Μεταπτυχιακό Πρόγραμμα Σπουδών "Μοριακή Βάση των Νοσημάτων του Ανθρώπου"

Graduate Program "The Molecular Basis of Human Disease"

Εργασία Μεταπτυχιακού Τίτλου Ειδίκευσης

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Ε.Κοντάκη

Επιστημονική Επιτροπή:

Κ.Παπαδάκης, MD

Π.Σιδηρόπουλος, MD

Ερευνητικό Εργαστήριο Αυτοανοσίας και Φλεγμονής

Τμήμα Ιατρικής

Πανεπιστήμιο Κρήτης

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Master Thesis Degree

NALP3 inflammasome and its role in inflammatory bowel diseases (IBD)

E.Kontaki

Scientific Committee:

K.Papadakis, MD

P.Sidiropoulos, MD

Research Laboratory of Autoimmunity and Inflammation

Department of Medicine

University of Crete

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Περίληψη

Η NALP3 πρωτεΐνη ανήκει σε μία νέα οικογένεια ενδοκυττάριων υποδοχέων, τους αποκαλούμενους NLRs (NOD-like receptors). Ως απάντηση σε ένα σύνολο ερεθισμάτων, τόσο εξωγενών όσο και ενδογενών, η NALP3 πρωτεΐνη σχηματίζει ένα κυτταροπλασματικό σύμπλοκο πρωτεϊνών, το ονομαζόμενο NALP3 φλεγμονόσωμα, το οποίο ενεργοποιεί την προφλεγμονώδη κασπάση-1 και η οποία με τη σειρά της καταλύει την ωρίμανση των προφλεγμονωδών κυτταροκινών, ιντερλευκινης 1β και 18. Στη νόσο του Crohn, η παραγωγή αυτών των κυτταροκινών είναι αυξημένη, ενώ πολυμορφισμοί της NOD2 πρωτεΐνης, μέλος της οικογένειας των NLRs, προδιαθέτουν για την ασθένεια. Πρόσφατα, πολυμορφισμοί του γονιδίου NALP3 καθώς και συνδυασμός ισομορφών του NALP3 και του CARDINAL - συστατικών του NALP3 - έχουν αναγνωριστεί ως παράγοντες κινδύνου για τη νόσο του Crohn. Τα παραπάνω δεδομένα υποδεικνύουν τη συμμετοχή του NALP3 φλεγμονοσώματος στην παθογένεια της νόσου. Στην παρούσα μελέτη βρέθηκε ότι η ενεργοποίηση του φλεγμονοσώματος χρησιμοποιώντας ως διεγέρτες το LPS και ATP δε διαφέρει μεταξύ υγιών εθελοντών και ασθενών με νόσο του Crohn, ενώ η ενεργοποίηση αυτού με τη χρήση MDP ήταν περισσότερο έκδηλη στους ασθενείς. Μεγαλύτερο ενδιαφέρον παρουσίαζει το εύρημα ότι η ενεργός κασπάση-1 εκφράζεται σε εντερικό ιστό ασθενών με νόσο του Crohn. Επιπλέον, ποντίκια με γενετική απαλοιφή του NALP3 γονιδίου είναι περισσότερο επιρρεπή στην πειραματική DSS διαμεσολαβούμενη κολίτιδα. Παρόλα αυτά, ο μηχανισμός που προκέλεσε την εντερική φλεγμονή χρίζει μεγαλύτερης διερεύνησης.

Summary

NALP3 protein belongs to the NLR (NOD-like receptor) subfamily, a newly identified cytosolic pattern recognition receptor (PRR) protein group. In response to various exogenous and endogenous danger signals, NALP3 forms a multiprotein platform, called NALP3 inflammasome, which activates the inflammatory caspase-1 leading to maturation of IL-1β and IL-18. In Crohn's disease (CD) the production of these two cytokines is elevated, while *NOD2*, another member of the NLR subfamily, is the main susceptibility locus for the disease. More interestingly, NALP3 polymorphisms as well as combined variants of NALP3 and CARDINAL- components of NALP3 inflammasome- have recently been identified as risk factors for developing CD. These data indicate the involvement of NALP3 inflammasome in the pathogenesis of CD. Here, we showed that NALP3 inflammasome activation upon LPS plus ATP has no difference between healthy controls and CD patients, while activation upon MDP was more evident in CD patients. Intriguingly, CD colonic tissue expressed bioactive caspase-1 contrary to normal colonic specimens. Furthermore, we found that mice lacking NALP3 gene were highly susceptible to DSS colitis, although the mechanism driving colonic inflammation was poorly understood.

1. Introduction

1.A. NALP3 inflammasome

1.A.1. NLR family

Innate immunity relies on the fast recognition of structural molecular patterns by a limited set of non-clonal germline encoded receptors, called "pattern recognition receptors" (PRRs). These molecular patterns comprise a heterogenous group of molecules, including lipids and nucleic acids present in pathogens (pathogen associated molecular patterns, so called PAMPs), as well as endogenous danger signals (danger associated molecular patterns, DAMPs), released into the extracellular space by cellular damage. Concerning the receptors, three major classes have been identified: (a) the Toll-like receptors (TLRs), which are transmembrane proteins sensing PAMPs on the cell surface or in endosomes, (b) the retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), which are cytosolic helicases, mediating antiviral immunity, and (c) the NOD-like receptors (NLRs), which are intracellular cytoplasmic sensors [1].

The NLR family is composed of 22 family members in humans, whereas the murine genome contains at least 34 NLR-encoding genes [2, 3]. Based on functional features, NLR family is seperared into distinct subfamilies as shown in Fig.1 [4].

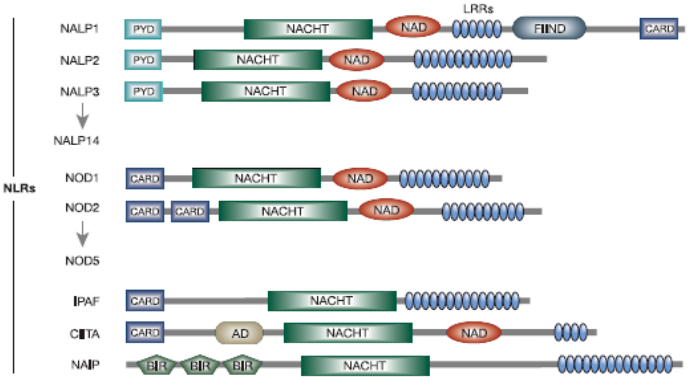

Fig.1 NLR family

CIITA (MHC class II transactivator) is the founding member of the family and regulates the transcription of MHC II genes. The NOD group, consisting of five members, activates the transcription factor NF-kB, via the recruitment of a serine/threonine kinase, called RICK. IPAF (inflammatory protease activating factor) and NAIP (neuronal apoptosis inhibitory protein), which are grouped together, seem to have opposite effects, since the former activates caspase-1, whereas the latter acts

as a caspase inhibitor. Finally, 14 NALPs constitute the largest NLR subfamily, with NALP3 being the most studied member [5].

NLRs, in contrast to TLRs that function through transcriptional activation of inflammatory genes and cytokines, are essential for the maturation of pre-existing inactive precursors of pro-inflammatory cytokines. In response to various signals, members of the NLR family assemble multiprotein cytoplasmic complexes, called inflammasomes, which are responsible for the activation of inflammatory caspases, leading in turn to the processing and secretion of IL-1 family cytokines [6]. Interestingly, NLRs detect not only pathogens, but also danger signals. The danger signal theory proposes that in addition to foreign objects, innate immunity can recognize abnormal self or danger signals, such as DNA, RNA or uric acid, which should not normally be present outside cells or at certain locations within the cell. For these danger signals, NALP3 inflammasome is thought to be their major sensor [7].

1.A.2. NALP3 protein: a member of NLR family

The full-length NALP3 protein consists of 920 aa and is encoded by *CIAS1* (cold -induced autoinflammatory syndrome 1), a nine-exon gene on chromosome 1q44 [8]. It is mainly expressed in peripheral blood leukocytes [8, 9], but it can also be induced in several cell types in response to inflammation. The structure of the NALP3 polypeptide includes: a C-terminal ligand sensing domain, a central nucleotide binding oligomerization domain and an N-terminal recruitment domain. The C-terminal domain, composed of multiple leucine-rich repeats (LRRs), is implicated, not only in sensing various pathogenic or danger signals, but also in autoregulation. Specifically, in the absence of an activating signal, the C-terminal segment interacts with the central domain, keeping the whole protein in an inactive conformation. The core NACHT (NAIP, CIITA, HET-E and TP-1 associated protein) domain is an oligomerization motif that can bind nucleotide, and therefore it is also called NOD (nucleotide binding oligomerization domain). NOD domain is member of the AAA+ (ATPase associated with diverse cellular activities) proteins, which are known to form oligomeric structures, involved in supporting large molecular complexes. The N-terminal PYD domain, member of the death-fold (DF) family, binds to others PYD-containing proteins that take part in the inflammasome assembly [10]. Apart from NALP3, other members of the NLR family, such as NALP1 and

IPAF, may form intracellular multiprotein platforms that modulate cytokine activation, each being activated by its own specific ligand.

1.A.3. Inflammatory caspases

Caspases are cytosolic cysteine proteases that cleave a limited number of substrates present after aspartic acid residues. Although their function have been mainly studied in apoptosis, there are several caspases, including human caspase-1, -4, -5 and mouse caspase-1,-11,-12, being involved in the processing and secretion of proinflammatory cytokines [11]. Caspase-1, the founding member of the "inflammatory caspase" family, is present in the cytosol of phagocytic cells as an inactive zymogen. After stimulation, procaspase-1 is self activated by proteolytic cleavage into its enzymatically active heterodimer, composed of two -10 and 20kDa-subunits. Caspase-1 mediates the maturation of pro-IL-1β, pro-IL-18 and possibly pro-IL-33 [10].

1.A.4. NALP3 inflammasome assembly

A critical step in caspase-1 activation is the assembly of the inflammasome, which includes NLR family members and the adaptor ASC (apoptosis associated speck-like protein). The inflammasome was initially described in extracts of human THP-1 cells and was found to contain NALP1, caspase-1, caspase-5 and the adaptor proteins ASC and CARDINAL. Specifically, ASC was found to recruit caspase-1, while NALP1 engaged caspase-5 [12]. Further overexpression studies in 293T cells revealed that NALP3 was a more potent activator of caspase-1, compared to other caspase-1 activating platforms, such as NALP1 and IPAF [13]. NALP1 is the only family member with a C-terminal extension, which makes it capable of interacting with casp-5 [14]. Since NALP3 lacks such a CARD extension, its interaction with an adaptor protein for caspase recruitment is obligatory. This is ASC that binds NALP3 via PYD-PYD interaction. Then, the CARD domain within ASC recruits caspase-1 to the inflammasome. ASC was not found to have affinity for caspase-5 [13]. The human genome, but not the murine, contains a gene, called *cardinal* that encodes a protein similar to the NALP1 C-terminus [15]. Cardinal was found to interact with the NALP3 inflammasome. However, NALP3-induced caspase-1 activation was not detectably increased by coexpression of cardinal and NALP3. So, cardinal seems to be important for inflammasome association with the cytoskeleton (fig.2 [16]) [13].

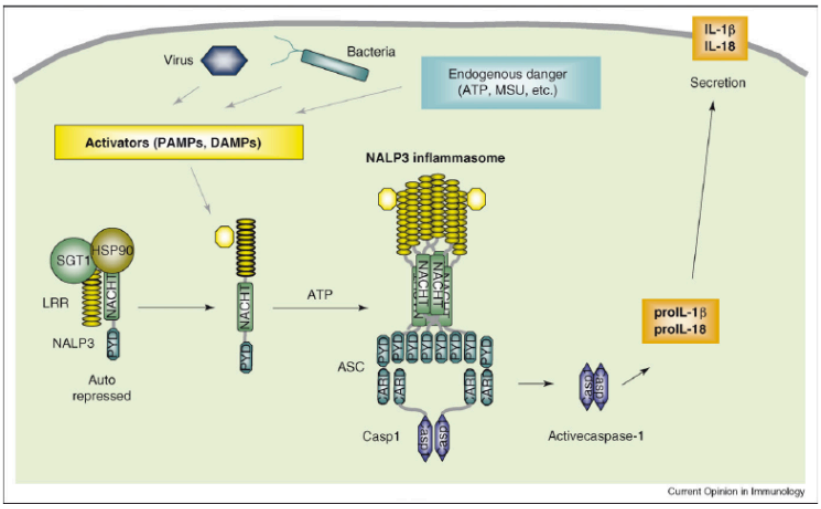

Fig.2 NALP3 inflammasome assembly

The necessity of each component of NALP3 inflammasome in its physiological function has been confirmed in experiments using deficient animals. As expected, studies in ASC-/- [17] and NALP3-/- [18] mice showed defective caspase-1-mediated IL-1 secretion in the absence of either of these proteins.

1.A.5. Regulation of NALP3 inflammasome assembly

Recent data have proposed the ubiquitin ligase-associated protein SGT1 and the heat shock protein 90 (HSP90) as potent regulators of NALP3 inflammasome assembly in humans. SGT1 and HSP90 are essential for NALP3 inflammasome activity, since knockdown of SGT1 by siRNA or chemical inhibition of HSP90 result in loss of caspase-1 and IL-1β processing, whereas inhibition of HSP90 renders the NALP3 inflammasome complex unstable and reduces NALP-3-mediated gout-like inflammation in mice. Moreover, SGT1 depletion affects HSP90 binding to the NACHT domain of NALP3, whereas HSP90 inhibition reduces SGT1 interaction with the LRR domain of NALP3. These data provide a model of action for both SGT1 and HSP90, in which SGT1 and HSP90 are associated with NALP3, keeping it inactive until the latter senses its agonists. Once activating signals are detected, HSP90 and SGT1 dissociate and allow NALP3 inflammasome assembly [19].

Apart from these regulatory molecules, NALP3 protein itself has an autoregulatory role on inflammasome assembly. In non-stimulated cells, NALP3 protein is located in the cytoplasm as an inactive monomer, in which LRR domain binds to the NACHT domain, inhibiting self-association and ASC recruitment. Recent studies revealed the crucial role of ATP in the oligomerization of NALP3 protein. In

particular, NALP3 binds ATP/dATP to its NACHT domain and exhibits ATPase activity, which is required for NALP-3 mediated IL-1β secretion. Analysis of mutant NALP3 protein, lacking ATP binding capacity, revealed reduced IL-1β producing activity, due to defective self-association, and association with the adaptor protein, ASC. So, LRR and NACHT domain seem to have a central regulatory role in NALP3 inflammasome assembly and activity [20].

1.A.6. The role of TLRs and NLRs in inflammasome activation

Based on experiments in either THP-1 cells or primary murine macrophages, it is generally accepted that activation and release of IL-1β requires two distinct signals. What constitutes these signals in vivo during an infection or during autoinflammatory responses remains unknown.

In vitro studies indicate that the first signal is provided by TLR activation, which leads to the synthesis of IL-1β proform. Specifically, TLR lignads induce the activation of the transcription factor NF-kB, via the MyD88 adaptor, leading to the IL-1β gene transcription and translation. Interestingly, TLR signaling is also required for the maturation of pro-IL-18, which unlike pro-IL-1β, is constitutively expressed [21]. Furthermore, TLR signaling is required for the induction of proteins that are essential for inflammasome function. NALP3 is expressed in a variety of resting immune cells, including macrophages, dendritic cells, neutrophils, and lymphocytes. Real-time PCR analysis carried out on cDNA isolated from LPS-stimulated macrophages demonstrated that the NALP3 message was maximally upregulated 1 hr after LPS stimulation [18], which correlates well with the expression of human NALP3 that is also induced by TLR agonists [22]. Additionally, the adaptor ASC and caspase-1 are constitutively expressed in macrophages and dendritic cells, while murine caspase-11, functional homolog of human caspases-4 and-5, has been found to be upregulated only upon TLR activation. The importance of caspase-11 for inflammasome activity is suggested by the observation that caspase-11-deficient macrophages are defective in IL-1β maturation under certain experimental conditions [23]. So, after priming the system with a first signal, a second one is needed for caspase-1 activation and IL-1β secretion. This second signal derives from activation of the purinergic $P2X_7$ receptor by ATP, which is thought to be its major endogenous ligand [24].

This two signal model, though well-established in macrophages, is inconsistent with studies in blood monocytes showing IL-1β release upon prolonged TLR stimulation [25]. Interestingly, a recent study, using primary human monocytes, revealed that LPS is able to induce IL-1β processing and release, due to constitutively active caspase-1 and release of endogenous ATP as well (fig. 3). This discrepancy between monocytes and macrophages serves an important biological role. Monocytes, circulating in a pathogen free environment, should be rapidly and efficiently activated by any pathogenic signal, while macrophages, constantly exposed to microbial stimuli, should be less sensitive to such signals; otherwise, uncontrolled and recurring inflammation would take place [26]. Consequently, the requirement for a second signal in the case of macrophages acts like a safeguard of systemic homeostasis.

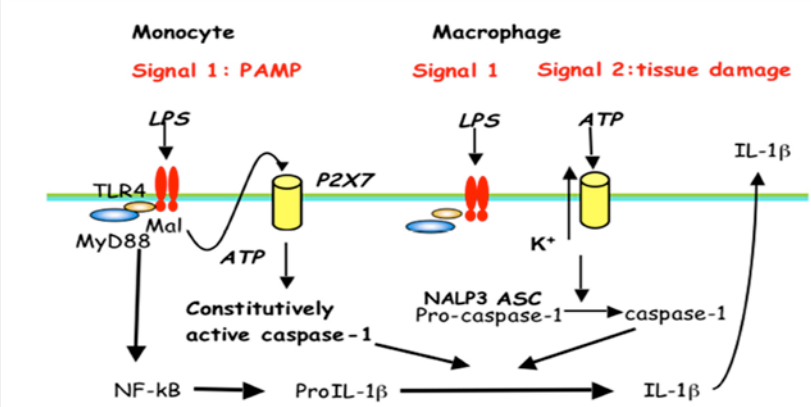

Fig.3 IL-1β activation pathway in monocytes and macrophages

1.A.7. NALP3 inflammasome activators

Characteristic of the NALP3 inflammasome is its capability to respond to a wide range of stimuli that are not necessarily microbial in origin, but also danger/stress products, as shown in table below [16].

NALP3	Microbial motifs (PAMPs) Peptidoglycan/muramyldipeptide [42] LPS[a] [41] Bacterial RNA[a] [41] Poly I:C[a] [41] Imidazolequinoline compounds (R837, R848) [41]
	Microbial toxins Nigericin (*Streptomyces hygroscopicus*) [31••] Listeriolysin O [31••] Aerolysin (*Aeromonas hydrophila*) [47] Maitotoxin (*Marina dinoflagellates*) [31••] Gramicidin (*Bacillus brevis*) [48] [b] α-Toxin (*Staphylococcus aureus*) [48] [b]
	Live bacteria *S. aureus* [31••] *Listeria monocytogenes* [31••] *Escherichia coli* [32]
	Viruses Sendai virus [40] Influenza virus [40]
	Danger-associated host components (DAMPs) ATP (via P2X7 receptor) [31,51] Reactive oxygen species, ROS [32] Monosodium urate crystals, MSU [51] Imidazolequinoline compounds (R837, R848) [32] Calcium pyrophosphate dihydrate deposition (CPPD) [51] TNCB, TNP-Cl [44,54] SDS [54] UVB [54,61]

Table 1 NALP3 inflammasome activators

1.A.8. Mechanisms of NALP3 inflammasome activation

A rather challenging aspect of research on NALP3 inflammasome is the identification of the mechanisms of its activation. Despite the fact that LRR domain of NLRs is supposed to be the sensor for cytosolic ligands, there is no evidence for direct interaction between them. Consequently, several hypotheses, whereby activating signals mediate the indirect inhibition of the autorepressive conformation of NALP3 protein have come out. These include K+ efflux, pore formation, ROS production and lysosome damage as shown in fig.4 [27].

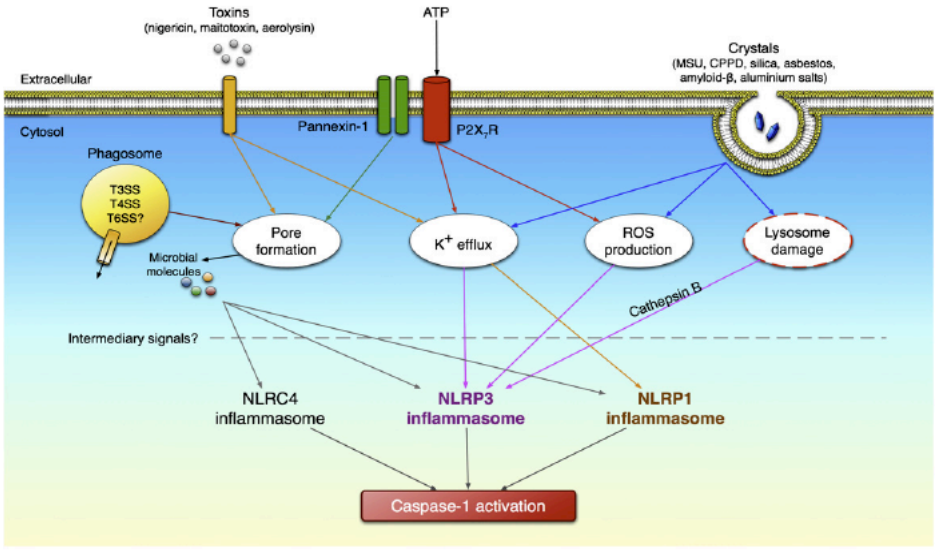

Fig.4 Mechanisms of inflammasome activation

***Potassium efflux*:** Potassium (K^+) efflux has been suggested to trigger activation of the NALP3 inflammasome. Early observations indicated that caspase-1 activators, such as ATP and nigericin, act through a mechanism dependent on K^+ efflux [28]. The recent identification of both ATP and nigericin as NALP3 inflammasome activators [29] triggered the investigation of the role of K^+ efflux in NALP3 inflammasome assembly. Indeed, incubation of THP-1 cells, previously stimulated with various NALP3 inflammasome activators, with K^+ buffer that potentiates K^+ exit from the cells, inhibited maturation and release of IL-1β. Consistently, high extracellular K^+ concentrations inhibited NALP3 inflammasome assembly in a cell-free system, using THP-1 cells lysates [30]. Further analysis on ATP-induced K^+ efflux revealed that K^+ depletion is necessary not only for pro-IL-1β processing, but also for mature IL-1β secretion. In more detail, K^+ drop results in the activation of Ca^{++} independent phospholipase (iPLA2), inhibition of which prevents IL-1β processing. Additionally, K^+ efflux is responsible for the intracellular Ca^{++} rise, ultimately leading to IL-1β exocytosis [31]. Despite its crucial role, K^+ efflux alone is not sufficient to drive NALP3 inflammasome activation [30], probably indicating the need for crosstalk between different mechanisms to engage NALP3 inflammasome.

***Pore-formation*:** As it has been mentioned above, microbes can activate NALP3 inflammasome not only via PAMPs, but also by virtue of their pore-forming toxins. Macrophages, cultured with *Listeria monocytogenes*, deficient in listeriolysin O, a pore-forming toxin, did not secrete IL-1β, indicating that cytosolic delivery of

the pathogen is required for NALP3 inflammasome activation. Notably, activation of NALP3 inflammasome by direct cytosolic delivery of heat-killed bacteria or selective PAMPs, confirm that the intracellular presence of bacteria rather than the consequences of pore formation (e.g. K^+ efflux) induces caspase-1 processing and IL-1β release. It is also interesting to note that the pore-forming model is consistent with pannexin-1, a hemichannel that associates with the $P2X_7$ receptor upon ATP stimulation and induces a large nonselective pore. Specifically, the ability of macrophages, incubated with various PAMPs and pulsed with ATP, to induce inflammasome activation is reversed in the presence of pannexin-1 inhibitor. On the other hand, inflammasome activation by direct delivery of heat-killed bacteria into the cytosol was not abolished even in the presence of pannexin-1 inhibitor. These data along with the cellular localization of pannexin-1 raise the possibility that pannexin-1 serves as a conduit for the delivery of microbial molecules to the host cytosol [32].

***ROS production*:** Reactive oxygen species were recently implicated in NALP3 inflammasome activation in response to silica and asbestos. Specifically, pharmacological inhibition of the NADPH oxidase complex in THP-1 cells and peritoneal macrophages as well as si-RNA-mediated downregulation of the $p22^{phox}$ subunit in THP-1 cells inhibited caspase-1 processing and IL-1β production [33, 34]. Cellular redox imbalance occurs not only upon exposure of macrophages to silica and asbestos dust, but also upon stimulation with MSU, ATP, alum or UVB [33, 35 36, 37]. The source of ROS involved in NALP3 inflammasome activation may be mitochondrial, since mice deficient in either specific components of the NADPH oxidase system ($gp91^{phox}$) [38] or the cytoplasmic antioxidant enzyme superoxide dismutase (SOD1) [39] are still capable of activating NALP3 inflammasome.

***Lysosome damage*:** Recent studies shed light on the mechanisms, by which particulate stimuli, such as silica crystals, aluminium salts and fibrillar amyloid-β (Aβ), set NALP3 inflammasome in motion [38, 40]. In particular, all these particles undergo the so-called frustrated phagocytosis, causing lysosomal swelling and dysfunction; lysosomal damage is followed by the release of the protease cathepsin B into the cytoplasm, which seems to be causally involved in NALP3 inflammasome activation. Pharmacological inhibition of cathepsin B disrupts the ability of particles to trigger IL-1β secretion. Notably, mutant-NALP3 has been implicated in a caspase-1-independent but cathepsin B-dependent cell death pathway [41].

1.A.9. NALP3 inflammasome associated diseases

A variety of inflammatory diseases has been associated with dysregulated inflammasome signaling. Particularly, genetic evidence links either inflammasome components, such as NALP3 and NALP1, or inflammasome associated molecules, NOD2, to periodic fever syndromes, vitiligo, Crohn's disease and Blau syndrome, respectively. Furthermore, inflammasome-mediated IL-1β production in response to specific stimuli seems to be implicated in the pathogenesis of inflammatory diseases such as gout and pseudogout [1].

1.B. Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) comprises two major forms of chronic inflammation of the gastrointestinal tract, Crohn's disease (CD) and ulcerative colitis (UC). Although the precise etiology of IBD remains unclear, it is thought to result from an aberrant immune response to a subset of commensal bacteria in genetically susceptible hosts, while environmental factors precipitate the onset or reactivation of disease. This complex theory involves four separate components that must intersect in multiple ways for disease to become clinically apparent [42].

1.B.1. Adaptive immune response in CD

The adaptive immune system was believed to play the primary role in IBD pathogenesis. In simplistic terms, CD seems to exhibit a Th1 cytokine profile. More specifically, intestinal CD4 T cells isolated from Crohn's patients produce large amounts of the Th1 cytokine IFN-a, and display marked overexpression of the Th1 cell-specific transcription factor, T-bet. Mucosal macrophages from Crohn's patients also produce large amounts of the Th1 inducing cytokines IL-12 and IL-18. Th1 cell resistance to apoptosis and increased cell cycling in CD inflammation appear to be sustained by cytokines. Blocking the pathways that confer resistance of Th1 cells to apoptotic stimuli and the use of drugs that enhance mucosal T cell death are effective in down-modulating intestinal inflammation [43].

More recent data have implicated the newly identified Th17 pathway, as playing a key role in CD pathogenesis. IL-17 mediates Th17 responses. The production of this cytokineis stimulated by the production of IL-6, TGFb and IL-23 by innate immune cells and APCs,especially dendritic cells. Bacterial colonization stimulates IL-23 expression by ileal dendriticcells. The levels of both IL-23 and IL-17 are increased in CD tissues and mostforms of experimental colitis.

Interestingly, IL-12–IFN-γ and IL-23–IL-17 pathways seem to be mutually exclusive, since IFN-γ suppresses IL-17, and vice versa [42]. Collectively, adaptive immune response mediating pathogenesis of CD is depicted in Fig.5 [44]

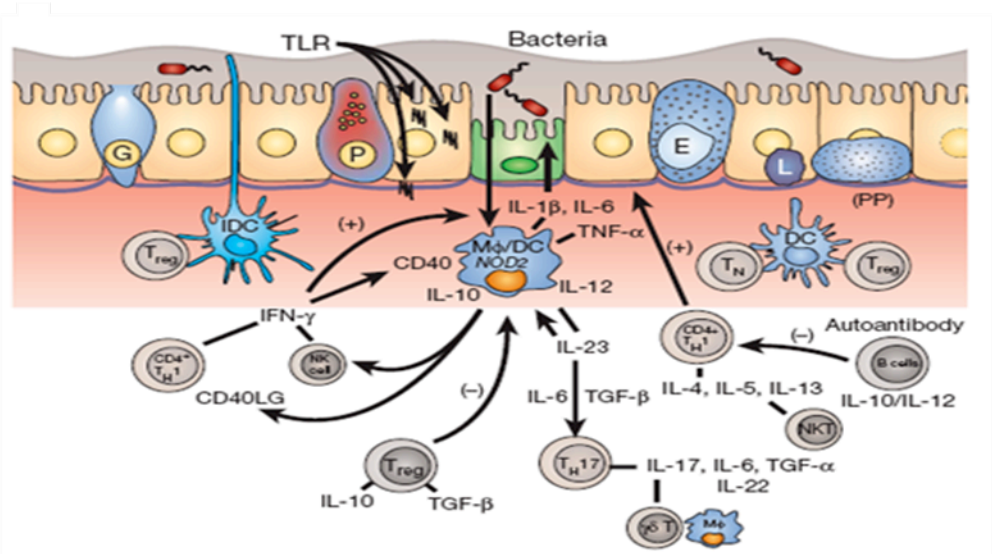

Fig.5 Adaptive immune responses in CD

1.B.2. Innate immune response in CD

Evidence of the role of the innate immune system comes mainly from the recently discovered association between CD and NOD2 gene. NOD2 protein is expressed in macrophages, dendritic cells, intestinal epithelial cells and Paneth cells, and may have cell-specific function. NOD2 is a cytoplasmic protein of the NLR family, which serves as a microbial sensor. More specifically, MDP, a fragment of peptidoglycan present in bacterial cell walls, is recognized by the LRR domain of NOD2 and leads to activation of the transcription nuclear factor (NF-kB), and induction of proinflammatory cytokines [45]. Specific mutations of the NOD2 gene have been definitively associated with increased susceptibility to ileal CD in Western population: Arg702Trp, Gly 908Arg and leu1007fsinsC. Heterozygous carriage of the risk alleles confers a 2-4 fold increased risk, while homozygotes or compound heterozygotes for any one of the three variants have as much as a 40-fold increased likelihood of developing CD [44]. More than 90% of all CD associated mutations are located in the LRR domain suggesting that these may affect the function of NOD2 with respect to bacterial recognition and signaling. Although, the mechanisms by which disease-associated NOD2 polymorphisms contribute to the intestinal inflammation are incompletely understood [45], two hypotheses have been proposed, as shown in Fig.13 [46].

Loss of function hypothesis:Transfection of wild-type but not mutant, NOD2 into intestinal epithelial cells inhibits uptake or growth of invasive bacteria. This suggests that the normal protein exerts antimicrobial activity, while CD-associated-variants are defective in this function. This loss of NOD2 function may be related to the inability to activate NF-kB and, hence, the expression of NF-kB-dependent antimicrobial gene products, such as β-defensins. Indeed, mice lacking NOD2 showed a defect in intestinal innate defense against oral infection with *L. monocytogenes*, which was paralleled by diminished expression of at least two Paneth cell-derived antimicrobial peptides, Defcr4 and Defcr-rs10. Moreover, CD patients with mutant NOD2 were shown to have decreased expression of the human Paneth cell α-defensins HD-5 and HD-6 in the small intestine, suggesting that CD-associated NOD2 mutations may be functionally equivalent to the loss of the protein in knockout mice. Alternatively, the loss of function may also affect the ability of NOD2 to attenuate signaling through TLR2 in macrophages, the net result being enhanced NF-kB activation and proinflammatory cytokine production. More specifically, incubation of normal murine splenic macrophages with MDP was shown to suppress IL-12 secretion induced by stimulation with peptidoglycan, a TLR2 ligand. This suppression did not occur either in cells lacking NOD2 or expressing a mutant NOD2 variant. These data suggest that inactivation of NOD2 enhances IL-12 production in macrophages because NOD2 normally limits TLR2 agonist-stimulated expression of this cytokine.

Gain of function hypothesis: Murine macrophages homozygous for a mutant NOD2 allele equivalent for the most common CD-associated allele were shown to secrete higher levels of proinflammatory cytokines upon MDP stimulation, compared with wild-type macrophages. In parallel, these mice exhibited greater colonic inflammation upon experimental challenge with dextrane sulfate sodium. However, these experimental data are not in accordance with findings from human studies showing that macrophages from CD patients homozygous for the (Leu1007fsinsC) mutation have a globally blunted transcriptional response to MDP. This discrepancy could be explained by the fact that human macrophages were derived from peripheral blood of patients with active inflammation, which might have altered their responsiveness to microbial products [47].

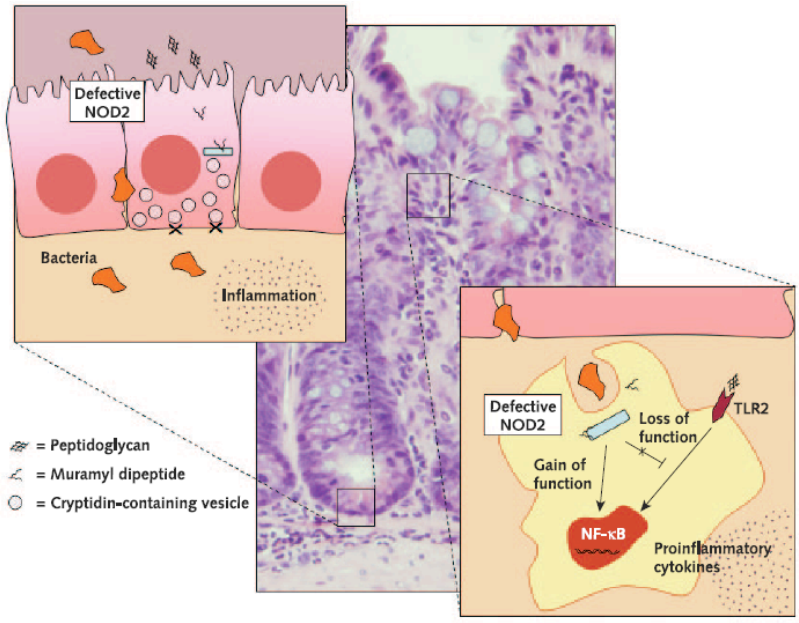

Fig.6 Proposed functional significance of NOD2 mutations in CD

Although the focus has been the NOD2 gene, as an NLR related to CD, recent evidence suggests that NALP3 is also involved in CD. More specifically, SNPs in a regulatory region downstream of NALP3 are associated with hypo-production of IL-1β and decreased NALP3 expression in healthy donors. Given that expression of NALP3 is elevated in CD and experimental colitis samples, this study suggests a role for NALP3 in either the initiation phase of the disease or in perpetuating chronic inflammation [48]. Moving a little closer to NALP3 inflammasome and CD, a recent study showed that men expressing the wild-type allele of NOD2, and simultaneously being carriers of combined polymorphisms in CARDINAL and NALP3, are at increased risk of developing CD [49].

Similarly, several other genes key players in host innate immune responses, including TLRs, ATG16L1 (which encodes autophagy related 16-like protein1) and IRGM (which encodes immunity-related GTPase family, M), have been found to confer susceptibility to CD [44].

Finally, another parameter of impaired innate immunity in CD was recently addressed by Mark et al. Experiments in CD patients, in full remission and under no medication, showed that these patients had profound and consistent abnormalities of acute inflammation, including failure of neutrophil accumulation and IL-8 production at sites of trauma both in the bowel and in the skin, unlike healthy controls and patients with other chronic inflammatory conditions (eg. ulcerative colitis and

rheumatoid arthritis), who had no such abnormalities. This diminished neutrophil recruitment into traumatized skin was not due to inherent motility defects, but rather due to reduced secretion of IL-8 by macrophages. This effect was also replicated *in vitro* by placing monocyte-derived macrophages into culture and exposing them to acute wound fluid. These findings suggested that in CD there might be a major defect in the handling of bowel flora. Indeed, when heat-killed bacteria were injected subcutaneously into the forearms of CD patients, there was decreased blood flow to the injection site, compared with healthy controls. However, IL-6 blood levels of CD patients were much higher than in healthy controls, suggesting that bacteria were taken up by macrophages, which produce IL-6. The last finding is consistent with the formation of granulomas and foci of chronic inflammation, characteristics of CD [50].

1.C. The link between NALP3 inflammasome and Crohn's disease

Unlike the well-established role of innate immunity in CD, there are only indications for a possible role of NALP3 inflammasome in the pathogenesis of the disease. These mainly come from genetic studies on NLRs (NOD2 and NALP3 are susceptibility loci for CD) as mentioned above [48, 49, 51], and the role of cytokines, and specifically NALP3 inlfammasome-dependent cytokines, in the pathogenesis of CD.

Regarding cytokines, their role in the pathogenesis of CD is based on data showing that the expression of most proinflammatory cytokines (IL-1, TNF-α, IL-6, IL-8, IL-18, and IL-23) is up-regulated [42], while blocking inflammatory cytokines has been proved effective in animal models and humans suffering from CD (anti-TNF-α (Infliximab) in humans [52], anti-IL-12, anti-IL-18, anti IL-1Ra in colitis animal models) [53-56]. Concerning IL-1β, its increased levels in the mucosa affected by active CD in humans may be the result of stimulation of colonic macrophages that can activate caspase-1 and hence release mature IL-1 into the colonic mucosa. Indeed, studies have shown that specific inhibition of caspase-1 leads to a significant reduction in the amount of mature IL-1β released [57]. Moreover, administration of IL-1-receptor antagonist (IL-1Ra) reduces disease severity in rabbit immune colitis [58]. Most recently, studies showed up-regulation of IL-18 expression during the course of CD, which is a co-stimulus for production of IFNγ and other Th-1 cytokines [54, 59], while blocking IL-18 in colitis mouse models results in a significant amelioration of intestinal inflammation [54]. Based on the above data it is obvious

that key players in CD are: (1) the cytokines IL-1β and IL-18, which are products of NALP3 inflammasome and (2) NOD2, a sensor of MDP, which is a possible in vivo trigger for intestinal inflammation in CD [60].

2. Objectives

In the present study, we sought to determine whether NALP3 inflammasome is implicated in Crohn's disease pathogenesis, since there are several findings that indicate a possible link between them as described above. To this end, experiments in human samples have focused on functional assessment of inflammasome activity in whole blood, while experiments performed in a murine model of experimental colitis were conducted to reveal the role of NALP3 in the innate immune aspect of CD.

Specific aim 1

- *Investigation of inflammasome activation in whole blood samples of CD patients versus healthy controls.*

For inflammasome activation two different stimuli were applied in whole blood samples : (a) pretreatment with ultra pure LPS to induce pro-IL-1β and pro-IL-18 synthesis and (b) ATP pulse (with the ATP being a cellular stress signal) treatment for inflammasome activation and production of functional IL-1β. Except from LPS plus ATP, MDP was also used as a trigger as well. MDP is a known NOD2 ligand, leading to pro-IL-1β synthesis via NF-kB pathway [51]. Recently, it is suggested to be a potent NALP3 inflammasome activator [61], while MDP-induced IL-1β processing requires both NOD2 and NALP3 [62]. These data combined with the fact that MDP is the bioactive molecule of the bacterial cell wall, being present in the intestinal lumen, made it an ideal stimulus for NALP3-inflammsome activation in terms of CD pathogenesis. Inducible inflammasome activity was assessed by measurement of IL-1β in supernatants of peripheral blood cells by ELISA.

- *Investigation of specificity of inflammasome activation*

In order to determine whether the results of the above experiment are specific to inflammasome activation two methods were used for verification: (a) the selective blockage of inflammasome activity by caspase-1 inhibitor (b) the measurement of non-inflammasome-dependent cytokine levels, such as TNF-α, by ELISA.

- *Analysis of NALP3 inflammasome components in colonic tissues from patients with CD and controls*

After having evaluated NALP3 inflammasome activation in peripheral blood cells, we next examined expression of NALP3 inflammasome components in the target tissue. Western blot analysis of NALP3, caspase-1 and IL-1β was performed in lysates of colonic specimens isolated from healthy controls and Crohn's disease patients.

Specific aim 2

The role of NALP3 in colonic homeostasis was investigated by using NALP3-deficient mice along with wild-type C57BL/6 mice. C57BL/6 wild type and NALP3-defecient mice were treated with 3,5% DSS in drinking water for 7 days, a model of acute innate immune mediated colitis.

- *Assessment of colitis severity in wild type and NALP3-/- mice*

Assessment of colitis severity included mortality of mice in defferent groups and body weight loss, measurement of colon length as an indicator of inflammatory intensity, histological analysis of distal colon sections and cytokine analysis in colonic tissues protein extracts by ELISA**.**

3. Materials and methods

3.A.1. Reagents

RPMI and ATP were obtained from Invitrogen, LPS and MDP from Sigma caspase-1 inhibitor from Calbiochem. The Abs used were anti-NALP3 from Alexis Biochemicals, anti-caspase-1 from Calbiochem, anti-IL-1β from Cell Signaling, anti-actin from Santa Cruz Biotechnology, anti-mouse HRP-conjugated Ab from Jackson Immunoresearch and anti-rabbit HRP-conjugated Ab from Sigma.

3.A.2. Experiments with peripheral blood cells

After fresh blood was collected in EDTA tubes, samples were processed as described below:

- ✓ RBC lysis solution was added to blood and incubated for 10 minutes at room temperature.
- ✓ Cells were centrifuged at 1800rpm for 12-13 min at RT, and supernatants were completely removed.
- ✓ Cells were washed by adding 10 ml cold PBS, centrifuged at 1500rpm for 8min at RT, and supernatant was removed .
- ✓ The cell pellet was resuspended in medium (500µl/condition) and cells were plated in 12-well cell culture plates.
- ✓ Cells were stimulated with LPS (1µg/ml or 250pg/ml), ATP (5mM), or MDP (10µg/ml or 1µg/ml) for various time periods at 37^0 C, and 5-7% CO_2.
- ✓ Cells were centrifuged at 1500rpm for 8min at RT
- ✓ Cell-free supernatant were collected and stored at -80^0 C, until assayed for cytokine levels by ELISA as described below.

3.A.3. Cytokine Determination via Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokines (IL-1β and TNF-α) levels were determined by specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturer (eBioscience, location, Country).

Briefly, purified anti-mouse capture antibody was used at a dilution of 1:250. to coat 96 well plates (Corrnig, city, country) with 100 µl/well of capture antibody in coating buffer and was incubated overnight at 4°C. After washing five times with washing buffer (PBS-0.05% Tween) plates were blocked with 1X Assay Diluent at room temperature for 1 hour. Following five more washes, standard and sample dilutions and were added to the appropriate wells (200µl/well) and incubated at room

temperature for two hours. A standard curve was constructed using recombinant mouse corresponding antibody. Each dilution of recombinant standard or sample was assayed in duplicate. After washing five times, wells were incubated with 100μl/well of biotin-conjugated anti-mouse antibody (100μl/well diluted 1:250 in 1X Assay Diluent) for 1 hour at room temperature. Subsequently, five more washes were performed and the detection enzyme Avidin-HRP (100μl/well diluted 1:250 in 1X Assay Diluent) was added. After a total of seven more washes, 100 μl/well of Substrate Solution was added to each well and the plate was sealed and incubated at room temperature for 15 minutes. The blue color produced by enzymatic activity was converted into yellow by adding 50 μl of Stop Solution to each well. The procedure was completed by reading the plate at 450 nm in an ELISA microplate reader (Biorad). The data were subsequently analysed in an Excel spreadsheet (Microsoft).

3.A.4. Homogenization of human intestinal tissues for Western Blotting

Colonic specimens were homogenized with a tissue grinder in suspension buffer containing an inhibitory cocktail of proteases (Roche Diagnostics, city, country). Samples were centrifuged at 1600rpm for 15min at 4^0 C and the cell-free supernatants were stored at -80^0 C until assayed.

3.A.5. Quantification of protein extracts with Bradford protein assay

The quantification of protein concentration was performed using the spectrophotometer and the Biorad Protein assay Dye Reagent (Biorad Lad, Ltd., Hemel Hempsted, UK) that alters its colour from light to dark blue, depending on the concentration of proteins. We dilute Bradford reagent 1:4 with H_2O and we add the appropriate amount of protein extracts (1-20μl) to prepare a homogenous suspension that is transferred into cuvettes. Samples were counted in a spectrophotometer at 595nm and protein concentration was calculated according to the equation of the standard curve, that links density (OD) (=y) and proteins amount in mg (=x).

3.A.6. Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

SDS-PAGE was used to fractionate proteins by size. All protein samples were mixed 3:1 with a 4 x sample buffer (5 mL Tris-Cl 1M pH 6.8, 1,6 mL β-

mercaptoethanol, 4 mL glycerol, 1,6 g SDS, 8 mg bromophenol blue, ddH_2O up to 20 mL), denatured at 100 °C for 5-10 minutes and loaded onto the gel. The BenchMark Prestained Protein Ladder (Invitrogen, city, country) was used as a size marker. In the present study 12.5 % acrylamide gels were used to detect and characterize NALP3, caspase-1 and IL-1β expression. The quantities used for the preparation of the gels are shown below:

Separating Gel	**12.5%**	**Stacking Gel**	
ddH_2O	3.2 mL	ddH_2O	3.6 mL
30% acrylamide	4.2 mL	30% acrylamide	900 µL
Separating buffer	2.5 mL	Separating buffer	1.5 mL
10% APS	160 µL	10% APS	60 µL
TEMED	8 µL	TEMED	6 µL
Final Volume	10 mL	Final Volume	6 mL

10x TGS	
Tris	30.3 g
Glycine	144.2 g
SDS	10 g
Final Volume	1 L

Separating Buffer	
Tris	18.165 g (1.5 M)
SDS	0.4 g (0.4% w/v)
pH adjusted with HCl	8.8
Final Volume	100 mL

Stacking Buffer	
Tris	6.05 g (0.5 M)
SDS	0.4 g (0.4% w/v)
pH adjusted with HCl	6.8
Final Volume	100 mL

Electrophoresis was performed in a Bio-Rad Mini-Protean II Vertical Electrophoresis apparatus in 1 x TGS buffer. The running conditions were 100 V until the samples had entered the stacking gel followed by 150 V.

3.A.7. Western Blot

Following electrophoresis the proteins were transferred from the gel to a nitrocellulose membrane, where they were detected using antibodies specific to the target protein. The transfer was performed in the Bio-Rad Mini-Protean II by electroblotting in 1 L transfer buffer (100 mL 10x TGS, 200 mL methanol and 700 mL ddH_2O) at 400 mA for 1,5 h. After blotting, the membrane was processed as described below:

- ✓ The membrane was washed with TBS-T 0,05 % (1x TBS + 0,05 % Tween 20) for 5 minutes at room temperature .
- ✓ Blocked with 5 % w/v milk for 1 h at room temperature.
- ✓ The membrane was washed with TBS-T 0,05 % for 5 minutes at room temperature and
- ✓ Incubated with the primary antibody according to the table below:

Primary antibody	Dilution	Diluted in	Incubation conditions
mouse α – NALP3	1:1000	1 % w/v milk in TBS-T 0,05 %	4°C, overnight
rabbit α – caspase-1	1:1000	1 % w/v milk in TBS-T 0,05 %	4°C, overnight
rabbit α – IL-1β	1:1000	1 % w/v milk in TBS-T 0,05 %	4°C, overnight
mouse α – actin	1:2000	1 % w/v milk in TBS-T 0,05 %	4°C, overnight

- ✓ The membrane was washed three times with TBS-T 0,05 % for 10 minutes at room temperature.
- ✓ Incubated with the secondary antibody according to the table below:

Secondary antibody	Dilution	Diluted in	Incubation conditions
α – rabbit HRP	1:2500	5 % w/v milk in TBS-T 0,05 %	RT, 1 h
α – mouse HRP	1:10000	5 % w/v milk in TBS-T 0,05 %	RT, 1 h

- ✓ The membrane was washed three times with TBS-T 0,05 % for 10 minutes at room temperature.
- ✓ Washed with 1x TBS for 5 minutes at room temperature.
- ✓ The membrane was placed on 1 mL of ECL reagent (SuperSignal West Pico Chemiluminescent Substrate, Pierce) per blot for 5 minutes.
- ✓ The membrane was drained, wraped in Saran Wrap, placed properly in cassette.
- ✓ And exposed to film in dark room.

3.B.1. Animal Experimentation

All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. Mice were housed in a temperature-controlled environment with a stable photoperiod of 12-hour light/12-hour dark cycle and allowed unrestricted access to standard mouse chow diet and tap water.

3.B.2. Induction of Dextran Sulfate Sodium (DSS)-induced Acute Colitis

For the induction of acute DSS colitis, mice were given 3.5 % (w/v) DSS (MW=36.000–50.000; MP Biomedicals, city, country) in drinking water for 7 days. The mice were monitored daily for survival and changes in body weight.

3.B.3. Histological Analysis

The extent and severity of colitis was also determined histologically. The isolated distal colons were fixed in 10% formalin. Tissues were embedded in paraffin, and consecutive 3μm sections were mounted on slides. Sections were stained with hematoxylin/eosin (H&E) for histological examination.

3.B.4. Homogenization of murine intestinal tissues for cytokine analysis by ELISA

Intestinal tissue samples were homogenized in PBS containing an inhibitory cocktail of proteases (Roche Diagnostics) and 10mM PMSF (Roche Diagnostics, city, country) with a tissue grinder. The homogenized tissues were centrifuged at 16000rpm for 20min at 4^0 C. The cell-free supernatants were stored at -80^0 C until assayed. Aliquots of cell extracts were analysed for protein content using the Bio-Rad colorimetric assay according to the method of Bradford (Bio-Rad). The amount of murine IL-1β and TNF-α in protein lysates were quantified by ELISA kits (eBioscience, city, country, only in first reference) and adapted to the protein content of the colon tissue probe.

4. Results

4.A. Human studies

4.A.1. NALP3 inflammasome activation

NALP3 inflammasome activation requires two signals, the first one provided by TLR agonists, such as LPS, while the second one is triggered by agents causing ionic perturbations, such as ATP. Therefore, in order to confirm NALP3 inflammasome activation, a pilot experiment was conducted. Whole blood cells from 4 healthy donors were either primed with LPS or treated with LPS plus ATP for 2 hours. Unlike LPS, which does not stimulate IL-1β secretion, LPS plus ATP leads to significant IL-1β production (LPS+ATP confers 121,5 fold increase) (Fig.7). Since IL-1β secretion is caspase-1 dependent, we also used caspase-1 inhibitor treatment to confirm inflammasome dependency of IL-1β production. Indeed, caspase-1 inhibitor decreased IL-1β (56% inhibition) secretion induced by LPS plus ATP treatment (Fig.7).

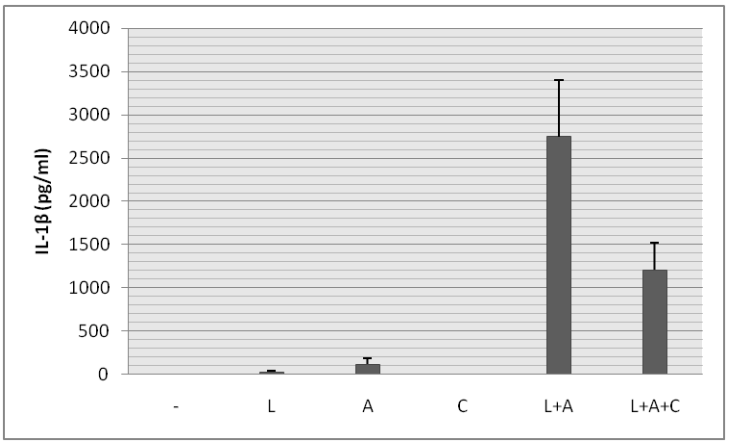

Fig.7 NALP3 inflammasome activation. Concentrations of mature IL-1β in supernatants of whole blood cells stimulated with LPS (250pg/ml) for 2h, ATP (5mM) for 20min and caspase-1 inhibitor (1mM) for 15min were determined by ELISA. Results are representative of five independent experiments.

4.A.2 MDP and NALP3 inflammasome activation

More recent data have suggested that MDP, the ligand for NOD2, can activate NALP3 inflammasome [61]. Therefore, the next experimental approach was to determine whether MDP can stimulate IL-1β secretion through NALP3 inflammasome activation in a time- and dose-dependent manner. To this end, whole blood cells were stimulated with MDP or MDP plus ATP for different time points and

concentrations (Fig.8, Fig.9). As shown in Fig.8, MDP plus ATP induced similar IL-1β induction at the three different tested time points (at 3h, 6h, and 12h). Based on these result we chose the shorter incubation time and continued with dose response experiment. Two different doses of MDP were used to stimulate IL-1β production in whole blood cells. As shown in Fig.9 both of the tested concentrations induced IL-1β production, with the highest dose causing a 6-fold increase of IL-1β secretion induced by MDP plus ATP. Interestingly, as shown in both figures MDP alone is a weak inducer of IL-1β production, which is in agreement with previous published data [62].

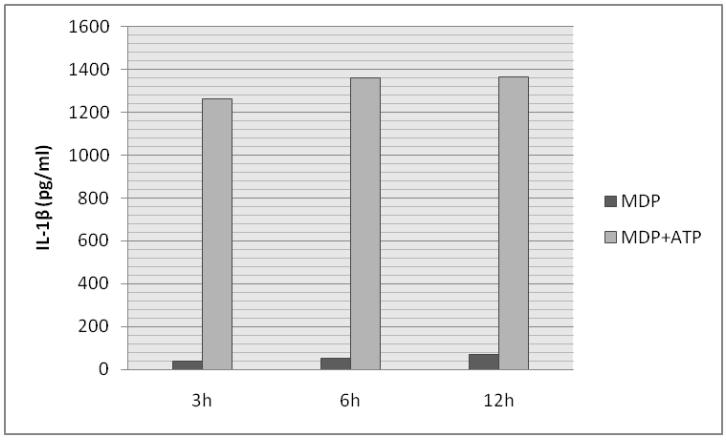

Fig.8 MDP time course experiment. Concentrations of mature IL-1β in supernatants of whole blood cells stimulated with MDP (10μg/ml) for 3h, 6h or 12h and pulsed with ATP (5mM) for 20min were determined by ELISA.

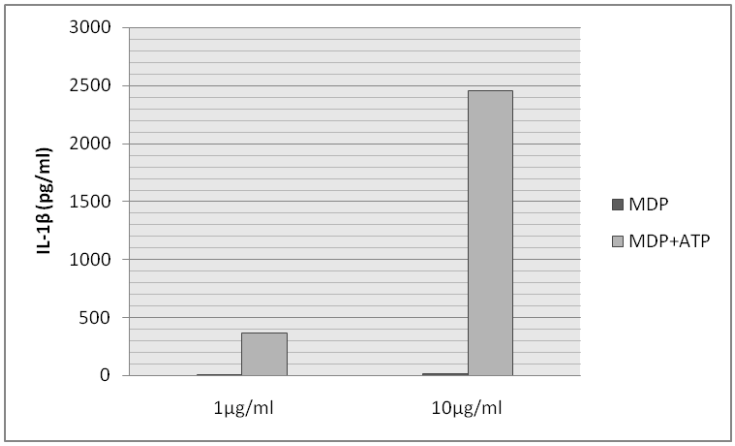

Fig.9 MDP dose response experiment. Concentrations of mature IL-1β in supernatants of whole blood cells stimulated with 1μg/ml or 10μg/ml of MDP for 3h and pulsed with ATP (5mM) for 20min were determined by ELISA.

4.A.3 Synergistic effect of LPS and MDP in NALP3 inflammasome activation

Since IL-1β production in response to MDP is rather poor, we next aimed to analyse the cytokine response of whole blood cells to combined stimulation with MDP plus LPS. To this end whole blood cells from one healthy donor were treated with both triggers using different combinations of MDP and LPS concentrations. As shown previously, MDP and LPS exhibited synergistic effects in inducing IL-1β production, which was rather evident at low concentration of both ligands [63]. Moreover, it is obvious that MDP is a less potent inducer of IL-1β production than LPS (Fig.10).

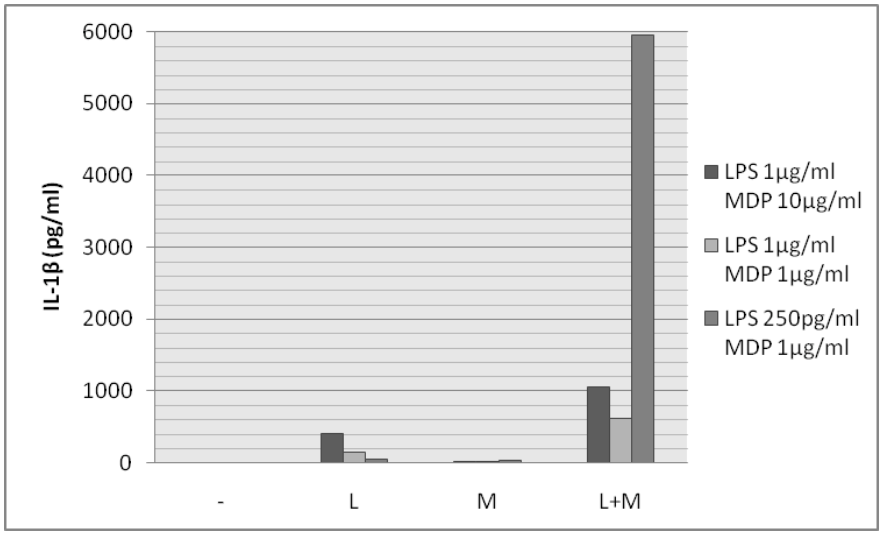

Fig.10 Synergistic effect of LPS and MDP in healthy donors. Concentrations of mature IL-1β in supernatants of whole blood cells stimulated with LPS and MDP at different concentrations were determined by ELISA.

4.A.4. NALP3 inflammasome activation in healthy controls versus IBD patients

4.A.4.1. NALP3 inflammasome activation upon LPS plus ATP

After having established NALP3 inflammasome activation upon LPS or MDP and ATP treatment, we compared basal and inducible NALP3 inflammasome activity in healthy donors and IBD patients. To this end, whole blood cells isolated from five healthy donors and four IBD patients were stimulated with LPS and pulsed with ATP. As shown in Fig.11 healthy controls and IBD patients produce comparable amounts of IL-1β both before and after treatment. In order to confirm that IL-1β production is inflammasome dependent, a caspase-1 inhibitor was also used. Whole blood cells were treated with caspase-1 inhibitor before the addition of ATP. Indeed, caspase-1

inhibitor decreases IL-1β induction upon LPS and ATP treatment. However, IL-1β was equally diminished in both groups that were tested (Fig.11).

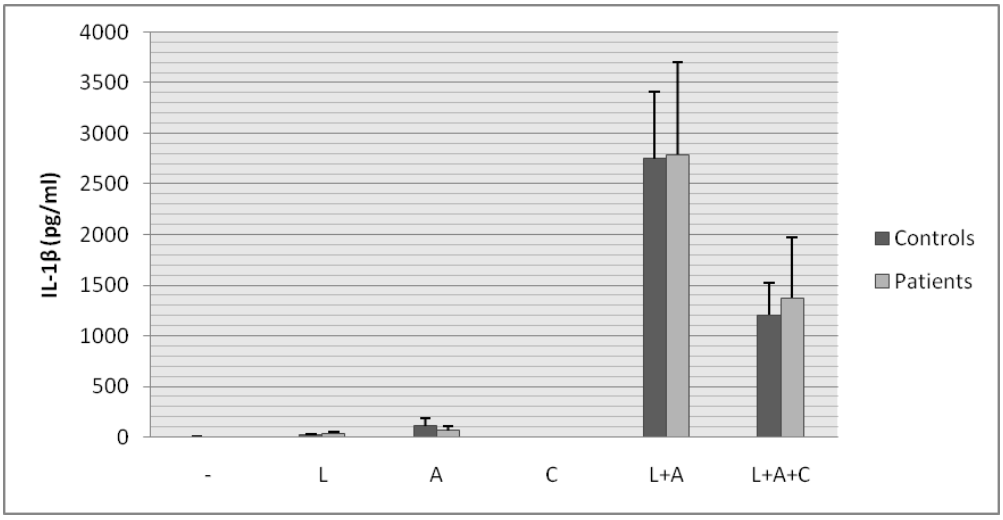

Fig.11 Classic NALP3 inflammasome activation in healthy donors versus IBD patients. Concentrations of mature IL-1β in supernatants of whole blood cells from healthy donors (n=5) and IBD patients (n=4) stimulated with LPS (250pg/ml) for 2h and ATP (5mM) for 20min in the presence or absence of caspase-1 inhibitor (1mM).

4.A.4.2. NALP3 inflammasome activation mediated by MDP

Next, we examined the basal and inducible NALP3 inflammasome activity upon MDP and/or ATP stimulation. Whole blood cells isolated from healthy donors (n=3) and IBD patients (n=3) were stimulated as indicated on Fig.12. As previously shown (Fig.11), IL-1β levels, produced by cells either left unstimulated or stimulated with LPS plus ATP, were comparable between the groups tested. On the other hand, it is clearly demonstrated that whole blood cells isolated from IBD patients secrete higher levels of IL-1β upon MDP plus ATP treatment compared to healthy controls (5,1 fold increase) (Fig.12). Interestingly, while synergistic effect of LPS and MDP is more evident in IBD patients compared to controls, maximum NALP3 inflammasome activation upon LPS plus MDP plus ATP stimulation showed no significant changes between the two groups (Fig.12). Furthermore, IL-1β levels produced upon LPS plus MDP stimulation were significantly decreased in the presence of caspase-1 inhibitor, confirming inflammasome dependency of IL-1β secretion. Specificity of inflammasome activation either upon LPS plus MDP stimulation or upon maximal inflammasome triggering was further confirmed by measuring TNF-α release, which is an inflammasome independent cytokine. Thus, TNFα secretion was not further up-

regulated upon ATP stimulation in cells pre-treated with LPS, MDP or both stimuli. Moreover, TNF-α secretion remained unchanged in the presence of caspase-1 inhibitor. Intriguingly, TNF-α levels were higher in patients after MDP and/or ATP stimulation when compared with healthy controls (Fig.13).

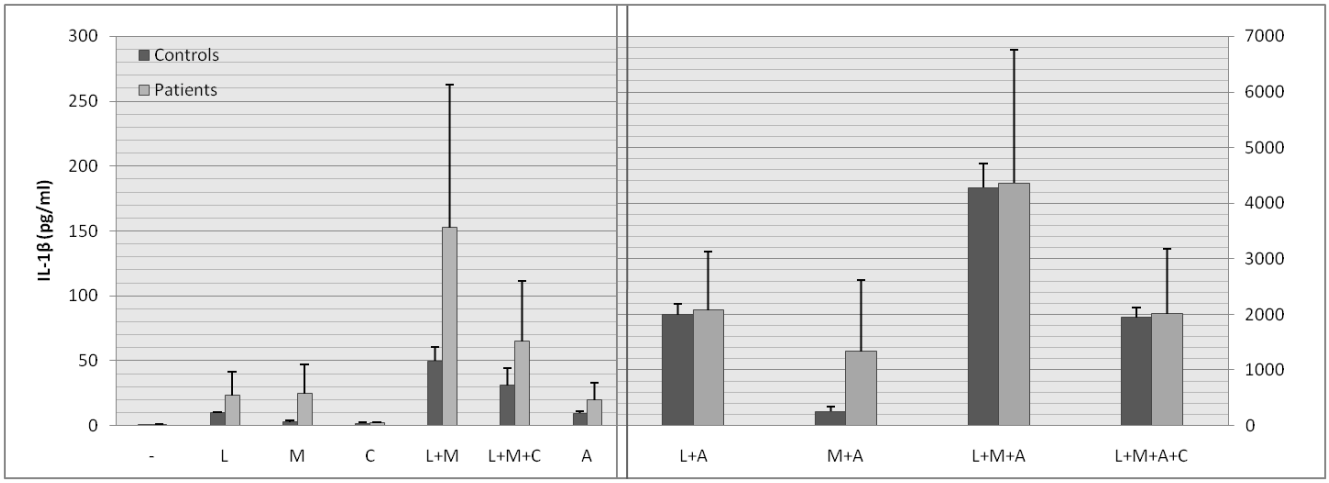

Fig.12 NALP3 inflammasome activation in healthy donors versus IBD patients. Concentrations of mature IL-1β in supernatants of whole blood cells from healthy donors (n=3) and IBD patients (n=4) stimulated with LPS (250pg/ml), MDP (1μg/ml) for 2h and ATP (5mM) for 20min in the presence of absence of caspase-1 inhibitor (1mM).

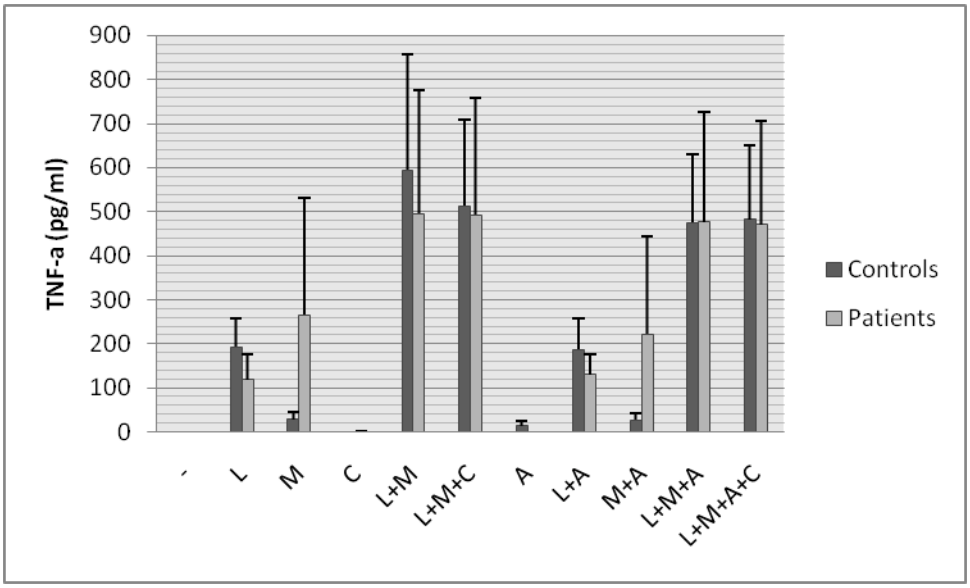

Fig.13 NALP3 inflammasome activation in healthy donors versus IBD patients. Concentrations of TNF-α in supernatants of whole blood cells from healthy donors (n=3) and IBD patients (n=4) stimulated with LPS (250pg/ml), MDP (1μg/ml) for 2h and ATP (5mM) for 20min in the presence of absence of caspase-1 inhibitor (1mM).

4.A.5. Expression of NALP3 inflammasome components in human colonic tissues

After having evaluated NALP3 inflammasome activation in peripheral blood cells, we next examined the expression of NALP3 inflammasome components at the protein level in colon tissues. To this end, colonic biopsy specimens from healthy donors and patients were used. Western blot analysis of lysates (from 3 normal colons, 1 pouchitis colon and 1 CD colon) showed equal levels of NALP3 protein and pro-IL-1β, while processed IL-1β was absent in all samples tested. Interestingly, while the precursor form of capsase-1 was present in all samples, a significant amount of the biologically active caspase-1 was present only in the CD sample. Inflammatory control was used to evaluate the specificity of findings in CD patient (Fig.14).

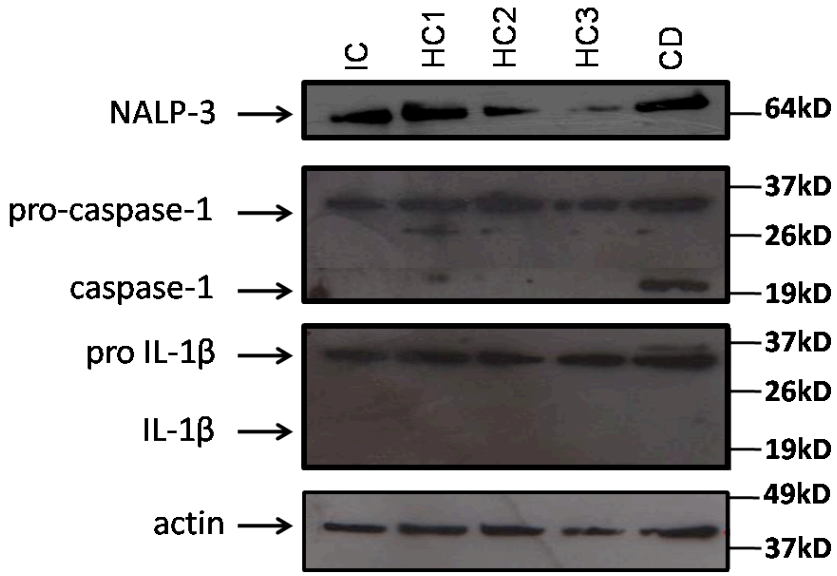

Fig.14 Expression of NALP3 inflammasome components in normal and diseased colons. Western blot analysis of protein extracts (40μg/ml) from colonic lysates of healthy controls and CD patient. (IC1: inflammatory control-pouchitis, HC: healthy control and CD: CD patient)

4.B. Mice experimentation

4.B.1. DSS-induced colitis in NALP3 deficient mice

One experimental animal model for human IBD is based on the oral administration of dextran sulfate sodium in mice. DSS-induced colitis is characterized by epithelial disruption resulting in luminal bacterial translocation and subsequent infiltration of neutrophils and other immune cells. This model can be generated in the absence of lymphocytes as evident by the development of the disease in SCID or RAG-deficient mice [64]. Therefore, DSS colitis can be considered an appropriate animal model to investigate aspects of the innate immunity contributing to disease pathogenesis.

Since aberrant expression of inflammatory cytokines, including IL-1β and IL-18 has been shown to be involved in the development of colitis, we assessed if Nalp-3 deficiency affects inflammation in DSS-induced experimental model of colitis. To this end, we compared DSS-induced colitis in NALP3-/- and wild type mice. Mice were randomized into four groups; NALP3-/- mice (n=6) receiving 3,5% DSS in drinking water, NALP3-/- receiving tap water (n=5), B57BL6 mice receiving 3,5% DSS in drinking water (n=4) and C57BL6 mice receiving tap water (n=2) for 7 days. It should be noted that C57BL6 mice show intermediate to high susceptibility to acute DSS colitis. Mice were killed with cervical dislocation on day 7.

We initially investigated the susceptibility of NALP3-/- mice to the development of acute DSS-induced colitis by analyzing mortality, loss of body weight, and histology of the distal colon. As illustrated in Fig.15a, NALP3-/- mice showed high mortality upon DSS administration. Strikingly, one NALP3-/- mouse died following 6 days of DSS exposure, while two more NALP3-/- mice died on the seventh day (50% mortality). On the other hand, all C57BL6 mice receiving DSS survived until the end of the experiment.

In accordance with the observed difference in survival, NALP3-/- mice showed more severe weight loss compared with the wild type control mice. In particular, significant weight loss occurred toward the end of DSS administration. Importantly, the difference between NALP3-/- and wild type mice treated with DSS became significant after day 5 of DSS administration (Fig.15b).

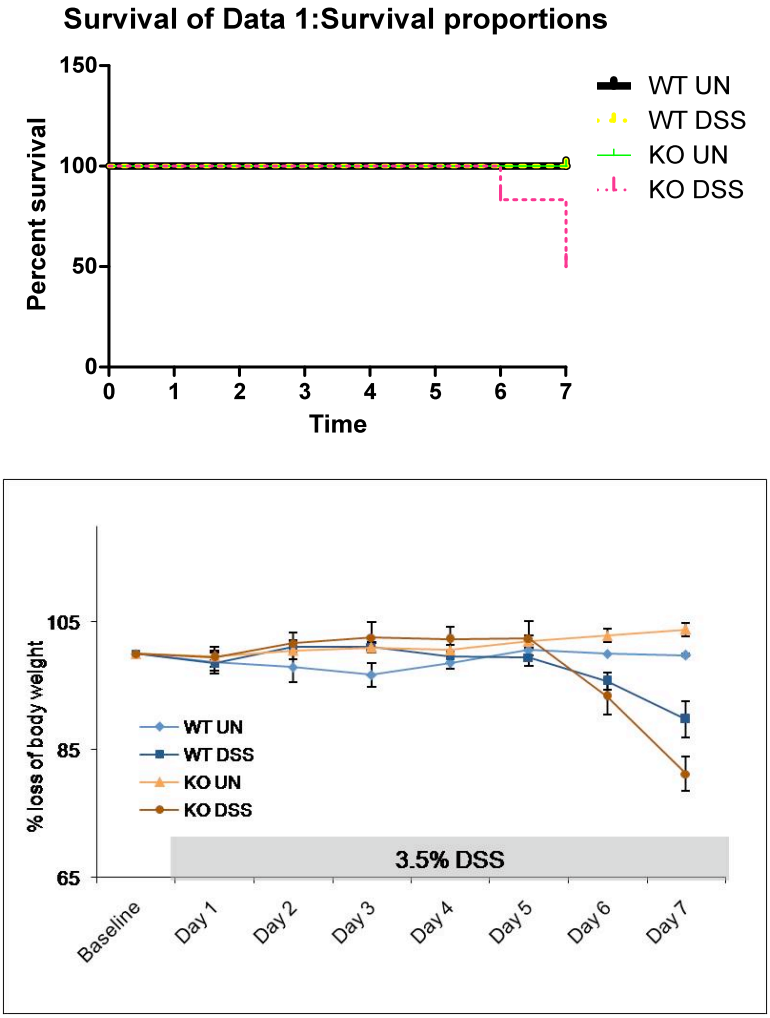

Fig.15 DSS-induced colitis in NALP3-/- mice. Mice were given 3,5% DSS in drinking water or drinking water for 7 days. The survival (a) and weight loss (b) of each mouse genotype was plotted.

The macroscopic examination of the colon on day 7 following DSS administration revealed that the colon in NALP3-/- mice was more hemorrhagic than that of wild type mice (Fig.16a) Although, the length of the DSS treated colons was shorter than that of untreated mice, no significant difference was observed between the colon length between NALP3-/- and wild type DSS treated mice. Histological analysis of the distal colon from NALP3-/- mice on day 7 after DSS administration showed an increased number of inflammatory cells and a general loss of architecture, including damage to both goblet cells and epithelial cells. No signs of colitis were seen in mice that were not treated with DSS (Fig.16b).

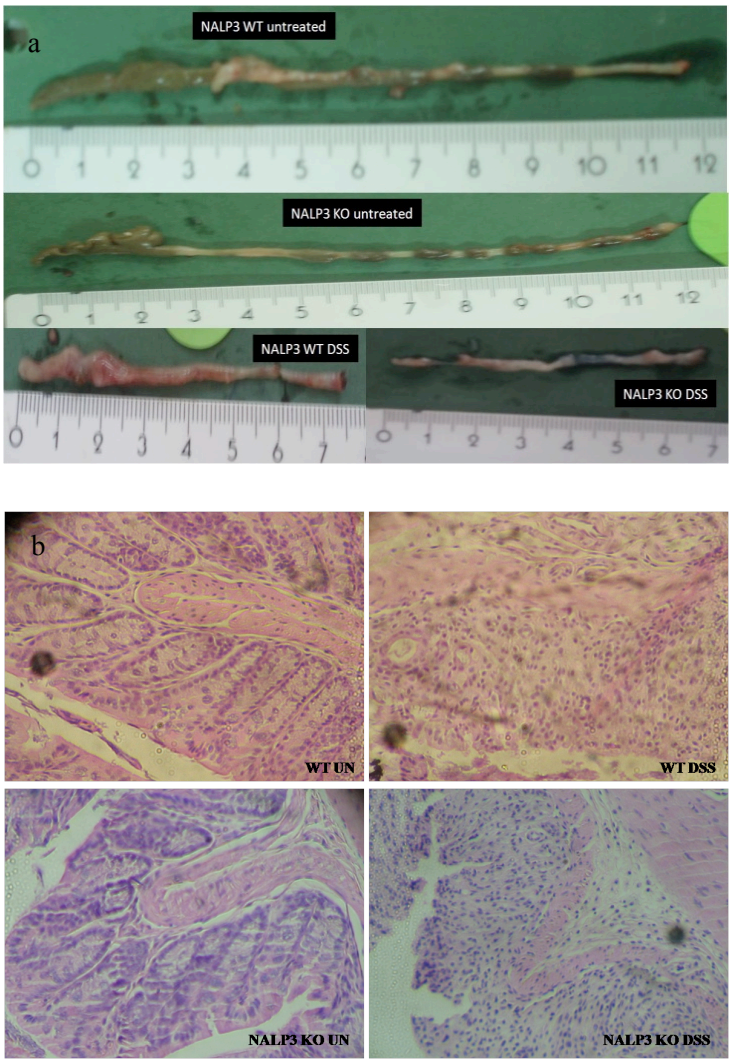

Fig.16 Macroscopic and microscopic appearance of colitis severity in NALP3-/- and wild type mice. (a) Typical colon appearance 7 days after DSS administration of each mouse genotype. (b) H&E staining of representative sections of the distal colon of each mouse genotype.

Since NALP3-/- mice seemed to be more susceptible to DSS-induced colitis, we reasoned that impaired innate immune response mediated by NALP3-dependent IL-1β may regulate the inflammatory cascade that drives colonic inflammation. Consequently, we measured the production of IL-1β and TNF-α in tissue samples, with the later being a well-established key player in colitis pathogenesis. As illustrated in Fig.17a wild type DSS-treated mice produced higher levels of IL-1β compared to their untreated counterparts. Notably, both untreated and DSS-treated NALP3-/- mice produced comparable amounts of IL-1β. More surprisingly, NALP3-/- DSS-treated mice produced equal levels of IL-1β compared to wild type DSS-

treated mice. Regarding TNF-α production, as indicated in Fig.17b NALP3-/- DSS-treated mice produced higher levels than wild type DSS-treated mice, albeit not so striking. Moreover, DSS-treated mice of each genotype produced lower amounts of TNF-α compared to their untreated counterparts.

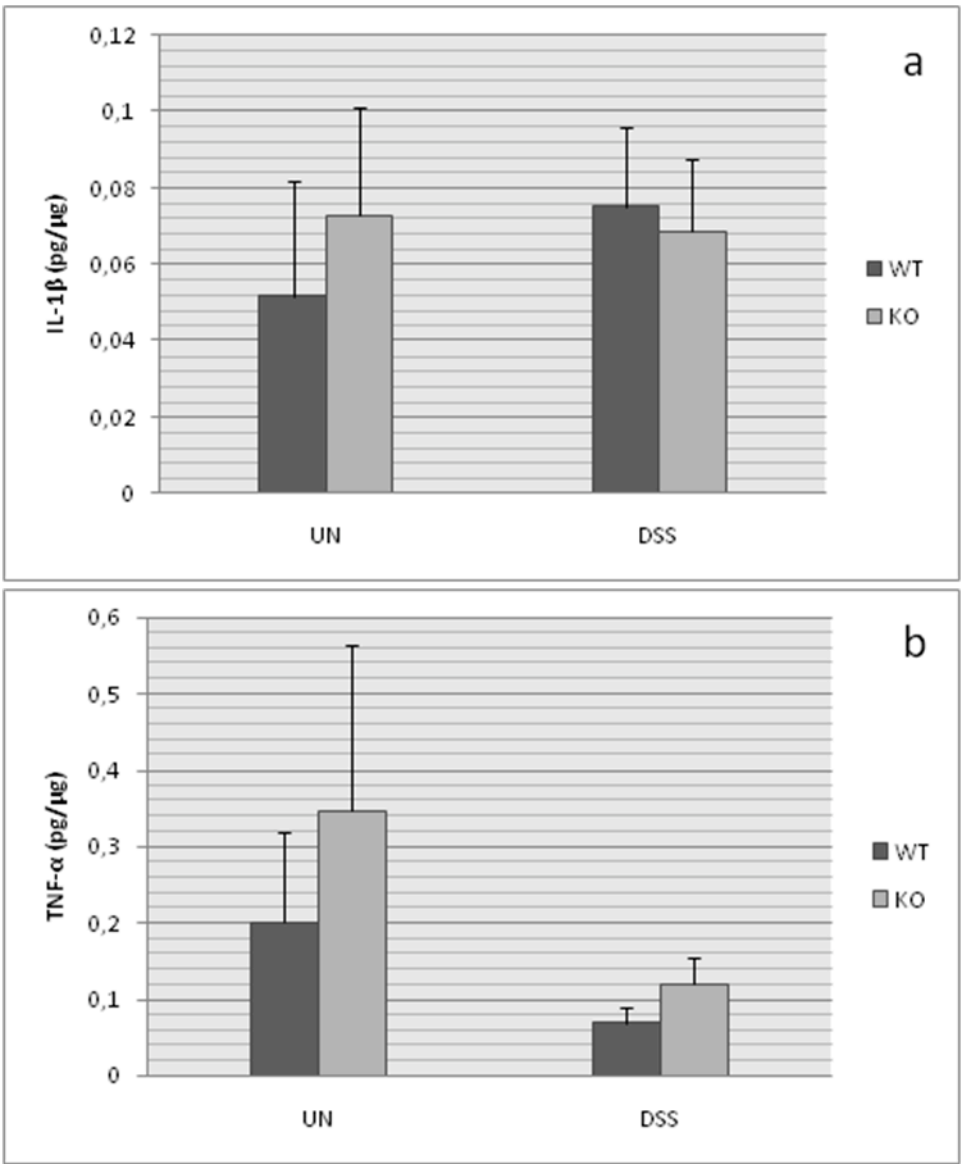

Fig.17 Expression levels of IL-1β and TNF-α in mouse colonic tissues. Concentrations of IL-1β (a) and TNF-α (b) in colonic lysates from untreated and DSS-treated mice of each genotype were determined by ELISA.

5. Discussion

Although the adaptive immune system has been the primary focus of research into the pathogenesis of inflammatory bowel disease (IBD), it is now clear that innate immune responses play an equally important or perhaps even more prominent role in disease initiation. This shift in focus is mainly based on the identification of Nod2 gene as an NLR related to CD susceptibility. However, recent evidence suggests that NALP3, the scaffold protein of NALP3-inflammsome, could also be involved in IBD pathogenesis. European investigators have found that NLRP3 SNPs are associated with 1.78 odds ratio of developing CD. Moreover, they observed a significant association between SNPs and NLRP3 expression and IL-1β production [48].

Consequently, in this study we examined the potential role of NALP3 inflammasome in CD pathogenesis. Our study comprise of two parts: The first part focused on the assessment of NALP3 inflammasome activity in human IBD, while the second one aimed to study the in vivo role of NALP3 inflammasome in an experimental model of acute colitis.

Regarding the first part of the study, we initially aimed to reproduce NALP3 inflammasome activation conditions in whole blood cells in healthy donors. We chose to stimulate whole blood cells ex vivo, since whole blood culture approximates the state of circulating cells in vivo and may be the most appropriate milieu to study ex vivo cell activation and cytokine production. Like previous studies [29], we activated NALP3 inflammasome in whole blood cells isolated from healthy donors, by priming peripheral blood cells with LPS and pulsing them with ATP (Fig.11). Unlike previous studies suggesting that MDP as an activator of both NFkB via NOD2 and caspase-1 via NALPs fulfills all the criteria for an optimal activation of NALP3 inflammasome [61], we were not able to detect significant amounts of IL-1β in supernatants of whole blood cells stimulated with low or high concentrations of MDP alone (Fig.9). Consistent with a more recent study [62], MDP induced IL-1β secretion after priming of cells with LPS (Fig.10). Furthermore, we confirmed that MDP and LPS have synergistic effects on induction of proinflammatory cytokines (IL-1β), especially at low LPS and MDP concentrations [63] (Fig.10).

After having established NALP3 inflammasome activation conditions, we next compared NALP3 inflammasome activation in healthy controls versus IBD patients. Based on our results, it was clear that no significant differences of IL-1β levels were obtained between healthy controls and IBD patients upon either classic (LPS+ATP) or

maximal (LPS+MDP+ATP) NALP3 inflammasome activation (Fig.11, Fig.12). On the other hand, patients tended to produce higher levels of IL-1β upon MDP+ATP stimulation compared to healthy controls (Fig.12). Notably, patients produced higher levels of TNF-α levels upon MDP+ATP compared to healthy controls (Fig.13), suggesting a positive loop between IL-1β and TNF-α secretion. In addition, while synergistic effect of LPS and MDP for IL-1β production was more evident in patients compared to controls, application of ATP dampened this difference between the two groups (Fig.12), suggesting that there is a differential NALP3 inflammasome activation in CD when only TLR and NOD2 is activated and not when a potent NALP3 trigger (like ATP) is applied.

Evaluation of NALP3 inflammasome activity in periphery was followed by the assessment of NALP3 inflammasome activity in the target organ-colon. To this end, western blot analysis of colonic lysates for the expression of NALP3 inflammasome components was performed. Western blot analysis of colonic lysates (from three healthy controls, one inflammatory control and one CD patient) showed the presence of the precursor of caspase-1 in all lysates. Surprisingly, in contrast to normal colonic lysates, CD patient tissue expressed a significant amount of the bioactive form of caspase-1. Consistent with a previous study, local inflammation is likely to be responsible for the process of immature caspase-1 to its active form [57]. However, mature IL-1β was not detected in any of the samples tested (Fig.14). Probably, mature IL-1β is difficult to detect in cell extracts given that it is rapidly secreted and enters the systemic circulation. Otherwise, the amount of mature IL-1β is below the sensitivity of western blot analysis shown here. The aforementioned results combined with the observation that NALP3 expression has no differences among the samples tested suggest that caspase-1 inflammasome, but not essentially the NALP3 one, could be implicated in Crohn's disease activity. Nevertheless, a larger number of samples from CD patients, inflammatory controls and UC patients need to be analyzed to draw any firm conclusions.

On the second part of the study, we examined the role of NALP3 in an animal model of acute colitis. We chose the DSS model of colitis, since we wished to examine the role of NALP3 in the early stages of colitis when the innate immune response may be more relevant. This model does not depend on the adaptive immune system, given that immunodeficient mice are still susceptible to DSS injury [64]. We induced DSS colitis in both C57BL6 and NALP3 deficient mice. We found that

NALP3-/- mice showed higher mortality when compared with their wild type DSS treated counterparts (Fig.15a). In agreement with the difference observed in survival, there was a trend of NALP3-/- mice losing more weight compared to wild type DSS treated mice. Notably, weight loss was more profound toward the end of DSS administration (Fig.15b). Taken together, these results indicated that NALP3-/- mice are more susceptible to DSS colitis, revealing a critical role of NALP3 in the early phase of experimental colitis. Following macroscopic and microscopic analysis of colon revealed more haemorrhage and increased numbers of infiltrating cells correlating with the intensity of inflammation. However, no significant differences were observed between NALP3-/- and wild type DSS treated mice (Fig.16a, b).

Since NALP3-/- mice seemed to be more susceptible to DSS-induced colitis, we reasoned that impaired innate immune response mediated by NALP3-dependent IL-1β may regulate the inflammatory cascade that drives colonic inflammation. Consequently, we measured the production of IL-1β and TNF-α as well, with the later being a well-established key player in colitis pathogenesis. Notably, the presence of mature IL-1β in colonic supernatants of NALP3-/- mice suggested that NALP3 independent pathways are responsible for the observed IL-1β production. Despite the increased mortality and body weight loss observed in NALP3-/- DSS treated mice, they produced equal levels of IL-1β compared to wild type DSS treated mice (Fig.17a), suggesting either that IL-1β is not the major proinflammatory cytokine driving the colonic inflammation or that IL-1β plays an important role at earlier stages of the disease, as has been previously shown for Atg16L1-/- DSS treated mice [65]. Regarding TNF-α production, DSS treated mice of each genotype produced lower levels of TNF-α compared to untreated mice, a result which is in contrast to previous studies showed increased TNF-α levels in wild type DSS-treated mice [66]. This discrepancy may be due to different protocols used in the two studies. More importantly, NALP3-/- DSS treated mice produced higher levels of TNF-α relative to their wild type counterparts, although the difference was not striking. Although increased lethality of NALP3-/- DSS fed mice compared to untreated mice could be attributed to decreased TNF-α production, meaning impaired immune response, the increased death rate of these mice compared to their wild type counterparts could not be explained.

The increased mortality could be explained through impaired innate immunity responses at the early phases of the disease as for example is seen with IL-18-/- mice.

More specifically, IL-18-/- animals that were given DSS, were defective in their ability to properly repair and restitute ulcerated epithelium, and there was an absence of IL-11 in restituting epithelium compared with wild type mice in which epithelium-derived IL-11 was potently up-regulated following DSS administration. These data support the protective role of IL-18 in the acute phase of colitis [67]. Importantly, NALP3 deficiency leads not only to IL-1β but to IL-18 deficiency as well, since the latter is also a NALP3 inflammasome substrate. Taken together these data suggest that NALP3 deficiency could cause a severe DSS colitis phenotype via an IL-18 deficient mechanism.

References

1. Chen, G., et al., *NOD-like receptors: role in innate immunity and inflammatory disease.* Annu Rev Pathol, 2009. 4: p. 365-98.
2. Harton, J.A., et al., *Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains.* J Immunol, 2002. 169(8): p. 4088-93.
3. Inohara, N. and G. Nunez, *NODs: intracellular proteins involved in inflammation and apoptosis.* Nat Rev Immunol, 2003. 3(5): p. 371-82.
4. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response.* Nature, 2006. 442(7098): p. 39-44.
5. Ting, J.P., et al., *The NLR gene family: a standard nomenclature.* Immunity, 2008. 28(3): p. 285-7.
6. Franchi, L., et al., *The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis.* Nat Immunol, 2009. 10(3): p. 241-7.
7. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. 296(5566): p. 301-5.
8. Hoffman, H.M., et al., *Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome.* Nat Genet, 2001. 29(3): p. 301-5.
9. Anderson, J.P., et al., *Structural, expression, and evolutionary analysis of mouse CIAS1.* Gene, 2004. 338(1): p. 25-34.
10. Martinon, F. and J. Tschopp, *Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases.* Cell, 2004. 117(5): p. 561-74.
11. Martinon, F. and J. Tschopp, *Inflammatory caspases and inflammasomes: master switches of inflammation.* Cell Death Differ, 2007. 14(1): p. 10-22.
12. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.* Mol Cell, 2002. 10(2): p. 417-26.
13. Agostini, L., et al., *NALP3 forms an IL-1βeta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder.* Immunity, 2004. 20(3): p. 319-25.
14. Tschopp, J., F. Martinon, and K. Burns, *NALPs: a novel protein family involved in inflammation.* Nat Rev Mol Cell Biol, 2003. 4(2): p. 95-104.
15. Razmara, M., et al., *CARD-8 protein, a new CARD family member that regulates caspase-1 activation and apoptosis.* J Biol Chem, 2002. 277(16): p. 13952-8.
16. Petrilli, V., et al., *The inflammasome: a danger sensing complex triggering innate immunity.* Curr Opin Immunol, 2007. 19(6): p. 615-22.
17. Mariathasan, S., et al., *Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf.* Nature, 2004. 430(6996): p. 213-8.
18. Sutterwala, F.S., et al., *Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1.* Immunity, 2006. 24(3): p. 317-27.
19. Mayor, A., et al., *A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses.* Nat Immunol, 2007. 8(5): p. 497-503.
20. Duncan, J.A., et al., *Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling.* Proc Natl Acad Sci U S A, 2007. 104(19): p. 8041-6.
21. Dinarello, C.A., *Interleukin-18.* Methods, 1999. 19(1): p. 121-32.
22. O'Connor, W., Jr., et al., *Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory mediator with NF-kappa B suppressive properties.* J Immunol, 2003. 171(12): p. 6329-33.

23. Wang, S., et al., *Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE.* Cell, 1998. 92(4): p. 501-9.
24. Ferrari, D., et al., *The P2X7 receptor: a key player in IL-1 processing and release.* J Immunol, 2006. 176(7): p. 3877-83.
25. Netea, M.G., et al., *The role of NLRs and TLRs in the activation of the inflammasome.* Expert Opin Biol Ther, 2008. 8(12): p. 1867-72.
26. Netea, M.G., et al., *Differential requirement for the activation of the inflammasome for processing and release of IL-1βeta in monocytes and macrophages.* Blood, 2009. 113(10): p. 2324-35.
27. Yu, H.B. and B.B. Finlay, *The caspase-1 inflammasome: a pilot of innate immune responses.* Cell Host Microbe, 2008. 4(3): p. 198-208.
28. Perregaux, D. and C.A. Gabel, *Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity.* J Biol Chem, 1994. 269(21): p. 15195-203.
29. Mariathasan, S., et al., *Cryopyrin activates the inflammasome in response to toxins and ATP.* Nature, 2006. 440(7081): p. 228-32.
30. Petrilli, V., et al., *Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration.* Cell Death Differ, 2007. 14(9): p. 1583-9.
31. Andrei, C., et al., *Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes.* Proc Natl Acad Sci U S A, 2004. 101(26): p. 9745-50.
32. Kanneganti, T.D., et al., *Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling.* Immunity, 2007. 26(4): p. 433-43.
33. Dostert, C., et al., *Innate immune activation through NALP3 inflammasome sensing of asbestos and silica.* Science, 2008. 320(5876): p. 674-7.
34. Cassel, S.L., et al., *The NALP3 inflammasome is essential for the development of silicosis.* Proc Natl Acad Sci U S A, 2008. 105(26): p. 9035-40.
35. Cruz, C.M., et al., *ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages.* J Biol Chem, 2007. 282(5): p. 2871-9.
36. Eisenbarth, S.C., et al., *Crucial role for the NALP3 inflammasome in the immunostimulatory properties of aluminium adjuvants.* Nature, 2008. 453(7198): p. 1122-6.
37. Bossi, O., et al., *UV irradiation increases ROS production via PKCdelta signaling in primary murine fibroblasts.* J Cell Biochem, 2008. 105(1): p. 194-207.
38. Hornung, V., et al., *Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization.* Nat Immunol, 2008. 9(8): p. 847-56.
39. Meissner, F., K. Molawi, and A. Zychlinsky, *Superoxide dismutase 1 regulates caspase-1 and endotoxic shock.* Nat Immunol, 2008. 9(8): p. 866-72.
40. Halle, A., et al., *The NALP3 inflammasome is involved in the innate immune response to amyloid-beta.* Nat Immunol, 2008. 9(8): p. 857-65.
41. Willingham, S.B., et al., *Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC.* Cell Host Microbe, 2007. 2(3): p. 147-59.
42. Sartor, R.B., *Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis.* Nat Clin Pract Gastroenterol Hepatol, 2006. 3(7): p. 390-407.
43. Macdonald, T.T. and G. Monteleone, *Immunity, inflammation, and allergy in the gut.* Science, 2005. 307(5717): p. 1920-5.
44. Xavier, R.J. and D.K. Podolsky, *Unravelling the pathogenesis of inflammatory bowel disease.* Nature, 2007. 448(7152): p. 427-34.
45. Yamamoto-Furusho, J.K. and J.R. Korzenik, *Crohn's disease: innate immunodeficiency?* World J Gastroenterol, 2006. 12(42): p. 6751-5.

46. Bamias, G., et al., *New concepts in the pathophysiology of inflammatory bowel disease.* Ann Intern Med, 2005. 143(12): p. 895-904.
47. Eckmann, L. and M. Karin, *NOD2 and Crohn's disease: loss or gain of function?* Immunity, 2005. 22(6): p. 661-7.
48. Villani, A.C., et al., *Common variants in the NLRP3 region contribute to Crohn's disease susceptibility.* Nat Genet, 2009. 41(1): p. 71-6.
49. Schoultz, I., et al., *Combined polymorphisms in genes encoding the inflammasome components NALP3 and CARD8 confer susceptibility to Crohn's disease in Swedish men.* Am J Gastroenterol, 2009. 104(5): p. 1180-8.
50. Marks, D.J. and A.W. Segal, *Innate immunity in inflammatory bowel disease: a disease hypothesis.* J Pathol, 2008. 214(2): p. 260-6.
51. Inohara, N., et al., *Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease.* J Biol Chem, 2003. 278(8): p. 5509-12.
52. Sands, B.E., et al., *Infliximab maintenance therapy for fistulizing Crohn's disease.* N Engl J Med, 2004. 350(9): p. 876-85.
53. Bamias, G. and F. Cominelli, *Novel strategies to attenuate immune activation in Crohn's disease.* Curr Opin Pharmacol, 2006. 6(4): p. 401-7.
54. Siegmund, B., *Interleukin-1beta converting enzyme (caspase-1) in intestinal inflammation.* Biochem Pharmacol, 2002. 64(1): p. 1-8.
55. Nakamura, K., et al., *Novel strategies for the treatment of inflammatory bowel disease: Selective inhibition of cytokines and adhesion molecules.* World J Gastroenterol, 2006. 12(29): p. 4628-35.
56. Cominelli, F., et al., *Interleukin 1 (IL-1) gene expression, synthesis, and effect of specific IL-1 receptor blockade in rabbit immune complex colitis.* J Clin Invest, 1990. 86(3): p. 972-80.
57. McAlindon, M.E., C.J. Hawkey, and Y.R. Mahida, *Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease.* Gut, 1998. 42(2): p. 214-9.
58. Cominelli, F. and T.T. Pizarro, *Interleukin-1 and interleukin-1 receptor antagonist in inflammatory bowel disease.* Aliment Pharmacol Ther, 1996. 10 Suppl 2: p. 49-53; discussion 54.
59. Neuman, M.G., *Immune dysfunction in inflammatory bowel disease.* Transl Res, 2007. 149(4): p. 173-86.
60. Sartor, R.B., *Microbial influences in inflammatory bowel diseases.* Gastroenterology, 2008. 134(2): p. 577-94.
61. Martinon, F., et al., *Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome.* Curr Biol, 2004. 14(21): p. 1929-34.
62. Pan, Q., et al., *MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3.* J Leukoc Biol, 2007. 82(1): p. 177-83.
63. van Heel, D.A., et al., *Muramyl dipeptide and toll-like receptor sensitivity in NOD2-associated Crohn's disease.* Lancet, 2005. 365(9473): p. 1794-6.
64. Pizarro, T.T., et al., *Mouse models for the study of Crohn's disease.* Trends Mol Med, 2003. 9(5): p. 218-22.
65. Saitoh, T., et al., *Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1βeta production.* Nature, 2008. 456(7219): p. 264-8.
66. Xiang, J.Y., et al., *Amelioration of murine dextran sulfate sodium-induced colitis by nuclear factor-kappaB decoy oligonucleotides.* Am J Surg, 2009. 197(6): p. 797-805.
67. Reuter, B.K. and T.T. Pizarro, *Commentary: the role of the IL-18 system and other members of the IL-1R/TLR superfamily in innate mucosal immunity and the pathogenesis of inflammatory bowel disease: friend or foe?* Eur J Immunol, 2004. **34**(9): p. 2347-55.