PhD thesis

Delineating novel innate inflammatory pathways involved in chronic autoinflammatory and autoimmune diseases such as Rheumatoid arthritis and its subtypes like Still's disease.

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Διερεύνηση νέων φλεγμονωδών μηχανισμών της έμφυτης ανοσίας σε χρόνια αυτοφλεγμονώδη και αυτοάνοσα νοσήματα όπως η Ρευματοειδής αρθρίτιδα και οι υπότυποι της όπως η νόσος του Still.

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Delineating novel innate inflammatory pathways involved in chronic autoinflammatory and autoimmune diseases such as Rheumatoid arthritis and its subtypes like Still's disease.

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Dedicated to my family (my parents Kostas-Maria and my husband Thomas)…..

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1. ABSTRACT

Rheumatoid arthritis (RA) is a chronic immune inflammatory disease characterized by synovial hyperplasia, joint destruction and extra-articular manifestations with a significant impact on both morbidity and mortality. Disease severity correlates with the presence of citrullinated proteins and immune complexes containing various citrullinated antigens which exhibit increased immunogenicity and arthritogenicity. The source of these proteins and immune complexes remains unknown.

RA presents local inflammation in the joints, which subsequently develops into a systemic disorder as a result of the loss of immune tolerance. Pathogenic T cells not only help B cells produce auto-Abs (rheumatoid factor (RF) and anti-citrullinated protein, anti-CCPs), but also actively mediate tissue destruction by secreting proinflammatory cytokines (IL-17, IL-6, and TNF-a) thus creating an inflammatory microenvironment that favors macrophage and neutrophil recruitment and osteoclast activation.

Dendritic cells (DCs) as professional antigen presenting cells (APCs), migrate to the lymph nodes where they process and present acquired antigens to naïve T cells and secrete cytokines resulting in skewing of naïve T cells toward T helper (Th1, Th17). Emerging data have revealed that the Th1 and Th17 are of the major T cell subsets during the course of RA but what drives their polarization and expansion is unknown.

In the clinical setting, neutrophils are present in high numbers in the synovial tissue during the initial stages of RA and persist in the synovial fluid during the perpetuation of the disease. A novel feature of neutrophils, recently described, is their ability to form neutrophil extracellular traps (NETs), a cell death mechanism that is referred as NETosis. NETs are composed of chromatin decorated with a variety of granular proteins. NETs may promote differential cell activation and cytokine release.

Aberrant formation of neutrophil extracellular traps (NETs) is a key feature in Rheumatoid arthritis (RA) and has been demonstrated to play a pivotal role in disease pathogenesis. However, the mechanism through which NETs shape the autoimmune response in RA remains elusive. Our aims were: (a) to delineate the role of NETs in shaping the RA autoimmune response, (b) to explore the mechanism involved in the NETs-mediated regulation of the autoimmune response.

In this study, we demonstrate that inhibition of peptidylarginine deiminases (PADs) activity in collagen-induced arthritis (CIA) mouse model significantly reduced NET formation, attenuated clinical disease activity and inhibited joint destruction. Importantly, blocking of PADs markedly reduced the frequency of collagen-specific IFN-γ producing Th1 cells in the draining lymph nodes (dLNs) of immunized animals whereas the levels of Th17 cells remained unaffected. Mechanistically, we showed that exposure of DCs to 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 support, CIA-NET-treated DCs promoted the induction of antigen-specific Th1 cells *in vitro*. Finally, RA-derived NETs showed an increased potential to induce the maturation of DCs from healthy individuals corroborating the findings obtained in CIA mouse model.

Collectively, our findings delineate an important role of NETs in the induction and expansion of Th1 pathogenic cell subsets in CIA through maturation of DCs. These findings reveal a novel role of NETs in shaping the RA autoimmune response that could be exploited therapeutically.

Key words: Autoimmunity, Rheumatoid arthritis, Neutrophil extracellular traps, Collagen-induced arthritis, Cl-amidine, dendritic cells, PMNs

1. ΠΕΡΙΛΗΨΗ

Η Ρευματοειδής Αρθρίτιδα (ΡΑ) είναι μια χρόνια φλεγμονώδης νόσος που χαρακτηρίζεται από υπερπλασία, καταστροφή των αρθρώσεων και εξωαρθρικές εκδηλώσεις με σημαντική επίδραση στο ρυθμό ανάπτυξης της νόσου και στη θνησιμότητα. Η σοβαρότητα της νόσου σχετίζεται με τη παρουσία αντισωμάτων έναντι κιτρουλλινοποιημένων πρωτεϊνών και ανοσοσυμπλεγμάτων που περιέχουν διάφορα κιτρουλλινοποιημένα αντιγόνα με μεγάλη ανοσογονικότητα και επίδραση στην καταστροφή της άρθρωση. Η πηγή αυτών των αυτοαντιγόνων και η αρχική αιτία απώλειας της ανοσολογικής ανοχής παραμένει άγνωστη.

Η ΡΑ παρουσιάζει τοπική φλεγμονή στις αρθρώσεις και σταδιακά αναπτύσεται σε μια συστηματική αυτοάνοση νόσο, αποτέλεσμα της απώλειας ανοχής του ανοσποιητικού συστήματος. Παθογόνα Τ κύτταρα όχι μόνο βοηθούν τα Β κύτταρα να παράγουν αυτοαντισώματα (έναντι κιτρουλλινοποιημένων πρωτεϊνών και ρευματοειδείς παράγοντες), αλλά επίσης μεσολαβούν ενεργά στη καταστροφή του ιστού εκκρίνοντας προ-φλεγμονώδεις κυτταροκίνες (IL-17, IL-6, and TNF-a) δημιουργώντας έτσι ένα φλεγμονώδες μικροπεριβάλλον που ευνοεί την προσέλκυση μακροφάγων και ουδετρόφιλων και την ενεργοποίηση των οστεοκλαστών.

Τα δενδριτικά κύτταρα ως εξειδικευμένα αντιγονοπαρουσιαστικά κύτταρα, μεταναστεύουν στους λεμφαδένες όπου επεξεργάζονται και παρουσιάζουν αντιγόνα σε μη ενεργοποιημένα Τ κύτταρα και εκκρίνουν κυτταροκίνες καταλήγοντας στη διαφοροποίηση των Τ κυττάρων σε Τ βοηθητικά κύτταρα (Th1, Th17). Διάφορα δεδομένα έχουν αναδείξει τα Th1 και Th17 ως τους κύριους παθογόνους Τ υποπληθυσμούς κατά τη διάρκεια της ΡΑ. Ωστόσο οι παράγοντες που οδηγούν στη πόλωση και στην ανάπτυξη τους παρανένουν άγνωστοι.

Σε κλινικό επίπεδο, τα ουδετερόφιλα βρίσκονται σε υψηλούς αριθμούς στον αρθρικό ιστό κατά τα αρχικά στάδια της PA και παραμένουν στο αρθρικό υγρό κατά τη πρόοδο της ασθένειας. Ένα νέο χαρακτηριστικό των ουδετερόφιλων που περιγράφηκε πρόσφατα, είναι η ικανότητα τους να σχηματίζουν εξωκυττάριες παγίδες ουδετερόφιλων (NETs), ένας μηχανισμός κυτταρικού θανάτου που αναφέρεται ως Νέτωση. Τα NETs αποτελούνται από DNA-χρωματίνη που είναι διακοσμημένη από μια ποικιλία πρωτεϊνών που βρίσκονται στα κοκκία των ουδετερόφιλων. Τα NETs μπορούν να προκαλέσουν κυτταρική ενεργοποίηση σε διάφορα κύτταρα και την έκκριση κυτταροκινών.

Ένα σημαντικό χαρακτηριστικό της PA είναι ο παθολογικός σχηματισμός NETs και έχει δειχθεί να παίζει σημαντικό ρόλο στη παθογένεια της ασθένειας. Ωστόσο, ο μηχανισμός μέσω του οποίου τα NETs συμμετέχουν στην αυτοάνοση απάντηση στη PA, παραμένει άγνωστος. Οι στόχοι της παρούσας ερευνητικής μελέτης ήταν: (α) η διερεύνηση του ρόλου των NETs στην αυτοάνοση απόκριση της PA,(β) η μελέτη τωνμηχανισμών ρύθμισης της αυτοάνοσης απόκρισης από τα NETs.

Στη παρούσα μελέτη, δείχνουμε ότι η καταστολή της δράσης των PAD ενζύμων (κύρια ένζυμα κατά τη κιτρουλλινοποίηση) στο μοντέλο ποντικού με αρθρίτιδα επαγώμενη από κολλαγόνο μείωσε σημαντικά τη δημιουργία των NETs, τη κλινική ενεργότητα της ασθένειας και παρεμπόδισε τη καταστροφή της άρθρωσης. Το σημαντικότερο είναι οτι αναστέλλοντας τα ένζυμα PAD μειώθηκε σημαντικά η συχνότητα των αντιγονοειδικών Τ κυττάρων που παράγουν IFN-γ (Th1) στους επιχώριους λεμφαδένες (dLNs) ανοσοποιημένων ποντικών ενώ τα επίπεδα των κυττάρων που παράγουν IL-17 (Th17) παρέμειναν σταθερά. Λειτουργικά δείξαμε οτι η έκθεση των δενδριτικών κυττάρων σε NETs προερχόμενα από ποντίκια με νόσο επέφερε την ενεργοποίηση των δενριτικών κυττάρων συνοδευόμενη από αύξηση των

μορίων συν-ενεργοποίησης CD80 και CD86 και από αυξημένη έκκριση της προφλεγμονώδους κυτταροκίνης IL-6. Επίσης, τα δενδριτικά κύτταρα που συγκαλλιεργήθηκαν με NETs (CIA-NETs) αύξησαν τα αντιγονοειδικά Th1 κύτταρα in vitro. Τέλος, τα NETs προερχόμενα από ασθενείς με PA αύξησαν την ενεργοποίηση υγιών δενδριτικών κυττάρων συμφωνώντας με τα ευρήματα στο πειραματικό μοντέλο PA.

Συνολικά, τα δεδομένα αυτά αποκαλύπτουν ένα σημαντικό ρόλο των NETs στην επαγωγή του Th1 κυτταρικού υποπληθυσμού στο μοντέλο ποντικού αρθρίτιδας επαγώμενης από κολλαγόνο (Collagen-induced arthritis) μέσω της ενεργοποίησης των δενδριτικών κυττάρων. Αυτά τα ευρήματα αποκαλύπτουν ένα καινοτόμο ρόλο των NETs στη δημιουργία αυτοάνοσων αποκρίσεων στη PA που μπορεί να διερευνηθεί θεραπευτικά.

Λέξεις κλειδιά: Αυτοανοσία, Ρευματοειδής αρθρίτιδα, εξωκυττάριες παγίδες ουδετερόφιλων, αρθρίτιδα επαγώμενη από κολλαγόνο, Cl-amidine, δενδριτικά κύτταρα, πολυμορφοπύρηνα-ουδετερόφιλα

2. ABBREVIATIONS

NETs	Neutrophil extracellular traps
RA	Rheumatoid arthritis
RF	Rheumatoid factor
anti-CCPs	anti-cyclic citrullinated peptides
ACPAs	anti-citrullinated protein antibodies
PADs	peptidylarginine deiminases
Cl-amidine	N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide
IL-17	Interleukin-17
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor-α
IL-10	Interleukin-10
TGF-β	transforming growth factor β
FoxP3	Forkhead box P3
IFN-γ	Interferon-γ
Th1	T helper 1
Th17	T helper 17
DCs	dendritic cells
APC	antigen-presenting cells
CIA	collagen-induced arthritis
dLNs	draining lymph nodes
TCR	T cell receptor
pDCs	plasmacytoid dendritic cells
DMARD	disease modifying antirheumatic drugs
nbDMARD	non-biologic disease modifying antirheumatic drugs

PMNs	polymorphonuclear cells
MPO	myeloperoxidase
NE	Neutrophil elastase
HLA	Human Leukocyte antigen
MHC	Major histocompatibility complex
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte antigen 4
STAT	Signal Transducers and Activators of Transcription
ROS	Reactive oxygen species
NADPH	Nicotamide Adenine Dinucleotide Phosphate

3. INTRODUCTION

3.1 Immunological tolerance and autoimmunity

The immune system has the ability to react with an enormous variety of foreign and self antigens with the ultimate goal of self protection. During the process of lymphocyte maturation lymphocytes with specificity to foreign or self antigens are being generated. A complex series of mechanisms, a phenomenon called immunological tolerance prevents the immune system form responding to self antigens. The breakdown of self tolerance and the activation of autoreactive lymphocytes lead to autoimmunity and autoimmune diseases resulting in deleterious inflammation in and destruction of self-tissues mediated by autoreactive T cells and autoantibodies (auto-Abs) (Goodnow, Sprent et al. 2005).

Self tolerance can be divided into central tolerance and peripheral tolerance. In central tolerance, immature lymphocytes that happen to recognize self antigens in generative lymphoid organs (the bone marrow for B cells and the thymus for T cells) die by apoptosis. In peripheral tolerance, mature self–reactive lymphocytes that have escaped from the protective mechanisms of central tolerance, encounter self antigens in peripheral tissues and are killed or shut off. Central and peripheral mechanisms of tolerance are discussed below.

3.1.1 Mechanisms of central and peripheral tolerance

Central tolerance

During ontogeny T and B lymphocytes are equipped with all possible antigen-specific receptors (lymphocyte repertoire). For B cells this process occurs within the bone

marrow and the receptors are surface-bound immunoglobulins which are capable of recognizing antigen in its native form. T cell repertoire occurs within the thymus and these antigen-specific receptors in T cells (TCR) interact with a peptide fragment of antigen when it is presented in the context of MHC class I or class II alleles on the surface of APC.

Central B cell tolerance may occur through two main mechanisms: A) immature B lymphocytes that encounter the native antigen in the bone marrow during their maturation undergo apoptotic cell death (Nossal 1983) or B) alternatively they change their receptor specificity (receptor editing) (Pelanda and Torres 2006).

Central T cell tolerance may occur through three main mechanisms: A) Death by neglect. Thymocytes with no affinity for peptide-MHC which are presented by cortical epithelial cells die of neglect (CD4+CD8+ lacking productive rearrangement of the TCRβ locus). B) Positive selection process. Thymocytes showing sufficient (intermediate) affinity for a peptide-MHC (CD4+ or CD8+) and contain clones with foreign and self peptide specificity. C) Negative selection. Thymocytes showing high affinity for a self peptide in the thymus and undergo apoptotic cell death. The CD4+ or CD8+ cells with TCR showing no high avidity for self peptides survive (Palmer 2003).

At the end of their development both mature B and T cells leave central lymphoid organs and migrate to secondary lymphoid organs (lymph node, spleen, mucosal lymphoid tissues) where they encounter their specific antigen presented by dendritic cells (DCs).

Peripheral tolerance

The existence of additional mechanisms in the peripheral tissues controlling the reactivity to self antigens dictates that not all self-antigens are presented in the thymus.

The surviving B and T cells from the central lymphoid organs migrate to the peripheral organs where additional mechanisms operate to maintain self tolerance.

Peripheral tolerance for mature B cell can occur under two main conditions: A) when B cell encounter the specific antigen in the absence of the specific T helper (Th) cell, B cell becomes incapable of activation (Bretscher and Cohn 1970) B) when B cell is partially activated resulting in its exclusion from lymphoid follicles (follicular exclusion) (Cyster, Hartley et al. 1994).

Peripheral T cell tolerance can occur through tree main mechanisms **(Figure 1).** A) Anergy. When T cell encounters its proper peptide but the presenting cell is not a professional antigen presenting cells and lacks the co-stimulatory molecules (CD80 and CD86) or the interaction of CD80 and CD86 co-stimulatory molecules with the activating CD28 on Th cell is disrupting by the interaction of co-stimulatory molecules with the suppressive cytotoxic T lymphocyte associated antigen (CTLA-4) instead of interacting with the CD28 molecule on the surface of Th cells. In both cases Th cell becomes functionally inactive (Powell 2006). B) Deletion. T cells encounter high antigen concentrations or they are heavily activated resulting in their apoptotic cell death through Fas (CD95)-Fas ligand interaction (activation-induced cell death) (Juo, Kuo et al. 1998). C) Immune suppression through the existence of T cells with suppressive activity (T regulatory cells). Treg cells ($CD4^{+}CD25^{high}$) can be generated at thymic level (natural occurring Tregs) and in the periphery (adaptive Tregs) mainly

acting via the production of cytokines, such as IL-10 or TGF-β (Bluestone and Abbas 2003). Both Treg cells are characterized by the expression of *FOXP3* transcription factor. Moreover, other Treg cells have been described called Tr1 and Th3 that lack Foxp3 expression but were found to be suppressive through the production of IL-10 and TGF-β respectively (Roncarolo, Bacchetta et al. 2001; Weiner 2001).

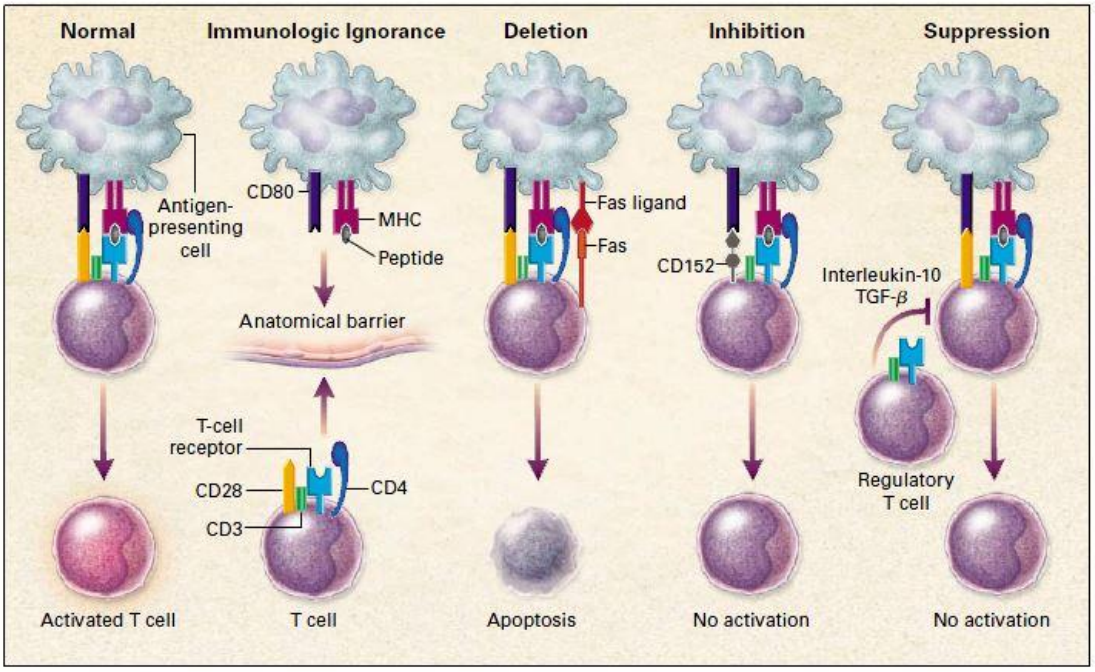


Figure1: Peripheral mechanisms of T cell tolerance. Different mechanisms contribute to the maintenance of T cell tolerance. In the immunological tolerance T cells are physically separated from the specific antigen. T cells are deleted by apoptosis when the binding of Fas with its ligand found on antigen presenting cells is achieved. Different molecules (e.g. CD152) expressed on the surface of T cells inhibit the T cell activation or T cell with regulatory function suppress the T cell activation. *(Source: Kamradt et al., N Engl J Med, 2001).*

3.1.2 Autoimmunity

Autoimmune responses represent an imbalance between effector and regulatory immune responses resulting to a defective elimination and/or control of self-reactive lymphocytes. Autoimmune diseases show phases of resolution (indicated by clinical remissions) and exacerbations (indicated by symptomatic flares). The major therapeutic goal for autoimmune diseases is to develop strategies for reestablishing the normal balance between effector and regulatory immune responses and reach the long-lived disease resolution.

The autoimmune diseases vary greatly in the organs they affect and their clinical manifestations with some being limited to particular tissues and other being systemic. All autoimmune diseases are believed to go through sequential phases of initiation, propagation and resolution.

Autoimmune diseases are believed to develop from a combination of genetic and environmental factors **(figure 2**). During the propagation phase clinical disease is evident in patients characterized by progressive inflammation and tissue damage. As a result of tissue damage new antigenic epitopes and alterations in self-proteins are developed (epitope spreading). Epitope spreading sets up a vicious cycle in which newly created antigenic epitopes activate more lymphocytes of different specificities and recruit these cells into the reaction, leading to more tissue damage and the emergence of even more novel epitopes targeted by autoreactive lymphocytes. Second, the autoimmune reaction creates an inflammatory environment in which multiple immune cells interact to produce cytokines and other mediators that amplify the reaction, creating a catastrophic inflammatory loop. Consistent with this notion is the finding that type I interferons, a product of plasmacytoid dendritic cells that is

produced during inflammatory reactions, is a biomarker for the progression of SLE and may be involved in the propagation of this disease (Banchereau and Pascual 2006). In the propagation phase different inflammatory cytokines play major role since treatments for cytokine inhibitors are effective. However, one third of patients do not respond and up to 50% of patients will eventually discontinue previously effective therapy due to adverse effects or lack of efficacy (Thalayasingam and Isaacs 2011). Moreover, therapeutic strategies which target the effector T cell/T reg balance area are appealing for the treatment of autoimmune diseases. The resolution phase usually implicates different regulatory mechanisms that limit the effector response and restore the effector/regulatory balance. In such regulatory mechanisms T regs with potent suppressive function are involved. Another attractive approach to reach the resolution phase is the existence of various inhibitory receptors such as CTLA-4 and PD-1 (members of the CD28 family) which exhibit suppressive function either on T effector cells or T regs.

All phases are associated with a failure of regulatory mechanisms with the resolution phase defined by a partial most times, short-term ability to restore the balance of effector and regulatory responses.

The precise mechanisms implicated in the autoimmune diseases remain unknown. Further studies needed to unravel specific signaling pathways that could exploited therapeutically.

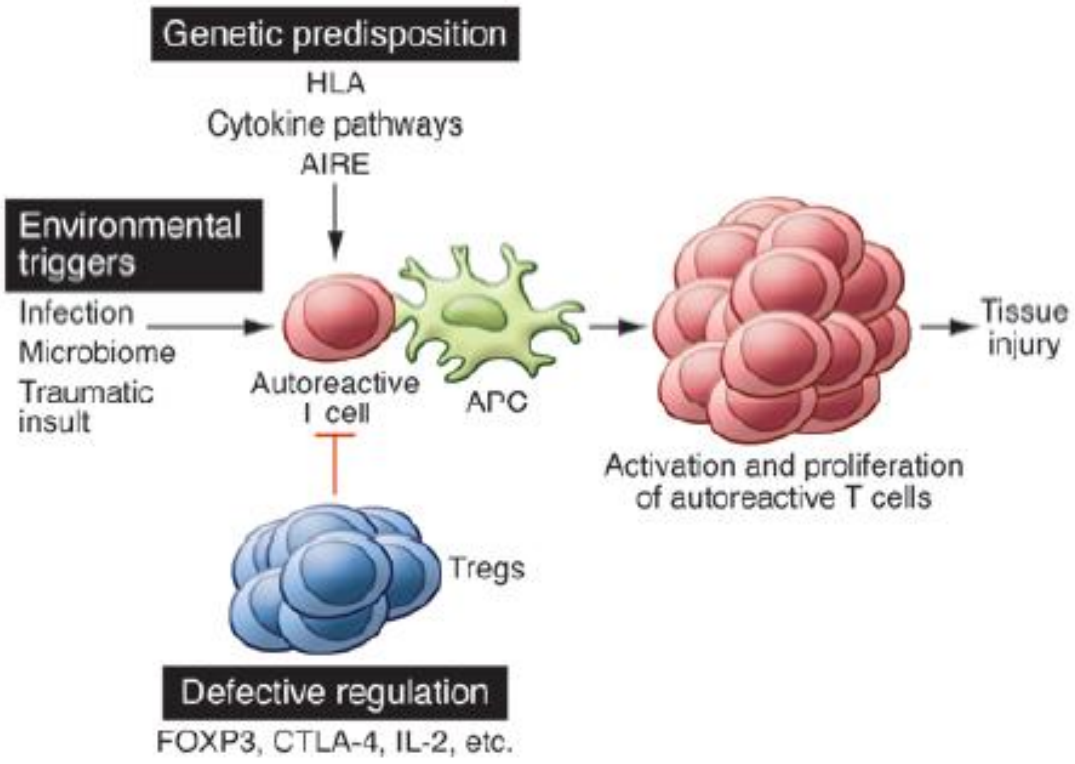


Figure 2: For the initiation of autoimmunity there are different responsible factors such as the genetic background, environmental factors and the defective regulation. Genetic polymorphisms may alter the threshold of autoreactive T cell activation. Moreover, environmental insults such as infections, the microbiome and tissue injury may contribute to the activation of autoreactive T cells. Deregulation in the suppressive function of T regs may render autoreactive T cells hyperactive. All these factors alone or in combination may contribute to the activation and proliferation of autoreactive responses resulting in tissue damage. *(Source: Rosenblum et al., J Clin Inves., 2015)*

3.2 Rheumatoid Arthritis (RA)

Rheumatoid arthritis is characterized by synovial inflammation and hyperplasia ("swelling"), autoantibody production (rheumatoid factor and anti–citrullinated protein antibody [ACPA]), cartilage and bone destruction ("deformity") and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders (McInnes and Schett 2011).

There is a long-established association of RA patients with the human leukocyte antigen (HLA)–DRB1, alleles that contain a common amino acid motif (QKRAA) in the HLA-DRB1 region, termed the shared epitope, that confers particular susceptibility (Gregersen, Silver et al. 1987). These findings suggest that some predisposing T-cell repertoire selection, antigen presentation, or alteration in peptide affinity has a role in promoting autoreactive adaptive immune responses.

Years before the clinical diagnosis pathogenic autoantibodies against citrullinated proteins (ACPAs) and rheumatoid factor (RF) are often detected and they are considered as serologic hallmark of the disease (Schellekens, Visser et al. 2000). Patients with ACPA-positive disease have a less favorable prognosis than those with ACPA-negative disease, which suggests that such molecular subsets are clinically useful.

Rheumatoid arthritis is considered to be a disease that is mediated by type 1 helper T cells. However, several studies have focused on the role of type 17 helper T cells (Th17), a subset that produces interleukin-17A, 17F, 21, and 22 and tumor necrosis factor α (TNF-α) (Chabaud, Fossiez et al. 1998; Miossec, Korn et al. 2009). Interleukin-17A, which synergizes with TNF-α to promote activation of fibroblasts and chondrocytes, is currently being targeted in clinical trials (Genovese, Van den Bosch et al. 2010). T regulatory (forkhead box P3 [Foxp3+]) are often detected in

tissues from patients with rheumatoid arthritis but appear to have limited functional capability (Behrens, Himsel et al. 2007). The early stages of RA manifest with local inflammation in the joints, which subsequently develops into a systemic disorder as a result of the loss of immune tolerance **(Figure 3)**. Pathogenic T cells not only help B cells produce auto-Abs (rheumatoid factor and anti-citrullinated protein) but also actively mediate tissue destruction by secreting proinflammatory cytokines (IL-17, IL-6, and TNF-a) and creating an inflammatory microenvironment that favors macrophage and neutrophil recruitment and osteoclast activation (Burmester, Rubbert-Roth et al. 2014). Synovitis occurs when leukocytes infiltrate the synovial compartment. Characteristics of early and established synovitis are neoangiogenesis which is induced by local hypoxic conditions and cytokines, and insufficient lymphangiogenesis, which limits cellular egress (Polzer, Baeten et al. 2008; Szekanecz, Pakozdi et al. 2009). The previous characteristics combined with local fibroblast activation construct the synovial inflammatory tissue in rheumatoid arthritis.

Different innate effector cells, including macrophages, mast cells, and natural killer cells, are found in the synovial membrane, whereas neutrophils reside mainly in synovial fluid.

Numerous murine models of RA (discussed below) have been used for elucidating the etiology and pathogenesis of this disease (Bevaart, Vervoordeldonk et al. 2010).

Restoration of immune regulation has been shown in RA patients upon effective treatment. Treatment strategy in RA has radically changed during the last 15 years. Early on, at the time of diagnosis, treatment with non-biologic disease modifying anti-rheumatic drugs (nbDMARDs) is initiated and combinations of nbDMARDs are often used in aggressive forms of the disease. The target of treatment is disease remission or

low disease activity (Smolen, Landewe et al. 2014). In those patients in whom residual disease activity still persists after optimal doses of nbDMARDs, then biologic DMARDs (bDMARDs) may be used. Biologic DMARDs applied in clinical practice are either cytokine inhibitors (TNFα or IL6 inhibitors), B-cell depleting agents (anti-CD20 antibodies), or T-cell co-stimulation inhibitors (CTLA4Ig). Real-life data from patients' registries have shown that about 50-60% of RA patients treated with those agents may respond to therapy, while the rest will stop treatment either due to inefficacy (primary or secondary) or due to toxicity (Flouri, Markatseli et al. 2014).

The different therapeutic approaches used for the treatment of RA dictates the several immune pathways that are involved in its pathogenesis. Understanding of such processes are targeting to potential avenues for therapeutic interventions in a personalized level.

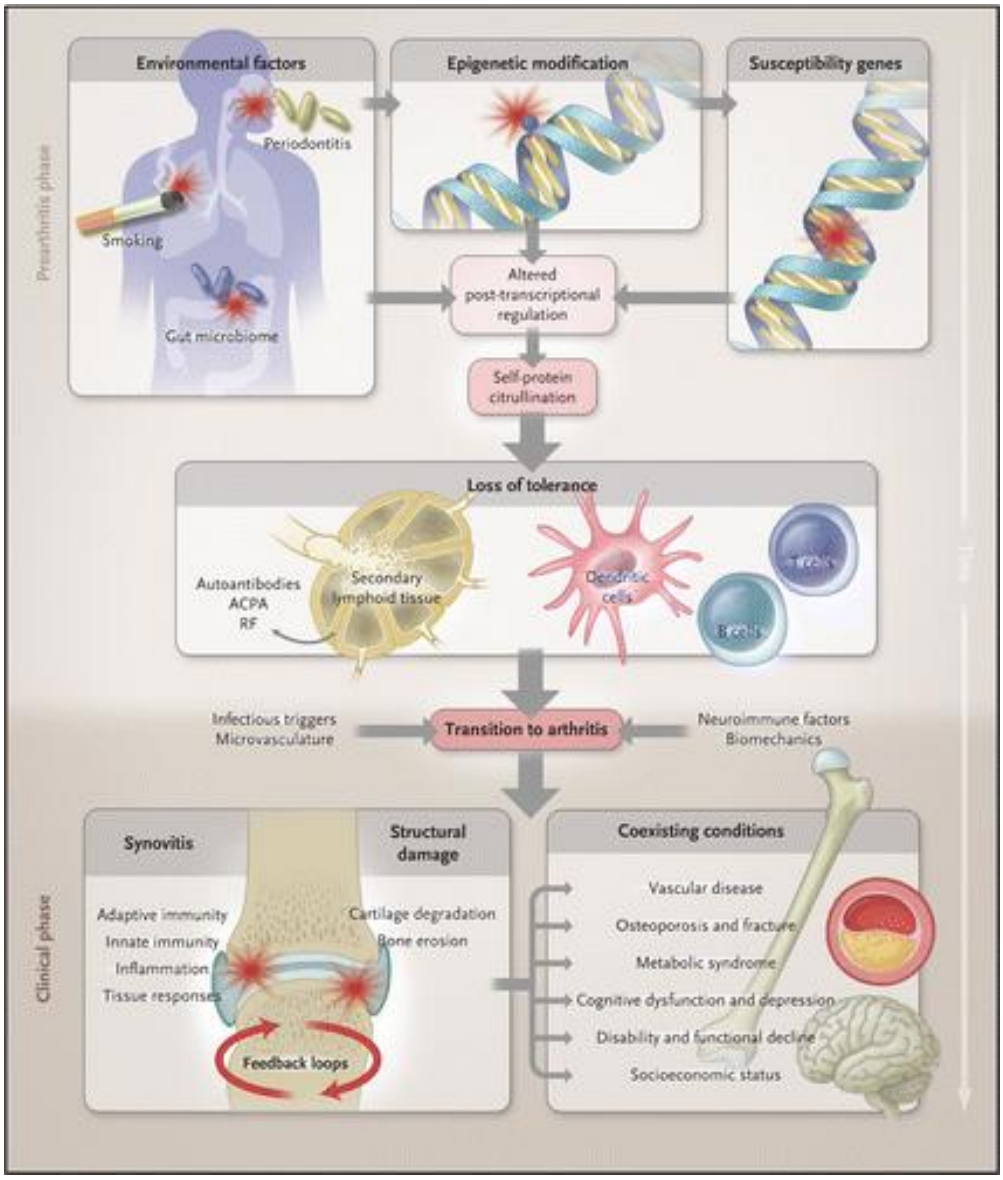


Figure 3: Rheumatoid arthritis is a multifactorial disease. Posttranslationally modified self-proteins contained a citrulline residue promote loss of tolerance. The inflammatory response in RA is detected in the joints where synovitis is iniated and perpetuated by positive feedback loops thus promoting systemic disorders. *(Source: McInnes and Schett, N Engl J Med, 2011)*

3.3 Animal models in RA

Rheumatoid arthritis is a multifactorial disease. Animal models of arthritis therefore provide important tools to dissect the various cellular and molecular mechanisms leading to RA. Despite the evolutionary distance between mice and man, clinical features of arthritis in many mouse models resemble those of human RA; therefore, the unraveling of the pathological pathways of arthritis in mice will provide insight into the mechanisms of human RA.

3.3.1 Induced-arthritis models

Collagen-induced arthritis

Collagen-induced arthritis (CIA) shares many similarities with human RA. Two characteristics of the CIA model – breach of tolerance and generation of autoantibodies toward self and collagen – make CIA the best in vivo model for RA studies. CIA was first described in rats (Trentham, Townes et al. 1977) and subsequently shown to be inducible in susceptible strains of mice (Courtenay, Dallman et al. 1980), following inoculation with type II heterologous collagen in complete Freund's adjuvant. Susceptibility has been linked to strains that have MHC Class II I-Aq haplotypes; however, it is clear that many mouse strains have variable degrees of susceptibility to CIA. Similarly restricted class II genotypes can be found in RA patients, for whom pathogenesis is associated with HLA-DR1 and HLA-DR4 (Brand, Kang et al. 2003). DBA/1 mice are most widely used in the CIA model. Clinical signs of disease typically develop 21–25 days after the initial inoculation and presents as a polyarthritis, which is most prominent in the limbs and characterized by synovial inflammatory infiltration, cartilage and bone erosion and synovial hyperplasia similar to human RA. The development of CIA is associated with both B-

and T-lymphocyte responses with the production of anti-collagen type II antibodies and collagen-specific T cells. The auto-antibody response in CIA is predominated by the IgG2 subclass with high levels of both IgG2a and IgG2b present at the peak of arthritis. Disease severity is expected to peak at approximately day 35, after which DBA/1 mice enter remission, marked by increased concentrations of serum IL-10 and a subsequent decrease in pro-inflammatory Th1 cytokines (Mauri, Williams et al. 1996).

Collagen-antibody-induced arthritis (CAIA).

In this mouse model the development of arthritis is induced by anti- collagen antibody cocktails. (Holmdahl, Rubin et al. 1986). Although the clinical development of arthritis is similar to that in CIA and RA, CAIA is characterized by macrophage and polymorphonuclear inflammatory cell infiltrate (Santos, Morand et al. 1997), but is not associated with a T- and B-cell response (Nandakumar, Backlund et al. 2004). Therefore, CAIA serve as a tool to assess the roles of innate and adaptive immune response separately, in the development of arthritis. Furthermore, disease develops within 48 h of antibody administration with 100% penetrance and is inducible regardless of the MHC class II haplotype. CAIA is well suited for studying the development of arthritis in genetically modified strains of mice.

Zymosan-induced arthritis

Zymosan is a polysaccharide from the cell wall of Saccharomyces cerevisiae which binds to TLR2 in macrophages leading to the induction of proinflammatory cytokines, arachidonate mobilization, protein phosphorylation and the activation of complement via the alternative pathway. Injection of zymosan intra-articularly into the knee joints

of mice results in a proliferative inflammatory arthritis with mononuclear cell infiltration, synovial hypertrophy and pannus formation with the peak of disease at about day 3 and inflammation subsiding by day 7 (Keystone, Schorlemmer et al. 1977). Recent data, however, demonstrate that the model is in fact biphasic, with both early and late phases (Frasnelli, Tarussio et al. 2005). The type of arthritis induced is of monoarthritic nature. Moreover, technical skill for an intra-articular injection in mice is required.

Antigen-induced arthritis

Priming of various strains with an antigen (e.g. methylated BSA in complete Freund's adjuvant) and subsequent intra-articular injection with the same antigen can result in the development of inflammatory arthritis (Brackertz, Mitchell et al. 1977; Brackertz, Mitchell et al. 1977). This is a useful model to investigate a hierarchical role for given factors in adaptive immune mediated articular damage. This pathology of this model involves immune complex mediated inflammation followed by articular T-cell-mediated responses but it does not recapitulate the endogenous breach of tolerance that is typical of RA pathogenesis. Adoptive transfer of transgenic ovalbumin-specific T cells followed by ovalbumin priming and later intra-articular challenge constitute a development of this model (Maffia, Brewer et al. 2004). The t mice develop arthritis, which is followed by the existence of auto-reactivity to collagen, and the presence over time of rheumatoid factors. This model has the advantage of facilitating imaging of the pathogenic T cells that in turn promote breach of self-tolerance to articular antigens (Nickdel, Conigliaro et al. 2009).

Other induced models of arthritis

Small amounts of pristane (a natural saturated terpenoid alkane) by a single injection leads to the development of an acute severe inflammation followed by a chronic relapsing phase in rats and mice (Wooley, Seibold et al. 1989). It is a T-cell dependent model which is characterized by edema accompanied by an acute phase response, infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation, and the erosion of cartilage and bone. The proteoglycan-induced arthritis model involves immunization of genetically susceptible mouse strains, such as BALB/c, with human cartilage-derived proteoglycans (Glant, Finnegan et al. 2003). These mice develop severe polyarthritis and spondylitis.

3.3.2 Genetically manipulated spontaneous arthritis models

TNF-α transgenic mouse model of inflammatory arthritis

Kollias and co-workers in 1991 developed a transgenic mouse over-expressing human TNF-a (Keffer, Probert et al. 1991). The mouse develops chronic inflammatory erosive polyarthritis which is eliminated by treatment with a monoclonal antibody against human TNF-α. Given the chronic progressive nature of the arthritis which is a characteristic of this model, it closely resembles to the human disease. This model has the advantage of evaluating the efficacy of novel therapies in RA, particularly in which novel targets are considered to operate downstream of TNF. Moreover, the model has proven particularly useful in defining the distinct contribution of effector cytokines that regulate inflammation and those that regulate cartilage and bone destruction, e.g. RANKL.

K/B_N model

These mice were generated by crossing the TCR transgenic KRN line with mice expressing the MHC class II molecule Ag7 (Kouskoff, Korganow et al. 1996). K/B_N mice develop severe and destructive inflammatory arthritis. They have high titers of autoantibodies recognizing glucose-6-phosphate isomerase, and serum from these mice induces arthritis in a wide range of normal recipient mouse strains (serum transfer model). The mechanism of action involves complement activation and mast cell degranulation, and is mediated not only by TNF but also by IL-1. The discovery of this model led to several studies investigating titers of pathogenic anti-glucose-6-phosphate isomerase antibodies in RA patients; however, to date the data remain controversial (Matsumoto, Lee et al. 2003; van Gaalen, Toes et al. 2004). Although this somewhat limits the utility of the model, it remains useful for the study of initial events involved in the induction of arthritis and in particular is invaluable in elucidating the contribution of discrete innate immune pathways in articular tissue damage.

SKG model

A point mutation in ZAP-70 induces inflammatory arthritis in part reflecting altered thymic T-cell selection (Sakaguchi, Takahashi et al. 2003). This SKG model is dependent upon environmental stimuli and is absent in germ-free mice but can be induced by injection of zymosan in a dectin-1- dependent manner.

Human/SCID chimeric mice

Several investigators have exploited the ability of SCID mice to tolerate xenografts by implanting them with human synovial tissue. In the first model, human synovial tissue from RA patients and normal cartilage were implanted under the renal capsule of

SCID mice (Geiler, Kriegsmann et al. 1994). After 35 days, focal erosions occurred at sites of synovial attachment to the cartilage. After 105 days, activated synovial fibroblast cells invaded the cartilage leading to cartilage destruction. Thus, this model allows the pathology of the human synovium with cartilage invasion and destruction mediated by the synovial fibroblast to be studied in an animal model.

Human DR4-CD4 mice

Genetic susceptibility to RA is associated with a group of HLA class II alleles, which all share a similar stretch of positively charged amino acids at the HLA-DRB1 locus. A mouse model that included four separate transgenes: HLA-DR_0401 and human CD4 molecules, a RA-related human auto-antigenic protein (HCgp-39), and a TCR (TCR-ab) transgene specific for an important HCgp-39 epitope, allowed the analysis of strong Th1 responses in the context of HLA-DR_0401 (Fugger, Michie et al. 1994; Eming, Visconti et al. 2002). This mouse has been particularly useful for the study of the mechanisms involved in the breach of self-tolerance that occurs in RA.

Other spontaneous transgenic models of Arthritis

Several other spontaneous models have also been reported. For example, mice with deficiency of IL-1 receptor antagonist similarly develop spontaneous arthritis that is dependent upon environmental stimuli and is mediated through a strong Th17-polarized response (Koenders, Devesa et al. 2008). A homozygous mutation in the gp130 receptor results in enhanced STAT3 activation and the emergence of an inflammatory destructive arthritis (Sawa, Kamimura et al. 2006). The foregoing provides opportunities to explore the interface of T-cell mediated and innate immunity in inducing arthritis. A recent model further implicates DNA recognition in this

process (Kawane, Ohtani et al. 2006). DNase II-/-IFN-IR-/- mice and mice with an induced deletion of the DNase II gene develop an inflammatory polyarthritis associated with high levels of anti-CCP antibody and rheumatoid factor. The model is in part TNF-a-dependent, suggesting that incomplete DNA disposal by macrophages may lead to the dysregulated cytokine release.

3.4 Neutrophils

Neutrophils (polymorphonuclear leukocytes, PMNs) are highly specialized white blood cells characterized by a multilobulated nucleus and a granular cytoplasm. Neutrophils are abundant in blood, where they have a short half-life if they are not recruited to a site of inflammation by specific chemokines and cytokines. Once recruited to an inflammatory site, neutrophils migrate rapidly from blood to tissue, which is otherwise devoid of neutrophils (Edens and Parkos 2003). The abundance of neutrophils in blood and the efficiency of their recruitment and antimicrobial action make these cells an essential first line of defense of the innate immune system.

3.4.1 Origin

Neutrophil precursors first develop into a myeloblast, a relatively small (10 mm) cell that does not express granule proteins. Myeloblasts give rise to promyelocytes, which further differentiate into myelocytes, metamyelocytes, band cells (which are generally considered immature immune cells), and finally segmented neutrophils, which are then able to leave the bone marrow and enter the bloodstream (Bainton, Ullyot et al. 1971). After the promyelocyte stage, the differentiating neutrophils exit the cell cycle. Granules are formed continuously during the differentiation process from the promyelocyte stage onward. Primary (or azurophilic) granules are made in

promyelocytes, secondary (or specific) granules in myelocytes, tertiary (or gelatinase) granules in band cells, and secretory vesicles in segmented neutrophils (Borregaard 2010). They contain different types of granules packed with molecules that allow them to fulfill their antimicrobial function. Neutrophils develop in the bone marrow and emerge as terminally differentiated cells in circulation, where they live a short life (Pillay, den Braber et al. 2010; Tak, Tesselaar et al. 2013), unless called into action at an inflammatory site (Nathan 2006; Amulic, Cazalet et al. 2012).

The production of neutrophils is controlled by granulocyte colony stimulating factor (G-CSF) (Lieschke, Grail et al. 1994), which is produced in response to interleukin-17A (IL-17A) synthesized by T cells that regulate neutrophils (γδ and natural killer T cells) (Ley, Smith et al. 2006). Release of IL-17A is in turn under the control of IL-23 originating from tissue-resident macrophages and dendritic cells. During inflammation the number of neutrophils in tissues increases, and with time the cells die apoptotically and are removed by macrophages and dendritic cells. This process results in downregulation of IL-23 synthesis by those cells and thus reduces G-CSF release (Stark, Huo et al. 2005; Ley, Smith et al. 2006). The process of neutrophil maturation is under the control of transcription factors (Nerlov and Graf 1998).

3.4.2 Granules and secretory vesicles

There are three fundamental types of granules in neutrophils (**Figure 4**). Azurophilic granules (also known as peroxidase-positive or primary granules) are the largest, measuring approximately 0.3 μM in diameter, and are the first formed during neutrophil maturation. They are named for their ability to take up the basic dye azure A and contain myeloperoxidase (MPO), an enzyme critical in the oxidative burst

(Nusse and Lindau 1988; Lacy 2005). Other cargo of this granule class include the defensins, lysozyme, bactericidal/permeability-increasing protein (BPI), and a number of serine proteases: neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G (CG) (Faurschou and Borregaard 2003). As such, these granules contain antimicrobial compounds and function as a primary repository for the molecular weaponry of neutrophils. However, mouse neutrophils do not produce defensins at all (Faurschou, Sorensen et al. 2002). The second class of granules, the specific (or secondary) granules are smaller (0.1 μM diameter), do not contain MPO, and are characterized by the presence of the glycoprotein lactoferrin. These granules are formed after azurophilic granules; they also contain a wide range of antimicrobial compounds including NGAL, hCAP-18, and lysozyme (Faurschou and Borregaard 2003; Lacy 2005). The third class, the gelatinase (tertiary) granules, are also MPO-negative, are smaller than specific granules, and contain few antimicrobials, but they serve as a storage location for a number of metalloproteases, such as gelatinase and leukolysin. These granules are also the last population of granules formed during neutrophil maturation (Borregaard 2010). Finally, a fourth set of structures, the secretory vesicles, are also commonly considered part of the neutrophil granule family. In contrast to the classical granules, these do not bud from the Golgi, but instead are formed through endocytosis in the end stages of neutrophil maturation (Borregaard, Sorensen et al. 2007). Consequently, their cargo consists predominantly of plasma-derived proteins such as albumin. The membrane of secretory vesicles serves as a reservoir for a number of important membrane-bound molecules employed during neutrophil migration.

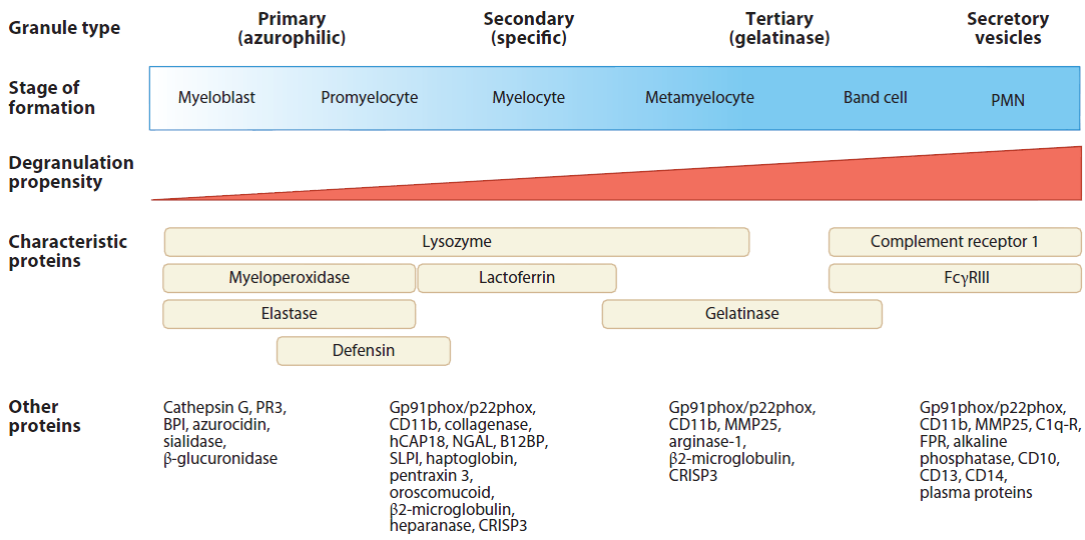


Figure 4: The stages of mature neutrophil generation. There are three different types of granules (azurophilic, secondary, tertiary) which are formed during the different stages of neutrophil maturation. Additionally, a fourth set of vesicles, the secretory exist but they are formed through endocytosis in the end stages of neutrophil maturation. Granules differ in their degranulation propensity with azurophilic granules exhibit the least one. *(Source: Amulic et al. Annu. Rev Immunol, 2012)*

3.5.3 Killing mechanisms

Neutrophils can eliminate pathogens by multiple means, both intra-and extracellular **(Figure 5)**. When neutrophils encounter microorganisms, they phagocytose them. After they are encapsulated in phagosomes, the cells kill the pathogens using NADPH oxygenase-dependent mechanisms (reactive oxygen species) or antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme) (Borregaard 2010; Hager, Cowland et al. 2010). The antibacterial proteins, as mentioned above, are released from the neutrophil granules either into phagosomes or into the extracellular milieu, thus acting on either intra-or extracellular pathogens, respectively. Highly activated neutrophils can eliminate extracellular microorganisms by releasing neutrophil extracellular traps (NETs). NETs are composed of a core DNA element to which histones, proteins (for example, lactoferrin and cathepsins) and enzymes (for example, MPO and neutrophil elastase) that are released from neutrophil granules are attached (Brinkmann, Reichard et al. 2004). NETs immobilize pathogens, thus preventing them from spreading but also facilitating subsequent phagocytosis of trapped microorganisms. They are also thought to directly kill pathogens by means of antimicrobial histones and proteases (Papayannopoulos and Zychlinsky 2009; Phillipson and Kubes 2011).

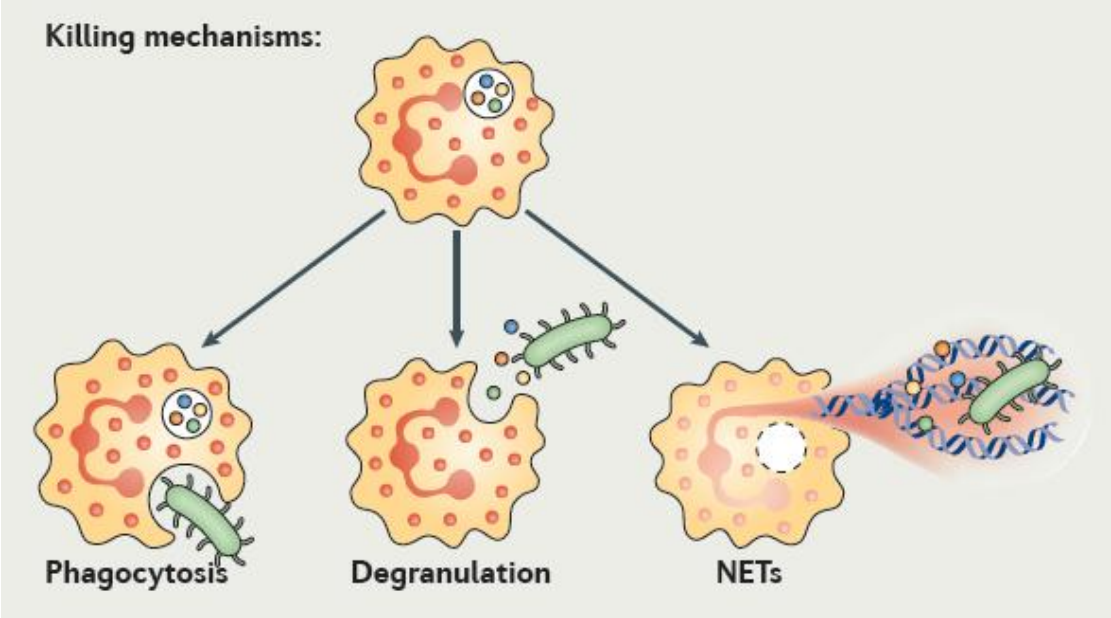


Figure 5: Killing mechanisms of neutrophils. Neutrophils employ different mechanism to combat a pathogen. Phagocytosis is the first mechanism of killing where neutrophils encapsulate the pathogen into phagosomes and kill either in a NADPH dependent mechanism or with antibacterial proteins. Antibacterial proteins are also able to externalize in the extracellular milieu during the process of degranulation. Another mechanism that neutrophils use to fight pathogens is neutrophil extracellular trap formation where the DNA complexed with the antimicrobial peptides is extruded to extracellular milieu and trap/kill the insults. *(Source: Kolaczkowskaet and Kubes, Nature reviews, 2013)*

3.5 Neutrophils in RA

Neutrophils are the most abundant cell type found in the synovial fluid. They also accumulate in the arthritic joints, where most of the tissue damage occurs (Mohr, Westerhellweg et al. 1981). Animal models of autoantibody-induced arthritis showed that neutrophils migrate to affected areas early in disease progression (Wipke and Allen 2001; Nandakumar, Svensson et al. 2003), where they produce enhanced oxidative responses to several stimuli (Dularay, Elson et al. 1988) and are, at least partially, responsible for the progression and severity of the disease. Several studies looking at cell recruitment to arthritic/inflamed joints in mice have revealed a signaling cascade initiated by the complement C5a receptor and Fcγ receptors, resulting in the release of the inflammatory mediator leukotriene B4 (LTB4) (Kim, Chou et al. 2006; Chou, Kim et al. 2010; Sadik, Kim et al. 2012) and IL-1b into the joint and subsequent neutrophil recruitment.

RA synovial neutrophils secrete a repertoire of cytokines and chemokines including TNF ligand superfamily member II (RANKL) (Chakravarti, Raquil et al. 2009) and TNFSF13B (also known as BLyS or BAFF) (Assi, Wong et al. 2007) which are implicated in the activation of osteoclasts and B lymphocytes, respectively. Activated neutrophils have been found in RA synovial fluid, synovial tissue and RA-associated skin disease (Barnhart, Riddle et al. 1967; Hughes, Erhardt et al. 1995; Ichikawa, Murata et al. 1998; Belcher, Doherty et al. 2002). Anti-granulocyte antibodies and ANCAs have also been described in RA (Cines, Passero et al. 1982; Lassoued, Sixou et al. 1991; Coremans, Hagen et al. 1993). Further, there is a prominence of neutrophil recruitment in RA animal models (Griffiths, Pettipher et al. 1995; Wipke and Allen 2001; Gal, Bajnok et al. 2005), with critical roles for these cells in initiating and maintaining joint inflammatory processes described in collagen-induced arthritis

(Matsubara, Yamamoto et al. 1991). Furthermore, various neutrophil function abnormalities have been reported in RA (Caccavo, Garzia et al. 2003). Exposure to immune complexes, rheumatoid factor and cytokines in synovial fluid results in neutrophil activation and granular content release that contributes to cartilage destruction (Mohr and Wessinghage 1978; Emery, Lopez et al. 1988).

The existence of these cells in RA progression is a fact but their exact role in disease initiation and perpetuation remains elusive.

3.6 Neutrophil extracellular traps (NETs)

NET formation, termed as NETosis in 2004, is a specific type of cellular death that differs from apoptosis and necrosis (Takei, Araki et al. 1996; Brinkmann, Reichard et al. 2004; Galluzzi, Vitale et al. 2012). NETs are produced predominantly by neutrophils, but also by other cell types of the innate immune system such as monocytes and macrophages (Chow, von Kockritz-Blickwede et al. 2010), eosinophils (Yousefi, Gold et al. 2008), basophils and mast cells (von Kockritz-Blickwede, Goldmann et al. 2008), in which the process is termed ETosis. Many inflammatory stimuli may trigger NETosis such as various bacteria, lipopolysaccharide (LPS), phorbol- 12-myristate-13-acetate (PMA), fungi or activated platelets. NETs are composed mainly of histones (account for 70% of total proteins of the traps), DNA and proteases, such as NE. In NETosis, neutrophils extrude large amounts of chromatin and granule proteins such as NE and MPO. This weapon can concentrate antibacterial substances and entrap invading microorganisms and eventually kill them.

NETs may have either the morphology of elongated thin filaments or can be cloud-like structures that occupy a 10-15 fold greater area compared to the initial cell size.

Several morphological modifications happen in the process of NETosis. Several minutes after activation, the cells lie flat, being tightly attached to the substrate. During the next hour the nucleus loses its lobules, chromatin is decondensed, and the inner and outer leaflets of the nuclear membrane separate. The disintegration of the granules occurs simultaneously. Within another hour, the nuclear membrane breaks up into separate vesicles, while the nucleoplasm and cytoplasm merge into a homogenous mass. Finally, the cells become rounded and seem to be contracted until the cytoplasmic membrane is broken. Then the cell contents are excreted to the exterior and form bundles of thin filaments.

On the molecular level, several successive steps happen a) ROS generation b) transport of neutrophil elastase and subsequently transport of the myeloperoxidase from the granules to the nucleus c) histone modification and d) disruption of cytoplasmic membrane and release of chromatin. Different mechanisms are active during NETosis. ROS are necessary for NETosis. ROS production is NADPH oxidase-dependent. In fact, the formation of hydrogen peroxide (H_2O_2) turns the cellular balance toward the inactivation of the tyrosine phosphatase throughout the oxidation of a cysteine residue. This enzyme inactivation seems to participate to the dismantling of the nuclear envelope and in the mixing of the NET components (Cui, Tan et al. 2012). Pharmacological inhibition of ROS using diphenyleneiodonium (DPI) or other inhibitors of NADPH oxidase completely abolished NETosis (Fuchs, Abed et al. 2007). Moreover, CGD (chronic granulomatus disease) patients who carry mutations in NADPH oxidase subunits cannot form NETs (Fuchs, Abed et al. 2007). The induction of Raf/MEK/ERK signaling pathway (Hakkim, Fuchs et al. 2011) and the Rac2 (a small GTPase of the Rho-family) mediated pathway are linked to NETosis induced by PMA (phorbol 12-myristate 13-acetate, a strong inducer of

NETosis) (Lim, Kuiper et al. 2011). During the initiation of NETosis neutrophil elastase (NE) and myeloperoxidase (MPO) (enzymes of azurophilic granules) move from the granules into the nucleus. NE, the first to be transported into the nucleus, catalyzes the cleavage of the linker histone H1 and modifies the core histones (Papayannopoulos, Metzler et al. 2010). Mice deficient in this enzymes were incapable of producing NETs (Papayannopoulos, Metzler et al. 2010). MPO which migrates later into the nucleus is involved in the chromatin decondensation, probably due to the synthesis of hypochlorous acid (Papayannopoulos, Metzler et al. 2010). Moreover, PAD enzymes and specifically PAD4 which catalyzes deimination of arginine residues (more details in 3.7) is also involved in the weaker binding of histones (3 out of 4) to DNA (Neeli, Khan et al. 2008; Neeli, Dwivedi et al. 2009; Wang, Li et al. 2009). Upon neutrophils activation by different stimuli, such as LPS, H_2O_2 or after calcium ionophore treatment in vitro, the PAD4 catalyzes, in a calcium dependent reaction, the conversion of arginine residues to citrulline in three of the four core histones (Mastronardi, Wood et al. 2006; Leshner, Wang et al. 2012).The citrullination prevents histone methylation and further transcription and contributes to the chromatin decondensation (Wang, Li et al. 2009). Another pathway that it seems to be important in NET formation is autophagy. Autophagy follows NADPH oxidase axtivation (Remijsen, Vanden Berghe et al. 2011). Under certain conditions stimulated NETs can be formed by mitochondrial DNA (Yousefi, Mihalache et al. 2009). These neutrophils exhibit an increased viability and survival rate compared to intact cells.

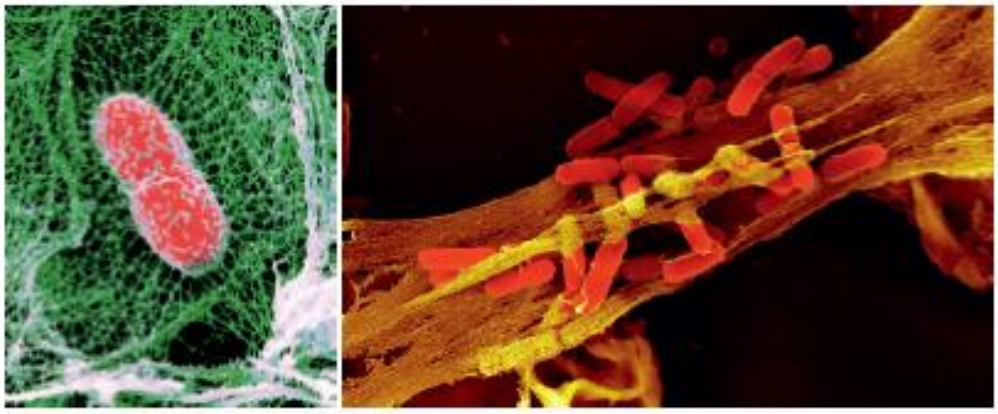


Figure 6: Neutrophil extracellular traps.

Left. A Klebsiella bacterium in the lung of a mouse has been caught in the NETs.

Right. NETs fight Shigella bacteria. The bacterial dysentery pathogens are killed by the antibacterial effect of the chromatin. (source: Brinkman and Zychlinsky).

3.7 Citrullination

Interestingly, more than 70% of patients with RA show a breach in tolerance for citrullinated proteins eliciting an anti–citrullinated protein antibody (ACPA) response (Vincent, de Keyser et al. 1999; De Rycke, Peene et al. 2004). This ACPA response is highly specific for RA and not found in other forms of autoimmune disease. The formation of ACPAs is closely related to certain HLA genotypes, like the shared epitope and environmental factors (such as smoking) (Harre, Georgess et al. 2012).

Citrullination is the post-translational modification of protein-bound arginine into the nonstandard amino acid citrulline, catalyzed by Ca2+ dependent peptidylarginine deiminases (PAD) enzymes. Each converted molecule (arginine to citrulline) leads to a 0.984 Da mass increase and the loss of one positive charge (Gyorgy, Toth et al. 2006) with substantial effect on the acidity of the amino acid side chain and a change of the iso-electric point (pI) (Orgovan and Noszal 2011). Moreover, it can influence

the hydrogen bond forming ability and the interaction with other amino acidic residues of the same protein or of another one leading to a conformational alteration and consequently to a possible functional alteration and change in the protein half-life. Finally, a new protein is created. Noteworthy, the modifications of proteins can generate new epitopes, thus causing the formation of new autoantigens.. Indeed, it is well established that small modifications can enhance the immunogenicity of the proteins, due to an enhanced protein unfolding and subsequent processing and exposure of the immunogenic epitopes (Carrasco-Marin, Paz-Miguel et al. 1998); to an increased uptake of the modified antigen by the antigen presenting cells (APC) (Wallberg, Bergquist et al. 2007); and to an improved presentation through enhanced recognition by the APC (Allison and Fearon 2000). Interestingly, it seems that certain citrullinated peptides fit better in the HLA-DRB1 antigen binding grooves – the so-called shared epitope (SE) – than the corresponding arginine containing peptides (Pratesi, Petit Teixeira et al. 2013). The conversion of peptidylarginine into peptidylcitrulline is catalyzed by PAD (Vossenaar, Zendman et al. 2003). To date, five isoforms of this enzyme have been identified with different tissue expression and consequently different functions (Baka, Gyorgy et al. 2012). PAD1 is predominantly expressed in the epidermis and the uterus. PAD2 has been found in muscle tissues, central nervous system (CNS), and hematopoietic cells, including mast cells and macrophages. PAD3 is localized in the hair follicles, while PAD4 has been found in neutrophils and eosinophils, spleen and secretory glands. Finally, PAD6 expression has been detected in eggs, ovaries, testis tissues, small intestine, spleen, lung, liver, skeletal muscle cells and in early embryos (Chavanas, Mechin et al. 2004; Zhang, Dai et al. 2004).

Various studies have described citrullination as an important mechanism in chromatin unfolding and NET formation (Neeli, Khan et al. 2008; Wang, Li et al. 2009).

N-α-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide or Cl-amidine (Luo, Arita et al. 2006), a pan-PAD inhibitor has previously been shown to inhibit PADs in mice without significant toxicity and to improve disease phenotypes in animal models of inflammatory arthritis and inflammatory bowel disease (Chumanevich, Causey et al. 2011; Willis, Gizinski et al. 2011). A PAD4-null strain of mice failed to produce NETs (Li, Wang et al. 2010; Hemmers, Teijaro et al. 2011). In addition, targeting PADs in two different lupus-prone mouse models have been shown to reduce NET formation *in vivo* and to protect against lupus-related organ damage including amelioration of rash, reduction of proteinuria and immune complex deposition in the kidneys, improvement of endothelial function and alteration of circulating autoantibody profiles and complement levels (Knight, Zhao et al. 2013; Knight, Subramanian et al. 2015).

Peptidylarginine deiminase 4 (PAD4) citrullinates proteins during RA progression. This process is crucial for the generation of NETs (Wang, Li et al. 2009). However, there is also a detrimental side to PAD4 activation, as RA patients have an abundance of ACPAs in their serum contributing to disease. Genetic studies show that RA patients with alleles linked to increased RA susceptibility have more stable PAD4 mRNA and as such may enhance PAD4 expression (Suzuki, Yamada et al. 2003).

3.8 Immunogenic role of NETs

Besides the beneficial role of NETs against pathogens, NETs can also lead to toxic effects in the host. In a susceptible individual, the release of potential autoantigens on

NETs (nucleic acid and proteins) in an inflammatory milieu, can initiate/propagate an autoimmune response (Grayson and Kaplan 2016).

Increasing evidence supports the immunogenic nature of NETs in different diseases. NETs contribute to immunogenicity by promoting the externalization of modified autoantigens, inducing synthesis of type I IFNs by pDCs, stimulating the inflammasome machinery in macrophages (Kahlenberg, Carmona-Rivera et al. 2013) and activating both the classic and alternative pathways of the complement system (Leffler, Martin et al. 2012). NETs may contribute directly to endothelial plaque burden and thrombosis (Knight, Zhao et al. 2013; Knight, Luo et al. 2014). Furthermore, NETs found in the marginal zone of the spleen can activate B cells in a T-cell independent manner and induce Ig class-switching and antibody production (Puga, Cols et al. 2012). Upon direct contact of NETs with T cells, T cells lower their activation thresholds and increase T cell responses to specific antigens (Tillack, Breiden et al. 2012). It is known that that there is a positive feedback loop involving NETosis and production of IFN-α. Netting PMNs were shown to activate pDCs in a TLR9 dependent manner, which in turn released IFN-α (Garcia-Romo, Caielli et al. 2011). LL37 present in NETs and NETs themselves trigger the inflammasome machinery by activation of caspase-1 in macrophages and the release of inflammatory cytokines. In ANCA-related pathogenesis immunization with NET-loaded DCs induced ANCA formation and vasculitis in murine models genetically predisposed to autoimmunity (Sangaletti, Tripodo et al. 2012).

Recently, it was proposed that neutrophils undergoing NETosis may also be the source of self antigens that give rise to autoantibodies in RA, such as ACPAs (Khandpur, Carmona-Rivera et al. 2013). Consistent with this hypothesis, analysis of NET components identified citrullinated vimentin, an important RA autoantigen that

decorated NETs (Khandpur, Carmona-Rivera et al. 2013) and neutrophils isolated from RA patients have been shown to exhibit enhanced NETosis (Khandpur, Carmona-Rivera et al. 2013). Independently, Pratesi et al. (2013) demonstrated that RA patients generated antibodies against histone H4 and that the source of this citrullinated protein was NETs (Pratesi, Dioni et al. 2014).

Moreover, in RA extracellular traps stimulated synovial fibroblasts to produce IL-6, IL-8, chemokines and adhesion molecules (Kim, Chou et al. 2006; Chou, Kim et al. 2010; Sadik, Kim et al. 2012). **However, their contribution in Rheumatoid arthritis pathogenesis regarding the autoreactive T cell responses remains elusive.**

3.9 T lymphocytes

T cells derive from hematopoietic stem cells and mature in the thymus. They are characterized by expression of a highly diverse antigen receptor, the TCR (Klausner, Lippincott-Schwartz et al. 1990). Unlike the B cell receptor antigen, TCR antigen recognition requires peptide presentation by human leukocyte antigen (HLA) molecules. The repertoire of TCRs is generated by somatic recombination at DNA level of TCR-encoding gene segments during T cell development. Since gene rearrangement occurs randomly, T cells that either express “nonself” TCR or a TCR with high affinity to self-antigens have to be selected through the mechanisms of central tolerance. Mature autoreactive T cells that escaped clonal deletion in the thymus can be inactivated in the periphery by different mechanisms (mechanisms of peripheral tolerance). Mature T cells express the pan-T cell marker CD3 and one of two co-receptors CD4 or CD8. Depending on the latter they are denoted as $CD4^+$ and

$CD8^+$ T cells. $CD8^+$ T cells exhibit cytoxic properties and therefore are also termed cytotoxic T cells. They are responsible for killing of virally infected cells and tumor cells by releasing cytotoxins such as perforin and granzyme (Peters, Borst et al. 1991; Heusel, Wesselschmidt et al. 1994). They recognize the cognate antigen associated with HLA class I molecules which is expressed by all nucleated cells in the body (Harty, Tvinnereim et al. 2000). $CD4^+$ T cells are also known as T helper (Th) cells and contribute mainly to the elimination of infectious agents by providing signals such as cytokines thus regulating the overall immune response. They are able to activate macrophages (Dalton, Pitts-Meek et al. 1993; Ethuin, Gerard et al. 2004), to provide B cell help (MacLennan 1994) as well as providing growth factors (Castellino and Germain 2006). Naïve T cells differentiate into different subsets of T helper cells, which secrete different cytokines and fulfill distinct effector function (Murphy and Stockinger 2010; O'Shea and Paul 2010). In contrast to $CD8^+$ T cells $CD4^+$ T cells bind to the cognate antigen associated with HLA class II molecule. HLA class II is mainly expressed in professional antigen-presenting cells (APCs), e. g. dendritic cells (DCs), monocytes, macrophages and B cells, but under certain conditions other cell types such as endothelial cells can also express HLA class II molecules (IFN-γ - induced) (Todd, Pujol-Borrell et al. 1985).

3.9.1 $CD4^+$ T cell activation

The antigen-specific activation of T cells requires three signals. The first one is provided by direct TCR-peptide/HLA class II contact (Doherty and Zinernagel 1975), the second signal by interaction of co-stimulatory molecules (Harris and Ronchese 1999) and the third signal is delivered by cytokines.

CD4+ T cells scan the environment of the local lymph node for the presence of professional APCs presenting antigens. After encounter with cognate antigen, both cells form a structure termed the “immunological synapse” (Grakoui, Bromley et al. 1999). The immunological synapse is a well organized structure within the T cell-APC contact area composed of accumulated TCR peptide/HLA complexes stabilized by adhesion molecules, e. g. lymphocyte-function associated antigen-1 (LFA-1) and inter-cellular adhesion molecule-1 (ICAM-1) binding (Dustin and Springer 1989; Seder, Germain et al. 1994). CD3 and co-receptors such as CD4, that are important for intracellular TCR signaling, mediate the specific binding of peptide/HLA class II complexes (Dianzani, Shaw et al. 1992; Zamoyska 1998). Co-stimulatory molecules (e. g. CD28:CD80/CD86 or cytotoxic T-lymphocyte antigen 4 (CTLA-4): CD80/CD86) (Linsley, Greene et al. 1992; Walunas, Lenschow et al. 1994; Kearney, Walunas et al. 1995; Harris and Ronchese 1999) are assembled in the immunological synapse to contribute to signal transduction and subsequent T cell activation. The formation of the immunological synapse results in phosphorylation of certain intracellular domains of the CD3- and TCR molecules and leads to further signaling events such as calcium-influx and gene expression (transcription factor, e. g. NFAT and NF-κB and subsequent secretion of e. g. cytokines such as IL-2). The magnitude of TCR stimulation is defined by the TCR-peptide/HLA binding strength (affinity), the sum of interactions of all co-stimulatory and adhesion molecules and co-receptors within the immunological synapse (avidity) and the duration of antigenic stimulation (Bachmann and Ohashi 1999; Iezzi, Scotet et al. 1999). The threshold of activation depends both on the affinities of these interactions and their timing (Viola and Lanzavecchia 1996). The threshold of activation can be further modulated e. g. by changes in the TCR-signaling pathways as shown in memory CD4+ T cells (Farber,

Acuto et al. 1997) which respond faster and stronger than naïve CD4+ T cells in response to a stimulus (Rogers, Dubey et al. 2000; McKinstry, Golech et al. 2007). $CD4^+$ T cells can also be activated by superantigens in an antigen- unspecific manner (e. g. staphylococcal enterotoxin B). Superantigens are often derived from bacteria and are able to cross-link the HLA-class II molecule and the TCR promoting T cell proliferation and cytokine production (Herman, Kappler et al. 1991).

3.9.2 $CD4^+$ T helper cell subsets and their functions

Due to the enormous variety of pathogens that enter the human body, the immune system has to provide T effector cells that elicit appropriate and successful immune responses. The differentiation of activated naïve T cells into distinct helper cell subsets is dependent on the cytokine milieu present at the time of activation and on the type of pathogen. The environment regulates the expression of characteristic transcription factors that drive the differentiation of naïve T cells into various T helper subsets termed Th1, Th2, Th17 or inducible regulatory T cells (iTreg). Macrophages produce IL-12 (Hsieh, Macatonia et al. 1993) that drives expression of signal transducer and activator of transcription (STAT)1/STAT4 (Jacobson, Szabo et al. 1995) and T-bet (Szabo, Kim et al. 2000) in response to intracellular pathogens. These are necessary for Th1 cell differentiation and the production of its major effector cytokine IFN-γ. Th1 cells are required for activation of macrophages (Dalton, Pitts-Meek et al. 1993; Ethuin, Gerard et al. 2004) and cytotoxic $CD8^+$ T cells (Castellino and Germain 2006). Infections with extracellular pathogens and parasites are controlled by Th2 cells and their effector cytokines IL-4, IL-5 and IL-13 (Mosmann, Cherwinski et al. 1986). Their differentiation is driven by IL-4 mediated by STAT6 (Kaplan, Schindler et al. 1996) expression that in turn induces GATA3 expression

(Zheng and Flavell 1997). TGF-β and IL-6 (Veldhoen, Hocking et al. 2006) mediate Th17 cell differentiation. They can be found at sites of infections with extracellular pathogens such as fungi. Th17 cells are also potent inducers of PMNs (Schwarzenberger, La Russa et al. 1998). The expression of RORγt (Ivanov, McKenzie et al. 2006) after activation of STAT3 (Yang, Panopoulos et al. 2007) governs Th17 differentiation and the production of IL-17A and IL-17F. Th1 and Th17 cells are involved in the development of organ-specific autoimmune diseases (Ghoreschi, Laurence et al. 2011), and Th2 cell can be associated with allergy. Besides these a fourth helper subset has been defined, i. e. regulatory T cells (Tregs) with immunomodulatory function.

3.9.3 Role of Th1 and Th17 in RA

RA pathogenesis is linked to autoreactive Th1 and Th17 immune responses and the release of proinflammatory cytokines such as TNF-a and IL-6 (Keffer, Probert et al. 1991; Feldmann, Brennan et al. 1996; McInnes, Leung et al. 1997; Burger, Zvaifler et al. 2001; Brennan, Hayes et al. 2002; Wong, Quinn et al. 2006). Dendritic cells (DCs) also play an important role in RA progression since they mediate the polarization of T cells. They are characterized as professional antigen presenting cells since they carry the Ags to the draining lymph nodes (dLNs) and promote activation, differentiation and polarization of naïve T cells into effector Th cell subsets.

Since different treatments (DMARDs and biological agents) have shown variable responses in individual patients with rheumatoid arthritis, the development of targeted treatment strategies constitute an unmet need. Notably, most treatments that have so far reached clinical practice target the innate part of the immune response. Only the last decade drugs targeting the adaptive immune response have been introduced in the

clinic. The first treatment was the TNF blockage followed by the combination of methotrexate treatment, a drug which acts by inhibition of adenosine metabolism (Montesinos, Takedachi et al. 2007; Wessels, Huizinga et al. 2008) and T-cell activation (Lange, Bajtner et al. 2005) and by affecting folate synthesis (Smolen, Beaulieu et al. 2008). This was followed by anakinra, an antagonist of interleukin-1 for binding to its receptor still less effective than TNF blockade and tocilizumab, a monoclonal antibody directed against the interleukin 6-receptor which reduces inflammation and erosions (Smolen, Beaulieu et al. 2008). Recently, different treatments that target T and B lymphocytes were developed. Among them are abatacept, a recombinant fusion protein that inhibits co-stimulatory signals-essential for T cell activation and rituximab, a monoclonal antibody that targets pre-B and mature B cells contributing in their elimination (Kremer, Westhovens et al. 2003; Edwards, Szczepanski et al. 2004). The above treatment strategies along with the long-established association of RA patients with the human leukocyte antigen (HLA)-DRB1 locus suggests the influence of T cell selection and antigen presentation in the induction and perpetuation of RA (Stastny 1976; Gregersen, Silver et al. 1987). Furthermore, in mice adoptive transfer of collagen specific T cells into SCID recipients can cause disease (Kadowaki, Matsuno et al. 1994). A local Th1 response is important in initiating inflammation (during the clinical disease onset) through recruiting cells into the joint and inducing mononuclear phagocytes to secret TNF-a (Fox 1997; Choy and Panayi 2001). Cytokine production kinetics have shown that during the initial response following CII/CFA challenge, Th1 response is dominated with IFN-γ being detectable in the LNs matched by the presence of IL-12 production by cells in the spleen and peritoneal cavity (Okamoto, Gotoh et al. 2000). On the other hand, many reports implicate (Kotake, Udagawa et al. 1999; Ziolkowska, Koc et al.

2000) IL-17 in RA and CIA pathogenesis. IL-17 producing Th17 cells are associated with late stages of the disease and the production of inflammatory mediators and this is supported by reports showing that Il-17 is increased at the site of inflammation (synovium) in patients with RA (Fossiez, Djossou et al. 1996). Mice genetically deficient in IL-17A were reported to be less susceptible to CIA and neutralization of IL-17 reduces disease severity (Nakae, Nambu et al. 2003).

These studies indicate that adaptive immune responses are definitely involved in RA. Thus, it is intriguing to delineate the effect of a novel innate immune mechanism such as NETosis in the autoreactive immune response underlying RA pathogenesis.

4. OBJECTIVES

The present study sought to delineate the role of Neutrophil extracellular traps in the pathogenesis of Rheumatoid Arthritis. Specifically, the objectives of the study were:

- **to evaluate the capacity of RA PMNs (derived from RA patients) to spontaneously release NETs**
- **to dissect the contribution of RA inflammatory environment (serum and synovial fluid) in NET generation**
- **identify molecules that mediate NETosis in the inflammatory environment of RA**

Results obtained from the previous objectives indicated that there is increased NET formation in RA patients and the RA inflammatory milieu favors the production of NETs. Therefore, we set the following questions:

- **What is the impact of the increased NET formation in RA pathogenesis?**
- **What are the functional consequences in the disease development?**
- **What is the underlying mechanism of NET formation in RA?**

To assess the previous questions we used the collagen-induced arthritis mouse model that shares many similarities with RA.

5. MATERIALS AND METHODS

5.1 Human subjects

Peripheral blood samples were obtained from patients diagnosed with RA according to the 1987 American College of Rheumatology (ACR) criteria(Arnett, Edworthy et al. 1988), followed by the Rheumatology Clinic of the University Hospital of Crete. SF was collected from active RA patients, centrifuged at 800 x g for 15min at 4°C and supernatants were stored at -80°C until used. Serum was also isolated from patients or healthy donors. The study was approved by the Ethics Committee of the University Hospital of Heraklion, University of Crete School of Medicine, and all participants gave informed consent.

5.2 Reagents

Fluorescent-conjugated monoclonal antibodies to CD80 (16-10A1), CD86 (GL-1), CD11c (N418), CD4 (RM4-5), CD25 (pc61), CD44 (IM7) were all from Biolegend, Ly6G (1A8) and IFN-γ (XMG1.2) from BD Pharmingen,, MHC class II (M5/114.15.2) from Miltenyi, IL-17 (eBio17B7) and brefeldin A from eBioscience.

Dulbecco's modified Eagle's medium (DMEM-glutamax, Gibco), RPMI-1640 (Gibco), fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml), all from Gibco, Carlsbad, CA; PMA, ionomycin, saponin and A23187 calcium ionophore were from Sigma-Aldrich. Murine PMNs were isolated with Percoll (Sigma) and cultured in RPMI-1640 supplemented with l-glutamine, 2% bovine serum albumin (BSA) (Gibco), and 10 mM HEPES (Gibco). PBS tablets were from Gibco.

For immunofluorescence the following primary antibodies were used: polyclonal anti-neutrophil elastase (anti-NE) Ab (Abcam), polyclonal anti-human myeloperoxidase (anti-MPO) (Dako), anti-citrullinated H3 (Abcam), CF488–labeled anti-rabbit Ab (Biotium), CF555-conjugated goat anti-rabbit IgG (H+L) Ab (Biotium) and DAPI (Sigma).

5.3 Mice

Male DBA/1J and C57BL/6 (B6) mice (7- to 12- wk old males) were obtained from the SPF facility of the Institute of Molecular Biology and Biotechnology (IMBB Heraklion Crete, Greece). OT-II TCR transgenic mice were obtained from Biomedical Research Foundation of the Academy of Athens. All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office.

5.4 CIA induction, treatment groups and scoring system

For induction of CIA, DBA/1J mice were injected intradermally (i.d.) at the base of the tail with 100 μl complete Freund's adjuvant (CFA, Sigma), containing 200 μg bCII (MD Biosciences) and 250 μg inactivated Mycobacterium tuberculosis (H37Ra; Difco) on d0. . On d21 mice were given a boost immunization intradermally (i.d.) at the base of the tail with 100 μl bCII emulsified 1:1 with incomplete Freund's adjuvant (IFA, Sigma) (Raptopoulou, Bertsias et al. 2010).

Each experimental group consisted of eight mice that were treated daily after the d21 booster injection through sacrifice on d35-45 with one of three interventions: no injection, PBS/DMSO (vehicle control) or 10 mg/kg Cl-amidine (kindly provided by

P. R. Thompson). All injections were given i.p. and doses were calculated for the average weight of the group (20 g). Mice were scored five times a week from d21 according to the following scoring key: 0 points if no visible clinical symptoms, 1 point per swollen toe [regardless of whether this includes one or two joints (exclusive digit I in both front paws), 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. Blood was collected by retro-orbital aspiration at the end of the study and the isolated plasma was stored at –80°C for later batch processing. At d35-45, all animals were sacrificed by anesthesia with diethyl-ester and cervical dislocation.

5.5 Histological examination

Tissue was fixed in 4% buffered formalin solution, routinely processed and embedded in paraffin. Bone was previously decalcified in Schaefer solution. Four µm-thickness sections were stained with hematoxylin & eosin (H&E) and evaluated by a Nikon Eclipse E-400 light microscope. Histological grading of arthritis of the proximal limb joint (knees) from each animal was performed according to the following five scale grading system 0= normal histology of the knee, 1= normal synovium or mild synovial reaction with scarce inflammatory cells, 2= moderate synovial reaction and moderate density of inflammatory cells, 3= severe synovial hyperplasia and dense population of inflammatory cells, 4= pannus formation associated with articular cartilage erosion. Histopathology pictures were captured using a Nikon Digital sight, DS-SM, photographic system.

5.6 Measurement of type II collagen-specific antibodies in plasma

Plasma samples were collected at the peak of the disease and the levels of anti-CII IgG were measured by ELISA as described(Raptopoulou, Bertsias et al. 2010). Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin IgG (Millipore) was used as secondary antibody and 3,3',5,5'-Tetramethylbenzidine (TMB) as substrate.

5.7 Isolation of PMNs

Human: peripheral blood (PB) PMNs were isolated from heparinized blood using density gradient separation according to the protocol (Kambas, Markiewski et al. 2008). Briefly, a double gradient was formed by layering an equal volume of histopaque-1077 (GE-healthcare) over histopaque-1119 (Sigma). Venous blood was collected in heparinized tubes and carefully layered onto the upper histopaque-1077. The blood was centrifuged at 700xg for 30 min. The granulocytes were found at the 1077/1119 interphase. The cells were collected, 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.

Mice: BM PMNs were isolated using modifications of published methods (Boxio, Bossenmeyer-Pourie et al. 2004; Ermert, Urban et al. 2009). Briefly, the BM was flushed out of the tibia and the femur using PBS with 15mM EDTA. After centrifugation at 400xg for 10min, cells were resuspended in 1ml PBS/EDTA. The cells were overlaid onto a 3-layer Percoll gradient 75%, 67% and 52% Percoll respectively, diluted in PBS (100% Percoll= 9 parts Pecoll and 1 part 10 x PBS) and

centrifuged at 1000xg for 30min. The neutrophis were harvested from the 67%/75% interface after carefully removing the cells from the upper phases. After one wash with PBS/EDTA-1%BSA remaining red cells in the PMN fraction were eliminated by hypotonic lysis. After a final wash with HEPES buffer (140mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES, pH 7.2) cells were resuspended in RPMI without phenol red supplemented with l-glutamine, 2% BSA, 10mM HEPES and used in further experiments. Cells were more than 85% Ly6G-positive by flow cytometry and had the typical segmented nuclei of mature PMNs by microscopy.

5.8 Generation, assessment and quantification of NETs (human, mouse)

Primary human PMNs (healthy or RA) or mouse PMNs from bCII-injected or control mice were seeded onto coverslips coated with 10% poly-l-lysine (Sigma-Aldrich). Primary human PMNs were cultured for 3h at 37°C, 5% CO_2 and murine BM-derived PMNs incubated for 4h at 37°C, 5% CO_2.. Cells were fixed with 4% parafolmadehyde (PFA), blocked with 5% BSA and were permeabilized with 0.5% triton X-100. DNA was stained with 300nM DAPI. Protein staining was with anti- NE or with anti- MPO primary Ab for 1 h at RT, followed by 1 h incubation (RT) with CF488–labeled or with CF555-conjugated secondary Ab. Three washes with 0.5% BSA/PBS were performed in-between all stainings. After staining, coverslips were mounted on Mowiol 4-88 (Sigma-Aldrich) and were observed under confocal microscope (Leica SP8). NETs were manually quantified by 2 blinded observers. Decondensed nuclei (stained with DAPI) which also stained positively for NE or MPO, were considered NETs. The percentage of NETs was calculated as the average of at least 5 fields,

normalized to the total number of cells. DNA was quantified with a PicoGreen dsDNA kit (Invitrogen).

For NETs isolation, human RA PMNs (1.5 x 10^6) or murine CIA BM-derived PMNs (2 x 10^6) were seeded in 6-well tissue culture plates and cultured for 3h or 4h, respectively. Wells were carefully washed once with pre-warmed medium and supernatants were vigorously collected and spun at 50g for 5min at 4°C to remove intact cells. Thus, the supernatant obtained after centrifugation (RA-NETs or CIA-NETs) were lyophilized resuspended in the appropriate volume of medium (3x condensed) and were used for the coculture experiments.

5.9 Generation of BMDCs and DC-NETs coculture experiments

DCs were generated from BM progenitors of DBA1/J mice and B6 mice(Lutz, Kukutsch et al. 1999) and RBC were lysed with NH_4Cl. On d 0, 3, 6, and 8, cultures were supplemented with fresh complete DMEM glutamax containing 20% X63Ag8 supernatant (derived from a murine granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting cell line; kindly provided by B. Stockinger, National Institute of Medical Research, London, U.K.)(Zal, Volkmann et al. 1994). 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 5% of concentrated supernatants derived from bCII-injected (CIA-NETs) or naïve (control) mice. Culture supernatants and cells were collected 18h after stimulation and cytokine levels were determined by ELISA. Cells were acquired after surface staining on a FACS Calibur.

5.10 Assessement of OVA specific T cell responses

LNs from OT-II transgenic mice were excised and single cell suspensions were prepared (Tiniakou, Drakos et al. 2015). LNCs were subsequently cultured in complete DMEM medium in flat-bottom 96-well plates at a density of 4 x 10^5 cells / 200μl / well in the presence or absence of DCs (10^5 cells) previously pulsed with OVA endotoxin free (20μg/ml, Hyglos) and/or 5% of concentrated CIA-NETs or control supernatants . LNCs were harvested 48 h after coculture and subjected to intracellular cytokine staining for IL-17 and IFN-γ.

5.11 Intracellular cytokine staining

Cultured OT-II LNCs or inguinal dLNCs isolated from CIA or CIA Cl-amidine administered mice stimulated with PMA (50ng/ml) - ionomycin (2μg/ml) for 6 h at 37°C, 5% CO_2 in complete DMEM medium. Brefeldin A (10μg/ml) was added for the last 2 h. Following staining of surface markers, cells were fixed with parafolmadehyde (PFA) and permeabilized with 0.5% saponin buffer (Alissafi, Hatzioannou et al. 2015). Cells were then incubated with IL-17 and IFN-γ mAbs . Cells were acquired on FACSCalibur (BD) and analyzed using FlowJo software (Tree Star).

5.12 Generation of moDCs and moDC-NETs coculture experiments

Monocytes were positively selected from human peripheral blood of healthy volunteers using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions and cells were cultured in complete RPMI 1640 medium for 5 days with

the addition of human GM-CSF and recombinant IL-4 (both from Peprotech) at d0 (100ng/ml) and d3 (50ng/ml). After 5 days of culture, non-adherent cells were harvested and moDCs were subsequently cultured in complete RPMI medium in flat-bottom 96-well plates at a density of 4 x 10^5 cells / 200µl / well in the presence or absence of 5% of concentrated supernatants derived from RA (RA-NETs) or healthy PMNs (control). Culture supernatants and cells were collected 18h after stimulation. Cytokine levels were determined by ELISA and the cells were acquired after surface staining on a FACS Calibur.

5.13 Western blot analysis

Control BM PMNs were lysed on ice in SDS lysis buffer (2% Sodium Dodecyl sulfate-SDS, 62.5mM Tris pH 6.8, 5% 2-Mercaptoethanol, 10% glycerol) supplemented with Complete Protease inhibitor cocktail (Roche) and centrifuged at 13,000 x g for 30min at 4°C. Whole-cell lysates (40 µg protein) were subjected to SDS-PAGE electrophoresis on 15% gels and then transferred to an Immobilon-P^{sq} membrane (Millipore). Membranes were blocked with 5% BSA in TBST and then incubated with anti–MPO (1:200) as loading control and anti-citrullinated-H3 (1:100). Detection was performed using HRP-linked Abs (Cell Signaling Technology) and enhanced chemiluminescent (Frasnelli, Tarussio et al.) detection reagents (Amersham Biosciences).

5.14 ELISA

Detection of mouse IL-6 and TNF-α Duo set (R & D systems) and human IL-6 and TNF-α (eBioscience) in culture supernatants harvested at the indicated time, were performed by sandwich ELISA following the manufacturer's recommendations. Light absorbance at 450 nm was measured using the ELx800 Biotek.

5.15 Flow cytometry

Cells were stained for extracellular markers for 20 min at 4°C in PBS/5% FBS. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

5.16 Statistics

Statistical analysis was performed with Prism software v6.01 (GraphPad Software). Student t test (two-tailed, 95 % confidence interval) was used, unless indicated otherwise. *p* values less than 0.05 were considered statistically significant.

6. RESULTS

6.1 Enhanced spontaneous NET formation in RA peripheral blood PMNs

NETs have been demonstrated to be a prominent feature in RA polymorphonuclear cells (PMNs) (Khandpur, Carmona-Rivera et al. 2013). Thus, we sought to assess NETosis in a cohort of RA patients with high inflammatory burden (clinical characteristics are in table 1). We found that RA PMNs display increased propensity to spontaneously form NETs compared to control PMNs isolated from healthy donors (percentage 8.09 ± 0.87 vs 3.53 ± 0.48 **Figure 1A, B**). Furthermore, we classified RA patients into RF (+) and RF (-) RA patients and we monitored whether there was a differential NET release between the two groups. Specifically, a significant increase in NET formation in RF (+) ve as compared to RF (-) ve RA patients, was found (extracellular DNA ng/ml 442.2 ± 68.42 vs 147.4 ± 11.49 **Figure 1C**). These results indicate that RA PMNs are more prone to NETosis and this is correlated with the levels of autoantibodies found in their sera.

6.2 RA inflammatory milieu triggers NET formation

We next assessed, whether RA inflammatory milieu could drive NET formation. In order to assess the capacity of soluble factors to induce NETosis, PMNs were isolated from healthy donors and treated with 2% serum or SF isolated from RA patients or healthy serum. It was found that both RA serum and SF are potent inducers of NETosis in healthy PMNs (percentage 14.36 ± 1.61 for RA serum, 10.23 ± 2.03 for RA SF vs 3.53 ± 1.61 for healthy serum **Figure 2A**). Furthermore, serum or SF derived from RF (+) ve patients induced higher NETosis as compared to the serum or SF from RF (-) ve RA patients (extracellular DNA fold increase normalized to

healthy serum 1.77 ± 0.13 vs 1.14 ± 0.29 for SF, 1.04 ± 0.08 vs 0.76 ± 0.09 for RA serum **Figure 2B, C**). Collectively, the RA inflammatory burden is associated with the increased rate of spontaneous NET release. In addition, the existence of autoantibodies further augments NETosis which is in accordance with previous reports (Khandpur, Carmona-Rivera et al. 2013).

Table 1. Demographic and clinical characteristics of RA patients

Variable	**RA (n= 30)**	**Control (n=15)**
Age (mean±SD)	63 ± 12,63	54± 22
Gender (% females)	67%	52%
Disease duration (months mean±SEM)	44,4 ± 6,55	
RF (% positive and mean titer±SEM, IU/mL)	47% (229,2 ± 15,77)	
ACPA (% positive and mean titer ±SEM, U/mL)	37% (329,1 ± 133,6)	
ESR (mean±SEM, mm/h)	40,6 ± 3,64	
CRP (mean ±SEM, mg/dL)	4,5 ± 1,64	
Disease activity score 28 joints (DAS28)	6,3 ± 0,78	
Medications (% taking)		
Naïve to treatment,	23%	
Methotrexate	45%	
Leflunomide	20%	
Biologic agents	45%	
Glucocorticoids	43%	

SD: standard deviation; SEM: standard error of mean; RF: rheumatoid factor; ACPA: anticitrullinated protein antibodies; ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

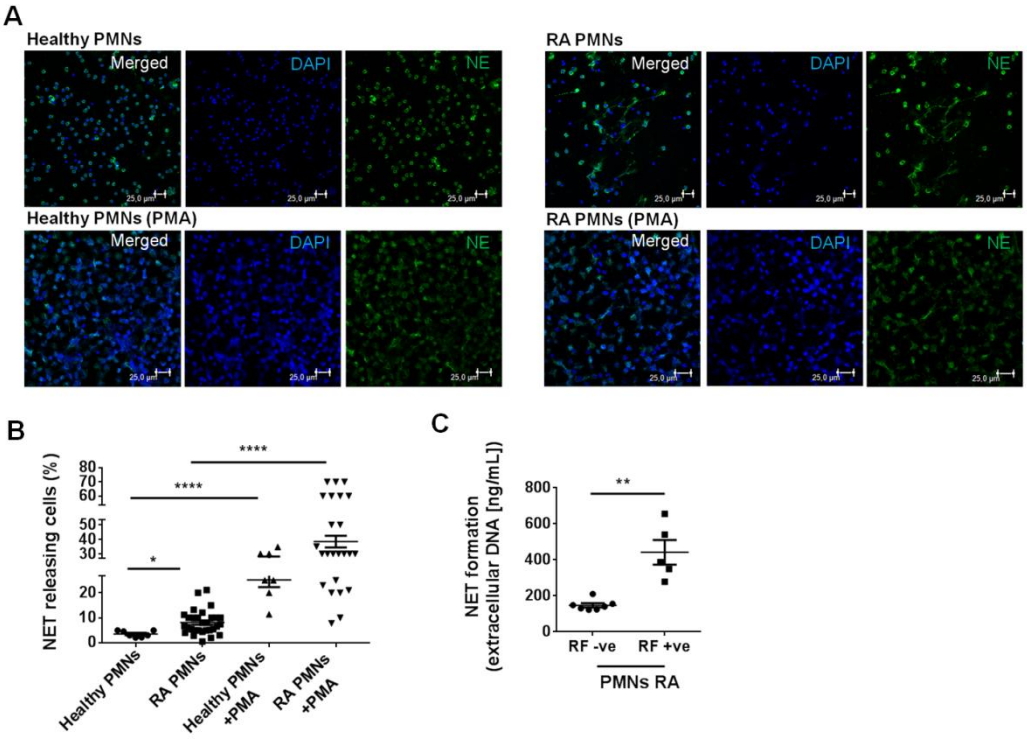


Figure 1. Enhanced spontaneous NET formation in RA peripheral blood PMNs. (A) Confocal microscopy images of RA (right panel) versus healthy PMNs (left panel). NETs were visualized as structures costained for NE (green) and DAPI (Bluestone and Abbas). Original magnification, 40x; scale bar, 25μm. Images are from a single experiment representative of ten independent experiments with 2-3 donors per experiment. (B) Quantification of NET release by RA PMNs (n=30) versus healthy untreated PMNs (n=7) under microscopy observation. PMA was used as positive control. Results are expressed as mean ± SEM and are combined from five independent experiments with 2-5 donor samples per experiment. (Unpaired *t*-test, **p*=0.018, ****p<0.0001). (C) Quantification of NETosis based on the spontaneous release of extracellular DNA between rheumatoid factor (RF) (+) ve (n=5) and RF (-) ve (n=7) RA PMNs and ***p*=0.0025.

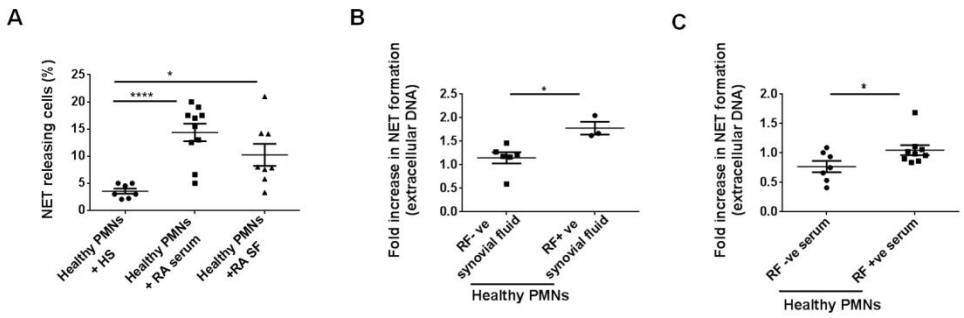


Figure 2. RA inflammatory environment favors NET formation. (A) Percentage of NET releasing cells upon treatment with RA serum (n=10) and RA synovial fluid (SF) (n=8). Healthy serum (HS) (n=7) was used as negative control. Results are expressed as mean ± SEM. Data are combined from three independent experiments with PMNs from 2-3 donors per experiment. (Unpaired *t*-test, **p*=0.010, ****p<0.0001). The release of NETs by healthy PMNs treated with RF (+) ve and RF (-) ve (B) synovial fluid (n=3 and 6, respectively) **p*=0.015 and (C) sera (n=7 and 9, respectively) **p*=0.04, is shown expressed as the amount of the released extracellular DNA normalized to the amount of extracellular DNA released by healthy PMNs treated with healthy serum (fold increase in NET formation). Results are expressed as mean ± SEM and are combined from three independent experiments (unpaired t-test).

6.3 BM PMNs from diseased CIA mice exhibit increased NETosis compared to control BM PMNs

To delineate the role of NETs in the adaptive immune responses we used the CIA mouse model which shares many similarities with human RA. It constitutes a well

defined model for experimental autoimmunity where both B- and T-cell mediated immune reactions appear to contribute the development of the disease (Trentham, Townes et al. 1977). We first sought to establish whether aberrant NETosis also occurs in CIA mouse model. Thus, we found that BM PMNs from CIA diseased mice (d35-45 post collagen injection) had higher numbers of spontaneously released NETs, characterized as DNA structures complexed with neutrophil elastase (NE) compared to BM PMNs isolated from naïve mice (percentage12.76 ± 0.4 vs 6.37 ± 0.51 **Figure 3A, B**). Moreover costaining of DNA with anti-citrullinated H3 revealed that externalized NETs contained citrullinated epitopes (**Figure 3C**). Together these data show that NETosis is also induced in CIA mouse model and these NETs may externalize citrullinated potential antigens.

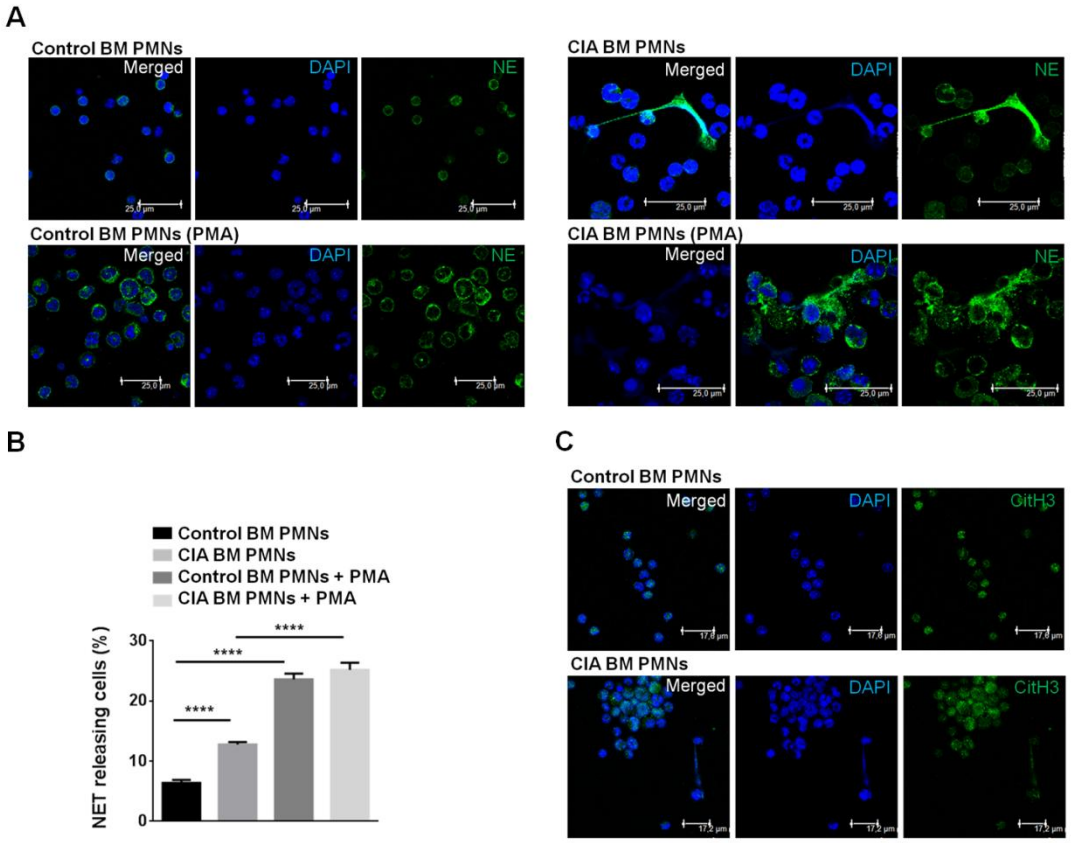


Figure 3. Increased NET release by BM PMNs derived from CIA diseased mice. (A) Confocal microscopy images of BM PMNs ($Ly6G^+$) derived from diseased CIA and control (naïve) mice. Original magnification, 63x; digital zoom, 3x. Scale bar, 25μm. (B) Quantification of NET release by BM PMNs derived from diseased CIA mice versus control mice (n=5 mice/group) under microscopy observation based on NE and DAPI costaining (Right panel). PMA was used as positive control. Results are expressed as mean ± SEM and are representative from four independent experiments with 8 mice/group per experiment. (Unpaired *t*-test, ****$p<0.0001$). (C) Representative confocal microscopy images display spontaneous NETosis in CIA BM PMNs versus control PMNs. NETs were positively stained with citrullinated H3 (citH3-green) and DAPI (Bluestone and Abbas). Original magnification, 63x; digital zoom, 3x. Scale bar; 17.2-17.6 μm. Representative results from four independent experiments.

6.4 CIA inflammatory environment triggers NETs by naïve BM PMNs.

Next, we asked whether the inflammatory environment of CIA could induce NET formation. To this end, BM PMNs from naïve DBA/1 mice were treated with 2% CIA or naïve mouse plasma. Interestingly, CIA plasma induced significantly elevated NET release compared to naïve plasma that failed to induce NETosis, as evidenced by the colocalization of DNA with NE (percentage 24.8 ± 1.39 vs 9.56 ± 0.45 **Figure 4A, B**). Collectively, these results provide evidence for an increased NET formation by PMNs in CIA model and corroborate human data that NETs are driven by CIA inflammatory milieu.

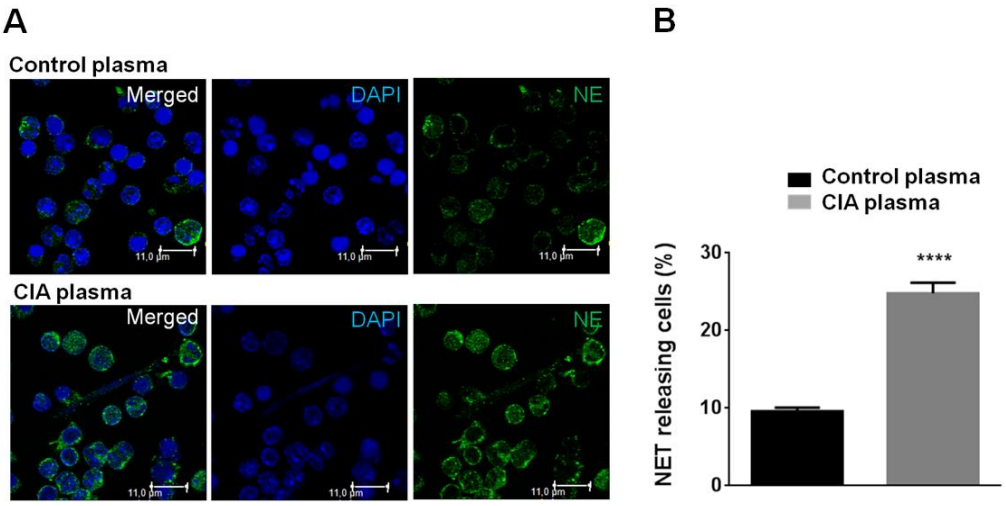


Figure 4. CIA inflammatory environment augments the formation of NETs. (A) Confocal microscopy analysis for NET formation by naïve BM PMNs treated with CIA diseased and naïve plasma. DAPI (Bluestone and Abbas) and NE (green) stainings are shown. Original magnification, 63x; digital zoom, 3x. Scale bar, 11µm. (B) Quantification of NETs released by naive BM PMNs treated with CIA diseased (n=5) or naïve plasma (n=5) under microscopy observation. Results are expressed as mean ± SEM and are representatives from four independent experiments using plasma isolated from five mice per group. (Unpaired *t*-test. ****p<0.0001).

6.5 Cl-amidine treatment during CIA reduces NETs release

PAD4 inhibition has been shown to prevent NETs (Knight, Zhao et al. 2013; Knight, Luo et al. 2014) and thus Cl-amidine is considered as an inhibitor for NET formation. Cl-amidine, is a highly specific inhibitor of PAD4 and related intracellular and extracellular PAD isozymes(Luo, Knuckley et al. 2006). Indeed, in vitro Cl-amidine

treatment prevented hypercitrullination of histone H3 in naïve PMNs as shown by western blot **(Figure 5)**.

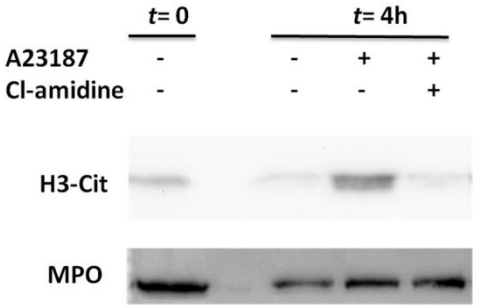


Figure 5. Cl-amidine treatment inhibits histone H3 citrullination *in vitro*. Control BM PMNs ($Ly6G^+$) were treated for 4 hours with 2μM A23187 calcium ionophore in the presence or absence of 200 μM Cl-amidine. Whole-cell lysates were resolved by SDS-PAGE. MPO expression was used as loading control. A Western blot representative of 3 independent experiments is shown. H3-Cit, citrullinated histone H3.

Given that citrullination (catalyzed by PAD4) is also an important mechanism during RA pathogenesis, we sought to investigate the phenotypic and immunological impact of PAD4 inhibition – as an inhibitor of NETosis – in the CIA model. We initially examined the effect of Cl-amidine administration in BM derived CIA-NETs. To address this, we administered Cl-amidine intraperitoneally (i.p.) (or DMSO as vehicle control) daily from d21 through d45 after priming with bovine type II collagen (bCII) **(Figure 6A)**. We found that NETs release was significantly decreased in CIA-Cl-amidine administered mice compared to CIA-DMSO animals as assessed by the costaining of DNA with NE (percentage 8.16 ± 1.67 vs 15.41 ± 1.68 **Figure 6B**).

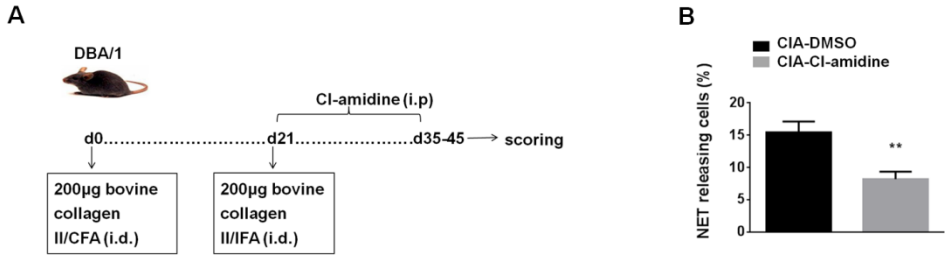


Figure 6. Cl-amidine treatment during CIA reduces NET formation.

(A) Outline of the experimental setup. DBA/1J mice were injected intradermally (i.d.) at the base of the tail with bovine collagen type II (bCII) emulsified in complete Freund's adjuvant (CFA) with the addition of inactivated Mycobacterium tuberculosis on d0, followed by a boost immunization of bCII emulsified in incomplete Freund's adjuvant (Klausner, Lippincott-Schwartz et al.) on d21. Each experimental group consisted of eight mice that were treated daily intraperitoneally (i.p.) with Cl-amidine or PBS/DMSO (vehicle control) after the d21 booster injection through sacrifice on d35-45 (B) Quantification of NET release by BM PMNs ($Ly6G^{+}$) derived from diseased CIA-DMSO mice (n=8) versus CIA-Cl-amidine administered mice (n=6) under microscopy observation based on NE and DAPI costaining. Results are expressed as mean ± SEM and are combined from three independent experiments with 2-3 mice/group per experiment. (Unpaired *t*-test, $**p<0.0065$).

6.6 Cl-amidine treatment during CIA reduces clinical disease activity and joint destruction

Importantly, CIA-Cl-amidine administered mice experienced significantly decreased severity and disease onset as compared to vehicle control administered mice.

Specifically, mice receiving daily Cl-amidine exhibited >50% decrease in clinical disease score on days 25-32 compared to mice administered vehicle control (CIA-DMSO) **(Figure 7)**. Represenative histological analyses of proximal knee joints were performed in 2 mice per group at the end of each experiment, at d32 and 39 post immunization (p.i.). Specifically, in the proximal knee joint severe arthritis with pannus formation was observed in CIA-DMSO mice (**Figure 8A** upper panel and lower panel; grade 4), as compared to moderate arthritis in CIA-Cl-amidine group (**Figure 8A** upper panel and lower panel; grade 1). In addition, CIA-Cl-amidine treated mice exhibited decreased intensity of inflammation and better integrity of bone structure in the proximal joint compared to the CIA-DMSO group **(Figure 8A)c**. Collectively, these results suggest that Cl-amidine treatment reduces NET formation by BM PMNs, attenuates clinical disease severity and decreases joint inflammation during the effector phase of the disease in CIA model.

Another hallmark of RA pathogenesis and CIA is the generation of autoantibodies. To this end, plasma from bCII-injected mice treated with Cl-amidine or vehicle control (DMSO) was analyzed for the presence of total IgG Ab to bCII. Notably, Cl-amidine treatment significantly attenuated the anti-bovine CII IgG Abs levels compared with DMSO vehicle control at the end-point of each experiment (d32 to 45 p.i.) (absorption 1.409 ± 0.1 vs 1.719 ± 0.09 **Figure 8B**). These results demonstrate that Cl-amidine treatment decreases the humoral response to bCII.

In summary, the above findings provide evidence that Cl-amidine administration in the CIA model inhibits NETosis, attenuates disease phenotype and arthritis progression.

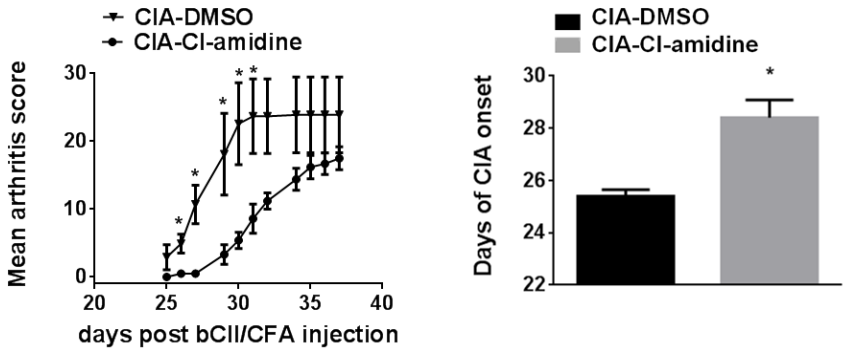


Figure 7. Cl-amidine treatment during CIA reduces clinical disease activity Evaluation of arthritis based on redness and swelling of the paws (n=5/group; day 26: **p* = 0.017, day 27: **p* =0.007, day 29: **p* =0.043 day 30: **p* = 0.02 day 31: **p* = 0.03, Multiple tests, unpaired *t*-test) and time (days) of CIA onset are shown. (Mann-Whitney test, **p* =0.023). Maximum score per mouse is 38. Results are expressed as mean ± SEM and are representatives of three independent experiments with 8 mice/group per experiment.

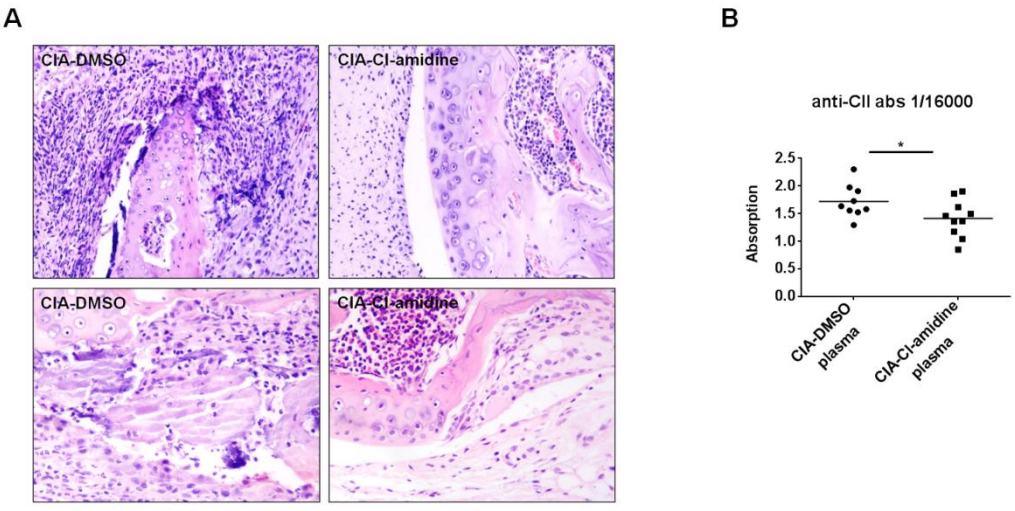


Figure 8. Cl-amidine treatment during CIA reduces joint destruction and anti-

collagen II antibody production (A) Representative H&E-stained sections of inflamed knee joints (proximal) from CIA-DMSO and CIA-Cl-amidine treated mice, are shown, at the peak of disease activity (d32-39) (original magnification, 400x). Two representative samples of CIA-DMSO and CIA-Cl-amidine administered mice, are shown. Data shown are from one experiment representative of three independent experiments with 2 mice/group per experiment. (B) Anti-bCII Ab production in the plasma of CIA-DMSO (n=9) or CIA-Cl-amidine (n=10) administered mice, as measured by ELISA. Results are expressed as absorption at 450nm. Each dot represents one mouse. Mean is depicted. Data are combined from three independent experiments with 3-4 mice/group per experiment. (Mann-Whitney test, $*p$ =0.043).

6.7 Cl-amidine treatment diminishes IFN-γ producing Th1 cells

Next we examined whether the attenuated disease pathology in the CIA after Cl-amidine administration, could be attributed in decreased Th1 and/or Th17 immune responses. To address this, flow cytometry was performed on draining lymph node cells (dLNCs) from CIA mice treated with Cl-amidine daily for 12 days after the first immunization with CII. Interestingly, the frequency of $CD4^+$ T cells was decreased in CIA-Cl-amidine administered mice compared to CIA-vehicle control mice (16.51 ± 0.44 vs 18.87 ± 0.01 **Figure 9)** and this was accompanied by significantly decreased frequencies of IFN-γ expressing $CD4^+$ T cells by dLNCs in CIA-Cl-amidine (percentage of $CD4^+$ IFN-γ^+ cells 1.99 ± 0.2 vs 2.77 ± 0.34 **Figure 9)**. IL-17 expressing $CD4^+$ T cells were also decreased but did not reach statistical significance.

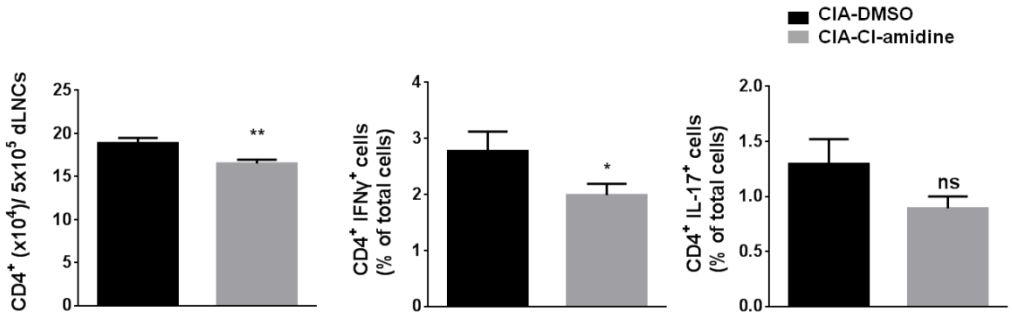


Figure 9. Cl-amidine treatment diminishes IFN-γ producing Th1 cells

dLNCs from bCII-injected DMSO and bCII-injected Cl-amidine treated mice were collected 12 d post collagen injection and analyzed by flow cytometry. Relative numbers of $CD4^+$ T cells/ 5 x 10^5 total LNCs (n=18-21/group) and the percentage of $CD4^+$ IFN-γ^+ and $CD4^+$IL-17^+ cells (n=14-17/group) between the two groups are shown. Results are expressed as mean ± SEM. Data are combined from four independent experiments with 4-5 mice/group per experiment. (Unpaired t-test, ***p*=0.0031, **p*=0.04; *ns*=non significant).

6.8 Cl-amidine treatment reduces the expression of MHC-II and CD86 costimulatory molecule on DCs

T cell activation, polarization and proliferation in vivo require Ag presentation by professional APCs. DCs serve as the best candidate that upon maturation provide the necessary costimulatory molecules and secrete proinflammatory cytokines to instruct T cell responses (Banchereau and Steinman 1998; Guermonprez, Valladeau et al. 2002). Therefore, we examined whether Cl-amidine administration in CIA could also

affect DC maturation. Interestingly, it was found a significantly decreased expression of MHC-II and CD86 costimulatory molecules expressed by $CD11c^+$ DCs isolated from dLNs upon Cl-amidine treatment compared to DMSO vehicle group (mean MFI fold change: MFI normalized to the average MFI of CIA group 0.66 vs 0.99 for MHC-II and 0.73 vs 0.99 for CD86 **Figure 10A, B**). Taken together, these data demonstrate that treatment of CIA mice with Cl-amidine attenuated the Th1 immune responses and the maturation of DCs in the dLNs.

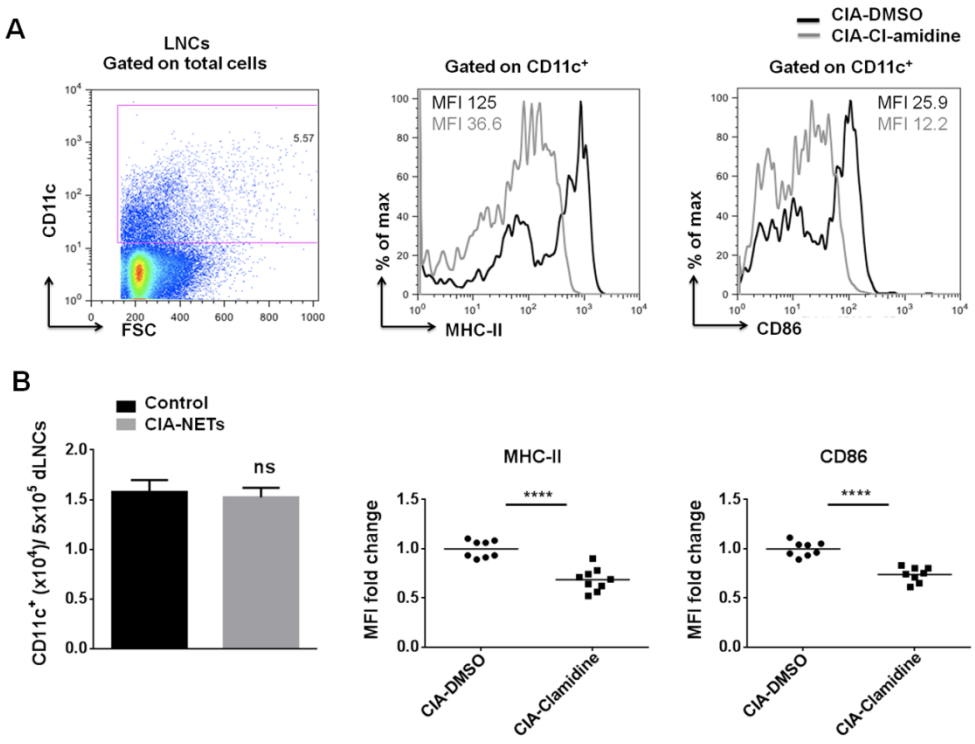


Figure 10. Cl-amidine treatment attenuates the expression of MHC-II and CD86 costimulatory molecules on DCs derived from draining lymph nodes (dLNs). (A) Representative flow cytometry analysis and relative numbers of $CD11c^+$ DCs/ 5 x 10^5 total LNCs are shown. Numbers in FACS plot denote frequency. Representative FACS histograms depicting the geometric mean fluorescence intensity (MFI) of MHC-II and CD86 expression between the two groups, are shown, as examined by

flow cytometry. Data shown are from one experiment representative of three independent experiments with 2-3 mice/group per experiment. (B) Relative numbers of $CD11c^+$ cells/ 5 x 10^5 total dLNCs (n=19-21/group) between the two groups are shown. Results are expressed as mean ± SEM. Data are combined from four independent experiments with 4-5 mice/group per experiment. (Unpaired t-test, *ns*=non significant). Dot plots represent the MFI of dLNCs of each CIA-DMSO or CIA-Cl-amidine administered mouse (n=8-9) normalized to the average MFI of CIA group (MFI fold change). Mean is depicted. Data are combined from three independent experiments with 2-3 mice/group per experiment. (Unpaired *t*-test, ****$p<0.0001$).

6.9 CIA-derived NETs increases the maturation of myeloid DCs

To provide direct evidence for a role of NETs in DC maturation we generated bone marrow-derived dendritic cells (BMDCs) from naïve DBA/1 and treated them with supernatants containing CIA-NETs (**Figure 11**) or control supernatants. We first examined the expression of costimulatory molecules on BMDCs ($CD11c^+$) in the presence or absence of CIA-NETs. We observed a significant up-regulation of both CD80 and CD86 costimulatory molecules in the presence of CIA-NETs as compared to control (mean fold induction: expression levels normalized to the expression of untreated BMDCs 1.275 vs 1.078 for CD80 and 1.773 vs 1.474 for CD86 **Figure 12A**). Moreover, assessment of proinflammatory cytokines in culture supernatants revealed a vast increase of IL-6 by DCs in the presence of NETs (fold induction: expression levels normalized to the secretion of untreated BMDCs 2.456 ± 0.41 vs 1.427 ± 0.05 **Figure 12B**). TNF-α production was also increased but did not reach

statistical significance (**Figure 12B**). These results indicate that NETs are capable of directly activating DCs as shown by the induction of costimulatory molecules and proinflammatory cytokine.

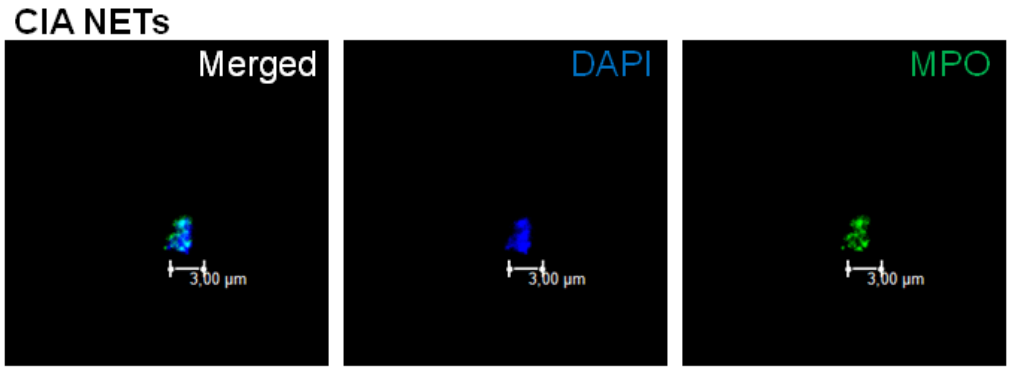


Figure 11. CIA BM-derived NETs. Representative confocal microscopy image from CIA-NETs after costaining with DAPI and MPO. Original magnification, 63x; digital zoom, 3x. Scale bar, 3µm. Microscopy image is representative of five independent experiments.

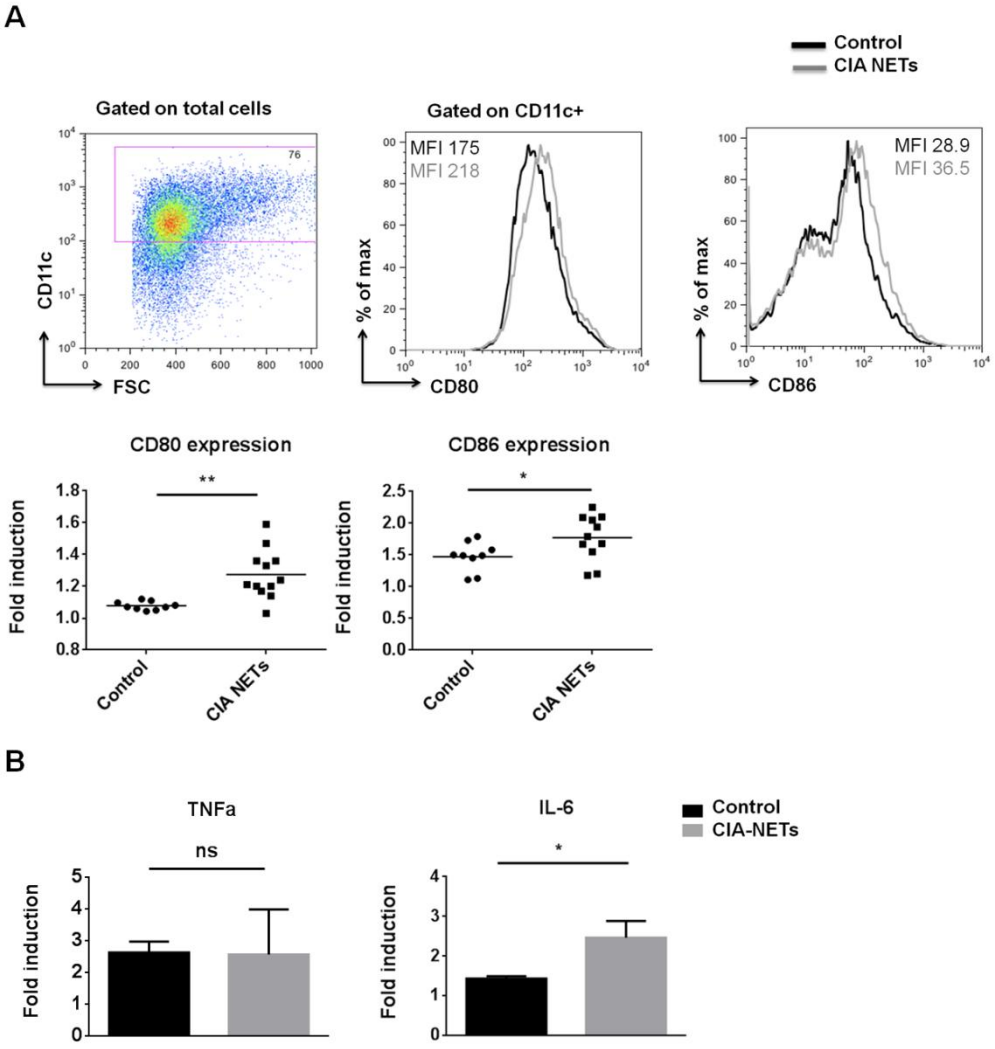


Figure 12. CIA BM-derived NETs increase the inflammatory properties of myeloid dendritic cells. Bone marrow-derived dendritic cells (BMDCs) from naïve mice were treated with condensed supernatants containing NETs (CIA-NETs) and control supernatants (control). (A) Representative flow cytometry analysis of $CD11c^+$ DCs. Numbers in FACS plot denote frequency. Representative FACS histograms are shown with the corresponding MFI of CD80 and CD86 expression levels on $CD11c^+$ cells. Data shown are from one experiment representative of four independent experiments. Dot plots represent expression levels of CD80 and CD86 of BMDCs treated with CIA-NETs or control (n=9-12) normalized against the expression of

untreated BMDCs (fold induction). Results are expressed as mean; data are combined from four independent experiments. (Unpaired *t*-test, ***p*=0.013, **p*=0.04). (B) TNF-α and IL-6 secretion in culture supernatants of BMDCs treated with CIA-NETs (n=12) or control (n=10) expressed as fold increase based on the secretion of the untreated BMDCs. Results are expressed as mean ± SEM; data are combined from four independent experiments. (Unpaired *t*-test, *ns*= non significant, **p*=0.038).

6.10 NETs potentiate the immunostimulatory effect of BMDCs in shaping Ag-specific Th1-immune responses

To assess the functional importance of our findings, we applied the OT-II mouse model. We performed coculture experiments of CIA-NETs treated ovalbumin (OVA)-pulsed BMDCs with OT-II dLNCs as shown in **Figure 13**. To this end, WT BMDCs were pulsed with OVA in the presence of CIA-NETs or control supernatants. Forty-eight hours later, the intracellular IL-17 and IFN-γ production by $CD4^+$ T cells was assessed. Interestingly, CIA-NETs treated OVA-pulsed BMDCs significantly induced IFN-γ production by $CD4^+$ OT-II-LNCs as compared with control treated OVA-pulsed BMDCs (percentages of $CD4^+IFN\text{-}\gamma^+$ cells 5.7 ± 0.36 vs 4.243 ± 0.328 **Figure 14**). Notably, no significant difference was observed in IL-17 production by $CD4^+$ T cells found in OT-II-dLNCs either in the presence of CIA-NETs or control (**Figure 14**). These findings provide evidence for the increased capacity of NET treated DCs to promote autoreactive Th1 immune responses.

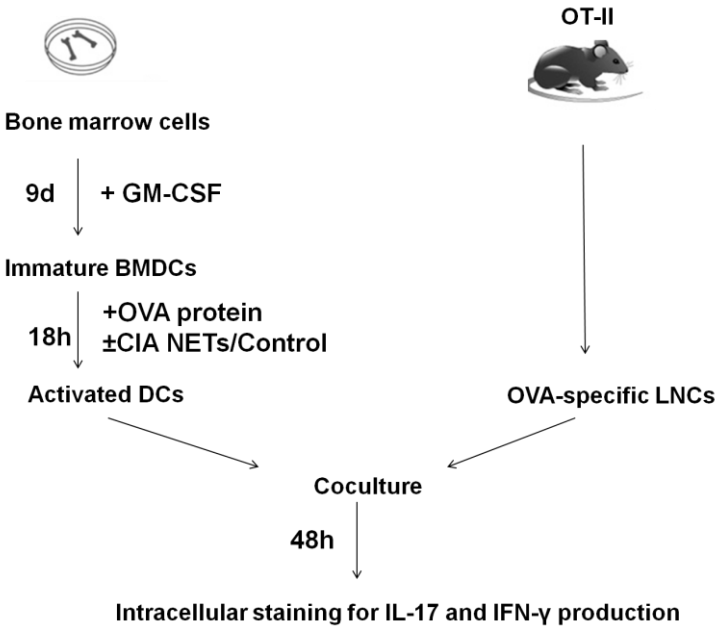


Figure 13. Outline of the coculture experimental setup. BM from C57BL/6 (B6) mice was differentiated into BMDCs ($CD11c^+$) in the presence of GM-CSF. At day 9 the immature BMDCs were pulsed with ovalbumin (OVA) protein in the presence or absence of CIA-NETs or control supernatants for 18hrs and subsequently cocultured with LNCs from OT-II transgenic mice. 48hrs later IFN-γ and IL-17 production was assessed by intracellular staining**.**

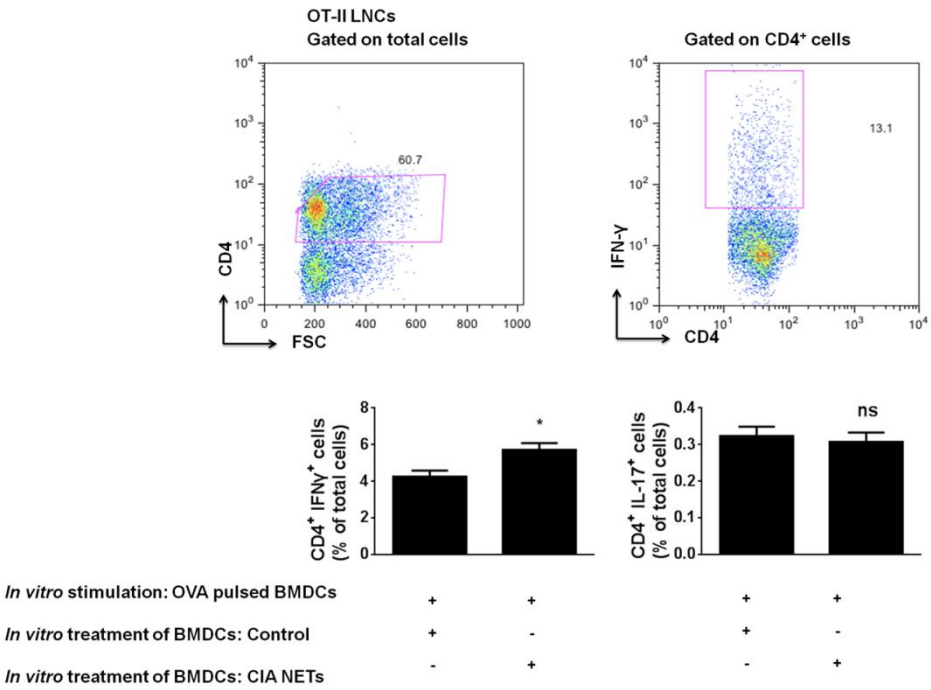


Figure 14. CIA-NETs treated BMDCs augments antigen (Ag)-specific Th1 responses in vitro. Gating strategy of $CD4^+$ T cells and $CD4^+IFN\text{-}\gamma^+$ cells are shown. Numbers on the gates denote frequencies. Gates were set as indicated. Percentages of $CD4^+$ $IFN\text{-}\gamma^+$ and $CD4^+$ $IL\text{-}17^+$ cells in total LNCs derived from OT-II mice pulsed with OVA and treated with CIA-NETs (n=4) or control (n=6), are depicted. Results are expressed as mean ± SEM; data are combined from two independent experiments. (Unpaired *t*-test, *ns*=non significant, **p*=0.02).

6.11 Human RA-NETs enhance the maturation of monocyte differentiated dendritic cells

Since NETs are a prominent feature in RA we sought to examine whether RA-NETs could induce the maturation of human DCs. To this end we exposed human monocyte

differentiated dendritic cells (moDCs) to RA-NETs and compared their effect to that from supernatants derived from control PMNs. RA-NETs increased the expression of HLA-DR and CD86 costimulatory molecule by DCs as compared to control (MFI fold change: expression normalized to MFI of untreated moDCs 1.55 vs 1.23 for HLA-DR and 1.15 vs 1.02 for CD86 **Figure 15**). Moreover, assessment of proinflammatory cytokines in culture supernatants revealed that RA-NETs significantly increased IL-6 and TNF-α cytokine release as compared to control supernatants (fold increase normalized to the secretion of the untreated moDCs 1.44 ± 0.13 vs 1.038 ± 0.05 for IL-6 and 1.43 ± 0.14 vs 0.95 ± 0.03 for TNF-α **Figure 16**). Overall, our data provide evidence for an immunostimulatory role of human RA-NETs in APCs.

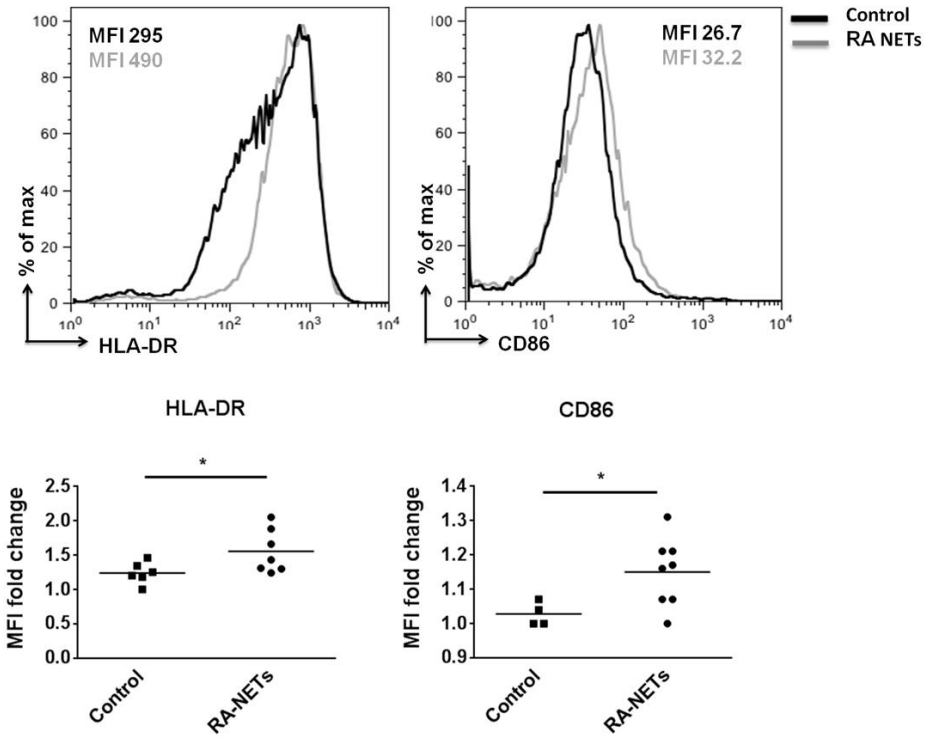


Figure 15. Human rheumatoid arthritis-NETs (RA-NETs) increase the expression of costimulatory molecules on human monocyte differentiated dendritic cells (moDCs). Representative FACs histograms are shown with the corresponding MFI of moDCs treated with RA NETs or control. Dot plots represent the MFI of HLA-DR and CD86 of moDCs treated with RA-NETs (n=7-8) or control supernatants (n=4-6) normalized against the average of MFI from untreated moDCs (MFI fold change). Results are expressed as mean; data are combined from three independent experiments. (Unpaired *t*-test, **p*=0.04).

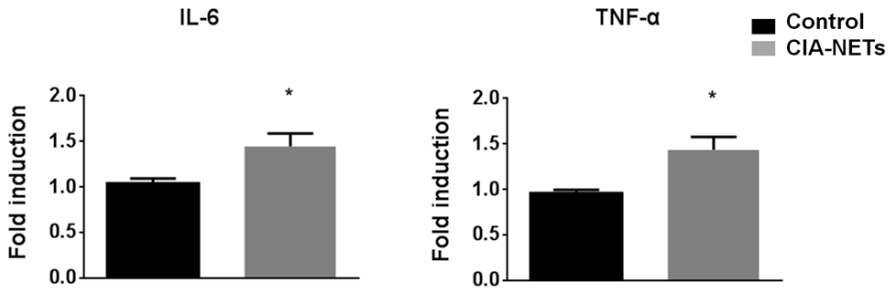


Figure 16. Human rheumatoid arthritis-NETs (RA-NETs) increase the secretion of proinflammatory cytokines by human monocyte differentiated dendritic cells (moDCs). IL-6 and TNF-α secretion in culture supernatants of moDCs treated with RA-NETs (n=7-9) or control supernatants (n=6) expressed as fold increase based on the secretion of the untreated moDCs. Results are expressed as mean ± SEM; data are combined from three independent experiments. [Unpaired *t*-test, **p*=0.01 (for TNF-α) and **p*=0.04 (for IL-6)].

7. DISCUSSION

Although it is becoming apparent that NETs have a significant impact on chronic autoimmune inflammatory diseases such as lupus, psoriasis, atherosclerosis and arthritis (Lande, 2011 #525;Doring, 2012 #852;Lin, 2011 #857;Skrzeczynska-Moncznik, 2012 #469;Vlachou, 2016 #1687;Khandpur, 2013 #546), the mechanism involved in NET-mediated adaptive autoimmune responses remains elusive. Herein, we provide new insights into the NETs-mediated induction of T cell autoreactivity that contribute to the immunopathogenesis of RA. Specifically, we identified that CIA-NETs favors the induction of Th1 immune responses through DC activation. In human, RA-NETs also augmented the inflammatory properties of moDCs. Importantly, inhibition of NETosis *in vivo* using Cl-amidine, a pan-PAD inhibitor, significantly diminished CIA severity and delayed disease onset. Additionally, Cl-amidine treatment decreased the expression of MHC-II and CD86 costimulatory molecules on dendritic cells and Th1 immune responses during the initial steps of the disease course.

The high importance of $CD4^+$ T cells in RA pathogenesis has been supported by genetic studies showing an association between genes important for T cell activation and function (such as *HLA-DR, PTPN22, CTLA4, CD40* etc) and the risk for RA (Stastny, 1976 #863;Gregersen, 1987 #881). In addition, the clinical effectiveness of T cell directed therapies (CTLA4Ig) (McInnes, 2011 #888) further supports the important role of these cells in disease progression. Finally, adoptive transfer of collagen specific T cells into SCID recipients can cause disease (Kadowaki, Matsuno et al. 1994). It is well-established that both Th1 and Th17 responses contribute in RA pathogenesis (Lubberts 2015). Th1 responses are important in initiating inflammation through recruiting cells into the joint and inducing mononuclear phagocytes to secret

TNF-α (Fox 1997; Choy and Panayi 2001) while IL-17 produced by Th17 cells stimulates fibroblasts, endothelial and epithelial cells to produce IL-6 and IL-8 (Shahrara, Pickens et al. 2009). Our study demonstrates that NETs promote induction of IFN-γ producing Th1 cells *in vitro* whereas they do not show to affect Th17-mediated responses. Of note, the clinical efficacy of anti-IL-17 agents has been highly variable, showing effectiveness in psoriasis and psoriatic arthritis but moderate to weak in RA (Genovese, Durez et al. 2013; Mease, McInnes et al. 2015). Although it has been shown that IL-17A may induce NETosis in the context of RA (Khandpur, Carmona-Rivera et al. 2013), that NETs may also be loaded with IL-17A in different human diseases (thrombosis, Alzheimer's disease) (de Boer, Li et al. 2013; Zenaro, Pietronigro et al. 2015) or that they may indirectly activate Th17 cells in atherosclerotic plaques (Warnatsch, Ioannou et al. 2015), in our study there is no evidence of a Th17 polarizing ability of NETs. Consistently, in our experiments Cl-amidine administered during the priming of the arthritogenic response resulted in the decrease of IFN-γ producing $CD4^+$ T cells in the dLNs but not of IL-17-producing cells. This is further supported by the increased IFN-γ production by antigen (OVA)-specific T cells cocultured with NET-treated BMDCs in the presence of OVA. Thus, we propose that recognition of NETs by APCs generate an inflammatory signal that favors Th1 induction.

DCs are characterized as professional APCs and they have been shown to be indispensable for T cell activation and proliferation (Guermonprez, Valladeau et al. 2002). Of note, it was previously demonstrated that PMNs prone to form NETs were shown to activate myeloid DCs ($CD11c^+$ $CD11b^+$ $CD45RA^-$) (Sangaletti, Tripodo et al. 2012). However, whether DC activation was mediated by NETs or other PMN-related molecules was not addressed. Our findings provide evidence for a direct

immunogenic ability of NETs to increase the DC costimulatory molecules, CD80 and CD86 as well as the secretion of IL-6. In addition, spontaneously released RA-NETs increased the maturation of human DCs. Finally, further evidence was obtained upon Cl-amidine administration during CIA that was accompanied by down-regulation of DC costimulatory molecule expression in dLNs. The molecular mechanism via which NETs are recognized by DCs remains unknown. It is likely, that NETs as DNA-containing structures may activate DCs via TLR9, or other endosomal DNA-sensors. In line with this, it has been shown that PMA-induced NETs efficiently triggered plasmacytoid DC activation through TLR9 (Lande, Ganguly et al. 2011).

It is well-established that NETosis could be specifically inhibited by Cl-amidine administration (PAD inhibition). PAD4 is a neutrophil enriched nuclear enzyme that targets histone arginine and mono- methylarginine residues for citrullination in a calcium dependent reaction (Nakashima, Hagiwara et al. 2002; Wang, Wysocka et al. 2004). Studies in murine lupus showed that Cl-amidine inhibited NETs and protected mice against disease related organ damage and associated autoimmunity, evidence that strengthens our findings (Knight, Zhao et al. 2013; Knight, Luo et al. 2014). In support, our findings demonstrate that Cl-amidine administration reduced NET formation, ameliorated arthritis severity and delayed disease onset and this was accompanied by reduced anti-CII antibody titers. The molecular mechanism through which Cl-amidine reduces CIA severity is currently under investigation. Accumulating evidence suggests that citrullination is an important mechanism in NET formation contributing to the chromatin decondensation and facilitate NET release (Neeli, Khan et al. 2008; Wang, Li et al. 2009). In support to this notion, it has been shown that a PAD4-null strain of mice failed to produce NETs (Li, Wang et al. 2010; Hemmers, Teijaro et al. 2011). In regard to citrullination, NETs may also function as

additional pool of cittrullinated antigens. It has been shown that active PAD isoforms are found attached on NETs from RA SF PMNs, which may contribute to the generation of extracellular autoantigens in RA (Spengler, Lugonja et al. 2015), and levels of NETosis correlate with the presence and levels of anti-citrullinated peptide antibodies (Khandpur, Carmona-Rivera et al. 2013). Our findings demonstrate that the levels of spontaneous NETosis in human are correlated with increased autoantibodies (RF positivity) and the RA inflammatory environment enriched in autoantibodies increases NETosis by control PMNs more avidly than an autoantibody poor environment. In support, it has been shown that RA sera, SF, rheumatoid factor and IgG fractions purified from RA patients with high levels of anti-citrullinated peptide antibodies significantly enhanced NET formation and led to distinct protein content in the NETs (Khandpur, Carmona-Rivera et al. 2013). Therefore, we propose that Cl-amidine inhibits NETs release and the externalization of citrullinated antigens which could activate citrulline Ag-specific autoreactive T cells thus leading to disease perpetuation.

The intracellular cascade that operates in PMNs and leads to post-translational Ag modification and generation of citrullinated antigens is not known. Of interest, it has been shown that the autophagy pathway in DCs is required for the processing and presentation of citrullinated Ags by APCs (Ireland and Unanue 2011). It is known that NETosis is also mediated through the autophagic pathway . Whether autophagy is also required for the processing of citrullinated Ags in neutrophils remains to be shown.

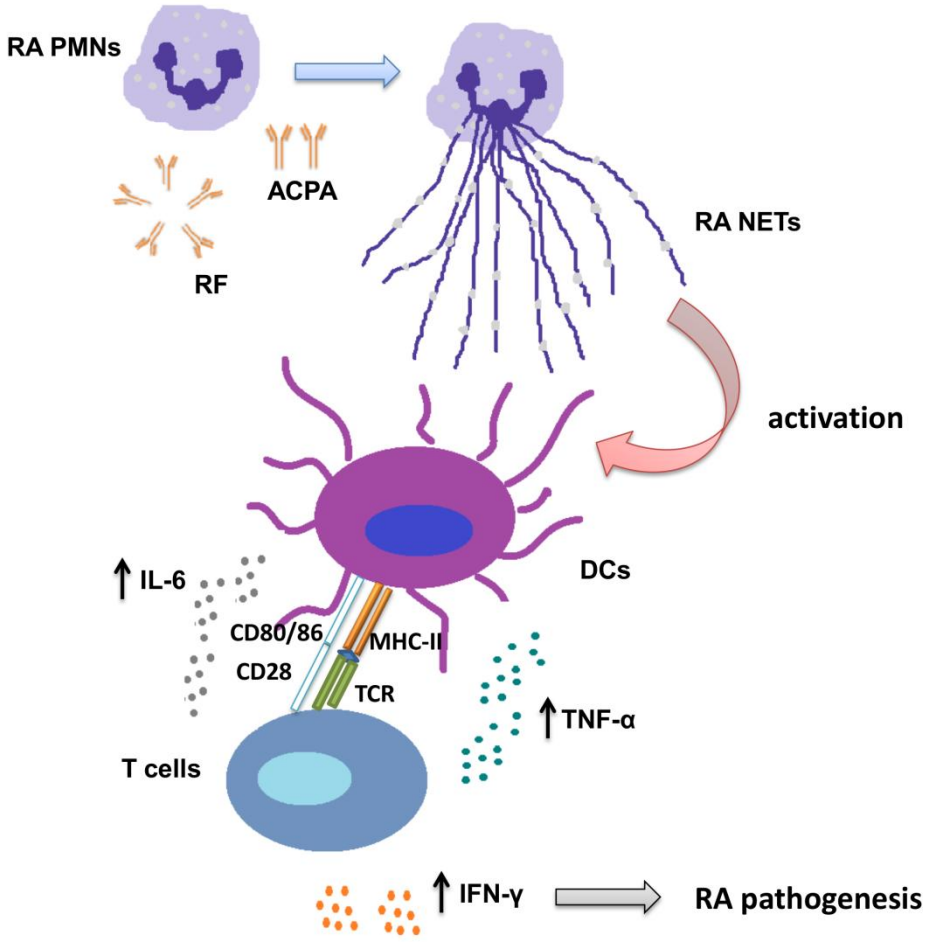
In summary, our findings provide evidence for the first time to our knowledge for a novel role of NETs in shaping the RA-related autoimmune responses *in vitro* by promoting induction of IFN-γ producing Th1 cells. Inhibition of NET release

significantly attenuated CIA and led to decreased stimulatory properties of antigen-bearing DCs. Our findings provide new insights into the pathogenic role of NETs in RA that could lead to the development of new targeted therapeutic approaches.

Clinical significance

Despite the well documented importance of NETs as effective antigenic first line defense mechanism, there is increasing evidence that NETs occur in various clinical settings in the absence of microbial infections. The increasing knowledge about NETs will strengthen the development of novel diagnostic and therapeutic strategies and tools in inflammatory and autoimmune disorders.

Proposed mechanism



Factors in the inflammatory milieu of rheumatoid arthritis induce PMNs to undergo NETosis. RA NETs serve as a source of neoantigens complexed with DNA to promote the activation and cytokine release by DCs, leading in the production of IFN-γ by T cells. This cascade contributes in the RA autoimmune responses.

8. FUTURE DIRECTIONS

Our data reveal a novel effect of neutrophil extracellular traps on immune cells implicated in RA pathogenesis. Specifically, we provide evidence for a key role of NETs on dendritic cells and the subsequent activation of Th1 cells. Based on our findings many interesting questions have been raised that could be further explored. Such questions are discussed below.

8.1. Identify potent RA related immunogenic molecules externalized on NETs

First we will investigate if the autoimmune inflammatory environment of RA affects NET protein or DNA load. Fundamental questions are listed next:

a) Which is the protein cargo of RA derived NETs? To answer this question, we will perform proteomic analysis: mass spectrometry to reveal new molecules externalized through NETs that may become immunogenic and their relevance with the disease progression. NETs derived from active RA patients but also NETs-induced by RA serum or synovial fluid will be used to reveal whether there is a differential NET content or not. The role of identified molecules could be further examined in functional experiments where inhibitors or neutralizing antibodies of these molecules will reveal their importance.

b) Is the externalized DNA oxidized (more immunogenic)?

According to a recent study NETs induced by RNP (ribonucleoprotein) immune complexes are enriched in oxidized mitochondrial DNA, become interferogenic and contribute to lupus-like disease (Lood, Blanco et al. 2016). Accordingly, are neutrophils stimulated with RA serum or synovial fluid- rich

in autoantibodies capable of releasing oxidized DNA? To this end, specific antibodies for 8-Oxo-2´ deoxyguanosine (8-OHdG) will be used to stain extruded NETs and examine their oxidation status using confocal microscopy.

c)Which is the origin of this DNA (mitochondrial-mtDNA versus chromosomal). To examine the origin of DNA we will perform immunoprecipitation of total oxidized DNA using anti-8-OHdG and quantify the relative abundance of mitochondrial (16S; MT-RNR2) and chromosomal (18S; RNA18S5) DNA by qPCR. Ratio 16S: 18S.

If our hypothesis is correct, it could provide additional evidence about the biological significance of NETs in the progression of RA.

8.2 Interplay of NETs with other cell populations with important role in joint pathology and the progression of the disease.

8.2.1 Study whether RA NETs could promote osteoclast activation and osteoclastogenesis

RA is characterized by excess of osteoclastic activity leading to an imbalance in bone remodeling which favours resorption. Osteoclasts are tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursors cells at or near the bone surface. The mature multinucleated osteoclast is activated by different signals, which leads to the initiation of bone remodeling resulting in the bone resorption.

Our hypothesis is that inflammatory mediators externalized during NETosis might increase osteoclast activation and differentiation thus leading to the resortion of the bone. To address this hypothesis:

a) Monocytes from PBMC fraction isolated from healthy donors will be cultures for 14 days in the presence of MCSF and RANKL to induce their differentiation into osteoclasts. Next, the differentiated osteoclasts will be cocultured with RA NETs and osteoclast differentiation will be assessed by staining cells for TRAP using a Leukocyte acid phosphatase kit.

During osteoclast maturation genes encoding tartrate-resistant acid phosphatase (TRAP), Cathepsin K (CATK), calcitonin receptor and β3-integrin are induced. Therefore the expression of these genes will be assessed in mRNA level using RTPCR.

b) If osteoclast activation is achieved after treatment with RA NETs, we aim to assess the functional importance of NET mediated osteoclast activation on bone resortion. To this end, NET treated osteoclasts will be cultured on bovine bone slides and resortion pit area will be assessed using staining with toluidine solution and subsequent observation and quantification by microscopy.

8.2.2 Interplay between NETs and plasmacytoid dendritic cells. Study whether RA NETs could promote pDCs isolated from patients responding to therapy towards an immunostimulatory phenotype

Previous reports using SLE NETs in coculture experiments with healthy plasmacytoid dendritic cells (pDCs) have shown that NETs are capable of inducing IFN-a producing pDCs (Garcia-Romo, Caielli et al. 2011; Lande, Ganguly et al. 2011). Previous studies from our lab have revealed that pDCs from RA patients responding to therapy (in remission) are capable of inducing IL-10 producing T regulatory cells through IDO expression. Our hypothesis is that NETs may trigger pDCs towards an activated status and subsequently activated pDCs may trigger T cell activation and

proliferation instead of exhibiting an immunosuppressive phenotype (reverse the suppressive function of pDCs). To assess our hypothesis:

a) We will isolate pDCs from the peripheral blood of patients with inactive RA and culture them in the presence of RA NETs. In parallel, cocultures of healthy pDCs with RA NETs will be placed to examine the capacity of NETs to activate pDCs in the RA autoimmune setting. Next, the maturation status of pDCs will be assessed using flow cytometry and stain for HLA-DR, CD80, CD86, CD40 and the chemokine receptor CCR7.

b) To identify pDC molecules that mediate this effect we will perform coculture experiments of naïve T cells and NET-treated pDCs in the presence of specific inhibitors (use neutralizing antibodies to block the secretion of cytokines). If our hypothesis that NETs promote pDC activation and maturation is correct pDCs in the presence of neutralizing antibodies are expected to promote poor T cell proliferation and decrease levels of CD69 (early activation marker) by proliferating T cells.

c) To detect the ability of matured pDCs to activate and expand naïve T cells , we will culture CFSE-labeled naïve CD4+ CD25- T cells from cord blood in the presence of NET treated pDCs and monitor cell proliferation based on dilution of CFSE staining after 6 days. As control CD40L-matured pDCs will be used in the cocultures with T cells. CD69 marker on CD4+ T cells will be also assessed by flow cytometry, as an early activation marker.

8.3 Assess the molecular mechanism of DC activation by CIA-NETs. How NETs can activate DCs? Are DNA-protein complexes incorporated into DCs?

a) To identify the route of NET incorporation into DCs we will try to inhibit receptor mediated endocytosis applying neutralizing antibodies against the Fc receptors on DCs prior to their treatment with CIA-NETs. Next, their activation and maturation status will be monitored using specific surface markers (expression levels by flow cytometry) or measuring the secretion of cytokines by ELISA.

b) To assess the transfer of NET-associated proteins (MPO, NE) to BMDCs, BM PMNs derived from CIA mouse (prone to NETosis) will be cocultured with DCs stained with PKH-26 dye onto coverslips in the presence of anti-MPO antibody conjugated with a fluorochrome and monitor the incorporation with confocal microscopy. The MPO conjugated antibody will allow deteting MPO in DCs without permeabilzation, thus ensuring that the signal only derives from material taken up from NETs.

8.4. To examine if RA NETosis is a ROS dependent process

To explore the relative contribution of NADPH oxidase and the mitochondrial electron chain in ROS generation and NETosis, we will stimulate normal human PMNs with RA serum or synovial fluid or healthy serum in the presence of ROS inhibitors and measure the DNA release either by MPO-DNA elisa or the extracellular DNA released (picogreen).

a) by using DPI (Diphenyleneiodonium), an inhibitor of NADPH oxidase

b) by using MitoSox Red, a triphenylphosphonium-linked dihydroethidium compound that concentrates within mitochondria and fluoresces red when oxidized by ROS

c) by using TTFA (thenoyltrifluoroacetone) a specific inhibitor of mitochondrial respiration

d) by using two specific inhibitors of NADPH oxidase, apocynin and VAS2870.

8.5 Assess whether RA inflammatory milieu modifies levels of autophagy

To determine the levels of autophagy in neutrophils from CIA and control mice, we will perform western blot analysis and confocal microscopy for LC3II expression (lipidated form, established marker of autophagy).

8.5.1 Demonstrate whether the CIA milieu influences autophagy and subsequent NET formation and externalization of citrullinated autoantigens

LC3II expression will be examined after treatment of control neutrophils with sera from CIA mice (at early and late stages).

8.5.2 Provide evidence for autophagy-mediated generation of citrullinated autoantigens

Neutrophils from $LysM^{Cre}$ $Atg5^{fl/fl}$ (defective autophagy in myeloid lineage) and from $LysM^{Cre}$ $Atg5^{fl/-}$ mice will be treated with CIA sera and will be examined for their ability to form NETs *in vitro* and to externalize citrullinated autoantigens using confocal microscopy and immunoblotting (anti-modified citrulline antibody).

8.6 How does the RA inflammatory environment trigger NETosis in healthy PMNs?

Our data revealed that RA inflammatory environment (RA serum/SF) trigger NETosis in healthy PMNs and this is positively correlated with the levels of RF/ACPAS autoantibodies. Moreover, in RA serum or SF are found different cytokines such as IL-17, IL-6, TNF-α which may be capable of inducing NET formation.

a) To this end, we are going to perform experiments using specific neutralizing antibodies for each cytokine (previously found elevated in RA serum) and examine levels of NETosis either with confocal microscopy or measuring the DNA extruded from NETs.

b) we will isolate RNA from neutrophils derived from seropositive (anti-CCPs/ RF) versus seronegative RA patients in active or remitting phase of the disease and perform DNA microarray analysis in order to compare the gene expression profile and the association with the levels of NETosis from each group (measure the spontaneous NETosis of PMNs with MPO-DNA elisa). Serum collection will be also performed from all the patients above in order to analyze the levels of antibodies against the neutrophil constituents and correlate with the levels of NETosis.

8.7 Deciphering CIA initiation and progression in a neutrophil elastase defective environment.

Additionally, to examine whether inhibiting specifically NET formation without disrupting any other process would ameliorate disease initiation and/ or propagation elastase knock-out mice will be back crossed with DBA/1 and apply the CIA mouse model to assess whether defective NET formation affects CIA progression.

Overall, our data as well as the proposed experiments will provide novel insights into the mechanism of RA pathogenesis and the mechanisms that limit inflammation during autoimmune diseases. Characterization of the immunostimulatory properties of neutrophils may open new avenues for the development of more specific cell-based therapies in patients with RA.

9. REFERENCES

Alissafi, T., A. Hatzioannou, et al. (2015). "De novo-induced self-antigen-specific Foxp3+ regulatory T cells impair the accumulation of inflammatory dendritic cells in draining lymph nodes." J Immunol **194**(12): 5812-5824.

Allison, M. E. and D. T. Fearon (2000). "Enhanced immunogenicity of aldehyde-bearing antigens: a possible link between innate and adaptive immunity." Eur J Immunol **30**(10): 2881-2887.

Amulic, B., C. Cazalet, et al. (2012). "Neutrophil function: from mechanisms to disease." Annu Rev Immunol **30**: 459-489.

Arnett, F. C., S. M. Edworthy, et al. (1988). "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis." Arthritis Rheum **31**(3): 315-324.

Assi, L. K., S. H. Wong, et al. (2007). "Tumor necrosis factor alpha activates release of B lymphocyte stimulator by neutrophils infiltrating the rheumatoid joint." Arthritis Rheum **56**(6): 1776-1786.

Bachmann, M. F. and P. S. Ohashi (1999). "The role of T-cell receptor dimerization in T-cell activation." Immunol Today **20**(12): 568-576.

Bainton, D. F., J. L. Ullyot, et al. (1971). "The development of neutrophilic polymorphonuclear leukocytes in human bone marrow." J Exp Med **134**(4): 907-934.

Baka, Z., B. Gyorgy, et al. (2012). "Citrullination under physiological and pathological conditions." Joint Bone Spine **79**(5): 431-436.

Banchereau, J. and V. Pascual (2006). "Type I interferon in systemic lupus erythematosus and other autoimmune diseases." Immunity **25**(3): 383-392.

Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-252.

Barnhart, M. I., J. M. Riddle, et al. (1967). "Immunocytology in arthritic joints." Ann Rheum Dis **26**(4): 281-296.

Behrens, F., A. Himsel, et al. (2007). "Imbalance in distribution of functional autologous regulatory T cells in rheumatoid arthritis." Ann Rheum Dis **66**(9): 1151-1156.

Belcher, C., M. Doherty, et al. (2002). "Synovial fluid neutrophil function in RA: the effect of pregnancy associated proteins." Ann Rheum Dis **61**(4): 379-380.

Bevaart, L., M. J. Vervoordeldonk, et al. (2010). "Evaluation of therapeutic targets in animal models of arthritis: how does it relate to rheumatoid arthritis?" Arthritis Rheum **62**(8): 2192-2205.

Bluestone, J. A. and A. K. Abbas (2003). "Natural versus adaptive regulatory T cells." Nat Rev Immunol **3**(3): 253-257.

Borregaard, N. (2010). "Neutrophils, from marrow to microbes." Immunity **33**(5): 657-670.

Borregaard, N., O. E. Sorensen, et al. (2007). "Neutrophil granules: a library of innate immunity proteins." Trends Immunol **28**(8): 340-345.

Boxio, R., C. Bossenmeyer-Pourie, et al. (2004). "Mouse bone marrow contains large numbers of functionally competent neutrophils." J Leukoc Biol **75**(4): 604-611.

Brackertz, D., G. F. Mitchell, et al. (1977). "Antigen-induced arthritis in mice. I. Induction of arthritis in various strains of mice." Arthritis Rheum **20**(3): 841-850.

Brackertz, D., G. F. Mitchell, et al. (1977). "Studies on antigen-induced arthritis in mice. III. Cell and serum transfer experiments." J Immunol **118**(5): 1645-1648.

Brand, D. D., A. H. Kang, et al. (2003). "Immunopathogenesis of collagen arthritis." Springer Semin Immunopathol **25**(1): 3-18.

Brennan, F. M., A. L. Hayes, et al. (2002). "Evidence that rheumatoid arthritis synovial T cells are similar to cytokine-activated T cells: involvement of phosphatidylinositol 3-

kinase and nuclear factor kappaB pathways in tumor necrosis factor alpha production in rheumatoid arthritis." Arthritis Rheum **46**(1): 31-41.
Bretscher, P. and M. Cohn (1970). "A theory of self-nonself discrimination." Science **169**(3950): 1042-1049.
Brinkmann, V., U. Reichard, et al. (2004). "Neutrophil extracellular traps kill bacteria." Science **303**(5663): 1532-1535.
Burger, J. A., N. J. Zvaifler, et al. (2001). "Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1- and CD106 (VCAM-1)-dependent mechanism." J Clin Invest **107**(3): 305-315.
Burmester, G. R., A. Rubbert-Roth, et al. (2014). "A randomised, double-blind, parallel-group study of the safety and efficacy of subcutaneous tocilizumab versus intravenous tocilizumab in combination with traditional disease-modifying antirheumatic drugs in patients with moderate to severe rheumatoid arthritis (SUMMACTA study)." Ann Rheum Dis **73**(1): 69-74.
Caccavo, D., P. Garzia, et al. (2003). "Expression of lactoferrin on neutrophil granulocytes from synovial fluid and peripheral blood of patients with rheumatoid arthritis." J Rheumatol **30**(2): 220-224.
Carrasco-Marin, E., J. E. Paz-Miguel, et al. (1998). "Oxidation of defined antigens allows protein unfolding and increases both proteolytic processing and exposes peptide epitopes which are recognized by specific T cells." Immunology **95**(3): 314-321.
Castellino, F. and R. N. Germain (2006). "Cooperation between CD4+ and CD8+ T cells: when, where, and how." Annu Rev Immunol **24**: 519-540.
Chabaud, M., F. Fossiez, et al. (1998). "Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines." J Immunol **161**(1): 409-414.
Chakravarti, A., M. A. Raquil, et al. (2009). "Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption." Blood **114**(8): 1633-1644.
Chavanas, S., M. C. Mechin, et al. (2004). "Comparative analysis of the mouse and human peptidylarginine deiminase gene clusters reveals highly conserved non-coding segments and a new human gene, PADI6." Gene **330**: 19-27.
Chou, R. C., N. D. Kim, et al. (2010). "Lipid-cytokine-chemokine cascade drives neutrophil recruitment in a murine model of inflammatory arthritis." Immunity **33**(2): 266-278.
Chow, O. A., M. von Kockritz-Blickwede, et al. (2010). "Statins enhance formation of phagocyte extracellular traps." Cell Host Microbe **8**(5): 445-454.
Choy, E. H. and G. S. Panayi (2001). "Cytokine pathways and joint inflammation in rheumatoid arthritis." N Engl J Med **344**(12): 907-916.
Chumanevich, A. A., C. P. Causey, et al. (2011). "Suppression of colitis in mice by Cl-amidine: a novel peptidylarginine deiminase inhibitor." Am J Physiol Gastrointest Liver Physiol **300**(6): G929-938.
Cines, D. B., F. Passero, et al. (1982). "Granulocyte-associated IgG in neutropenic disorders." Blood **59**(1): 124-132.
Coremans, I. E., E. C. Hagen, et al. (1993). "Autoantibodies to neutrophil cytoplasmic enzymes in Felty's syndrome." Clin Exp Rheumatol **11**(3): 255-262.
Courtenay, J. S., M. J. Dallman, et al. (1980). "Immunisation against heterologous type II collagen induces arthritis in mice." Nature **283**(5748): 666-668.
Cui, B. B., C. Y. Tan, et al. (2012). "Neutrophil extracellular traps in sterile inflammation: the story after dying?" Autoimmunity **45**(8): 593-596.
Cyster, J. G., S. B. Hartley, et al. (1994). "Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire." Nature **371**(6496): 389-395.

Dalton, D. K., S. Pitts-Meek, et al. (1993). "Multiple defects of immune cell function in mice with disrupted interferon-gamma genes." Science **259**(5102): 1739-1742.
de Boer, O. J., X. Li, et al. (2013). "Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction." Thromb Haemost **109**(2): 290-297.
De Rycke, L., I. Peene, et al. (2004). "Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations." Ann Rheum Dis **63**(12): 1587-1593.
Dianzani, U., A. Shaw, et al. (1992). "Physical association of CD4 with the T cell receptor." J Immunol **148**(3): 678-688.
Doherty, P. C. and R. M. Zinernagel (1975). "Capacity of sensitized thymus-derived lymphocytes to induce fatal lymphocytic choriomeningitis is restricted by the H-2 gene complex." J Immunol **114**(1 Pt 1): 30-33.
Dularay, B., C. J. Elson, et al. (1988). "Enhanced oxidative response of polymorphonuclear leukocytes from synovial fluids of patients with rheumatoid arthritis." Autoimmunity **1**(3): 159-169.
Dustin, M. L. and T. A. Springer (1989). "T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1." Nature **341**(6243): 619-624.
Edens, H. A. and C. A. Parkos (2003). "Neutrophil transendothelial migration and alteration in vascular permeability: focus on neutrophil-derived azurocidin." Curr Opin Hematol **10**(1): 25-30.
Edwards, J. C., L. Szczepanski, et al. (2004). "Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis." N Engl J Med **350**(25): 2572-2581.
Emery, P., A. F. Lopez, et al. (1988). "Synovial fluid neutrophils of patients with rheumatoid arthritis have membrane antigen changes that reflect activation." Ann Rheum Dis **47**(1): 34-39.
Eming, R., K. Visconti, et al. (2002). "Humanized mice as a model for rheumatoid arthritis." Arthritis Res **4 Suppl 3**: S133-140.
Ermert, D., C. F. Urban, et al. (2009). "Mouse neutrophil extracellular traps in microbial infections." J Innate Immun **1**(3): 181-193.
Ethuin, F., B. Gerard, et al. (2004). "Human neutrophils produce interferon gamma upon stimulation by interleukin-12." Lab Invest **84**(10): 1363-1371.
Farber, D. L., O. Acuto, et al. (1997). "Differential T cell receptor-mediated signaling in naive and memory CD4 T cells." Eur J Immunol **27**(8): 2094-2101.
Faurschou, M. and N. Borregaard (2003). "Neutrophil granules and secretory vesicles in inflammation." Microbes Infect **5**(14): 1317-1327.
Faurschou, M., O. E. Sorensen, et al. (2002). "Defensin-rich granules of human neutrophils: characterization of secretory properties." Biochim Biophys Acta **1591**(1-3): 29-35.
Feldmann, M., F. M. Brennan, et al. (1996). "Role of cytokines in rheumatoid arthritis." Annu Rev Immunol **14**: 397-440.
Flouri, I., T. E. Markatseli, et al. (2014). "Comparative effectiveness and survival of infliximab, adalimumab, and etanercept for rheumatoid arthritis patients in the Hellenic Registry of Biologics: Low rates of remission and 5-year drug survival." Semin Arthritis Rheum **43**(4): 447-457.
Fossiez, F., O. Djossou, et al. (1996). "T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines." J Exp Med **183**(6): 2593-2603.
Fox, D. A. (1997). "The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives." Arthritis Rheum **40**(4): 598-609.
Frasnelli, M. E., D. Tarussio, et al. (2005). "TLR2 modulates inflammation in zymosan-induced arthritis in mice." Arthritis Res Ther **7**(2): R370-379.

Fuchs, T. A., U. Abed, et al. (2007). "Novel cell death program leads to neutrophil extracellular traps." J Cell Biol **176**(2): 231-241.
Fugger, L., S. A. Michie, et al. (1994). "Expression of HLA-DR4 and human CD4 transgenes in mice determines the variable region beta-chain T-cell repertoire and mediates an HLA-DR-restricted immune response." Proc Natl Acad Sci U S A **91**(13): 6151-6155.
Gal, I., E. Bajnok, et al. (2005). "Visualization and in situ analysis of leukocyte trafficking into the ankle joint in a systemic murine model of rheumatoid arthritis." Arthritis Rheum **52**(10): 3269-3278.
Galluzzi, L., I. Vitale, et al. (2012). "Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012." Cell Death Differ **19**(1): 107-120.
Garcia-Romo, G. S., S. Caielli, et al. (2011). "Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus." Sci Transl Med **3**(73): 73ra20.
Geiler, T., J. Kriegsmann, et al. (1994). "A new model for rheumatoid arthritis generated by engraftment of rheumatoid synovial tissue and normal human cartilage into SCID mice." Arthritis Rheum **37**(11): 1664-1671.
Genovese, M. C., P. Durez, et al. (2013). "Efficacy and safety of secukinumab in patients with rheumatoid arthritis: a phase II, dose-finding, double-blind, randomised, placebo controlled study." Ann Rheum Dis **72**(6): 863-869.
Genovese, M. C., F. Van den Bosch, et al. (2010). "LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study." Arthritis Rheum **62**(4): 929-939.
Ghoreschi, K., A. Laurence, et al. (2011). "T helper 17 cell heterogeneity and pathogenicity in autoimmune disease." Trends Immunol **32**(9): 395-401.
Glant, T. T., A. Finnegan, et al. (2003). "Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics." Crit Rev Immunol **23**(3): 199-250.
Goodnow, C. C., J. Sprent, et al. (2005). "Cellular and genetic mechanisms of self tolerance and autoimmunity." Nature **435**(7042): 590-597.
Grakoui, A., S. K. Bromley, et al. (1999). "The immunological synapse: a molecular machine controlling T cell activation." Science **285**(5425): 221-227.
Grayson, P. C. and M. J. Kaplan (2016). "At the Bench: Neutrophil extracellular traps (NETs) highlight novel aspects of innate immune system involvement in autoimmune diseases." J Leukoc Biol **99**(2): 253-264.
Gregersen, P. K., J. Silver, et al. (1987). "The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis." Arthritis Rheum **30**(11): 1205-1213.
Griffiths, R. J., E. R. Pettipher, et al. (1995). "Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis." Proc Natl Acad Sci U S A **92**(2): 517-521.
Guermonprez, P., J. Valladeau, et al. (2002). "Antigen presentation and T cell stimulation by dendritic cells." Annu Rev Immunol **20**: 621-667.
Gyorgy, B., E. Toth, et al. (2006). "Citrullination: a posttranslational modification in health and disease." Int J Biochem Cell Biol **38**(10): 1662-1677.
Hager, M., J. B. Cowland, et al. (2010). "Neutrophil granules in health and disease." J Intern Med **268**(1): 25-34.
Hakkim, A., T. A. Fuchs, et al. (2011). "Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation." Nat Chem Biol **7**(2): 75-77.
Harre, U., D. Georgess, et al. (2012). "Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin." J Clin Invest **122**(5): 1791-1802.

Harris, N. L. and F. Ronchese (1999). "The role of B7 costimulation in T-cell immunity." Immunol Cell Biol **77**(4): 304-311.
Harty, J. T., A. R. Tvinnereim, et al. (2000). "CD8+ T cell effector mechanisms in resistance to infection." Annu Rev Immunol **18**: 275-308.
Hemmers, S., J. R. Teijaro, et al. (2011). "PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection." PLoS One **6**(7): e22043.
Herman, A., J. W. Kappler, et al. (1991). "Superantigens: mechanism of T-cell stimulation and role in immune responses." Annu Rev Immunol **9**: 745-772.
Heusel, J. W., R. L. Wesselschmidt, et al. (1994). "Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells." Cell **76**(6): 977-987.
Holmdahl, R., K. Rubin, et al. (1986). "Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies." Arthritis Rheum **29**(3): 400-410.
Hsieh, C. S., S. E. Macatonia, et al. (1993). "Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages." Science **260**(5107): 547-549.
Hughes, J. R., C. C. Erhardt, et al. (1995). "Neutrophilic dermatosis in association with rheumatoid arthritis." Clin Exp Dermatol **20**(2): 168-170.
Ichikawa, M. M., Y. Murata, et al. (1998). "Rheumatoid neutrophilic dermatitis." Eur J Dermatol **8**(5): 347-349.
Iezzi, G., E. Scotet, et al. (1999). "The interplay between the duration of TCR and cytokine signaling determines T cell polarization." Eur J Immunol **29**(12): 4092-4101.
Ireland, J. M. and E. R. Unanue (2011). "Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells." J Exp Med **208**(13): 2625-2632.
Ivanov, II, B. S. McKenzie, et al. (2006). "The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells." Cell **126**(6): 1121-1133.
Jacobson, N. G., S. J. Szabo, et al. (1995). "Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4." J Exp Med **181**(5): 1755-1762.
Juo, P., C. J. Kuo, et al. (1998). "Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade." Curr Biol **8**(18): 1001-1008.
Kadowaki, K. M., H. Matsuno, et al. (1994). "CD4+ T cells from collagen-induced arthritic mice are essential to transfer arthritis into severe combined immunodeficient mice." Clin Exp Immunol **97**(2): 212-218.
Kahlenberg, J. M., C. Carmona-Rivera, et al. (2013). "Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages." J Immunol **190**(3): 1217-1226.
Kambas, K., M. M. Markiewski, et al. (2008). "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 **180**(11): 7368-7375.
Kaplan, M. H., U. Schindler, et al. (1996). "Stat6 is required for mediating responses to IL-4 and for development of Th2 cells." Immunity **4**(3): 313-319.
Kawane, K., M. Ohtani, et al. (2006). "Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages." Nature **443**(7114): 998-1002.
Kearney, E. R., T. L. Walunas, et al. (1995). "Antigen-dependent clonal expansion of a trace population of antigen-specific CD4+ T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4." J Immunol **155**(3): 1032-1036.
Keffer, J., L. Probert, et al. (1991). "Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis." EMBO J **10**(13): 4025-4031.

Keystone, E. C., H. U. Schorlemmer, et al. (1977). "Zymosan-induced arthritis: a model of chronic proliferative arthritis following activation of the alternative pathway of complement." Arthritis Rheum **20**(7): 1396-1401.

Khandpur, R., C. Carmona-Rivera, et al. (2013). "NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis." Sci Transl Med **5**(178): 178ra140.

Kim, N. D., R. C. Chou, et al. (2006). "A unique requirement for the leukotriene B4 receptor BLT1 for neutrophil recruitment in inflammatory arthritis." J Exp Med **203**(4): 829-835.

Klausner, R. D., J. Lippincott-Schwartz, et al. (1990). "The T cell antigen receptor: insights into organelle biology." Annu Rev Cell Biol **6**: 403-431.

Knight, J. S., W. Luo, et al. (2014). "Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis." Circ Res **114**(6): 947-956.

Knight, J. S., V. Subramanian, et al. (2015). "Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice." Ann Rheum Dis **74**(12): 2199-2206.

Knight, J. S., W. Zhao, et al. (2013). "Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus." J Clin Invest **123**(7): 2981-2993.

Koenders, M. I., I. Devesa, et al. (2008). "Interleukin-1 drives pathogenic Th17 cells during spontaneous arthritis in interleukin-1 receptor antagonist-deficient mice." Arthritis Rheum **58**(11): 3461-3470.

Kotake, S., N. Udagawa, et al. (1999). "IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis." J Clin Invest **103**(9): 1345-1352.

Kouskoff, V., A. S. Korganow, et al. (1996). "Organ-specific disease provoked by systemic autoimmunity." Cell **87**(5): 811-822.

Kremer, J. M., R. Westhovens, et al. (2003). "Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig." N Engl J Med **349**(20): 1907-1915.

Lacy, P. (2005). "The role of Rho GTPases and SNAREs in mediator release from granulocytes." Pharmacol Ther **107**(3): 358-376.

Lande, R., D. Ganguly, et al. (2011). "Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus." Sci Transl Med **3**(73): 73ra19.

Lange, F., E. Bajtner, et al. (2005). "Methotrexate ameliorates T cell dependent autoimmune arthritis and encephalomyelitis but not antibody induced or fibroblast induced arthritis." Ann Rheum Dis **64**(4): 599-605.

Lassoued, S., L. Sixou, et al. (1991). "Antineutrophil cytoplasmic antibodies and antibodies to myeloperoxidase in rheumatoid arthritis." Arthritis Rheum **34**(8): 1069-1070.

Leffler, J., M. Martin, et al. (2012). "Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease." J Immunol **188**(7): 3522-3531.

Leshner, M., S. Wang, et al. (2012). "PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures." Front Immunol **3**: 307.

Ley, K., E. Smith, et al. (2006). "IL-17A-producing neutrophil-regulatory Tn lymphocytes." Immunol Res **34**(3): 229-242.

Li, P., D. Wang, et al. (2010). "Coordination of PAD4 and HDAC2 in the regulation of p53-target gene expression." Oncogene **29**(21): 3153-3162.

Lieschke, G. J., D. Grail, et al. (1994). "Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization." Blood **84**(6): 1737-1746.
Lim, M. B., J. W. Kuiper, et al. (2011). "Rac2 is required for the formation of neutrophil extracellular traps." J Leukoc Biol **90**(4): 771-776.
Linsley, P. S., J. L. Greene, et al. (1992). "Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes." J Exp Med **176**(6): 1595-1604.
Lood, C., L. P. Blanco, et al. (2016). "Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease." Nat Med **22**(2): 146-153.
Lubberts, E. (2015). "The IL-23-IL-17 axis in inflammatory arthritis." Nat Rev Rheumatol **11**(7): 415-429.
Luo, Y., K. Arita, et al. (2006). "Inhibitors and inactivators of protein arginine deiminase 4: functional and structural characterization." Biochemistry **45**(39): 11727-11736.
Luo, Y., B. Knuckley, et al. (2006). "A fluoroacetamidine-based inactivator of protein arginine deiminase 4: design, synthesis, and in vitro and in vivo evaluation." J Am Chem Soc **128**(4): 1092-1093.
Lutz, M. B., N. Kukutsch, et al. (1999). "An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow." J Immunol Methods **223**(1): 77-92.
MacLennan, I. C. (1994). "Germinal centers." Annu Rev Immunol **12**: 117-139.
Maffia, P., J. M. Brewer, et al. (2004). "Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells." J Immunol **173**(1): 151-156.
Mastronardi, F. G., D. D. Wood, et al. (2006). "Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation." J Neurosci **26**(44): 11387-11396.
Matsubara, S., T. Yamamoto, et al. (1991). "Complement C4-derived monocyte-directed chemotaxis-inhibitory factor. A molecular mechanism to cause polymorphonuclear leukocyte-predominant infiltration in rheumatoid arthritis synovial cavities." Am J Pathol **138**(5): 1279-1291.
Matsumoto, I., D. M. Lee, et al. (2003). "Low prevalence of antibodies to glucose-6-phosphate isomerase in patients with rheumatoid arthritis and a spectrum of other chronic autoimmune disorders." Arthritis Rheum **48**(4): 944-954.
Mauri, C., R. O. Williams, et al. (1996). "Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis." Eur J Immunol **26**(7): 1511-1518.
McInnes, I. B., B. P. Leung, et al. (1997). "Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis." Nat Med **3**(2): 189-195.
McInnes, I. B. and G. Schett (2011). "The pathogenesis of rheumatoid arthritis." N Engl J Med **365**(23): 2205-2219.
McKinstry, K. K., S. Golech, et al. (2007). "Rapid default transition of CD4 T cell effectors to functional memory cells." J Exp Med **204**(9): 2199-2211.
Mease, P. J., I. B. McInnes, et al. (2015). "Secukinumab Inhibition of Interleukin-17A in Patients with Psoriatic Arthritis." N Engl J Med **373**(14): 1329-1339.
Miossec, P., T. Korn, et al. (2009). "Interleukin-17 and type 17 helper T cells." N Engl J Med **361**(9): 888-898.

Mohr, W. and D. Wessinghage (1978). "The relationship between polymorphonuclear granulocytes and cartilage destruction in rheumatoid arthritis." Z Rheumatol **37**(3-4): 81-86.

Mohr, W., H. Westerhellweg, et al. (1981). "Polymorphonuclear granulocytes in rheumatic tissue destruction. III. an electron microscopic study of PMNs at the pannus-cartilage junction in rheumatoid arthritis." Ann Rheum Dis **40**(4): 396-399.

Montesinos, M. C., M. Takedachi, et al. (2007). "The antiinflammatory mechanism of methotrexate depends on extracellular conversion of adenine nucleotides to adenosine by ecto-5'-nucleotidase: findings in a study of ecto-5'-nucleotidase gene-deficient mice." Arthritis Rheum **56**(5): 1440-1445.

Mosmann, T. R., H. Cherwinski, et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-2357.

Murphy, K. M. and B. Stockinger (2010). "Effector T cell plasticity: flexibility in the face of changing circumstances." Nat Immunol **11**(8): 674-680.

Nakae, S., A. Nambu, et al. (2003). "Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice." J Immunol **171**(11): 6173-6177.

Nakashima, K., T. Hagiwara, et al. (2002). "Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes." J Biol Chem **277**(51): 49562-49568.

Nandakumar, K. S., J. Backlund, et al. (2004). "Collagen type II (CII)-specific antibodies induce arthritis in the absence of T or B cells but the arthritis progression is enhanced by CII-reactive T cells." Arthritis Res Ther **6**(6): R544-550.

Nandakumar, K. S., L. Svensson, et al. (2003). "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 **163**(5): 1827-1837.

Nathan, C. (2006). "Neutrophils and immunity: challenges and opportunities." Nat Rev Immunol **6**(3): 173-182.

Neeli, I., N. Dwivedi, et al. (2009). "Regulation of extracellular chromatin release from neutrophils." J Innate Immun **1**(3): 194-201.

Neeli, I., S. N. Khan, et al. (2008). "Histone deimination as a response to inflammatory stimuli in neutrophils." J Immunol **180**(3): 1895-1902.

Nerlov, C. and T. Graf (1998). "PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors." Genes Dev **12**(15): 2403-2412.

Nickdel, M. B., P. Conigliaro, et al. (2009). "Dissecting the contribution of innate and antigen-specific pathways to the breach of self-tolerance observed in a murine model of arthritis." Ann Rheum Dis **68**(6): 1059-1066.

Nossal, G. J. (1983). "Cellular mechanisms of immunologic tolerance." Annu Rev Immunol **1**: 33-62.

Nusse, O. and M. Lindau (1988). "The dynamics of exocytosis in human neutrophils." J Cell Biol **107**(6 Pt 1): 2117-2123.

O'Shea, J. J. and W. E. Paul (2010). "Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells." Science **327**(5969): 1098-1102.

Okamoto, Y., Y. Gotoh, et al. (2000). "Characterization of the cytokine network at a single cell level in mice with collagen-induced arthritis using a dual color ELISPOT assay." J Interferon Cytokine Res **20**(1): 55-61.

Orgovan, G. and B. Noszal (2011). "The complete microspeciation of arginine and citrulline." J Pharm Biomed Anal **54**(5): 965-971.

Palmer, E. (2003). "Negative selection--clearing out the bad apples from the T-cell repertoire." Nat Rev Immunol **3**(5): 383-391.

Papayannopoulos, V., K. D. Metzler, et al. (2010). "Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps." J Cell Biol **191**(3): 677-691.

Papayannopoulos, V. and A. Zychlinsky (2009). "NETs: a new strategy for using old weapons." Trends Immunol **30**(11): 513-521.
Pelanda, R. and R. M. Torres (2006). "Receptor editing for better or for worse." Curr Opin Immunol **18**(2): 184-190.
Peters, P. J., J. Borst, et al. (1991). "Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes." J Exp Med **173**(5): 1099-1109.
Phillipson, M. and P. Kubes (2011). "The neutrophil in vascular inflammation." Nat Med **17**(11): 1381-1390.
Pillay, J., I. den Braber, et al. (2010). "In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days." Blood **116**(4): 625-627.
Polzer, K., D. Baeten, et al. (2008). "Tumour necrosis factor blockade increases lymphangiogenesis in murine and human arthritic joints." Ann Rheum Dis **67**(11): 1610-1616.
Powell, J. D. (2006). "The induction and maintenance of T cell anergy." Clin Immunol **120**(3): 239-246.
Pratesi, F., I. Dioni, et al. (2014). "Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps." Ann Rheum Dis **73**(7): 1414-1422.
Pratesi, F., E. Petit Teixeira, et al. (2013). "HLA shared epitope and ACPA: just a marker or an active player?" Autoimmun Rev **12**(12): 1182-1187.
Puga, I., M. Cols, et al. (2012). "B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen." Nat Immunol **13**(2): 170-180.
Raptopoulou, A. P., G. Bertsias, et al. (2010). "The programmed death 1/programmed death ligand 1 inhibitory pathway is up-regulated in rheumatoid synovium and regulates peripheral T cell responses in human and murine arthritis." Arthritis Rheum **62**(7): 1870-1880.
Remijsen, Q., T. Vanden Berghe, et al. (2011). "Neutrophil extracellular trap cell death requires both autophagy and superoxide generation." Cell Res **21**(2): 290-304.
Rogers, P. R., C. Dubey, et al. (2000). "Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen." J Immunol **164**(5): 2338-2346.
Roncarolo, M. G., R. Bacchetta, et al. (2001). "Type 1 T regulatory cells." Immunol Rev **182**: 68-79.
Sadik, C. D., N. D. Kim, et al. (2012). "Neutrophils orchestrate their own recruitment in murine arthritis through C5aR and FcgammaR signaling." Proc Natl Acad Sci U S A **109**(46): E3177-3185.
Sakaguchi, N., T. Takahashi, et al. (2003). "Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice." Nature **426**(6965): 454-460.
Sangaletti, S., C. Tripodo, et al. (2012). "Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity." Blood **120**(15): 3007-3018.
Santos, L. L., E. F. Morand, et al. (1997). "Anti-neutrophil monoclonal antibody therapy inhibits the development of adjuvant arthritis." Clin Exp Immunol **107**(2): 248-253.
Sawa, S., D. Kamimura, et al. (2006). "Autoimmune arthritis associated with mutated interleukin (IL)-6 receptor gp130 is driven by STAT3/IL-7-dependent homeostatic proliferation of CD4+ T cells." J Exp Med **203**(6): 1459-1470.
Schellekens, G. A., H. Visser, et al. (2000). "The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide." Arthritis Rheum **43**(1): 155-163.

Schwarzenberger, P., V. La Russa, et al. (1998). "IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines." J Immunol **161**(11): 6383-6389.
Seder, R. A., R. N. Germain, et al. (1994). "CD28-mediated costimulation of interleukin 2 (IL-2) production plays a critical role in T cell priming for IL-4 and interferon gamma production." J Exp Med **179**(1): 299-304.
Shahrara, S., S. R. Pickens, et al. (2009). "IL-17 induces monocyte migration in rheumatoid arthritis." J Immunol **182**(6): 3884-3891.
Smolen, J. S., A. Beaulieu, et al. (2008). "Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial." Lancet **371**(9617): 987-997.
Smolen, J. S., R. Landewe, et al. (2014). "EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update." Ann Rheum Dis **73**(3): 492-509.
Spengler, J., B. Lugonja, et al. (2015). "Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid." Arthritis Rheumatol **67**(12): 3135-3145.
Stark, M. A., Y. Huo, et al. (2005). "Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17." Immunity **22**(3): 285-294.
Stastny, P. (1976). "Mixed lymphocyte cultures in rheumatoid arthritis." J Clin Invest **57**(5): 1148-1157.
Suzuki, A., R. Yamada, et al. (2003). "Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis." Nat Genet **34**(4): 395-402.
Szabo, S. J., S. T. Kim, et al. (2000). "A novel transcription factor, T-bet, directs Th1 lineage commitment." Cell **100**(6): 655-669.
Szekanecz, Z., A. Pakozdi, et al. (2009). "Chemokines and angiogenesis in rheumatoid arthritis." Front Biosci (Elite Ed) **1**: 44-51.
Tak, T., K. Tesselaar, et al. (2013). "What's your age again? Determination of human neutrophil half-lives revisited." J Leukoc Biol **94**(4): 595-601.
Takei, H., A. Araki, et al. (1996). "Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis." J Leukoc Biol **59**(2): 229-240.
Thalayasingam, N. and J. D. Isaacs (2011). "Anti-TNF therapy." Best Pract Res Clin Rheumatol **25**(4): 549-567.
Tillack, K., P. Breiden, et al. (2012). "T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses." J Immunol **188**(7): 3150-3159.
Tiniakou, I., E. Drakos, et al. (2015). "High-density lipoprotein attenuates Th1 and th17 autoimmune responses by modulating dendritic cell maturation and function." J Immunol **194**(10): 4676-4687.
Todd, I., R. Pujol-Borrell, et al. (1985). "Interferon-gamma induces HLA-DR expression by thyroid epithelium." Clin Exp Immunol **61**(2): 265-273.
Trentham, D. E., A. S. Townes, et al. (1977). "Autoimmunity to type II collagen an experimental model of arthritis." J Exp Med **146**(3): 857-868.
van Gaalen, F. A., R. E. Toes, et al. (2004). "Association of autoantibodies to glucose-6-phosphate isomerase with extraarticular complications in rheumatoid arthritis." Arthritis Rheum **50**(2): 395-399.
Veldhoen, M., R. J. Hocking, et al. (2006). "TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells." Immunity **24**(2): 179-189.

Vincent, C., F. de Keyser, et al. (1999). "Anti-perinuclear factor compared with the so called "antikeratin" antibodies and antibodies to human epidermis filaggrin, in the diagnosis of arthritides." Ann Rheum Dis **58**(1): 42-48.
Viola, A. and A. Lanzavecchia (1996). "T cell activation determined by T cell receptor number and tunable thresholds." Science **273**(5271): 104-106.
von Kockritz-Blickwede, M., O. Goldmann, et al. (2008). "Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation." Blood **111**(6): 3070-3080.
Vossenaar, E. R., A. J. Zendman, et al. (2003). "PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease." Bioessays **25**(11): 1106-1118.
Wallberg, M., J. Bergquist, et al. (2007). "Malondialdehyde modification of myelin oligodendrocyte glycoprotein leads to increased immunogenicity and encephalitogenicity." Eur J Immunol **37**(7): 1986-1995.
Walunas, T. L., D. J. Lenschow, et al. (1994). "CTLA-4 can function as a negative regulator of T cell activation." Immunity **1**(5): 405-413.
Wang, Y., M. Li, et al. (2009). "Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation." J Cell Biol **184**(2): 205-213.
Wang, Y., J. Wysocka, et al. (2004). "Human PAD4 regulates histone arginine methylation levels via demethylimination." Science **306**(5694): 279-283.
Warnatsch, A., M. Ioannou, et al. (2015). "Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis." Science **349**(6245): 316-320.
Weiner, H. L. (2001). "Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells." Immunol Rev **182**: 207-214.
Wessels, J. A., T. W. Huizinga, et al. (2008). "Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis." Rheumatology (Oxford) **47**(3): 249-255.
Willis, V. C., A. M. Gizinski, et al. (2011). "N-alpha-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis." J Immunol **186**(7): 4396-4404.
Wipke, B. T. and P. M. Allen (2001). "Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis." J Immunol **167**(3): 1601-1608.
Wong, P. K., J. M. Quinn, et al. (2006). "Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis." Arthritis Rheum **54**(1): 158-168.
Wooley, P. H., J. R. Seibold, et al. (1989). "Pristane-induced arthritis. The immunologic and genetic features of an experimental murine model of autoimmune disease." Arthritis Rheum **32**(8): 1022-1030.
Yang, X. O., A. D. Panopoulos, et al. (2007). "STAT3 regulates cytokine-mediated generation of inflammatory helper T cells." J Biol Chem **282**(13): 9358-9363.
Yousefi, S., J. A. Gold, et al. (2008). "Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense." Nat Med **14**(9): 949-953.
Yousefi, S., C. Mihalache, et al. (2009). "Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps." Cell Death Differ **16**(11): 1438-1444.
Zal, T., A. Volkmann, et al. (1994). "Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen." J Exp Med **180**(6): 2089-2099.
Zamoyska, R. (1998). "CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses?" Curr Opin Immunol **10**(1): 82-87.

Zenaro, E., E. Pietronigro, et al. (2015). "Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin." Nat Med **21**(8): 880-886.
Zhang, J., J. Dai, et al. (2004). "cDNA cloning, gene organization and expression analysis of human peptidylarginine deiminase type VI." Acta Biochim Pol **51**(4): 1051-1058.
Zheng, W. and R. A. Flavell (1997). "The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells." Cell **89**(4): 587-596.
Ziolkowska, M., A. Koc, et al. (2000). "High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism." J Immunol **164**(5): 2832-2838.

APPENDIX I

Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation

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Abstract

Aberrant formation of neutrophil extracellular traps (NETs) is a key feature in rheumatoid arthritis (RA) and plays a pivotal role in disease pathogenesis. However, the mechanism through which NETs shape the autoimmune response in RA remains elusive. In this study, we demonstrate that inhibition of peptidylarginine deiminases activity in collagen-induced arthritis (CIA) mouse model significantly reduces NET formation, attenuates clinical disease activity, and prevents joint destruction. Importantly, peptidylarginine deiminase 4 blocking markedly reduces the frequency of collagen-specific IFN-γ-producing T helper 1 (Th1) cells in the draining lymph nodes of immunized mice. Exposure of dendritic cells (DCs) to CIA-derived NETs induces DC maturation characterized by significant upregulation of costimulatory molecules, as well as elevated secretion of IL-6. Moreover, CIA-NET-treated DCs promote the induction of antigen-specific Th1 cells in vitro. Finally, NETs from RA patients show an increased potential to induce the maturation of DCs from healthy individuals, corroborating the findings obtained in CIA mouse model. Collectively, our findings delineate an important role of NETs in the induction and expansion of Th1 pathogenic cells in CIA through maturation of DCs and reveal a novel role of NETs in shaping the RA-autoimmune response that could be exploited therapeutically.

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APPENDIX II

Elimination of Granulocytic Myeloid-Derived Suppressor Cells in Lupus-Prone Mice Linked to Reactive Oxygen Species–Dependent Extracellular Trap Formation

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Abstract

Objective. Emerging evidence supports a crucial role of myeloid-derived suppressor cells (MDSCs) in the regulation of autoimmune diseases, however their role in systemic lupus erythematosus (SLE) remains unknown. In this study we address the role of MDSCs in the pathogenesis of SLE.

Methods. The NZB/W F1 lupus-prone mouse model was used to assess MDSC phenotype by flow cytometry and function through *in vitro* T-cell proliferation assay and real-time quantitative PCR. Extracellular Trap formation was evaluated by immunofluoresence and confocal microscopy. The production of reactive oxygen species (ROS) by $Ly6G^+$ cells was determined by FACS analysis.

Results. Herein, we demonstrate an impaired expansion and defective function of MDSCs in the lymphoid organ of NZB/W F1 lupus-prone mice with established disease that involved predominantly the granulocytic MDSC cell subset (G-MDSCs). More specifically, we found increased elimination of G-MDSCs due to extracellular trap (ET) formation driven by the inflammatory milieu of lupus and we demonstrate a role of cytokines such as IFN-α, IFN-γ and IL-6 in this process. Induction of ET release by G-MDSCs was mediated by production of ROS, since inhibition of ROS generation significantly reduced ET release.

Conclusion. Collectively, our findings reveal the elimination of a crucial regulatory immune cell subset in SLE microenvironment and provide new insights into the pathogenetic mechanisms of the disease.

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