Master Thesis Tittle

# Delineating molecular mechanisms of Dendritic cell/monocyte activation mediated by neutrophil extracellular traps underlying Rheumatoid Arthritis pathogenesis

Eirini Gonianaki (1060171)

**Project supervisor:** Garyfalia Papadaki, Postdoctoral research fellow

**Supervising Professors:** Prodromos Sidiropoulos, MD

George Bertsias, MD

Panayiotis Verginis, Associate Professor

Research Laboratory of Autoimmunity and Inflammation, School of Medicine, University of Crete

Τίτλος Μεταπτυχιακής Εργασίας

# Διερεύνηση των μοριακών μηχανισμών της ενεργοποίησης των μονοκυττάρων/δενδριτικών κυττάρων που διαμεσολαβούνται από εξωκυτταρικές παγίδες ουδετερόφιλων και συμβάλουν στην παθογένεια της Ρευματοειδούς Αρθρίτιδας

Ειρήνη Γωνιανάκη (Α.Μ.1060171)

**Επιβλέπουσα Μελέτης:** Γαρυφαλλιά Παπαδάκη, Μεταδιδακτορική ερευνήτρια

**Τριμελής επιτροπή :** Πρόδρομος Σιδηρόπουλος, MD

Γεώργιος Μπερτσιάς, MD

Παναγιώτης Βεργίνης, Αναπληρωτής Καθηγητής

Ερευνητικό Εργαστήριο Αυτοανοσίας και Φλεγμονής, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

**Acknowledgements**

I would like to thank Dr Prodromos Sidiropoulos, Dr George Bertsias and Pr Panayiotis Verginis who accepted me in their lab and provided me with the appropriate research guidance during this year. Special thanks to Dr Prodromos Sidiropoulos who gave me the chance to participate in the biobanking project of Rheumatology Clinic.

Also, I would like to express my gratefulness to my supervisor, Garyfalia Papadaki who trusted me and let me fully participate in her postdoctoral project and guided me with plenty of patience and understanding. Also, I want to say that I feel more than fortunate and happy to have worked with Panayiota Goutakoli and Elpida Neofotistou. Elpida and Giota have been much more than lab partners through this challenging period, they have been my friends.

Moreover, I want to express my gratitude to the rest of the lab members that guided me and provided me with helpful suggestions. Many thanks to Spyros Georgakis, Despoina Kosmara, Dimitra Nikoleri and Panayiotis Sidiropoulos.

Finally, I would also like to express my gratitude to the hospital personnel of Blood Donation Department for their help and also Dr Augoustidis and Lena from Rheumatology Clinic for their help with RA samples.

## TABLE OF CONTENTS

## ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease that involves multiple immune cell types and biological processes. Complexity of RA in combination with inadequate treatment responses and frequent patient relapses, underscore the necessity to delineate the molecular mechanisms underlying the disease.

Neutrophils are the most abundant white blood cell type and the first cells that reach the inflamed synovium. Besides releasing several inflammatory cytokines, neutrophils also externalize chromatin fibers called extracellular traps. RA patients present increased spontaneous formation of NETs, which indicates an important role in pathogenesis of the disease. Moreover, our previous work revealed that RA-derived NETs show an increased potential to induce the maturation of dendritic cells (DCs) from healthy individuals. Mechanistically, we showed that exposure of DCs to Collagen induced Arthritis (CIA)-derived NETs induced DC maturation characterized by significant up-regulation of CD80 and CD86 costimulatory molecules as well as elevated secretion of the inflammatory cytokine IL-6. In agreement, CIA-NET-treated DCs promoted the induction of antigen-specific Th1 cells *in vitro*. In the present study, our main purpose was to delineate the mechanisms implicated in NET-induced activation of DCs. For this purpose, we focused on monocytes as they represent the progenitor cells of monocyte derived dendritic cells (moDCs) and exhibit similar properties with DCs.

To address our hypothesis, first, we provoked NET formation with IgG antibodies isolated from sera of active RA patients and observed increased NET release on healthy neutrophils as compared with neutrophils treated with IgG isolated from healthy volunteers. Next, we focused on the effect of RA IgG-induced NETs on the activation of monocytes. We measured the secreted protein levels of cytokines in monocytes treated with NETs and observed increased expression of Tumor Necrosis Factor alpha (TNF-α) indicative of monocytes activation.

According to recent evidence, induction of autophagy is highly linked with the activation of monocytes. For that reason, we focused on the autophagic machinery of monocytes. Interestingly, monocytes treated with RA IgG-induced NETs showed decreased expression of the autophagic marker LC3 and increased expression of p62 in confocal microscopy. In Western blotting, p62 levels also tend to increase. The above evidence suggests impaired autophagy in monocytes treated with RA IgG NETs.

Next, we sought to determine the intracellular signaling events mediating the NET-dependent downregulation of monocyte autophagic machinery. To address this, we performed flow cytometry on monocytes treated with NETs for the phosphorylated intracellular markers of the mTOR pathway, pmTOR and pAkt and the downstream molecules of the mTORC1 pathway, pS6 and p4EBP1 and observed increased expression of the PI3K/Akt/mTOR axis.

To extend our findings *in vivo*, we used the collagen induced arthritis mouse model (CIA). CIA bone marrow neutrophils (PMNs) present increased spontaneous NET formation as compared with control BM PMNs. Treatment of bone marrow derived dendritic cells (BMDCs) with CIA NETs revealed increased antigen presenting capacity as compared with the control. Collectively, our findings indicate that RA IgG induced NETs may promote monocyte activation through decreased autophagy and increased PI3K/Akt/mTOR signaling pathway.

# ΠΕΡΙΛΗΨΗ

Η ρευματοειδής αρθρίτιδα (PA) είναι μια χρόνια αυτοάνοση ασθένεια στην οποία εμπλέκονται πολλαπλοί κυτταρικοί τύποι και μονοπάτια. Η πολυπλοκότητα της ασθένειας σε συνδυασμό με την μη επιτυχή θεραπεία και τις υποτροπές των ασθενών επιδεικνύουν την ανάγκη για διερεύνηση των μοριακών μηχανισμών που διέπουν την ασθένεια.

Τα ουδετερόφιλα αποτελούν τον πολυπληθέστερο τύπο λευκών αιμοσφαιρίων και είναι η πρώτη ομάδα κυττάρων που φτάνουν στην άρθρωση. Εκτός από την απελευθέρωση αρκετών φλεγμονωδών κυτταροκινών, τα ουδετερόφιλα απελευθερώνουν επίσης ίνες χρωματίνης που ονομάζονται εξωκυτταρικές παγίδες (NETs). Μελέτες σε ασθενείς με PA έδειξαν αυξημένη αυτόματη απελευθέρωση NETs, γεγονός που υποδηλώνει το σημαντικό τους ρόλο στην παθογένεια της νόσου. Επιπλέον, προηγούμενη μελέτη του εργαστηρίου μας έδειξε ότι τα NETs που προέρχονται από PA ασθενείς παρουσίασαν αυξημένη δυνατότητα να προκαλέσουν ωρίμανση δενδριτικών κυττάρων (DCs) υγιών ατόμων. Μηχανιστικά, δείξαμε ότι η έκθεση των DCs σε NETs απομονωμένα από CIA προκάλεσε ωρίμανση των DCs η οποία χαρακτηρίστηκε από σημαντική αύξηση των CD80 και CD86 συνδιεγερτικών μορίων καθώς και αυξημένη έκκριση της φλεγμονώδους κυτταροκίνης IL-6. Ακόμη, τα DCs που ενεργοποιήθηκαν με CIA- NETs προκάλεσαν την ενεργοποίηση αντιγονο-ειδικών Th1 κυττάρων *in vitro*. Στην παρούσα μελέτη, ο κύριος στόχος μας ήταν να περιγράψουμε τους μηχανισμούς που εμπλέκονται στην ενεργοποίηση των DCs από τα NETs. Για το σκοπό αυτό, εστιάσαμε στα μονοκύτταρα καθώς θεωρούνται προγονικά κύτταρα των DCs που προέρχονται από μονοκύτταρα (moDCs) και παρουσιάζουν παρόμοιες ιδιότητες με τα πρώτα.

Για να επιβεβαιώσουμε την υπόθεση μας, πρώτα, προκαλέσαμε απελευθέρωση των NETs ενεργοποιώντας υγιή ουδετερόφιλα με IgG αντισώματα που απομονώθηκαν από ορούς ενεργών ασθενών με PA και παρατηρήσαμε αυξημένη απελευθέρωση NETs στα ουδετερόφιλα που ενεργοποιήθηκαν με IgG αντισώματα απομονωμένα από ασθενείς σε σχέση με εκείνα που απομονώθηκαν από υγιείς εθελοντές. Στη συνέχεια της μελέτης μας, εστιάσαμε στην επίδραση των NETs στην ενεργοποίηση των μονοκυττάρων. Όταν μετρήσαμε τα επίπεδα πρωτεΐνης των κυτταροκινών σε μονοκύτταρα που καλλιεργήθηκαν με NETs και παρατηρήσαμε αυξημένη έκφραση της προφλεγμονώδους κυτταροκίνης (TNF-α), ενδεικτική της ενεργοποίησης των μονοκυττάρων.

Σύμφωνα με πρόσφατες δημοσιεύσεις, η επαγωγή της αυτοφαγίας φαίνεται να συνδέεται με την ενεργοποίηση των μονοκυττάρων. Γι' αυτόν τον λόγο, εστιάσαμε στο μηχανισμό αυτοφαγίας των μονοκυττάρων. Τα μονοκύτταρα που καλλιεργήθηκαν με NETs, παρουσίασαν μειωμένη έκφραση του αυτοφαγικού δείκτη LC3 και αυξημένη έκφραση της p62 στη συνεστιακή μικροσκοπία. Επίσης, σύμφωνα με τη μέθοδο Western Blotting, τα επίπεδα της p62 έδειξαν ήπια αύξηση. Τα παραπάνω δεδομένα υποδηλώνουν μειωμένη αυτοφαγία.

Στη συνέχεια, προσπαθήσαμε να προσδιορίσουμε τα ενδοκυττάρια σήματα που ευθύνονται για την μείωση της αυτοφαγίας σε μονοκύτταρα που καλλιεργήθηκαν με NETs. Για να απαντήσουμε σε αυτό το ερώτημα, χρησιμοποιήσαμε τη μέθοδο της κυτταρομετρίας ροής για τους φωσφορυλιωμένους δείκτες του mTOR μονοπατιού, pmTOR και pAkt και τα κάτωθι μόρια του mTORC1 μονοπατιού pS6 και p4EBP1 σε μονοκύτταρα που καλλιεργήθηκαν με NETs και παρατηρήσαμε αυξημένη έκφραση του PI3K / Akt / mTOR άξονα.

# INTRODUCTION

## Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease with a prevalence of 1% of the population varying among regions and genders. More specifically, the female to male ratio is higher than 2, rising in the elderly[2]. Main characteristic of the disease is persistent inflammation of the synovial joints causing articular cartilage damage, resulting in stiffness, swelling, pain and loss of mobility. Moreover, increased cardiovascular disease risk has been reported for people diagnosed with autoimmune diseases such as RA and systemic lupus erythromatosus (SLE) [3]. Early diagnosis of the disease is considered as a determining factor for the improvement of life quality of the patients and can be accomplished with descriptive review of the family history and detection of pathogenic autoantibodies against citrullinated proteins in their sera.

The majority of RA patients develop autoantibodies against various factors, including rheumatoid factor (RF), detected approximately in 80% of the patients and anti-citrullinated peptide antibodies (ACPAs), present in 50-70% of the patients [4]. Recently, researchers have characterized the production of autoantibodies to carbamylated proteins (anti-CarP) as another detrimental factor in the prognosis of erosive RA. Genetic risk factors that have been associated with RA, predominately implicate the HLA-DRB1 locus of the major histocompatibility complex (MHC) and some individual genetic loci like PTPN22 and CTLA4, while the main treatments that are used upon RA patients are disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, Biologics like Abatacept and Adalimumab and Janus associated Kinase inhibitors like Tofacitinib [5-7].

## Pathogenesis of the disease

Rheumatoid arthritis, such as other autoimmune diseases are characterized by the presence of auto-antibodies, antibodies against proteins that are not recognized as self-antigens. In rheumatoid arthritis, this process commences with the citrullination of type II collagen and vimentin. Post-translational modification of these proteins in combination with the presence of susceptibility genes, HLA-DR1 and HLA-DR4 are responsible for the activation of CD4 T-helper cells and B -cells and the production of autoantibodies. Activated T cells and antibodies produced by B cells reach the joints and secrete cytokines such as Interferon-gamma (INF-γ) and Interleukin-17 (IL-17) to recruit macrophages in the joint space. Macrophages that release tumor necrosis factor alpha (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6), provoke the proliferation of synovial cells. Consequently, increased levels of immune cells in combination with proliferation of synovial cells leads to the formation of the Pannus, a thick, swollen synovial membrane that consists of fibroblasts, myofibroblasts and inflammatory cells. Pannus formation is responsible for cartilage damage and bone erosion since

activated synovial cells produce Proteases, proteolytic enzymes that break down the cartilage [8-10]. On the other hand, inflammatory cytokines activate T cells to express RANKL that binds RANK to activate osteoclasts, monocyte derived multinucleated cells, responsible for bone erosion [8]. Additionally, the presence of Rheumatoid Factor (RF) and anti- Citrullinated cyclic protein anti-CCP in the joint space, promotes the activation of the Complement System, an immune complex of the innate immune system that consist of 9 proteins. Persistent inflammation of the joint leads to angiogenesis and the arrival of more inflammatory cells in the problematic site. Chronic production of inflammatory cytokines may cause several issues in the organism such as atheromatic plaque formation [11].

**Innate immune system**

Innate immune system was initially considered to be the first line of defense against invading microbial pathogens. After a long time of controversy, innate immunity was also found to contribute to the pathogenesis of autoimmune and inflammatory diseases. It consists of four elements: epithelial cells, immune cells like neutrophils, monocytes, macrophages, dendritic cells, natural killers and more, protein groups like the complement system and cytokines. The complement system, an essential mediator of inflammation, is composed of a family of proteins that cooperate in order to opsonize pathogens or dying cells, enhance phagocytosis and recruit effector cells to the inflammation site. Monocyte progenitor cells play a key role in the innate immune system since they have the ability to differentiate to macrophages, osteoclasts and dendritic cells. Macrophages, together with B cells and DCs perform antigen presentation to T cells and trigger adaptive immune responses. DCs that are activated by Toll-like receptor (TLR) signaling produce cytokines or express critical co-stimulatory molecules, CD80 and CD86, that interact with CD28 on T cells. In RA, the constant/successive expression of macrophage-derived cytokines such as tumor necrosis factor a (TNF-a), interleukin-1 (IL-1) and interleukin-6 (IL-6) produced by monocytes, macrophages and DCs, indicates persistent activation of the innate immune system. Moreover, the solid presence of these population in the inflammatory milieu such as the synovium, describe the role of innate immune system in the progression of the disease [12].

Neutrophils

Neutrophils represent the first line of defense in response to invading microorganisms as the most abundant type of white blood cells reaching approximately 60% of the white blood cell count. They also represent the majority of leukocytes in inflamed joints and they are the first cells that reach the synovium. Activated neutrophils have been shown to secrete immunologic factors such as

interleukins (IL-1, IL-6, IL-12), cytokines like transforming growth factor B (TGF-β), tumor necrosis factor alpha (TNF-α) and other molecules that result in acute and persistent inflammation. Thus, they have been firmly connected to more than one autoimmune disease including SLE and psoriasis [13].

Neutrophils are extensively studied for their ability to release Extracellular Traps (NETs), networks consisting of chromatin fibers and antimicrobial proteins. Except for their capability to trap bacteria, NETs have also been shown to trigger autoimmunity via cytokine release and differential cell activation. Peripheral blood neutrophils from RA patients show greater capacity in NET formation than neutrophils isolated from healthy donors and enhanced NETosis has been also linked with the presence of ACPAs and RF in RA sera and the production of IL-17A and TNF cytokines. Moreover, neutrophils express protein arginine deiminases (PAD4), an enzyme that catalyzes the hydrolysis of peptidyl-arginine to citrulline, a post-translational modification on histones, enolase, vimentin, filaggrin and other proteins. These citrullinated proteins exposed in NETs can be utilized as autoantigens, recognized by ACPAs [14-16].

### Neutrophils in RA

Neutrophils are the most abundant cell type located in the synovial fluid that accumulate in the arthritic joints. In animal studies of autoantibody-induced arthritis, neutrophils have been reported to migrate to affected areas early in the disease progression where they perform inflammatory responses. Similar findings have been proposed in CIA model studies. Additionally, RA synovial neutrophils secrete various cytokines and chemokines including TNF ligand superfamily member II (RANKL) and TNFSF13 or BAFF that activate osteoclasts and B cells. Overall, strong presence of neutrophils in the inflamed sites suggests a remarkable role in disease progression, however, their role in disease initiation remains elusive [14, 15].

### Dendritic cells

In RA pathogenesis, dendritic cells (DCs) seem to significantly contribute to the initiation of joint inflammation. They express high levels of class I and II MHC molecules and act as antigen-presenting cells, inducing T cell differentiation. According to clinical research, RA patients present decreased number of DCs circulating in the periphery and increased number of DCs in the inflammatory sites. Myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are the two more prominent subpopulation of dendritic cells located in the synovial fluid. However, mDCs are more efficient in antigen presentation than pDCs. Both subpopulations contribute to disease pathogenesis and progression by secreting various cytokines including TNF, IL1, IL-12, IL-6, Interferons (IFNs) as well as differentiation factors, including macrophage colony stimulating factor (MCSF) and fibroblast growth factor (FGF).

Monocyte-derived DCs (moDCs) are an acceptable and widely used DCs model. Peripheral blood monocytes are isolated with CD14+ beads and cells are differentiated in vitro into DCs when cultured with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 [17].

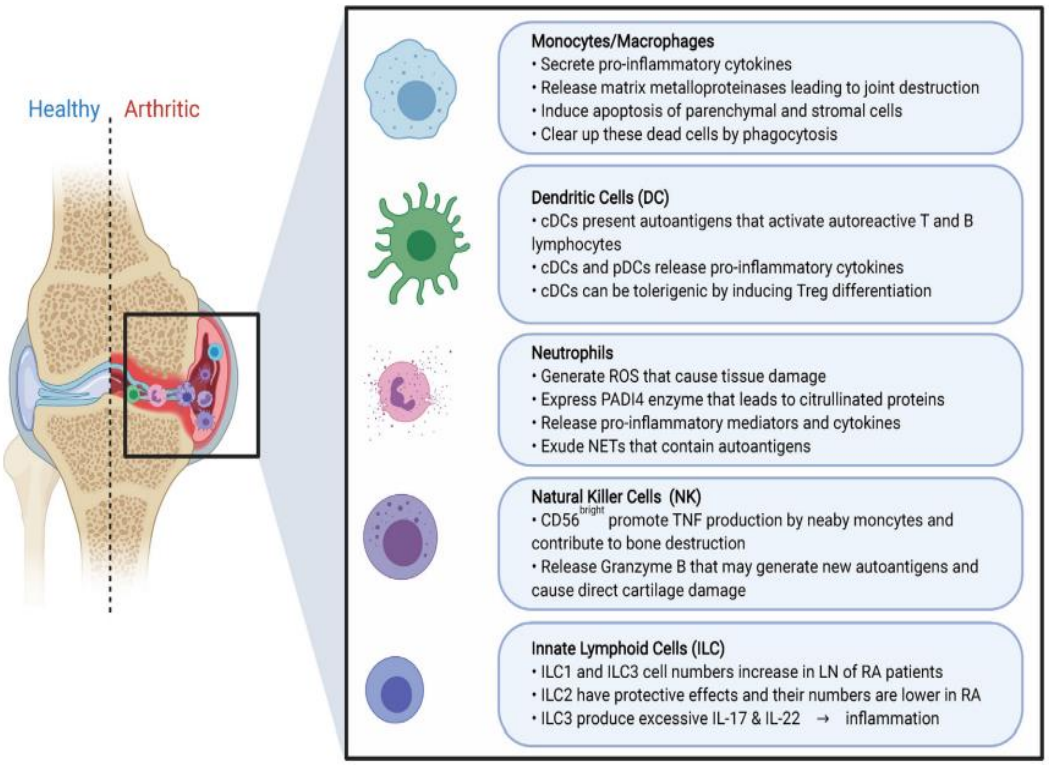


*Image 1: Ali A. Abdul-Sater, Review in Biomedical Journal, 2020*

*Contribution of various innate immune cells to RA pathogenesis. Monocytes, macrophages, dendritic cells, neutrophils, natural killer cells and innate lymphoid cells play a key role in the early stages as well as in the progression of rheumatoid arthritis.*

## Cytokines implicated in RA pathogenesis

### TNF alpha

Cytokines such as TNF-a that are highly detected in the serum and the synovial fluid of RA patients as compared to control groups, seem to regulate multiple inflammatory processes related to the pathogenesis of the disease and have been linked to the promotion of osteoclastogenesis and bone erosion. TNF is a proinflammatory cytokine that is mostly secreted by monocytes, macrophages, T cells, NK cells, mast cells and neutrophils and binds to TNFR1 (p55) and TNFR2 (p75), two functionally distinct receptors. TNF that is secreted in the inflammatory site by synovial macrophages and lymphocytes infiltrating the synovium, induces the proliferation of fibroblast like synoviocytes (FLS) and mediates joint destruction by

stimulation of matrix degrading enzyme production (MMPs). Anti-TNF therapies like Infliximab, Adalibumab and Etanercept have been widely used for the treatment of RA patients particularly, for those that disease-modifying antirheumatic drugs (DMARDs) have failed [18].

### Interleukin-6

Interleukin-6 has been proven to act as both proinflammatory and anti-inflammatory cytokine. In general, IL-6 expression participates in host defense against infectious agents and tissue injuries inducing immunological and hematopoietic responses. This cytokine is produced by macrophages, neutrophils, T cells, B cells and other cells. Nevertheless, uncontrolled and excessive expression of this cytokine might contribute to the development of various immune-mediated diseases. In a study that enrolled. RA patients, IL-6 was detected in elevated levels in their serum and synovial fluid [19]. Additionally, IL-6 levels are correlated with the activity of the disease, since it can mediate chronic inflammation interacting with macrophages, neutrophils and T cells, support persistent activation of autoimmunity through B-cell maturation and Th-17 differentiation and promote synovitis and joint destruction by stimulating neutrophil migration and osteoclast maturation. In general, decreased levels of IL-6 are used as a prognostic marker for better clinical outcome. Treatment with DMARDs or TNF inhibitors reduced IL-6 levels in patients' sera [18].

## Autophagy

Autophagy is an important mechanism responsible for the degradation of cytoplasmic components. Except from providing additional energy, autophagy is necessary for the degradation of intracellular pathogens, damaged organelles and protein aggregates. The steps of autophagy are initiation, elongation, autophagosome formation and fusion of the autophagosome with the lysosome for the formation of autolysosome. Protein aggregates and pathogens will be degraded in the lysosome. However, the most significant step of autophagy is the autophagosome formation through the coupling of microtubule-associated protein light chain 3 (LC3) with phosphatidylethanolamine. LC3 consists of a soluble form (LC3-I, 18kDa) and a lipidated form (LC3-II, 16kDa). The LC3-binding adaptor, sequestosome 1 or p62, binds ubiquitinated substrates and carries them to autophagosome for their degradation. Decreased p62 levels have been related with autophagy activation. Overall, dysfunctional autophagy has been connected in many scientific reports with various autoimmune diseases including Rheumatoid Arthritis [1, 20, 21].

### PI3K/Akt/mTOR pathway

The mammalian target of rapamycin (mTOR), is a phosphatidylinositol 3-kinase-related kinase that can be found in two structurally different complexes, mTORC1 and mTORC2. In general terms, mTOR acts upstream of various transcription factors

and controls pathways linked with protein synthesis, degradation and cellular growth. Phosphorylation of mTOR is accomplished by the serine/threonine protein kinase Akt or Protein Kinase B that is involved in various cellular survival pathways. mTORC1 is also responsible for the phosphorylation of p70 ribosomal kinase (S6) and 4E-binding protein 1 (4EBP1), key regulators of mRNA translation that are frequently upregulated in breast cancer [22]. Autophagy, has been tightly connected with the mTORC1 complex since inhibition of mTORC1 induces autophagy by controlling the activity of the ULK, a protein kinase complex that is essential for autophagosome formation. More specifically, the transcription factor EB (TFEB) has been reported to drive expression of autophagic and lysosomal genes in a MTORC-1 depended manner [23].

**Treatments**

Early treatment of Rheumatoid Arthritis is critical in order to control the progression of the disease and prevent cartilage and bone erosion and joint destruction. Nowadays, several classes of drugs with distinct models of action have been developed. The main treatments that are used for RA are DMARDs such as methotrexate, Biologics like Abatacept and Adalimumab and Janus associated Kinase inhibitors like Tofacitinib. Methotrexate is targeting adenosine signaling, in order to downregulate the production of TNF and NF-kB and suppress the activation of T-lymphocytes. Abatacept is a fusion protein consisting of the extracellular domain of human cytotoxic T lymphocyte-associated antigen 4 (CTL4) and a genetically modified fragment of the Fc region of IgG1. This drug specifically blocks the CD80/CD86 binding on T cells in order to inhibit T cell activation and restrain immune responses. On the other hand, Adalimumab (ADA) is a recombinant human IgG1 monoclonal antibody that binds to both soluble and membrane form of TNF-a with high affinity and suppress their signaling. Tofacitinib is an inhibitor of JAK 1 and, effective in moderating severe RA as monotherapy or in combination with MTX. However, multiple side effects and increased risk of thrombosis and pulmonary embolism or incomplete effectiveness of these drugs may lead to the disruption of the treatment. To this end, it is of high importance the delineation of the mechanisms implicated in RA pathogenesis for therapeutic intervention in a personalized level. [6, 24-26].

**Collagen induced Arthritis**

Collagen induced arthritis represents the most usual in vivo model for the study of RA since it shares various similarities with human RA. The main common characteristics are generation of autoantibodies against self and collagen and tolerance rupture. Since CIA model was introduced initially in rats, many mouse strains that carry MHC Class II I-Aq haplotypes, have been reported to be susceptible in CIA induction presenting variable degree of susceptibility. (In RA patients, HLA-DR1 and HLA-DR4 serotypes have been linked with disease pathogenesis.) Moreover, CIA development is related with both B- and T- lymphocyte responses including the production of anti-collagen type II antibodies and collagen-specific T cells. DBA/1 mice are more widely used in the *in vivo* studies of arthritis in mice [27]. Approximately, 21-25 days after initial inoculation, mice present several symptoms such as cartilage and bone erosion and synovial hyperplasia. The peak of the disease inflammation and symptom severity is visible up today.

## AIM OF THE STUDY

To delineate the mechanism of NET induced monocyte activation. Specifically, to study the autophagic pathway of monocytes upon NET treatment.

## OBJECTIVES

To explore the autophagic machinery of net induced monocyte activation in human and in Collagen induced arthritis CIA mouse model

# MATERIALS AND METHODS

## Human monocyte isolation from peripheral blood

Heparinized blood (20ml) was collected from healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated on Lymposep cell separation medium (Biowest) density gradient. Briefly, peripheral blood from healthy donors was diluted 1:1 with phosphate-buffered saline (PBS). and overlayed gently onto Lymposep cell separation medium 2:1. Density-gradient centrifugation was performed at 1800 rpm for 30 minutes, at room temperature (RT), without brake to obtain PBMC layer. The intermediate white phase that contains the PBMC fractions was collected with a poire. Cells were washed using sterile PBS and erythrocytes were eliminated according to hypotonic lysis method. Lysis was performed by resuspending the cells in water for injection for 30-60 seconds and erythrolysis was blacked with NaCl 1,8%. For each wash sterile PBS was used. Cell viability was validated with Trypan Blue dye. Monocytes were magnetically isolated with positive selection using CD14 MicroBeads (Miltenyi Biotec) according to MACS cell separation protocol.

## Isolation of PMNs

Human peripheral blood (PB) PMNs were isolated from heparinized blood using density gradient separation according to the protocol [28]. Briefly, a double gradient was formed by layering an equal volume of Lymphosep (Biowest) over histopaque-1119 (Sigma). Venous blood was collected in heparinized tubes and carefully layered onto the upper Lymphosep. The blood was centrifuged at 1950rpm for 30 min, without brake. Ganulocytes were collected from the bottom interphase. The cells were washed with PBS and erythrocytes were eliminated by hypotonic lysis (0.5ml $ddH_2O$ for 35s and 0.5ml 1.8% NaCl). Viability was measured 99% by trypan blue dye exclusion.

## Formation of Neutophil extracellular traps (NETs)

Isolated neutrophils were plated in 12-well plates ($1,5*10^6$ cells/ well) and cultured in RPMI 1640 without phenol (Pan Biotech), 1% Fetal Bovine Serum (FBS), 1% penicillin/ strepdavidin. Neutrophils treated/activated with IgGs isolated from healthy or RA sera for 3 hours at 37$^o$C, 5% $CO_2$, in order to achieve NET formation. For the isolation of NETs, 300μl out of 400μl medium were discarded and renewed. The plate was placed on a shaker for 3 min, 800 rpm to dislodge NETs. The total content of the wells was transferred into 1,5ml eppendorfs and centrifuged for 5min, 4$^o$C, at 120g. The supernatants, that contain the NETs, were kept in -80$^o$C until further use.

### Isolation and purification of healthy and RA IgGs

Isolation of IgGs from healthy and RA sera was performed according to Melon Gel IgG Spin Purification Kit instructions (Thermo Scientific). Purification Buffer from Melon Gel purification kit was removed with Amicon Ultra Centrifugal Filter Devices (Millipore).

### NET quantification with Sytox green

Neutrophils were cultured in RPMI without phenol red. Isolated NETs were applied in 96-well dark plate. Sytox Green nucleic acid stain (Thermofisher) was used in 0.2μM concentration. Fluorescence (excitation 485 nm, emission 520 nm) was measured in a fluorescence plate reader and results were reported as DNA fluorescence (RFU). Bacterial phage lamda DNA in 0 to 1000ng/ml concentrations was used as a control.

## PRIMARY CELL CULTURE

### Monitoring NET-mediated monocyte activation using flow cytometry

$2{,}5*10^5$ magnetically isolated healthy monocytes were cultured in flat-bottom 96-well and cultured in RPMI-1640 supplemented with 10% FBS, 100 IU/mL penicillin and 100μg/mL streptomycin and kept at 37°C and 5% $CO_2$. Monocytes were treated with different concentrations (500 ng/ml, 200ng/ml, 150 ng/ml) of isolated NETs for 18 hours. Lipopolysaccharide (LPS) was used as a positive control of monocyte activation. Culture supernatants were collected and stored at -80ºC for measuring cytokine secretion using ELISA, while monocytes were detached with cold PBS. Cells were centrifuged at 400g for 10 min and prepared for flow cytometry.

**Flow cytometry**

Human single-cell suspensions were prepared and cells were stained for surface markers for 20 min at 4°C in 5% FBS/PBS. Fluorochrome- conjugated antibodies against human: CD14 (Biolegend) was used to identify monocyte population. anti-HLA-DR, anti-CD80, anti-CD86, anti-CD40 conjugated antibodies (Biolegend) were used as maturation-activation markers.

For intracellular phospho protein staining, cells were fixed with 1,5% PFA for 10 min and iced cold methanol for another 10 min. Monocytes we washed with 1% PBS/BSA buffer and stained with conjugated antibodies against human pmTOR, pAKT1, pS6 and p4EBP1 (Invitrogen). Samples were acquired on a FACS Calibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Mice BMDCs were prepare and stained for surface markers CD11c, CD80, CD86 and MHC II.

*Table 1 : Antibodies used for flow cytometry*

| Marker | Color | Reactivity | Company | Lot number |
|---|---|---|---|---|
| CD14 | FITC | Human | Biolegend | B268830 |
| CD40 | FITC | Human | BD Biosciences | 19533 |
| CD80 | APC | Human | Biolegend | B297923 |
| CD86 | PE | Human | Biolegend | B307923 |
| HLA-DR | PerCP | Human | Biolegend | B273764 |
| pmTOR | PE | human, mouse | Invitrogen | 12-9718-42 |
| pAkt | APC | human, mouse | Invitrogen | 17-9715-42 |
| pS6 | APC | human, mouse | Invitrogen | 17-9007-41 |
| p4EBP1 | PE | human, mouse | Invitrogen | 12-9107-41 |
| CD80 | PE | Mouse | BioLegend | 104707 |
| CD86 | PerCP | Mouse | BioLegend | 105025 |
| MHC II | FITC | Mouse | BD | 553551 |
| CD11c | APC | mouse | BioLegend | 127614 |

**Detection of cytokines**

Detection of human IL-6 and TNF-α levels derived from freshly isolated human monocytes was performed by sandwich ELISA, following the manufacturer's recommendations (Invitrogen). Light absorbance at 450 nm was measured using the ELx800 Biotek plate reader. All samples were assessed in duplicates.

**Immunofluorescence**

Magnetically isolated monocytes were seeded onto coverslips coated with 10% poly-L-Lysine and cultured at 37$^{o}$C, 5% $CO_2$. Cells were treated with the isolated NETs overnight (18h). Then, coverslips were washed with PBS and cells were fixed with 100% methanol for 5min in RT, blocked and permeabilized with blocking buffer (2% BSA, 0.1% Saponin) for 30min in RT. Primary anti-human antibodies for LC3 (host: mouse) and p62 (host: rabbit) were applied for 1h. Secondary antibodies anti-mouse 555 (for LC3) and anti-rabbit 488 (for p62) were also incubated for 1h. Three washes with 0.5% BSA/PBS were performed between all stainings. For visualization of the nuclei DAPI (Sigma-Aldrich) was used. After staining, coverslips were mounted with Mowiol and visualized using inverted confocal live cell imaging system Leica SP8. Intensity/cell was calculated using the Leica software. Puncta/cell were calculated using a macro developed in Fiji software.

**Western blot analysis**

$2*10^6$ magnetically isolated healthy monocytes were placed in 24-well plates and treated with NETs overnight. The next day, culture medium was discarded and cells were lysed with RIPA buffer after supplementation of protease and phosphatase inhibitors), for 15 minutes on ice. The mixture was transferred to 1,5ml Eppendorf tubes and centrifuged at 13.000g for 15 min at 4$^{o}$C. Lysates were collected and protein concentration was measured with Pierce BCA Protein Assay Kit – Thermo Fischer Scientific. 40 μg protein/ sample were subjected to SDS-PAGE electrophoresis on 12% gels and then transferred to a PVDF Immobilon-Psq membrane (Millipore). Membranes were blocked with 5% BSA in TBST and then incubated with anti-LC3 1:1000 (Novous Biologicals), anti-P62 (Abnova), and anti-actin (1:2000). Detection was performed using HRP-linked Abs and Immobilon Forte Western HRP substrate (Sigma Aldrich).

**CIA induction and scoring system**

For the induction of Collagen-induced Arthritis (CIA), C57BL/6 mice were injected intradermally at the base of the tail with 100 μl complete Freund's adjuvant (Sigma-Aldrich), containing 5mg/ml inactivated Mycobacterium tuberculosis (H37RA Difco) and 2mg/ml type II chicken collagen diluted in 10mM acetic acid. The first day of injections is counted as day 0 followed by a boost immunization on d21 with 100μl of incomplete Freud's adjuvant containing also type II chicken collagen. Animals were sacrificed on d35–45 by anesthesia with isoflurane and cervical dislocation. Before euthanasia, blood was collected by retro-orbital aspiration and the isolated plasma was stored at –80°C.

The pointing system of CIA scores 0 points if no visible clinical symptoms, 1 point per swollen toe, 5 points for involvement of knuckles (metatarsal/metacarpal), or 5 points for involvement of wrist (tarsal/carpal), giving a total score of 9 and 10 per paw for front and hind paws, respectively.

**Isolation of Bone Marrow PMNs from CIA mice**

Femur and tibia bone marrow was flushed with PBS-EDTA buffer using a 25G 5/8 needle. A 20G needle was used afterwards to separate bone marrow clumps. Cells were centrifuged for 10 min in 400g and the pellet was resuspended in 2 ml PBS-EDTA. The suspension was gently overlayed on a percoll density gradient of three layers, 75%, 67% and 52% percoll. The gradient was centrifuged for 30 min, 1000g, RT and the 75% 67% interface were collected. After one wash with PBS-EDTA +1%BSA, red blood cells were lysed with deionized water. Lysis was blocked with 1.8% NaCl and cells were washed again.

**Generation of Neutrophil extracellular traps from CIA mice**

Typically, $2*10^6$ bone marrow derived neutrophils were seeded on a six-well plate in 1.2 ml 1640 RPMI without phenol, 2% BSA and 10mM HEPES. After 4h culture, 1 ml of medium was discarder and 600 ul of fresh medium was added. The plate was shaked for 3 min, 800 rpm and total volume was centrifuged for 5min, 4$^o$C, 50g. The supernatant was collected and kept at -80$^o$C. NETs were quantified with Sytox assay. Bone marrow derived PMNs from healthy mice were used as control.

**Generation of BMDCs and DC-NETs coculture experiments**

Dendritic cells were generated from BM progenitors of C57BL/6 mice. Red blood cells were lysed with NH4Cl. On days 0, 3, 6, and 8, cultures were supplemented with fresh complete DMEM containing 20% X63Ag8 supernatant (derived from a murine granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting cell line. After 9 days of culture, nonadherent cells were harvested and purity was assessed based on CD11c expression by flow cytometry. DCs were subsequently cultured in the presence or absence of 10% of supernatants containing NETs derived from

collagen injected or healthy mice. Culture supernatants and cells were collected 18 h after stimulation cells were acquired after surface or intracellular staining on a FACS Calibur (BD Bio sciences).

## RESULTS

### Increased NET formation induced by RA IgGs versus HS IgGs.

In order to induce NET formation from healthy PMNs and study the mechanism of NET mediated monocyte activation, we isolated IgGs from RA patients and healthy volunteers. More specifically, pooled sera samples from healthy volunteers and RA active patients were processed with Melon gel IgG isolation kit. The purity of the immunoglobulins isolated by the Melon Gel IgG spin purification was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were either stained with Coomassie Blue (Figure 1A) in order to confirm successful isolation of the heavy and light chains. NET formation was provoked by treatment of healthy volunteer isolated PMNs with IgGs isolated from RA and healthy (HS) sera. Using Sytox green, a nucleic acid stain that binds DNA, we observed that IgGs isolated from RA active patients, induce higher number of released NETs as compared with HS IgGs. As a positive control we used phorbol myristate acetate (PMA) (Figure 1B).

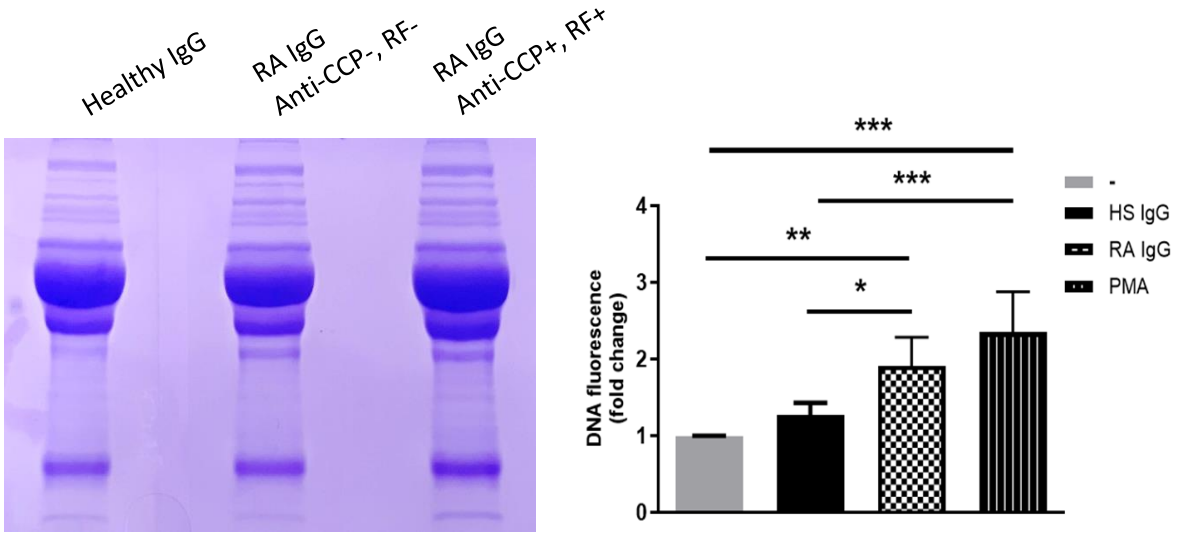


*Figure 1 : A) increased NET formation upon RA IgG treatment*

*Staining with Coomassie Blue of immunoglobulins isolated by the Melon Gel IgG spin purification and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4·5% stacking and a 10% running gel. Twenty µg of the immunoglobulin preparations were applied to each lane. B) fold change of DNA fluorescence quantified as NETs released by neutrophils upon treatment with RA IgG, HS IgG and PMA. Healthy neutrophils were isolated and cultured for three hours with 100µg/ml IgG isolated from RA serum, IgG isolated from healthy serum and PMA 50ng/ml as a positive control.*

**NETs activate monocytes**

Recent evidence suggest that NETs participate in antigen specific immune responses of T-cells through activation of DCs. Our basic aim was to delineate the molecular mechanism of dendritic cell activation by NETs. DCs demand several days of differentiation. For that reason, we utilized monocytes as a progenitor of DCs. To address the above question, NETs derived from healthy PB PMNs, activated with RA IgGs and HS IgGs, were cultured with healthy monocytes for 18 hours.

Assessment of TNF-α protein levels in RA IgG NETs-treated monocytes cultured supernatants demonstrated markedly increased secretion of TNF-α compared to supernatants collected from HS IgG NETs-treated monocytes. In addition, we measured IL-6 and IL-1β protein levels and we concluded that RA IgG NETs seem to increase IL-6 and Il-1β, in cultured supernatants of monocytes.

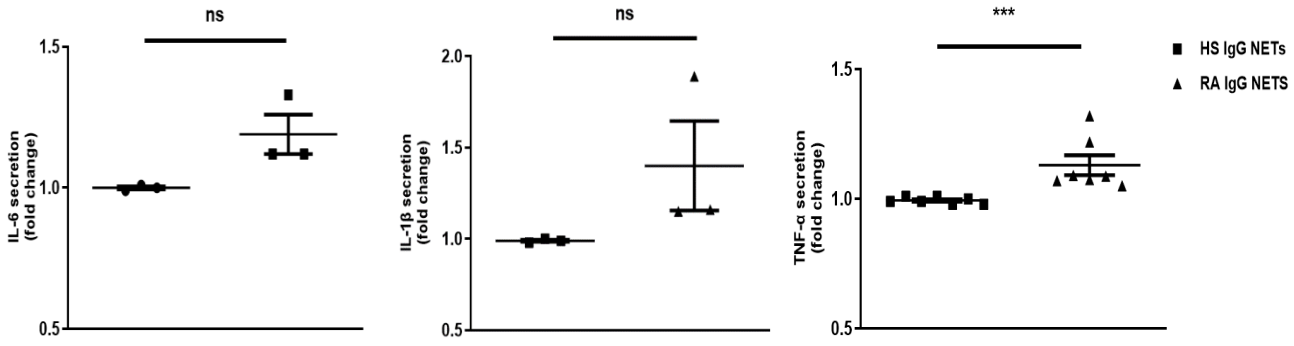


*Figure 2: NETs stimulate the production of pro-inflammatory cytokines released by primary monocytes.*

*IL-6, IL-1b and TNF-a measured in culture supernatants of monocytes treated for 18 hours with 100ng/ml RA IgG or HS IgG generated NETs. Results are expressed as fold change over the cytokines released by monocytes treated with HS IgG generated NETs.***P=0.0006 ; ns=non-significant.*

**Evidence for decreased autophagy after treatment of monocytes with NETs**

Accumulating data underline an essential role of autophagy during antigen presentation by APCs and priming of adaptive immune responses. In order to investigate whether NETs formed by RA IgG NETs affect the autophagic machinery of healthy monocytes, we assessed the expression of two autophagic markers, LC3 and p62. For this purpose, monocytes isolated from PB were cultured overnight with NETs. Our confocal microscopy data, demonstrated decreased levels of LC3 and increased levels of p62 suggest decreased autophagy and accumulation of p62 in these cells (Figure 3).

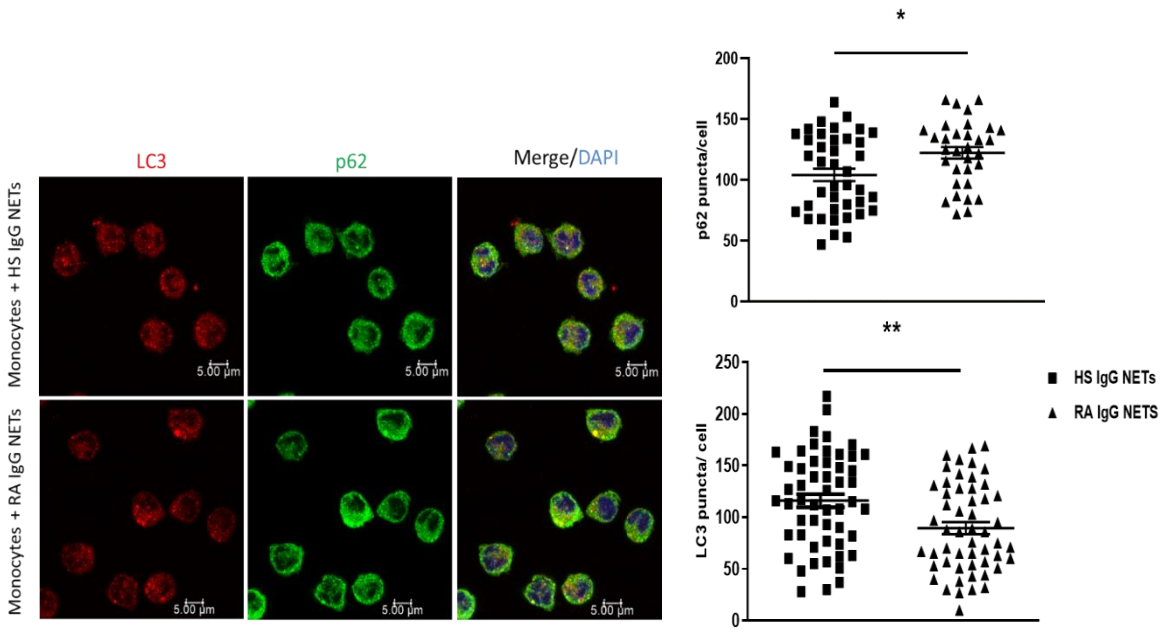


*Figure 3 Treatment of monocytes with NETs results to decreased expression of LC3 and accumulation of p62 according to confocal microscopy.*

*PB healthy monocytes were cultured overnight with 100ng/ml RA IgG NETs or HS IgG NETs. Cells were stained for LC3 (red), p62 (green) and DAPI (blue). Immuofluorescence confocal microscopy images were analyzed with Fiji and the numbers of LC3 puncta/cell and p62 puncta /cell were calculated using a macro developed in fiji software as described [1]**P=0.0038, *P=0.019.*

In order to confirm the data that emerge from our confocal microscopy experiments, we performed Western Blotting in monocytes treated with RA IgG NETs and HS IgG NETs for 18 hours. We found increased levels of p62 expression in monocytes treated with RA IgG NETs as compared to our HS control. However, we didn't manage to conclude any results for the LC3 –I and LC3-II protein levels.

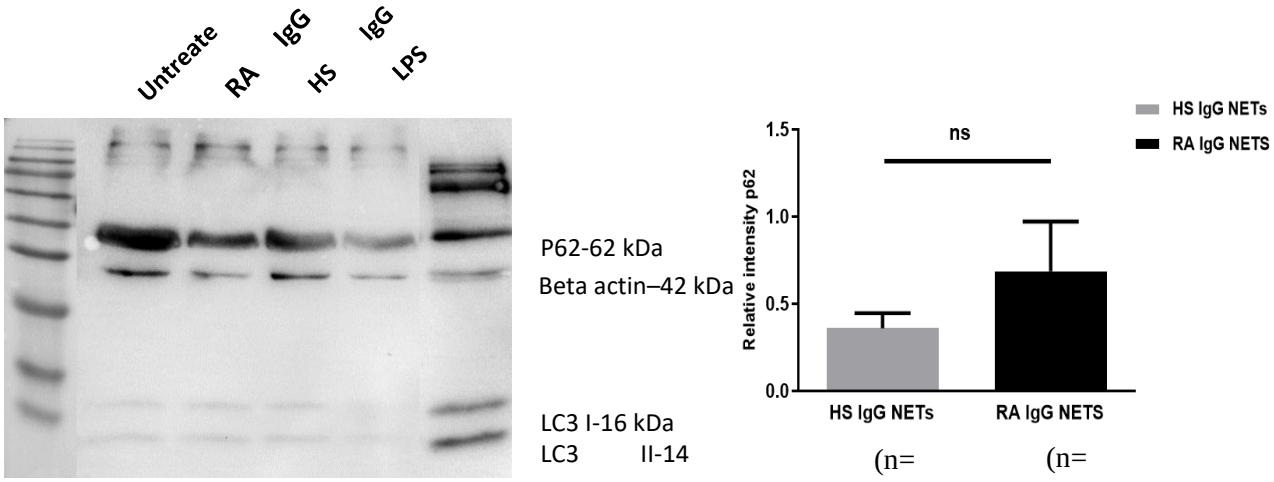


*Figure 4 : NETs stimulate increased expression of p62.*

*Healthy monocytes were cultured overnight with 100ng/ml RA IgG NETs and HS IgG NETs. Cells were lysed with RIPA buffer and western blot analysis for the expression of LC3I/LC3II and p62 in monocytes lysates of indicated conditions was performed. One representative experiment of 3 is depicted. Relative intensity of p62 is depicted.*

Next, we sought to determine the intracellular signaling events mediate the NET-dependent downregulation of monocyte autophagy. The kinase mTOR-dependent pathway is the best characterized regulator of autophagy, and activation of the PI3K/Akt/ axis is an upstream modulator of mTOR activity (ref). To this end, in order to assess the effect of NETs on this pathway, we treated monocytes with RA IgG NETs and HS IgG NETs for four hours and performed intracellular staining for the expression of phosphorylated levels of mTOR and Akt using flow cytometry.

Our data claim that pmTOR and pAkt are upregulated in monocytes upon treatment with RA IgG NETs compared with HS IgG NETs treated monocytes. Overall, we suggest that RA IgG generated NETs affect the PI3K/Akt/mTOR signaling pathway of monocytes therefore decreasing their autophagic machinery.

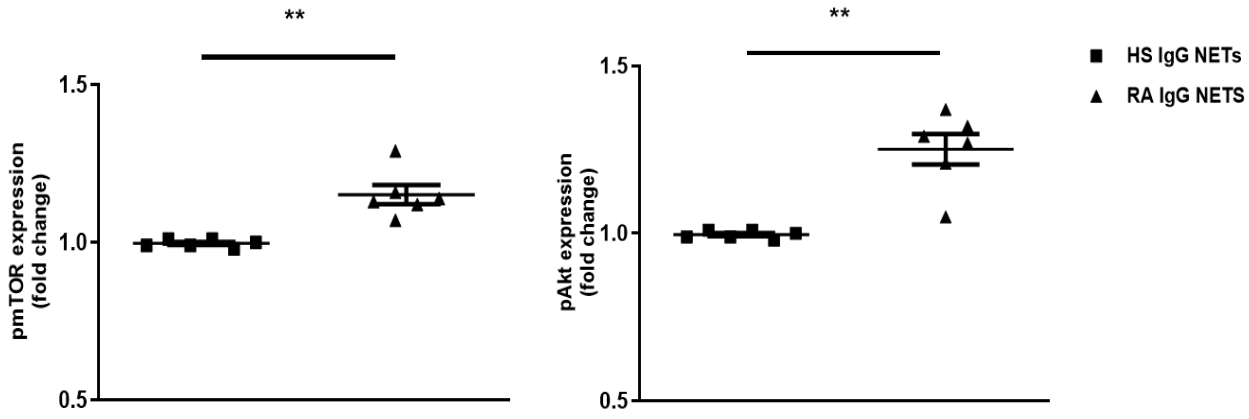


*Figure 5 : NETs impair monocyte autophagy via modulation of the PI3K/ Akt/mTOR axis*

*fold change expression of phosphorylated autophagy markers mTOR and Akt upon treatment with RA IgG based on the levels of phosphorylated levels of monocytes treated withHS IgG generated NETs. Monocytes were treated for 4 hours with RA IgG NETs and HS IgG NETs and stained intracellularly against the corresponding antibodies using flow cytometry. **P=0.0022*

Moreover, activation of mTOR leads to phosphorylation of the ribosomal protein S6 and phosphorylation of the eukaryotic initiation factor 4E-binding protein (4EBP1). Monocytes treated with RA IgG induced NETs, compared with HS IgG induced NETs, provide evidence for an increased phosphorylation of S6 and 4EBP1, confirming the activation of the mTORC1 signaling pathway in NET exposed monocytes.

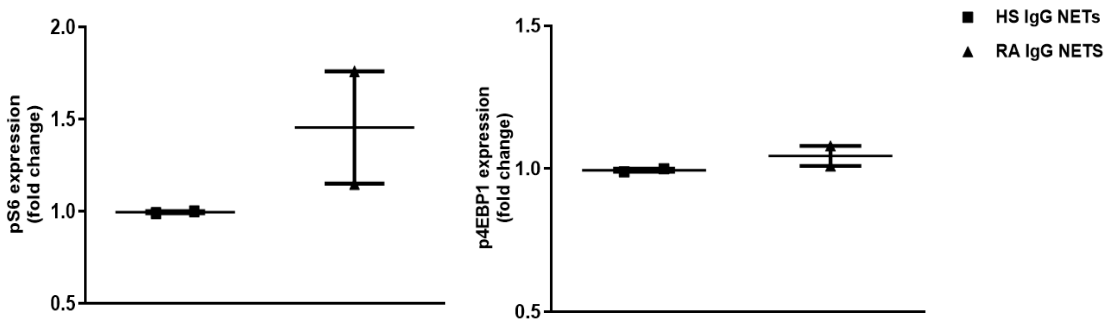


*Figure 6 :NETs may affect the downstream molecules of mTOR pathway, pS6 and p4EBP1*

*fold change expression of phosphorylated S6 and 4EBP1 proteins upon treatment with RA IgG or HS IgG generated NETs. Monocytes were treated for 4 hours with RA IgG NETs and HS IgG NETs and stained intracellularly against the corresponding proteins using flow cytometry.*

**The effect of Neutrophil Extracellular Traps on Bone Marrow derived Dendritic Cells**

Recent findings suggest that NETosis also occurs in CIA mouse model. In order to assess this, we isolated bone marrow PMNs from CIA diseased mice (d35-45 post collagen injection) using Percoll gradient method. According to Sytox measurement method, PMNs isolated from CIA diseased mice presented higher number of spontaneously released NETs as compared with PMNs NETs isolated from naïve uninjected mice.

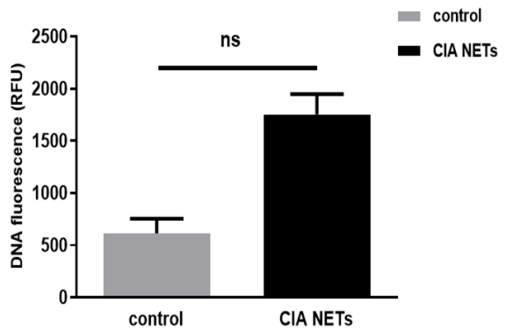


*Figure 7 : CIA mice present enhanced spontaneous NET formation.*

*PMNs isolated from BM of CIA mice were cultured for four hours without triggers. NETs were collected and measured with Sytox. As a negative control we used NETs isolated from naïve mice.*

Next, we asked whether these DNA structures affect the immune responses of BM derived DCs. To address this question, we isolated total BM cells from naïve mice and used the GM-CSF differentiation protocol in order to differentiate them into Dendritic cells within 8 days. In order to understand the effect of CIA formed NETs on naïve BM DCs, we treated these cells with NETs for 18 hours and assessed the expression of co-stimulatory molecules and activation markers.

We conclude that DCs (CD11c positive cells) treated with CIA NETs have a trend for increased expression of CD80, CD86 and MHC II. Collectively, induction of arthritis in CIA mouse model, leads to increased NET formation and these NETs have the ability to activate BM DCs.

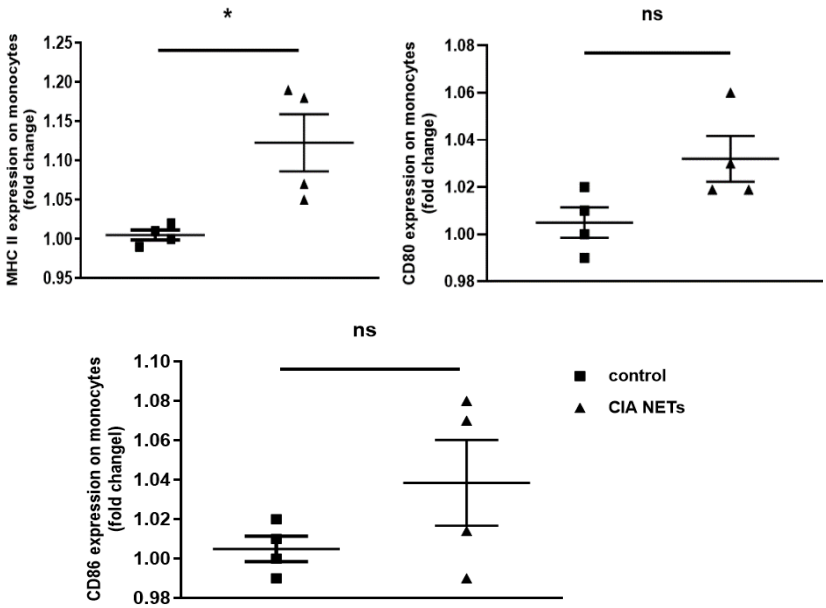


*Figure 8: BM DCs treated with CIA NETs show a trend for increased activation.*

*Fold change expression of phosphorylated expression of MHC II, CD80, CD86 and surface markers in BM derived DCs upon 18hour treatment with CIA or control NETs *P=0.028.*

## DISCUSSION

It is known that NETs have a significant impact on chronic autoimmune inflammatory diseases such as lupus, psoriasis, atherosclerosis and arthritis [15, 16, 29, 30]. In support, previous work from our lab revealed collagen-induced arthritis (CIA)-NETs favors the induction of Th1 immune responses through DC activation. In human, there is increased NET formation in RA patients and the RA inflammatory milieu favors the production of NETs. Moreover, RA-NETs also augments the inflammatory properties of moDCs. Our previous findings provide new insights into the pathogenic role of NETs in RA.[16] Herein we observed that RA autoAbs (RA isolated IgGs) potently induce NETosis. In support, other autoAbs (ANCA for SVV and anti RNP for SLE) have been reported to induce NETs [15] [31, 32].

The intracellular cascade that operates in dendritic cells and leads to their activation induced by NETs is not known. Of interest, induction of autophagy facilitates the delivery of antigenic peptides to the MHC class II–loading compartment and subsequent presentation to CD4+ T cells [23, 33-36]. In this study we focused on monocytes as they consist the progenitor cells of monocyte derived dendritic cells (moDCs) and exhibit similar properties with DCs. Therefore, we focused on the autophagic machinery of monocytes. We provide evidence for decreased autophagy of monocytes treated with RA IgG induced NETs as compared with HS IgG induced NETs (control). Following the updated guidelines for the assessment of autophagy we examined with confocal microscopy the formation of functional autophagolysosomes based on the expression of LC3 and p62. It was found decreased expression of LC3 and increased accumulation of p62 in RA IgG NETs treated monocytes as compared with HS IgG NETs treated monocytes. In accordance, western blot analysis for the expression of p62 in monocytes lysates revealed increased p62 levels in monocytes treated with RA IgG induced NETs as compared with control NETs. Due to technical hardships, we didn't manage to assess the expression of LC3II/LC3I in monocytes lysates.

Next, we sought to determine the intracellular signaling events mediating the NET-dependent downregulation of monocyte autophagic machinery. Given that, the mTOR-dependent pathway is the best characterized regulator of autophagy, we sought to determine the phosphorylation levels of mTOR as well as the phosphorylation levels of Akt as an upstream modulator of mTOR activity[37] Autophagy is primarily mediated by signals that activate ULK1/2 and recruitment of Atg and LC3-II proteins on the autophagosomes. mTORC1 activation suppresses autophagy by blocking ULK1/2 and LC3-II recruitment. Thus, activation of Akt and subsequent induction of mTORC1 results in inhibition of autophagy. Interestingly, we found increased levels of phosphorylated mTOR and Akt in monocytes upon RA IgG-induced NET treatment versus control as well as a trend for increased phosphorylated levels of S6 and 4EBP1 the downstream molecules of the mTOR, thus confirming the activation of the mTORC1 signaling pathway in RA IgG NETs exposed monocytes. However, it is known that activation of the PI3K/Akt pathway is critical in restricting

proinflammatory and promoting anti-inflammatory responses in TLR-stimulated macrophages [38], and has been considered as a negative regulator of TLR and NF-kB signaling in macrophages [39] [40]. One explanation for this discrepancy in our findings could be that in most studies, the PI3K/Akt/mTOR pathway has been studied as a single entity, and the effect of different isoforms of PI3K or Akt kinases has not been defined. For example, activation of PI3Kγ and PI3Kδ has been implicated in alveolar macrophage activation by immune complexes via Fcγ receptor [41], thus involved in proinflammatory signaling. Moreover, individual Akt isoforms could be activated by specific PI3K members in macrophages. Nevertheless, isoform-specific effects on macrophage function have been reported both for Akt and PI3K.

To extend our findings we used the collagen induced arthritis mouse model (CIA). CIA bone marrow PMNs exert increased spontaneous NET formation as compared with control BM PMNs. Moreover, treatment of bone marrow derived dendritic cells (BMDCs) with these NETs revealed increased antigen presenting capacity as compared with the control. In the future we aim to characterize in depth the autophagic machinery and the PI3K-Akt-mTOR signaling pathway of BMDCs and perform functional experiments.

Overall, our data will provide novel insights into the mechanism of RA pathogenesis and the mechanisms that provoke inflammation during autoimmune diseases with the ultimate goal the development of more specific therapies in patients with RA.

# REFERENCES


1. Alissafi, T., et al., *Tregs restrain dendritic cell autophagy to ameliorate autoimmunity.* J Clin Invest, 2017. **127**(7): p. 2789-2804.
2. Alamanos, Y. and A.A. Drosos, *Epidemiology of adult rheumatoid arthritis.* Autoimmun Rev, 2005. **4**(3): p. 130-6.
3. Tsokos, G.C., *Autoimmunity and organ damage in systemic lupus erythematosus.* Nat Immunol, 2020. **21**(6): p. 605-614.
4. van Delft, M.A.M. and T.W.J. Huizinga, *An overview of autoantibodies in rheumatoid arthritis.* J Autoimmun, 2020. **110**: p. 102392.
5. Okada, Y., et al., *Genetics of rheumatoid arthritis contributes to biology and drug discovery.* Nature, 2014. **506**(7488): p. 376-81.
6. Geiler, J., M. Buch, and M.F. McDermott, *Anti-TNF treatment in rheumatoid arthritis.* Curr Pharm Des, 2011. **17**(29): p. 3141-54.
7. Kohler, B.M., et al., *Current Therapeutic Options in the Treatment of Rheumatoid Arthritis.* J Clin Med, 2019. **8**(7).
8. D'Amico, L. and I. Roato, *Cross-talk between T cells and osteoclasts in bone resorption.* Bonekey Rep, 2012. **1**: p. 82.
9. Muller-Ladner, U., et al., *Mechanisms of disease: the molecular and cellular basis of joint destruction in rheumatoid arthritis.* Nat Clin Pract Rheumatol, 2005. **1**(2): p. 102-10.
10. Wright, H.L., et al., *Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps.* Front Immunol, 2020. **11**: p. 584116.
11. McInnes, I.B. and G. Schett, *The pathogenesis of rheumatoid arthritis.* N Engl J Med, 2011. **365**(23): p. 2205-19.
12. Edilova, M.I., A. Akram, and A.A. Abdul-Sater, *Innate immunity drives pathogenesis of rheumatoid arthritis.* Biomed J, 2021. **44**(2): p. 172-182.
13. McInnes, I.B., C.D. Buckley, and J.D. Isaacs, *Cytokines in rheumatoid arthritis - shaping the immunological landscape.* Nat Rev Rheumatol, 2016. **12**(1): p. 63-8.
14. Fousert, E., R. Toes, and J. Desai, *Neutrophil Extracellular Traps (NETs) Take the Central Stage in Driving Autoimmune Responses.* Cells, 2020. **9**(4).
15. Khandpur, R., et al., *NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis.* Sci Transl Med, 2013. **5**(178): p. 178ra40.
16. Papadaki, G., et al., *Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation.* Eur J Immunol, 2016. **46**(11): p. 2542-2554.
17. Yu, M.B. and W.H.R. Langridge, *The function of myeloid dendritic cells in rheumatoid arthritis.* Rheumatol Int, 2017. **37**(7): p. 1043-1051.
18. Wei, S.T., et al., *Serum Levels of IL-6 and TNF-alpha May Correlate with Activity and Severity of Rheumatoid Arthritis.* Med Sci Monit, 2015. **21**: p. 4030-8.
19. Houssiau, F.A., et al., *Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides.* Arthritis Rheum, 1988. **31**(6): p. 784-8.
20. Vomero, M., et al., *Autophagy and Rheumatoid Arthritis: Current Knowledges and Future Perspectives.* Front Immunol, 2018. **9**: p. 1577.
21. Alissafi, T., et al., *Autophagy orchestrates the regulatory program of tumor-associated myeloid-derived suppressor cells.* J Clin Invest, 2018. **128**(9): p. 3840-3852.

22. Karlsson, E., et al., *The mTOR effectors 4EBP1 and S6K2 are frequently coexpressed, and associated with a poor prognosis and endocrine resistance in breast cancer: a retrospective study including patients from the randomised Stockholm tamoxifen trials.* Breast Cancer Res, 2013. **15**(5): p. R96.
23. Dengjel, J., et al., *Autophagy promotes MHC class II presentation of peptides from intracellular source proteins.* Proc Natl Acad Sci U S A, 2005. **102**(22): p. 7922-7.
24. Kremer, J., et al., *Tofacitinib in combination with nonbiologic disease-modifying antirheumatic drugs in patients with active rheumatoid arthritis: a randomized trial.* Ann Intern Med, 2013. **159**(4): p. 253-61.
25. Keystone, E.C., et al., *Long-term use of adalimumab as monotherapy after attainment of low disease activity with adalimumab plus methotrexate in patients with rheumatoid arthritis.* RMD Open, 2018. **4**(1): p. e000637.
26. Guo, Q., et al., *Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies.* Bone Res, 2018. **6**: p. 15.
27. Nandakumar, K.S., L. Svensson, and R. Holmdahl, *Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes.* Am J Pathol, 2003. **163**(5): p. 1827-37.
28. Kambas, K., et al., *C5a and TNF-alpha up-regulate the expression of tissue factor in intra-alveolar neutrophils of patients with the acute respiratory distress syndrome.* J Immunol, 2008. **180**(11): p. 7368-75.
29. Lim, M.B., et al., *Rac2 is required for the formation of neutrophil extracellular traps.* J Leukoc Biol, 2011. **90**(4): p. 771-6.
30. Lande, R., et al., *Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus.* Sci Transl Med, 2011. **3**(73): p. 73ra19.
31. Kessenbrock, K., et al., *Netting neutrophils in autoimmune small-vessel vasculitis.* Nat Med, 2009. **15**(6): p. 623-5.
32. Garcia-Romo, G.S., et al., *Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus.* Sci Transl Med, 2011. **3**(73): p. 73ra20.
33. Paludan, C., et al., *Endogenous MHC class II processing of a viral nuclear antigen after autophagy.* Science, 2005. **307**(5709): p. 593-6.
34. Schmid, D., M. Pypaert, and C. Munz, *Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes.* Immunity, 2007. **26**(1): p. 79-92.
35. Lee, H.K., et al., *In vivo requirement for Atg5 in antigen presentation by dendritic cells.* Immunity, 2010. **32**(2): p. 227-39.
36. Aichinger, M., et al., *Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance.* J Exp Med, 2013. **210**(2): p. 287-300.
37. Martina, J.A., et al., *MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB.* Autophagy, 2012. **8**(6): p. 903-14.
38. Lopez-Pelaez, M., et al., *Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression.* Eur J Immunol, 2011. **41**(6): p. 1733-41.
39. Troutman, T.D., J.F. Bazan, and C. Pasare, *Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K.* Cell Cycle, 2012. **11**(19): p. 3559-67.
40. Fukao, T. and S. Koyasu, *PI3K and negative regulation of TLR signaling.* Trends Immunol, 2003. **24**(7): p. 358-63.

41. Konrad, S., et al., *Phosphoinositide 3-kinases gamma and delta, linkers of coordinate C5a receptor-Fcgamma receptor activation and immune complex-induced inflammation.* J Biol Chem, 2008. **283**(48): p. 33296-303.