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University of Siena – Department of Medicine, Surgery and Neuroscience

Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

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"Role of granulocytes activation in the pathogenesis of Systemic juvenile idiopathic arthritis and Adult onset Still's disease"

SSD: MED/16 - Rheumatology

Candidate
Flora Magnotti

Tutor
Prof. Mauro Galeazzi

Coordinator
Prof. Alessandra Renieri

Academic Year 2015/2016

Westfälische Wilhelms-Universität Münster

UKM Universitätsklinikum Münster

Universitätsklinikum Münster . Klinik für Pädiatrische Rheumatologie und Immunologie . 48129 Münster

To whom it may concern

Department of Pediatric Rheumatology and Immunology

Univ.-Prof. Dr. med. Dirk Föll
Direktor

Laboratory: Albert-Schweitzer-Campus 1, Gebäude D3
Domagkstraße 3, 2nd floor
48149 Münster

Patients: Albert-Schweitzer-Campus 1, Building W30
Waldeyerstraße 30
48149 Münster

Contact:
Cornelia Marinca

T +49 251 83-58178
F +49 251 83-58104
Vermittlung: T +49 251 83-0

c.marinca@uni-muenster.de
www.ukmuenster.de

Münster, 07.06.2016

Subject: PhD Thesis of the Flora Magnotti at the University of Siena, Italy with the title

„Role of granulocytes activation in the pathogenesis of systemic juvenile idiopathic arthritis and adult onset Still's disease"

Flora Magnotti presents a PhD thesis that focuses on key aspects of innate immune dysregulation in the pathogenesis of the most severe form of juvenile idiopathic arthritis called "systemic juvenile idiopathic arthritis (sJIA)" and also of its counterpart in adults, which is called "adult onset Still's disease (AOSD)". The thesis is also special with regard to international collaboration. Flora Magnotti performed the scientific experiments in different laboratories, which all focus on clinical translational research related to the diseases that are in the focus of the thesis. The work was performed in the laboratories of Prof. Mauro Galeazzi, Dr. Luca Cantarini, Prof. Rolando Cimaz, Dr. Alexandre Belot, and also our own laboratory here in Muenster. It has to be taken in mind that it is quite an endeavour to perform such a project on patients with rare diseases, and it shows the ambition of the PhD student that she followed her topic at

different departments in several European countries to be able to get a broad expertise and also sufficient patients and samples to perform her research.

The basis of Flora Magnotti's thesis is the hypothesis that in sJIA and AOSD neutrophils may be key cellular components involved in the pathogenesis of the diseases. This is also due to the typical neutrophilia and tissue infiltration that is seen in patients with the disease. Following this hypothesis, Flora Magnotti analysed the neutrophil activation status in patients at baseline and also the response of neutrophils to proinflammatory stimulation. Flora Magnotti found that neutrophils from patients with the disease showed little or no difference in activation state and IL-1 β production compared to healthy controls. On the contrary, monocytes appear to be the main components responsible for the release of IL-1 β concentrations into cell supernatants *in vitro*, and probably also these cells are most important for IL-1 β production *in vivo*.

After realizing this, Flora Magnotti switched from a focus on neutrophils to a focus on monocyte activation. She therefore analysed monocyte activation in sJIA, AOSD and also from patients with Familial Mediterranean Fever and Cryopyrin Associated Periodic Syndrome. She found that patients from the latter syndromes, which are due to mutations in inflammasome-related components, IL-1 β secretion is indeed significantly different from healthy controls. On the contrary, monocytes from patients with sJIA or AOSD did not show significant differences, leaving some doubts about the cellular source of IL-1 β in these syndromes.

In addition, Flora Magnotti also analysed concentrations of the danger molecule S100A12, which has previously been described to be elevated in patients with sJIA. She found that neutrophils from patients in fact produced higher levels of S100A12 *in vitro* after stimulation in comparison to healthy control cells. Therefore, neutrophil derived S100A12 could indeed contribute to hyperactivation of myeloid cells in sJIA and also AOSD, which may then contribute to enhanced IL-1 β production from monocytes.

The thesis is very interesting and also well written. Flora Magnotti presents a well designed introduction that presents both the diseases and also the molecular pathways that are involved in it. She also clearly sets the ground for understanding the research that was involved in her project. Objectives, materials and methods are clearly presented. The results are also presented in a manner that makes it easy to comprehend them. The discussion is pretty concise, it clearly puts the results that are presented into context and also discusses them properly with regard to existing literature and background knowledge. The only minor comment I would have here is

that limitations of the study, including the rather small patient number and also the observed obstacles and changes with regard to the primary objectives, could have been discussed a little bit further. However, this does not prevent the work from being very interesting and well presented, and the results could indeed trigger future research endeavours to further analyse neutrophil and monocyte dysfunction in sJIA and AOSD in a broader fashion.

Apart from this I can only congratulate Flora Magnotti and her mentors for the very impressive achievements. I find that the presented thesis is well appropriate for a PhD dissertation and can only support the acceptance of this work as a very good ground for obtaining the PhD doctoral grade.

With kind regards

Prof. Dr. med. Dirk Foell

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Siena, June 8th 2016

To:
Prof. Dr. med. Dirk Foell
Department of Pediatric Rheumatology and Immunology
Universitätsklinikum Münster

Dear Prof. Foell,
I have really appreciated your comments on my work, and I would like to thank you for reviewing my thesis.

I'm herein including the response to your comments:

1. *"The only minor comment I would have here is that limitations of the study, including the rather small patient number and also the observed obstacles and changes with regard to the primary objectives, could have been discussed a little bit further."*

Page 71, line 22: I have better discussed about the encountered difficulties and experimental choices made while working on the project.

UNIVERSITA' DEGLI STUDI DI SIENA
DIPARTIMENTO DI SCIENZE MEDICHE, CHIRURGICHE E NEUROSCIENZE
DIRETTORE: Prof. Ranuccio Nuti
Policlinico "Santa Maria alle Scotte" – Viale Bracci, 16 – 53100 Siena
Tel.0577-233425 e-mail: ranuccio.nuti@unisi.it

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I would like to take the opportunity to thank you for your time and your suggestions that will improve my thesis.

Best regards,

Flora Magnotti

UNIVERSITA' DEGLI STUDI DI SIENA
DIPARTIMENTO DI SCIENZE MEDICHE, CHIRURGICHE E NEUROSCIENZE
DIRETTORE: Prof. Ranuccio Nuti
Policlinico "Santa Maria alle Scotte" – Viale Bracci, 16 – 53100 Siena
Tel.0577-233425 e-mail: ranuccio.nuti@unisi.it

GROUPEMENT HOSPITALIER EST
Hôpital Femme Mère Enfant
59 boulevard Pinel
69677 Bron cedex – France
N° FINESS : 690 007 539

Hospices Civils de Lyon

SERVICE DE PEDIATRIE
***Chef de Service* : Pr Pierre Cochat**
Télécopie 04 27 85 67 68

FILIERES MALADIES RARES
ORKID – *Orphan Kidney Diseases*
FAI²R – *Maladies auto-immunes et auto-inflammatoires rares*
OSCARD – *Os-Calcium/Cartilage-Rein-Dent*

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Dr Justine Bacchetta, PHU
Secrétariat - Annick Berthet 04 27 85 61 30
Dr Odile Basmaison, PH
Secrétariat - Christel Jacquelin 04 27 85 61 28
Dr Christian Grand, PH attaché
Secrétariat - Annick Berthet 04 27 85 61 30

RHUMATOLOGIE & MEDECINE INTERNE
Dr Alexandre Belot, MCU-PH
Dr Marine Desjonquères, PH
Dr Agnès Duquesne, PH attaché
Secrétariat - Raphaëlle Deborde 04 27 85 61 26

DERMATOLOGIE
Dr Alice Phan, MCU-PH
Secrétariat - Raphaëlle Deborde 04 27 85 61 26

HOSPITALISATION
Assistant - Chef de Clinique
Dr Marie-Caroline Chastang
Secrétariat - Raphaëlle Deborde 04 27 85 61 26

Cadres infirmiers
Danielle Gustin 04 27 85 60 42
Delphine Michallon (adjointe) 04 27 85 50 80

Hôpital de jour
Rendez-vous 04 27 85 61 34
Secrétariat 04 27 85 61 28

Hospitalisation
Secrétariat 04 27 85 61 30

Site internet : www.chu-lyon.fr

To whom it may concern

Lyon, 2016 June the 9th

Re: Flora Magnotti PhD thesis assessment

I received the PhD thesis of Flora Magnotti, focusing on the role of PMN and inflammasome in autoinflammatory diseases.
The topic is of interest and the explorations on the implication of PMNs in autoinflammatory diseases are very clear. I had the opportunity to supervise some experiments on PMNs/monocytes in Lyon and Flora Magnotti is very efficient and a good worker at the bench.

Regarding the document transmitted, I think that several points need to be answered.

1/ Regarding the whole content, the English should be cautiously reviewed and the reading is often difficult secondary to syntax anomalies along the manuscript, several repetitions ("in fact")…
few exemples: page 2 analysis instead of analys. P5 manifestation instead of manafestation… T-specific lymphocytes -> autoreactive T cells…(Inteleukin replaced by interleukin). The capital letters should be proposed for acronym (with a list of all abbreviation at the beginning of the manuscript).

2/ Abstract:
Conclusions are not supported by the results. Indeed, there is no evidence that PMN play a role in Still disease.

3/ Introduction:

-The figures 1 and 5 are not called in the text. In general, tables and figures should be call in the text and these element should be cautiously reviewed.
-The figure 4 is not described in the introduction.
-The IL-1 secretion is not well detailed and it is unclear how autophagy interferes with the secretion. A figure or additional explanation is mandatory.
-Autoinflammatory diseases could be presented in a table with a precision on the genetic and non-genetic forms.
-In the CAPS paragraph, it is unclear whether CAPS could be or not genetics (page 22).
-The author should precise what is a somatic mutation (by reading the manuscript, it is unclear if the student has understood the mechanism).
-Regarding FMF description, a new function of pyrin has been recently reported (Masters et al. 2016). Related information should be reporter here.
-In the treatment section, cautious information should be given regarding the efficiency of the drugs. Anakinra has never been compared to other biologics and can't be considered (on a scientific basis) as the most effective drug (p34). In addition, the situation in FMF is not a lack of efficacy of biologics but a very good effect of an oral, low-cost drug (colchicine).

4/ In the result section, the author should be cautious on conclusions. The higher release of cytokine (p51-52) does not provide evidence on the sensitivity to treatment.

-In Figure 15, the author states that IL1 production is no more related to caspase 1 but the panel B still suggest some reduction of IL1 production after YVAD treatment. Considering the very few level of IL1 production, inhibitory test are not helpful to conclude.
- The results on S100A12 production are interesting and could be better highlighted.
-The 4.8 section could be presented before the S100A12 paragraph because it is still related to the demonstration that PMN do not produce IL1b.

5/ In the discussion, the author suggests granulocytes remain "bad " IL-1b producer. The data do not support any production of IL1 by PMN upon stimulation.
-PMNs from one AOSD patient are reported to secrete high IL1b release upon activation. This conclusion is not supported by the data (no intracellular staining, probably contaminating monocyte) so this statement is somehow contrary to the previous demonstration that PMN do not produce IL1b. It is necessary to clarify this point.
-The author cannot conclude on IL1b production by PMN and its supposed link to caspase 1. Indeed in the context of no / very few IL1, inhibitory experiments cannot show a difference, and it is not possible to conclude on the caspase 1 involvement.
In the last paragraph (p69), the conclusion of the author is the total opposite of the presented results "confirm the importance of studying PMNs in autoinflammatory disorders…". Strictly, the author should point that this work demonstrates that PMN do not display a competent inflammasome and that IL1 is produced by monocytes in AID.

Answering these comments will improve the overall quality of the thesis.

Sincerely,

Alexandre BELOT
Associate Professor of Pediatric Rheumatology, Department of Pediatrics, Hospices Civils de Lyon
Chair of the National Network on Rare Autoimmune and Autoinflammatory Diseases (FAI2R)
INSERM U1111, "innate immunity in infectious and autoimmune diseases"

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DIPARTIMENTO DI SCIENZE MEDICHE, CHIRURGICHE E NEUROSCIENZE

Siena, June 11th 2016

To:
Dr. Alexandre Belot
INSERM U1111, "Innate immunity in infectious and autoimmune diseases"
Université Lyon 1

Dear Dr. Belot,
I have really appreciated your comments on my work, and I would like to thank you for reviewing my thesis.

I'm herein including a detailed response to your comments:

1) *" Regarding the whole content, the English should be cautiously reviewed and the reading is often difficult secondary to syntax anomalies along the manuscript, several repetitions ("in fact")… few exemples: page 2 analysis instead of analys. P5 manifestation instead of manafestation… T-specific lymphocytes -> autoreactive T cells…(Inteleukin replaced by interleukin). The capital letters should be proposed for acronym (with a list of all abbreviation at the beginning of the manuscript)."*

   I've carefully reviewed the text and corrected the typing errors, avoiding repetitions and syntax anomalies. Furthermore I've added the list of abbreviation at the beginning of the manuscript.

UNIVERSITA' DEGLI STUDI DI SIENA
DIPARTIMENTO DI SCIENZE MEDICHE, CHIRURGICHE E NEUROSCIENZE
DIRETTORE: Prof. Ranuccio Nuti
Policlinico "Santa Maria alle Scotte" – Viale Bracci, 16 – 53100 Siena
Tel.0577-233425 e-mail: ranuccio.nuti@unisi.it

2) Abstract:

"Conclusions are not supported by the results. Indeed, there is no evidence that PMN play a role in Still disease."

Page 9, line 9: I've modified the conclusions in the abstract, better specifying that until now there is no evidence of the pathogenic role of PMNs in Still's disease, as indicated by our results on the activation state and inflammasome activity analysis. But our observations also suggest a different action mechanism, not related to the IL-1β production, and we think it deserves further investigations.

3) Introduction:

"- The figures 1 and 5 are not called in the text. In general, tables and figures should be call in the text and these element should be cautiously reviewed.

- The figure 4 is not described in the introduction."

I've called figure 1, 4 and 5 in the text (Page 12, line 10; Page 17, line 8; Page 33, line 7, respectively) .

"- The IL-1 secretion is not well detailed and it is unclear how autophagy interferes with the secretion. A figure or additional explanation is mandatory."

Pages 21 and 22: I've better described the IL-1 secretion mechanism and clarified how autophagy interferes with its the secretion.

"- Autoinflammatory diseases could be presented in a table with a precision on the genetic and non- genetic forms."

Page 24: I've added a table (Table 1) presenting the different autoinflammatory syndromes and their genetic or non-genetic base.

"- In the CAPS paragraph, it is unclear whether CAPS could be or not genetics (page 22).

- The author should precise what is a somatic mutation (by reading the manuscript, it is unclear if the student has understood the mechanism)."

Page 25, line 6: I've better described the genetic base of the CAPS syndromes, indicating also the involvement of somatic mosaicism as a major cause of these diseases.

"- Regarding FMF description, a new function of pyrin has been recently reported (Masters et al. 2016). Related information should be reporter here."

Page 30, line 10: as you have suggested, I've reported the recent study about a new identified *MEFV* gene mutation, associated to a new pyrin-associated autoinflammatory disease.

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"- In the treatment section, cautious information should be given regarding the efficiency of the drugs. Anakinra has never been compared to other biologics and can't be considered (on a scientific basis) as the most effective drug (p34). In addition, the situation in FMF is not a lack of efficacy of biologics but a very good effect of an oral, low-cost drug (colchicine)."

Page 39, line 14: I've specified that Anakinra is one of the biological drugs used for the treatment of autoinflammatory syndromes, together with other biologics like canakinumab or rilonacept.

Page 39, line 25: I've specified the efficacy of the colchicine administration in the major part of FMF patients.

4) *In the result section, the author should be cautious on conclusions. The higher release of cytokine (p51-52) does not provide evidence on the sensitivity to treatment.*

Page 57, Figure 9: to show the higher sensitivity to the treatment in PMNs from affected patients, I've added the Figure 9A, representing the fold change of IL-1β concentration relatives to the untreated sample. The figure shows the higher fold increase in IL-1β release, after LPS stimulation, in affected patients compared to healthy donors.

"- In Figure 15, the author states that IL1 production is no more related to caspase-1 but the panel B still suggest some reduction of IL1 production after YVAD treatment. Considering the very few level of IL1 production, inhibitory test are not helpful to conclude."

Page 62, line 9: as you have suggested, I've specified that IL-1β release resulted dependent on the caspese-1 activity just in the healthy controls, and that the very few levels of the detected cytokine don't lead to a definite conclusion (Figure 15C).

"- The results on S100A12 production are interesting and could be better highlighted."

Page 65, Paragraph "Granulocytes from sJIA patients produce higher levels of S100A12": I've tried to better highlight the result on S100A12, that will be better discussed in the section "Discussion".

"- The 4.8 section could be presented before the S100A12 paragraph because it is still related to the demonstration that PMN do not produce L-1b."

Pages 63-65: as you have suggested, I've changed the paragraphs presentation.

5) *" In the discussion, the author suggests granulocytes remain "bad" IL-b producer. The data do not support any production of IL1 by PMN upon stimulation."*

Page 61, Figure 14: I've modified the figure 14 indicating all the detected statistical significances, in order to show that the stimulation on ultra pure PMNs enhances the IL-1β release. This result is also supported by the Figures 15 (page 63) and 20 (page 68),

that show the significant increase in IL-1β release after LPS / LPS+ATP and S100A12 stimulation respectively.

"- PMNs from one AOSD patient are reported to secrete high IL1b release upon activation. This conclusion is not supported by the data (no intracellular staining, probably contaminating monocyte) so this statement is somehow contrary to the previous demonstration that PMN do not produce IL1b. It is necessary to clarify this point."

Page 70, line 25: I've better clarified that this comment refers to the experiments with less than the 0.2% of contaminating monocytes, in order to be sure about the specific granulocytic response. We have decided to highlight the result obtained in this specific AOSD patient, to speculate about a possible implication of the pharmacological treatment on the PMNs inflammatory response, but we have also specified that to better clarify this aspect, it will be necessary to recruit more patients and separate them according to the therapy.

"- The author cannot conclude on IL1b production by PMN and its supposed link to caspase-1. Indeed in the context of no/very few IL1, inhibitory experiments cannot show a difference, and it is not possible to conclude on the caspase-1 involvement."

Page 71, line 16: I've better specified that it is not yet possible to make conclusions about caspase-1 involvement, both because of the small number of patients analyzed, and because of the few levels of the detected cytokine.

"In the last paragraph (p69), the conclusion of the author is the total opposite of the presented results "confirm the importance of studying PMNs in autoinflammatory disorders…". Strictly, the author should point that this work demonstrates that PMN do not display a competent inflammasome and that IL1 is produced by monocytes in AID."

Page 76: I've better specified that our findings exclude the original hypothesis of a different PMNs activation state in sJIA and AOSD, and, above all, they don't represent the major IL-1β producers in these syndromes.

The results obtained in monocytes experiments, indicate that in monogenic disorders, it is possible to distinguish a specific inflammasome functional phenotype. On the contrary, monocytes from sJIA and AOSD patients didn't manifest important differences in comparison with healthy ones, indicating that neither monocytes have shown a direct implication in these syndromes, regarding to the inflammasome complexes activation. Therefore, considering the result shown in the figure 20 (page 68) about the effects of S100A12 stimulation on healthy monocytes, and a previous study showing that S100A12 stimulation can have a stronger effect on monocytes respect LPS (ref. 144), we could just conclude with a new hypothesis on the PMNs involvement in polygenic autoinflammatory syndromes, assuming that they don't intervene directly with the production of IL-1β, but they could mediate their action through the production of S100A12 (resulted higher in sJIA in our experiments. Figure 16), whose action could fall, in turn, on monocytes which are much more capable in the IL-1β production and release.

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I would like to take the opportunity to thank you for your time and your suggestions that will improve my thesis.

Best regards,

Flora Magnotti

UNIVERSITA' DEGLI STUDI DI SIENA
DIPARTIMENTO DI SCIENZE MEDICHE, CHIRURGICHE E NEUROSCIENZE
DIRETTORE: Prof. Ranuccio Nuti
Policlinico "Santa Maria alle Scotte" – Viale Bracci, 16 – 53100 Siena
Tel.0577-233425 e-mail: ranuccio.nuti@unisi.it

Table of contents

Abbreviation Index

AOSD: Adult Onset Still's Disease

ASC: apoptosis-associated speck like protein

ATP: Adenosine triphosphate

BMDM: Bone-marrow-derived macrophages

CAPS: Cryopyrin associated periodic syndrome

CARD: caspase recruitment domain

CG: cathepsin G

CIAS1: cold-induced autoinflammatory syndrome 1

CINCA: chronic infantile neurologic cutaneous articular syndrome

CPPD: calcium pyrophosphate dehydrate

CRP: C-reactive protein

CSF: colony stimulating factors

DAMPs: Damage-associated molecular pattern molecules

dsDNA: double-stranded DNA

ER: Endoplasmic reticulum

ESR: erythrocytes sedimentation rate

FCAS: Familial Cold Autoinflammatory Syndrome

FCS: fetal calf serum

FMF: Familial Mediterranean Fever

HC: healthy controls

HIDS: Hyperimmunoglobulinemia D

HPFs: Hereditary periodic fever syndromes

IFN: Interferon

IL: Interleukin

IL-1R: IL-1 receptor

JIA: juvenile idiopathic arthritis

JNK: c-Jun N-terminal protein kinases

LPS: Lipopolysaccharide

LRRs: Leucine-rich repeats

mAbs: monoclonal antibodies

MDP: muramyl dipeptide

MIF: migration inhibitory factor

MKD: Mevalonate Kinase Deficiency

MSU: monosodium urate

MWS: Muckle-Wells syndrome

NACHT: NAIP (neuronal apoptosis inhibitor protein), C2TA (MHC class 2 transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein)

NE: neutrophil elastase

NLR: Nucleotide oligomerization domain-like receptor

NOD: Nucleotide oligomerization domain

NOMID: Neonatal Onset Multisystem Inflammatory Disorder

PAAND: pyrin-associated autoinflammation with neutrophilic dermatosis

PAMPs: Pathogen-associated molecular pattern molecules

PAPA: Pyogenic arthritis with pyoderma gangrenosum and acne syndrome

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PFTs: pore-forming toxins

PGN: peptidoglycan

PMA: Phorbol 12-myristate 13-acetate

PMNs: Polymorphonuclear leukocyte

PR3: proteinase-3

PRR: Pattern recognition receptors

PYD: pyrin domain

RBC: red blood cell

RLRs: Retinoic acid-inducible gene I-like receptors

ROS: Reactive Oxygen Species

SAPHO: Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis syndrome

sJIA: systemic juvenile idiopathic arthritis

T3SS: type III secretion systems

TACE: Tumor Necrosis Factor α converting enzyme

TLR: Toll-like receptor

TNF: Tumor Necrosis Factor

TRAPS: Tumor Necrosis Factor receptor–associated periodic syndrome

WBC: White blood cells

YVAD: z-Val-Ala-Asp fluoromethyl ketone

Abstract

The autoinflammatory syndromes are a group of disorders defined as “unmotivated recurrent inflammatory events” characterized by an apparently spontaneous manifestations of inflammation, without autoreactive T cells or auto-antibody involvement. These conditions usually present aberrant responses associated to molecular pathogenic patterns and deregulated production of inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-18 (IL-18) and Tumor Necrosis Factor-α (TNF- α).

Systemic juvenile idiopathic arthritis (sJIA) and Adult onset Still’s disease (AOSD) are particular kinds of autoinflammatory disorders with still unknown etiology, mainly associated to the possible pathogenic role of IL-1β, since the efficiency of pharmacological treatments with IL-1β blockers agents.

The typical patients’ neutrophilia would presume the PMNs involvement at the onset of these syndromes. For this reason we have analyzed the PMNs activation state at baseline, together with their response to extracellular pro-inflammatory stimuli.

In comparison with healthy granulocytes, sJIA and AOSD PMNs didn’t present differences in term of cellular activation state and IL-1β production, and our results also revealed that neither healthy nor sJIA/AOSD PMNs can produce elevated levels of this cytokine, as opposed to monocytes, which can release IL-1β concentrations more than 10 fold higher than PMNs.

We have also analyzed the inflammatory response mediated by monocytes, after activation of three different inflammasome complexes, in sJIA, AOSD patients and patients affected by the hereditary periodic fever syndromes (HPFs) Familial Mediterranean Fever (FMF) and Cryopyrin associated periodic syndrome (CAPS). Monocytes from HPFs affected patients have shown the clear involvement of one specific inflammasome complex. sJIA and AOSD monocytes, instead, have not

presented significant differences with healthy monocytes, leaving some doubts about the principal cellular source of IL-1β in these syndromes, also considering previous experiments which indicated that sJIA monocytes released even lower IL-1β concentrations than healthy monocytes.

An important difference has been observed about the S100 calcium-binding protein A12 (S100A12) production. Indeed, patients PMNs have produced higher levels of S100A12 after stimulation in comparison with healthy ones. This result could be related to the typical elevated S100A12 serum concentration observed in affected patients.

Taken together, our observations indicate that the role of PMNs in sJIA and AOSD is not directly related to the inflammasome activity and IL-1β production, but it deserves further investigations about a possible different action mechanism, involved in the pathogenesis of these syndromes.

1. Introduction

1.1 The inflammatory response

Inflammation is a defence mechanism which acts to protect an organism from infections and injuries, triggered by the innate immune system, able to localize and eliminate the injurious agent and remove damaged tissue components.

Innate immune cells localized in tissues, like macrophages, fibroblasts, mast cells and dendritic cells, together with circulating leukocytes, such as monocytes and neutrophils, recognize pathogens or damaged cells by means of intracellular and surface-expressed pattern recognition receptors (PRRs). In particular, PRRs recognize pathogen-associated molecular pattern molecules (PAMPs) or damage-associated molecular pattern molecules (DAMPs). PAMPs are molecules pathogen-derived and usually essential for microbe survival such as bacterial and viral nucleic acids, fungal β-glucan and α-mannan cell wall components, the bacterial protein flagellin, components of the peptidoglycan bacterial cell wall and lipopolysaccharide (LPS) from Gram-negative bacteria.

DAMPs, instead, are endogenous molecules released by injured cells. They cause the sterile inflammation and include adenosine triphosphate (ATP), the cytokine Interleukin (IL)-1α, uric acid, the calcium-binding proteins S100A8 and S100A9, the DNA-binding protein HMGB1.

PRRs include receptors located on the cell surface, such as Toll-like receptors (TLRs), which are type I trans-membrane proteins containing leucine-rich repeats (LRRs) that recognize bacterial and viral PAMPs in the extracellular environment (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11) or endolysosomes (TLR3, TLR7, TLR8, TLR9, and TLR10); but one can distinguish also cytosolic PRRs, localized within distinct intracellular compartments, such as:

- the retinoic acid-inducible gene I-like receptors (RLRs), which specifically

detect RNA species derived from viruses and coordinate anti-viral programs via the type I Interferon (IFN) induction;

- the nucleotide oligomerization domain-like receptors (NLRs), strongly associated with host responses to intracellular invasion by bacteria or the intracellular presence of specific bacterial products;
- cytosolic DNA sensors activated after infection with DNA viruses or certain bacteria through TLR9-independent and RLR-independent pathways (1-3).

However, the major cellular PRRs are members of the TLR family. TLRs, with the exception of TLR3, engage the adaptor MyD88 leading to a pathway which ends with the activation of transcription factors such as NF-κB (Figure 1), CREB, c/EBPb and AP1, inducing the expression of several genes encoding for chemokines (Cxcl1, Cxcl2), cytokines (IL-10, IL-12β, IL-1α, IL-1β, TNF) and regulators of the extracellular matrix remodelling (Mmp13) and cell adhesion (Vcam1) (4-7).

The induction of the cytokines IL-1β and TNF by PRRs activation, amplifies the inflammatory response. In fact, after binding to specific receptors, IL-1 Receptor (IL-1R) for IL-1β (8) and TNF receptor I (TNF-RI) for TNF (9), they promote NF-kB activation, too.

Moreover, an important step during the inflammatory response is the vasodilatation, when the blood flow changes to increase the permeability of blood vessels allowing the migration of fluids, proteins and leukocytes from the blood to the damaged tissues. Indeed, LPS, TNF and IL-1β signalling in endothelial cells induce the surface expression of trans-membrane proteins involved in cell adhesion such as P-selectin, E-selectin, ICAM1 and VCAM1 which promote the recruitment of leukocytes in the interested region (10).

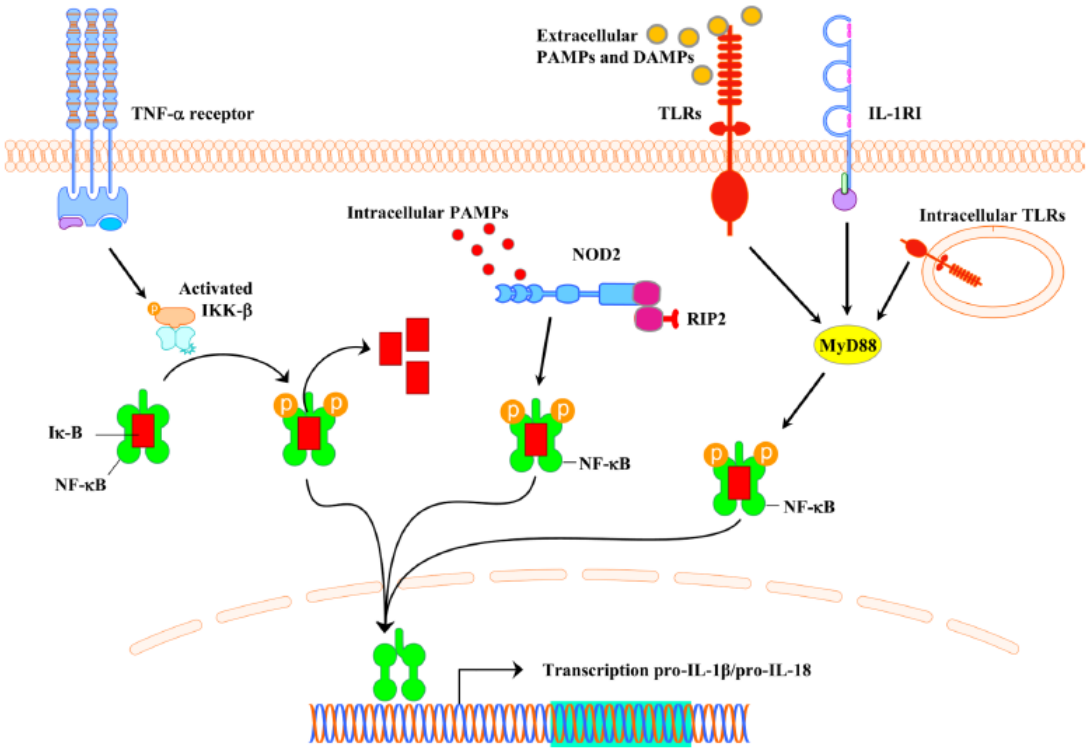

Figure 1. Induction of pro-IL-1β and pro-IL-18 occurs via TLR, NOD or TNFR signaling in response to extracellular PAMPs, cytoplasmic PAMPs, or TNF-α[7].

The abovementioned cytokines, once produced are secreted through tightly controlled processes. One can distinguish the canonical secretory pathway, specific for cytokines presenting signal peptides, which provides first the migration into the endoplasmic reticulum (ER), then the traffic into the Golgi complex for further processing, with final loading into vesicles or carriers and the delivery to the cell surface or other organelles. This is the most common secretory process, different from the "non-canonical" one, which interests particular cytokines such as IL-1β and IL-18, pro-inflammatory cytokines crucial mediators of inflammation and host response to infections. They lack the N-terminal signal sequence required for ER entry, so they are synthesized in the cytoplasm and released directly into the extracellular milieu. In particular, they are first generated as biologically inactive pro-IL-1β and pro-IL-18, later processed into the

mature and biologically active proteins after cleavage mediated by caspase-1, and finally released outside the cells (11).

Therefore, for these particular cytokines the functioning mechanism is twofold: the priming phase and the activation phase. The priming phase is represented by the binding of PAMPs and DAMPs to the PRRs on the cellular surface, which allow the production of pro-IL-1β and pro-IL-18. But, once produced, they need to be activated in a second step mediated by the Inflammasome complex.

1.2 The Inflammasome structure

An inflammasome is a multiprotein complex necessary for the activation of caspase-1. There are different kind of inflammasome complexes, with different structures but common final purpose to activate IL-1 and IL-18 (12, 13).

The most studied inflammasome complexes NLRP1, NLRP3, NLRC4 and NLRP12 are constituted by NOD-like receptors (NLRs) family members, particular PRRs located in the cytoplasm, able to recognize microbial components only after intracellular infections (14).

Basically, these kinds of inflammasome are complexes of NLRs, organized in oligomers, and the NLRs, in turn, are made of protein complexes acted to recruit the pro-caspase-1, since also this protease requires an activation process.

The basic structure of a NLR is made of:

- The Leucine-rich repeat (LRR), able to recognize PAMPs;
- The NACHT domain, able to oligomerize;
- The N-terminal effector domain, necessary for the recruitment of pro-caspase-1.

The effector domain changes in the different complexes. In particular, the effector

domain of NLRP3 and NLRP12 is a pyrin domain (PYD), which associates through a PYD–PYD interaction with the adaptor protein ASC (apoptosis-associated speck like protein) containing the CARD domain (caspase recruitment domain) involved in the activation of pro-caspase-1 (15).

NLRC4, instead, at the N-terminal of the NLR presents the CARD domain, to recruit the pro-caspase-1 by itself, without the necessity of the adaptor protein ASC (16, 17).

NLRP1, present both: at the N-terminal the PYD domain and at C-terminal the CARD domain (Figure 2) (18).

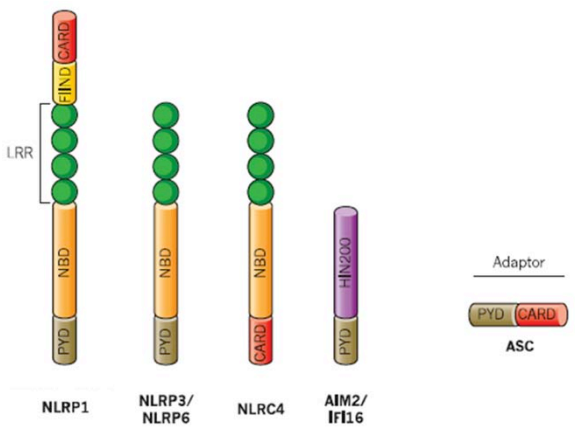

Figure 2. Domain organization of inflammasome proteins.
The NLR family members all contain a nucleotide-binding domain (NBD or NACHT domain), a carboxy-terminal leucine-rich repeat (LRR), and can contain either a PYD or a caspase activation and recruitment domain (CARD) or both. AIM2 has, in addition to a PYD, a HIN200 domain, which is involved in the ligand binding. If the complex doesn't present the CARD domain, such as NLRP3, it needs the adaptor ASC to interact with caspase-1[13].

One of the first described NLR is the Nucleotide oligomerization domain 1 (NOD1), which has, together with the Nucleotide oligomerization domain 2 (NOD2), the CARD domain. NOD1 and NOD2 can oligomerize with the protein RIP2, through CARD, determining the activation of the NF-κB transcription factor pathway. It is a particular aspect for a NLR, usually involved in the second step of the inflammatory response, but

in this case involved also in the priming phase (19).

Besides the inflammasome complexes constituted by NLRs proteins, there are also non-NLRs proteins. Pyrin, for example, presents a very similar structure with NLRs but it is composed by a N-terminal PYD, followed by two central B-box zinc-finger and coiled coil domains and a C-terminal B30.2/rfp/SPRY domain (20). In this case, PAMPs are recognised by the SPRY domain and the protein oligomerizes with ASC through PYD.

Another non-NLR protein is AIM2, constituted by a C-terminal DNA-binding HIN domain and a N-terminal PYD. The HIN domain binds the double-stranded DNA (dsDNA), in a non-sequence-specific manner due to the electrostatic attraction between its positively charged residues and the dsDNA sugar-phosphate backbone (21). PYD, as already described, is necessary to bind ASC and recruit the pro-caspase-1 through CARD (22).

Despite of the differences in the monomeric structure of the inflammasome components it is important to highlight that the main function for an inflammasome is to form a platform to put in contact pro-caspase-1 enzymes that will activate each other through proteolytic cleavage (Figure 3) (12, 17, 23).

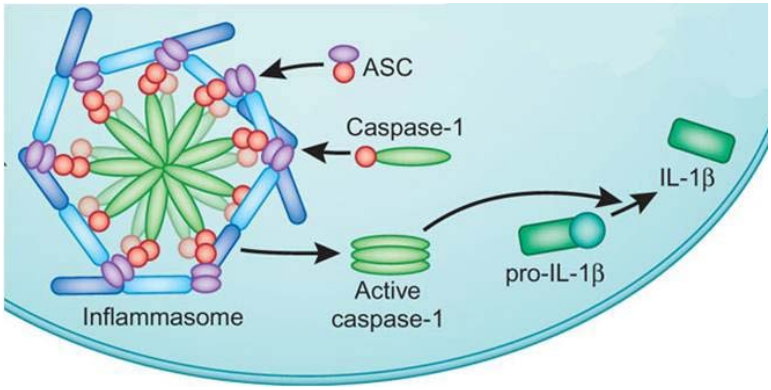

Figure 3. The inflammasome platform.
Once activated the inflammasome proteins oligomerize to constitute a structure able to recruit and put in contact different molecules of pro-caspase 1, that will activate each other.

1.3 Inflammasome activation mechanisms

The inflammasome activation process is finely regulated and the assembly of its monomers usually take place after an activation signal, named “signal two”, consecutive to the recognizing of PAMPs and DAMPs which represent, instead, the first signal which determines the synthesis, through the NF-κB and JNK kinase pathways, of pro-IL-1β, pro-IL-18 and the inflammasome components (24, 25). The second activation signal can be represented by various factors, different according to the inflammasome type (Figure 4).

The NLRP3 inflammasome, the best studied until now, can be activated in response to a wide array of stimuli, classified in microbial activators and sterile activators. Numerous microbes including various bacteria, viruses, fungi, and protozoan parasites can activate NLRP3, such as the microbial toxin nigericin or bacterial pore-forming toxins (PFTs). However, in addition to microbial activators, also endogenous danger signals have been demonstrated to activate the NLRP3 inflammasome such as ATP; monosodium urate (MSU) crystals and calcium pyrophosphate dihydrate (CPPD) crystals, causative agents of gout and pseudo-gout respectively; silica and asbestos particles which cause the fibrotic lung disorders silicosis and asbestosis; aggregated beta-amyloid, associated with the pathogenesis of Alzheimer’s disease, and aluminium salts (26-28).

In particular, ATP is a danger signal released by damaged, stressed or necrotic cells. It binds the purinergic receptor P2X7 (P2X7R) triggering the formation of a pannexin-1 hemichannel which results in the activation of the NLRP3 inflammasome. High extracellular ATP levels activate and open the leukocytic P2X7R, allowing the massive cytoplasmic K^+ efflux and Ca^{2+} influx. High intracellular Ca^{2+} levels determine, in turn, the P2X7R/NLRP3 interaction and allow the NLRP3 recruitment and activation (29, 30). Moreover, the Reactive Oxygen Species production (ROS), enhanced by ATP (31),

has been associated to the NLRP3 activation, since inflammasome activation defects have been detected in case of mitochondrial dysfunctions (32).

Anyway, it still remains unclear how these stimuli activate NLRP3 and since its activators are chemically and structurally unrelated factors, one can hypothesize that NLRP3 does not directly detect these factors but they probably induce a common intracellular signal. Probably the K^+ efflux represents the key event. In fact, it has been demonstrated that K^+-free medium activates the NLRP3 inflammasome in Bone-marrow-derived macrophages (BMDM), even in the absence of other NLRP3 agonists. Therefore, K^+ depletion alone seems necessary and sufficient to activate the NLRP3 inflammasome (33).

NLRC4 can be activated and oligomerize after detection of two kinds of proteins, sharing a similar structure: the bacterial flagellin and the rod component of the type III secretion systems (T3SS) of gram-negative bacteria (e.g. PrgJ for *S. typhimurium*; EprJ and Esci for *E. coli*; Pscl for *P. aeruginosa*) (34, 35).

The NRLP1 activation mechanism is not very clear, particularly in humans. Probably it is very different from NLRP3 or NLRC4, since in murine macrophages NLRP1 is present as a multiprotein complex even without activation (36). NLRP1 activity is probably NOD2-related because it takes part to the NOD2-mediated bacterial killing, together with NLRP3. In particular NOD2 is an intracellular sensor for the muramyl dipeptide (MDP), a fragment of peptidoglycan (PGN) from Gram-negative and Gram-positive bacterial cell walls. As previously mentioned NOD2, once activated, contributes to the NF-κB and MAPK activation (the priming phase) but it also participates to the second phase of the inflammasome formation, together with NALP1 and NLRP3. Indeed, it has been demonstrated that NLRP1, NLRP3 and ASC are required for the NOD2-induced inflammasome activation and IL-1β secretion, after

exposure to MDP (37, 38).

NLRP12 triggers remain to be identified. It has a quite singular behaviour, and this receptor was initially considered a negative regulator of inflammation because it can suppress the response to *Salmonella typhimurium* infections, inhibiting the TLR-induced NF-κB activation by dampening the phosphorylation of IκBα and ERK, enhancing in this way the intracellular bacterial survival (39). But it has also been shown its involvement in periodic fevers (40) and in the host resistance to *Yersinia pestis* (41).

Just like NLRP12 inflammasome, also Pyrin presents a controversial function. Pyrin interacts with tubulin and colocalizes with microtubules (42). Recently it has been shown that it works as a cytosolic PRR, detecting pathogen modifications of the Rho GTPases, frequently targeted by bacteria able to manipulate host actin cytoskeleton and inhibit their phagocytosis. An example of Rho modifications is represented by the Rho-glucosylation activity of the cytotoxin TcdB, the major virulence factor of *Clostridium difficile*. Moreover, additional Rho-inactivating toxins also include the FIC-domain adenylyltransferases (*Vibrio parahaemolyticus* VopS and *Histophilus somni* IbpA) and the *Clostridium botulinum* ADP-ribosylating C3 toxin, which all target the Rho subfamily and modify a switch-I residue. Once these modifications have been detected, Pyrin becomes active and induces the ASC-dependent but NLRP3-independent IL-1β activation (43). In particular, Pyrin works as a trimer and probably, thanks to this structure, it can constitute the inflammasome platform for the pro-caspase-1 activation (20, 44).

However, it has also been demonstrated that Pyrin has an anti-inflammatory activity. The B30.2/rfp/SPRY domain can interact, with NLRP3 and other inflammasome components (45) but also with Caspase-1, preventing its activation (46).

Finally, AIM2, even if it doesn't present the NRLs' structure, forms a platform for the pro-caspase-1 like the other ones. Its major trigger is the cytoplasmic double-stranded DNA (dsDNA). Indeed, it can bind dsDNAs of viral, bacterial or mammalian origin, which induce its oligomerization (22, 47, 48).

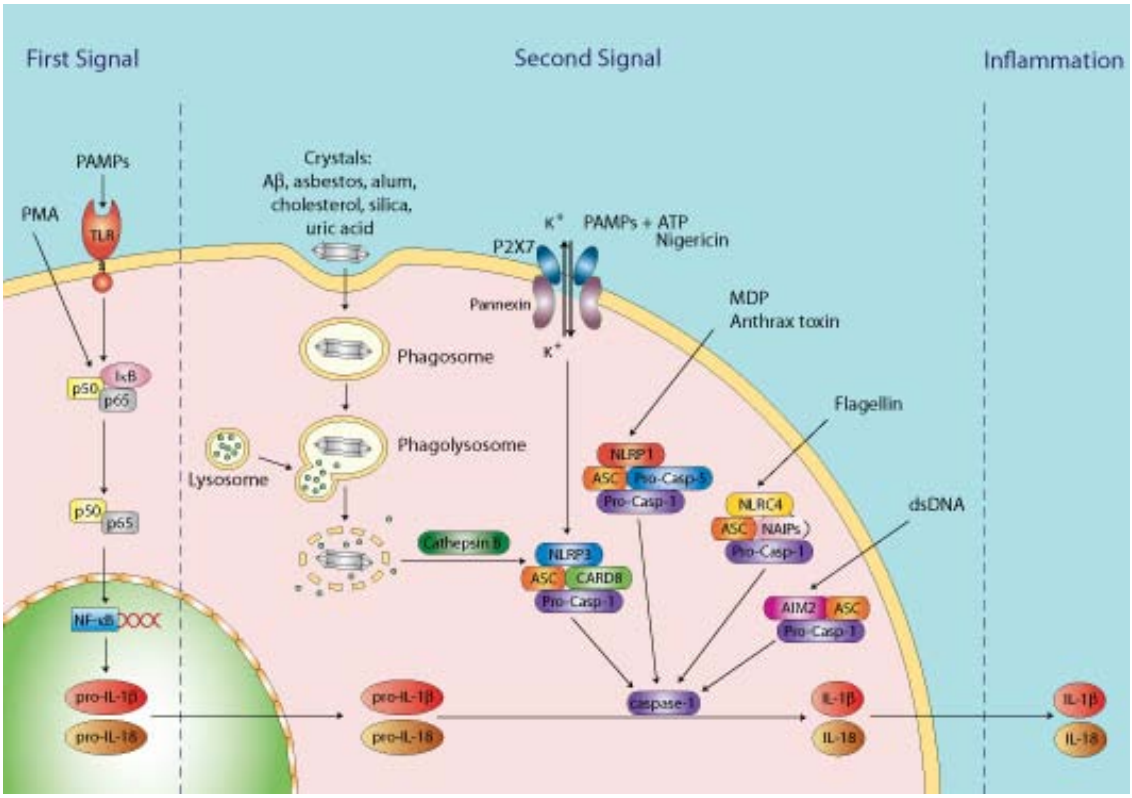

Figure 4. The different signals and mediators of an inflammatory response.

1.4 Cytokines secretion

The priming phase after detection of pro-inflammatory stimuli, induces the synthesis of inflammatory mediators, including cytokines. One can distinguish different kind of cytokines, characterized by different activation and secretion mechanisms. In most cases they are secreted through the canonical pathway, typical of TNF-α, IL-2 and IL-12. These proteins contain a signal peptide, a hydrophobic peptide 20 amino acids long,

necessary to translocate in the ER. Here, they lose the signal peptide and are subjected to post-traslational modifications like the oligomerization or the folding in the tertiary structure. From the ER, they translocate in the trans-Golgi apparatus, to be finally guided toward the plasma membrane, directly or via secretory granules that release their content in case of specific signals. The mechanism for the TNF-α release is of particular interest. Its precursor doesn't lose the signal peptide during the passage through the ER, but it accumulates in the Golgi and once transported to the plasma membrane it is cleaved by the Tumor Necrosis Factor α converting enzyme (TACE) in the ectodomain, representing the mature TNF-α cytokine, finally released (49).

A different secretory mechanism interests the cytokines IL-1β and IL-18. It is the so called non-canonical pathway, independent from the ER and the Golgi apparatus and for this reason it interests "leaderless" proteins, i.e. proteins lacking the hydrophobic signal sequence. The non-canonical mechanism comprises different ways of reaching the extracellular milieu, described below.

Most of the active IL-1β localizes in the cytosol, although a fraction resides in endolysosomal vesicles, containing the pro-IL-1β/pro-IL-18 and the pro-caspase-1 (49, 50). Endolysosomes represent the site of pro-IL-1β/pro-IL-18 processing, in fact when the active forms of IL-1β or IL-18 are released from the cells, usually caspase-1 is released, too (51, 52).

The mobilization of secretory lysosomes is P2X7R and Ca^{2+}-dependent, and it is blocked by phospholipase A_2 and phosphatidylcholine-specific phospholipase C inhibitors (53). In particular, phospholipase A_2 seems to be ultimately responsible for the lysosome exocytosis, whereas phospholipase C seems to mediate the phospholipase A_2 activation by allowing the increase of intracellular Ca^{2+} concentration (54).

Besides the lysosomal exocytosis, IL-1β can also be sorted through the shedding of

microvesicles from the plasma membrane. IL-1β contained in microvesicles is bioactive and may be released following the contact with IL-1 receptor (IL-1RI) expressing cells. ATP-stimulation of these microvesicles induces the release of their contents (55) and also this process has been related to the P2X7R activation (52).

Also pyroptosis could represent a mechanism for IL-1β and IL-18 secretion. Pyroptosis is a pro-inflammatory caspase-1-dependent form of cell death used by infected macrophages to kill themselves and release at the same time pro-inflammatory cytokines (56). This process prevents the pathogen growth and occurs after the caspase-1-dependent formation of pores in the plasma membrane, which causes the dissipation of ionic gradients and the osmotic lysis of the cell. These caspase-1-dependent pores may also represent a conduit for the passage of IL-1β in the extracellular space (57, 58).

Finally, IL-1β secretion process is also regulated by mechanisms which determine its degradation. One of them is the autophagy process. Autophagy is a mechanism used to eliminate damaged organelles and proteins from the cytosol and it works by encapsulating the molecules to eliminate, in a double membrane structure, the autophagosome. It fuses with lysosomes forming autophagolysosomes, determining the proteolytic degradation of their contents. Pro-inflammatry stimuli induce the recruitment of IL-1β and other inflammatory response components to autophagosomes, suggesting that autophagy contributes to maintain the homeostasis, controlling the pro-IL-1β degradation (59, 60).

1.5 Autoinflammatory disorders

The inflammatory response, the synthesis and the release of pro-inflammatory cytokines are highly regulated processes, sequentially and temporally orchestrated. When these

regulatory mechanisms are defective and the ability to clear damaged tissues is impaired or the inflammatory response is prolonged, one can encounter tissue destruction and the onset of autoinflammatory disorders (10, 11).

The concept of autoinflammation arose quite recently in 1999, following the identification of mutations in the Tumor Necrosis Factor receptor–associated periodic syndrome, TRAPS (61).

TRAPS is a monogenic autoinflammatory disease, classified today as Hereditary Periodic Fever Syndrome (HPFs) together with: Familial Mediterranean Fever (FMF), Hyperimmunoglobulinemia D (HIDS)/Mevalonate Kinase Deficiency (MKD) and Cryopyrin-associated periodic syndromes (CAPS) (62, 63).

Besides the HPFs, the autoinflammatory group also includes: Pyogenic arthritis with pyoderma gangrenosum and acne syndrome (PAPA), Blau syndrome, Crohn disease, osteoarthritis, gout and pseudogout (64, 65).

These disorders are usually characterized by the disruption of the homeostasis of the canonical cytokine cascades and seemingly unprovoked episodes of systemic inflammation. When the defects affect the functioning of the inflammasome complexes, one can talk about “inflammasomopathies” or IL-1β activation disorders, classified in: intrinsic inflammasomopathies, when molecular lesions affect the constituent proteins of the inflammasome complex, such as in CAPS; and extrinsic inflammasomopathies, when the defects affect proteins upstream or downstream the inflammasome activity, such as in FMF, MKD, PAPA and Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis (SAPHO) syndrome (64).

In the Table 1, we have summarized the most known autoinflammatory disorders, indicating also their genetic base.

Table 1. Clinical classification of selected Autoinflammatory Diseases	
Disease	**Gene (Protein)**
Hereditary Recurrent Fevers	
Familial Mediterranean Fever (FMF)	MEFV (Pyrin)
TNF receptor-associated periodic syndrome (TRAPS)	TNFRSF1A (TNFR1)
Mevalonate Kinase Deficiency (MKD)	MVK (Mevalonate Kinase)
Familial Cold Autoinflammatory Syndrome (FCAS)	NLRP3/CIAS1 (NLRP3/cryopyrin)
Muckle-Wells syndrome (MWS)	NLRP3/CIAS1 (NLRP3/cryopyrin)
Neonatal Onset Multisystem Inflammatory Disorder (NOMID)	NLRP3/CIAS1 (NLRP3/cryopyrin)
Idiopathic Febrile Syndromes	
Systemic onset juvenile idiopathic arthritis (sJIA)	Complex
Adult-onset Still's disease (AOSD)	Complex
Pyogenic Disorders	
Pyogenic Arthritis with pyoderma gangrenosum and acne syndrome (PAPA)	PSTPIP1/CD2BP1 (PSTPIP1/CD2BP1)
Granulomatous Diseases	
Chronic granulomatous synovitis with uveitis and cranial neuropathy (Blau syndrome)	NOD2/CARD15 (NOD2/CARD15)
Crohn's disease	Complex (NOD2, ATG16L1, IRGM)
Autoinflammatory disorders of skin and bone	
Deficiency in IL-1 receptor antagonist (DIRA)	IL1RN (IL-1Ra)
Majeed syndrome	LPIN2 (Lipin-2)
Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis syndrome (SAPHO)	Complex
Metabolic Disorders	
Gout (monosodium urate deposition)	Complex (SLC2A9/GLUT9, ABCG2)
Pseudogout (calcium pyrophosphate dehydrate deposition)	Complex
Type 2 diabetes mellitus	Complex
Vasculitis	
Behçet's disease	Complex

1.5.1 Cryopyrin-associated periodic syndromes

The Cryopyrin-associated periodic syndromes (CAPS) are autoinflammatory diseases due to autosomal dominant or the novo mutations in the exon 3 of the *NLRP3* gene (also known as *CIAS1*, cold-induced autoinflammatory syndrome 1), encoding for the NACHT domain of the NLR (66, 67).

Although heterozygous germline gain-of-function *NLRP3* mutations are a known cause of this diseases, conventional genetic analyses fail to detect disease-causing mutations in ~40% of patients. Indeed, somatic *NLRP3* mosaicism has been detected in several mutation-negative CAPS patients, and considering that a mutation of *NLRP3* in the 10% of the cells is sufficient to cause a distinct disease phenotype, it has been estimated that the somatic mosaicism is a major cause of NOMID/CINCA syndrome (68, 69).

In the CAPS group are classified three disorders, with different severity:

- Familial Cold Autoinflammatory Syndrome (FCAS), with blander phenotype usually manifested before the age of 6 months. It is characterized by recurrent intermittent episodes of fever and skin rash, after cold exposure, whose duration is less than 24 hours. The attacks present also arthralgia and other symptoms including myalgia, conjunctivitis, sweating, drowsiness, headache, extreme thirst and nausea. Although these symptoms impair the quality of life, they are not associated with organ damage or physical disability (70, 71);
- Muckle-Wells syndrome (MWS) has intermediate phenotype. It determines spontaneous or cold triggered "flare-ups" which begin during infancy or early childhood. Affected individuals typically manifest skin rash, mild to moderate fever, painful and swollen joints, and in some cases conjunctivitis. During the adolescence it is possible to develop the sensorineural deafness caused by the

cochlear inflammation, and one-third of affected people presents also abnormal deposits of the protein amyloid, which progressively damages kidneys and other organs (72, 73);

- Neonatal Onset Multisystem Inflammatory Disorder (NOMID) also known as chronic infantile neurologic cutaneous articular syndrome (CINCA) is the most severe. It causes, like the others: fever, fatigue, loss of energy, myalgias and skin manifestations. In this case the risk of amyloidosis is higher in Europe than United States, indicating the involvement of environmental factors. Also NOMID could presents eye involvement with conjunctivitis, anterioruveitis, corneal infiltrates, papilledema and uveitis. These lesions can result in a permanent damage and progressive loss of visual field. Sensorineural hearing loss in most patients with NOMID becomes clinically evident in the first decade of life, differently from MWS, where it develops later. The most severe implications interest the Central Nervous System, with aseptic meningitis and increased intracranial pressure, cognitive impairment, seizures, stroke and other vascular occlusive events. Moreover, patients could also manifest severe bone deformities resulting in limb length discrepancies and joint contractures, associated with significant loss of mobility and, in some patients, the inability to walk. Physical disability in patients with NOMID is also caused by the severe growth retardation (74, 75).

The typically related activating mutations in the abovementioned ATP-binding cassette are probably responsible for the constitutive activation of the NLRP3 inflammasome, which in turn, is associated to the constitutive release of IL-1β, typical in peripheral blood mononuclear cells (PBMCs) from CAPS patients. Indeed, functional studies on PBMCs purified from affected patients have shown that they result active after the

exposure to the first signal, such as LPS, but they don't need the second stimulus, such as ATP, to determine the release of the cytokines, indicating that the inflammasome complex is already active and ready to complete the activation of the inflammatory response (76). However, IL-1β activation through the NLRP3 inflammasome seems dependent on the NF-κB pathway, necessarily activated by the signal one (77).

It has also been demonstrated an overproduction of IL-1α from CAPS PBMCs, not yet related to the NLRP3 inflammasome. Moreover, oxidative stress could also play an important role in the inflammasome activation typical of these disorders, because in CAPS patients blood monocytes display higher ROS production than healthy subjects and ROS are able to lower the threshold of inflammasome activation. IL-1β, IL-18 and IL-1α secretions in patients monocytes have been induced, indeed, by markedly lower LPS concentrations, unable to activate the cytokines secretion in monocytes from healthy subjects. Furthermore, the presence of antioxidants restored the secretion of the anti-inflammatory cytokine IL-1Ra (Receptor antagonist), which is inhibited by oxidative stress (78).

The major anomaly founded in CAPS patients is the excessive production of IL-1β. For this reason the most effective treatment for them, available today, is the administration of IL-1 blocker agents, like anakinra, rilonacept or canakinumab, described below. Recently, it has also been identified a new small molecule, named MCC950, that could be promising for the development of new pharmacological treatments, thanks to its ability to block the oligomerization of NLRP3 with the adaptor ASC, in a selective manner for this inflammasome (79).

1.5.2 Familial Mediterranean Fever

Familial Mediterranean Fever (FMF) is a periodic fever syndrome, associated to autosomal recessive mutations in the Pyrin encoding gene *MEFV*, usually located in the exons 2, 3, 5 and 10 (80). It has been described for the first time in 1802 (81) and affected patients usually manifest unprovoked, recurrent fever attacks and painful polyserositis, together with abdominal, chest or joint pain. Pericarditis is rare and the affected skin is generally erythematous with erysipelas-like lesions, reminiscent of cellulitis. Untreated FMF patients may suffer decreased fertility but the most significant complication of this condition is the risk of amyloidosis with amyloid deposition that primarily occurs in kidneys but can also affects the gastrointestinal tract, liver, spleen and thyroid. However, there are also FMF cases of fever without other symptomatology, especially in younger children.

FMF usually manifests in childhood or, in any case, before reaching the age of 20. Each attack may last from 24 to 72 hours and can be a consequence of viral illness, emotional stress, intense physical activity, high-fat diet, extremes of temperature and menstruation in women (82).

To date, more than 170 *MEFV* mutations have been associated with FMF (80). The most common variants are: E148Q, E167D, T267I, P369S, F479L, I591T, M680I, I692del, M694I, M694V, K695R, V726A, A744S, R761H. Among them the M694V, the most frequently encountered in FMF patients, or mutations in position 680 to 694 on the exon 10 of the gene, have been associated to a more severe FMF phenotype. E148Q in the exon 2 is one of the most frequent alterations in the *MEFV* gene, but frequently encountered in the general population and for this reason it is considered a polymorphism, not useful to support the diagnosis of FMF.

Indeed FMF has mainly a clinical diagnosis. Its clinical criteria are usually distinguished in major and minor criteria:

- Major criteria are: peritonitis, pleuritis or pericarditis, monoarthritis, recurrent febrile episodes, amyloidosis of the AA type;
- Minor criteria are: incomplete attacks involving chest and/or joint, exertional leg pain, response to colchicine.

Following these parameters, the diagnosis is consequent to the manifestation of one major criteria or two minor criteria. Genetics is crucial only to support the diagnosis, but it should never substitute the clinical diagnosis (83). Indeed, it is possible to diagnose the syndrome without a *MEFV* gene mutation (84).

The FMF defective protein is the Pyrin, which is expressed primarily in neutrophils, eosinophils, cytokine-activated monocytes and synovial fibroblasts (85, 86). Pyrin function is quite controversial. According to the structure described in the paragraph "The Inflammasome structure", it has a pro-inflammatory activity, because it can bind the adaptor protein ASC through the PYD domain and consequently activate the pro-caspase-1, probably constituting a platform thanks its trimeric form (20, 43, 44).

Bone marrow $CD11b^{+}$ cells from $Mefv^{V726A/V726A}Asc^{-/-}$ but not from $Mefv^{V726A/V726A}Nlrp3^{-/-}$ mice, showed complete ablation of IL-1β secretion and caspase-1 activation after LPS stimulation, even with ATP treatment, indicating that ASC, but not NLRP3, is critical for the caspase-1 activation mediated by FMF-associated pyrin mutations (87).

However, it has also been demonstrated the Pyrin anti-inflammatory activity. The B30.2/rfp/SPRY domain can interact with NLRP3 and Caspase-1, inhibiting the NLRP3 inflammasome assembly. This domain is frequently mutated in FMF and the consequence of this mutation could be the Pyrin loss-of-function, its decreased

interaction with NLRP3 and Caspase-1, and the hyper activation of IL-1β because of the lack of its inhibitory effect on NLRP3 (45, 46).

Differently from murine models studies, more recent investigations on monocytes freshly isolated from FMF patients carrying *MEFV* gene mutations, have shown that the downregulation of NLRP3 in these cells, inhibits the IL-1β secretion, demonstrating the opposite of the previous hypothesis (88). Actually, the functional relationship between Pyrin and NLRP3 remains unclear and deserves further investigation, but the last observations do not preclude the existence of a Pyrin inflammasome triggered by different stimuli, not necessarily related to the NLRP3 inflammasome (43).

Recently it has been identified a new mutation in the exon 2 of the *MEFV* gene, which determines a serine-to-arginine substitution at position 242 (S242R), resulting in the loss of a 14-3-3 binding motif, usually not perturbated in the B30.2/rfp/SPRY domain mutations. The S242R mutation has been associated to a new autoinflammatory disease, clinically different from FMF, named pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND). The 14-3-3 motif regulates the pyrin activity and its loss of function allows a spontaneous inflammasome activation, determining the ASC speks formation into the cytoplasm, the caspase-1 activation and the NLRP3 independent IL- 1β secretion (89).

Despite of the evidence of the involvement of IL-1β in this disease, treatment with anti-IL-1 drugs didn’t give a broad-spectrum effect like in CAPS patients. In FMF, in fact, the conventional treatment is represented by the colchicine, a microtubule-inhibitor, effective with the majority of patients, probably because of the interaction of Pyrin with cytoskeleton microtubules. IL-1 inhibitors, in these cases, represent the most valid alternative for patients unresponsive or intolerant to colchicine (90-92).

1.6 Focus: Systemic juvenile idiopathic arthritis and Adult onset Still's disease

1.6.1 Syndromes description and diagnosis

This thesis will focus particular attention to two autoinflammatory disorders with still unknown etiology but suspected inflammasome implication: Systemic juvenile idiopathic arthritis (sJIA) and Adult onset Still's disease (AOSD).

sJIA is a rare inflammatory disorder, described for the first time by Sir George Frederick Still in 1897, who identified three forms of arthritis in children: rheumatoid arthritis, Jaccouds' arthropathy and the systemic-onset variant (93). The systemic variant is considered today a subtype of juvenile idiopathic arthritis (JIA), characterized by onset before the age of 16 and symptoms persisting for more than 6 weeks (94).

AOSD, described by Eric Bywaters in 1971, presents very similar features with sJIA and often it is considered the sJIA adult counterpart (95, 96). In both conditions, affected patients usually manifest a set of signs and symptoms that don't refer to one specific pathogenic mechanism and for this reason it is more correct to define them as syndromes rather than diseases.

It has been shown the resemblance of clinical and laboratory outcomes. In particular, young and adult patients present arthritis, of course, but mainly systemic implications, like quotidian high spiking fever, salmon-colored erythematous rash, lymphadenopathy, hepatosplenomegaly, serositis. The systemic inflammation is highlighted also by laboratory tests, with quite elevated C-reactive protein concentration (CRP) and erythrocytes sedimentation rate (ESR). Moreover, in the active phase, patients can also present leukocytosis, with high number of circulating neutrophils and monocytes/macrophages (97).

Comparing sJIA and AOSD clinical and laboratory features, Pay et al. revealed some differences, such as fever, skin rash, myalgia or liver dysfunctions more common in adult patients; a higher AOSD incidence in women, probably related to a hormonal influence, not yet present in children which don't manifest differences depending on sex; the seasonality incidence, more frequent in young patients. Probably these differences are due to a different immune reaction in young and adult subjects but it is still possible to consider them as two expressions of the same syndrome.

Since the lack of specific laboratory criteria for both of them, the diagnosis usually remains a clinical one and requires the exclusion of other diseases (98, 99).

1.6.2 Genetic assessment

The prominent activity of the innate immune system and the involvement of pro-inflammatory cytokines, allow to classify sJIA and AOSD as autoinflammatory disorders (100, 101), but unlike the majority of such disorders, they don't present a defect in a single gene. sJIA and AOSD are considered multigenic disorders associated to polymorphisms in pro-inflammatory genes or their promoter elements. In sJIA these polymorphisms have been found in the genes encoding TNF-α, IL-6, IL-10, macrophage migration inhibitory factor (MIF) and IL-1 family proteins (102-105). While, in AOSD they have been mainly identified in IL-18 and MIF genes (106, 107).

In few cases it has been supposed a correlation between severe systemic JIA and *MEFV* mutations, in particular in a subgroup of Turkish patients (108) and Egyptian children, which present a significantly higher frequency of *MEFV* mutations than healthy population (109). Moreover, Turkish AOSD patients have shown an increase in the *MEFV* mutation rate (110). Furthermore, sJIA patients from the southern region of Saudi Arabia present a homoallelic missense mutation in the *LACC1* gene, which

encodes the enzyme laccase (multicopper oxidoreductase) domain-containing 1 (111). However, supplementary clinical studies with large number of patients are needed to confirm these genetic associations.

1.6.3 Pathogenesis hypothesis and pharmacological treatments

Many features of sJIA and AOSD seem to be explained by the effects of innate pro-inflammatory cytokines IL-1, IL-6, TNF and IL-18 (Figure 5). Serum or synovial fluids from affected patients usually present quite high levels of these proteins.

TNF-α and soluble TNF receptor levels are increased in both syndromes, but treatment with TNF-blockers, like etanercept, failed with either sJIA and AOSD patients, indicating that TNF-α doesn't play a major role in these syndromes and patients require alternative treatments (112, 113).

IL-6, instead, correlates with clinical manifestations. Indeed, its levels result markedly elevated in the blood and synovial fluid during fever spikes, arthritis and the other systemic manifestations typical of the active phase. Treatment with interleukin-6 inhibitors, like tocilizumab, is effective in severe, persistent and unresponsive systemic JIA (114) and represent a suitable option for the therapy of refractory AOSD patients (115).

IL-1β role is not very well understood. It has been hypothesized that it is involved in the early sJIA, because the pharmacological treatment with anti-IL-1, particularly anakinra, solves systemic symptoms and arthritis when administered as first line drug but it results less effective in patients with refractory arthritis. It is still unknown how IL-1β intervenes. Serum from active sJIA patients can induce the expression of innate immune genes, including IL-1β, in healthy PBMCs but it has not been shown a gene expression profile related to the IL-1β activity in PBMCs from active patients (116, 117).

Therefore, the main evidence of IL-1 involvement in the pathogenesis of sJIA is represented by clinical improvements after treatment with IL-1 inhibitors, also seen in AOSD patients (118). But the principal cellular source of IL-1 in these disorders remains unknown.

The typical systemic and local manifestations together with the other possible sJIA complications, are due to the activation of the above-mentioned cytokine network mediated by activated phagocytes and endothelial cells (119). Cytokines can be produced by a wide range of cells, including immune cells (monocytes, macrophages, granulocytes, B and T lymphocytes, mast cells), endothelial cells, fibroblasts and stromal cells.

Leukocytosis typical of sJIA and AOSD patients, would suggest an exuberant innate inflammation that, primarily in children, could be consequent to the exposure to infectious agents typically encountered in childhood but, also in adults subjects, pathogens could represent an activation trigger. However in both cases, it is not correct to define them infectious diseases.

The first hypothesized source of IL-1β were monocytes. It has been shown that PBMCs from sJIA patients don't present a spontaneous release of cytokines but, once activated with Phorbol 12-myristate 13-acetate (PMA), they release higher levels of IL-1β in comparison with healthy cells (116). This result was not confirmed in other studies. In particular, Macaubas et al. have shown that sJIA monocytes respond to LPS stimulation with higher intracellular production of IL-1β than healthy controls (detected by intracellular staining), but they don't secrete the cytokine into the supernatant, where IL-1β concentration is even lower in sJIA PBMCs versus healthy ones, confirming what has been observed in another study by Gattorno et al. (120, 121). Probably this difference was due to the kind of stimulation and the different signaling pathways

induced by LPS and PMA. LPS is a glycolipid component of Gram-negative bacteria cell walls, able to activate monocytes through intracellular events which mediate the activation of specific protein kinases and the consequent cytokines production. PMA, instead, is a protein kinase C activator, able to induce immunomodulatory cytokines secretion (122).

Often sJIA and AOSD are compared with CAPS (123, 124). There is no evidence of the same involvement of the inflammasome complex NLRP3 in these syndromes, but they share clinical phenotypes and also molecular markers. In particular, proteins like IL-18 and S100 proteins result up-regulated. Gene expression profiling have also shown the up-regulation of innate immunity pathways, with a good correspondence between elevated levels of total IL-18 protein in serum and elevated transcript levels of IL-18, similarly to what happen for S100A8/9 and S100A12. Moreover, another common aspect is the efficacy of IL-1 blockers in the three syndromes.

However, differently from what has been observed in CAPS, sJIA monocytes involvement in IL-1 release seems controversial. For this reason one can suppose neutrophils implication. Also neutrophils, in fact, could be an IL-1β cellular source. It is well known that monocytes, respect neutrophils, represent the major source of IL-1β in physiological conditions, but also neutrophils express inflammasome components and produce IL-1β (125) in an inflammasome dependent way (126). Nevertheless, IL-1β activation in neutrophils can also be mediated by proteases different from caspase-1, including neutrophil elastase (NE), cathepsin G (CG) and proteinase-3 (PR3) (127).

Some experiments were performed on neutrophils isolated from peripheral blood of CAPS patients. Although older experiments, above mentioned, on PBMCs have shown the constitutively increased NLRP3 inflammasome activity, neutrophils presented only minor changes in the baseline production of IL-1 compared with age-matched healthy

control cells and, even upon inflammasome activation, the protein release of IL-1 and IL-18 in neutrophils resulted lower in CAPS patients compared with controls. Probably *NLRP3/CIAS1* gene mutations, lead to the constitutive over activation of the inflammasome at baseline conditions, rendering neutrophils unresponsive toward exogenous inflammasome activators, because of the consumption of the pro-IL-1 protein (126).

Instead, regarding to sJIA and AOSD, there is no information about the possible neutrophils role or their activation state and it is possible to assume their pathogenic involvement, considering the high number of circulating neutrophils and elevated serum levels of S100A12 in affected patients. S100A12 is a protein with a cytokine-like action, typically produced by neutrophils and its overproduction in sJIA and AOSD confirms neutrophils systemic activation in this syndromes (128, 129).

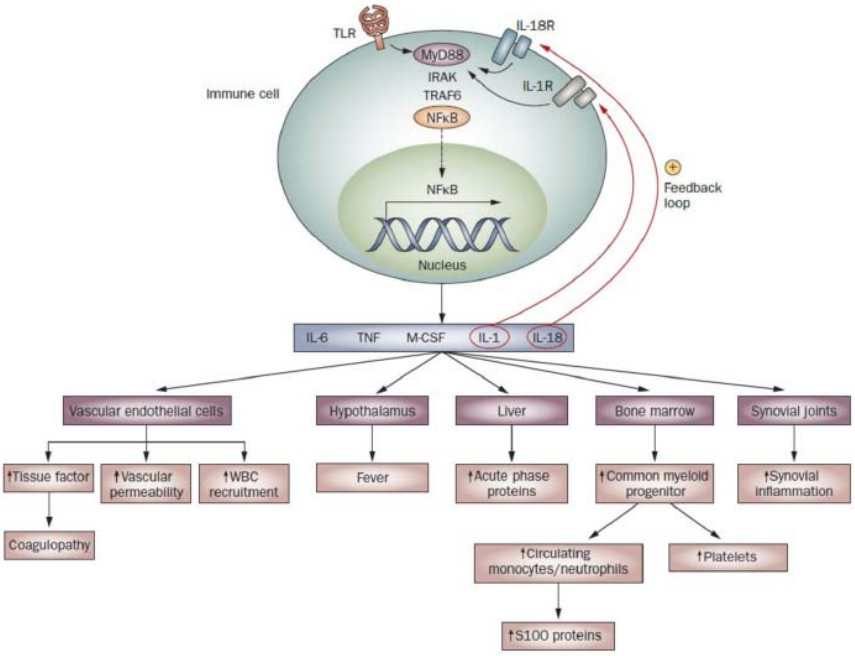

Figure 5. Perpetuation of innate immune responses in sJIA.
Innate immune pathways activated in sJIA are normally triggered by the recognition of PRRs by TLRs expressed on innate immune cells, but can also be triggered by endogenous ligands in inflammatory conditions. NFκB activation and proinflammatory cytokines production, initiate the inflammatory cascade through effects on the hypothalamus, bone marrow, liver and vascular endothelial cells. IL-1R and IL-18R share the downstream portion of the TLR4 signaling pathway, therefore IL-1 and IL-18 provide positive feedback loops that further contribute to perpetuation of the inflammatory responses in sJIA[136].

1.7 Biological drugs in IL-1β activation disorders

Biological drugs are particular pharmaceuticals used for treatment, prevention and cure of human diseases. They act on specific steps of cellular pathways, defective in certain diseases. Therapeutic targets of these kind of drugs are always genes or proteins which are part of the altered pathway, today more easily identified thanks to the increasing knowledge of genetics and cell processes.

Differently from the most common drugs chemically synthesized, which are more pure and have a well-defined structure, biological products derive from living material such as humans, animals or microorganisms and their complex structure is usually not fully characterized. Chemical drugs, in fact, can be produced in uniform large quantity and are usually subjected to chemical tests for identity and purity. Biologics, instead, present a complicated production process, are produced in small quantities and require stability assessments because they are often sensitive to temperature, light or shear forces.

Biologics include biological substances, hormones, nucleic acids and monoclonal antibodies (mAbs) able to modulate the immune system and to act on inflammation and cell proliferation (Table 2).

Since the complexity of their molecular structure and production process, they result more expensive than chemicals and usually they are administered when there are no other options, for example in case of failure of alternative therapies (130, 131).

One can distinguish:

- Monoclonal antibodies: mouse IgG immunoglobulins, in which the murine fraction is decreased in order to reduce their immunogenicity. Their names end with the suffix –mab, preceded by a syllable indicating the degree of humanization:
    - -ximab: for chimeric mAb, which contains the 25% of the murine

fraction in the Fab fragment of the immunoglobulin;

- -zumab: for humanized mAb, which contains 2%-5% of the murine fraction in the Fab fragment;
- -mumab: for human mAb.

- Fusion proteins: derive from the binding of receptors or cell ligands to part of the Fc fragment of IgG1. This binding is necessary to increase solubility and half-life of the proteins and their names end with the suffix –cept;
- Cytokines: interferons (IFNs), interleukins (IL) and colony stimulating factors (CSF).

Autoinflammatory syndromes are usually treated by administration of specific biological drugs including receptor blocking agents, cytokine blocking antibodies, and fusion receptors. As previously mentioned, for these disorders the main cellular target is the IL-1β pathway, which results defective. In these cases, it is possible to intervene with different biologics, acting on this defective pathway. Anakinra, for example, is a recombinant form of the naturally occurring IL-1 receptor antagonist and it works as competitive inhibitor of IL-1α and IL-1β by binding the IL-1R without transducing any signal. There are also other drugs able to block IL-1β action such as canakinumab and rilonacept. Canakinumab is a fully humanized IgG1 monoclonal antibody, acting against IL-1β; rilonacept is a fusion protein constituted by the human extracellular portions of IL-1R and IL-1R accessory protein linked to the Fc portion of human IgG1. Both inhibit IL-1β signaling but rilonacept also acts as a soluble decoy receptor which binds IL-1β preventing its interaction with cell surface receptors. In some cases, also treatment with tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, can result effective, such as in MKD (Figure 6).

Among inflammasomopathies the only exception regarding to the administration of

biological agents, is represented by FMF patients. For them, in fact, the mainstay of therapy remains colchicine, able to control recurrent attacks and to prevent amyloidosis. It is preferred to biologics because it is a low-cost drug (132, 133).

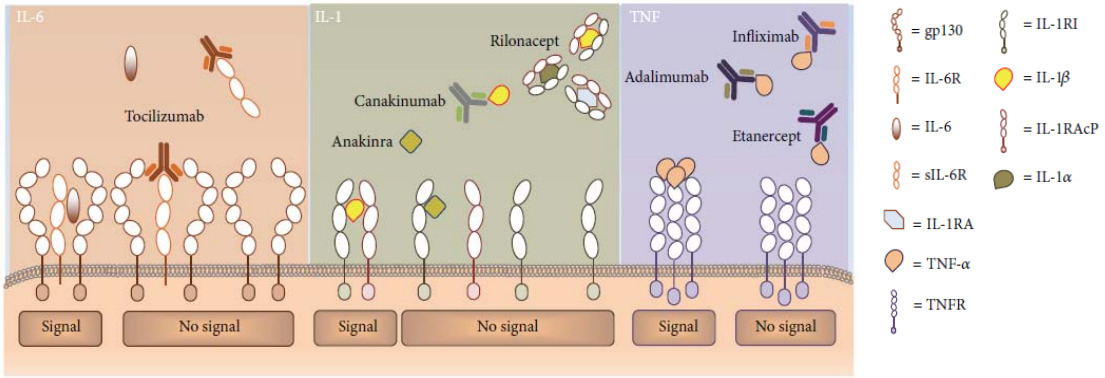

Figure 6. Mechanisms of IL-1, IL-6, and TNF-α targeted therapies.
Tocilizumab, a recombinant humanized anti-IL-6 receptor antibody, inhibits the binding of IL-6 to IL-6R or soluble IL-6R (sIL-6R), thus blocking IL-6 inflammatory response. IL-1-targeted therapy includes anakinra (IL-1R receptor antagonist), canakinumab (anti-IL-1β IgG1 mAb), and rilonacept (soluble IL-1 receptor that binds IL-1β, IL-1α, and IL1RA). Adalimumab, infliximab, and etanercept are anti-TNF blockers. Adalimumab is a fully human monoclonal anti-TNF antibody. Infliximab is a mouse/human chimeric monoclonal anti-TNF antibody. Etanercept is a dimeric fusion protein of TNFR2 (p75) linked with the Fc region of human IgG1.

Table 2. Categories of biologic agents
Hormone (growth hormone, parathyroid hormone, insulin): A substance, usually a peptide or steroid, produced by one tissue and conveyed by the bloodstream to another to effect physiological activity, such as growth or metabolism.
Interferons: Proteins that are normally produced by cells in response to viral infection and other stimuli.
Interleukins: A large group of cytokine proteins. Most are involved in directing other immune cells to divide and differentiate.
Growth factor: A substance such as a vitamin B12 or an interleukin that promotes growth, especially cellular growth.
Monoclonal antibodies (MAbs): A single species of immunoglobulin molecules produced by culturing a single clone of a hybridoma cell. MAbs recognize only one chemical structure, i.e., they are directed against a single epitope of the antigenic substance used to raise the antibody.
Polypeptides: Peptides containing ten or more amino acids. Typically, a peptide consists of fewer than 50 amino acids, while a protein has more than 50 amino acids.
Proteins: Naturally occurring and synthetic polypeptides having molecular weights greater than about 10.000 (the limit is not precise).
Vaccine: An agent containing antigens produced from killed, attenuated or live pathogenic microorganisms, synthetic peptides or by recombinant organisms. Used for stimulating the immune system of the recipient to produce specific antibodies providing active immunity and/or passive immunity in the progeny.

2. Objectives

In this work we evaluated the potential pathogenic involvement of granulocytes in sJIA and Adult Still's disease patients, analyzing the behaviour of these cells in comparison with granulocytes purified from healthy donors. In particular, our aim was to analyze granulocytes' responsiveness to the extracellular environment and their activation state.

Neutrophils activation process is finely regulated. In fact, this cell type is able to regulate and also to limit itself activity, ensuring a correct innate immunity response. The major steps that occur in case of their activation are: the priming phase represented by the exposure on the cellular surface of proteins necessary for neutrophils recruitment and phagocytosis (in particular, the complex CD11b/CD18 and CD66b); NADPH-oxidase dependent ROS generation, which represent potent microbicidal agents; the absence of the normal progression of neutrophils apoptosis (134, 135).

Since sJIA and AOSD are classified as autoinflammatory syndromes, we have suspected a defect in the immunological response mediated by granulocytes and for this reason we tried to evaluate their activation state, analyzing all the principal steps related to their activation, together with their response in term of cytokines and chemokines production usually determined by activated neutrophils, such as IL-1β and S100A12. All these parameters have been measured at baseline and after treatment with pro-inflammatory stimuli.

Moreover, we have also studied the inflammatory response mediated by monocytes purified from sJIA and AOSD patients, and from patients affected by the monogenic autoinflammatory syndromes FMF and CAPS, with the purpose of distinguishing a specific functional phenotype, according to the different inflammasome complex related to each disease.

3. Materials and Methods

3.1 Patients

In this study have been enrolled 18 sJIA patients, 14 AOSD patients, 18 FMF patients and 2 CAPS patients. Clinical characteristics, therapy and laboratory examinations values are shown in the Table 3.

Together with samples obtained from affected patients it was processed, at the same time, at least one sample from a healthy subject, with a total of 59 enrolled healthy controls.

Table 3. Patients clinical characteristics

Patient	Sex	Age	Gene Mutations	WBC	%Lympho	%Neutro	%Mono	Joints involvement	Systemic Symptoms	Therapy	ESR	CRP
sJIA 1	F	17	-	5280 cells/mmc	24,3	66,1	6,3	-	-	NSAID	68	1,68
				7470 cells/mmc	19,5	70.1	6,1	-	-	NSAID	64	2,56
sJIA 2	M	2	-	25280 cells/mmc	19,9	72,2	5,4	-	+	-	42	14
				9480 cells/mmc	43,3	38,8	9,3	-	-	Canakinumab	6	<0,29
sJIA 3	F	4	-	9460 cells/mmc	34,2	51	6,5	-	+	Anakinra	12	<0,29
sJIA 4	F	15	-	5320 cells/mmc	27,2	64,2	5,2	-	-	Tocilizumab	2	<0,29
sJIA 5	M	10	-	7600 cells/mmc	33	53	0,6	-	-	Anakinra	5	<0,29
sJIA 6	F	4	-	8570 cells/mmc	53,3	32,9	7,1	-	-	Methotrexate	5	<0,29
sJIA 7	M	18	-	5,85 x10^9/l	46	35	12	+	-	Rituximab, Methotrexate, Steroids	-	0
				7,5 x10^9/l	36,1	57,3	6,6	-	-	Methotrexate	-	0
				7,8 x10^9/l	32,1	52,5	15,4	+	-	Methotrexate	2	0
sJIA 8	F	9	-	11,3 x10^9/l	13,9	75,8	9,1	-	-	Methotrexate, Steroids	2	0
				11 x10^9/l	25,5	61,8	12,7	-	-	Methotrexate, Steroids	-	0
				13,7 x10^9/l	29	56	10	+	-	Methotrexate, Steroids	-	0
sJIA 9	F	16	-	7,6 x10^9/l	35,5	52,6	11	+	-	Methotrexate	0	0
				5,06 x10^9/l	48	37	14	+	-	Methotrexate	2	0
				6,83 x10^9/l	47,6	36,4	5,5	+	-	Methotrexate	2	0
				5 x10^9/l	60	34	6	-	-	Methotrexate	2	0
				-	-	-	-	-	-	Steroids	-	-
sJIA 10	F	5	*LACC1*	6 x10^9/l	39	50	7	+	-	Methotrexate, Steroids	4	0
				6,5 x10^9/l	26	60	10	-	-	Methotrexate, Steroids	0	0
				5,55 x10^9/l	38	48	11	-	-	Steroids	-	0
sJIA 11	F	20	*LACC1*	-	-	-	-	+	+	-	-	15
				7,5 x10^9/l	29,3	62,7	8	+	-	Methotrexate, Steroids	-	3,6

Patient	Sex	Age	Gene Mutations	WBC	%Lympho	%Neutro	%Mono	Joints involvement	Systemic Symptoms	Therapy	ESR	CRP
sJIA 12	F	11	-	-	-	-	-	-	-	Methotrexate, Steroids	2	0
				-	-	-	-	-	-	Methotrexate, Steroids	-	-
sJIA 13	F	16	-	-	-	-	-	-	-	-	-	52
sJIA 14	F	13	-	-	-	-	-	-	-	Anakinra	-	0
				9,6 x10^9/l	13	77	6,5	-	+	-	-	0
				14,5 x10^9/l	-	86,2	-	+	-	Canakinumab, Steroids	42	105
sJIA 15	M	10	-	4,3 x10^9/l	53,4	34,9	11,6	Remission		Methotrexate, Steroids	-	0
sJIA 16	F	11	-	-	-	-	-	+	-	Methotrexate	-	0
sJIA 17	M	11	-	6,4 x10^9/l	34,4	57,8	7,8	Remission		NSAID	12	0
sJIA 18	M	9	-	8,5 x10^9/l	43,6	47	9,4	Remission		-	18	0
AOSD 1	M	45	-	-	-	-	-	+	+	Methotrexate, Steroids	-	-
AOSD 2	M	36	-	4400 cells/mmc	34,9	55,2	8,4	-	-	Methotrexate	15	0,29
AOSD 3	F	65	-	3510 cells/mmc	31,1	53,2	10,5	+	-	Steroids, NSAID	31	<9
AOSD 4	F	62	-	5870 cells/mmc	24,4	64,4	10,7	-	-	-	4	<9
AOSD 5	M	52	-	1240 cells/mmc	21,9	70,2	5,7	+	+	Methotrexate, Steroids	25	12
AOSD 6	F	67	-	6010 cells/mmc	2,65	45,6	0,45	-	-	Methotrexate, Steroids	10	<9
AOSD 7	F	44	-	8220 cells/mmc	15,9	77,7	5,8	-	-	-	17	<9
AOSD 8	F	45	-	6460 cells/mmc	16,7	67,9	9,9	-	-	-	19	<9
AOSD 9	F	33	-	4820 cells/mmc	30,5	57,3	7,9	-	+	Steroids, Tocilizumab	18	<9
AOSD 10	F	26	-	-	-	-	-	-	-	Methotrexate, Steroids	-	-
AOSD 11	F	52	-	-	-	-	-	-	-	Methotrexate, Steroids	-	-
AOSD 12	F	17	-	7 x10^9/l	35,7	55,7	8,6	Remission		Canakinumab	-	0,7
AOSD 13	M	50	-	14,9 x10^9/l	5,4	85,9	8,7	+	-	Colchicine, Steroids	0	147
AOSD 14	M	47	-	-	-	2,6	-	+	-	Methotrexate	2	-
FMF 1	M	6	M608I/M694V	7,4 x10^9/l	53	39	4,9	-	+	Colchicine	-	1
FMF 2	F	4	M608I/M694V	8 x10^9/l	54	36	7	-	+	Colchicine	-	6
FMF 3	M	7	on hold *	7,5 x10^9/l	28	60	9,4	-	+	-	-	8

Patient	Sex	Age	Gene Mutations	WBC	%Lympho	%Neutro	%Mono	Joints involvement	Systemic Symptoms	Therapy	ESR	CRP
FMF 4	M	17	-	11 x10^9/l	32	60	5,5	-	+	Colchicine	-	-
FMF 5	M	7	M694V/M694V	7,5 x10^9/l	37,5	51	7,3	-	-	Colchicine	-	1,5
FMF 6	F	3	E148Q/WT	-	-	-	-	-	-	Colchicine	-	1
FMF 7	F	6	on hold *	9,97 x10^9/l	46	45	8,55	-	+	Colchicine	-	24,3
FMF 8	M	2	on hold *	11,5 x10^9/l	70	19,4	10,6	-	+	-	-	82
FMF 9	F	13	on hold *	8,6 x10^9/l	31,4	61,6	7	-	+	Colchicine	-	2
FMF 10	M	69	on hold *	-	-	-	-	Remission		Colchicine	-	-
FMF 11	F	7	M694V/M694V	5,6 x10^9/l	48,3	42,8	8,9	-	+	Colchicine	-	3
FMF 12	M	13	M694V/M694V	6,5 x10^9/l	46,7	49,2	7,7	-	+	Colchicine	-	7
FMF 13	F	11	V726A/E148Q	8,7 x10^9/l	47,2	45,9	6,9	-	+	Colchicine	24	1,7
FMF 14	M	8	V726A/WT	5,5 x10^9/l	52,3	40	7,7	Remission		Colchicine	16	2,2
FMF 15	F	42	M694V/M694V	-	-	-	-	Remission		Colchicine	-	0
FMF 16	M	28	M694V/M694V	-	-	-	-	Remission		Colchicine	-	0
FMF 17	M	13	M694V/M694V	4,6 x10^9/l	45,7	45,6	8,7	Remission		Colchicine	-	2,2
FMF 18	-	-	-	-	-	-	-	-	-	-	-	-
CAPS 1	F	17	D303N/WT	4,9 x10^9/l	6	85,7	8,36	Remission		Canakinumab	3	1
CAPS 2	F	5	Q703K/WT	9,2 x10^9/l	41,3	52,2	6,5	-	+	Colchicine	-	3,8

Clinical characteristics, therapy and laboratory examinations: In red we have indicated the presence, at the moment of the blood sampling, of joints involvement and/or systemic symptoms, together with the values beyond the following reference ranges: White Blood Cells (WBC): 4500-8500 cells/mmc or 4-11 x10^9 cells/l (Neutrophils: 40-75%; Monocytes: 2-10%; Lymphocytes 25-55%); Erthyrocyte Sedimentation Rate (ESR): Men under 50 years old: < 15 mm/hr, Men over 50 years old: < 20 mm/hr, Women under 50 years old: < 20 mm/hr, Women over 50 years old: < 30 mm/hr, Children (newborn to puberty): 3-13 mm/hr; C Reactive Protein (CRP): 0-0.5 mg/dl.
* Patients pending the results of the genetic test, with clinical diagnosisof FMF.

3.2 Chemicals

Dextran, Lipopolysaccharide (LPS), adenosine triphosphate (ATP) and z-Val-Ala-Asp fluoromethyl ketone (YVAD, Caspase-1 inhibitor) were purchased from Sigma-Aldrich; Nigericin (Invivogen, ref. tlrl-nig); TcdB (ABCAM, ref. 124-001); LFN-PrgI was produced by IBCP (Institut de Biologie et Chimie des protéines - Lyon) and used associated to PA (Calbiochem, ref. 176905-100UG); LPS-free recombinant tag–free human S100A12 was expressed in E. coli and produced by the Department of Pediatric Rheumatology and Immunology Laboratories of Münster University.

3.3 Isolation of human polymorphonucleates (PMNs) and treatments

PMNs from patients and controls were obtained from 4 mL of heparinised venous blood. Blood was diluted in PBS (without Ca^{2+} and Mg^{2+}), and applied on Lympholite Human (Euroclone) for a density gradient centrifugation. Granulocytes were separated from red blood cells (RBCs) by Dextran and residual RBCs were lysed with ice cold bi-distilled water. Obtained PMNs were resuspended in RPMI 1640 medium (supplemented with 10% FCS and 1% penicillin/streptomycin) and plated with a density of $1x10^6$ cells/mL. After 1 hours of stay at 37°C in 5% CO_2, cells were pre-treated with the YVAD caspase-1 inhibitor (30 µM for 30 minutes) and then activated with 10 ng/mL of LPS for 4 hours. During the last 30 minutes of LPS stimulation, ATP (5 mM) was added. In other experiments PMNs were treated with S100A12 (10 µg/ml) for 4 hours, with or without ATP addition during the last 30 minutes.

3.4 Cell surface staining for CD11b and CD66b

PMNs surface expression levels of CD11b and CD66b were evaluated by immune-staining and flow cytometry. After stimulation leukocytes were stained with PhycoErythrin-labeled anti-CD11b mAb (1:11 dilution) (Miltenyi Biotec) or FITC-labeled anti-CD66b mAb (ready to use) (BD Pharmingen™) for 30 minutes on ice. After washing with PBS, a minimum of 10000 gated PMNs (forward scatter versus side scatter) were analyzed using a CyFlow Cube 8 PARTEC or a BD LSR II flow cytometer and the FlowJo V. 10.0.7 software.

3.5 Measurement of intracellular ROS production

General Oxidative Stress Indicator 5 - (and - 6) – chloromethyl - 2', 7' – dichloro dihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) (Life Technologies), was added after LPS stimulation (10 µM for 30 minutes at 37°C). After washing with PBS, a minimum of 10000 gated PMNs (forward scatter versus side scatter) were analyzed using a CyFlow Cube 8 PARTEC or a BD LSR II flow cytometer and the FlowJo V. 10.0.7 software.

3.6 Evaluation of apoptosis

Isolated PMNs were treated with LPS (anti-apoptotic stimulus) and incubated at 37°C in 5% CO_2 for 20 hours, before staining with annexin V and 7-aminoactinomycin D (7-AAD) from the PE Annexin V Apoptosis Detection kit I (BD Biosciences). A minimum of 10000 gated PMNs (forward scatter versus side scatter) were analyzed using a CyFlow Cube 8 PARTEC or a BD LSR II flow cytometer and the FlowJo V. 10.0.7 software.

3.7 Measurement of S100A12 levels in PMNs supernatant

S100A12 content in PMNs supernatants was determined using a human Luminex assay by the Department of Pediatric Rheumatology and Immunology Laboratories of Münster University.

3.8 IL-1β intracellular staining on whole blood

White blood cells (WBC) were obtained from 4 mL of heparinised venous blood from a healthy subject. RBCs were immediately lysed with pre-wormed lysis buffer (NH_4Cl 1.5 M, $KHCO_3$ 0.1 M, EDTA 0.5 M), added to the blood sample (1:10) for 5 minutes at 37°C. After washing with PBS, WBC were counted through the Accuri C6 flow cytometer and plated with a density of $1x10^6$ cells/mLin RPMI 1640 medium (supplemented with 10% FCS and 1% penicillin/streptomycin). WBC cells were cultured for 1 hour and then stimulated with LPS (10 ng/ml) or LPS + ATP (5 mM). After two hours, protein transport was blocked with Golgi Plug (BD Biosciences) for additional two hours. Cells were stained with anti-CD3 (Beckman,#A94680), anti CD-14 (Beckman,#PN IM2707U), anti CD-20 (BD, #555623), anti CD-16 (BD, #558122) antibodies for cellular phenotyping. After fixation and permeabilization (BD, #554722), cells were stained with the antibody against IL-1β (BD, #340515). Stained WBC were captured by flow cytometry with a BD LSR Fortessa flow cytometer and data were analyzed with FlowJo V. 10.0.7 software.

3.9 Isolation of ultra pure PMNs

PMNs were obtained from 4 mL of heparinised venous blood. As previously described, blood was diluted in PBS (without Ca^{2+} and Mg^{2+}), and applied on Lympholite Human (Euroclone) for a density gradient centrifugation. Granulocytes were separated from

RBCs by Dextran and residual RBCs were lysed with ice cold bi-distilled water. The obtained not pure PMNs were washed with the Automacs solution (PBS 1X, FCS 0.5%, EDTA 2Mm) and negatively selected through the monocytes depletion by means of HLA-DR Human MicroBeads (eBioscience, ref. 13-9956) and the Automacs Pro Separator instrument (Miltenyi Biotec). The obtained ultra pure PMNs (99%) were counted through the Accuri C6 flow cytometer, resuspended in RPMI 1640 medium (supplemented with 10% FCS and 1% penicillin/streptomycin) with a density of $1x10^6$ cells/mL and finally stimulated as already described.

3.10 Isolation of human monocytes and treatments

Monocytes from patients and controls were obtained from 9 mL of heparinised venous blood.

Blood was diluted in PBS (without Ca^{2+} and Mg^{2+}), and applied on a Lymphocyte-separation-medium (Eurobio, ref. CMSMSL01-01) for a density gradient centrifugation. The obtained peripheral blood mononuclear cells were washed with the Automacs solution (PBS 1X, FCS 0.5%, EDTA 2Mm) and monocytes were selected with the CD14 Human MicroBeads (Miltenyi Biotec, ref. 130-050-201) by means of the Automacs Pro Separator instrument (Miltenyi Biotec). The obtained cells were counted through the Accuri C6 flow cytometer and resuspended in RPMI 1640 medium (supplemented with 10% FCS and 1% penicillin/streptomycin) with a density of 50000 cells/mL. LPS (10 ng/ml) was added for 3 hours and to induce the activation of different inflammasome complexes, a different “signal two” was added for additional 1.5 hours: Nigericin (5 µg/ml); ATP (2.5 mM); TcdB (125 ng/ml and 12.5 ng/ml); PA-LFN-PrgI (50 ng/ml).

In other experiments monocytes, with a density of $1x10^6$ cells/ml, were treated with

S100A12 (10 µg/ml) for 4 hours, with or without ATP addition (5 mM) during the last 30 minutes.

3.11 Measurement of IL-1β levels in leukocytes supernatants

IL-1β content in PMNs and monocytes supernatants was determined by ELISA assay (Human IL-1β/IL-1F2 R&D Systems, DuoSet Elisa DY 201).

3.12 Statistical Analysis

Statistical calculations were performed with GraphPad Prism software, version 6.0. Differences between the various conditions were analyzed by Mann-Whitney U test, Wilcoxon's matched pairs test or unpaired student's t test, according to the different cases. Differences were considered statistically significant at P values less than 0.05.

4. Results

4.1 Granulocytes from affected patients don't present different activation state in comparison with healthy ones.

To verify the involvement of granulocytes in the pathogenesis of sJIA and AOSD, we have analyzed the main factors determining neutrophils activation during human infections: expression levels of the surface activation markers CD11b and CD66b, ROS production and apoptosis (134, 135).

PMNs, isolated from healthy donors (HC), sJIA and AOSD patients together with FMF patients considered as disease control, were stimulated with LPS for 4 hours to measure CD11b and CD66b levels by cytometry (Figure 7). The stimulation increased the expression of these factors, as expected, but there are no differences between healthy controls and affected patients.

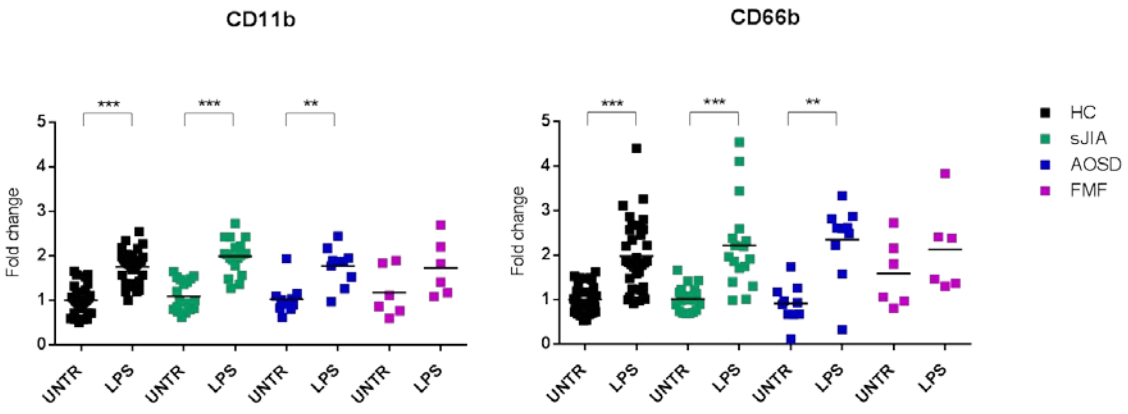

Figure 7. CD11b and CD66b levels at baseline and after LPS stimulation in PMNs purified from healthy controls (HC, n=34), sJIA patients (n=19), AOSD patients (n=10) and FMF patients (n=6). Data are expressed as fold change of the Mean Fluorescence Intensity relatives to the untreated control. Statistical significance was determined using the Mann-Whitney non-parametric U-test (**$p \leq 0.01$, ***$p \leq 0.001$).

Moreover, ROS production and apoptosis levels were analyzed, after 4 hours and 20 hours of LPS stimulation respectively (Figure 8). No significant differences have been observed about ROS production (Figure 8A). LPS reduced the apoptotic cell death, confirming its anti-apoptotic effect, but at baseline PMNs from affected patients don't

manifest a boost viability as originally expected (Figure 8B).

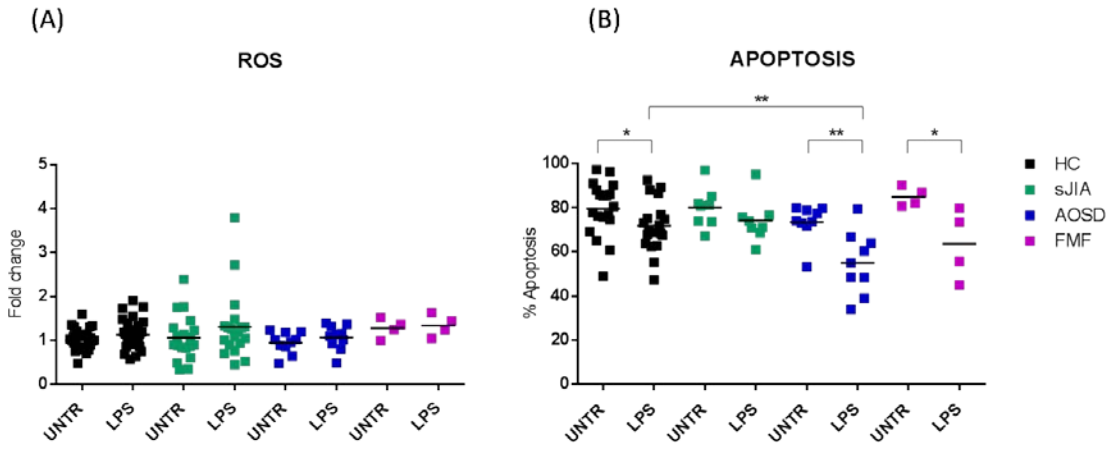

Figure 8. (A) ROS levels at baseline and after 4 hours of LPS stimulation in healthy controls (n=32), sJIA patients (n=19), AOSD patients (n=10) and FMF patients (n=4). Data are expressed as fold change of the Mean Fluorescence Intensity relatives to the untreated control. (B) Apoptosis levels at baseline and after 20 hours of LPS stimulation in healthy controls (n=19), sJIA patients (n=8), AOSD patients (n=9) and FMF patients (n=4). Data are expressed as percentage of annexin V-positive cells. Statistical significance was determined using the Mann-Whitney non-parametric U-test (*$p \leq 0.05$, **$p \leq 0.01$).

4.2 Increased IL-1β production in sJIA and AOSD PMNs, dependent on caspase-1 activity.

IL-1β is the pivotal alarm cytokine during inflammation and the clinical improvements in sJIA and AOSD patients after treatment with IL-1 inhibitors are the major proof of the IL-1β involvement in the pathogenesis of these disorders, but the principal cellular source of this cytokine in these syndromes is still unknown (118, 136). Therefore, we verified if granulocytes from affected patients could present differences in IL-1β production compared with healthy ones, measuring the amount of IL-1β in PMNs-derived supernatants.

After purification, cells were treated with LPS (the first signal in the two steps-activation pathway) or LPS+ATP (signal 1 + signal 2) to induce granulocytes activation. Supernatants were analyzed by ELISA assay. The results have shown a significant higher release of the cytokine after stimulation in sJIA and AOSD patients (Figure 9A),

and their higher sensitivity to the treatment (Figure 9B).

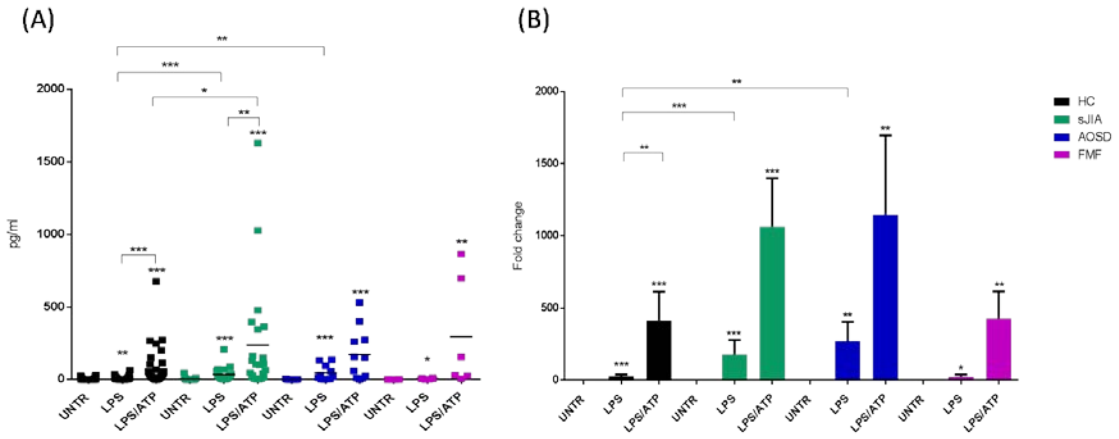

Figure 9. IL-1β levels in PMNs supernatant at baseline and after LPS / LPS+ATP stimulation in healthy controls (n=36), sJIA patients (n=21), AOSD patients (n=11) and FMF patients (n=6). (A) Data are expressed as pg/ml. (B) Data are expressed as the fold change of IL-1β concentration relatives to the untreated sample ± s.e.m.
Statistical significance was determined using the Mann-Whitney non-parametric U-test
(*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

To assess if the release of IL-1β was dependent on the caspase-1 enzymatic activity, we have also pre-treated purified PMNs with YVAD, a selective caspase-1 inhibitor. IL-1β levels in PMNs supernatants are lower after YVAD pre-treatment, indicating the correlation with caspase-1 activity (Figure 10).

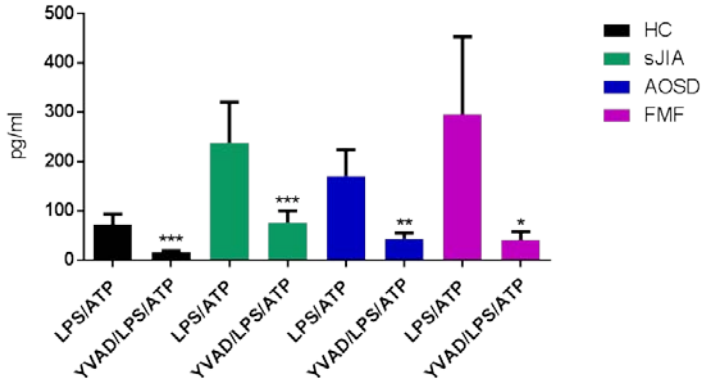

Figure 10. IL-1β levels in PMNs supernatant after LPS+ATP stimulation with or without the YVAD pre-treatment, in healthy controls (n=36), sJIA patients (n=21), AOSD patients (n=11) and FMF patients (n=6). Data are expressed as mean ± s.e.m. Statistical significance was determined using the Wilcoxon non-parametric U-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

4.3 High experimental variability

Comparing the results obtained in these different experiments, purifying PMNs from blood samples of the same subject (patient or healthy control) collected and analyzed in different moments, we noticed a high variability between the different experiments, either for the same healthy donor or the same sJIA patient even if in the same phase of the disease (Figure 11, Table 4).

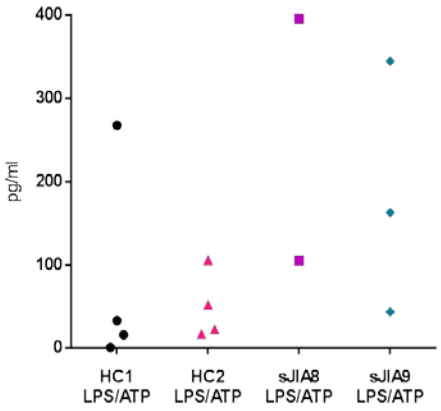

Figure 11. IL-1β concentrations in PMNs-derived supernatants after LPS+ATP stimulation in two HC and two sJIA patients, analyzed more than one time, in different moments.

Table 4. Clinical and laboratory features relative to the Figure 11

Patient	WBC ($x10^9$/l)	Joints Involvement	Systemic symptoms	Therapy	ESR	CRP	Disease activity
sJIA8	11,3	-	-	Methotrexate, Steroids	2	0	INACTIVE
	11	-	-	Methotrexate, Steroids	-	0	INACTIVE
sJIA9	7,6	+	-	Methotrexate	0	0	ACTIVE
	5,06	+	-	Methotrexate	2	0	ACTIVE
	6,83	+	-	Methotrexate	2	0	ACTIVE

4.4 IL-1β levels correlate with the percentage of contaminating Monocytes identified in the population of purified PMNs

To explain the above mentioned variability, we have supposed the presence of contaminating monocytes in the purified granulocytes population. Therefore, besides the percentage of PMNs after staining with an anti-CD66b antibody, we also determined the percentage of possible contaminating monocytes after staining with an anti-CD14 antibody. In the Figure 12 we have reported two different PMNs purifications, obtained using the same purification procedure, to highlight the variability obtained in different experiments on the percentage of purified PMNs and contaminating monocytes. Thus, in some experiment we obtained a good PMNs purity without contaminating monocytes (Figure 12A), but in others there were also monocytes, whose amount was different for each experiment (Figure 12B).

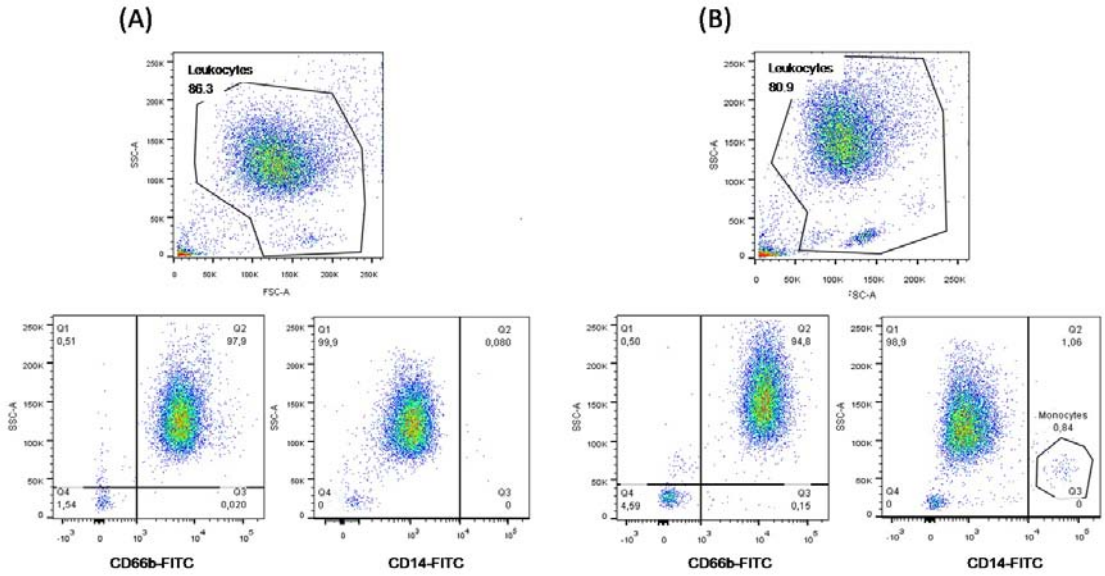

Figure 12. FACS analysis of two different PMNs purifications, obtained using the same protocol. (A) 97,9% of $CD66b^+$ cells (granulocytes) and 0% of $CD14^+$ cells (monocytes). (B) 94.8% of $CD66b^+$ cells and 0,84% of $CD14^+$ cells.

Then, we calculated the correlation between the concentration of IL-1β released after LPS+ATP stimulation and the percentage of contaminating monocytes detected in the same stimulated sample. This analysis indicates that rising IL-1β levels correspond to rising percentages of contaminating monocytes, in a significative measure in HC and sJIA. The slope of the curves doesn't indicate significant differences in IL-1β release between the three groups (Figure 13).

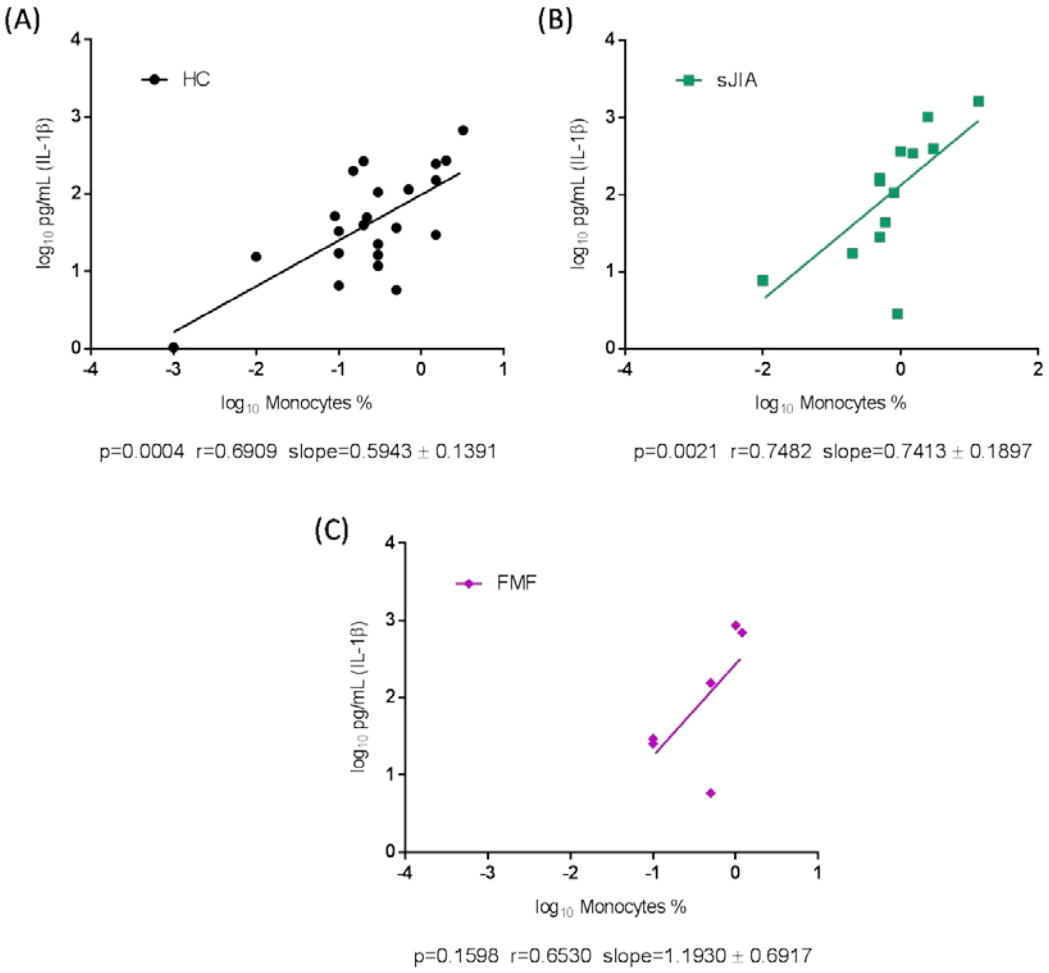

Figure 13. Statistical correlation between IL-1β levels measured into the PMNs supernatant after LPS+ATP stimulation and the percentage of $CD14^+$ cells relieved in the granulocytes purified population used for the same stimulation. Data are expressed as log_{10} of IL-1β concentrations and monocytes percentages.

4.5 The 0,2% of contaminating monocytes is enough to enhance the release of IL-1β

To define how many monocytes are necessary to interfere with the evaluation of PMNs IL-1β release, we reproduced the monocytes contamination of the previous experiments, purifying PMNs from a healthy donor and adding, in an arbitrary way, different percentages of monocytes to the purified population (137). We determined that ultra pure PMNs are sensitive to the LPS+ATP treatment, which enhaces the release of IL-1β, but only the 0,2% of contaminating monocytes interferes in a significant measure with our assay (Figure 14).

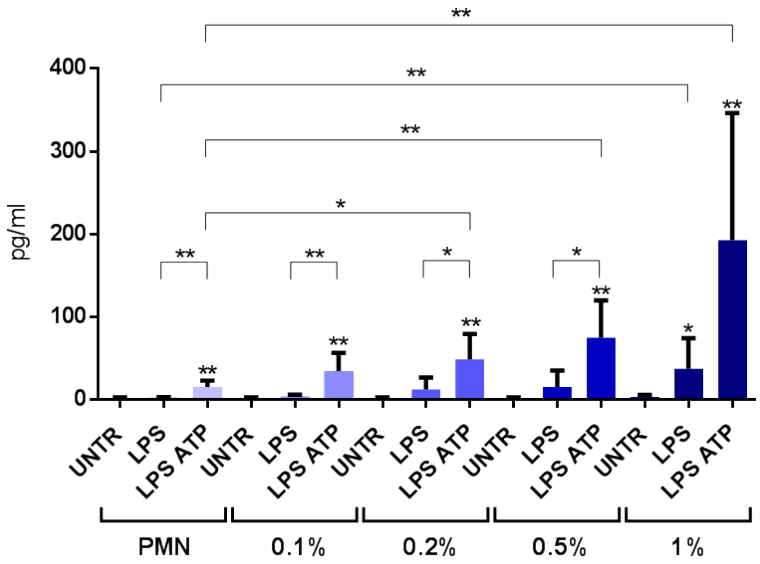

Figure 14. IL-1β levels measured in the supernatant of 99% pure healthy PMNs (PMN in the figure) and the same healthy PMNs contaminated with increasing percentages of healthy monocytes (0,1%; 0,2%; 0,5%; 1%). Cells supernatant has been collected at baseline and after LPS / LPS+ATP treatment. Data are representative of three independent experiments and are expressed as mean ± s.e.m. Statistical significance was determined using the Mann-Whitney non-parametric U-test (*$p \leq 0.05$, **$p \leq 0.01$).

4.6 Granulocytes from sJIA and AOSD patients don’t produce higher IL-1β levels

Taking into account the last described assessments, we have reanalyzed the previous results, just considering granulocytes purifications with less than the 0,2% of contaminating monocytes, which correspond in our experiments to more than the 97% of CD66b positive cells. Patients PMNs don’t present anymore the higher cytokine release observed in the Figure 9A (Figure 15A), and neither the higher sensitivity to the treatment (Figure 15B), confirming that the previous results were just an artefact of the presence of monocytes. Moreover, the IL-1β release results dependent on the caspese-1 activity just in the healthy controls, probably indicating also the involvement of other granulocytes proteases, different from caspase-1, as previously described. Anyway the very few levels of the detected cytokine don’t lead to a definite conclusion (Figure 15C).

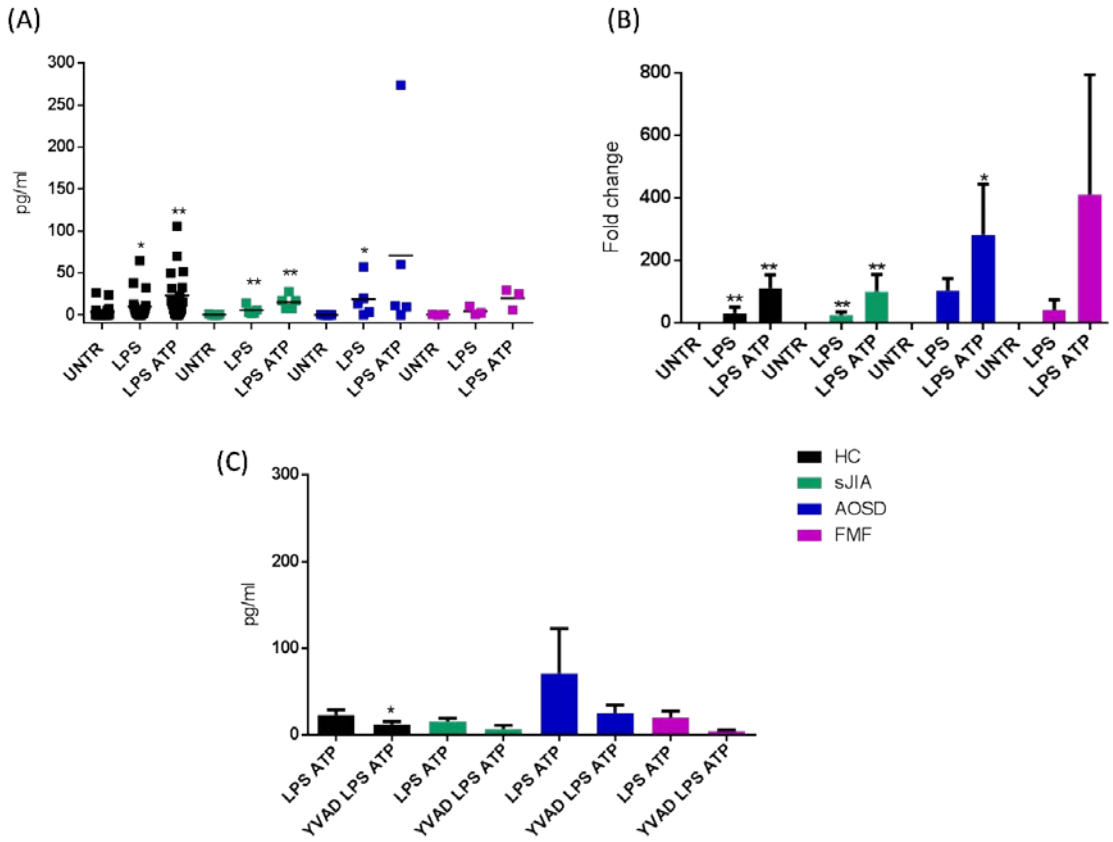

Figure 15. IL-1β levels in PMNs-derived supernatant in HC (n=20), sJIA patients (n=5), AOSD patients (n=5) and FMF patients (n=3). (A) Results obtained at baseline and after LPS / LPS+ATP stimulation. Data are expressed as pg/ml. Statistical significance was determined using the Mann-Whitney non-parametric U-test. (B) Results obtained at baseline and after LPS / LPS+ATP stimulation. Data are expressed as the fold change of IL-1β concentration relatives to the untreated sample ± s.e.m. Statistical significance was determined using the Mann-Whitney non-parametric U-test. (C) Results obtained after LPS+ATP stimulation with or without YVAD pre-treatment. Data are expressed as mean ± s.e.m. Statistical significance was determined using the Wilcoxon non-parametric U-test (*$p \leq 0.05$, **$p \leq 0.01$).

4.7 Monocytes represent the best candidates for IL-1β production

To compare the capability of IL-1β production among the different kind of white blood cells, we made an intracellular staining on whole blood from a healthy donor. We verified that monocytes represent very good producers of IL-1β, differently from PMNs, which produce a few amount of the cytokine, and lymphocytes which determine an IL-1β production very close to the zero (Figure 16) (137).

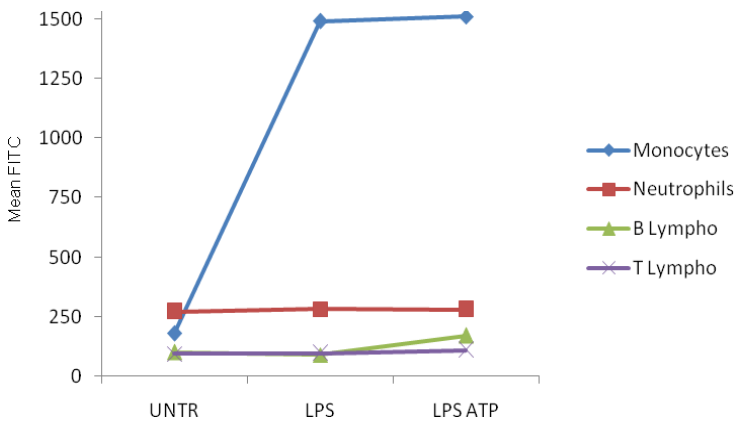

Figure 16. Intracellular cytokine production by $CD14^+$ monocytes, $CD16^+$ neutrophils, $CD3^+$ T-lymphocytes and $CD20^+$ B-lymphocytes. White blood cells were cultured in LPS or LPS+ATP for 2 hours, then brefaldin A was added for additional 2 hours. Data are expressed as MFI and represent one single experiment reproducing literature results[137].

Furthermore, it is possible to easily induce a good release of IL-1β in healthy monocytes stimulating a few number of cells (50.000 cells/ml), after activation of different inflammasome complexes. In our experiments we activated NLRP3 through ATP and Nigericin stimulations, NLRC4 through PA-PRGI stimulation and Pyrin through TcdB stimulation (Figure 17).

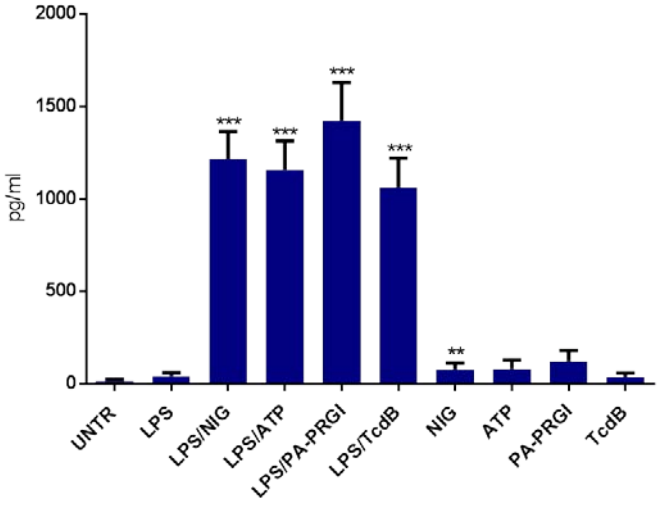

Figure 17. IL-1β levels in healthy monocytes-derived supernatant collected at baseline and after LPS / LPS+Nigericin (NIG) / LPS+ATP / LPS+PA-PRGI / LPS+TcdB stimulation, to induce the activation of different inflammasome complexes. Data are representative of three independent experiments and are expressed as mean ± s.e.m. Statistical significance was determined using the Mann-Whitney non-parametric U-test ($^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

4.8 Granulocytes from sJIA patients produce higher levels of S100A12

S100A12 represents another factor with pro-inflammatory properties. It is a calcium-binding protein mainly expressed in granulocytes, useful as biomarker for monitoring the disease activity thanks to its elevated serum levels in affected patients (128, 129). S100A12 function is related to the inflammatory response mediated by neutrophils. Therefore, since PMNs were found ineffective in IL-1β production, we decided to evaluate the release of S100A12, comparing affected patients and healthy donors, to verify eventual anomalies. We measured S100A12 levels in granulocytes supernatants, at baseline and after LPS stimulation.

sJIA patients' granulocytes seem more sensitive to the stimulation, because they release a significantly higher amount of this protein. About AOSD, we have analyzed just one patient, so it is too early to make conclusions (Figure 18).

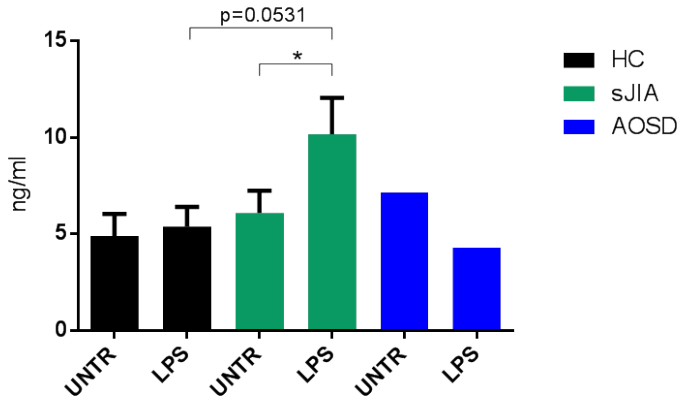

Figure 18. S100A12 levels in PMNs-derived supernatants after LPS stimulation in HC (n=11), sJIA patients (n=15) and AOSD patients (n=1). Data are expressed as mean ± s.e.m. Statistical significance was determined using the Mann-Whitney non-parametric U-test (*$p \leq 0.05$).

4.9 Inflammasome complex activation analysis in monocytes of monogenic and polygenic autoinflammatory disorders

To assess the functional phenotype of monocytes' inflammasome complexes in autoinflammatory disorders, we have analyzed IL-1β release from monocytes purified from FMF, CAPS, sJIA and AOSD patients, in comparison with healthy donors.

FMF patients have revealed the hyper-activation of Pyrin, also with lower concentrations of TcdB (12 ng/ml) or with TcdB alone, without the pre-treatment with LPS (Figure 19A); CAPS monocytes manifested different behaviour according to the mutation in the *NLRP3* gene. We enrolled one patient presenting the mutation Q703K and one patient carrying the D303N mutation. CAPS monocytes didn't show particular differences with the healthy donors after the different stimulations, but it has been confirmed the constitutive activation of the NLRP3 inflammasome complex in the D303N mutated monocytes, by the release of the cytokine just after the exposure to the signal 1 (LPS) without the need of the inflammasome activating signal 2 (Figure 19B). The Q703K mutated monocytes, instead, have responded the same way as the healthy controls.

sJIA derived monocytes have shown a significant higher IL-1β release after LPS+Nigericin stimulation, probably indicating an involvement of the NLRP3 inflammasome, not confirmed by the LPS+ATP treatment, which should determines the same result (Figure 19C). AOSD patients didn't show significant differences with the healthy controls (Figure 19D).

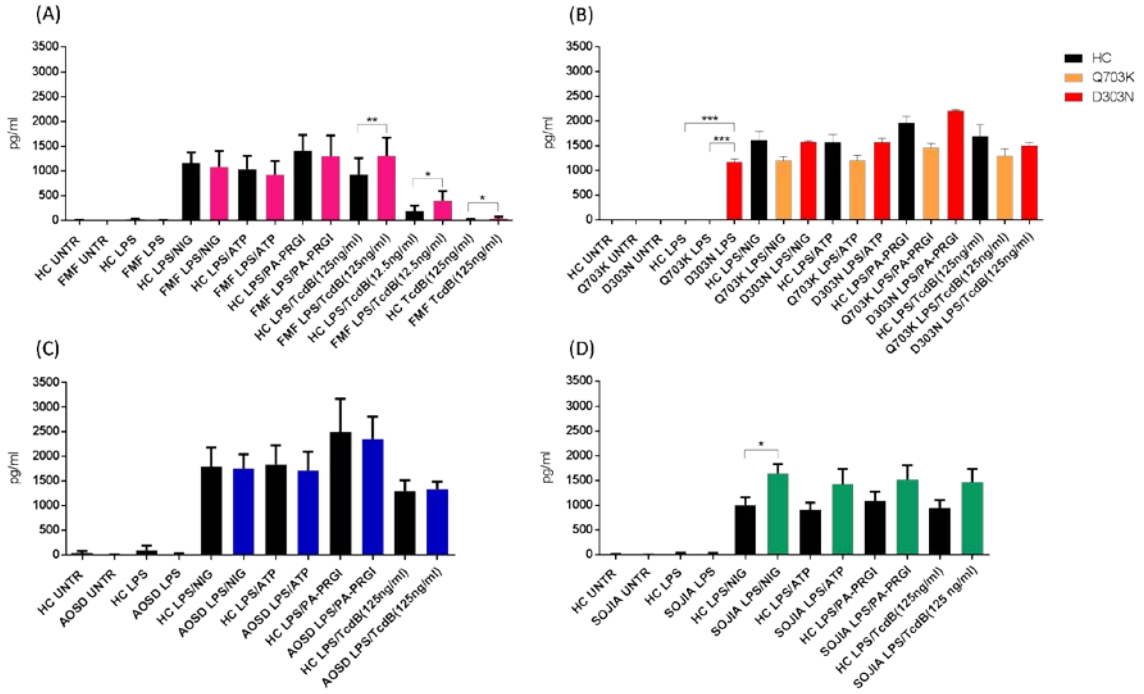

Figure 19. IL-1β levels in monocytes-derived supernatant collected at baseline and after LPS / LPS+Nigericin / LPS+ATP / LPS+PA-PRGI / LPS+TcdB stimulation. (A) Results obtained in HC (n=12) and FMF patients (n=12). (B) Results obtained in HC (n=2), Q703K patient (n=1) and D303N patient (n=1). (C) Results obtained in HC (n=13) and sJIA patients (n=13). (D) Results obtained in HC (n=4) and AOSD patients (n=4). Data are expressed as mean ± s.e.m. Statistical significance was determined using the Wilcoxon non-parametric U-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

4.10 IL-1B levels in PMNs and Monocytes supernatants after S100A12 stimulation

To evaluate the effects of S100A12 on granulocytes and monocytes, we stimulated purified white blood cells with the S100A12 recombinant protein and measured the release of IL-1β after 4 hours. PMNs resulted also in this case, the worst producer of this cytokine, in term of pg/ml, in comparison with monocytes and we didn't detect differences between sJIA patients and healthy donors. Monocytes, indeed, are stimulated very well after S100A12 exposure (Figure 20).

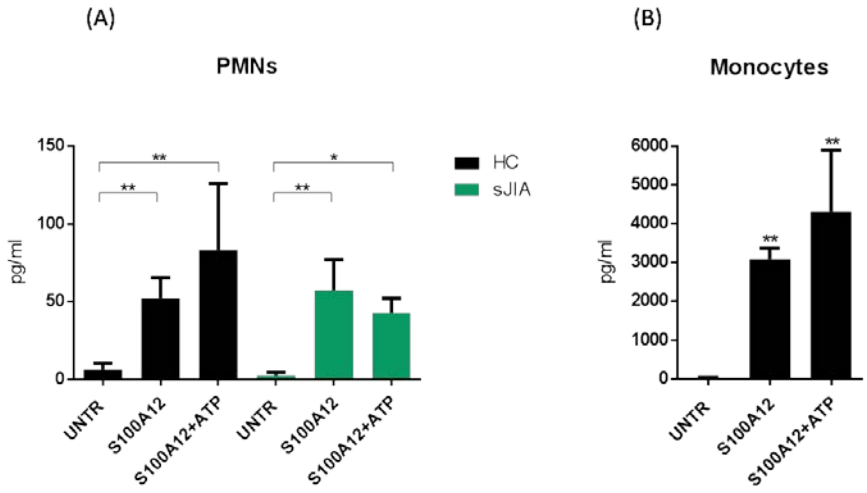

Figure 20. (A) IL-1β levels in PMNs-derived supernatant collected at baseline and after S100A12 / S100A12+ATP stimulation in HC (n=6) and sJIA patients (n=5). Data are expressed as mean ± s.e.m. Statistical significance was determined using the MannWhitney non-parametric U-test. (B) IL-1β levels in monocytes-derived supernatant collected at baseline and after S100A12 / S100A12+ATP stimulation in HC (n=3). Data are expressed as mean ± s.e.m. Statistical significance was determined using the unpaired Student's T-test (*$p \leq 0.05$, **$p \leq 0.01$).

5. Discussion

This study was undertaken to investigate role and function of PMNs in the pathogenesis of sJIA and AOSD syndromes. In particular, one of the major objectives of this work was to identify the principal IL-1β cellular source in affected patients. It is clear the important role of this cytokine in these syndromes, since its elevated serum levels in affected patients and, above all, the therapeutic efficacy of IL-1 blockers agents (112, 114-116). Previous studies excluded sJIA monocytes because, once stimulated, they produced even lower IL-1β levels in comparison with healthy monocytes (120, 121). Besides IL-1β, serum from affected patients also presents high S100A12 levels (128, 129).

All these factors, together with the typical patients neutrophilia, lead us to hypothesize granulocytes involvement in the pathogenesis of sJIA and AOSD, also as potential IL-1β cellular source.

In our experiments PMNs purified from affected patients didn't present a hyper-active state as expected, because surface activation markers levels (including CD11b/CD18 and CD66b), intracellular ROS production and apoptosis levels seem comparable with healthy PMNs.

In order to evaluate granulocytes IL-1β production we applied the Ficoll density gradient centrifugation isolation method, considered a good option for PMNs purification, with purity of isolates around the 96% ± 2% (138) and for this reason used in different works (139, 140). However, this purification procedure seems to be not appropiated for our purposes, because just the 0.2% of contaminating monocytes is enough to determine enhanced IL-1β production. Therefore, this purification protocol could be probably more adequate for different kind of assays such as cytometric analysis, since in that case the cellular population can be selected with a specific gate. Anyway, considering just the experiments with less than the 0.2% of contaminating

monocytes, granulocytes remain bad IL-1β producers in either healthy donors or affected patients. They release very low concentrations of IL-1β after stimulation, without significant differences between the analyzed groups. Furthermore, our results indicate that PMNs from just one AOSD patient, after stimulation, manifested a higher IL-1β release (around 270 pg/ml) in comparison with the other analyzed patients (Figure 15). This subject was in inactive disease phase and without a specific therapy to modulate the inflammatory response (no biologics, steroids or Methotrexate). On the contrary, PMNs from another AOSD patient, in the active disease phase, but treated with steroids and Methotrexate, didn't respond to the stimulation, indicating that probably the pharmacological treatment could interfere with the PMNs inflammatory response.

This observation has not been confirmed by the results obtained in sJIA patients due to the lack of responses to the stimulation, even in one active and not treated patient.

In order to better clarify this aspect, it will be necessary to recruit more patients and separate them according to the therapy.

Moreover, the release of IL-1β mediated by PMNs seems not dependent on the caspase-1 activity in affected patients, since the pre-treatment with a selective caspase-1 inhibitor didn't reduce in a significant manner the release of the protein. It is coherent with the fact that IL-1β activation in granulocytes is not mediated just by caspase-1, but also other proteases are involved such as PR3, NE or cathepsin G (127). But, also in this case, the number of patients analyzed for this aspect is probably not enough.

We acknowledge the limitations of our small samples size. Above all this study has interested rare syndromes and presented objective difficulties about patients recruitment, but we have encountered technical difficulties, too with the PMNs purification method. Therefore a further study involving larger cohorts of patients and

healthy donors, with use of different technical approaches, is being planned.

Driven by the results obtained from contaminating monocytes detected in our experiments on PMNs and considering the aspect that monocytes release very high levels of IL-1β after stimulation (120, 137), we decided to focus our attention also to monocytes activation, not only in sJIA and AOSD, but also in monogenic autoinflammatory disorders.

In the second part of this work we have analyzed the functional phenotype in monocytes' inflammasome complexes of monogenic and polygenic autoinflammatory disorders. Comparing the results obtained from the activation of three different inflammasome complexes (NLRP3, NLRC4 and Pyrin), our results confirm the involvement of a specific inflammasome type in monogenic disorders. Indeed, FMF patients presented hyper-active monocytes than healthy cells, after Pyrin activation, as we expected, since the mutations usually interest the gene encoding for Pyrin. The effects of Pyrin activation after TcdB treatment have been observed, until now, just in mouse BMDM (87). In our experiments, we have observed that the same activation mechanism also works in humans and the *MEFV* gene mutations amplify the Pyrin mediated inflammatory response. This response was different according to the kind of stimulation and probably, in our case, it was not related to the NLRP3 inflammasome activity indicating, as suggested by Omenetti el al., that Pyrin could have different triggers (88). The major part of the analyzed patients were treated with Colchicine and the Pyrin activation was independent from the disease activity, because also inactive patients have responded to the stimulation.

A different monocytic response, according to the *MEFV* gene mutation, is possible. Indeed, patients carryng M694V homozygotes mutation, known to be associated to severe disease phenotype, showed a higher response in comparison with the low-

penetrance mutations E148Q and V726A (141). But in our cohort, the number of patients for each mutation is too small and there are still patients pending the results of genetic tests, which can't be included in any group. Therefore, for the moment, we can just speculate on the observed trend, that has to be confirmed extending the number of cases.

Moreover, in agreement with other studies (76, 142), monocytes from CAPS patient carrying D303N mutation showed enhanced IL-1β release after LPS stimulation, confirming that in this syndrome the inflammasome NLRP3 is already active. This patient was in complete remission at the moment of the sampling and treated with canakinumab, indicating that pharmacological treatment and disease activity seem not to influence the response.

The Q703K mutated monocytes, instead, presented similar characteristics with healthy ones, confirming the weak clinical and functional effect of this variant (143).

On the contrary, sJIA and AOSD monocytes didn't manifest the involvement of a specific kind of inflammasome. Indeed, AOSD monocytes have not shown differences with healthy monocytes while sJIA monocytes, presented just a trend, with higher IL-1β release after all the different stimulations, but not statistically significant in comparison with healthy monocytes results.

An important result has been obtained from the evaluation of S100A12 in PMNs supernatant. sJIA patients seem more sensitive to LPS stimulation, because they release higher levels of this protein than healthy PMNs. This difference seems independent on the disease activity and therapy, because PMNs from inactive patients also release higher levels of S100A12, even from patients treated with Methotrexate and steroids. This result, together with the typical patients neutrophilia, could explain the high serum S100A12 levels observed in affected patients (128, 129). In a previous study it has been

proven that S100A12 can have a stronger effect on monocytes respect LPS, concerning the expression of some genes such as IL-15RA, IL-7 and IL-18 (144). In our experiments, PMNs still remain bad IL-1β producers, not just after LPS stimulation but also after S100A12 stimulation, and no differences have been observed between healthy and sJIA granulocytes in this case either. Monocytes from healthy donors, instead, produce very high levels of IL-1β when treated with S100A12. This last result leaves hypothesize that IL-1β production in sJIA and AOSD could be still associated to monocytes, but with a granulocytes upstream action, which could mediate a physiological monocytes activation through the release of high levels of S100A12.

6. Conclusions and Future perspectives

In conclusion, our findings exclude the original hypothesis of a different PMNs activation state in sJIA and AOSD. These cells, obtained from affected patients, manifest a behaviour strictly comparable to healthy granulocytes and, above all, they don't represent the major IL-1β producers in these syndromes. But, an important difference has been observed in the S100A12 release. Therefore, before excluding the PMNs pathogenic role, one can suppose they don't intervene directly with the production of IL-1β, as originally hypothesized, but they could mediate their action through the production of S100A12, whose action fall, in turn, on a different type of innate immune cells, such as monocytes which are much more capable in the IL-1β production and release, as demonstrated both in healthy donors and patients affected by autoinflammatory syndromes.

Our small samples size, just leads to new hypothesis and to intriguing questions for future hypothesis-driven studies, addressed for example to the analysis of S100A12 stimulation effects on monocytes purified from sJIA and AOSD patients, or to the most interesting evaluation of the S100A12 production involvement in the regulation of monocytes activation. These results might contribute to better understand the sJIA and AOSD pathogenesis, since they could allow another step forward in the studies on PMNs potential role in these disorders.

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