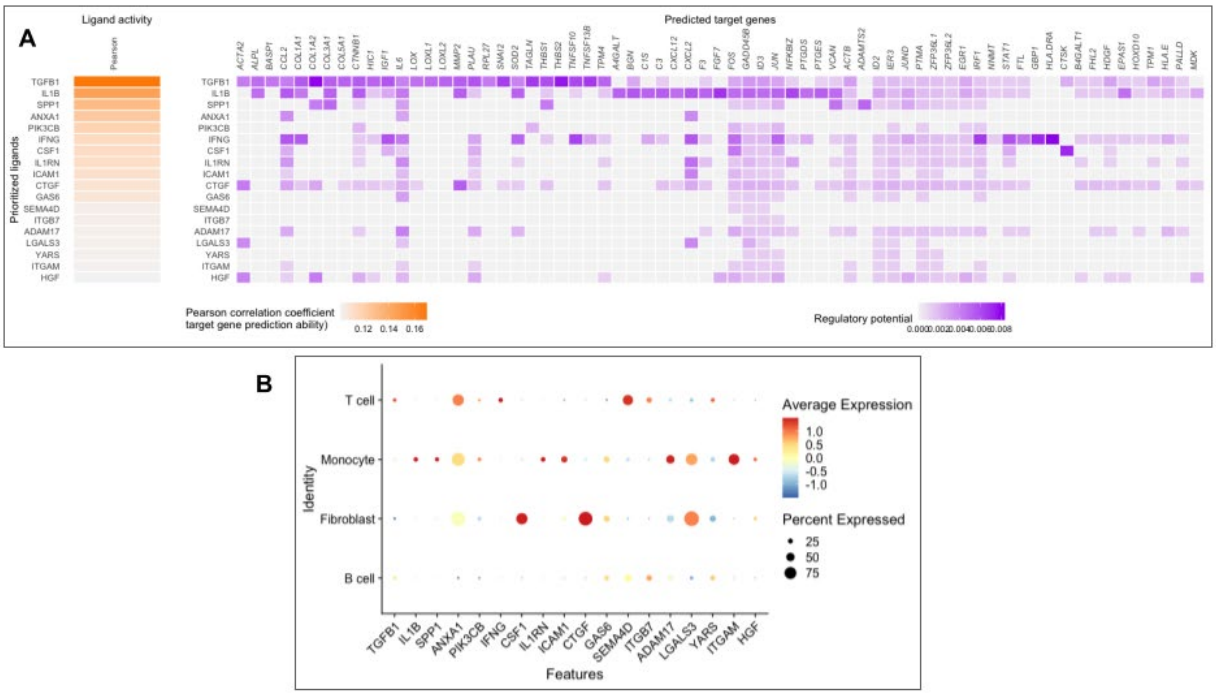

Figure 2. Monocyte-derived IL1B drives chemokine production by synovial fibroblasts. (**2A**) Ligand Activity Chart of Pearson correlation coefficients (left) and heatmap of predicted ligand-target genes with fibroblasts as receiver cell type (right). Indicates the highest signaled ligands and the genes that each ligand is responsible for inducing expression of in fibroblasts. (**2B**) Dot plot showing expression of top ligands signaling through RA fibroblasts among other cell types. Color indicates average expression and dot size represents percent of cells expressing ligand.

Fibroblast-derived CCL2 promotes accumulation of T cells in RA Joints

T cells are a key cell type in driving RA pathogenesis (3). Therefore, I next examined the signaling networks enriched among T cells in the joints of RA patients. NicheNet analysis identified CCL2 among the highest confidence signaling ligands enriched in RA T cells compared to OA T cells (Fig 3A). This CCL2 is largely produced by fibroblasts and to a lesser extent by monocytes (Fig 3B). Interestingly, CCL2 is a chemokine which directs cellular trafficking, however,

prediction of transcriptional alterations resulting from CCL2 signaling suggests it is involved in T cell activation based on up-regulation of IFNG, JUN and JUNB.

In addition to CCL2, HLA-DRA and CD86 showed strong ligand activity in RA T cells. Both molecules are involved in T cell activation and highly expressed by monocytes (Fig 3B). Prediction of ligand-target gene expression suggests that HLA-DRA and CD86 synergize with CCL2 to promote T cell activation based on JUN, JUNB and IFNG expression. Therefore, it is likely that CCL2-mediated recruitment of T cells brings them into proximity of monocytes, thus promoting cell-cell interaction and ultimately T cell activation. Together, these data suggest that fibroblast and monocyte-derived CCL2 promote T cell recruitment and interaction with monocytes in the joints of RA patients via TCR: HLA-DRA resulting in T cell activation and IFNG production, contributing to disease pathogenesis.

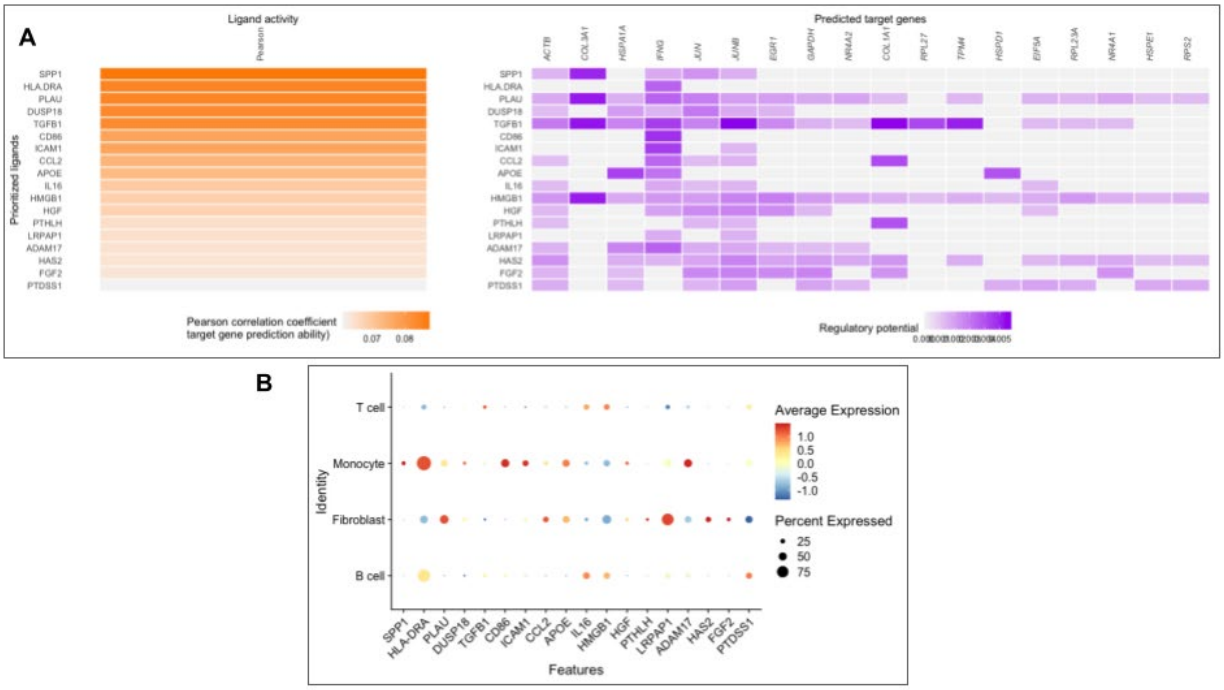

Figure 3. Fibroblast-derived CCL2 promotes accumulation of T cells in RA joints and drives interactions with monocytes. **(3A**) Ligand Activity Chart of Pearson correlation coefficients (left) and heatmap of predicted ligand-target genes with T cells as receiver cell type (right). Indicates the highest signaled ligands and the genes that each ligand is responsible for inducing expression of in T cells. **(3B)** Dot plot showing expression of top ligands signaling through RA T cells among other cell types. Color indicates average expression and dot size represents percent of cells expressing ligand.

Fibroblast and T cell-derived factors drive monocyte activation in RA Joints

I have previously shown that the monocyte-derived IL1B signals to the fibroblasts in order to promote the expression of CCL2, which likely drives recruitment of T cells and interaction with monocytes. Therefore, I next examined the signaling pathways altered in monocytes of RA patients. IFNG, HAS2, and TNF exhibit the highest confidence signaling activity among all ligands in monocytes of RA patients (Fig 4A). IFNG and TNF are predominantly expressed by T cells, whereas HAS2 is expressed almost exclusively by fibroblasts. Together, IFNG, HAS2, and TNF drive monocyte activation (JUN, JUNB, and FOS) and enhance antigen presentation abilities (B2M, CD74 and HLA-E).

Additionally, this activation seems to result in a pro-inflammatory monocyte phenotype based on up-regulation of SOD2.

CCL2 has previously been shown to contribute to RA pathogenesis through promoting osteoclastogenesis, migration of effector T cells to the RA synovium tissue, and angiogenesis (6). CCL2 is also known as monocyte chemoattractant protein-1 (MCP-1), and it can induce the locomotion and recruitment of monocytes to the site. In addition to IFNG, HAS2 and TNF, CCL2 is also among the highest confidence signaling ligands among RA monocytes (Fig 4A). While NicheNet did not identify any osteoclast-specific markers activated downstream of CCL2 signals in monocytes, the genes predicted to be upregulated by this signaling pathway largely overlap with those of IFNG, HAS2 and TNF (Fig 4B). Thus, CCL2 likely functions to promote further recruitment of monocytes to the site of inflammation, allowing for activation downstream of interactions with fibroblasts and T cells in the joints of RA patients.

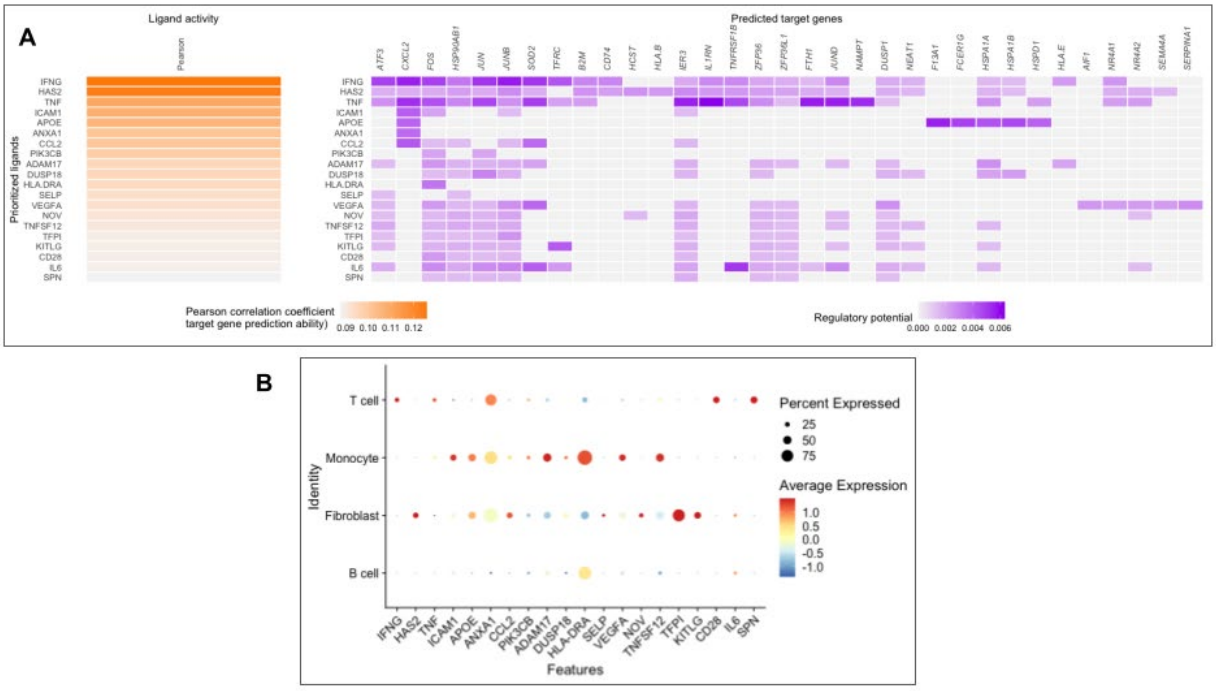

Figure 4. T cell-derived pro-inflammatory cytokines enhance monocyte activation in RA joints. **(4A**) Ligand Activity Chart of Pearson correlation coefficients (left) and heatmap of predicted ligand-target genes with monocytes as receiver cell type (right). Indicates the highest signaled ligands and the genes that each ligand is responsible for inducing expression of in monocytes. **(4B)** Dot plot showing expression of top ligands signaling through RA monocytes among other cell types. Color indicates average expression and dot size represents percent of cells expressing ligand.

Integrated feedback loops may drive progressive inflammation in RA

A feedback loop of the signaling pathways between monocytes, fibroblasts, and T cells can now be proposed (Fig 5). The feedback loop begins with the monocytes producing IL1B which signals through fibroblasts. Once in the fibroblast, IL1B activates CCL2, which signals to both T cells and monocytes. At the same time, monocytes induce expression of HLA-DRA and CD86, promoting T cell activation. Once the CCL2 from the fibroblasts and the HLA-DRA and CD86 ligands from the monocytes arrive in the T cells, INFG is produced by T cells and signals to both the fibroblasts and the monocytes. The T cells also activate TNF, which signals back to the monocytes, and it activates

TGFB1, which signals to the fibroblasts. Together, this study identifies a previously unappreciated integrated signaling network in which ligands produced by monocytes, fibroblasts and T cells cooperate to promote immune activation and cell-cell interactions contributing to RA disease pathogenesis.

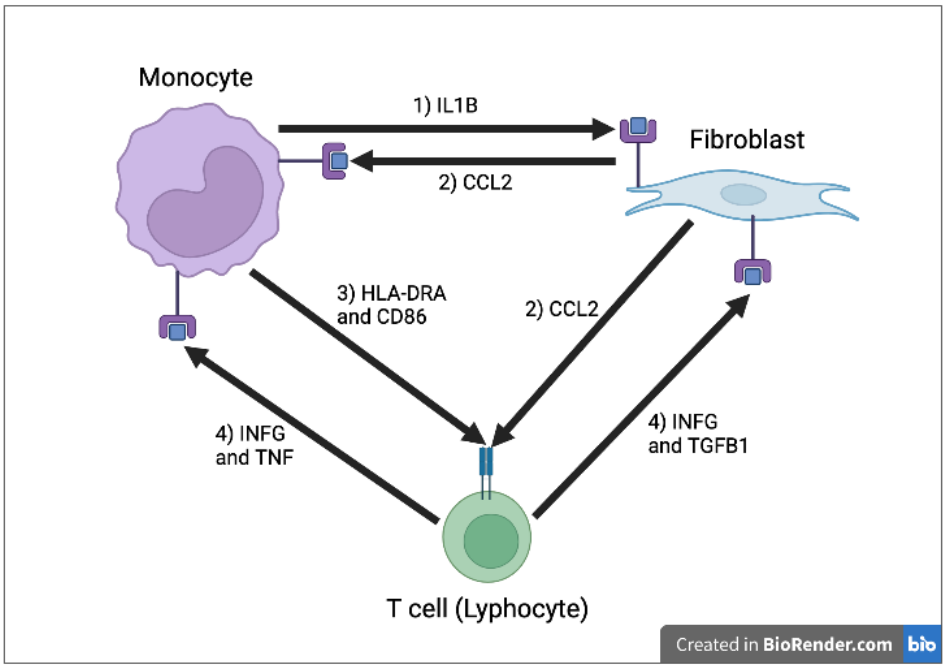

Figure 5. Proposed cell-cell communication network underpinning RA disease pathogenesis. Graphical depiction of key cell-cell communication events underpinning RA disease pathogenesis.

Discussion

Through this study I have identified a novel cell-cell communication network potentially contributing to RA disease pathogenesis. This pathway involves IL-1B production by monocytes acting on fibroblasts to promote the expression of CCL2. CCL2 strongly signals through both monocytes and T cells, likely driving their recruitment to the inflamed joint. This recruitment seems to promote T cell-monocyte interactions based on the strong ligand activities of HLA-DRA and CD86 upon T cells. HLA-DRA and CD86 are key components expressed by antigen presenting cells which synergize to promote TCR-mediated activation of T cells. These interactions drive the production of IFNG, TNF, and TGFB1 by T cells, which act upon both monocytes and fibroblasts promoting activation of each cell type.

As previously mentioned, IFNG is one of the genes induced by CCL2, HLA-DRA, and CD86 ligands in T cells. Furthermore, this dataset demonstrates that IFNG is exclusively expressed by T cells (Fig 4B). IFNs, also known as interferons, are pleiotropic cytokines, which means that they mediate both immunostimulatory and immunosuppressive effects of the disease.[8] Interferons are typically found within the synovial fluid and tissue of RA patients.[8] Studies regarding the deficiency of IFNG in RA indicate that IFNG inhibits inflammatory cells.[16] Based on the ligands driving T cell IFNG expression, CCL2 likely functions to promote T cell recruitment and interaction with HLA-DRA and CD86 expressed by monocytes driving TCR-mediated IFNG production. IFNG activates the expression of CXCL12, CCL2, JUN, and JUNB in monocytes and fibroblasts. CXCL12, also known as SDF1 (Stromal cell-derived factor 1), plays a major role in the pathogenesis of RA[17] and its expression is activated by both IL1B and INFG (Fig 2A). While not identified as having differential signaling activity between RA and OA patients, CXCL12 likely synergizes with CCL2 to promote recruitment of immune cells such as T cells and monocytes to the RA joint. Based on

these findings suggesting pleotropic effects of IFNG production by T cells on both monocytes and fibroblasts, IFNG blockade is an attractive potential therapeutic approach to treat RA. However, more detailed studies will be required to determine the effects of such an intervention.

IL1B expression plays a role in different RA symptoms. In fact, evidence shows that IL1B overexpression is associated with clinical and historical features characteristics of RA, while underexpression is associated with reduced joint damage.[7] Additionally, RA patients have elevated levels of IL1B concentration in comparison to other interleukin-1 (IL-1) cells, including IL1A and IL1Ra.[7] In my analysis and proposed signaling pathway, IL1B is created in the monocytes and migrates to the fibroblasts, where it activates the CCL2 ligand. Furthermore, IL1B can be recognized as one of the initial ligands, as its creation in the monocytes helps to trigger the creation of further ligands. As such, it is likely that IL1B overexpression leads to the clinical and historical feature characteristics of RA because the increased amount of IL1B leads to the creation of ligands such as CCL2, IFNG, TNF, and TGFB1, which all help to demonstrate the key characteristics of RA.

In addition to T cells, monocytes, and fibroblasts, B cells play a key role in disease pathogenesis.[11] While these cells were present in the dataset, virtually no transcriptional differences between RA and OA B cells could be attributed to cell-cell communication (data not shown). Due to their importance in RA pathogenesis and a lack of cell-cell communication differences between RA and OA samples it is possible that the events which promote B cell-mediated disease pathogenesis occur outside the joints. Alternatively, B cells may play very similar roles in both RA and OA or similar signaling pathways may be utilized by B cells in both diseases. In either case, based on the limited scope of this data set, effects of cell-cell communication in B cells cannot be determined. Perhaps a more detailed assessment of cell-cell communication in joints, blood and lymph nodes of RA and OA patients may provide a deeper understanding of the molecular pathways involved in the B cell components of RA pathogenesis.

Together, my analyses identify a novel signaling network which distinguishes immune cell-cell communication networks of arthritic diseases caused by mechanical damage (OA) and autoimmune reactions (RA). The use of a non-autoimmune joint inflammation group (OA) as a control allowed for the discernment of cell-cell communications driven simply by inflammation from those resulting from autoimmune reactions. However, this study focused exclusively on predictive computational assessment of the differences between RA and OA immune reactions, therefore, any findings will require experimental testing. Nonetheless, the T cell-monocyte-fibroblast signaling network identified provides numerous potential avenues of therapeutic intervention for RA.

Conclusion

Rheumatoid Arthritis is a debilitating autoimmune disease which results in the destruction of affected joints. Many cell types involved in this process have been previously described; however, the mechanisms by which these cells cooperate to drive disease remain poorly understood. Utilizing previously published scRNA-seq datasets generated from the joints of RA patients and predictive algorithms we identify a previously unappreciated cell-cell communication network underpinning disease pathogenesis. Specifically, we found that monocyte-derived IL-1β drives the production of chemoattractants such as CCL2 by synovial fibroblasts. These chemoattractants promote the recruitment of monocytes and T cells into inflamed joints facilitating cell-cell interactions and T cell activation. Ultimately these interactions propagate inflammatory reactions underpinning disease pathogenesis. These findings identify numerous potential targets for therapeutic intervention to inhibit disease progression.

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