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Laboratory of Immunomics

(Center for Translational Research on Autoimmune and Allergic Diseases-CAAD)

IN VITRO STUDY OF A STORE-OPERATED CALCIUM ENTRY (SOCE) NEGATIVE MODULATOR IN A 3D SYNOVIAL MODEL OF RHEUMATOID ARTHRITIS

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Summary

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent synovial inflammation, progressive cartilage destruction, and bone erosion driven by complex interactions between immune cells and fibroblast-like synoviocytes (FLS). Dysregulated intracellular calcium signaling, particularly through store-operated calcium entry (SOCE), plays a key role in immune cell activation and cytokine production, making SOCE an attractive therapeutic target. In this study, we developed an 3D *in vitro* model of inflamed RA synovium by co-encapsulating RA-derived FLS and activated peripheral blood mononuclear cells (PBMCs) in a GelMA hydrogel to better recapitulate the cellular crosstalk of the synovial microenvironment. Using this model, we evaluated the immunomodulatory potential and safety profile of a novel SOCE negative modulator, CIC-39, in comparison with clinically used RA drugs, including methotrexate (MTX), adalimumab (Humira), and celecoxib. Our results showed that the none of the tested drugs, including CIC-39, significantly affected cell viability, supporting their biocompatibility under inflammatory 3D conditions. Multicytokine analysis revealed distinct profiles among the treatments. While Humira and celecoxib showed limited modulation of cytokine secretion and MTX induced a broad and unexpected early cytokine upregulation, CIC-39 elicited a unique immunomodulatory signature characterized by a significant increase in the anti-inflammatory cytokine interleukin (IL)-13. In addition, CIC-39 downregulated key immune mediators such as CD163 and Tumor Necrosis Factor Receptor 1(TNF-R1), indicating a regulatory rather than broadly suppressive effect on immune activation. By promoting anti-inflammatory pathways while preserving cell viability, CIC-39 potentially emerges as a promising SOCE targeting immunomodulatory agent for RA.

1. INTRODUCTION

1.1 Immune system overview

The immune system is a complex and highly adaptive biological network finely tuned to detect, respond to, and eliminate invading pathogens. It comprises two functionally distinct yet interconnected components: the innate and adaptive immune systems. The innate immune system, known as the body's first line of defence against foreign agents, provides immediate, non-specific responses (Wang *et al.*, 2024). The adaptive immune system is distinguished by its ability to tailor responses to specific infections and to create immunological memory, making it more effective, highly specific, and long lasting than innate immunity (Vivier and Malissen, 2005). The cells that act in the adaptive immunity are B and T lymphocytes. Thanks to their wide variety of specific receptors, they can distinguish many types of foreign antigens that enter the body (Parkin and Cohen, 2001). Each lymphocyte clone is specific to a single antigen and this clonal specificity determines the precision of the immune response.

B lymphocytes recognize the antigen in its native form, whereas T lymphocytes require antigen-presenting cells (APCs)—such as macrophages, dendritic cells, or B lymphocytes—that display peptide fragments of protein antigens on their surface in association with major histocompatibility complex (MHC) molecules. These peptide–MHC complexes are specifically recognized by the T-cell receptor (TCR), a transmembrane heterodimeric receptor expressed on the surface of T cells (Parkin and Cohen, 2001).

In contrast to T cells, B cells can directly recognize antigens in their native form with their B cell receptor or Immunoglobulin (Ig), which then allows them to secrete antibodies in a soluble form upon B cell activation and differentiation into plasma cells.

Activation of T cells require three distinct signals to fully activate and differentiate into effector T-helper (Th) cells (Morales-Martínez *et al.*, 2024). The first signal is the recognition of the antigenic peptide presented by the appropriate MHC molecule by the TCR; the second signal is a costimulatory signal triggered by the binding of costimulatory molecules on T cells to costimulatory ligands expressed on APCs activated by inflammation; and the third signal is mediated by cytokines that modulate the differentiation of activated T cells into effector cells (Szeto *et al.*, 2020). Among them, interleukin (IL)-4 drives differentiation of Th2 cells, which support B cell activation and antibody production (Pelly *et al.*, 2016). In contrast, interferon

(IFN)-γ drives the differentiation of Th1 cells, which primarily secrete IL-2 and support the activation of macrophages, natural killer (NK) cells, and cytotoxic T lymphocytes (Castro *et al.*, 2018).

Furthermore, the activation of B cells also plays an important role in the adaptive immune response. B cell activation begins when the membrane-bound immunoglobulin known as the B cell receptor (BCR) binds to its specific antigen, triggering receptor-mediated endocytosis and antigen processing. After undergoing clonal expansion, activated B cells develop into memory B cells and plasma cells that secrete antibodies. Initially producing IgM antibodies, plasma cells can subsequently undergo class-switch recombination to switch antibody classes (e.g., IgG, IgA, and IgE), thereby improving the specificity and efficacy of the immune response. The outcome is a strong humoral immune response with long-term immunological memory and high-affinity antibody generation (Cappellano et al., 2019; Crotty, 2019).

Dysregulation of immune system can result in loss of self-tolerance, leading to autoimmunity(Song, Li and Wu, 2024).

1.2 Autoimmune diseases (ADs)

Autoimmunity develops when self-tolerance is disrupted, leading B cells to produce antibodies against self-antigens and T cells to mount aberrant responses against host tissues. Disruption of both B cell and T cell tolerances therefore contributes to the initiation and progression of autoimmune diseases (ADs) (Yasmeen *et al.*, 2024). Immune tolerance is a mechanism by which the immune system does not respond to self-antigens to protect the body's own tissues from damage. This is maintained through central and peripheral mechanisms; failures in these can result in the development of autoimmunity (Ashiq *et al.*, 2023). Central tolerance occurs in the primary lymphoid organs where autoreactive lymphocytes are eliminated. Nevertheless, though some cells may escape into the periphery, peripheral tolerance mechanisms (i.e. deletion, anergy, suppression) control these autoreactive cells by inactivating them, for example via regulatory T cell(Tregs)-mediated suppression (Rosenblum, Remedios and Abbas, 2015).

Failures in the tolerance mechanisms can lead to the survival of *self*-reactive B cells, which secrete autoantibodies (Abs) involved in the pathogenesis of several autoimmune diseases (Collet *et al.*, 2025). They are not merely byproducts of the disease but actively participate in pathogenesis. For example, in systemic lupus erythematosus (SLE), immune complexes formed by Abs deposit in tissues and trigger complement activation (Galindo-Feria, Wang and

Lundberg, 2022). Abs can also act as antagonists or agonists and activate the Thyroid Stimulating Hormone (TSH) receptor on the thyroid, which leads to Graves' illness (Morshed and Davies, 2015). Moreover, Abs which block or internalize Acetylcholine (ACH) receptors at the neuromuscular junctions contribute to the disease myasthenia gravis (Fichtner *et al.*, 2020). In addition, Abs such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (anti-CCP) form immune complexes that deposit in synovial fluid, enhancing innate immune activation contributing to chronic inflammation, joint damage and systemic manifestations of rheumatoid arthritis (RA) (Johnson and Jiang, 2023; Yasmeen et al., 2024).

1.3 Rheumatoid arthritis (RA)

RA is a chronic, systemic autoimmune disorder characterized primarily by persistent inflammatory synovitis, which leads to the progressive destruction of cartilage and bone within joints (Gao, Zhang and Liu, 2024a). This loss of self-tolerance and subsequent attack on host tissues arises from a complex interplay of genetic predisposition, environmental triggers, immune dysregulation, and epigenetic modifications (Di Matteo, Bathon and Emery, 2023).

Globally, RA affects approximately 0.5-1% of the adult population, with a higher prevalence in women and certain ethnic groups, underscoring its significant public health burden (Ashiq *et al.*, 2023). The disease typically presents with clinical symptoms including symmetric joint pain, swelling, morning stiffness lasting more than an hour, and systemic features such as fatigue and fever. If left untreated or poorly managed, the chronic inflammation leads to severe consequences, including irreversible joint deformity, functional disability, chronic pain, and a reduced quality of life. Furthermore, systemic inflammation accelerates atherosclerosis, increasing the risk of cardiovascular comorbidities and contributing to increased mortality rates (Gao, Zhang and Liu, 2024a; Radu and Bungau, 2021).

The risk of RA development is substantially increased by genetic factors, particularly variants in human leukocyte antigen (HLA) genes, most notably the HLA-DRB1 allele (Gao, Zhang and Liu, 2024a; Radu and Bungau, 2021). These variants impact immune system responses, which in turn affect antigen presentation (Radu and Bungau, 2021). Individuals who are genetically prone, exposure to environmental pollutants acts as essential catalyst for disease development. Particularly, smoking and occupational exposure to silica dust are considered important environmental contributors to the development of RA (Chen, Zhao and Meng, 2025).

Vitamin D deficiency is also reported to disrupt the immune system function (Gao, Zhang and Liu, 2024). Additionally, because of their pro-inflammatory properties and potential to damage joint tissues, exposure to sulphur oxides, ozone and nitrates has also been associated with RA (Radu and Bungau, 2021).

Furthermore, changes in the gut microbiome have become more widely identified as an essential contributor to the aetiology of RA. By stimulating toll-like receptors (TLRs) and NOD-like receptors (NLRs) on APCs, dysbiosis or an imbalance in the bacterial population in the gut can initiate autoimmune reactions and increase the risk of widespread inflammation (Radu and Bungau, 2021).

Moreover, a dysregulated immune response in which immune cells attack their own synovial tissues is a characteristic feature of RA (Gao, Zhang and Liu, 2024). Abs such as RF and anti-CCP antibodies, are common in RA (Gao, Zhang and Liu, 2024a; Radu and Bungau, 2021) and generate immune complexes that pile up in the synovial fluid, inducing joint damage and constant inflammation (Radu and Bungau, 2021). Moreover, in RA, pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-a), interleukin-6 (IL-6) and interleukin-1 (IL-1) are secreted by synovial macrophages and other immune cells, which destroys the joint tissues (Gao, Zhang and Liu, 2024)(Radu and Bungau, 2021). These cytokines accelerate the tissue degeneration by increasing the number and activity of osteoclasts and fibroblast-like synoviocytes (FLS) (Radu and Bungau, 2021). It has been proposed that self-antigens mimic microbial or environmental antigens by molecular mimicry. Given these commonalities, the immune system attacks its own body's tissues, leading to chronic inflammation and joint destruction in RA (Radu and Bungau, 2021).

1.3.1 RA pathogenesis

The pathogenesis of RA involves a complex interplay of cellular and molecular systems that result in chronic inflammation and destruction to the joints through multiple mechanisms. As a consequence of a decreased immunological tolerance, autoreactive B cells release Abs such as RF and ACPA that can be identified years before the signs and symptoms appear, highlighting their effect on the early development of this disease (Volkov, Van Schie and Van Der Woude, 2020). Pro-inflammatory cytokines like TNF-α and IL-6 are secreted after the

immune cells become activated. Pain and swelling are two of the prominent signs of RA, caused by inflammation in synovial tissue and the secretion of TNF-a and IL-6 (Gao, Zhang and Liu, 2024b; Mariani et al., 2023).

Chronic synovitis, triggered by inflammation, predominantly affects the synovial membrane. This leads to abnormal proliferation of synovial cells and formation of pannus tissue, which contributes to bone and cartilage destruction (Wu *et al.*, 2025). The persistent inflammatory environment not only causes ongoing tissue injury but also impairs normal repair mechanisms, preventing restoration of tissue homeostasis. This failure to re-establish homeostasis represents a major hurdle in the treatment of RA (Alivernini, Firestein and McInnes, 2022).

Importantly, the chronic inflammatory environment in RA is not only sustained by cytokine secretion and autoreactive immune responses but is also deeply influenced by intracellular signaling pathways. Among these, calcium signaling plays a central role in modulating immune cell activation, differentiation, and effector functions. Dysregulation of calcium homeostasis, particularly through store-operated calcium entry (SOCE), has been closely linked to the aberrant immune activation observed in RA (Vig and Kinet, 2009; Izquierdo et al., 2014).

1.3.2 Calcium signalling in RA

RA has a characteristic feature of dysregulated calcium channel (Ca^{2+}) signalling which plays a key role in sustaining chronic inflammation as well as autoimmunity. In RA, dysregulation of SOCE which is a main pathway, along with input from other calcium channels, drives the abnormal activation of T and B lymphocytes. In the synovial joints, the T cells receptors (TCRs) on infiltrating T cells recognize the autoantigens triggering the classical SOCE pathway, which in turn activates the phospholipases Cγ1 (PLCγ1), thus cleaving PIP_2 to produce IP_3. The release of stored Ca^{2+} into the cytosol is triggered by binding of IP_3 to its receptor on the endoplasmic reticulum (ER) (Vig and Kinet, 2009).

In RA, stromal interaction molecule 1 (STIM1) detects the ER Ca^{2+} store depletion and aggregates with and activates Orai1 channels on the plasma membrane, creating Ca^{2+} release-activated Ca^{2+} (CRAC) channels. The Calcineurin-NFAT signalling cascade is activated by this subsequent and continuous influx of extracellular Ca^{2+} (Park et al., 2020). Once NFAT is translocated to the nucleus, it drives the expression of pro-inflammatory cytokines (IL-2, IFN-γ, TNF-α) along with genes that promote T cell growth, differentiation and survival, which

enhances the “autoimmune” activity in the RA synovium (Izquierdo et al., 2014; Hogan and Rao, 2015).

Along with SOCE, other calcium channels also contribute to immune cell hyperactivation in RA. Lymphocytes and FLS in RA joints express certain transient receptor potential channels (TRP), like TRPV and TRPM channels, which respond to inflammatory cues in the RA joints, such as lipids and reactive oxygen species, enabling alternative pathways for Ca^{2+} influx. This leads to a further increase in intracellular Ca^{2+}, thus intensifying NFAT action as well as cytokine production (Froghi et al., 2021). In addition to this, Purinergic P2X receptors, stimulated by extracellular ATP abundant in inflamed RA tissues also enhance Ca^{2+} entry, which adds to T cell activation and pain signalling.

The importance of SOCE in RA pathogenesis is emphasized by genetic associations. Gain of function mutations in STIM1 and Orai1 are associated with autoimmunity whereas loss of function mutations that impair CRAC channel activity cause significant immunodeficiencies. Through dysregulated SOCE signalling, RA promotes T cell activation and increased follicular helper T (Tfh) support for B cells, which in turn drives the production of autoantibodies such as RF and anti-citrullinated protein antibodies (ACPAs), which are defining features of RA (Hogan and Rao, 2015; Aprile et al., 2021).

In summary, abnormal Ca^{2+} signalling is a key driver for hyperactivation of T and B cells in RA. Though SOCE hyperactivity appears to be the main contributor, TRP and P2X receptors provide additional Ca^{2+} influx leading to activation of transcriptional programmes that causes chronic inflammation, autoantibody generation, and joint damage. All this points to the fact that these pathways are promising therapeutic targets.

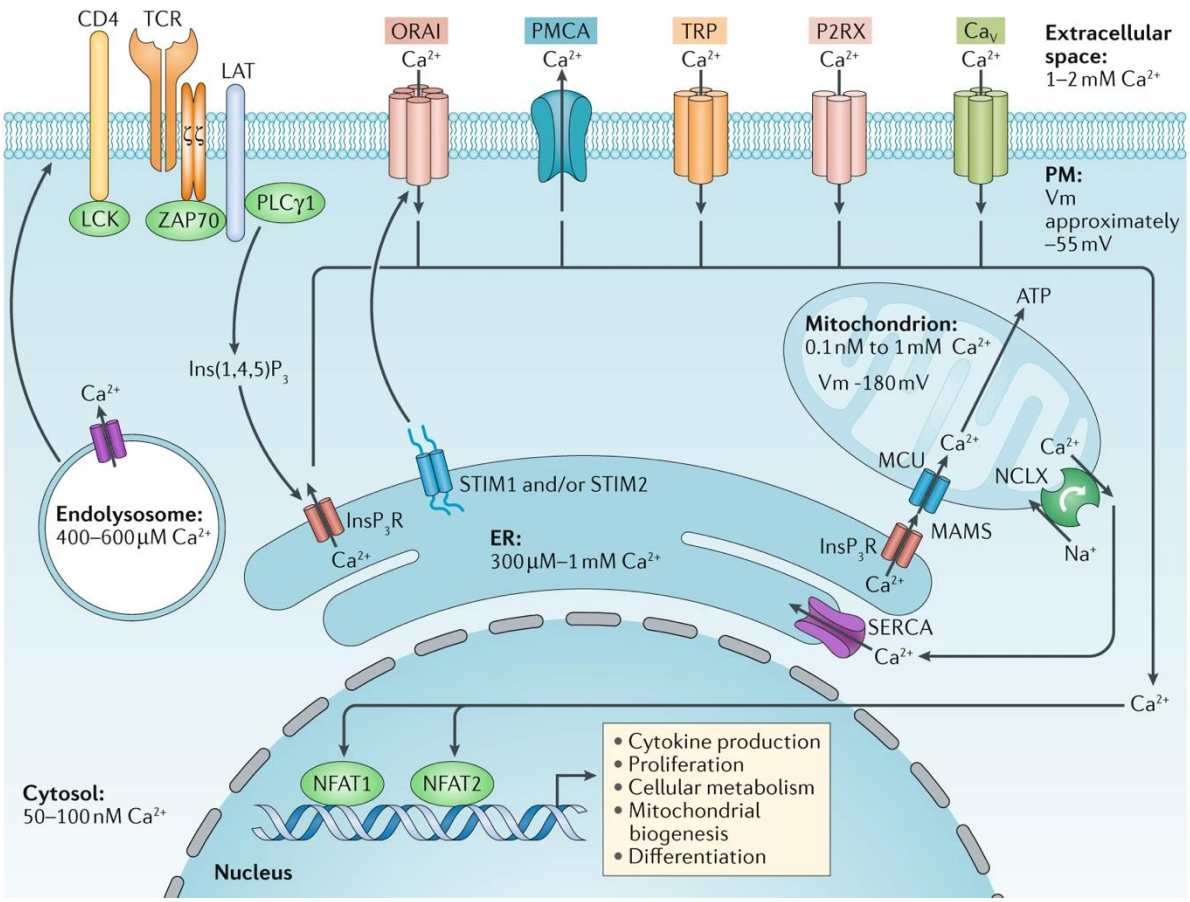


Figure 1: TCR activation in RA synovial T cells triggers ER Ca2+ release and STIM-mediated ORAI1 opening, inducing SOCE. Ca2+ signalling regulates NFAT activation, mitochondrial energy production, and vesicular trafficking, essential for T cell function (Trebak and Kinet, 2019).

1.3.3 Therapeutic advancements in RA

RA, without appropriate treatment can inevitably cause irreversible damage to the structural joints and is often accompanied by multiple organ damage (Gao, Zhang and Liu, 2024). Therapeutic strategies aim to reduce inflammation, control symptoms, and halt disease progression (Gao, Zhang and Liu, 2024). Historically, treatment has relied on broad-spectrum immunosuppressants; however, advances in immunology and molecular medicine have paved the way for more targeted and individualized interventions. The way RA is treated has changed dramatically in the twenty-first century (Smolen et al., 2023).

Traditional treatments for RA, such as methotrexate (MTX), sulfasalazine, hydroxychloroquine, NSAIDs, and glucocorticoids, may help in reducing inflammation but

have non-specific mechanisms, often leading to systemic side effects and varying patient responses (Smolen et al., 2023). In contrast, biologic and synthetic disease modifying anti-rheumatic drugs (DMARDs) like TNF-a inhibitors (e.g., adalinumab, infliximab), IL-6 receptor blockers (e.g., tocilizumab), and JAK inhibitors target specific immune pathways, resulting in improved clinical outcomes and better safety profiles (Fraenkel et al., 2021; Smolen et al., 2023). However, these newer therapies still face challenges, including patient heterogeneity, high cost, and drug resistance (Smolen et al., 2023)(Table 1).

Supportive and symptomatic therapies for RA include NSAIDs like ibuprofen, naproxen, and celecoxib, which reduce pain and inflammation but do not affect disease progression. Corticosteroids such as prednisone and methylprednisolone provide short-term symptom relief and are often used as a bridge until DMARDs take effect, though their long-term use is limited due to significant side effects (Gao, Zhang and Liu, 2024a; Smolen et al., 2023).

Despite significant therapeutic advancements, the management of RA continues to face substantial challenges. Achieving drug-free remission remains elusive for most patients, and a considerable proportion exhibit partial, intermittent, or complete non-responsiveness to currently available treatments. Furthermore, the efficacy of these therapies is often offset by adverse effects, which may compromise patient adherence and delay the onset of meaningful clinical improvement (Gao, Zhang and Liu, 2024). Because of the heterogeneity of RA pathogenesis and patient-specific immune pathways, treatment selection in routine clinical practice often follows a trial-and-error approach, with sequential switching between different DMARDs and biologics until an adequate clinical response is achieved (Smolen et al., 2023).

Synovial biopsies from patients with RA reveal distinct histopathological patterns, ranging from lymphoid aggregates rich in B and T cells to myeloid-dominant, fibroid, or pauci-immune phenotypes, which reflect underlying differences in immune activation pathways and cytokine networks and are thought to contribute to variability in therapeutic responses (Smolen et al., 2023).

Category	Class / Drug	Examples	Role
1.Conventional Synthetic DMARDs (Smolen *et al.*, 2023)	Dihydrofolate reductase inhibitors, Dihydroorotate dehydrogenase inhibitors	Methotrexate, Leflunomide, Hydroxychloroquine, Sulfasalazine	Broad immune suppression; reduce inflammation & prevent joint damage; MTX is first-line due to efficacy & safety
2. Biologic DMARDs (bDMARDs) (Fraenkel *et al.*, 2021; Smolen *et al.*, 2023)	TNF inhibitors	Etanercept, Infliximab, Adalimumab, Certolizumab, Golimumab	Block TNF-α, a key inflammatory cytokine
	Risks:		Infection risk, requires monitoring
3. Targeted Synthetic DMARDs (tsDMARDs) (Smolen *et al.*, 2023)	JAK inhibitors	Tofacitinib, Baricitinib, Upadacitinib	Block JAK enzymes, disrupt cytokine signaling; oral; used when other DMARDs insufficient; risks include infection, blood clots, ↑ cholesterol
4. Supportive & Symptomatic Therapies (Smolen *et al.*, 2023)	NSAIDs	Ibuprofen, Naproxen, Celecoxib	For pain & inflammation; do not modify disease progression
	Corticosteroids	Prednisone, Methylprednisolone	Short-term symptom control; bridge until DMARDs take effect; avoid long-term use due to side effects

Table 1. Classification of therapeutic strategies in rheumatoid arthritis (RA). The table outlines conventional synthetic (csDMARDs), biologic (bDMARDs), and targeted synthetic DMARDs (tsDMARDs), along with supportive therapies, representative drugs, and their clinical roles. (DMARDs-Disease-Modifying Anti-Rheumatic Drugs; csDMARDs-Conventional Synthetic DMARDs; bDMARDs-Biologic DMARDs; tsDMARDs-

Targeted Synthetic DMARDs; TNF-Tumor Necrosis Factor; IL-Interleukin; JAK-Janus Kinase; NSAIDs-Nonsteroidal Anti-Inflammatory Drugs.).

Given these challenges, there is a growing need for a personalised medicine approach in RA to improve treatment effectiveness and reduce response variability. By allowing the *in vitro* reproduction of patient-specific injured tissue, emerging technologies like organ-on-chip models would offer a viable platform for customized treatment in RA (Leung *et al.*, 2022). By incorporating autologous patient-derived cells, including synovial fibroblasts, macrophages, lymphocytes, and endothelial cells, these systems can recreate the unique immunopathological microenvironment of each patient. Such personalized models enable direct testing of therapeutic agents within a physiologically relevant context, providing predictive insights into drug efficacy and toxicity for the individual.

1.4 SOCE Modulators

SOCE modulators have two main roles: they either enhance or inhibit SOCE, providing tools to study calcium signalling pathways and potential therapeutic strategies for diseases related to SOCE dysregulation (Aprile et al., 2021). The process of SOCE relies on STIM and ORAI proteins, and the activity of the proteins can be altered by such modulators (Shaw and Feske, 2013).

Numerous compounds have been described as SOCE inhibitors. BTP2 (YM-58483), for example, has been shown in preclinical models to reduce autoimmune reactions and inflammations by inhibiting T cell activation and cytokine production (Liang *et al.*, 2021). Novel SOCE modulators CIC-37 and CIC-39 have demonstrated the capacity to block gain-of-function mutations in STIM1 and ORAI1, which are linked to elevated SOCE activity (Riva *et al.*, 2022). Synta66 has also been reported to inhibit SOCE and reduce immune cell activation (Liang et al., 2021; Skopin et al., 2024).

Most SOCE inhibitors remain in the preclinical phase for various conditions including autoimmune diseases such as inflammatory bowel disease (IBD), where SOCE inhibition has been shown to reduce proinflammatory cytokine production. Although preliminary findings suggest that SOCE modulators are generally tolerable in clinical settings, further studies are required to define their long-term safety and efficacy (Skopin et al., 2024; Riva et al., 2022).

Currently, no SOCE modulators has been approved for the treatment of ADs, further research is needed to determine their therapeutic potential and long-term safety profiles in these conditions (Riva *et al.*, 2022).

1.5 3D *in vitro* model

2D cultures do not accurately reproduce the intricate 3D structure and cell–cell interactions present in the tissue. While the animal models are closer to the physiological conditions, they often exhibit species-species differences which restrict the application of the results in humans. A 3D model is *in vitro* culture system where cells are incorporated into biomaterial scaffold that mimics the extracellular matrix (ECM) which enables proliferation and interaction in all the three spatial dimensions. This arrangement facilitates important cell-cell interaction and cell-ECM interaction which are critical for tissue function but lack the conventional flat 2D monolayer culture.

3D models offer significant advantages over traditional 2D cultures which are particularly important for RA research. 3D Cells cultures exhibit characteristics which are close to their in vivo behaviour including morphology, gene expression, protein secretion, and metabolic activity. Along with this 3D systems also offer more reliable platform for drug screening thus providing better insights into drug efficacy, penetration, and toxicity (Kapałczyńska *et al.*, 2016).

1.5.1 Type of hydrogels

Hydrogels are 3D polymers which are hydrophilic in nature which form networks which has the capacity to absorb and hold large amount of water usually several hundred times their dry weight. They are recognized for their highly porous structure and polar functional groups making them well matched and biocompatible for various biomedical applications such as targeted therapeutic delivery, ocular implants, wound dressing, regenerative scaffolds, hygiene products (Karoyo and Wilson, 2021). Depending on the intended use, hydrogels can be synthesized from natural polymers like alginate, gelatin, hyaluronic acid, and chitosan, or from synthetic materials such as polyethylene glycol (PEG), polyacrylamide, and polyvinyl alcohol. They exhibit various physical properties based on the combination of their molecules, ranging from stable to reversible. In addition hydrogels are heterogeneous which contain pore of various size suitable for loading solutions and cells. despite attempts to categorize hydrogel

embolic agents, the present classification remains uncertain in bridging material science and clinical demand (Ho *et al.*, 2022). They possess different categories depending on their characteristics and properties.

Natural hydrogels are produced from natural sources like proteins, polysaccharides and other biological substances. They are considered suitable for its use in biomedical applications as they often show acceptable biocompatibility (Oliveira *et al.*, 2021; Yi *et al.*, 2022) and biodegradability (Yi *et al.*, 2022). Also, they imitate the extracellular matrix (ECM) which is found in the living tissues (Oliveira *et al.*, 2021). For instance, hyaluronic acid (HA) and chondroitin sulfate, they have the function to increase the hydrogel's activity when added to it (Yi *et al.*, 2022).

Synthetic hydrogels are produced using synthetic polymers through chemical methods. They are engineered to attain exact chemical and mechanical functions, which facilitates customization for tissue engineering and drug delivery uses (Oliveira *et al.*, 2021; Yi *et al.*, 2022). Semi-synthetic hydrogels are produced by altering natural polymers which contains both favourable functions of natural materials and synthetic. For instance, the inclusion of short-chain chitosan into poly(ethylene glycol) (PEG) hydrogel to enhance mechanical functions and biocompatibility (Yi *et al.*, 2022). Smart hydrogels have the ability to adapt to environmental stimuli such as temperature, pH and light. They are appropriate for regulated drug delivery systems as they have the ability to alter their characteristics in response to environmental factors (Oliveira *et al.*, 2021). Composite hydrogels are formed by putting together different kinds of hydrogels or inserting with other materials such as nanoparticles to improve their properties. This compilation can enhance their drug delivery ability, strength and bioactivity. (Oliveira *et al.*, 2021). Injectable hydrogels are developed with low viscosity which makes it easier for injecting and can crosslink rapidly under physiological environments. They are extremely beneficial for delivering therapeutic medications and cells in RA therapy. (Yi *et al.*, 2022)Drug-loaded hydrogels are specially created to secure and release drugs over a period of time. For example, hydrogels loaded with DMARDs have exhibited favourable results in lowering dosages without losing its efficacy (Yi *et al.*, 2022). Lastly, cell-loaded hydrogels are designed to deliver cells like adipose-derived stem cells to any affected regions. They can assist in managing the risks imposed by the inflammatory conditions in RA. (Yi *et al.*, 2022).

2. OBJECTIVES

This internship was carried out at the Immunomics laboratory (Head Prof. Annalisa Chiocchetti), and it is part of the NODES project code ECS00000036 financed by the MUR with PNRR MUR-M4C2 funds, Investment 1.5, "Innovation Ecosystems". Prof. Giuseppe Cappellano is responsible for a research module that aims to develop a synovia-on-a-chip starting from the biopsies of patients with RA in order to test the effects of anti-inflammatory drugs within this chip. He has a collaboration with the laboratory of Prof. Tracey Pirali (Department of Pharmaceutical Sciences) and ChemiCare, a startup of UPO, who developed and patented the molecule called CIC-39, which represents a new negative modulator of SOCE. It is in a pre-clinical development phase and has demonstrated both optimal safety tolerability in good laboratory practice (GLP) non-clinical studies and efficacy in rare disorders associated with SOCE over-activation.

The synovial membrane undergoes various structural changes during the pathogenesis of RA. Activated synovial FLSs are believed to play a crucial role in the destruction of cartilage in this disease. Ca^{2+} signaling in FLSs can influence the production of inflammatory cytokines, MMPs, and other factors involved in RA progression. SOCE, a major mechanism by which cells regulate Ca^{2+} influx, plays a critical role in cellular processes linked to inflammation. We hypothesized that SOCE modulation in RA-FLSs might represent an efficient pharmacological target approach to reduce inflammation in the RA synovium.

We embedded FLS together with peripheral blood mononuclear cells (PBMC) in an 3D hydrogel, to recreate the immune–stromal interactions of the synovial microenvironment, making the model more physiologically relevant than 2D cultures of FLS alone. In this off-chip model, we evaluated the effects of CIC-39 in comparison with used RA drugs on cell viability and cytokine secretion.

3. MATERIALS AND METHODS

3.1 Patients and samples

Primary RA FLS were obtained from synovial tissue of four RA patients at Queen Mary University of London (QMUL) Hospital, UK. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors. All patients and donors provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Eastern Piedmont (protocol OCEANIA 9/21).

3.2 Isolation of PBMCs

PBMCs were isolated from whole blood of healthy donors using density gradient centrifugation method. Blood was diluted 1:1 with sterile phosphate-buffered saline (PBS) and carefully layered over the density gradient medium Lympholyte. Samples were centrifuged at 1800 rpm for 20 minutes with the brake off to allow clear separation of the cellular layers. The PBMC ring was collected, washed with PBS 1X, and centrifuged at 1500 rpm for 5 minutes. The resulting cell pellet was resuspended in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.1% gentamicin. Cell viability counts were determined using trypan blue.

For T cell activation, 96-well flat-bottom plates were coated with anti-human CD3 antibody (1 µg/mL) and incubated overnight at 4°C. After 24 hours, plates were washed with PBS, and PBMCs were seeded at a density of 2×10^5 cells per well in 100 µL of complete RPMI medium along with recombinant human IL-2 (100 U/mL) to support T-cell survival and proliferation. Soluble anti-human CD28 antibody (2 µg/mL) was added directly to the appropriate wells. Cells were incubated for 72 hours at 37°C in a humidified 5% CO_2 incubator prior to encapsulation.

3.3 FLS culture

RA FLS were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.1% gentamicin after counting the cells using trypan blue, media was changed every two days. Once reached to more than 80% of confluency, the RA FLS cells were detached from the platwe by trypsinization and used in the experiments. All the experiments were performed between passage (P)3 and P7 of the cell culture.

3.4 Bioink preparation

Gelatin methacryloyl (GelMA) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) were used for hydrogel preparation. For each experiment, GelMA and LAP were prepared at 20% and 2% respectively, in 2 separate sterile tubes. In brief, the required amounts of GelMA and LAP were weighed in their solid forms. Both were sterilized under ultraviolet (UV) light for 20 minutes and then dissolved in the solution of PBS 1X supplemented with 10% FBS. Later, both were incubated in a 60 °C water bath for 1 hour, with LAP kept protected from light.

After stock preparation, a hydrogel working mixture was then freshly prepared by combining the appropriate volumes of GelMA and LAP stocks (to obtain final concentrations of 5% GelMA and 0.3% LAP) with the PBMC (2×10^6 cells/ml) and FLS (5×10^6 cells/ml) and additional complete RPMI medium supplemented with IL-2 (100 U/ml) to adjust the final volume. The mixtures were maintained at 37 °C in 5% CO_2 before manually printing.

3.5 In vitro inflamed synovia 3D model

To establish an in vitro inflamed synovia 3D model, RA FLS cells (5×10^6 cells/ml) and healthy PBMCs (2×10^6 cells/ml) were encapsulated together in a hydrogel mix of GelMA 5% and LAP 0.3%. Prior to encapsulation in hydrogel, PBMCs were stained with Cell Tracker™ Blue (Thermo Fisher Scientific) using a 20 µM working concentration, following the manufacturer's instructions, allowing their identification during co-culture. The following formulation drop of bioink was dispensed manually on a flat-bottom culture plate and photocrosslinked under UV for 1 minute to stabilize the hydrogel structure. At the end, complete RPMI media with IL-2 (100 U/ml) was added and cells were incubated at 37 °C in 5% CO_2. After 18 hours of encapsulation, a cytokine cocktail consisting of IL-1β 10 ng/ml, IL-6 10 ng/ml, and TNF-α 20 ng/ml was added in the complete media. After 2 days of cytokine exposure, cell viability was performed using Calcein-AM/propidium iodide (PI) staining.

3.6 Drug testing on 3D inflamed synovia

To evaluate drug responses in a 3D inflammatory synovia system, RA FLS and healthy PBMCs were encapsulated together in a hydrogel matrix composed of 5% (w/v) GelMA and 0.3% (w/v) LAP. After 18 hours of encapsulation, an inflammatory stimulus was applied by supplementing the culture with a cytokine cocktail containing IL-1β (10 ng/mL), IL-6 (10 ng/mL), and TNF-α (20 ng/mL). At 24 hours post-encapsulation, therapeutic compounds were introduced to assess anti-inflammatory effects. The treatment groups included CIC-39 (3 μM), MTX (0.1 μM), Humira (10 ng/mL), Celecoxib (25 μM), and a DMSO-treated group as the control. Drug exposure was maintained for 48 hours. At the end, the hydrogel constructs were subsequently stained using a Live/Dead viability assay and imaged with a Thunder microscope to evaluate cell viability and structural integrity within the 3D environment. Whereas, culture supernatants were harvested for cytokine analysis using 2 different cytokine arrays, including the Bio-Plex Pro™ Human Cytokine 37-Plex Assay and the Bio-Plex Pro™ Human Th17 Cytokine Assay (Bio-Rad Laboratories, Hercules, CA, USA).

3.7 Viability Assay

To assess the viability of RA-FLSs and healthy PBMCs in the 3D inflamed synovia model after drug response, Calcein-AM/PI staining was performed. At the endpoint, the medium was collected, and the hydrogel constructs were washed with 1× PBS. Calcein-AM (1 μM) and PI (50 μg/mL) were added to each well and incubated for 20 minutes in the dark at room temperature. Calcein-AM is converted to green-fluorescent calcein by intracellular esterases in live cells, while PI enters only membrane-compromised cells, binding DNA and emitting red fluorescence. After staining, hydrogel constructs were washed with PBS and imaged using the Thunder Imaging System (Leica Microsystems, Germany). PBMCs were pre-stained with a blue cell tracker prior to encapsulation with FLS. Following live/dead staining, PBMCs appeared as blue+green (live) or blue+red (dead), while FLS appeared only as green (live) or red (dead). Cell viability analysis was performed using ImageJ software (NIH, USA). Images were processed to enhance contrast and threshold to distinguish individual cells. The number of live (green) and dead (red) cells was quantified using the "Analyze Particles" function, and viability was calculated as the percentage of live cells relative to the total cell count.

3.8 Multicytokine Assay

To evaluate the cytokine and chemokine concentrations in supernatants of 3D co-culture FLSs and PBMCs among different treatments, we performed the Bio-Plex Pro™ Human Cytokine 37-Plex Assay and the Bio-Plex Pro™ Human Th17 Cytokine Assay (Bio-Rad Laboratories, Hercules, CA, USA). Both assays are bead-based multiplex immunoassays built on Luminex xMAP® technology, enabling simultaneous quantification of multiple analytes in a single well using magnetic beads conjugated to analyte-specific capture antibodies.

At the endpoint of the experiment, as described in chapter 3.6, supernatants were collected and stored at –80 °C until cytokine measurement. Both multiplex assays were performed according to the manufacturer's instructions. Briefly, standards were reconstituted and serially diluted to generate eight-point standard curves. In each well of a 96-well flat-bottom plate, 50 µL of antibody-conjugated magnetic beads was incubated with 50 µL of either standards, controls, or samples for 1 h at room temperature on a microplate shaker (850 ± 50 rpm, protected from light). After three washes with assay buffer, 25 µL of biotinylated detection antibody solution was added and incubated for 30 min, followed by three additional washes. Next, 50 µL of streptavidin–phycoerythrin (SA-PE) reporter solution was added and incubated for 10 min. Wells were washed, and beads were resuspended in 125 µL of assay buffer prior to acquisition. Plates were read on a Bio-Plex® 200 system (Bio-Rad Laboratories). A minimum of 50 beads per analyte were collected per well. Median fluorescence intensity (MFI) values were recorded, and cytokine concentrations were interpolated from the standard curves using a five-parameter logistic (5-PL) regression model in Bio-Plex Manager™ software.

To generate a non-redundant dataset, cytokines measured in both the 37-plex and Th17 panel were identified, and duplicate analytes were excluded from the final analysis. The resulting combined panel included unique cytokines and chemokines across both kits, covering pro-inflammatory, anti-inflammatory, Th17-associated cytokines, chemokines, and growth factors. Concentrations were reported as pg/mL.

3.9 Statistical analysis

Data are shown as mean ± SEM. Data was analysed with normality and lognormality tests, repeated measures one-way Anova test, Wilcoxon test, Student's two-tailed t-test, repeated measures one-way ANOVA, Friedman test. P-values < 0.05 were considered statistically significant. Statistical analysis was performed with GraphPad Prism software (GraphPad Software Inc., USA).

4. RESULTS

4.1. Development of 3D synovia model

The first step was to determine the optimal FLS concentration that would ensure the highest viability and functionality of cells within the hydrogel. The viability assay and morphological evaluation of patient-derived FLS embedded in 5%. GelMA at a concentration of 2×10^6 cells/mL at both 24h and 4 days are shown in Figure 1. At 24 hours post-seeding, we found an increasing number of dead cells figuring in red (Figure 1A) as well as with appearing rounded and not elongated (Figures 1B). The same behaviour was observed at 4 days (Figures 1C, D).

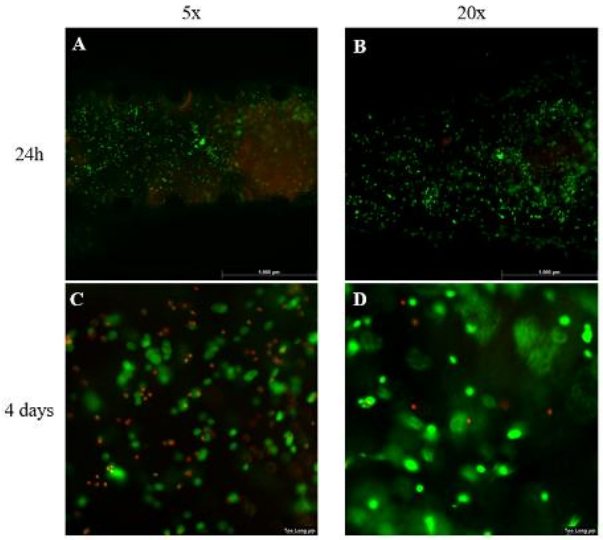


Figure 1. Viability assay of FLS derived from RA patient at 2×10^6 cells/mL, embedded in 5% GelMA after 24 hours (A, B) and after 4 days (C, D) of culture. THUNDER (Leica Biosystem), with a 5X lens (A, C) and a 20X lens (B, D). (Green: Calcein; Red: PI).

When we seeded FLS at a concentration of 5×10^6 cells/mL, we observed a notable improvement in both the viability and morphology of the encapsulated cells, detected through the emergence of their characteristic elongated shape. Importantly, this morphological change was evident at both 24 hours and 4 days of culture, as depicted in Figures 2A-D. Based on the results, this concentration was chosen for the following experiments.

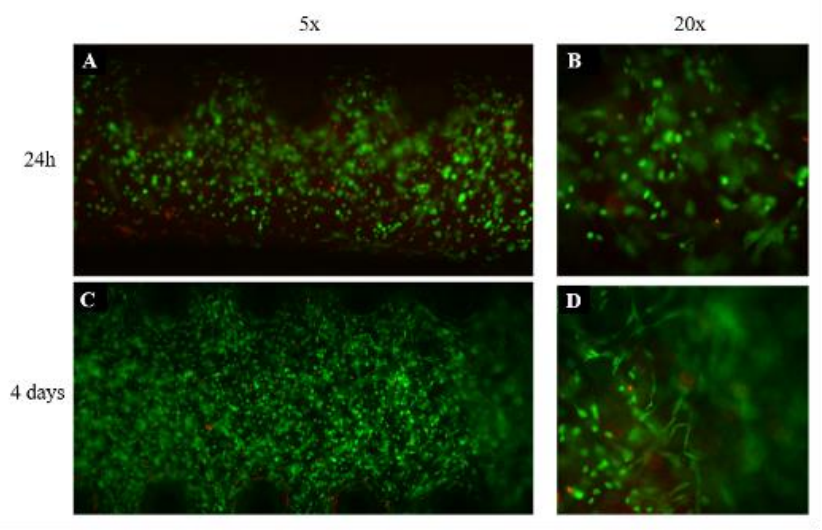


Figure 2. Viability assay and morphology evaluation of FLSs originating from RA patients embedded at 5×106 cells/mL in 8% GelMA, on the chip in microfluidic conditions after 24 hours (A, B) and after 4 days of culture (C, D). THUNDER, Leica, 5X magnification (A, B) and 20X magnification (C, D). (Green: Calcein; Red: PI).

In order to mimic *in vitro* the the cellular complexity of RA synovia microenvironment, RA-derived FLS (5×10^6 cells/ml) and activated PBMCs (2×10^6 cells/ml) were encapsulated in hydrogel (GelMA 5% and LAP 0.3%) in the presence of a proinflammatory cytokine cocktail (IL-1β, IL-6, and TNF-α). After 72 hours of incubation, we performed calcein/PI (Live/Dead) staining under Thunder imaging systems to evaluate cell viability.

Immunofluorescence staining combined with ImageJ-based quantification was performed to evaluate the viability of PBMC and FLS under inflammatory stimulation in a 3D model. In the merged cultures, PBMC were distinguished by blue nuclear staining, while overall cell viability was assessed using green fluorescence for live cells and red fluorescence for dead cells. FLS were present in either the live (green) or dead (red) populations, whereas PBMC were primarily visualized through the blue channel in combination with viability markers. In PBMC, cytokine stimulation led to an apparent improvement in viability. Non-stimulated PBMC cultures displayed a mean viability of 32%, whereas cytokine treatment increased viability to 50%, corresponding to an 1.5-fold enhancement. Immunofluorescence images reflected this trend, with more intense green fluorescence and relatively fewer red-stained cells observed in cytokine-stimulated PBMC compared to non-stimulated controls. However, this increase did not reach statistical significance. In contrast, FLS exhibited the opposite response to cytokine stimulation. Non-stimulated FLS demonstrated high viability (74%), but this decreased to 60% following cytokine exposure, representing an approximate 20% reduction (0.8-fold).

Immunofluorescence images were consistent with this finding, showing a visibly higher proportion of red-stained FLS in cytokine-treated cultures compared to controls (Figure 3).

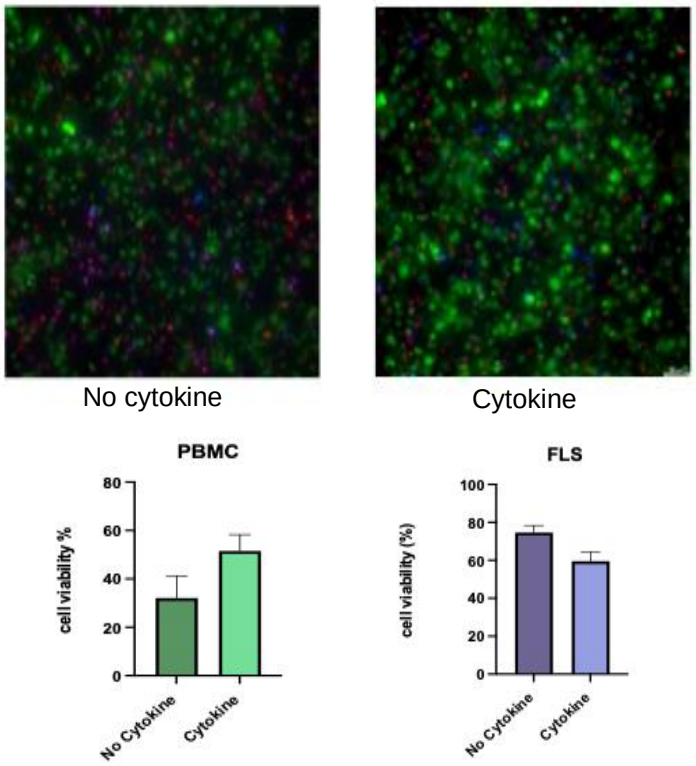


Figure 3. Immunofluorescence images of RA-FLSs and PBMC co-encapsulated in GelMA 5% in the presence or absence of 2-day cytokine stimulation and stained with Calcein-AM (green)/PI (red) and visualized with Thunder microscope. Histograms show the Viability of PBMC and FLS in the absence or presence of 2-day cytokine stimulation quantified by ImageJ.

4.2. Impact of CIC-39 on cell viability in the 3D synovial model

Currently approved therapies for RA include conventional DMARDs such as methotrexate, biological agents such as anti-TNF antibodies (e.g., adalimumab), and anti-inflammatory drugs such as NSAIDs. In this study, these drugs were employed as reference treatments to benchmark the activity and safety of CIC-39, a newly developed compound.

RA FLS and activated PBMCs were encapsulated in GelMA (5%) with LAP (0.3%) following the addition of the cytokine cocktail. Subsequently, cells were treated for 48 hours with CIC-39 (3 µM), MTX (0.1 µM), Humira (10 µg/mL), or celecoxib (25 µM). To determine whether these compounds affected cell survival, the viability of both PBMCs and FLS was evaluated using live/dead immunofluorescence staining (Figure 4A), combined with quantitative viability analysis. CIC, MTX, and Humira consistently induced slight increases in viability in both PBMCs and FLS compared with control conditions, whereas celecoxib reduced PBMC

viability while producing only a marginal increase in FLS viability. However, all observed variations were modest (<20%) and did not reach statistical significance. These findings indicate that, at the tested concentrations, CIC, MTX, Humira, and celecoxib do not significantly impact the viability of PBMCs and FLS in the 3D inflammatory synovial model (Figures 4B,C).

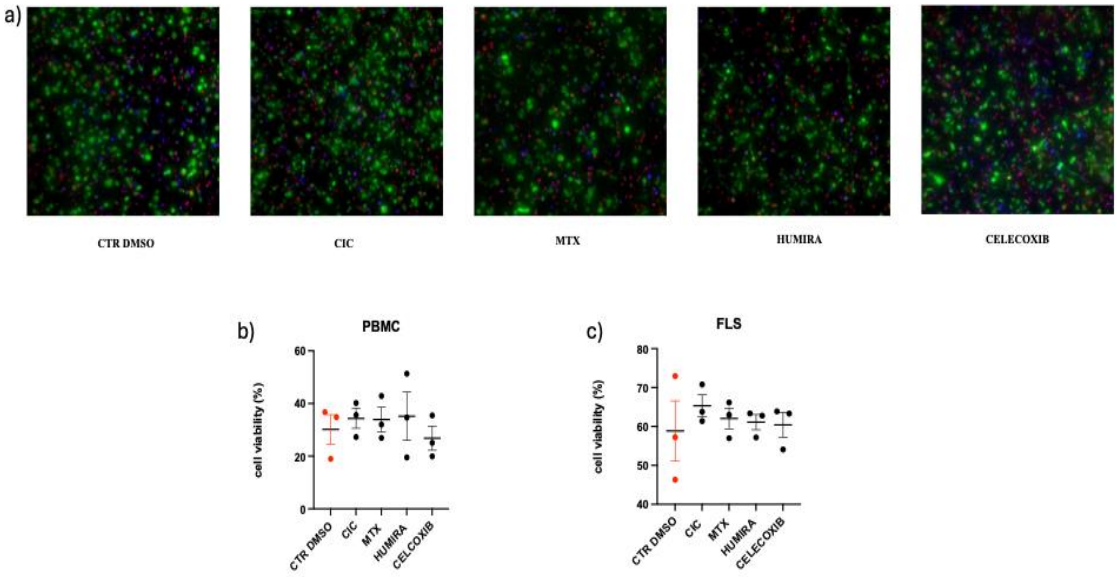


Figure 4. A) Immunofluorescence images of RA-FLSs and PBMC encapsulated within GelMA 5% under cytokine stimulation and drug treatment after 72-hour encapsulation, stained with Calcein-AM (green)/PI (red) and acquired with Thunder microscope, B) scatter dot plot graph of PBMC viability under drug treatment, C) scatter dot plot graph of FLS viability under drug treatment.

4.3. Impact of CIC-39 on cytokine secretion in a 3D synovial model

To investigate the effects of different therapeutic interventions, in a 3D co-culture synovia model, a comprehensive multi-cytokine analysis was performed using both the Bio-Plex Human Cytokine 37-Plex and Th17 Cytokine panels. All the cytokines were categorised according to their biological fucntions as summarized in Table 2. This approach allowed for the simultaneous assessment of numerous inflammatory, regulatory, and tissue-associated cytokines. After excluding 7 overlapping analytes between the two assays, including IL-2, IL-6, IL-8, TNF-a, IL-10, IL-17A, IFN-y and IL-12(p70), and cytokines, which were not able to be evaluated due to out-of-range values, a non-redundant dataset of 47 cytokines was obtained.

Data were normalized, averaged across replicates, and analyzed to compare baseline, cytokine-stimulated, and drug-treated conditions.

CATEGORY	ANALYTES
PRO-INFLAMMATORY CYTOKINES	IL-1β, IL-6, TNF-α, IL-12 (p70), IL-12 (p40), IL-17A, IL-32, IL-35
ANTI-INFLAMMATORY / REGULATORY	IL-10, IL-27 (p28), sTNF-R1, sTNF-R2, sCD163
T CELL GROWTH & DIFFERENTIATION	IL-2, IL-7, TSLP, IL-4, IL-5, IL-13
B CELL SURVIVAL / AUTOANTIBODY AXIS	APRIL / TNFSF13, BAFF / TNFSF13B, sCD30 / TNFRSF8
INTERFERONS	IFN-α2, IFN-β, IFN-γ, IL-28A / IFN-λ2, IL-29 / IFN-λ1
IL-6 FAMILY (GP130-DEPENDENT)	IL-6, IL-11, IL-27 (p28), gp130 / sIL-6Rβ, sIL-6Rα
IL-10 FAMILY (BARRIER/EPITHELIAL IMMUNITY)	IL-19, IL-20, IL-22, IL-26
COLONY-STIMULATING FACTORS	G-CSF, GM-CSF, IL-34
CHEMOKINES (CELL RECRUITMENT)	IL-8 (CXCL8), MCP-1 (CCL2), MIP-1β (CCL4)
TNF SUPERFAMILY (COSTIMULATION / REMODELING)	LIGHT / TNFSF14, TWEAK / TNFSF12
TISSUE REMODELING & DAMAGE MARKERS	MMP-1, MMP-2, MMP-3, Chitinase-3-like 1 (YKL-40), Osteocalcin, Osteopontin, Pentraxin-3

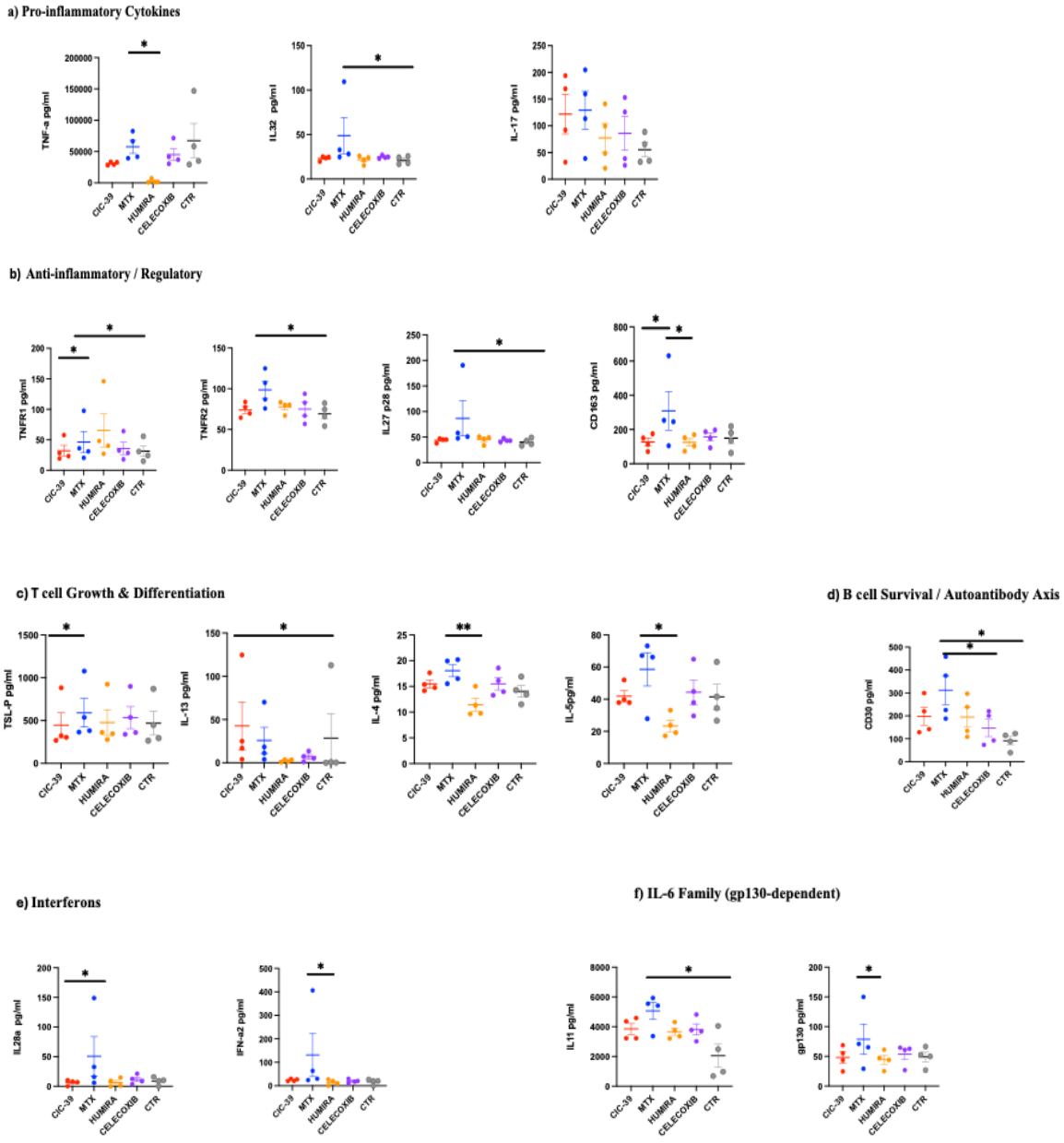
Table 2. Immune-related analytes from 17- and 37 multiplex assays are categorized by function, including cytokines, chemokines, growth factors, and tissue remodelling markers.

When CIC-39 was compared with the CTR, IL-13, belonging to the T-cell growth and differentiation category, was the only cytokine significantly upregulated. Among the pro-inflammatory cytokines, IL-17A showed a slight increasing trend in CIC-39–treated samples; however, this change did not reach statistical significance. All other measured cytokines remained largely unchanged compared with CTR, indicating that CIC-39 had a minimal impact on the overall cytokine profile under these conditions. In contrast, significant differences emerged when CIC-39 was compared with MTX, particularly within the anti-inflammatory/regulatory cytokine group, which includes TNFR1 and CD163 ($p < 0.05$). CIC-39 treatment resulted in a 1.44-fold downregulation of TNFR1 relative to MTX. Similarly, CD163 expression was reduced to 41% of MTX levels, corresponding to a 2.43-fold downregulation. Furthermore, CIC-39 significantly decreased the expression of thymic stromal lymphopoietin (TSLP) to approximately 75% of MTX levels, representing a 1.33-fold reduction ($p < 0.05$). An even more pronounced effect was observed for IL-28A, whose expression was suppressed to 12.5% of MTX levels, corresponding to an 8-fold downregulation ($p < 0.05$). Collectively, these findings indicate that while CIC-39 induces a selective increase in IL-13 compared with CTR, it also exerts a marked regulatory effect on several key immune mediators when compared with MTX.

Compared to CTR, MTX treatment significantly upregulated multiple cytokines. The proinflammatory cytokine IL-32 significantly increased by approximately 2.3-fold. IL-11 and IL-27 p28, both from the IL-6 family, were upregulated by about 2.47- and 2.16-fold, respectively. The anti-inflammatory/regulatory cytokines TNFR1 and TNFR2 increased by around 1.47- and 1.43-fold, and IL-22 (e.g. an IL-10 family cytokine) by 1.59-fold. CD30, involved in B cell survival and the autoantibody axis, showed a 3.4-fold increase. All changes were statistically significant. MTX also significantly increased colony-stimulating factors: G-CSF by 3.53-fold, GM-CSF by 5.29-fold, and IL-34 by 2.94-fold. It significantly upregulated LIGHT (TNFSF14), a TNF superfamily member, by 2.35-fold, and MMP-3, a marker of tissue remodelling and damage, by 2.82-fold.

Compared to Humira, MTX treatment significantly reduced TNF-α expression by 15% ($p < 0.05$). It significantly increased GP130 expression by 1.61-fold and CD163 expression by 2.07-fold ($p < 0.05$). IL-4 ($p<0.001$) and IL-5 ($p<0.05$) were also significantly upregulated by 1.59-

fold and 2.46-fold, respectively. Additionally, MTX markedly increased IFN-α2 expression by 8.98-fold. Expression of G-CSF and MIP-1β increased by 2.30-fold and 1.54-fold, respectively. LIGHT, also known as TNFSF14, was upregulated by 2.35-fold. In comparison with Celecoxib, MTX significantly increased CD30 expression by 2.12-fold. Although the changes in other cytokines did not reach statistical significance, several exhibited consistent trends in expression, indicating potential biological relevance that may warrant further investigation. When either Humira or Celecoxib was compared to CTR, no statistically significant differences in cytokine expression were observed, although both treatments showed variable trends, with cytokine levels either similar to control or exhibiting slight fluctuations (Figure 5).



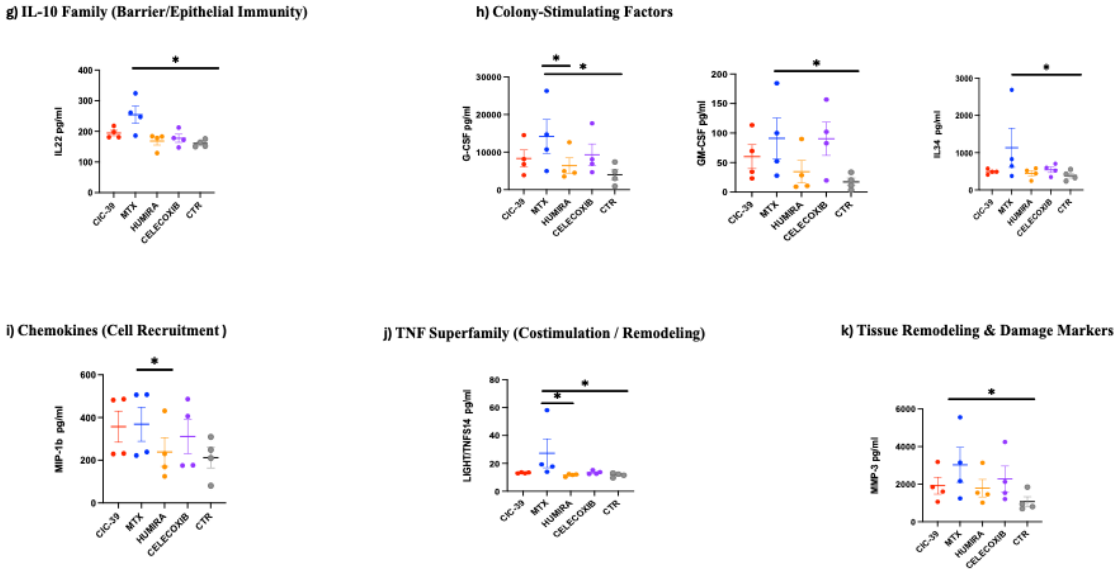


Figure 5. Graphs depicting cytokines and immune markers that were significantly altered in the multicytokine analysis. Only analytes showing statistically significant changes between experimental conditions are displayed, highlighting key inflammatory and regulatory responses (ANOVA, *p<0.05, **p<0.01).

5. DISCUSSION

RA is a chronic AD characterized primarily by persistent inflammatory synovitis, which leads to the progressive destruction of cartilage and bone within the joints (Gao, Zhang and Liu, 2024). Synovitis involves the infiltration of T cells into the synovial membrane, which is the tissue lining the joint, and the proliferation of FLS within the joint's synovial membrane. T cells, along with other immune cells, form organized structures and interact with FLS, contributing to joint inflammation, damage, and the release of pro-inflammatory cytokines (Tu *et al.*, 2022).

The pathogenesis of RA involves a complex interplay of cellular and molecular systems that result in chronic inflammation and destruction of the joints through multiple mechanisms. Aberrant activation of adaptive immunity, particularly autoreactive T and B cells, leads to the production of Abs such as rheumatoid factor (RF) and anti-cyclic citrullinated peptides (anti-CCP) and pro-inflammatory cytokines (e.g. TNF-α, IL-6, IL-1, IL-17 among the others) (Nandakumar *et al.*, 2023). These mediators activate FLS and macrophages, promoting persistent inflammation, pannus formation, and recruitment of additional immune cells. The inflamed synovium drives cartilage degradation and bone erosion through matrix metalloproteinases and osteoclast activation, resulting in progressive joint damage and disability (Maeda *et al.*, 2022).

Inflammation is not only sustained by cytokine secretion but is also deeply influenced by intracellular signaling pathways. Among them, Ca^{2+} signaling plays a central role in modulating immune cell activation, differentiation, and effector functions. Dysregulation of calcium homeostasis, particularly through SOCE, has been closely linked to the aberrant immune activation observed in RA. Dysregulation of SOCE, which is a main pathway, along with input from other calcium channels, drives the abnormal activation of T and B lymphocytes (Vig and Kinet, 2009; Izquierdo *et al.*, 2014).

In this thesis, we developed in vitro a 3D model of inflamed RA synovia, where we evaluated any potential immunomodulatory effects of a SOCE negative modulator, e.g. CIC-39 in comparison to RA clinically used drugs, employed as reference treatments to benchmark its activity and safety. By co-encapsulating RA FLS and activated PBMCs in GelMA, we attempted to mimic the complicated cellular crosstalk present in the synovial microenvironment.

Our main findings showed that: 1) the 3D inflammatory model efficiently mimicked the cell-type-specific responses upon cytokine stimulation; 2) CIC-39 and the other RA drugs did not

affect cell viability at the concentrations used; and 3) CIC-39 elicited a distinct and potentially beneficial immunomodulatory cytokine profile, notably a significant upregulation of IL-13, setting it apart from other treatments.

The successful establishment of our 3D co-culture model was a critical first step. Initial observations indicated a differential trend in cell viability: cytokine stimulation was associated with a higher average readout in PBMCs and a lower average readout in FLS compared to untreated controls. The inflammatory milieu, enriched in TNF-α, IL-1β, and IL-6, cytokines promotes the survival and activation of immune cells (Alivernini, Firestein, and McInnes, 2022; Gao, Zhang, and Liu, 2024a). Conversely, the same cytokines can induce metabolic stress and pro-apoptotic signaling in FLS (Mariani et al., 2023).

The primary challenge with any new therapeutic compound is it's safety profile. Our viability tests revealed that survival of both PBMCs and FLS encapsulated in hydrogel was not significantly affected by the 48 hour treatment with either CIC-39, or MTX, Humira or celecoxib. The absence of significant changes in viability is particularly relevant for CIC-39, as the primary goal of SOCE modulation is not to induce cytotoxicity but to regulate inflammatory signaling pathways. SOCE plays a crucial role in calcium-dependent activation, cytokine production, and cellular functions in both immune cells and synovial FLS. Therefore, the maintenance of cell viability in the presence of CIC-39 supports its potential as a safe immunomodulatory agent rather than a cytotoxic compound. Moreover, the comparison with clinically approved drugs strengthens the translational relevance of our findings. MTX and humira are well-established RA treatments with known safety profiles, and their similar impact on cell viability in our model provides an internal validation of the system, confirming its suitability for preclinical drug screening studies.

The most interesting results, however, emerged from the comprehensive multicytokine analyses. While humira and celecoxib exhibited minimal significant changed as compared to the inflamed control, MTX treatment led to a broad and significant upregulation of a wide range of cytokines such as pro-inflammatory (IL-32), growth factors (G-CSF, GM-CSF, IL-34), and tissue remodeling markers (MMP-3). This was unexpected, since MTX is an important DMARD with long-lasting anti-inflammatory and disease-modifying effects *in vivo*. Although, its immediate *in vitro* effect may be more complex. MTX can induce the initial cellular stress and "flare" responses in specific systems before the full onset of its anti-metabolite effects, potentially explaining the unexpected cytokine spike MTX can cause initial cellular stress and "flare" responses in some systems before its anti-metabolite functions take full effect, potentially explaining this paradoxical cytokine surge (Smolen et al., 2023).

Humira, as expected for an anti-TNF monoclonal antibody, primarily affected TNF-dependent pathways, confirming its highly specific mechanism of action. Celecoxib, which mainly acts through cyclooxygenase-2 inhibition, showed a more limited influence on cytokine production, consistent with its role as a symptomatic anti-inflammatory drug rather than a disease-modifying agent.

With regard to CIC-39, one of the significant finding was the strong upregulation of IL-13, which is an anti-inflammatory cytokine mainly produced by Th2 cells and type 2 innate lymphoid cells (ILC2)s. IL-13 is known to inhibit the production of pro-inflammatory cytokines like IL-1β, TNF-α, and IL-6 from macrophages and to counteract the effects of Th1 and Th17 responses (Souza *et al.*, 2013). In the context of RA, which is traditionally characterized by a Th1/Th17 imbalance, a therapeutic shift towards a Th2 response, mediated by cytokines like IL-13 and IL-4, would be considered beneficial.

Additionally, CIC-39 significantly downregulated several key mediators, including the macrophage marker CD163 and the TNF receptor TNF-R1, compared with MTX (Skytthe, Graversen and Moestrup, 2020). CD163 is a marker associated with macrophages, cells that play a central role in sustaining inflammation in RA, while TNF-R1 is a receptor involved in TNF-α signaling, one of the most important inflammatory pathways in the disease (Cousin *et al.*, 2025). By reducing the levels of these mediators, CIC-39 seems to dampen excessive inflammatory activity without completely blocking immune function.

In conclusion, we developed a 3D in vitro model of RA synovia that is responsive to both inflammatory stimulation and pharmacological intervention. Using this platform, we provide the first evidence that the novel SOCE modulator CIC-39 displays a favourable safety profile and exerts a distinct mechanism of action, characterized by the induction of the anti-inflammatory cytokine IL-13. These findings would suggest that targeting SOCE represents a promising therapeutic strategy in RA. CIC-39 may therefore serve as a complementary approach to current pharmaceutical therapies, contributing to a more balanced modulation of the inflammatory response rather than its complete suppression.

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Finally, and most importantly, I dedicate this work to my parents. My mumma and papa were immensely proud and happy when I received the opportunity to study abroad, even though it meant their only kid would be far away from home. “Amma, njan entha parayende, enth paranjalum ath korava. Ningal randu perum enik vendi kore sacrifices cheythu, enne ivide vere ethikaan. Papa, njan eppazhum papade mol aa. Ningale proud cheypikaan aan ente aim. Ningale santhoshipikkan, ningal pashe eppazhum ente santhosham aa nokeett olle. Ningal aan ente ettavum valiya strength. Ningal randuperdeyum prayers aan enne ivide vere ethiche. Ee thesisum ee graduation ninak randu perkum dedicated aan. Thankyou, ella divasum enne ormimpichennu, eshuappachan koode ind enn.”

Love and gratitude
Keziya george

References

Alivernini, S., Firestein, G.S. and McInnes, I.B. (2022) "The pathogenesis of rheumatoid arthritis," *Immunity*, 55(12), pp. 2255–2270. Available at: https://doi.org/10.1016/j.immuni.2022.11.009.

Aprile, S. *et al.* (2021) "1,2,4-Oxadiazole-Bearing Pyrazoles as Metabolically Stable Modulators of Store-Operated Calcium Entry," *ACS Medicinal Chemistry Letters*, 12(4), pp. 640–646. Available at: https://doi.org/10.1021/acsmedchemlett.1c00034.

Ashiq, K. *et al.* (2023) "An Updated Review on Rheumatoid Arthritis (RA): Epidemiology, Pathophysiology, Diagnosis, and the Current Approaches for Its Treatment," *Sudan Journal of Medical Sciences*, 18(4), pp. 539–551. Available at: https://doi.org/10.18502/sjms.v18i4.14742.

Cao, U.M.N. *et al.* (2023) "Microfluidic Organ-on-A-chip: A Guide to Biomaterial Choice and Fabrication," *International Journal of Molecular Sciences*, 24(4), p. 3232. Available at: https://doi.org/10.3390/ijms24043232.

Cappellano, G. *et al.* (2019) "Exploiting PLGA-Based Biocompatible Nanoparticles for Next-Generation Tolerogenic Vaccines against Autoimmune Disease," *International Journal of Molecular Sciences*, 20(1), p. 204. Available at: https://doi.org/10.3390/ijms20010204.

Castro, F. *et al.* (2018) "Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion," *Frontiers in Immunology*, 9, p. 847. Available at: https://doi.org/10.3389/fimmu.2018.00847.

Centola, M. *et al.* (2013) "Development of a Multi-Biomarker Disease Activity Test for Rheumatoid Arthritis," *PLoS ONE*. Edited by O. Frey, 8(4), p. e60635. Available at: https://doi.org/10.1371/journal.pone.0060635.

Chaplin, D.D. (2010) "Overview of the immune response," *Journal of Allergy and Clinical Immunology*, 125(2), pp. S3–S23. Available at: https://doi.org/10.1016/j.jaci.2009.12.980.

Chen, L., Zhao, J. and Meng, Q. (2025) "From genetic variants to therapeutic targets: insights into understanding rheumatoid arthritis," *Frontiers in Immunology*, 16, p. 1556971. Available at: https://doi.org/10.3389/fimmu.2025.1556971.

Collet, A. *et al.* (2025) "Autoreactive B cells in autoimmune diseases: Mechanisms, functions and clinical implications," *Autoimmunity Reviews*, 24(8), p. 103851. Available at: https://doi.org/10.1016/j.autrev.2025.103851.

Cousin, A. *et al.* (2025) "CD163, a novel receptor for TNF, was revealed in situ by proximity ligation assay," *Heliyon*, 11(3), p. e42194. Available at: https://doi.org/10.1016/j.heliyon.2025.e42194.

Crotty, S. (2019) "T Follicular Helper Cell Biology: A Decade of Discovery and Diseases," *Immunity*, 50(5), pp. 1132–1148. Available at: https://doi.org/10.1016/j.immuni.2019.04.011.

Deng, L. and Mariuzza, R.A. (2007) "Recognition of self-peptide–MHC complexes by autoimmune T-cell receptors," *Trends in Biochemical Sciences*, 32(11), pp. 500–508. Available at: https://doi.org/10.1016/j.tibs.2007.08.007.

Di Matteo, A., Bathon, J.M. and Emery, P. (2023) "Rheumatoid arthritis," *The Lancet*, 402(10416), pp. 2019–2033. Available at: https://doi.org/10.1016/S0140-6736(23)01525-8.

Fichtner, M.L. *et al.* (2020) "Autoimmune Pathology in Myasthenia Gravis Disease Subtypes Is Governed by Divergent Mechanisms of Immunopathology," *Frontiers in Immunology*, 11, p. 776. Available at: https://doi.org/10.3389/fimmu.2020.00776.

Fraenkel, L. *et al.* (2021) "2021 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis," *Arthritis Care & Research*, 73(7), pp. 924–939. Available at: https://doi.org/10.1002/acr.24596.

Froghi, S. *et al.* (2021) "New Insights on the Role of TRP Channels in Calcium Signalling and Immunomodulation: Review of Pathways and Implications for Clinical Practice," *Clinical Reviews in Allergy & Immunology*, 60(2), pp. 271–292. Available at: https://doi.org/10.1007/s12016-020-08824-3.

Galindo-Feria, A.S., Wang, G. and Lundberg, I.E. (2022) "Autoantibodies: Pathogenic or epiphenomenon," *Best Practice & Research Clinical Rheumatology*, 36(2), p. 101767. Available at: https://doi.org/10.1016/j.berh.2022.101767.

Gao, Y., Zhang, Y. and Liu, X. (2024) "Rheumatoid arthritis: pathogenesis and therapeutic advances," *MedComm*, 5(3), p. e509. Available at: https://doi.org/10.1002/mco2.509.

Ho, T.-C. *et al.* (2022) "Hydrogels: Properties and Applications in Biomedicine," *Molecules*, 27(9), p. 2902. Available at: https://doi.org/10.3390/molecules27092902.

Hogan, P.G. and Rao, A. (2015) “Store-operated calcium entry: Mechanisms and modulation,” *Biochemical and Biophysical Research Communications*, 460(1), pp. 40–49. Available at: https://doi.org/10.1016/j.bbrc.2015.02.110.

Izquierdo, J.-H. *et al.* (2014a) “Calcio, canales, señalización intracelular y autoinmunidad,” *Reumatología Clínica*, 10(1), pp. 43–47. Available at: https://doi.org/10.1016/j.reuma.2013.05.008.

Izquierdo, J.-H. *et al.* (2014b) “Calcium, Channels, Intracellular Signaling and Autoimmunity,” *Reumatología Clínica (English Edition)*, 10(1), pp. 43–47. Available at: https://doi.org/10.1016/j.reumae.2013.11.004.

Johnson, D. and Jiang, W. (2023) “Infectious diseases, autoantibodies, and autoimmunity,” *Journal of Autoimmunity*, 137, p. 102962. Available at: https://doi.org/10.1016/j.jaut.2022.102962.

Kapałczyńska, M. *et al.* (2016) “2D and 3D cell cultures – a comparison of different types of cancer cell cultures,” *Archives of Medical Science* [Preprint]. Available at: https://doi.org/10.5114/aoms.2016.63743.

Karoyo, A.H. and Wilson, L.D. (2021) “A Review on the Design and Hydration Properties of Natural Polymer-Based Hydrogels,” *Materials*, 14(5), p. 1095. Available at: https://doi.org/10.3390/ma14051095.

Leung, C.M. *et al.* (2022) “A guide to the organ-on-a-chip,” *Nature Reviews Methods Primers*, 2(1), p. 33. Available at: https://doi.org/10.1038/s43586-022-00118-6.

Liang, X. *et al.* (2021) “Development of Store-Operated Calcium Entry-Targeted Compounds in Cancer,” *Frontiers in Pharmacology*, 12, p. 688244. Available at: https://doi.org/10.3389/fphar.2021.688244.

Maeda, K. *et al.* (2022) “Inflammation and Bone Metabolism in Rheumatoid Arthritis: Molecular Mechanisms of Joint Destruction and Pharmacological Treatments,” *International Journal of Molecular Sciences*, 23(5), p. 2871. Available at: https://doi.org/10.3390/ijms23052871.

Mariani, F.M. *et al.* (2023) “Pathogenesis of rheumatoid arthritis: one year in review 2023,” *Clinical and Experimental Rheumatology* [Preprint].

Morales-Martínez, M. *et al.* (2024) “Identification of potential new T cell activation molecules: a Bioinformatic Approach,” *Scientific Reports*, 14(1), p. 22219. Available at: https://doi.org/10.1038/s41598-024-73003-9.

Morshed, S.A. and Davies, T.F. (2015) “Graves’ Disease Mechanisms: The Role of Stimulating, Blocking, and Cleavage Region TSH Receptor Antibodies,” *Hormone and*

Metabolic Research, 47(10), pp. 727–734. Available at: https://doi.org/10.1055/s-0035-1559633.

Nandakumar, K.S. *et al.* (2023) "Aberrant Activation of Immune and Non-Immune Cells Contributes to Joint Inflammation and Bone Degradation in Rheumatoid Arthritis," *International Journal of Molecular Sciences*, 24(21), p. 15883. Available at: https://doi.org/10.3390/ijms242115883.

Oliveira, I.M. *et al.* (2021) "Hydrogels in the treatment of rheumatoid arthritis: drug delivery systems and artificial matrices for dynamic in vitro models," *Journal of Materials Science: Materials in Medicine*, 32(7), p. 74. Available at: https://doi.org/10.1007/s10856-021-06547-1.

Park, Y.-J. *et al.* (2020) "The Role of Calcium–Calcineurin–NFAT Signaling Pathway in Health and Autoimmune Diseases," *Frontiers in Immunology*, 11, p. 195. Available at: https://doi.org/10.3389/fimmu.2020.00195.

Parkin, J. and Cohen, B. (2001) "An overview of the immune system," *The Lancet*, 357(9270), pp. 1777–1789. Available at: https://doi.org/10.1016/S0140-6736(00)04904-7.

Pelly, V.S. *et al.* (2016) "IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection," *Mucosal Immunology*, 9(6), pp. 1407–1417. Available at: https://doi.org/10.1038/mi.2016.4.

Radu, A.-F. and Bungau, S.G. (2021) "Management of Rheumatoid Arthritis: An Overview," *Cells*, 10(11), p. 2857. Available at: https://doi.org/10.3390/cells10112857.

Riva, B. *et al.* (2022) "STIM1 and ORAI1 mutations leading to tubular aggregate myopathies are sensitive to the Store-operated Ca2+-entry modulators CIC-37 and CIC-39," *Cell Calcium*, 105, p. 102605. Available at: https://doi.org/10.1016/j.ceca.2022.102605.

Rooney, I.A. (no date) "Protection of human amniotic epithelial cells (HAEC) from complement-mediated lysis: expression on the cells of three complement inhibitory membrane proteins."

Rosenblum, M.D., Remedios, K.A. and Abbas, A.K. (2015) "Mechanisms of human autoimmunity," *Journal of Clinical Investigation*, 125(6), pp. 2228–2233. Available at: https://doi.org/10.1172/JCI78088.

Sharma, S. and Bluett, J. (2024) "Towards Personalized Medicine in Rheumatoid Arthritis," *Open Access Rheumatology: Research and Reviews*, Volume 16, pp. 89–114. Available at: https://doi.org/10.2147/OARRR.S372610.

Shaw, P.J. and Feske, S. (2013) "Physiological and pathophysiological functions of SOCE in the immune system."

Skopin, A.Y. *et al.* (2024) "Vulnerability of Store-Operated Calcium Entry to Inhibitors and Microenvironment in Cells of Different Breast Cancer Subtypes," *Life*, 14(3), p. 357. Available at: https://doi.org/10.3390/life14030357.

Skytthe, M.K., Graversen, J.H. and Moestrup, S.K. (2020) "Targeting of CD163+ Macrophages in Inflammatory and Malignant Diseases," *International Journal of Molecular Sciences*, 21(15), p. 5497. Available at: https://doi.org/10.3390/ijms21155497.

Smolen, J.S. *et al.* (2023) "EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2022 update," *Annals of the Rheumatic Diseases*, 82(1), pp. 3–18. Available at: https://doi.org/10.1136/ard-2022-223356.

Song, Y., Li, J. and Wu, Y. (2024) "Evolving understanding of autoimmune mechanisms and new therapeutic strategies of autoimmune disorders," *Signal Transduction and Targeted Therapy*, 9(1), p. 263. Available at: https://doi.org/10.1038/s41392-024-01952-8.

Souza, P. *et al.* (2013) "IL -4 and IL -13 inhibit IL -1β and TNF -α induced kinin B_1 and B_2 receptors through a STAT6 -dependent mechanism," *British Journal of Pharmacology*, 169(2), pp. 400–412. Available at: https://doi.org/10.1111/bph.12116.

Szeto, C. *et al.* (2020) "TCR Recognition of Peptide–MHC-I: Rule Makers and Breakers," *International Journal of Molecular Sciences*, 22(1), p. 68. Available at: https://doi.org/10.3390/ijms22010068.

Taha, S. *et al.* (2025) "Progressive Elevation of Store-Operated Calcium Entry-Associated Regulatory Factor (SARAF) and Calcium Pathway Dysregulation in Multiple Sclerosis," *International Journal of Molecular Sciences*, 26(10), p. 4520. Available at: https://doi.org/10.3390/ijms26104520.

Togashi, T. *et al.* (2025) "Rheumatoid Factor: Diagnostic and Prognostic Performance and Therapeutic Implications in Rheumatoid Arthritis," *Journal of Clinical Medicine*, 14(5), p. 1529. Available at: https://doi.org/10.3390/jcm14051529.

Trebak, M. and Kinet, J.-P. (2019) "Calcium signalling in T cells," *Nature Reviews Immunology*, 19(3), pp. 154–169. Available at: https://doi.org/10.1038/s41577-018-0110-7.

Tu, J. *et al.* (2022) "Two Main Cellular Components in Rheumatoid Arthritis: Communication Between T Cells and Fibroblast-Like Synoviocytes in the Joint Synovium," *Frontiers*

in Immunology, 13, p. 922111. Available at: https://doi.org/10.3389/fimmu.2022.922111.

Vig, M. and Kinet, J.-P. (2009) "Calcium signaling in immune cells," *Nature Immunology*, 10(1), pp. 21–27. Available at: https://doi.org/10.1038/ni.f.220.

Vivier, E. and Malissen, B. (2005) "Innate and adaptive immunity: specificities and signaling hierarchies revisited," *Nature Immunology*, 6(1), pp. 17–21. Available at: https://doi.org/10.1038/ni1153.

Volkov, M., Van Schie, K.A. and Van Der Woude, D. (2020) "Autoantibodies and B Cells: The ABC of rheumatoid arthritis pathophysiology," *Immunological Reviews*, 294(1), pp. 148–163. Available at: https://doi.org/10.1111/imr.12829.

Voswinkel, J. and Gause, A. (no date) "From immunoglobulin gene fingerprinting to motif-specific hybridization: advances in the analysis of B lymphoid clonality in rheumatic diseases," 4(1).

Wang, R. *et al.* (2024) "The interaction of innate immune and adaptive immune system," *MedComm*, 5(10), p. e714. Available at: https://doi.org/10.1002/mco2.714.

Wu, D. *et al.* (2025) "Synovial macrophages drive severe joint destruction in established rheumatoid arthritis," *Scientific Reports*, 15(1), p. 12111. Available at: https://doi.org/10.1038/s41598-025-93784-x.

Yasmeen, F. *et al.* (2024) "Understanding Autoimmunity: Mechanisms, Predisposing Factors, and Cytokine Therapies," *International Journal of Molecular Sciences*, 25(14), p. 7666. Available at: https://doi.org/10.3390/ijms25147666.

Yi, J. *et al.* (2022) "Hydrogels for the treatment of rheumatoid arthritis," *Frontiers in Bioengineering and Biotechnology*, 10, p. 1014543. Available at: https://doi.org/10.3389/fbioe.2022.1014543.

Zhang, X. *et al.* (2024) "The promise of Synovial Joint-on-a-Chip in rheumatoid arthritis," *Frontiers in Immunology*, 15, p. 1408501. Available at: https://doi.org/10.3389/fimmu.2024.1408501.