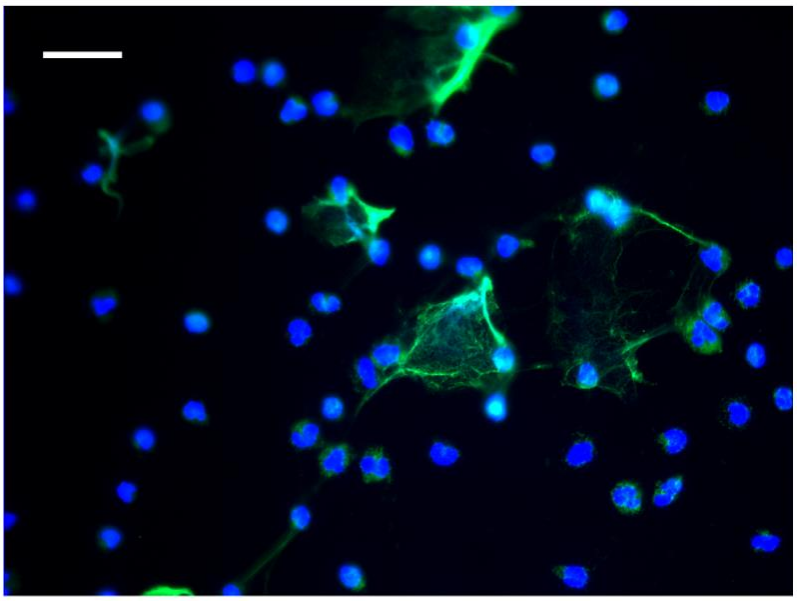
The expression of IL-1β in NETs in neutrophils stimulated with monosodium urate crystals



Department of Molecular Biology & Genetics

Bouti Panagiota

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ΕΥΧΑΡΙΣΤΙΕΣ

Η παρούσα πτυχιακή εργασία πραγματοποιήθηκε στο Εργαστήριο Μοριακής Αιματολογίας του Πανεπιστημιακού Γενικού Νοσοκομείου Αλεξανδρούπολης και αποτελεί την κορύφωση των σπουδών μου στο τμήμα Μοριακής Βιολογίας & Γενετικής στο Δημοκρίτειο Πανεπιστήμιο Θράκης.

Η ολοκλήρωση της πτυχιακής εργασίας θα ήταν αδύνατη χωρίς την πολύτιμη στήριξη του επιβλέποντα καθηγητή μου κ. Κωνσταντίνου Ρίτη, τον οποίο και ευχαριστώ βαθύτατα για όλη τη βοήθεια και τις ουσιώδεις συμβουλές που μου προσέφερε.

Χρωστάω ένα μεγάλο ευχαριστώ στον κ. Καμπά Κωνσταντίνο για την άριστη συνεργασία που είχαμε στα πλαίσια εκπόνησης της εργασίας, την προθυμία και την βοήθεια που ποτέ δε δίστασε να μου δώσει. Δε θα μπορούσα να ξεχάσω την κ. Βικτώρια Τσιρονίδου, την οποία και ευχαριστώ βαθύτατα για την υπομονή της αλλά και την ενθάρρυνση που μου παρείχε όλο αυτό το διάστημα. Ευχαριστώ επίσης τον κ. Κωνσταντινίδη Θεοχάρη και την κ. Ηλιάνα Αγγελίδου για τις συμβουλές και την όμορφη επικοινωνία, καθώς και τον Αραμπατζιόγλου Θάνο, από τον οποίο έλαβα μεγάλη βοήθεια στην πραγματοποίηση της πτυχιακής εργασίας. Ευχαριστώ θερμά τους συμφοιτητές μου Μήτσιο Αλέξανδρο και Συγκούνα Αθηνά με τους οποίους μοιράστηκα το άγχος και την αγωνία μου για την τελειοποίηση της εργασίας καθώς και όλα τα υπόλοιπα μέλη για το κλίμα και την ευχάριστη ατμόσφαιρα στο χώρο του εργαστηρίου. Επίσης, θα ήθελα να ευχαριστήσω τις φίλες μου για την άμετρη υποστήριξη και συμπαράσταση που μου έδειξαν στα 4 χρόνια της φοιτητικής μας πορείας.

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Αλεξανδρούπολη, 2015

ΠΕΡΙΛΗΨΗ

Σκοπός: Τα ουδετερόφιλα είναι κύτταρα της φυσικής ανοσίας και αποτελούν την πρώτη γραμμή άμυνας κατά των παθογόνων μικροοργανισμών. Ένας από τους μηχανισμούς που τα κύτταρα αυτά χρησιμοποιούν για να ασκήσουν την αντιμικροβιακή τους δράση είναι οι εξωκυττάριες παγίδες (ExtracellularTrapsEts). Η συμμετοχή των NETs στην ουρική αρθρίτιδα, καθώς και η έκφραση επάνω σε αυτά της IL-1β σε αυτοφλεγμονώδεις παθήσεις, αποτέλεσε την αφορμή για να εξετάσουμε την έκφραση της IL-1β επάνω σε NETs στο περιβάλλον της ουρικής αρθρίτιδας.

Μέθοδοι: Η παρουσία της IL-1β ελέγχθηκε μετά από διέγερση ουδετεροφίλων από φυσιολογικούς δότες με κρυστάλλους ουρικού οξέος ή αρθρικό υγρό. Η αναστολή της IL-1β σε διεγέρσεις μονοπυρήνων πραγματοποιήθηκε με το anakinra, το οποίο δρα ως αναστολέας του υποδοχέα της κυτταροκίνης. Για την πραγματοποίηση των πειραμάτων χρησιμοποιήθηκαν ανοσοϊστοχημεία, ανοσοφθορισμός καθώς και καλλιέργειες ανθρώπινων κυττάρων.

Αποτελέσματα: Τα αποτελέσματα ανέδειξαν την παρουσία της IL-1β σε NETs από ουδετερόφιλα τα οποία είχαν διεγερθεί με κρυστάλλους ουρικού οξέος είτε αρθρικό υγρό. Αναστολή της λειτουργίας της IL-1β μέσω anakinra σε διεγέρσεις μονοπυρήνων με NETs έδειξε ότι η κυτταροκίνη είναι στην βιοενεργή μορφή της και μπορεί να σηματοδοτήσει παρουσία της επάνω στα NETs..

Επίλογος: Η εξαγωγή των παραπάνω αποτελεσμάτων αναδεικνύει την IL-1β ως μια βασική προφλεγμονώδη κυτταροκίνη η οποία εντοπίζεται στα ουδετερόφιλα, δηλαδή το βασικό κυτταρικό πληθυσμό της φλεγμονής στην ουρική αρθρίτιδα.

ABSTRACT

Backround/Aim: Neutrophils are cells of the innate immune system and consist the first line of defense against pathogens. These cells exert their antimicrobial action through development extracellular traps (ExtracellularTrapsEts). The participation of NETs in gout, a condition characterized by uncontrolled inflammation and overproduction of IL-1 gave rise to consider the study of development mechanisms of NETs with IL-1β- stimulated with uric acid crystals (MSU crystals) and in vitro inhibition with anakinra.

Methods: The presence of IL-1 was detected after stimulation of cells with uric acid crystals, or synovial fluid. The suppression of IL-1 was performed in the presence of anakinra, which acts as a suppressor of cytokine receptor. To perform the experiments were used immunoblotting, immunofluorescence and cultured primary human cells.

Results: The results revealed the presence of IL-1β in NETs from neutrophils stimulated with uric acid crystals or synovial fluid as well as the upregulation of IL-1 production in mononuclear from the same pathophysiology. Finally, it demonstrated the inhibition of IL-1 production after stimulation of cells with anakinra.

Conclusion: The export of these results demonstrates the IL-1 as a key pro-inflammatory cytokine that is localized in the basal cell population of inflammation in gout.

INTRODUCTION

Gout- Patients

Gout is the most common inflammatory arthritis disease in men older than 40 years. The rate at which rheumatoidis arthritis occurs is 1.4 in women and 4.0 in men per 1,000 person-years (1).Although researches have shown that women become increasingly susceptible to gout after menopause because of increased uricemia parallel with decreasing of estrogen levels(5). It is an acute inflammatory arthritis characterized by the deposition of monosodium urate (MSU) crystals in the joints and periarticular tissue, leading to acute attacks or chronic arthritis. Hyperucaemia is another symptom that accompanies gout. Increased uric acid is resulting in the oversaturation of blood or synovial fluid with urate above the critical level for crystal formation (2).

Gout can be classified as primary or secondary, depending on the presence / absence of an identified cause of hyperuricemia. Primary gout is more common, without identifiable underlying disease causing of the hyperuricemia (5) .Secondary gout, which is less common, can be a result of many conditions. Alcohol intoxication causes lactic acidemia and reduces renal urate excretion .Thiazide and loop diuretics (antihypertensives), increase serum urate levels by interfering with renal tubular ion transport. Several immunosuppressant like cyclosporine, used in patients undergoing solid organ transplantation, substantially reduces the renal clearance of serum urate (6).

Surprisingly, the prevalence of hyperuricaemia is 10 to 20% in the Western population, although a substantial number of these patients stay asymptomatic during life. As it has already been mentioned, hyperuricemia is a primary risk factor for the development of gout, although it is likely that two–thirds or more of hyperuricemic individuals will remain asymptomatic (incidence rate less than 50/1000/year) (2).There are many risk factors which could induce gout such as obesity, metabolic syndrome, diuretic therapy, high-purine diet and alcohol intake.

Acute gout is characterized by sudden outbreak, intensely painful, self-limited inflammatory attacks of mono- or oligoarticular arthritis. The duration of disease's flares varies from days to weeks, while an increased frequency and duration of attacks is observed in patients who did not follow a complete treatment. Except from this type of attacks, some patients develop a chronic, refractory to treatment arthritis, characterized by the formation of tophi (1).

The diagnosis of this disorder is correlated with the presence of needle-shaped monosodium urate (MSU) crystals in synovial fluid, often inside neutrophils, the predominant cell population in synovial fluid during acute attacks (1).

The treatment of gout includes the changes in lifestyle, nutrition, and adjunctive therapies as well as different classes of drugs which are approved for lowering urate levels. For the latter, there are xanthine oxidase inhibitors, uricosuric agents, uricase agents and some which are still under development, such are inhibitors of urate transporter, purine nucleoside phosphorylase and interleukin-1 antagonists. However, the security and viability of combination therapies for the treatment of gout still stayed hazy. Sadly, bringing down of urate concentration does not relive gout side effects and urate lowering down sufficiency is poor and additionally clinical routcomes in gout patients (4).

MSU Crystals

In contrast with other mammals where uric acid (UA) is metabolized to allantoin by uricase, UA is the end product of purine metabolism in humans. The consumption of purines in the diet and the biosynthesis of UA from endogenous purines and renal balance determine the amount of UA in blood. Also it is mentioned that up to 90% of the filtered UA is reabsorbed (3).

Degradation of purine nucleotides (Figure 1) starts with nucleotidase activity in reaction which releases phosphate from nucleotides and produces nucleosides, adenosine and guanosine (3). Adenosine is than deaminated into inosine in reaction catalyzed by adenosine deaminase. Purine nucleotide phosphorilase hydrolyses ribose group from inosine and guanosine to produce xypoxsanthine and guanine, respectively (3). Guanine is deaminated to xsanthine. Xsanthine oxidoreductase is widely distributed enzyme. It oxidases hypoxantine to xsanthine and finally xsanthine to uric acid in the liver, gut, lung, kidney, heart, brain and plasma (4).

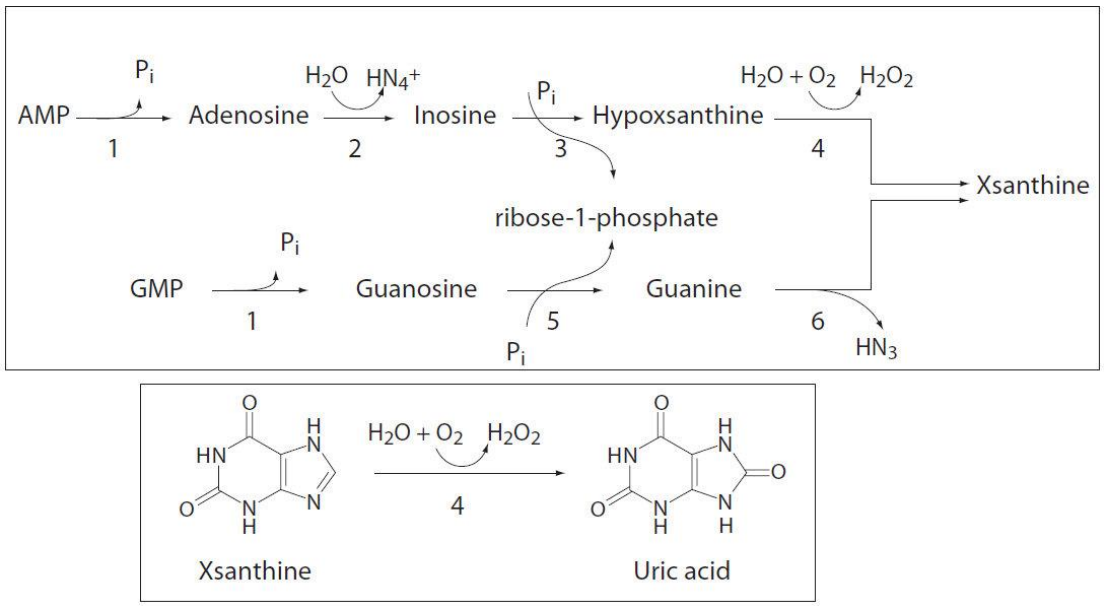


Figure 1. Degradation of purine nucleotides and formation of uric acid. 1 - Nucleotidase; 2 - Adenosine deaminase; 3 - Inosine phosphorilase; 4 - Xsanthine oxidase; 5 - Purine nucleosidase phosphorilase; 6 -

In the promoter site of uricase there are two deletion which are responsible for the high uric levels in humans compared with other mammals. This causes the uric acid plasma levels to be around 300 mM. Also, there is a remarkable balance among the production and the disposal of uric acid. In other words, a small increment could cause supersaturation and crystal formation. Thus, the deposition of monosodium urate monohydrate microcrystals or uric acid in various parts of the body could lead to the development of gout (5).

Whenever the level of uric acid in the synovial fluid fluctuates and the synovial fluid gets supersaturated with uric acid the risk for crystal formation increases. The solubility of urate in synovial fluid is influenced by more factors than solely the concentration, including temperature, level of dehydration and the presence of nucleating agents. These factors in part explain the predilection of gout in the first metatarsal phalangeal joint (podogra, low temperature), in osteoarthritic joints (nucleating debris) and the nocturnal onset (dehydration) (Fig. 2) (2). The likelihood of such diseases increases when urate concentrations exceed 380 μmol/L.

Crystallization of uric acid and the release of MSU crystals by soft tissue deposits are the initiatory pathogenic event in gout.

Uric acid has been identified as a danger signal that triggers a cytosolic sensor, the inflammasome, a signalling platform that is required for the activation of various inflammatory mediators in inflammatory cells, such as interleukin-1, a cytokine that is critical to the initiation of acute inflammation in gout and is described above(2). The characterization of MSU as a danger-associated molecular pattern, the description of intracellular pathways implicated in its recognition and the subsequent production of inflammatory cytokines by innate immune cells resulted in categorization of gout in the group of autoinflammatory disorders (17).

However, UA is not only a waste product of purine metabolism or an inert compound. It has been demonstrated that it also has an important role in many biological functions (3).

Figure 2 Formation of tophi in gout and its uric acid deposition

IL-1β

IL-1 is an important proinflammatory cytokine which has multiple properties and affects almost all cell types. It is a mediator of the acute phase of inflammation by induction of local and systemic responses. IL-1 is strongly expressed by monocytes, tissue macrophages and dendritic cells, but is also produced by B lymphocytes, NK cells and epithelial cells. IL-1 induces pain sensitivity, fever, vasodilation and hypotension. The activity of both IL-1α and IL-1β exert by binding and signalling through IL-1 receptor type I (IL-1RI). This receptor type is present in almost all cell types (15).More than any other cytokine family, the IL-1 family of ligands and receptors is primarily associated with acute and chronic inflammation. The cytosolic segment of each IL-1 receptor family member contains the Toll-IL-1-receptor domain.

Each Toll like receptor has also the domain that mentioned above, and they respond to microbial products and viruses. Since Toll-IL-1-receptor domains are functional for both receptor families, responses to the IL-1 family are fundamental to innate immunity. Of the 11 members of the IL-1 family, IL-1has emerged as a therapeutic target for a remarkable number of systemic and local inflammatory conditions called autoinflammatory diseases (13).

IL-1β is a main pro-inflammatory cytokine of IL-1 family. It is involved in multiple inflammatory pathways that ultimately lead to the production of Tumor Necrosis Factor, IL-6, prostaglandin E2, nitricoxide, and adhesion molecules that are involved in inflammation diseases (14).

ProIL-1β cannot bind to IL-1RI. Prior proteolytic processing of the propeptide is required for receptor binding and activation. This process is achieved in most cases intracellularly by the protease caspase-1. Caspase-1 is the intracellular cysteine protease which converts the inactive precursor to the active “mature” cytokine by cleaving the N-terminal 116 aa from the IL-1 precursor. Caspase-1 exists in tissue macrophages and dendritic cells as an inactive zymogen and requires conversion to an active enzyme by autocatalysis. However, in circulating human blood monocytes, caspase-1 is

present in an active state (13). Nevertheless, caspase-1 is considered to be the master protease required for activation of IL1-β. As ProIL-1β and several other proteins lacking a signal peptide, tent to be called as "leaderless", thus they are secreted by a non-canonical pathway called the unconventional protein secretion pathway (15).

NLRP3 (also known as cryopyrin and NALP3) is expressed by myeloid cells and is upregulated in response to stimulation of macrophages with pathogen-associated molecule patterns (PAMPs) . The gene encodes an amino-terminal pyrin domain, a central NBD, and a C-terminal LRR. NLRP3 lacks a caspase recruitment domain (CARD) and cannot recruit procaspase-1 except in the presence of the adaptor molecule ASC. NLRP3 interacts with ASC via pyrin domain homophilic interactions. HSP90 and SGT1 have been shown to interact with NLRP3 LRRs to maintain an inactive but stabilized structure. In vitro ectopic expression systems have demonstrated that NLRP3 can activate caspase-1 in the presence of ASC. Activation of the NLRP3 inflammasome results in IL-1β processing, and various conditions can induce forms of programmed inflammatory cell death (16).

This inflammatory cascade is counter balanced by a naturally occurring IL-1β receptor antagonist (IL-1Ra), produced by macrophages, which competitively binds to the IL-1β receptor (14). The balance between IL-1β and IL-1Ra released in neutrophils during different phases of gout could participate in the progression of inflammation towards resolution (1).

Several studies have described the use of IL-1receptor antagonism with anakinra in cases of acute gout flares. Anakinra is approved for reducing the signs and symptoms of rheumatoid arthritis and slows the progressive joint destructive characteristics of the disease (14). These data further reinforce the proposed key role of IL-1β by the experimental models in the pathogenesis of this disorder.

During the past years, based on in vitro studies and in vivo animal models, gout has been identified as a prototype IL-1β-dependent autoinflammatory disease, an observation that has been also confirmed by clinical studies, addressing the therapeutic effect of IL-1 blockage (18). The modesteffectiveness and the daily use of anakinra precluded the widespread introduction of the agent in the treatment for RA. Clinical trials using canakinumab are ongoing, trying to gain a role for IL-1 inhibition in the treatment of this disease, if proven successful, and offer an additional targeted therapy (17).

Neutrophils & NETosis

Neutrophils are the foot soldiers of the immune system necessary to maintain homeostasis of the organism. They are short-lived polymorphonuclear granulocytes which constitute a primary defence against microbial infections (7). In the absence of infection, these short-lived, terminally differentiated cells will leave the bone marrow and die in the confines of the bloodstream. Upon infection, tissue-resident macrophages and other sentinel cells, secrete inflammatory cytokines and chemoattractants that are able to recruit and prime neutrophils. In response to these molecules, neutrophils leave the bloodstream and invade the infected tissues in a selectin- and integrin-mediated process known as extravasation (8).

At the inflammatory site, activated immune cells acquire the ability to kill pathogens. To fulfill that task, neutrophils use a number of strategies such as phagocytosis,

degranulation and the recently discovered formation of extracellular traps. During phagocytosis, internalized pathogens are translocated to phagosomes where the antimicrobial factors derived from granules and reactive oxygen species (ROS) create a killing environment for pathogens. The second mechanism, degranulation, is

similar to phagocytosis, but rather than being engulfed the pathogens are killed extracellularly by the same antimicrobial factors which are in part released outside the cell. The neutrophil extracellular traps (NETs) can be released by neutrophils in a process called netosis. NETs are a special kind of trap formed by decondensed chromatin fibres decorated with antimicrobial factors delivered by the granules. The main function of NETs is trapping and killing of pathogens (9).

NETs are web-like structures that are composed of decondensed chromatin in complex with over 30 different neutrophil proteins that can capture, neutralize, and kill a variety of microbes (8). To date, two major NET release mechanisms have been

described. In the first mechanism, neutrophils release NETs via a slow lytic cell death mechanism. This appears to be a major route for NET release. In addition, Pilsczek et

al. have described that a small number of neutrophils rapidly expulse their nuclear content via vesicular secretion, yielding NETs and live intact cytoplasts that continue to crawl and digest microbes (10).

To date, because of the short life of neutrophils, very little is known about the mechanisms of NETosis, terminally differentiated leukocytes,which are unable to divide, and genetic manipulations with them are difficult.Although, NETs have a unique composition: DNA, granular components, histones, and some cytoplasmic proteins. No more than 30 different proteins are detected in NET composition most of these proteins are of granular origin; only a few types of proteins get into NETs from the nucleus (histones) and very few come from the cytoplasm. As reported previously, NET formation is a gradual process with several successive steps:

1) ROS generation

2) Transport of neutrophil elastase (NE), and, later, myeloperoxidase (MPO) from the granules to the nucleus

3) Histone modification, and,

4) Disruption of cytoplasmic membrane and release of chromatin.

It would be worth dwelling on these steps in more detail, because together they compose a unique mechanism of cell death, i.e. NETosis (11).

Like many other physiological processes in an organism, NET formation plays not only positive but also negative role. The negative effect of NETs is related to their participation in the development of autoimmune diseases based on self-sustained adaptive immune response to self-antigens, which results in cell and tissue damage. The initial basis for the induction of adaptive response is chronic activation of cells of the innate immune system and synthesis of antiinflammatory cytokines by these cells (12).

Neutrophils & Gout

Neutrophil identification in the synovial fluid and uptake of MSU crystals by these cells is the hallmark for the diagnosis of acute gout. In contrast to acute inflammatory arthritis, neutrophils are not present in the synovial fluid in normal joints (Figure 3) (1).

Neutrophil recruitment in gout

Several lines of evidence have shown a clear-cut correlation between IL-1 signaling and neutrophil infiltration at the affected joints. It has been recently shown that NLRP3 inflammasome-dependent IL-1β production promoted neutrophil recruitment, which relied on the production of CXCR2- acting chemokines, mainly CXCL1(19).

Neutrophils infiltrating the inflamed joints encounter and recognize MSU crystals, which results in their activation and the subsequent amplification of inflammation. Neutrophil activation by MSU crystals has been proposed to be mediated by FcgRIIIB and the b2-integrin CD11b receptors, as demonstrated by studies assessing the inhibitory effect of antibodies targeting these receptors on neutrophil functional responses (1).

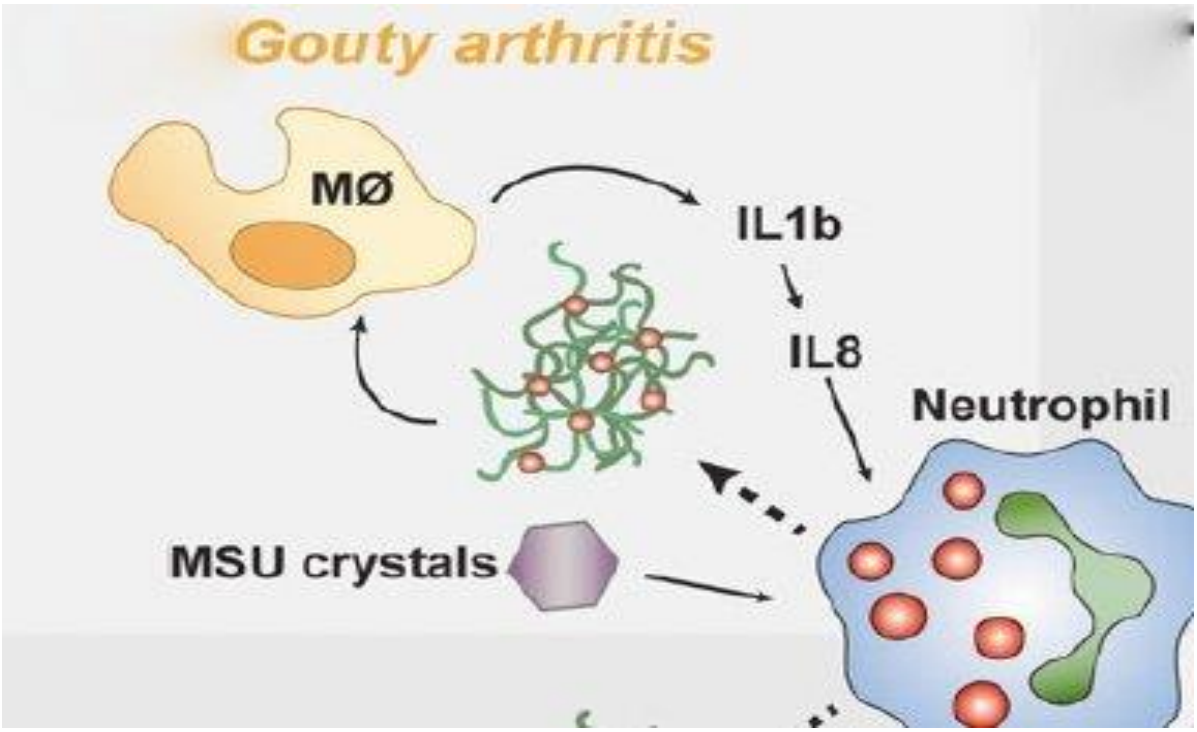


Figure 3. NETs are implicated in the onset of inflammatory and autoimmune diseases. Deposition of MSU leads to release of IL-1β by monocytes and induction of IL-8, with subsequent neutrophil recruitment. MSU needles induce release of NETs that stimulate a feedback loop of IL-1β production by monocytes (8).

Functional activity of neutrophils in response to MSU crystals

Several lines of evidence have demonstrated the function role of neutrophils in the amplification and maintenance of MSU crystal-dependent inflammatory attacks. This role is mediated by the release of a variety of mediators known to inflict tissue damage or promote inflammation, including ROS, antimicrobial peptides, the myeloid-related peptides S100A8 and S100A9, and cytokines/chemokines, including IL-1 and IL-8.

Neutrophils & IL-1β

The link between IL-1β and neutrophils in gout is bidirectional; neutrophils produce IL-1β, while IL-1β regulates neutrophil functions. Several lines of evidence suggest that neutrophils are an important source of IL-1β acute inflammatory disorders. Recently, the secretion of IL-1β in a NLRP3 inflammasome-dependent has been shown (1). However, previous reports indicated that neutrophil proteases are able for pro-IL-1β processing in an inflammasome-independent manner.

Several studies imply that the recruitment of high numbers of activated neutrophils, which produce significant amounts of IL-1β, may be essential for acute IL-1β-driven inflammation, including gout. However, considering that neutrophils are not detected in the synovial fluid in noninflamed joints or at tophi, the role of resident macrophages in the production of IL-1β at the initial phase of gout remains indispensable (1).

Except from the indirect effect of IL-1β in the recruitment of neutrophils at the site of inflammation, there is experimental evidence that IL-1β is directly involved in neutrophil function. IL-1β signaling interferes with cell death pathways in neutrophils.

IL-1β has been also shown to induce autophagy in human neutrophils, further

implying a possible anti-apoptotic effect in IL-1β-related disorders.

NET release has been recently demonstrated in the context of gout. It has observed the formation of NETs by synovial fluid and peripheral neutrophils from patients with acute gouty arthritis. This was also observed in neutrophils treated with MSU crystals and synovial fluid or sera from such patients. IL-1β was partially involved in this process, while inhibition of autophagy with 3-MA and bafilomycin A1 attenuated this process (1).

In conclusion, neutrophil elastase, a dominant component of neutrophils, has been identified as the culprit for the processing of pro-IL-1β.Data also imply that IL-8 could play a significant role as a chemotactic factor in MSU-dependent arthritis, not during the initiation, but for the evolution of inflammation (1).

SCOPE OF THE STUDY

The formation of Neutrophil Extracellular Traps constitutes among others one main research field of the Laboratory of Molecular Hematology of DUTH. Bearing in mind that IL-1β is a major pro-inflammatory cytokine in several diseases included gout and neutrophils are a vital population in inflammation, we investigated whether NETs derived from neutrophils are decorated with this protein.

In addition, considering that anakinra is the major inhibitor of the receptor of IL-1β, we examined whether the stimulation of cells with anakinra tends to inhibit the expression of the two forms of IL-1β(pro-IL-1β and mature IL-1β).

MATERIALS & METHODS

Patients and sampling

Synovial fluid was collected from six patients with active gout attack, centrifuged at 800x g to separate cells from supernatant. Supernatants were stored at 220uC until used. Synovial fluid cell populations were characterized by May-Grunwald and indicated a neutrophil predominance (90%). Synovial cells were used immediately for determination of NET formation. Furthermore, PMNs were isolated from heparinized blood from both patients and healthy donors by Histopaque double-gradient density centrifugation as previously described. Serum was also isolated from patients with gout. The study protocol design was in accordance of the Declaration of Helsinki and the procedures have been approved by the local ethics committee (Scientific Committee of the University Hospital of Alexandroupolis, Greece). Informed, written consent has been obtained from all participants involved in the present study.

The use of synovial fluid demands defrost from 80 ºC, while MSU crystals warmed in 50 ºC.

MSU crystal and PMA preparation

MSU crystals were prepared under pyrogen-free conditions. Urate acid sodium salt (Sigma-Aldrich) was dissolved in 1 M NaOH (25 mg/ml) and boiled for 2 hours at 200uC prior to crystallization. The solution was left to cool at room temperature and filtered through a 0.2 mM filter. It was then incubated at room temperature for 7 days. The resulting crystals were washed with ethanol and acetone and allowed to air dry under sterile conditions. Triclinic MSU crystals were needle-shaped, between 5 and 20 mm in length and verified to be free of detectable LPS contamination by the

Limulus amebocyte cell lysate assay (Sigma- Aldrich). Before each use of crystals MSU, preparation is made by stirring for at least 15 minutes at 50 ^{o}C. It was also made use of PMA (40ng/mL, Sigma-Aldrich, St. Luois, MO, USA), the preparation of which was after dilution with PBS (1X). In the well-plate was added 3 μl/500 well and 7,2 μl/1200 well.

Separation of peripheral blood cells

For isolation of polymorphonuclear cells of peripheral blood from healthy donors were used blood collection tubes with heparin. The separation of the mononuclear and polymorphonuclear cells peripheral blood was as follows: 6ml total blood was dissolved in an equal volume of saline and epistoivachthikan in 3ml Ficoll-histopaque 1119 and 3ml Ficoll-Histopaque 1077 . This was followed by centrifugation at 500xg for 30 min at room temperature. The separating 1077 separates the mononuclear layer, while 1119 separates the layer of polymorphonuclear cells. Then the layer of polymorphonuclear washed with sterile PBS followed by centrifugation for 10 minutes at 300xg. The supernatant was removed and the cells were counted and tested for purity and viability. Then, the cells were stimulated as described below.

Stimulation and inhibition studies

Neutrophils were incubated at 37uC in a total volume of 500 ml of RPMI (Gibco BRL, Gaithersburg, MD) in the presence of 2% serum from healthy donor and different stimulatory agents. In the set of experiments evaluating the effect of treatment with MSU crystals, neutrophils were treated for 4 h. Neutrophils were treated with MSU crystals (250 mg/ml) or synovial fluid supernatant (20 ml). The doses were selected according to optimization experiments. PBMCs were also stimulated with NETs structures from acute gout fluid , MSU crystals as well as synovial fluid. IL-1β inhibition was performed by Anakinra (Kineret; Amgen/ Biovitrum AB) at a concentration of 100 ng/ml, according

to neutralizing optimization experiments. For the study of the formation of extracellular chromatin networks, cells were grown in 400 μl RPMI for 3 hours and 30 minutes. For the isolation of proteins (IL-1β) of extracellular chromatin networks, cells grown in 1000 μl PΠMI for 3 hours and 30 minutes. The final volume was set at 1,2 ml for stimulations performed in 6-well dish (~ 0.5 x 10^6 cells / well) and 500 μl for those which held on microscope slides.

All experiments were performed in duplicates. Viability of cells treated with MSU for 3 h was assessed by flow cytometry using propidium iodide (PI, Sigma-Aldrich) to stain necrotic cells and an antibody against Annexin-V (BD Biosciences) as an apoptotic marker. Cells were analyzed in a FACScan flow cytometer (BD Biosciences). All the materials in this study were endotoxin free, as determined by a Limulus amebocyte assay.

Immunofluorescence

To visualize IL-1β and NE (neutrophil elastase) in NETs formation, after incubation of the cells with appropriate stimuli, isolated neutrophils were seeded in lysine-coated glass coverslips and prepared as previously described. In brief, cells, after incubation, were fixed in 4% paraformaldehyde for 2–4 hours at room temperature.PFA stock (40%) was used. Fixation of samples Control PMNs, Control PMNs + PMA, was diluted 8% to H_2O while the fixation of samples Control PMNs + MSU, Control PMNs + Synovial Fluid was diluted to 2% H_2O. All samples were prepared in duplicates. Then, after removing of the solutions was added 1 ml PBS (1X) and the well plate stored at 4 ^oC until immunofluorescence. The next day, the coverslips were placed in a special mounting pole and the process begun as follows:

Table 1. Immunofluorescence		
Washes	Number of washing	Time
PBS	2x	5 min
Triton	1x	1 min
PBS	3x	1 min
Blocking	1x	30 min
Primary Ab	1x	1 h
PBS	3x	5 min
1st Secondary Ab	1x	1 h
PBS	3x	5 min
2nd Secondary Ab	1x	1 h
PBS	3x	5 min
DAPI	1x	2,5 min
H_2O	1x	30 sec

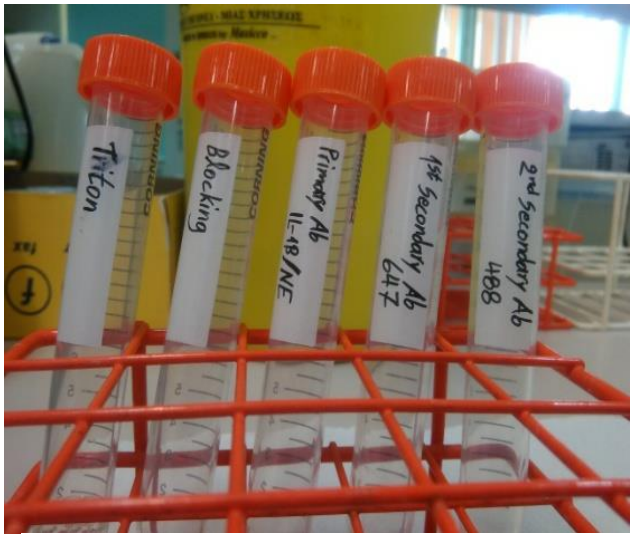


Figure 4. Eppendorfs of immunofluorescence

Nonspecific binding sites were blocked with 5% rabbit serum in PBS. A polyclonal rabbit anti-mouse Alexa Fluor 488 antibody was utilized as secondary. DNA was counterstained using DAPI. Then, the coverslips were placed on a slide, using 5 μl Mowiol per coverslip. Cell preparations were visualized in a fluorescence microscope (Leica DM2000). The percentage of cells undergoing NET release was determined by examining 200 cells per sample in a double blind fashion.

Protein purification from NETs

The proteins of neutrophil extracellular chromatin structures (NET proteins) formed from $1.5x10^6$ neutrophils. In brief, neutrophils were seeded in 6-well culture plates (Corning Incorporated) in RPMI medium (Gibco BRL). Cells were incubated for 3 h and 30 m at 37uC in a 5% CO2 atmosphere. Supernatant was removed and each well was washed twice with 1 mL of pre-warmed RPMI and incubated at 37uC for 10 min. Extracellular DNA formations were then digested with 10 U/ml DNase-1 (Fermentas, 798 Cromwell Park, USA) in 1 ml RPMI for 20 min. The activity of DNase-1 was blocked with 5 mM ethylenediaminetetraacetic acid (EDTA; Applichem, GmbH, Stockholm, Sweden). Samples were sequentially centrifuged at 300xg to remove whole cells and at 16000xg to remove cellular debris.Then, the collected supernatant phase, which contained the aforementioned structures, separated into individual eppendorfs to 250 μl. In 3 out of 4 eppendorfs for each sample was added 750 μl of cold acetone. The samples were stored overnight at -20 ° C. Next day the samples with acetone centrifuged at 13.000 rpm for 15 minutes. Proteins found in the precipitate. After careful removal of the supernatant, the precipitate was dissolved in lysis buffer [1% Triton X-100 in 150 mM NaCl / 20 mM HEPES (pH 7.5)] containing protease inhibitors (Complete Protease Inhibitor tablets, Roche. The amount was proportional pellet (15-20 μl). After 10 minutes waiting on ice, the samples were placed for 15-20 min at -80 ° C. Then, the samples were kept in ice to thaw and then centrifuged at 20.000 rpm for 7 minutes. After pooling, the samples remained at -20 ^oC until quantification of proteins.

Quantification of proteins

Analytically, samples were:

1st Process:

1. Control PMNs
2. Control PMNs + PMA
3. Control PMNs + MSU
4. Control PMNs + Synovial Fluid

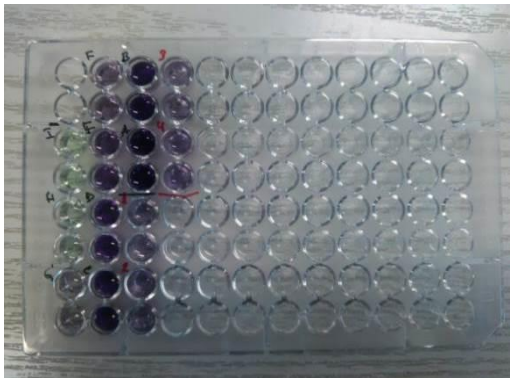


Figure 5. Quantification of proteins

2nd Process:

1. Untreated PBMCs

2. PBMCs + Control NETs

3. PBMCs + NETs derived from MSU Crystals

4. PBMCs +NETs derived from MSU Crystals + Anakinra

5. PBMCs + NETs derived from Synovial Fluid

6. PBMCs + NETs derived from Synovial Fluid + Anakinra

The process of proteins quantification conducted in accordance to the manufacturer's instructions (BCA Protein Assay Kit THERMO Scientific). In brief, were used nine successive dilutions albumin required to generate the standard curve. The samples were tested in duplicates and the total volume of each reaction is 225 μl.

Specifically, used 96well ELISA Microplate, PS, U-buttom in which the following quantities were added:

Table 2. Quantification of proteins		
96 well ELISA	Samples (1-4)	Standards (A-I)
PBS	24 µl	-
Mix	200 µl	200 µl
Samples	1 µl	-
Standards	-	25 µl

Table 3. 1st Quantification Curve

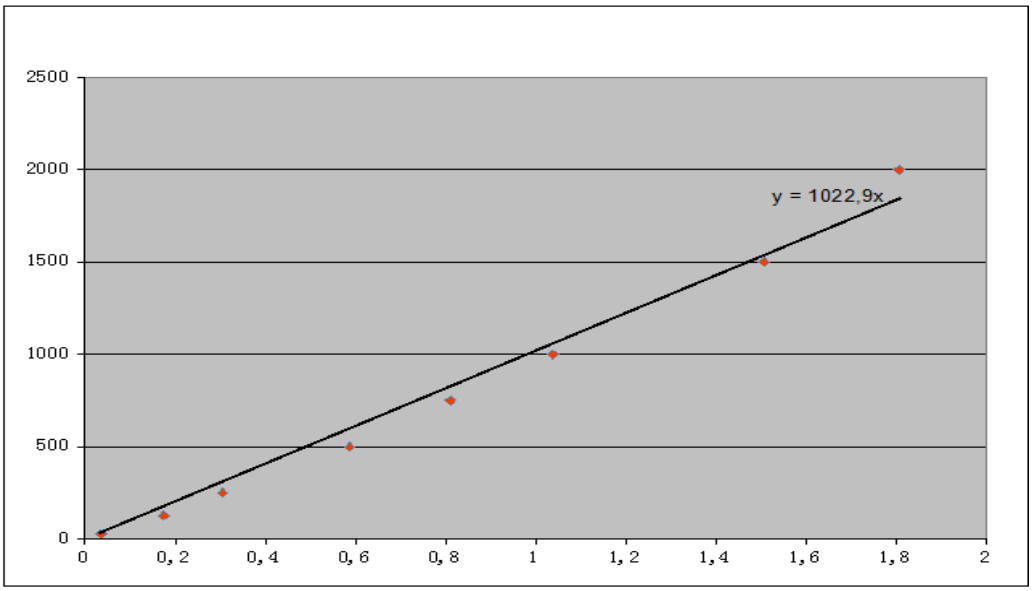
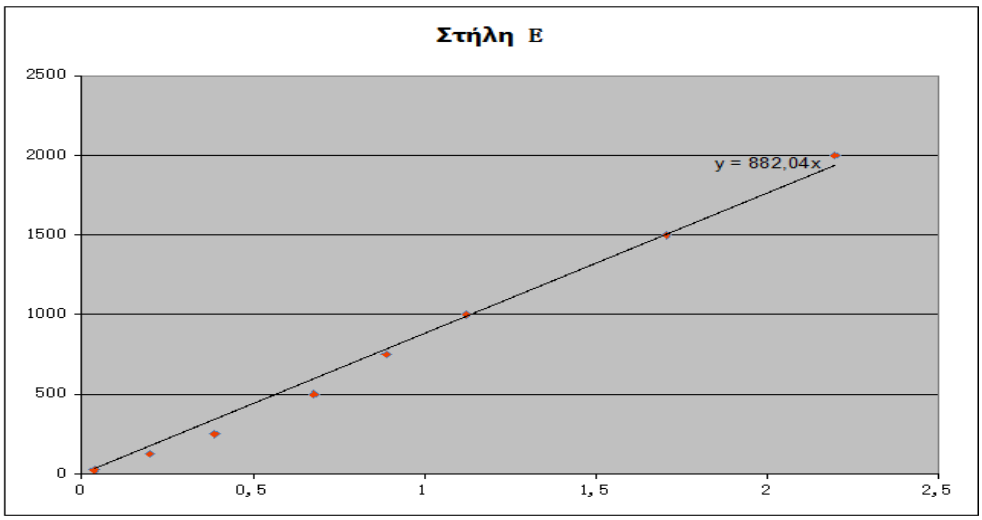


Table 4. 2nd Quantification Curve



Samples after covered with PARAFILM, incubated for 30 minutes at 37 ºC while stirring and then followed by measuring them in a spectrophotometer at a wavelength 550 nm.

After calculating the curve, proper amounts to effect the process of the immunoblot were calculated. These specific amounts were boiled in 95ºC for 7 minutes. Then the quantities were ready for immunoblotting.

Immunoblotting (Western Blot)

40 mg protein was dissolved in a buffered solution (120 mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 10% b-mercaptoethanol and 0.02% bromophenol blue) and heated at 95˚C for 10 min to completely denature. Proteins were analyzed on acrylamide gel 13% and membranes PVDF (Biorad, CA) (Figure 6).

The membranes were preincubated for nonspecific binding for 45 minutes in 3% skimmed milk diluted in TBS with 0.1% Tween 20 (TBS-T). Followed by overnight incubation at 4˚C with anti-human IL-1β polyclonal antibody (stock c: 500mg/mL). The preparation of the antibody was made for 6 ml and included 600μl Blocking and 5.400 μl TBS-T. After thorough washing with TBS-T, the membranes were incubated with anti-mouse antibody (R & D Systems) as has already described, for 45 minutes at room temperature. The immunoreactive proteins were detected using the ECL detection system (enhanced chemiluminescence, Supersignal West Pico Chemiluminescent Substrate, Pierce Biotechnology Inc., Rockford, IL) and were recorded in the X-ray film (Fuji medical x-ray film, Fujifilm Co LTD, Tokyo).

In conclusion, after immunoblotting of IL-1β protein, the membrane was also incubated using housekeeping gene called GAPDH. The first antibody binded for 45 minutes. The second antibody

(antirabbit) was incubated also for 45 minutes. After well washing with TBS-T, the housekeeping gene was detected using the ECL detection system as it has already been mentioned.

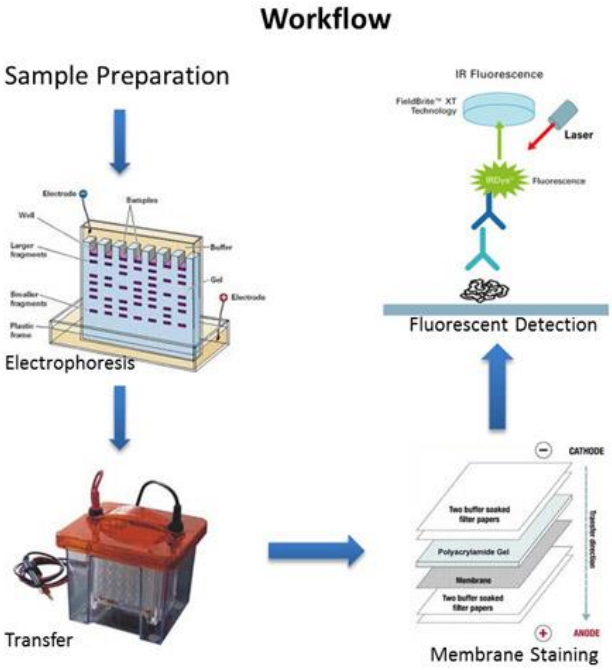


Figure 6. Western blot process

RESULTS

IL1-b is localized on NETs stimulated with monosodium urate crystals or synovial fluid from gout attack

To investigate the presence of IL1-b on NETs in gout, control PMNs were treated with MSU crystals or synovial fluid collected from patients with active disease. PMA was used as a generic inducer of NETs. There was IL-1β localization on NETs generated by PMNs treated with MSU crystals or synovial fluid (Figure 7.). In contrast, IL-1β was not detected in PMA-treated or control PMNs (Figure 7.).

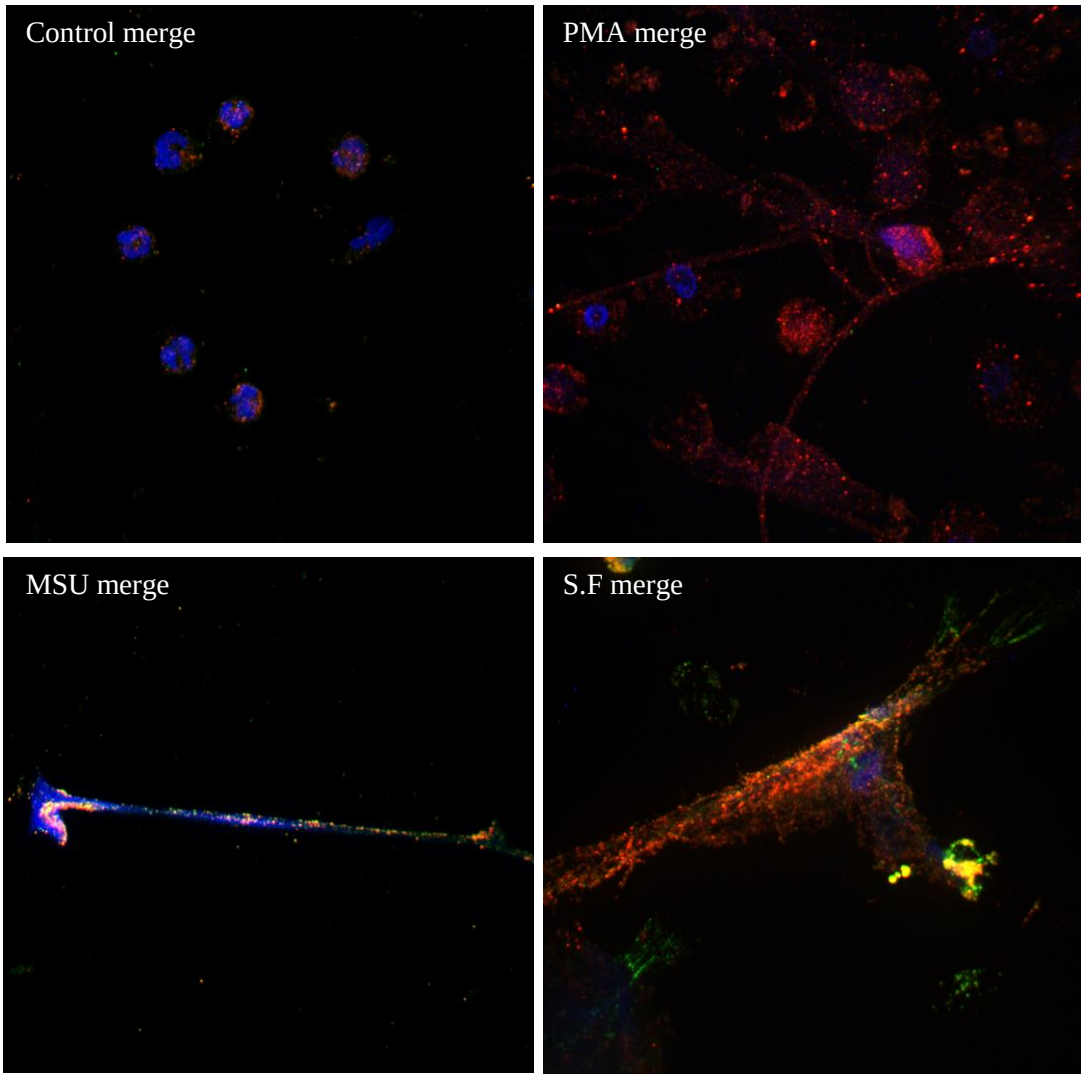


Figure 7. Presence of IL-1β on NETs stimulation with MSU crystals or synovial fluid, as observed by confocal microscopy. (Green - IL-1β, Red - Neutrophil Elastase, Blue - DNA/DAPI.

This was also verified on NET proteins derived from these stimulation by immunoblotting (Figure 8). Both forms of IL-1β (premature and mature) were detected.

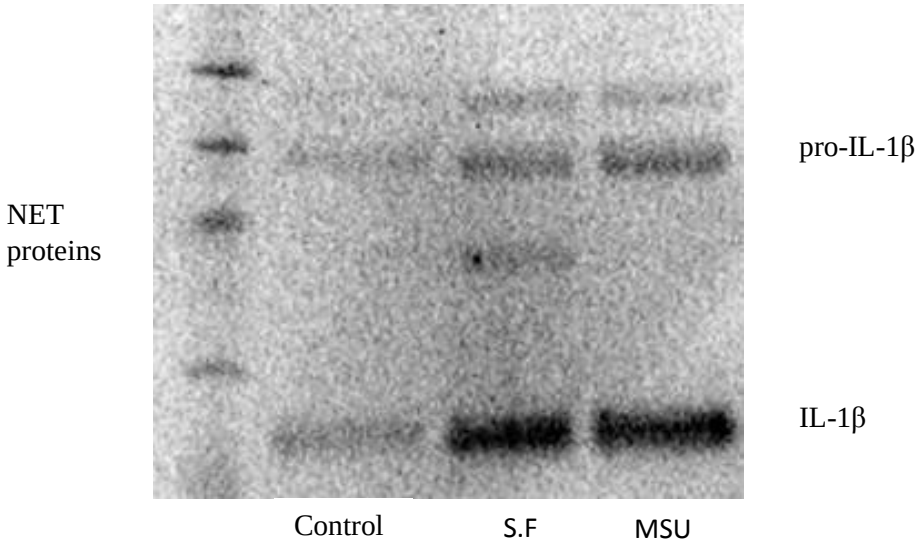


Figure 8. NET proteins as detected by immunoblotting

Bioactivity of NET bound IL-1β

Since mature IL-1β can induce further production of IL-1β in monocytes we sought to investigate the bioactivity of NET-bound IL-1β. In order to do so, we stimulateδ control PBMCs with NETs structures derived from previously described stimulations. We observed increased IL-1β production by PBMCs stimulated with MSU crystals-induced or synovial fluid-induced NETs. To assess whether this increase was IL-1β dependent, we used anakinra, an inhibitor of IL-1β receptor. Pre-treatment of PBMCs with anakinra attenuated the NET- dependent induction of IL-1β (Figure 9.).

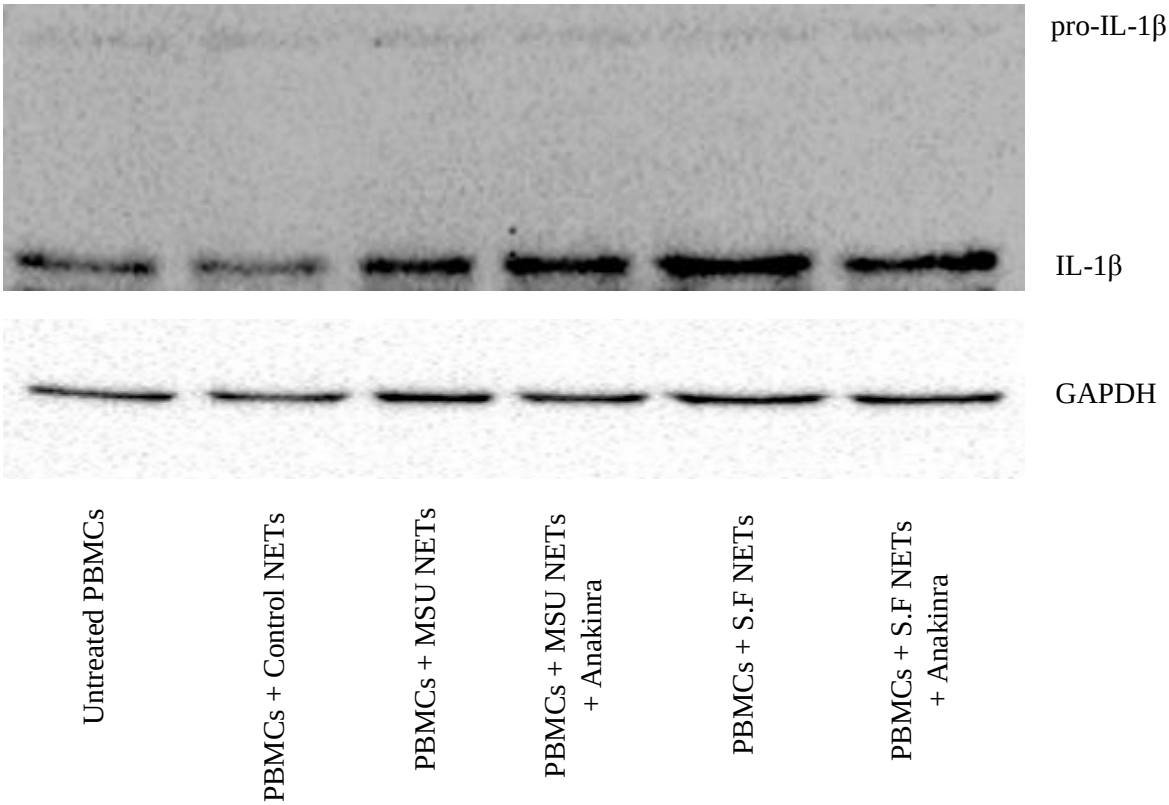


Figure 9. IL-1β functionality as detected by immunoblotting

DISCUSSION

According to the references, IL-1β is an essential protein that is demanded in order to induce inflammatory diseases. Considering that neutrophils are the major factor of inflammatory joints in inflammatory diseases, we realized that it could be interesting to search about the correlation between IL-1β and neutrophils. For the first time, it was investigated whether IL-1β is localized on NETs after stimulation with monosodium urate crystals or synovial fluid. Our results demonstrate that IL-1β is indeed localized on NETs derived from neutrophils as previously described.

Furthermore, to examine the activity of both premature and mature IL-1β, we stimulated PBMCs with NETs derived from MSU crystals or synovial fluid. A significant positive regulation of IL-1β was detected in each of these stimulations. Concurrently, we stimulated these cells with anakinra, an inhibitor of IL-1β receptor. We showed that there is a prominent inhibition in the expressionof IL-1β.

So, we have demonstrated that a vital protein in inflammatory diseases, such as IL-1β, is located in the basic cell population of inflammation, neutrophils. Thus, we showed that neutrophils are responsible for the production of IL-1β.

These results above could be used for further investigation and future perspectives. More specifically, it could be interesting to investigate which mechanisms are utilized for IL-1β maturing process. Furthermore, an interesting approach it could be to examine the ways that IL-1β behaves in the specific environment. Also, a possible effect of tackling NETs in the disease of acute gout could be innovative. This particular process it could be achieved by DNases or by other NETs- inhibitors. However, this approach is quite difficult, due to the absence of clinical tries.

To conclude, these series of experiments have demonstrated a dominant mechanism of IL-1β production in the inflammatory disease of gout by neutrophils and the release of this cytokine through NETs. This demonstration could be a beginning in several examinations that are correlated to inflammatory diseases, IL-1β and neutrophils.

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