TRANSCRIPTIONAL REGULATION
AND
FUNCTION OF NLRP12

PhD Thesis

SINEM TUNCER

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2014

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ABSTRACT

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are evolutionarily conserved intracellular PRRs (Pattern Recognition Receptors) that play an important role in host defense and physiology. NLRP12 is a NLR protein which is expressed predominantly in cells of myeloid origin. Because NLRP12 expression is restricted to immune cells and its expression is down-regulated in response to pathogens, pathogen products and inflammatory cytokines, it has been predicted that NLRP12 functions in regulating inflammation and immunity. Although NLRP12 was one of the earliest identified NLRPs, its precise function(s) is not fully understood as yet. Despite the increasing number of publications, the role of NLRP12 remains ambiguous because of conflicting results.

In this PhD thesis, the regulation of *NLRP12* promoter activity by NF-κB, the transcriptional modulation of NLRP12 expression during myeloid cell lines and primary monocytes stimulation/differentiation as well as the effects of NLRP12 over-expression on the growth of monocytic cell line U937 are reported.

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LIST OF ABBREVIATIONS

AD: Transactivator domain

AIM2: Absent in Melanoma2

AML: Myeloid leukaemia

AP-1: Activator protein-1

Arg: Arginine

ASC: Apoptosis-associated speck-like protein containing a CARD domain

Asp: Aspartic acid

ATP: Adenosine triphosphate

BAFF: B cell activating factor

BIR: Baculoviral inhibitor of apoptosis repeat

Birc: Baculoviral inhibitors of apoptosis repeat containing protein

Blimp-1: B lymphocyte-induced maturation protein-1

BLS: Bare lymphocyte syndrome

BMDC: Bone marrow derived dendritic cells

βTrCP: β-transducin repeat-containing protein

C-terminal: Carboxy-terminal

CIITA: MHC class II transcriptional activator

CARD: Caspase recruitment domain

CATERPILLER: Caspase activation and recruitment domains (CARD), transcription enhancer, R (purine)-binding, lots of leucine repeats

CBP: CREB binding protein

CD40L: CD40 ligand

CDS: Cytosolic DNA sensor

cIAP: Cellular inhibitor of apoptosis

CLR: C-Type lectin receptor

CO$_2$: Carbon dioxide

CREB: cAMP response element-binding protein

CRM1: Chromosome region maintenance 1

CREB: cAMP response element-binding protein

Cys: Cysteine

CXCL12: Chemokine (C-X-C motif) ligand 12

CXCL13: Chemokine (C-X-C motif) ligand 13

CXCR4: Chemokine (C-X-C motif) receptor 4

DAI: DNA dependent activator of IFN-regulatory factor

DAMPs: Damage-associated molecular patterns

DC: Dendritic cell

ds: double strand

EDTA: Ethylenediaminetetraacetic Acid

EGFP: Enhanced green fluorescent protein

EGTA: Ethylene glycol tetraacetic acid

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

Glu: Glutamic acid

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HGNC: HUGO Gene Nomenclature Committee

HLA: Human leukocyte antigen

HRP: Horseradish peroxidase

HSP: Heat-shock protein

Hsp70: Heat shock protein 70

Hsp90: Heat shock protein 90

IBD: Inflammatory bowel disease

IFI16: Gamma-interferon-inducible protein Ifi-16

IFN: Interferon

IgG: Immunoglobulin G

IKK: IκB kinase

IL: Interleukin

IKK: Inhibitor of NF-κB kinase

IPS-1: IFN-β Promoter Stimulator-1

IRAK1: Interleukin-1 receptor-associated kinase

IRF: Interferon regulatory factor

IκB: Inhibitor of NF-κB

kb: Kilo base

kDa: Kilo dalton

LPS: Lipopolysaccharide

LRR: Leucine-rich repeats

LRRFIP1: Leucine rich repeat in FLII interacting protein 1

Luc: Luciferase

MAL: MYD88-adaptor-like protein

MAPK: Mitogen-activated protein kinase

M-CSF: Macrophage Colony-Stimulating Factor

MDA5: Melanoma Differentiation-Associated protein 5

MHC: Major Histocompatibility Complex

MyD88: Myeloid differentiation factor 88

N-terminal: Amino-terminal

NaF: Sodium fluoride

NAIP: NLR family, apoptosis inhibitory protein

NALP: NACHT domain-, leucine-rich repeat-, and pyrin-containing protein

NBD: Nucleotide binding domain

NEMO: NF-kappa-B essential modulator

NF-Y: Nuclear Factor-Y

NF-κB: Nuclear factor kappa B

NIK: NF-κB inducing kinase

NLR: Nucleotide binding domain leucine-rich repeat

NOD: Nucleotide-binding and oligomerization domain

PAMPs: Pathogen-associated molecular patterns

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PGN: Peptidoglycan

PI: Propidium Iodide

PKR: Protein kinase regulated by RNA

PMA: Phorbol 12-myristate 13-acetate

PMSF: Phenylmethylsulfonyl fluoride

PRR: Pattern recognition receptors

PYD: Pyrin domain

qRT-PCR: Quantitative reverse transcriptase PCR

R protein: Disease resistance protein

RFX5: Regulatory factor X, 5

RIG-I: Retinoic acid inducible gene 1

RLH: RIG-like helicases

RLR: RIG-I-like receptor

rno: Regulated by Nitric Oxide

ROS: Reactive oxygen species

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

STING: Stimulator of interferon genes

TAB: TAK1-binding protein

TAK1: Transforming growth factor beta-activated kinase 1

TIR: Toll/IL-1β receptor

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

TRAF: TNF receptor associated factor 6

TRAM: TIR domain-containing adaptor protein

Val: Valine

WT: Wild type

CHAPTER 1

INTRODUCTION

Vertebrates have the immune system to protect themselves against infectious agent. The innate and adaptive immune systems developed during evolution for immune detection. All vertebrates use the innate immune system, however, only jawed vertebrates use both innate and adaptive immune systems. Adaptive immune cells, such as T and B lymphocytes, in jawed vertebrates can express nearly an unlimited number of antigen specific receptors[1]. Antigen recognition by these receptors causes lymphocytes to undergo clonal proliferation, to generate daughter cells with the potential to recognize the same pathogen[2]. These lymphocytes form effector cells for clearing the infection and memory cells to react quickly upon secondary exposure to the same antigen. While the adaptive immune system responses take days to occur, innate immune responses occur within minutes to hours[3], therefore the innate immune system is the first line of host defense during infection since the early recognition and subsequent triggering of a pro-inflammatory response to invading pathogens is crucial. However, the adaptive immune system functions in the

elimination of pathogens in the late phase of infection and in the generation of long-lasting protective immunity[4].

Innate receptors are able to detect highly conserved microbial components and respond quickly to a wide range of microbial diversity[5]. This strategy is called "pattern recognition" and relies on a germline encoded, limited set of pattern recognition receptors (PRRs). The molecular patterns which are detected by PRRs are broadly classified into two categories on the basis of their origin: pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[6]. PAMPs refer to exogenous molecules which are found particularly on a group of pathogens. These include patterns such as cell wall components, flagella, lipoproteins, and nucleic acids of bacterial, fungal, and viral origin[5, 6, 7]. In contrast to PAMPs, DAMPs are endogenous molecules which are released or modified by the host cell upon stress or damage, like DNA-binding proteins, heat-shock proteins (HSPs), extracellular ATP, and uric acid crystals[7, 8].

1.1. PRRs Families

PRRs are expressed by cells of innate immunity including monocytes, macrophages, dendritic cells, endothelial cells and neutrophils, as well as cells of the adaptive immunity[9]. PRRs located on the cell surface recognize the microbes present in the extracellular environment[10]. Cytoplasmic PRRs recognize

intracellular cytosolic pathogens and their derivatives, endosomal PRRs interact with microbes that have entered the phagolysosomal degradation pathway[5]. The recognition of PAMPs or DAMPs by the PRRs initiates an inflammatory response including cytokines and chemokines secretion, the induction of host defense peptides, pyroptotic cell death and the recruitment of phagocytes[11, 12].

The PRRs families of the innate immune system are C-type lectin receptors (CLRs), RIG-I-Like receptors (RLRs), Cytosolic DNA sensors (CDSs), Toll-Like receptors (TLRs) and Nucleotide binding leucine rich repeat containing receptors which also known as NOD-like receptors (NLRs). Each PRRs family has a diverse structure and acts differently to detect pathogens and generate the appropriate immune responses [13] (**Fig. 1.1**).
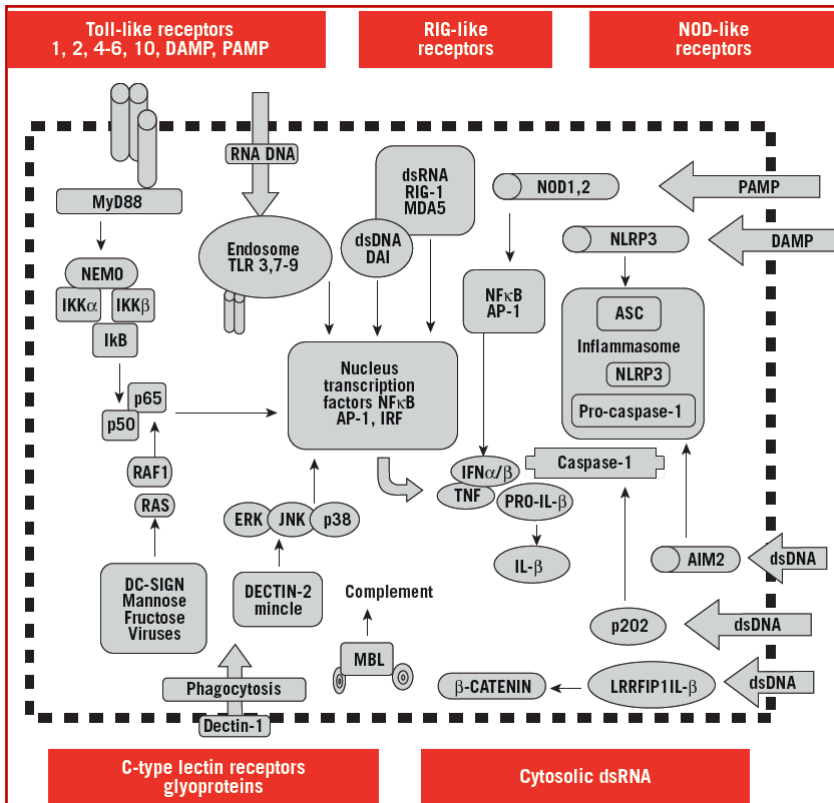
Fig. 1.1. Schematic representation of PRRs and the downstream signaling events: The PRRs families of the innate immune system are C-type lectin receptors, RIG-I-Like receptors, cytosolic DNA sensors, Toll-Like receptors and NOD-like receptors. They can recognize PAMPs or endogenous danger molecules, DAMPs, and cause activation of several signalling events to mediate immun response (Adapted from Shaw, SG. and Tsui, J., 2013)[13].

1.1.1. C-Type lectin receptors (CLRs)

CLRs, also called the C-type lectins, act as phagocytic receptors which are critical in recognition of antigens which can be of extrinsic origins, such as viruses or other microbes as well as

intrinsic origins such as altered self lipids or proteins[14]. CLRs are found in cells which phagocytize various glycoproteins and microbes for the purposes of clearance or antigen presentation[12] (Fig. 1.2).
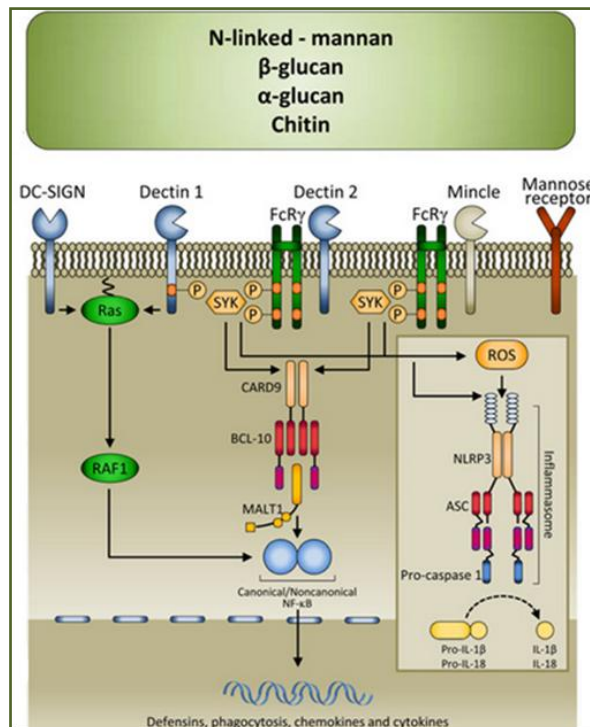
Fig. 1.2. Signalling pathways through CLRs: CLR signaling can be activated by various numbers of PAMPs. Upon ligand binding, the signaling pathways are triggered to activate NF-κB and NLRP3 inflammasome resulting in the production of defensins, chemokines, cytokines, and reactive oxygen species (Modified from Cunha, C. *et al*., 2012)[15].

1.1.2. RIG-I-like receptors (RLRs)

Another family of cytosolic PRRs is the RIG-I-like receptors (RLRs), which are the cytoplasmic sensors for RNA viruses. Recognition of viral dsRNA by Toll-Like receptor 3 (TLR3) or by RLRs is cell-type dependent[16].

The members of RLR family contain a DExH-box helicase domain that can detect the presence of RNA from a broad range of viruses[17]. Following recognition of viral RNA, the subsequent signaling cascade results in the induction of transcription factors like interferon regulatory factor 3 (IRF3), IRF7, and NF-κB (Nuclear Factor κB) which leads to the production of type I IFN (Interferon). Type I IFN binds to the IFN receptor to initiate the expression of interferon-stimulated genes like antiviral proteins, immune-proteasome components, members of the TLR family, transcription factors like IRF7, various pro-inflammatory cytokines and chemokines[4, 16, 17] **(Fig. 1.3)**.
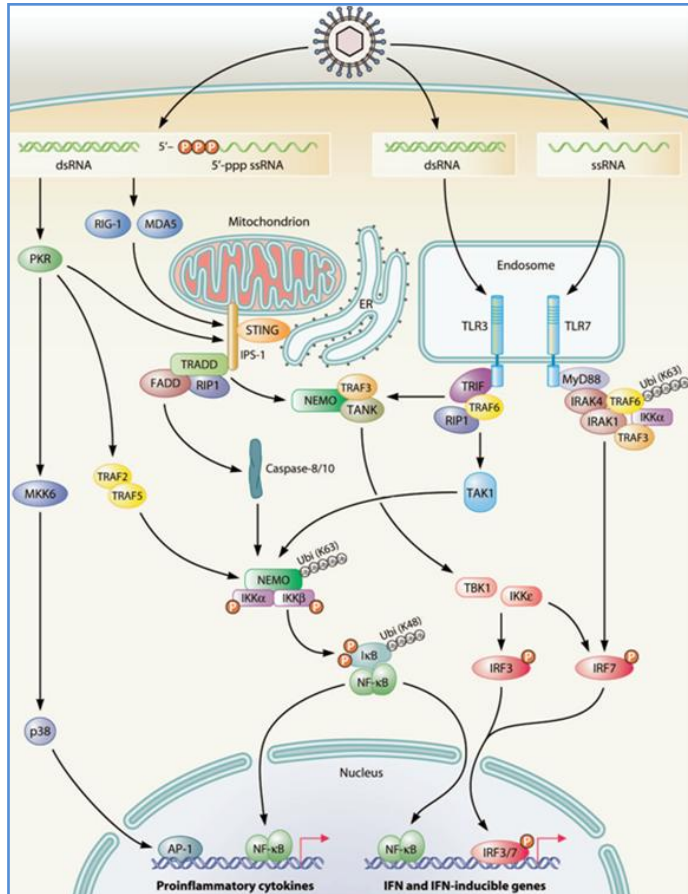
Fig. 1.3. RLRs signaling: Cytosolic dsRNA and 5'-triphosphate ssRNA is recognized primarily by RIG-I (Retinoic acid-Inducible Gene 1) and MDA5 (Melanoma Differentiation-Associated protein 5) which then interact with mitochondria localized IPS-1 (IFN-β Promoter Stimulator-1). The interaction triggers NF-κB and IRF3 activation. PKR (protein kinase regulated by RNA) is another RLR which recognizes dsRNA and activates MAPKs signalling. dsRNA and ssRNA can also be recognized by the Toll like Receptors TLR3 and TLR7/8, respectively (Adapted from Mogensen, TH., 2009)[4].

1.1.3. Cytosolic DNA sensors (CDSs)

The recognition of cytosolic DNA requires several sensors which trigger different signaling pathways depending on cell types[12].

DNA dependent activator of IFN-regulatory factor (DAI) binds cytosolic dsDNA and triggers the production of type I IFNs. AIM2 (Absent in Melanoma 2) is a cytosolic DNA receptor that associates with ASC (Apoptosis-associated speck-like protein containing a CARD) through CARD-CARD (Caspase Recruitment Domain) interactions to form an inflammasome and triggers caspase-1 activation and production of IL-1β (Interleukin-1β) and IL-18 (Interleukin-18). In contrast to AIM2, the cytosolic dsDNA sensor p202 is a negative regulator of inflammasome activity. LRRFIP1 (leucine rich repeat in FLII interacting protein1) is another cytosolic dsDNA sensor which leads to production of IFN-β in a β-catenin-dependent manner. Although RIG-I was a candidate receptor for cytosolic DNA recognition, it has been recently shown that rather than the cytosolic DNA, a double-stranded RNA intermediate is responsible for RIG-I activation[15,16,18] **(Fig. 1.4)**.
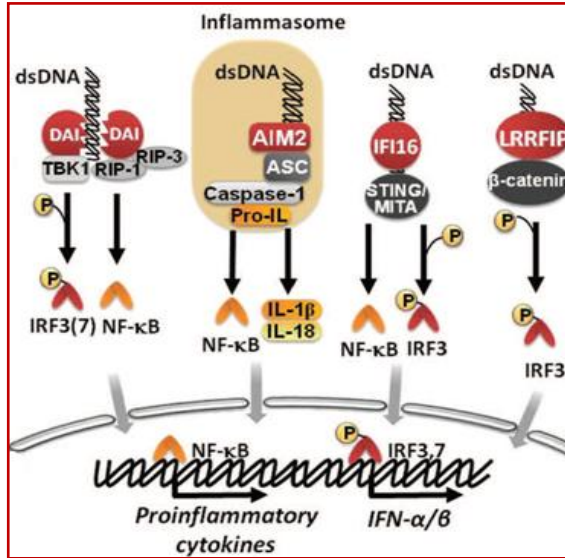
Fig. 1.4. Signaling pathways of innate immunity involved in DNA-sensing: DNA can be recognized by different sensors, including AIM2, LRRFIP1, DAI, and IFI16. Cytosolic DNA from invading viruses and bacteria activate AIM2 binding to the adaptor ASC for caspase-1 activation and subsequent IL-1β and IL-18 production which are important mediators of inflammatory responses to infection. LRRFIP1 recognizes dsDNA to induce IFNβ via a β-catenin-IRF3 trans-activator pathway. DAI induces Tank-binding kinase (TBK1)-IRF3-dependent IFNβ production following activation by ds B-form or a-typical Z-form DNA. IFI16 binds to viral DNA, directly and initiate IFNβ induction via mitochondria located STING protein (Modified from Herrada, AA. *et al*., 2012)[19].

1.1.4. Toll-like receptors (TLRs)

Among PRRs families, Toll-Like Receptors (TLRs) are the first identified and most extensively studied receptors. TLRs recognize a variety of bacterial structures like different bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipoprotein, as well as bacterial DNA and viral RNA or DAMPs which include intracellular proteins such as heat shock proteins and extracellular matrix proteins released by tissue injury. Stimulation of TLRs by the corresponding PAMPs or DAMPs triggers signaling cascades to activate transcription factors like AP-1 (activator protein 1), NF-κB and IRFs. The activated transcription factors subsequently bind to specific DNA sequences and mediate the production of effector molecules such as cytokines, inflammatory enzymes, chemokines, and type I IFNs which are important for the triggering of the immune response [5] (**Fig. 1.5**).

The large number of ligands which activate TLRs signalling include peptidoglycan, lipoteichoic acid, and zymosan (TLR2), lipopolysaccharide (TLR4), double-stranded RNA (TLR3), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8) and bacterial DNA containing non-methylated CpG motifs (TLR9)[20].
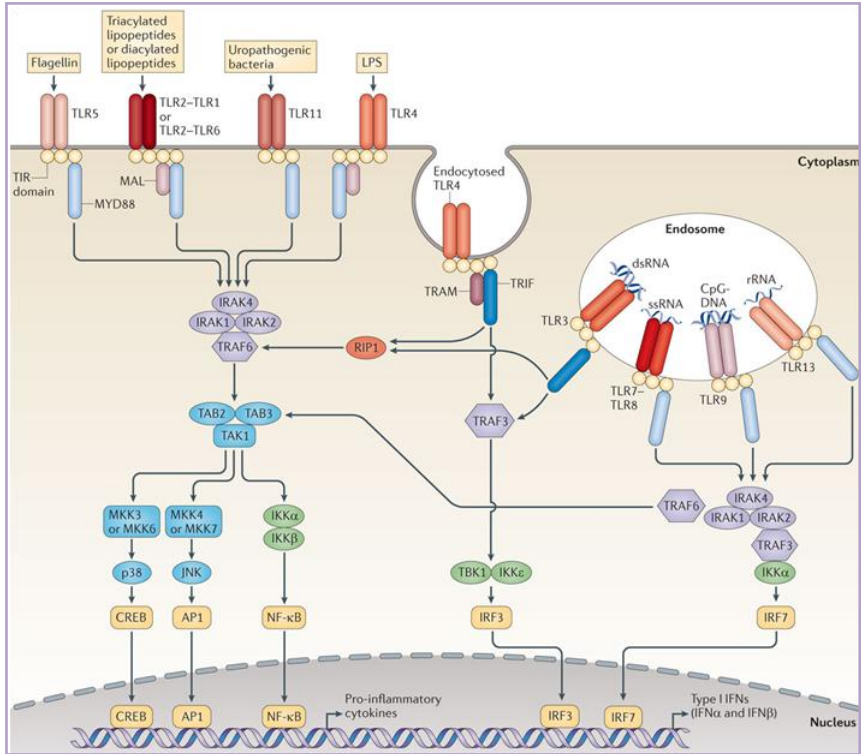
Fig. 1.5. TLR signalling: TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are located on the cell surface and TLR3, TLR7, TLR8, and TLR9 are localized to the endosomal/lysosomal compartment. TLR signalling is initiated by ligand-induced dimerization of TLRs. Following the dimerization, the cytoplasmic TIR (Toll/IL-1 receptor) domain of the TLR associates with the MyD88 via adaptor protein MAL (MYD88-adaptor-like protein) or TRAM (TIR domain-containing adaptor protein). Engagement of the signalling adaptor molecules triggers downstream signalling pathways which induces the production of pro-inflammatory cytokines and Type I IFNs through the activation of transcription factors NF-κB, IRFs, CREB (cAMP Response Element-Binding Protein) and AP1 (Adapred from O'Neill, LA., Golenbock, D., and Bowie, AG., 2013)[21].

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1.1.5. The NLR family

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are evolutionarily conserved intracellular PRRs that play an important role in host defense and physiology[22]. Mammalian NLRs share structural homology to plant disease resistance R proteins which mediate plants defense response against infection. The similarity between plant R and animal NLR proteins hints that the NLR family represents an ancient family of immune defense genes[23, 24].

The NLR proteins, in general, have a tripartite domain organization: they consist of a central nucleotide-binding and oligomerization (NACHT) domain (also known as Nucleotide Binding Domain, NBD) which enables the activation of the signaling complex via ATP-dependent oligomerization, a C-terminal leucine-rich repeats (LRRs) domain which functions in ligand sensing and autoregulation and a variable N-terminal interaction domain which mediates homotypic protein-protein interactions for downstream signaling[25, 26]. The mammalian NLRs can be divided into four subfamilies, based on different N-terminal effector domains. The effector domains found in NLRs are CARDs, pyrin domains (PYDs), baculoviral inhibitor of apoptosis repeat (BIR) domains, or the transactivator domain (AD). A standardized nomenclature system categorizes the NLR family into four subfamilies based on the initial of the domain name: NLRC (formerly known as NODs), NLRP (formerly

known as NALPs), NLRB (formerly known as NAIP or Birc) and NLRA[27, 28] (**Fig. 1.6**).

NLRs have been most recently discovered family member of PRRs and the research field is very dynamic. The research on agonists, interaction partners, and signaling pathways of NLRs are rapidly increasing. However, different studies may report contradicting results and many aspects related to the divergent functions of NLRs are not fully understood as yet[29].
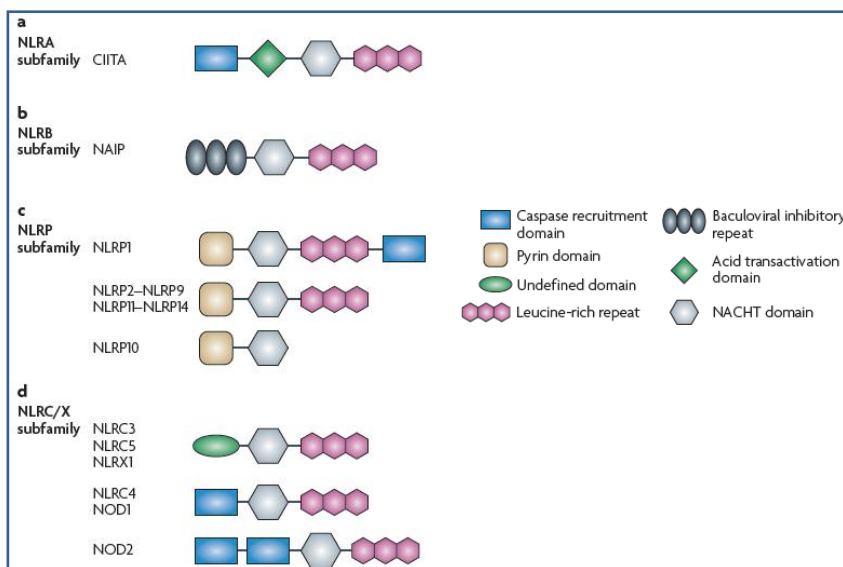
Fig. 1. 6. Members of human NLRs family: All the members NLRs family contains central nucleotide-binding and oligomerization (NACHT) domain, a carboxy-terminus leucine-rich repeat domain and a variable amino terminal domain. NLRs can be divided into four subfamilies, based on their amino terminal domain: **(a)** The unique NLRA family member is CIITA, **(b)** the NLRB subfamily consists of one member, NAIP (NLR family, apoptosis inhibitory protein), **(c)** members of the NLRP subfamily express an N-terminal

pyrin domain, **(d)** the NLRC/X subfamily members have either an N-terminal caspase recruitment domain (CARD), or an undefined domain that has no apparent homology with other proteins (Adapted from Geddes, K. *et al*., 2009)[30].

1.2. Mechanism of Action of NLRs

NLR receptor family consists of more than 20 members and only about half of them have been characterized in any detail[31]. Although it is well known that NLRs play a critical role in host defense, it remains unclear how NLRs can recognize diverse ligands[22] even if there is not much evidence to support that NLRs directly bind and/or recognize pathogens or pathogen-derived products[25]. It has been also that the upstream signaling receptors or effectors direct the activation of NLRs. Furthermore, NLRs might interact with co-receptors or dimerize with additional sensors to execute their functions[22].

NLRs can be broadly categorized into four groups depending on their functions: (1) Trans-activators of transcription, (2) Activators of NF-κB and mitogen activated protein kinase (MAPK), (3) Inflammasome activators, (4) Inhibitors of inflammatory signaling[30]. It should be also considered that some NLRs are likely to have overlapping functions. Furthermore, several NLRs function in pre-implantation and prenatal development which indicates that they can play multiple roles within inflammation or development and some NLRs might have cell type specific alternative roles or they can be activated by

multiple activation mechanisms with distinct downstream effects[30, 32-37].

1.2.1. CIITA and NLRC5 are the trans-activators of MHC expression

The class II transactivator (CIITA) was the first mammalian NLR to be characterized[35]. CIITA is expressed in macrophages, B and T lymphocytes and dendritic cells (DCs)[36]. It functions as a transcriptional coactivator at the promoter of MHC (Major Histocompatibility Complex) class II genes. In macrophages and DCs, the alternative splicing mechanism allows the production of a CIITA variant equipped with an N-terminal CARD domain. The presence of CARD domain is suggested to enhance MHC class II gene expression in professional antigen-presenting cells (APCs)[38].

N-terminal activation domain of CIITA is responsible for its recruitment to the enhanceosome, a protein complex consisting of several nuclear factors including CBP (CREB Binding Protein), RFX5 (Regulatory Factor X, 5), NF-Y (Nuclear Factor-Y) and CREB that binds cis-acting elements in the MHC class II promoter[38]. CIITA is the master regulator of MHC class II expression in the enhanceosome, driving transcription of MHC class II and MHC class II-linked antigen presentation accessory genes like the invariant chain and HLA-DM genes[38, 39] **(Fig. 1.7)**.

CIITA is constitutively expressed by immune cells, and its expression is significantly induced by IFN-γ[40]. Type II Bare

Lymphocyte Syndrome (BLS), a severe immunodeficiency disorder, is caused by the lack of MHC class II expression on the cell surface which is the result of the loss of function mutation in CIITA[41].

Recently, the NLR family member NLRC5 (Nucleotide Binding domain and Leucine Rich Repeat Containing 5) was shown to transcriptionally activate MHC class I genes as well as β2M, TAP1 and LMP2, essential components of MHC class I antigen processing and presentation[42].
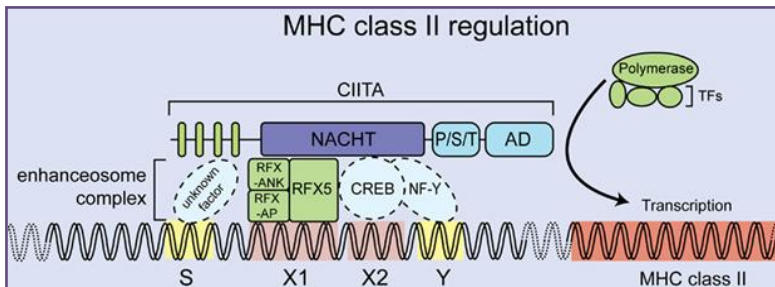
Fig. 1.7. Assembly of CIITA mediated enhanceosome: RFX proteins, CREB and NF-Y bind to SXY module which is characteristic of the MHC class II gene promoters. The assembly of enhanceosome constitutes a platform for the recruitment of CIITA to mediate transcription (Modified from Neerincx, A. *et al.*, 2013)[43].

1.2.2. NLRs as activators of NF-κB and MAPK

NOD1 (NLRC1 or CARD4) and NOD2 (NLRC2 or CARD15) are the members of NLRs which regulate NF-κB and MAPK[31]. NOD1 and NOD2 recognize the iE-DAP and MDP bacterial peptidoglycans, respectively[44]. Upon ligand binding, NOD1 and NOD2 undergo conformational changes and self-oligomerization via the central NOD domain, followed by the recruitment and activation of the serine threonine kinase RICK (RIP2) by CARD-CARD interactions[25]. Assembly of NOD1 and NOD2 signalosomes result in the activation of NF-κB and MAPKs which drives the up-regulation of pro-inflammatory genes[45].

NOD2 has recently been described as having other roles besides its well characterized role in RIP2-dependent NF-κB activation. There are multiple reports which demostrate that NOD2 can respond to cytosolic RNA during viral infection and potentiates antiviral signaling[38, 44]. In addition, NOD2 has been shown to interact with c-Rel in T lymphocytes, resulting in the decreased nuclear accumulation of c-Rel and impaired IL-2 transcription[38]. NOD1 and NOD2 also stimulate autophagy in response to invasive bacteria independently of RIP2 and NF-κB signaling[6]. These observations indicate the relevance of NOD1 and NOD2 in immunity. Indeed, NOD1 and NOD2 have been implicated in a number of chronic inflammatory diseases[46]. Mutations in NOD2 have been linked to Crohn's disease, an

inflammatory disease of the intestine, and Blau's syndrome, a familial granulomatous disease characterized by inflammation of the eyes, joints and skin[47]. Morever, mutations in NOD1 are associated with several inflammatory disorders such as inflammatory bowel disease (IBD), asthma, and sarcoidosis[22].

1.2.3. NLRs and the Inflammasome

The sensing of PAMPs and DAMPs by NLR proteins can also result in the assembly of a caspase-1 activating multiprotein complex referred as inflammasome[48]. Inflammasome formation leads to auto-activation of caspase-1 which proteolytically activates the potent pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18[49]. IL-1β and IL-18 activate NF-κB, JNK, and p38 MAPK signaling pathways to induce the expression of pro-inflammatory cytokines and chemokines that recruit immune cells to the site of infection or injury and lead to pathogen phagocytosis or removal of danger signal, resulting in the resolution of infection and/or cell death[50].

A number of NLR family members have been shown to exhibit inflammasome activity *in vitro*; but only a few NLR proteins have clear functions *in vivo* in the inflammasome formation[9]. NLRP1, NLRP3, NLRC4 (also known as IPAF) and NAIP5 (also called as BIRC1 or NLRB1) are the best known NLRs playing a role in inflammasome[48 ,51, 52] (**Fig. 1.8**). The particular NLR involved in inflammasome formation appears to be stimulus-specific. For example, the NLRC4 inflammasome is

formed in response to multiple Gram-negative bacteria expressing flagellin while NLRP3 inflammasome has been shown to respond to a variety of pathogens and host derived molecules[53]. Recently, an inflammasome complex consisting of NOD2, NLRP1 and caspase-1 has been revealed. Considering the well known role of NOD2 in NF-κB activation, it has been hypothesized that this heterogeous inflammasome couples transcriptional activation of inflammatory genes with IL-1β production. Additional studies will be likely to reveal the presence of other heterogenous inflammasomes and to describe the interactions between certain elicitors and NLR proteins[48].
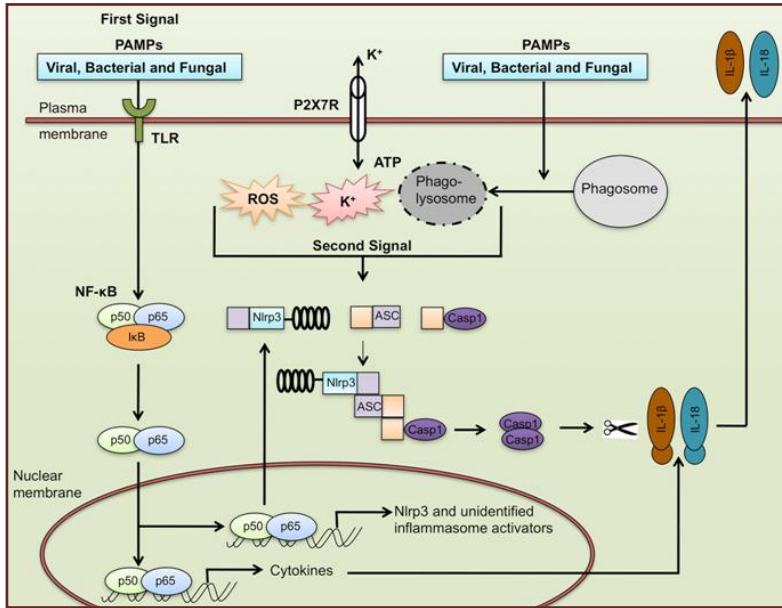
Fig. 1.8. Inflammasome activation: The various inflammasomes are activated by a wide spectrum of DAMPs and PAMPs. NLRP3 activation and inflammasome formation is depicted as an example. Signal 1 is represented by microbial molecules or endogenous cytokines and is required for the up-regulation of NLRP3 and pro-IL-1β by NF-κB. NLRP3 inflammasome is activated by various mechanisms like K$^+$ efflux, lysosomal damage and reactive oxygen species (ROS) production (second signal). Once activated, NLRP3 undergoes a conformational change, NLRP3 binds ASC via pyrin domain. ASC acts as an adaptor protein and binds to procaspase-1 through CARD domain. This large complex is termed the inflammasome and provides the platform for the autoactivation of caspase-1 by proteolytic cleavage. Active caspase-1 then cleaves pro-forms of IL-1β and IL-18 for their secretion and biological activity (Adapted from Anand, PK. *et al*., 2011)[54].

1.2.4. NLRs with inhibitory functions

Among the members of the NLRP subfamily, NLRP2, NLRC3, NLRP4, NLRP10, NLRP6 and NLRP12, play inhibitory roles during inflammation by suppressing distinct arms of inflammatory signaling pathways[31, 55]. How these proteins are activated or perform their inhibitory functions is not well understood as yet[31].

1.3. NLRP12

1.3.1. Identification and expression

NLRP12, also named RNO, PYPAF7, and Monarch-1, is a pyrin-containing NLR protein[56]. A partial 3' portion of the gene was first identified in the HL60 human leukemic cell line. Since the gene was upregulated when HL60 cells were stimulated with nitric oxide, it was first named *rno* (Regulated by Nitric Oxide)[56, 57]. The full-length gene was cloned by two groups and named as Monarch-1 and PYPAF7, subsequently[58, 59]. The HUGO Gene Nomenclature Committee (HGNC) approved the designation *NLRP12* for this gene.

NLRP12 encodes an intracellular protein which contains an N-terminal pyrin domain, a NACHT-associated domain, and a C-terminus leucine-rich repeat region, encoded by 10 exons[60]. The full-length human NLRP12 cDNA is 3186-bp long (NCBI Reference Sequence: NM_144687.3). There are four known alternatively spliced transcript variants encoding distinct isoforms

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of NLRP12, however if these splice forms are differentially expressed and/or serve different functions from the full-length product, has not been determined[61]. Human NLRP12 is expressed exclusively in cells of myeloid lineage: neutrophils, eosinophils, monocytes, macrophages, and immature dendritic cells[62]. NLRP12 expression is down-regulated in response to pathogens, pathogen products, and inflammatory cytokines[58, 64]. Partial down-regulation of *NLRP12* promoter after TLR stimulation is achieved by binding of B lymphocyte-induced maturation protein-1 (Blimp-1)[65]. NLRP12 has been shown to interact with Hsp70 and Hsp90 chaperones and these interactions are important for NLRP12 stability[66].

1.3.2 Function

Since the expression of NLRP12 is restricted to immune cells and its expression is down-regulated in response to pathogens, pathogen products, and inflammatory cytokines, it has been predicted that NLRP12 functions in regulating inflammation and immunity[59, 64, 67].

The very first report describes that, when it is over-expressed in non-immune cells, NLRP12 co-localizes with ASC and activates NF-κB and caspase-1, leading to IL-1β secretion[59]. However, all subsequent publications suggested a negative regulatory role. In one of these studies, endogenous NLRP12 expression was silenced in the human monocytic cell line THP-1, using siRNA. Compared to cells treated with a control siRNA,

NFκB activation is enhanced in NLRP12-silenced cells. Furthermore, when stimulated with TNFα (Tumor Necrosis Factor α), TLR ligands and whole bacteria, NLRP12-silenced cells produced a greater amount of NF-κB -regulated pro-inflammatory cytokines, such as IL-6, IL-8, IL-1β and TNFα[52].

There are two signaling pathways leading to the activation of NF-κB known as the canonical pathway (or classical) and the non-canonical pathway (or alternative pathway)[63] **(Fig. 1.19)**. Biochemical studies showed that NLRP12 suppresses pro-inflammatory cytokine and chemokine production by regulating both canonical and non-canonical NF-κB activation. Stimulation through TLRs leads to the recruitment of cytoplasmic adaptor protein MyD88 that then recruits the kinase IRAK1 (Interleukin-1 receptor-associated kinase 1). The autophosphorylation of IRAK1 leads to downstream NF-κB activation. Exogenous expression of NLRP12 reduces IRAK1-induced activation of an NF-κB luciferase reporter plasmid by associating with IRAK1[64]. In addition to suppressing canonical NFκB activation, it has been also shown that NLRP12 inhibits the non-canonical NF-κB pathway. Although nuclear translocation of the canonical NF-κB subunits RelA (p65) and p50 proceeds normally after stimulation with TLR agonist followed by CD40L, p52 processing from NF-κB2/p100 and nuclear translocation of p52 is abolished in NLRP12 over-expressing THP-1 cells. It has been revealed that NLRP12 exerts this inhibitory activity by associating with NIK

(NF-κB-inducing kinase) and inducing its degradation via the proteasome[68]. Accordingly, NIK, p52 and p52-regulated cytokines and chemokines including CXCR4, CXCL12 and CXCL13 levels are elevated in NLRP12-silenced cells[68].

The inhibitory role of NLRP12 on inflammation and NF-κB signaling was also supported by *in vivo* studies. It has been shown that *Nlrp12⁻/⁻* mice are highly susceptible to colon inflammation and tumorigenesis with increased production of inflammatory cytokines, chemokines, and tumorigenic factors due to an excessive NF-κB and ERK (Extracellular signal-regulated protein kinase) activation in the macrophages[69]. Furthermore, the role of NLRP12 in maintaining intestinal homeostasis and providing protection against tumorigenesis were also shown in colitis-associated colon cancer model. It has been demonstrated that *Nlrp12⁻/⁻* mice were highly susceptible to colitis and colitis-associated colon cancer because of the elevated activation of non-canonical NF-κB, ERK and AKT (also known as protein kinase B or PKB) signaling, and increased expression of cancer associated target genes including *Cxcl12* and *Cxcl13*[70]. Depending on these results, NLRP12 is suggested to be a checkpoint of non-canonical NF-κB signaling, inflammation, and tumorigenesis.
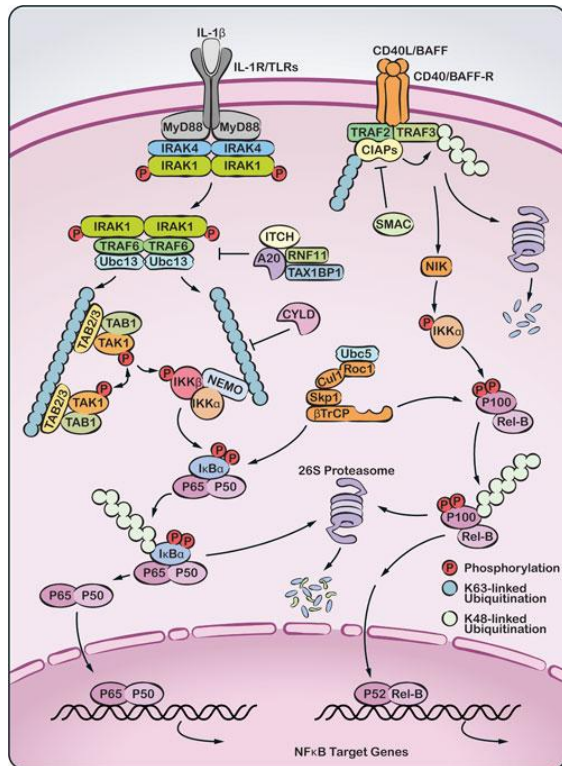
Fig. 1.9. Canonical and non-canonical NF-κB pathways: The activation of canonical NFkB pathway, represented by IL-1R/TLR stimulation on the left, starts with the recruitment of the adaptor protein MyD88 and the kinases IRAK1 and IRAK4, leading to oligomerization of E3 ubiquitin ligase TRAF6 (TNF receptor associated factor 6). Together with the E2 enzyme complex Ubc13, TRAF6 synthesizes unanchored K63-polyubiquitin chains that bind to the TGF-β-activated kinase 1 (TAK1)-binding protein (TAB)2 and TAB3 subunits of the TAK1 kinase complex and the NEMO subunit of the IKK complex. This binding brings the kinases into proximity for phosphorylation and activation. IKK subsequently phosphorylates IκBα. Phosphorylated IκBα is targeted for ubiquitin-mediated proteasomal degradation, causing release of the NF-κB p65/p50 into the nucleus to regulate the transcription of target genes. The activation of non-canonical NF-κB pathway is represented by

CD40L/BAFF stimulation on the right. Stimulation of the surface receptors leads to the recruitment of the E3 ligases TRAF2, TRAF3 and cIAPs. TRAF2 catalyzes K63 polyubiquitination of cIAPs, which in turn target TRAF3 for degradation by promoting its K48 polyubiquitination. As a result, TRAF3 level in the cell drop below a critical threshold, and NIK can no longer be recruited to the cIAPs:TRAF2 complex. In the absence of TRAF3, NIK is stabilized, leading to the activation of IKKα which phosphorylates the NF-κB precursor p100. Phosphorylated p100 is recognized by the βTrCP E3 complex and targeted for ubiquitin-mediated proteasomal processing to form the mature subunit p52. p52 forms a complex with RelB, which migrates to the nucleus to control gene transcription (Adapted from Liu, S. and Chen, ZJ., 2011)[63].

Additionally, NLRP12 has been linked to hereditary periodic fever syndrome. The p.Arg284X mutation, located within the NBD and the p.Val635ThrfsX12 mutation, located between the NBD and LRRs domain of NLRP12, caused lower NF-κB suppression property compared with the wild type NLRP12[71]. The NLRP12 missense mutation p.Asp294Glu was also identified in familial cold-induced autoinflammatory syndrome. The missense mutation p.Asp294Glu is found within an evolutionarily conserved NBD which is fundamental for ATP binding. In contrast to the previous findings, no clear reduction of the inhibitory properties of the p.Asp294Glu mutant of NLRP12 on NF-κB signaling was observed[72]. Furthermore, another missense mutation (pArg352Cys) within the NBD of NLRP12 in periodic fever syndrome patients has also no direct effect on NF-κB signaling. The mutation, which does not alter the inhibitory effect

of NLRP12 on NF-κB activation, increases speck formation and activates caspase-1 signaling[73].

NLRP12 has also been implicated as an inflammasome component recognizing *Yersinia pestis*. NLRP12 inflammasome was shown as an important regulator of IL-18 and IL-1β production after *Y. pestis* infection. NLRP12 also directed IFN-γ production, but had minimal effect on signaling of the transcription factor NF-κB[74]. However, in an other study, it has been demonstrated that there was no difference in IL-1β production in *Nlrp12*^{−/−} vs wild type bone marrow (BMCs) or bone marrow derived dendritic cells (BMDCs) of mice stimulated with LPS, LPS together with ATP, other TLR ligands or TNFα[56]. In contrast to previous finding which indicated that silencing of NLRP12 with small hairpin RNA leads to increased production of IL-6 and TNFα in human cells of monocytic lineage[64] , NLRP12 did not affect the production of pro-inflammatory cytokines IL-12, IL-6 and TNFα in response to TLR stimulation[56].

In vivo studies on the functional role of NLRP12 in dendritic cells revealed a novel role of NLRP12 in cellular migration: dendritic cells and neutrophils from *Nlrp12*^{−/−} mice showed reduced migration (attenuated contact hypersensitivity) from the periphery to the draining lymphonodes *in vivo* and failed to respond to chemokines *in vitro*. On the other hand, NLRP12 did not affect dendritic cell maturation neither antigen presentation in NLRP12 deficient mice[56]. Despite the findings pointing to an anti-inflammatory role and the effect on the contact hypersensitivity,

NLRP12 did not play a vital role in allergic airway inflammation using common model systems that are physiologically relevant to human disease[75].

The role of NLRP12 during the *in vivo* host immune response to *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* (*Mtb*) was also characterized. As mentioned before, NLRP12 has been shown to negatively regulate both canonical and non-canonical NF-κB pathways *in vitro* and, more recently, has been found to display this role *in vivo* in colitis and colon cancer models. Despite the known effects of both *K. pneumoniae* and *Mtb* infections on the activation of canonical NF-κB signaling, no significant differences were observed between *Nlrp12*^{-/-} mice and wild type animals after *K. pneumoniae* or *Mtb* infections[76]. Together with the *in vitro* findings which indicate NLRP12 as a negative regulator of NF-κB signaling after LPS stimulation or *MTb* infection in human THP-1 monocytic cell line[64], recent studies suggest that NLRP12 does not significantly contribute to the *in vivo* host innate immune response to LPS stimulation or *Mtb* infection[76]. Additionally, in response to LPS stimulation, Nlrp12 deficient mice showed a slight decrease in IL-1β and a slight increase in IL-6 production which were not statistically significant[76]. These data are consistent with the other *in vivo* findings that also failed to support a role for NLRP12 in inflammasome formation[75].

Finally, as distinct from the indicated roles, NLRP12 has been suggested to control the expression of classical and non-classical MHC class I genes *in vitro*[58]. These findings are reminiscent of CIITA and NLRC5 which are the trans-activators of MHC class II and MHC class I genes, respectively.

1.4 Aim of the Work

Despite the large number of studies, the role of NLRP12 remains ambiguous. The conflicting results about the functional role of NLRP12 can be caused by the differences in the experimental systems: NLRP12 can function through unpredictable mechanisms, or other proteins can compensate for the loss of NLRP12 in an *in vivo* environment[76]. Furthermore, it is also possible that NLRP12 acts temporarily or in a cell type-, tissue-, dose- or stimuli-specific manner. Nevertheless, the transcriptional regulation of NLRP12 is clear: NLRP12 transcription is down-regulated following TLR activation or exposure of cells to inflammatory cytokines. It is hypothesized that the down-regulation of NLRP12 expression is necessary for an appropriate immune response. Once the infection has been cleared, NLRP12 expression turns back to basal level to prevent excessive inflammation and to maintain immune quiescence[38]. Based on these statements, I asked whether NF-κB-driven negative feedback mechanism induces the down-regulation of NLRP12 transcription. Since the expression of NLRP12 is

restricted within the immune cells, I have also focused my attention on the NLRP12 regulation during myeloid cell differentiation.

CHAPTER 2

MATERIALS and METHODS

2.1 Cell Culture and Differentiation

HEK293T (Human Embryonic Kidney 293T) and HeLa (derived from human cervical cancer cells) cell lines were maintained in DMEM (Dulbecco's Modified Eagle's Medium, *Biochrom*) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 25 U/ml penicillin and 25 U/ml streptomycin. Treatment with trypsin-EDTA (*Biochrom*) was used in the passaging of the adherent cell lines.

The leukemic monocytic cell line *U937* was cultured in RPMI 1640 (*Biochrom*) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin. KG1, the human acute myelogenous leukemia cell line, was maintained in IMDM, (Iscove's Modified Dulbecco's Medium, *Biochrom*) containing 20% heat-inactivated FBS, 2 mM L-glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin. The cells were cultured in a humidified atmosphere of 7% CO_2 at 37°C.

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy donors using Ficoll density gradient centrifugation. Monocytes were enriched from freshly isolated PBMC using MACS Monocyte Isolation Kit II and MACS LS Columns (*Miltenyi Biotec*) by depletion of non-monocytes (negative selection), according to the supplier's instruction. The isolated monocytes were adjusted to a concentration of 10^6 cells/ml and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 25 U/ml penicillin and 25 U/ml streptomycin in cell culture plates at 37°C in a humidified 7% CO_2 incubator. Macrophages were obtained by incubation of the monocytes for 7 days with 100 ng/ml of GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor). Half of the medium was replaced every two days with complete fresh medium containing 100 ng/ml GM-CSF (*Miltenyi Biotec*). The differentiation of the myeloid cell lines was achieved by the incubation with Phorbol 12-myristate 13-acetate (PMA, *Sigma-Aldrich*) for the indicated times and concentrations. When indicated, the proteasome inhibitor MG-132 (*Sigma*) was used at a concentraion of 10 µM, for 6 hours.

2.2. Construction of the Plasmids, Transformation and Plasmid DNA Isolation

2.2.1. Generation of *NLRP12* promoter constructs

NLRP12 promoter region was determined by using *Gene2Promoter*, Genomatix Software. The 765 bp long sequence of the human *NLRP12* promoter was amplified using 5'-CTGGGTGGCAGAGTGAGAC-3' as forward and 5'-TGCCGTGAGCCCCAAAGGAG-3' as reverse primers, and the PCR product cloned into pGL4.10 [*luc2*] (*Promega*) promoterless luciferase reporter vector by KpnI and XhoI. The construct was named as pGL4-*NLRP12*- full length. The promoter region was then cloned into pGL4.10 [*luc2*] as three separate fragments which are named as pGL4-*NLRP12*-299, pGL4-*NLRP12*-530 and pGL4-*NLRP12*-765.

Illustra GFX PCR, DNA and gel band purification kit (*GE Healthcare*) was used for the gel isolation of PCR products and digested plasmids, and for the reaction purifications after enzymatic digestions, according to the manufacturer's instructions.

All the ligations were performed overnight at 13°C by T4 DNA ligase (*Bioline*). The ligations were transformed into *E. coli* DH10β electro competent cells by electroporation using *Bio-Rad Gene Pulser*.

2.2.2. Cloning of human NLRP12

Full lenght NLRP12 (3186 bp) was amplified from healty individual monocyte cDNA by forward 5'-ATGCTACGAACCGCAGG-3' and reverse 5'-CAGCAGCCAATGTCCAAATAAG-3' primers, using Phusion high fidelity DNA polymerase (*New England Biolabs, Thermo Scientific*). HyperLadder™ 1kb (*Bioline*) was used in this thesis to determine the sizes of DNA samples.

The purified PCR product was ligated by T4 DNA ligase (*Bioline*) to pcDNA3 or pEGFP-N1 expression plasmids, maintaining the reading frame. The ligation was performed at 13°C for overnight. *E. coli* DH10β electrocompetent cells were used for transformation.

2.2.3. Plasmid DNA isolation

The transformed *E. coli* DH10β colonies were grown in LB (Luria-Bertani) broth medium containing the appropriate antibiotics. All the plasmid DNA isolations were performed using Genopure Plasmid Midi Kit (*Roche*), following the manufacturer's protocols.

2.3. Identification of Potential NF-κB Binding Site in the *NLRP12* Promoter and Site-directed Mutagenesis

To identify if there was a potential NF-κB binding site inside the promoter region, *MatInspector*, Genomatix Software was used. The putative NF-κB binding site was then mutated using nested primers, containing mismatched bases. The construct was called as pGL4-*NLRP12Mut*-765. In addition, NF-κB "consensus sequence" containing construct was generated, and the plasmid construct (pGL4-*NLRP12*-NFκB-765) was used as a positive control in response to NF-κB.

2.4. Cell Transfections

2.4.1. Transfection of NLRP12 promoter constructs and luciferase assay

HEK 293T cells were transiently transfected with the promoter constructs or empty pGL4.10 [*luc2*], together with expression plasmids for RelA, RelB or a RelA mutant "RelA YA ED" which is unable to bind κB site. Lipofectamine 2000 transfection reagent (*Invitrogen*) was used for the transient transfection of HEK 293T cells.

pRL-TK (*Promega*) renilla luciferase vector was used as an internal transfection efficiency control. 48-hours post transfection, cells were lysed with Passive Lysis Buffer (*Promega*) and luciferase activity was measured by VICTOR light

luminescence counter (*Perkin Elmer*) using the Dual-Luciferase Assay kit (*Promega*) according to manufacturer's instructions. The luciferase readings were normalized to renilla luciferase. Since the pGL4.10 [*luc2*] vector luciferase expression is up-regulated by NF-κB, the normalized luciferase values are plotted as an average fold increase over pGL4.10 [*luc2*] vector.

2.4.2. Transient and stable transfection of NLRP12 expression constructs

For the expression analysis of pEGFP-N1-NLRP12 plasmid construct, HeLa cells were transfected transiently with pEGFP-N1 (Mock) or pEGFP-N1-NLRP12 plasmids, using Lipofectamine 2000 reagent (*Invitrogen*) according to manufacturer's instructions.

The transfection of U937 cells was achieved by using polymer-based Xfect™ Transfection Reagent (*Takara*). Following the gene transfers, cells were cultivated in the culture medium containing 2 mg/ml of the selective antibiotic G-418 (*Calbiochem*) for 4 weeks. The bulk (mixed population of the resistant cells) cells were then also single-cell cloned under G418 selection. The stable transfectants were maintained in the complete growing medium with 1,5 mg/ml G418. NLRP12 expression was controlled by flow cytometry analysis and qRT-PCR (Real-Time Quantitative Reverse Transcription PCR*).*

2.5. RNA Isolation and qRT-PCR

Total RNA was isolated using the Trizol reagent (*Invitrogen*) according to the manufacturer's protocol. Total RNA was used to perform reverse transcription using High-Capacity cDNA Reverse Trascription kit (*Applied Biosystems*) using random primers. NLRP12 transcript was analyzed by qRT-PCR using sequence-specific TaqMan probe (Hs00536435_m1, *Applied Biosystems*). qRT-PCR performed in ABI PRISM 7300 Sequence Detection Systems (*Applied Biosystems*). The housekeeping 18S rRNA (TaqMan probe: Hs99999901_s1, *Applied Biosystems*) was used as the endogenous control.

2.6. Protein Extraction and Western Blotting

Cell extracts were obtained by incubation of the cell pellets in NP40 lysis buffer (20 mM Tris-HCl, pH 8; 137 mM NaCl; 10% Glycerol; 1% NP40; 2 mM EDTA) containing protease inhibitors (1 mM EGTA; 0,5 mM Na_3VO_4; 50 mM NaF; 1 mM PMSF; 1µg/ml Aprotinin; 1µg/ml Leupeptin) for 30 minutes on ice. The cellular debris were removed by centrifugation at 13000 rpm for 15 minutes at 4°C. Protein concentrations were determined by Bradford assay (*BioRad*) and equilibrated amount of samples loaded into 8-12% SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) gels after denaturation in SDS sample buffer, at 100°C, for 5 minutes. The separated proteins were transferred to a nitrocellulose membrane

(AmershamHybond ECL Nitrocellulose Membrane, *GE Healthcare*), which was then blocked in 5% (w/v) non fat dry milk powder in 0,1% PBST (v/v, Tween20 PBS) and subsequently incubated with the primary antibodies. After several washing steps, membranes were incubated with proper secondary antibodies conjugated with horseradish peroxidase (HRP). When necessary, the membranes were treated with stripping buffer (65,5 mM Tris-HCl pH 6,8; 100 mM 2-Mercaptoethanol; 2% SDS in dH$_2$O) for 30 minutes at 55°C, followed by serial washing steps in 0,1% PBST.

The signals were detected by ECL (Enhanced chemiluminescence, *Amersham*) and the images were acquired using Molecular Imager ChemiDoc XRS+ System (*Bio-Rad*). ProSieve QuadColor Protein Marker (*Lonza*) was used as standart. For the densitometric analyses, ImageJ 1.46r (*NIH*) software was used.

The primary antibodies used are the followings: GFP Antibody (B-2): sc-9996 (*Santa Cruz*), cyclin B1 Antibody (GNS1): sc-245 (*Santa Cruz*), cyclin A Antibody (H-432): sc-751 (*Santa Cruz*), cyclin D1 Antibody (A-12): sc-8396 (*Santa Cruz*), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) Antibody (FL-335): sc-25778 (*Santa Cruz*). Anti-rabbit IgG, peroxidase-linked species-specific F(ab′)$_2$ fragment, from donkey (*Amersham*) and Peroxidase-AffiniPure Goat Anti-Mouse (*Jacksonn ImmnoReserach Laboratories*) were used as secondary antibodies.

2.7. Sample Preparation for Fluorescence Microscopy

EGFP (Enhanced Green Fluorescent Protein) or EGFP fused NLRP12 expressing HeLa cells were grown on the poly-L-lysine (Sigma) treated cover slips. The cells were fixed-permeabilized by cold (-20°C) absolute methanol (MetOH) treatment for 6 minutes at -20°C. After removal of MetOH, the nuclei were stained with DAPI (0.1 µg/ml) for 2 minutes. DAPI was removed and the cover slips were rinsed in 1x PBS and inverted onto the microscope slides containing 5 µl of mounting media (VECTASHIELD mounting media*; Vector Laboratories*). The slides were then allowed to dry and the edges of each coverslip were sealed with a regular transparent nail polish and air dried. The same sample preparation protocol was used also for the pEGFP-N1-NLRP12 expressing U937 cells after "cyto-centrifuge" which is described below.

2.8. Immunostaining and Fluorescence Microscopy

2.8.1. Fixation and permeabilization

U937 cells were washed in 1x PBS and the cells were suspended in 1 ml of 1x PBS containing 10^6 cells. To attach the suspension growing U937 cells to poly-L-lysine treated coverslip surface, "cyto-centrifuge" method was used. The cells were pipetted onto coverslips and centrifuged starting with 2000 rpm. The spin-speed was slowed down to 1000 rpm in 2 minutes, then

the cells were centrifuged for additional 10 minutes at 1000 rpm. At the end of the centrifuge, PBS was removed and the slides were treated with 3,7% formaldehyde (FA), for 15 minutes at room temperature (RT). Following the removal of FA, the samples were washed 5 minutes in 1xPBS and 5 minutes in 0.25% TritonX, subsequently. The samples were then treated with cold (-20°C) absolute MetOH at -20°C for 10 minutes. MetOH was removed and the samples were washed in 1xPBS, for 5 minutes. Since the PMA treated (differentiated) U937 cells are adherent, the cells were gently detached by a scrapper in cold 1xPBS, before the cytocentrifuge.

2.8.2. Cellular Staining

After fixation and permeabilization procedures, the cells were covered with the blocking buffer composed of 3% BSA (Bovine serum albumin) and 0,05% PBST and incubated at RT for 30 minutes. The blocking buffer was aspirated and the cells were incubated with the diluted cyclin B1 antibody (GNS1: sc-245, *Santa Cruz*) for 45 minutes at RT. After removing the primary antibody, the samples were washed three times for 5 minutes in 0,05% PBST and subsequently incubated with the secondary antibody (Texas Red anti-mouse, IgG, *Vector Laboratories*) for 30 minutes at RT. The nuclei were stained with 0,1 µg/ml DAPI solution for 2 minutes at RT. The samples were then washed three times in 0,05% PBST, 5 minutes each wash. The cover slips were inverted onto the microspe slides containing 5 µl of mounting

media (VECTASHIELD mounting media; *Vector Laboratories*) and the slides prepared as above.

2.8.3. Fluorescence microscopy

Cyclin B1 stained samples or GFP expressing cells were analyzed using a Nikon Eclipse 90i microscope equipped with a Qicam Fast 1394 CCD camera (*Qimaging*), at Nikon Reference Centre, CNR Institute of Molecular Biology and Pathology. An oil immersion 100x (N.A. 1.3) objective was used and the image acquisitions were performed using NIS-Elements AR 3.2 (*Nikon*).

2.9. Intracellular Cyclin B1 Staining, DNA Content and Cell Cycle Analyses

2.9.1. Intracellular cyclin B1 staining and DNA content analysis

10^6 cells were transfered into FACS tubes (*Becton, Dickinson*), washed twice in 1xPBS and suspended in ice cold 300 μl of 1xPBS. 700 ul of cold (-20ºC) absolute ethanol (EtOH) added onto the cells in a dropwise manner and vortexed gently. The EtOH fixed cells were incubated at 4ºC for at least 2 hours. Before the intracellular staining, the fixed cells were washed 3 times in 0,5% PBST, for 10 minutes each. To minimize the non-specific antibody bindings, the cells were incubated in 5% goat serum (in 0,5% PBST) for 1 hour at RT. After blocking, the samples were incubated with diluted cyclin B1 antibody (H-433:

sc-752, *Santa Cruz*) for 1 hour at RT, then washed three times in 0,5% PBST. Following the removal of PBST, the cells were incubated with diluted secondary antibody (Alexa Fluor 488, FITC conjugated anti rabbit IgG, *Life Technologies*) for 30 minutes at RT. The secondary antibody was then removed by 3 times washing in 0,5% PBST, and the samples were incubated in 25 µg/ml of Propidium iodide (PI, *Sigma*) in 0,5% PBST for 30 minutes in the dark at RT, for the DNA content analyses together with cyclin B1 expression. Ten thousand events were acquired for each sample using a FACStar Plus fluorescence-activated cell sorter (*Becton, Dickinson*) flow cytometer and analyzed by FACS WinMDI software.

2.9.2. Cell cycle analysis

$3x10^5$ cells were washed in 1xPBS and suspended in 300 µl of PI/Triton X-100 staining solution (50 µg/ml PI; 0,1% Triton X-100 in 1xPBS) containing 0,5 mg/ml of DNAse free RNase A (*Thermo Scientific*). The samples were incubated for 15 min at 37°C in the dark. FACS Calibur (*Becton, Dickinson*) flow cytometer were used for acquisition of the samples. The results were analyzed by CellQuest software (*Becton, Dickinson*).

2.10. Surface staining and flow cytometry analysis

The cells were washed in 1xPBS and the samples were incubated with FITC conjugated CD11b antibody (*Immunotools*)

for 45 minutes, at 4ºC, in the dark. After the incubation, the cells were washed in 1xPBS and analyzed by FACS Calibur (*Becton, Dickinson*) flow cytometer. Analyses were performed using CellQuest software (*Becton, Dickinson*).

2.11. Statistical Analysis

The *one-way* Analysis of Variance (ANOVA) was used to determine significance. The asterisks indicate the range of the different p values (one asteriks indicates $p \leq 0.05$; two asterisks for $p \leq 0.005$).

CHAPTER 3

RESULTS

In this chapter, results reporting the effect of NF-κB on the *NLRP12* promoter activity, the transcriptional modulation of NLRP12 during myeloid cell differentiation and the effects of NLRP12 over-expression on monocytic cell line U937 are presented.

3.1. Analyses of *NLRP12* Promoter Activity in Response to NF-κB

3.1.1. Predicted *NLRP12* promoter sequence enhances luciferase expression

NLRP12 promoter region is predicted by using *Gene2Promoter*, Genomatix software and the region was cloned into pGL4.10 [*luc2*] promoterless luciferase reporter vector. The cloned DNA sequence greatly stimulated the luciferase expression which indicates that the sequence has promoter activity **(Fig 3.1)**.

(A)


```
                                          1 CT GGGTGGCAGA GTGAGACTCT GACTCAAAGA AAAAAAAAAA
        43 AAAAAAAGCC AGGGACGGTG GCTCACACCT GTAATCCTAG CACTTTGGAA GGCTGAGGCG
       103 GGTGGATCAC CTGAGGTCAG GAGTTCGAGA CCAGCCTGAC CAACATGGTG AAACCCTGTC
       163 TCTTCTAAAA CCACAAAAAT TAGCTGGGTG TGGTGTCCGG CGCCTGTAAT CCCAGCTACT
       223 CAAGGGAAGC TGAGGTGGGA GAATCGCTTG AACCTGGGAG GCAGAGGTTG CGGTGAGCCA
       283 AGATCGCAGA GCCAGGGCCA CAGAGTAAGA CTCTGTCTCA AAAAAAAAAA AATTCTCCCT
       343 GGAAGAGGCG CAGTCGCAGT TTTGATAATA CACTCTACCA CACACACTAG TGAAATGAGG
       403 AAAACAAGAG GAAAGCGGAA AGAGAGTGAC TGTTTTGTCA AGAGATCCAG GAAGAGACAG
       463 CAGAAGTGAA AATCTTTTTC ATCAGCCAAG TGGCTCCTCG GATTTCTTAA TCTCCCCCCG
       523 AACTTCCTCT TCTCCACCCA GAACAGCCAT TGGTGAGTGG GGCAGGGCAG GAGGGAACTG
       583 AAGAGTGAGA AAGCATTATT TCAGCAAAAG GTCTTTCCTC CCTTGCTCTC TCCTCCAACC
       643 ACTGGCTCAG CCTCTCCGCC CGCTGCCTGT GAATGATGCA ATGGAAGGTG TGCTGGGGTC
       703 GCCCTGTGTC CCGTGCATAG GAGCATCTCA GCCTCCAGGT CCTCTCCTTT GGGGCTCACG
       763 GCA
```

	pGL4.10[luc2]
luciferase [luc2]	

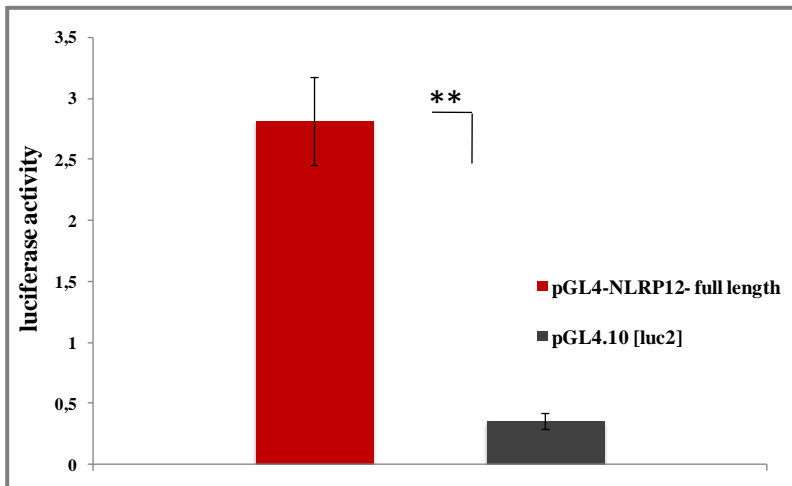| 765 bp NLRP12 promoter | luciferase [luc2] | pGL4-NLRP12-full length |

(B)

Fig. 3.1 *NLRP12* promoter activity: (A) The cloned *NLRP12* promoter region and the schematic representation of luciferase reporter construct generated to assess *NLRP12* promoter luciferase activity. The arrow indicates the transcription start site. **(B)** HEK 293T cells were transfected with the promoter

construct or pGL4.10 [*luc2*]. 48-hours post transfection, cells were lysed and the luciferase activities were determined. pRL-TK renilla luciferase vector was used as an internal transfection efficiency control. The error bars represent the mean from four independent experiments (** $p \leq 0.005$).

3.1.2. RelA causes down-regulation of the *NLRP12* promoter activity

To determine if there is an effect of NF-κB on the *NLRP12* promoter, we co-transfected the promoter construct (pGL4-*NLRP12*-full length) with NF-κB expression plasmids into HEK293T cells. Our results showed that RelA causes down-regulation of the promoter activity while the presence of RelA YA ED, which is unable to bind canonical κB sites, did not modify the luciferase expression. Furthermore, we did not observe any modulation in the *NLRP12* promoter activity in response to RelB (**Fig. 3.2**).

To identify the region that mediates the RelA-mediated down-regulation, we transfected the luciferase reporter gene plasmids containing different regions of the promoter (**Fig. 3.3, A)**, together with RelA or mutant RelA expression plasmids. Inside the analyzed promoter region, DNA sequence between the bases 530-745 was found to be responsible for the observed down-regulation induced by RelA (**Fig. 3.3**).

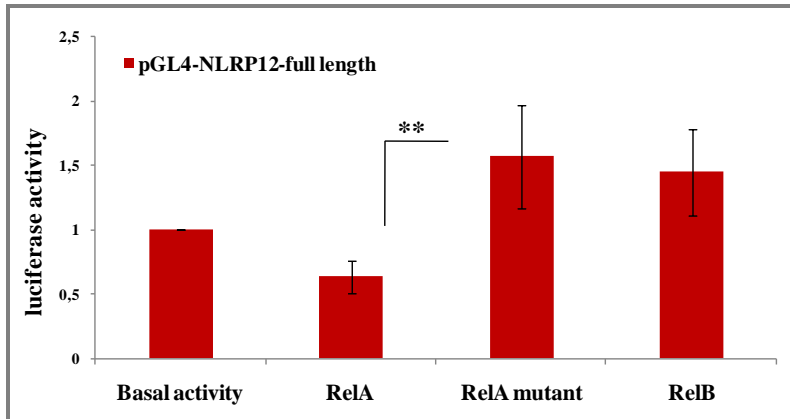**Fig. 3.2. Effect of NF-κB on the *NLRP12* promoter:** The plasmid containing the 745 bp promoter region (pGL4-NLRP12- full length) co-transfected with RelA, RelB or mutant RelA expressing plasmids into HEK 293T cells. After 48 hours, cells were lysed and the relative luciferase levels were determined. The error bars represent the mean of four independent experiments (*$p \leq 0.05$; **$p \leq 0.005$).

(A)
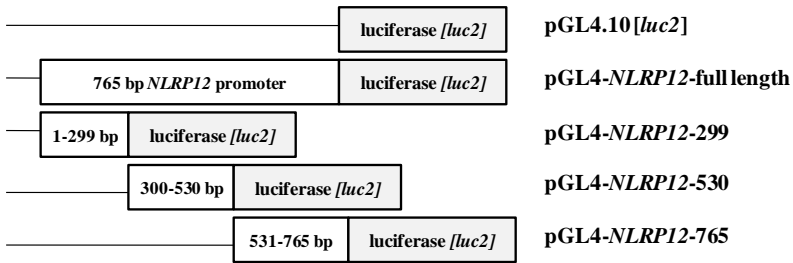
(B)
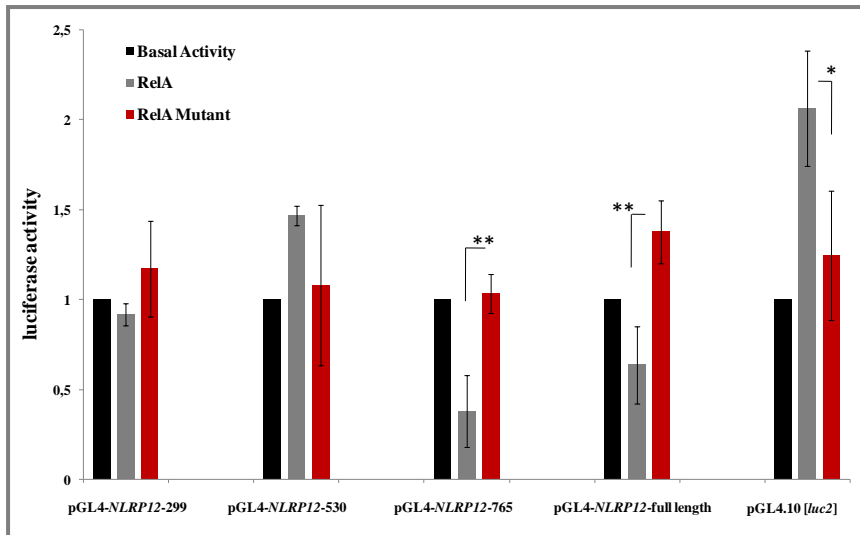
Fig.3.3 Effect of RelA on the NLRP12 promoter: HEK 293T cells were transfected with the full length (pGL4-*NLRP12*- full length), pGL4-*NLRP12*-299, pGL4-*NLRP12*-530, pGL4-*NLRP12*-765 or control pGL4.10 [*luc2*] together with RelA, RelB or with the mutant RelA expressing plasmids. 48 hours later, cells were lysed and the relative luciferase levels were determined as described in "Materials and Methods". The error bars represent the standard error of four separate experiments (*$p \leq 0.05$; ** $p \leq 0.005$).

3.1.3. Characterization of NF-κB binding site on the *NLRP12* promoter

Sequence analysis revealed that *NLRP12* promoter region contains a "non-consensus", putative NF-κB binding site. Therefore, to verify whether this sequence (between the bases 611 and 620) was mediating the inhibitory effect of RelA, the sequence was mutated by "site-directed mutagenesis" **(Fig. 3.4)**. This mutation however, was not able to counteract the effect of RelA **(Fig. 3.5)**. As a positive control, another mutation was generated to create a consensus NF-κB response sequence inside the analyzed *NLRP12* promoter region **(Fig. 3.4)**, and this mutation did indeed induce a significant up-regulation in response to RelA **(Fig. 3.5)**.

(A)
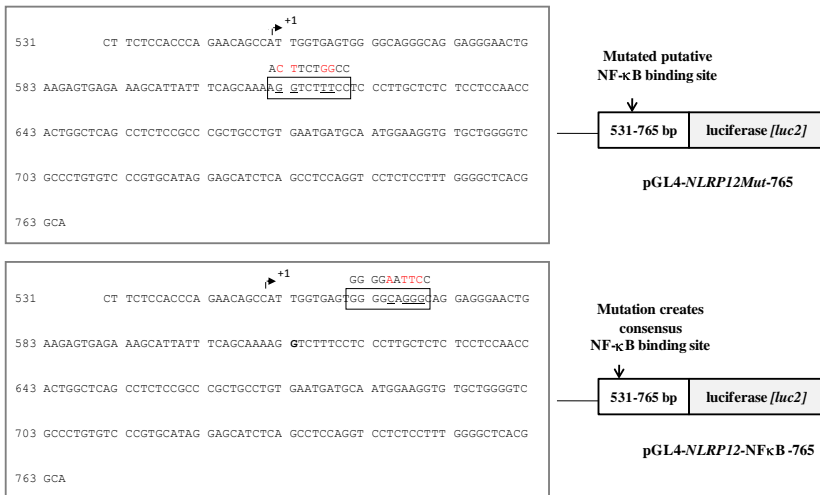
(B)

Canonical NF-κB binding site	**GGGRNNYYCC**
The putative NF-κB binding site inside the promoter	**AGGTCTTTCC**
The putative NF-κB binding site mutated	**ACTTCTGGCC**
Consensus NF-κB binding site generated from	**GGGGCAGGGC**
to obtain	**GGGGAATTCC**

Fig. 3.4. Site directed mutagenesis of the NLRP12 promoter: (A) The putative NF-κB binding site was mutated and the promoter region containing the mutation (between the bases 530 and 745) was cloned into pGL4.10 [*luc2*]. The construct is named pGL4-*NLRP12Mut*-765. To create a consensus NF-κB binding site on the promoter, the underlined bases were mutated and the region was cloned into pGL4.10 [*luc2*]. The construct, which was used as a positive control, called as pGL4-*NLRP12*-NFκB-765. **(B)** The "consensus" NF-κB response sequence and the generated mutations on the promoter are shown.
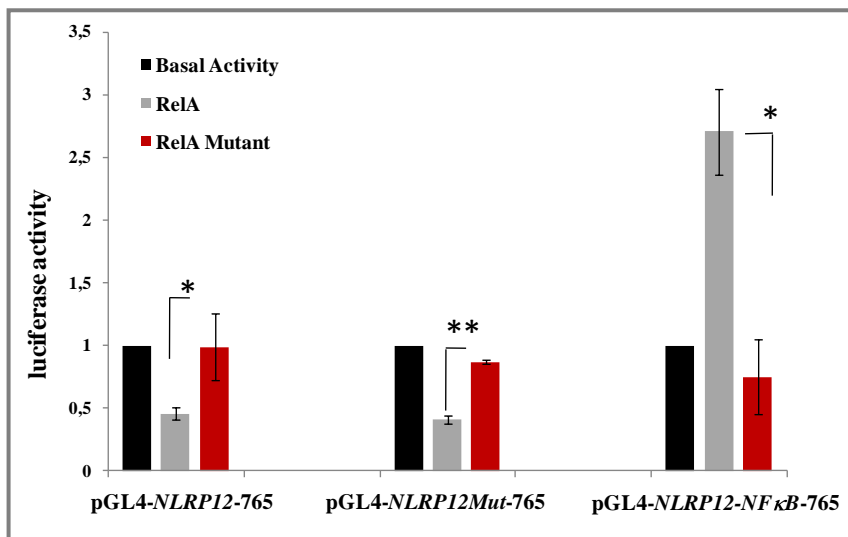
Fig. 3.5. The activity of mutated NLRP12 promoter region: HEK 293T cells were transfected with the wild type (pGL4-*NLRP12*-765) or mutated (pGL4-*NLRP12Mut*-765 and pGL4-*NLRP12*-NFκB-765) promoter constructs together with RelA or mutant RelA expressing plasmids. 48 hours post-transfection, cells were lysed and the relative luciferase activity was determined as described in "Materials and Methods". The error bars represent the standard *error* of three separate experiments (*$p \leq 0.05$; ** $p \leq 0.005$).

3.2. NLRP12 mRNA Level is Decreased by LPS and IFN-γ in Macrophages

As mentioned before, NLRP12 expression is down-regulated following activation by TLR agonists or exposure of cells to inflammatory cytokines. We show here that NLRP12 transcription is down-regulated in human macrophages differentiated *in vitro* from primary human monocytes by GM-

CSF and treated with the TLR4 agonist LPS (*Escherichia coli* liposaccharide) or stimulated by the cytokine IFN-γ for 9 hours (**Fig. 3.6**).

(A) **(B)**
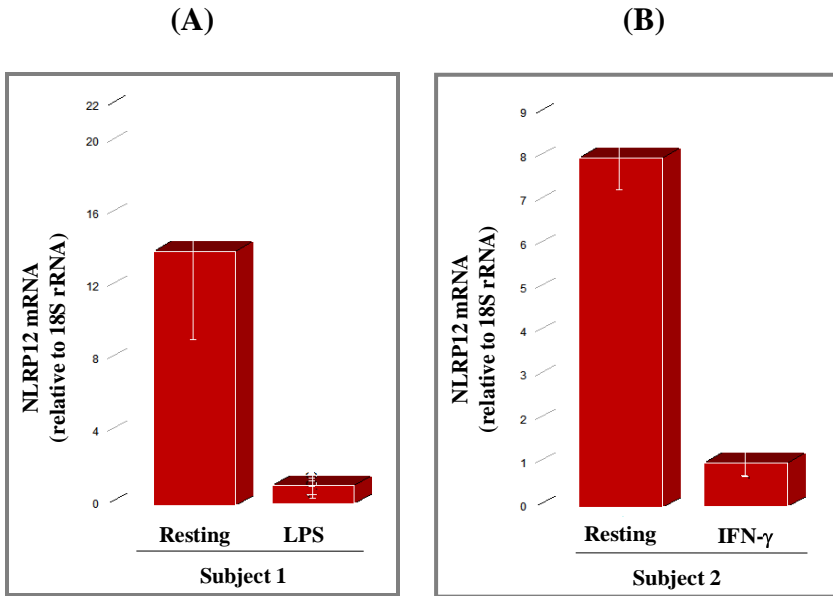
Fig. 3.6. NLRP12 transcription in human primary macrophages: Primary human monocytes from two healthy donors were differentiated into macrophages by GM-CSF treatment for 7 days, then incubated with **(A)** 50 ng/ml of LPS or **(B)** 100 U/ml IFN-γ for 9 hours, or retained unstimulated (indicated as "Resting"). The changes in the NLRP12 transcription were analyzed by qRT-PCR. The error bars represent the standard error of three replicates.

3.3. NLRP12 Transcription Changes During Myeloid Cell Differentiation

To further investigate the dynamic of the NLRP12 transcription during activation and/or differentiation, we used monocytic cell lines as model systems. U937 cells (Human histocytic leukemia cell line) were differentiated into macrophage-like cells by 80 nM, KG1 cells (Human acute myelogenous leukemia cell line) were differentiated into dendritic cells by 16 nM of PMA.

There are clearly two different time-course of the NLRP12 down-regulation in response to PMA: while KG1 were responding rapidly and the NLRP12 mRNA was already strongly down-modulated after 4 hours treatment **(Fig. 3.7)**, U937 cells were instead up-regulating the expression of NLRP12 after 24 hours treatment that was then returning below the basal level after 72 hours, when the differentiation to macrophages was completed and cells were not cycling anymore **(Fig. 3.8).**

Fig. 3.7. NLRP12 mRNA levels during dendritic cell differentiation: NLRP12 transcription during differentiation of KG1 monocytic cells into dendritic cells by PMA treatment was analyzed by qRT-PCR. The untreated KG1 cells are indicated as "Unt". The error bars represent the standard error of three separate experiments with three replicates for each sample (*$p \leq 0.05$; ** $p \leq 0.005$).
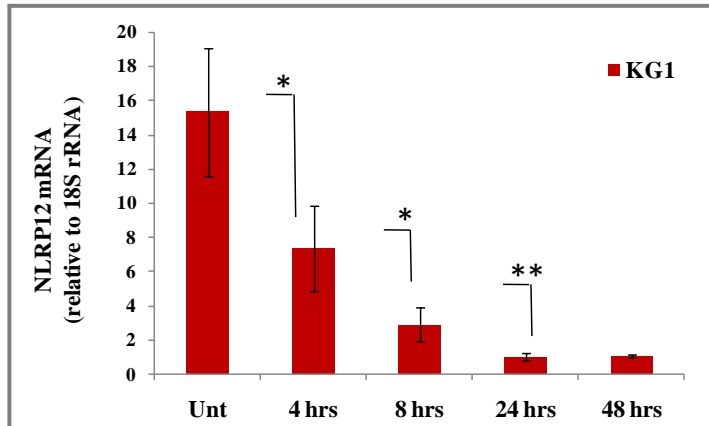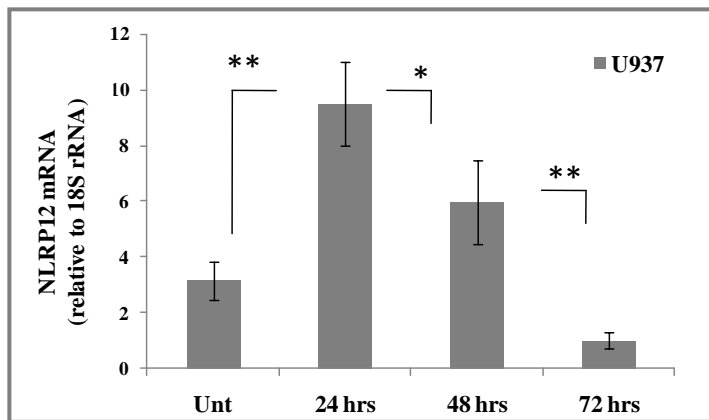

Fig. 3.8. NLRP12 mRNA levels during macrophage differentiation: NLRP12 transcription during differentiation of U937 monocytic cells into macrophage-like cells by PMA treatment was analyzed by qRT-PCR. The untreated cells were indicated as "Unt". The error bars represent the standard

error of three separate experiments with three replicates for each sample (*$p \leq 0.05$; ** $p \leq 0.005$).

In addition, we asked also whether primary human monocytes undergoing stimulation by PMA were also regulating NLRP12 expression since PMA is not a differentiative agent for primary monocytes[77]. Accordingly, primary human monocytes isolated from three healthy donors were stimulated with 80 nM of PMA for three hours. As shown in **Fig. 3.9**, in all the cases there was a consistent down-modulation of the NLRP12 mRNA expression.

Fig. 3.9. NLRP12 transcription in human primary monocytes treated with PMA: Primary human monocytes from different healthy donors were incubated with 80 nM of PMA or were left untreated (Unt) for 3 hours. The changes in the NLRP12 transcription level were analyzed by qRT-PCR. The error bars represent the standard error of three replicates.

3.4. U937 Cells Over-expressing NLRP12 Show Different Cell Cycle Distribution After PMA Treatment
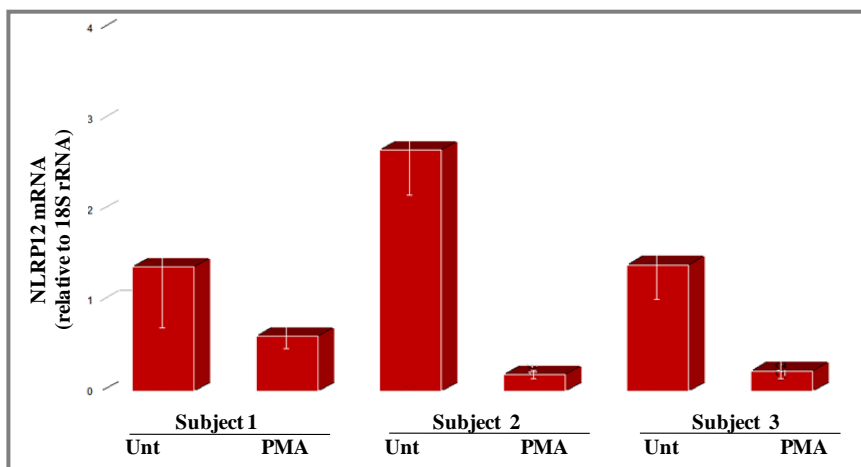
It is already known that NLRP12 is expressed in peripheral blood leukocytes, predominantly in eosinophils and granulocytes, and at lower levels in monocytes. To assess the role of NLRP12 in monocytes, we investigated the effect of NLRP12 over-expression in monocytic cell line U937. To achieve this, NLRP12 was cloned into pcDNA3 expression vector and the green fluorescent protein (GFP)-expressing plasmid pEGFP-N1. Using GFP expression system, its expression verified in a transient transfection in HeLa cells. The expression of 120 kDa NLRP12 protein was shown by western blotting, and the cytoplasmic localization was observed by fluorescent microscopy **(Fig. 3.10)**.

U937 cells were then transfected with NLRP12 expressing plasmid constructs as described in "Materials and Methods". pcDNA3 and pEGFP-N1 plasmids were used to deliver NLRP12 into U937 cells. The stable NLRP12 expression was verified by qRT-PCR **(Fig. 3.11)** and flow cytometry analysis **(Fig. 3.12)**. The NLRP12 expressing U937 cells were then incubated with PMA to analyze the effect of NLRP12 on PMA mediated macrophage differentiation. PMA treated U937 cells are adherent, non-replicative cells with characteristics of tissue macrophages, and this is mediated by a profound change in transcriptome[90]. Although the different morphological features allow to distinguish the differentiated cells, there are markers that allow to quantify

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this process such as CD11b, an integrin associated with cellular attachment which is strongly up-regulated by PMA treatment in U937 cell line[78]. Moreover, the differentiated cells undergo growth arrest in late G1 phase of the cell cycle and express a higher amount of the cyclin-dependent kinase inhibitor p21[79]. Accordingly, we compared CD11b surface marker and cell cycle progression in both Mock-transfected and NLRP12 over-expressing cells after PMA treatment.

(A)

PCR amplified NLRP12 cDNA
Negative control
DNA ladder

— 4 kb
— 3 kb

(B)

pEGFP-N1
pEGFPN1-NLRP12
Protein marker

— 140 kDa

— 42 kDa

— 25 kDa

(C)

HeLa cells

pEGFP-N1 (Mock)
transfected

pEGFPN1-NLRP12
transfected
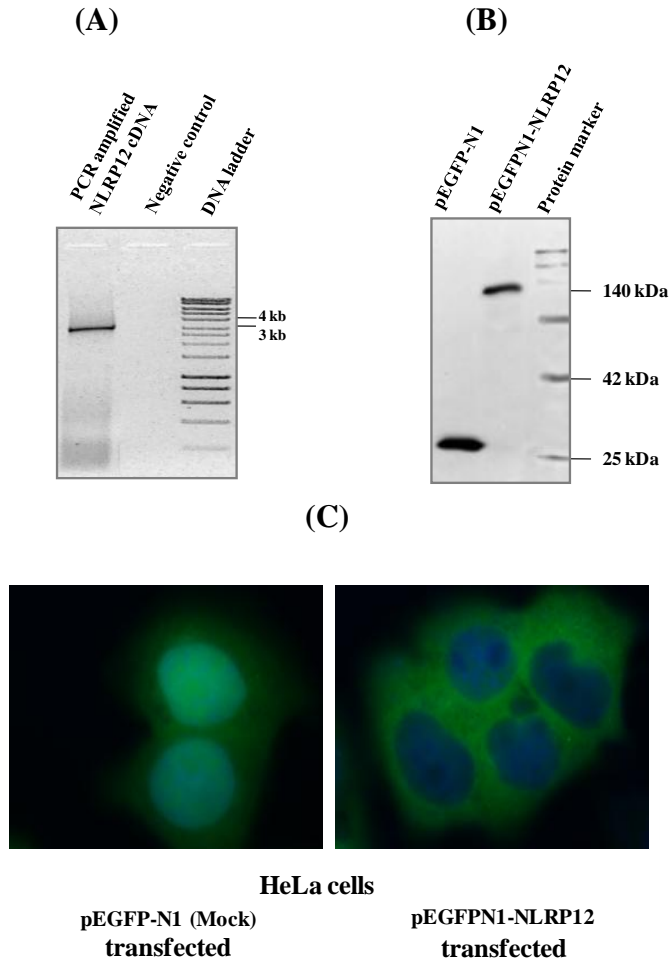
Fig. 3.10. Cloning and expression of human NLRP12: (A) Full length NLRP12 (3186 bp) was amplified from healthy individual monocyte cDNA by PCR, and **(B)** cloned into enhanced green fluorescent protein (EGFP)-N1 (pEGFP-N1) vector as C-terminal fusion. NLRP12 expression was confirmed by western blot analysis using anti-GFP antibody, and **(C)** by fluorescence microscopy of transient transfected HeLa cells. The EGFP expression is shown in the panel, EGFP fused NLRP12 expressing cells are shown in the right in the panel. Nuclei are stained blue with DAPI.

(A)   **(B)**
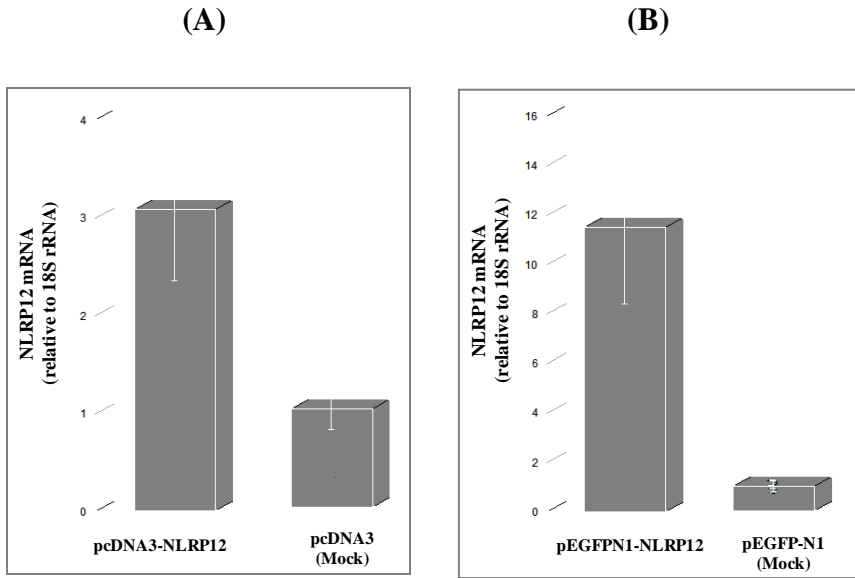
Fig. 3.11. NLRP12 mRNA expression in stably transfected U937 cells: NLRP12 expression was analyzed by qRT-PCR to confirm the stable expression of NLRP12 in U937 cells transfected with **(A)** pcDNA3, or **(B)** pEGFP-N1 constructs. The bars indicate the standard deviation of the three replicates.

(A)
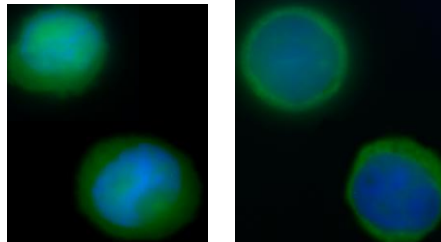
U937 cells

pEGFP-N1 (Mock) transfected   **pEGFPN1-NLRP12 transfected**

(B)
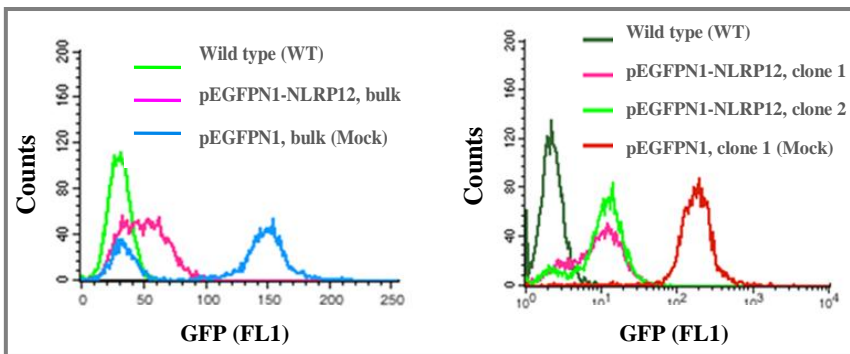
Fig. 3.12. Expression of NLRP12-GFP in U937 cells: (A) EGFP fused NLRP12 (pEGFPN1-NLRP12) is observed by fluorescence microscope 24 hours post-transfection of U937 cells. Nuclei are stained blue with DAPI. **(B)** Flow cytometry analysis of EGFP expression to evaluate NLRP12 expression level in stably transfected U937 cells. The "bulk" populations are given on the left, and the expression in the cell clones are shown on the right, as histograms.

The results show that NLRP12 over-expression did not alter CD11b expression after PMA treatment **(Fig. 3.13)**. However, NLRP12 over-expressing cells have shown different cell cycle phase distribution after PMA treatment: NLRP12

caused a higher percentage of cells accumulating in G2/M phase of the cell cycle, which was unexpected **(Fig. 3.14)**. Higher concentrations of PMA caused an even higher increase in the number of cells in G2/M phase **(Table 3.1)**. An unrelated protein (a MHC class I protein) over-expressing, stably transfected U937 cells was also used as control **(Table 3.2)**.
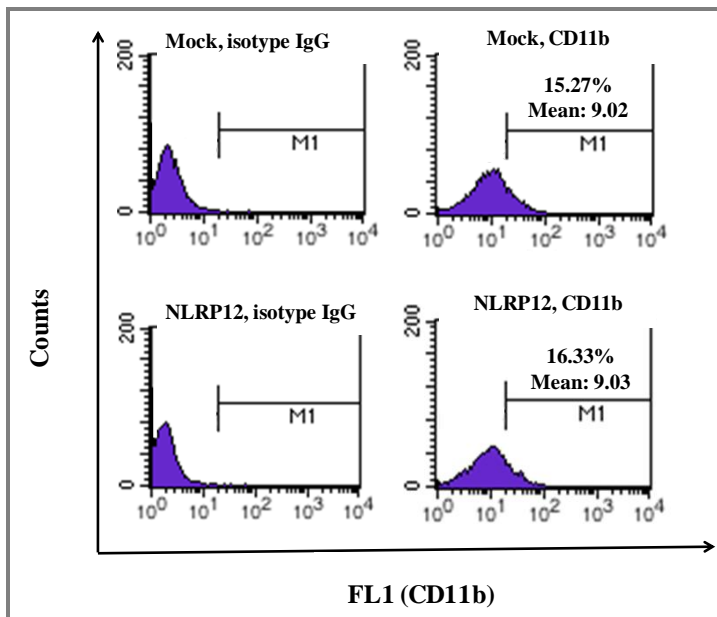
Fig. 3.13. CD11b expression in PMA treated NLRP12 over-expressing U937 cells: pcDNA3 (Mock) or pcDNA3-NLRP12 transfected U937 cells treated with 40 nM of PMA for 72 hours. The histograms show CD11b expression (on the right) relative to isotype controls (on the left).
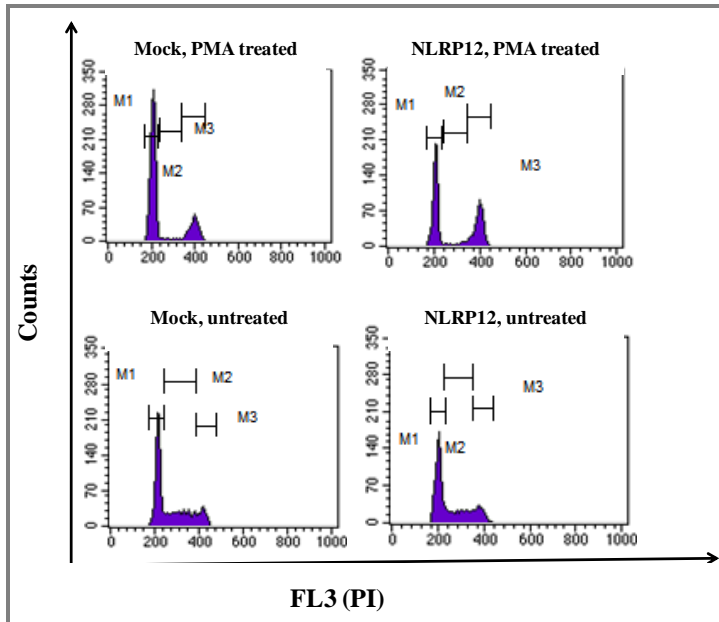
U937 cells	G1 phase % (gate: M1)	S phase % (gate: M2)	G2/M phase % (gate: M3)
Mock, PMA treated	78,24	2,82	18,91
NLRP12, PMA treated	52,69	4,51	42,75
Mock, untreated	53,55	34,28	11,76
NLRP12, untreated	56,77	30,41	14,07

Fig. 3.14. Analysis of cell cycle progression in NLRP12 over-expressing and Mock-transfected cells: pcDNA3-NLRP12 or pcDNA3 (Mock) transfected U937 cells were analyzed for cell cycle progression following treatment with 40 nM PMA for 72 hours. The percentage of cells in G1, S and G2/M phase of the cell cycle are indicated in the table. The histograms are representative of three independent experiments.

Untreated	U937 cells	G1 phase %	S phase %	G2/M phase %
	Mock, untreated	72,55	9,11	14,25
	NLRP12, untreated	67,22	11,78	16,80

48 hrs	U937 cells	G1 phase %	S phase %	G2/M phase %
	Mock, 20 nM PMA treated	70,25	4,93	22,64
	NLRP12, 20 nM PMA treated	58,63	5,65	32,42
	Mock, 40 nM PMA treated	70,32	4,88	22,60
	NLRP12, 40 nM PMA treated	54,55	5,66	36,20

72 hrs	U937 cells	G1 phase %	S phase %	G2/M phase %
	Mock, 20 nM PMA treated	84,89	2,39	11,90
	NLRP12, 20 nM PMA treated	66,46	3,79	28,01
	Mock, 40 nM PMA treated	84,67	1,84	12,92
	NLRP12, 40 nM PMA treated	62,32	4,34	31,80

Table 3.1. Cell cycle distribution of NLRP12 expressing or Mock-transfected cells after PMA treatment: After treatment with different doses of PMA for 48 or 72 hours, the pcDNA3-NLRP12 or pcDNA3 (Mock) stably transfected cells were analyzed for cell cycle distribution by PI staining. The percentage of cells in each phase is given.

U937 cells	G1 phase %	S phase %	G2/M phase %
Mock, PMA treated	78,89	6,60	15,25
NLRP12, PMA treated	66,78	2,93	30,70
Unrelated, PMA treated	76,55	5,51	18,62
Mock, untreated	64,91	15,60	20,99
NLRP12, untreated	60,89	19,56	21,19
Unrelated, untreated	67,46	14,11	19,68

Table 3.2. Cell cycle distribution of NLRP12 or unrelated protein over-expressing cells: After 48 hours treatment with 40 nM of PMA, DNA stained with PI and the percentage of cells in each phase of the cell cycle was determined by flow cytometry analysis. An unrelated protein over-expressing U937 cells were used as control.

3.5. Immunostaining Reveals Higher Percentage of Cyclin B1 Positive Interphase Cells in NLRP12 Over-expressing Cells

The G2/M phase accumulation in NLRP12 over-expressing cells was also shown by cyclin B1 immunostaining. It is already known that as a G2/M phase specific cell cycle marker, cyclin B1 expression begins to increase during G2, peaks in mitosis, and is rapidly degraded before the cell cycle is completed[80]. We have seen that the percentage of cyclin B1 positively stained cells in interphase was higher in NLRP12 over-expressing cells when compared with the Mock-transfected cells, in both untreated and PMA treated cases (**Table 3.3**).

Number of counted cells	U937 cells	Cyclin B1 (+) cells in interphase %	Cells in mitosis %
618	**Mock, untreated**	4,85	2,52
601	**Mock, PMA treated**	7,32	0,50
620	**NLRP12, untreated**	6,94	2,05
521	**NLRP12, PMA treated**	11,9	0,00

Table 3.3. Immunostaining for cyclin B1: U937 cells were treated with 40 nM PMA for 72 hours. The percentages of positively stained interphase cells are given.

3.6. DNA Content Analysis Shows Unscheduled Cyclin B1 Expression in Cells Over-expressing NLRP12

Intracellular cyclin B1 staining was combined with DNA content analysis to investigate if the cyclin B1 expression is restricted to very late S and G2/M phases of the cell cycle. The result show that NLRP12 over-expressing, untreated U937 cells express cyclin B1 in an "unscheduled" manner (unrestricted to particular phases of the cycle) **(Fig. 3.15)**.
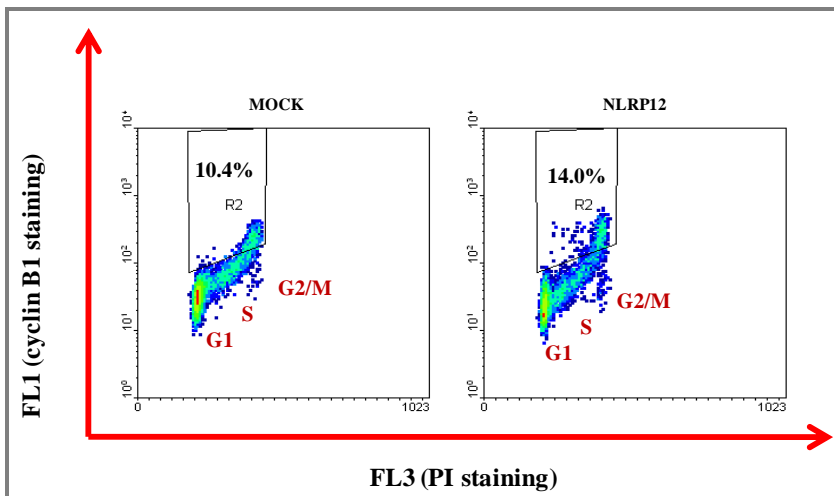

Fig. 3.15. Cyclin B1 expression during cell cycle progression: During cell cycle progression, cyclin B1 expression in pcDNA3-NLRP12 or pcDNA3 (Mock) transfected U937 cells was analyzed by flow cytometry. The G1, S and G2/M populations were gated (R2) based on the cells stained only with FITC conjugated secondary antibody. The DNA content was analyzed by PI staining.

3.7. Cyclin B1 and Cyclin A Expression Increase in NLRP12 Over-expressing cells

The expression of cyclin B1 was also analyzed by western blotting. The protein analyses show higher cyclin B1 expression in cells transfected with different (pcDNA3 or pEGFP-N1) NLRP12 expression plasmids, and also in different cell clones, obtained from "bulk" cell population **(Fig. 3.16; Fig. 3.17)**.
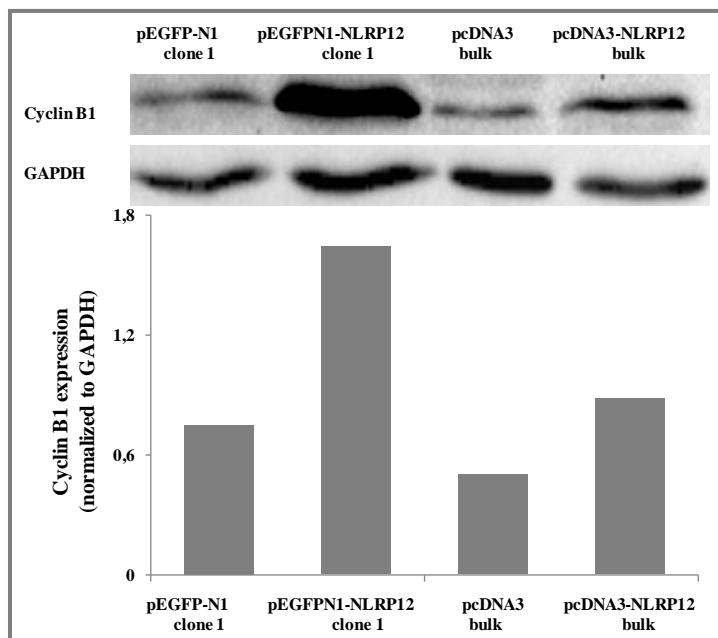
Fig. 3.16. Cyclin B1 expression in NLRP12 over-expressing U937 cells: Cyclin B1 expression in Mock-transfected (pEGFP-N1, clone 1 and pcDNA3, bulk), NLRP12 over-expressing bulk cell population (pcDNA3-NLRP12, bulk) and in GFP fused NLRP12 expressing clone cells (pEGFPN1-NLRP12, clone 1) was analyzed by western blotting. Densitometric analysis of the cyclin B1 bands are normalized to corresponding GAPDH bands and illustrated in the graph.
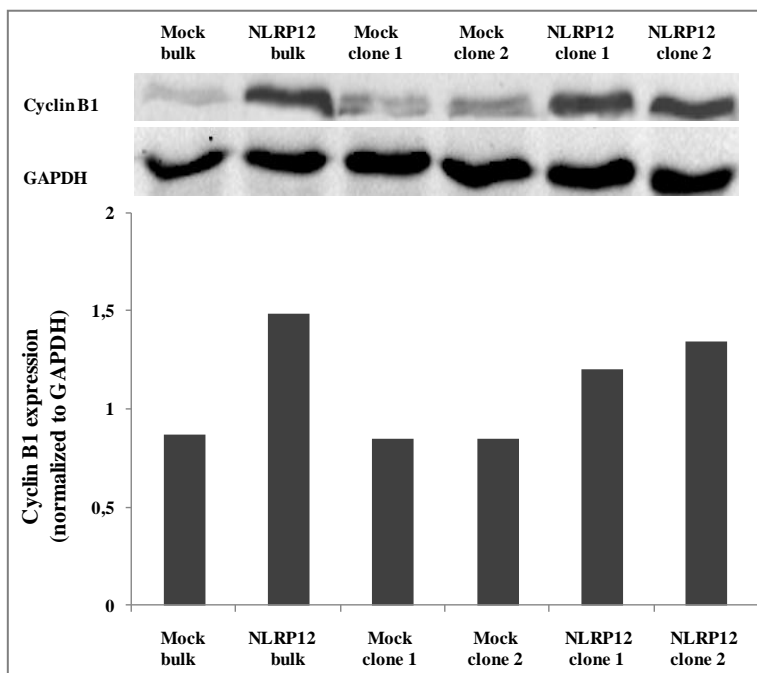
Fig. 3.17. Cyclin B1 expression in NLRP12 over-expressing U937 cell clones: Western blot shows expression of cyclin B1 in pEGFP-N1 (Mock) or pEGFPN1-NLRP12 transfected bulk cell populations or cell clones. The band intensities of cyclin B1 shown in the graph were normalized to that of GAPDH.

Cyclin A is the other G2/M cyclin whose expression begins to be detectable as cells enter S phase, and its level increases throughout S and G2 phase, peaking in early mitosis[81]. Therefore, the expression of cyclin A was also analyzed in NLRP12 over-expressing cells, and likewise cyclin B1, found to be upregulated **(Fig. 3.19)** whereas the expression of G1/S phase cyclin, cyclin D1[82] **(Fig. 3.18)** remained stable. This observation suggests that the over-expression of NLRP12 in U937 monocytic cells affects specifically the G2/M phase cyclins.

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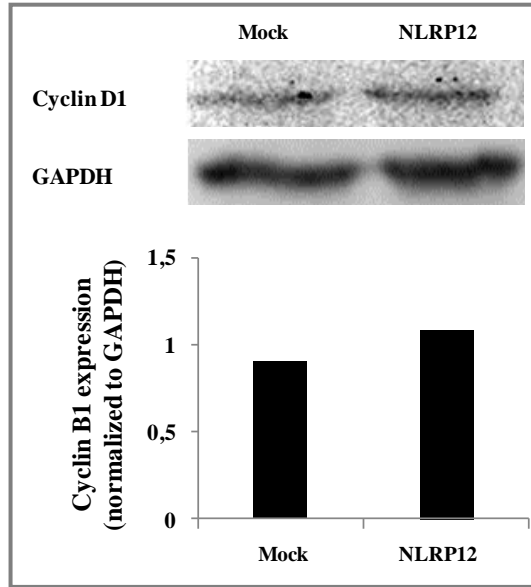
Fig. 3.18. Cyclin D1 expression: Cyclin D1 expression in pEGFP-N1 (Mock) and pEGFN1-NLRP12 stably transfected cells was analyzed by western blot. Densitometric analysis of cylin D1 bands normalized with GAPDH as loading control is shown.

To understand the mechanisms behind the increased levels of G2/M cyclins, we asked whether it is related with the proteasome function, since proper progression of the cell cycle requires ubiquitin-mediated degradation of the cyclins[86]. The proteasome inhibitor MG-132 was used to block proteasome activity. The effect of proteasome inhibition on cyclin B1 and cyclin A protein levels were analyzed by western blot shown in **Figure 3.19**.
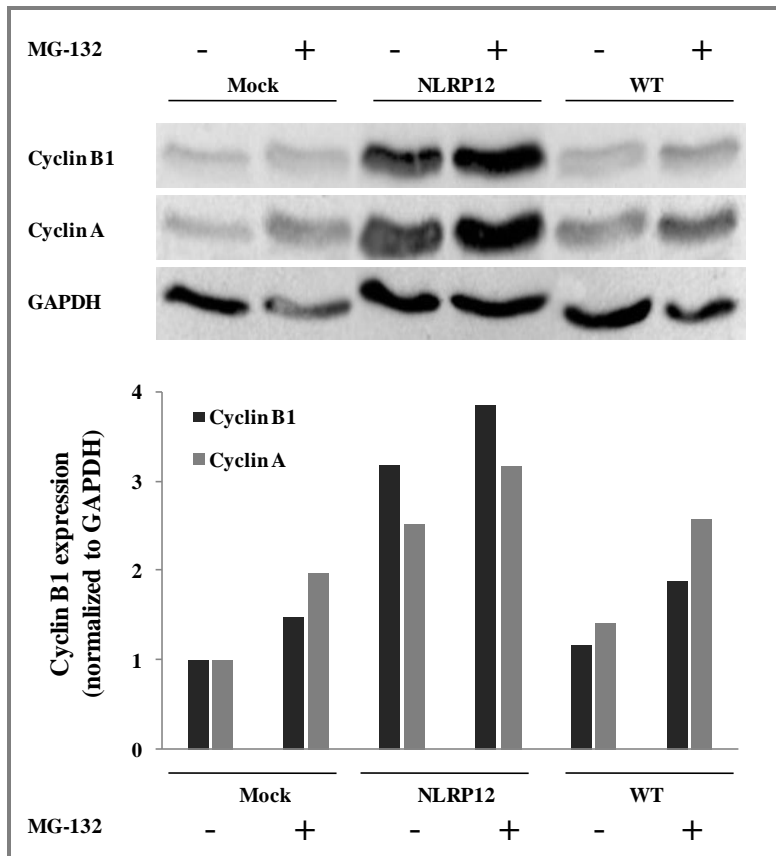
Fig. 3.19. Modulation of cylin B1 and cyclin A levels after MG-132 treatment: pEGFPN1 (Mock) and pEGFN1-NLRP12 stably transfected or wild type (WT) U937 cells treated with the proteasome inhibitor MG-132 for 6 hours or left untreated. Western blot analysis showing the expression of cyclin B1 and cyclin A in the MG-132 treated and untreated (indicated with + and -, respectively) U937 cells. Densitometric analysis of the western blot. immunoreactive bands were normalized to GAPDH.

CHAPTER 4

DISCUSSION

NLRP12 is a NLR (Nucleotide-binding domain and leucine-rich repeat containing) protein which is expressed predominantly in cells of myeloid origin. Its expression is down-regulated in response to pathogens, pathogen products, and inflammatory cytokines, and therefore it has been predicted that NLRP12 functions in modulating inflammation and immunity. Since NLRP12 has been described as a negative regulator of both canonical and non-canonical NF-κB activation we asked whether NF-κB has a modulatory effect on the *NLRP12* promoter.

Our results suggest that RelA (p65) causes a down-regulation of the promoter activity, and the region between the bases 530-745 appears to be responsible of this down-regulation. The *NLRP12* promoter region that we have analyzed contains a "non-consensus", putative NF-κB binding site. However, when this region was mutagenized, no effect in the promoter expression was observed. We therefore concluded that the reduced activity in the *NLRP12* promoter in response to RelA, is not due to a direct

effect of RelA itself but rather mediated by other, unknown factors, possibly induced by RelA.

Detailed analysis of the canonical and non-canonical NF-κB pathways in NLRP12 over-expressing monocytic THP-1 cells has revealed that nuclear translocation of the canonical NF-κB subunits RelA and p50 proceeds normally after stimulation with TLR agonist followed by CD40L, however p52 processing from NF-κB2/p100 and nuclear translocation of p52 is abolished[68]. To exert this effect, NLRP12 has been shown to target NF-κB-inducing kinase (NIK), the kinase responsible for activation of the non-canonical pathway [63 68, 70]. In accordance with these results, p52 levels are shown elevated in NLRP12-silenced cells[64]. Thus, it can be hypothesized that the stimulation of canonical NF-κB pathway can cause the down-regulation of NLRP12 transcription which interferes with the inhibitory effect of NLRP12 on the non-canonical NF-κB pathway (**Fig. 4.1**).

The canonical NF-κB pathway is activated rapidly by a number of upstream kinases through the IKK (IκB kinase) complex[67]. The activation of the canonical pathway results in nuclear translocation of RelA which induces a subset of early immune response genes. In contrast, the non-canonical activation of NF-κB displays slower kinetics and is often triggered in response to a second signal through TNF (Tumor Necrosis Factors) receptor super-family members[67, 68]. Once activated, the non-canonical NF-κB pathway drives later events in innate

immunity by inducing a different set of inflammatory genes to promote the ongoing immune response[83]. In this manner, the inhibition of NLRP12 transcription by canonical NF-κB may be crucial for the activation of non canonical NF-kB pathway in time, for the proper inflammatory response to occur. Further characterization of the *NLRP12* promoter will likely lead to a better understanding of the role of NF-κB on the NLRP12 transcription.
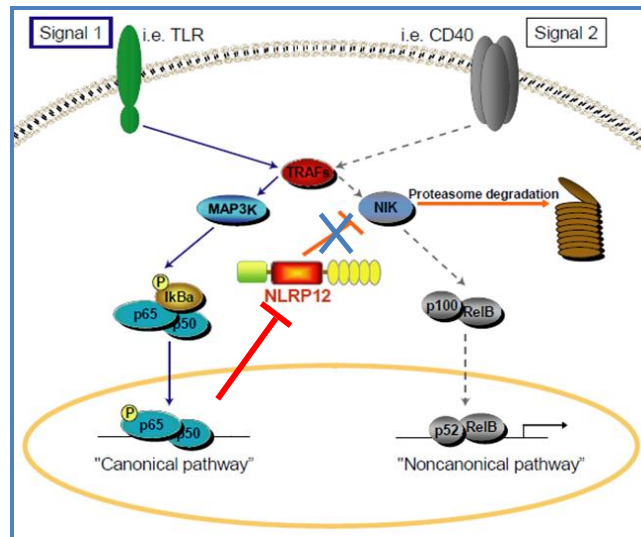
Fig. 4.1. NLRP12 and NF-κB pathway: NLRP12 negatively controls the non-canonical NF-κB pathway by interacting with and inducing proteasome-mediated degradation of NIK. As a result, NF-κB2/p100 to p52 processing is abolished, and p52-regulated genes are suppressed until NLRP12 expression fades. Transcriptional suppression of NLRP12 by RelA can allow the activation of non-canonical NF-κB pathway (Modified from Arthur , JC., 2009)[68].

As mentioned before, functions of NLRs range from antigen presentation to pathogen/damage sensing besides suppression or modulation of inflammatory signalling. In addition, some NLR proteins are also involved in embryonic development. NLRP12 is a member of NLR proteins family and its expression is restricted to myeloid cells. Although NLRP12 was one of the earliest identified NLRPs[60], the precise function(s) of NLRP12 is not fully understood as yet. Despite the studies which indicate a role for NLRP12 in regulating NF-κB, the results are conflicting. Therefore it can be considered that the function of NLRP12 in NF-κB regulation can be dependent on the nature of the stimulus and the specific context in which it is given[84].

According to its immune cells restricted expression, we asked how the NLRP12 expression is modulated during myeloid cell differentiation. As *in vitro* model system for monocyte to macrophage differentiation, U937 cells were differentiated into macrophage-like cells by PMA treatment. Interestingly, we have observed an up-regulation in NLRP12 transcription 24 hours post treatment with PMA. The up-regulation of NLRP12 transcription has been so far only reported to be induced by NO (nitric oxide) treatment[57]. As mentioned before, a partial 3' portion of the gene encoding NLRP12 was first identified in the human acute myeloid leukaemia (AML) cell line HL60, and called as *rno* (regulated by nitric oxide). Polymorphonuclear leucocytes were shown as the primary source of *rno* in the peripheral blood, and mononuclear cells were specified to express the gene constitutively but less

than polymorphonuclear leukocytes. When HL-60 cells were treated with NO, *rno* was up-regulated within 24 hours and the expression returned to baseline by 48 hours[57]. NO have been shown to inhibit the growth of HL-60, and induce HL-60 cells to differentiate with an enhanced expression of CD11b, CD14 and HLA-DR[85]. Based on these findings, enhancement of *rno* expression has been suggested as having a role in differentiation and growth inhibition of leukaemia cells as induced by NO[57]. Our study showed that PMA causes the up-regulation of NLRP12 transcription after 24 hours treatment in U937. However, treatment of primary human monocytes with PMA, which is not a differentiative agent for primary monocytes but rather causes cellular stress[77], determined the down-regulation of NLRP12 transcription after three hours treatment. In addition, LPS and IFN-γ treatment of human primary macrophages also caused down-regulation of the transcription, similar to the previous findings showing the NLRP12 transcriptional suppression in human primary adherent PBMC or granulocytes after treatment with TLR agonists and various cytokines[58, 64].

Human monocytes can be induced *in vitro* to differentiate to macrophages or to dendritic cells depending on the agents used. To study the NLRP12 transcriptional modulation during differentiation through these two pathways we took advantage of two widely used model systems to study differentiation of myeloid cells *in vitro*: the before mentioned U937 cell line in which PMA induces differentiation to macrophage and the AML

cell line KG1 in which PMA treatment induces differentiation towards dendritic cells. PMA treatment of KG1 cells caused an immediate down regulation of the transcription which proceeded through all the differentiation period whereas in the case of U937 cell line, there was an early up-regulation of NLRP12 mRNA followed by down-regulation. These data suggest that the modulation of NLRP12 transcription is dependent on the state of the myeloid differentiation: NLRP12 transcription is up-regulated in the first stages of the macrophage differentiation while is down-regulated when the differentiation is completed, whereas down-regulation of the transcription during dendritic cell differentiaton is immediate and persistent. Our results obtained from *in vitro* differentiation of primary human monocytes to macrophages by GM-CSF or M-CSF (Macrophage Colony-Stimulating Factor) treatment, and dendritic cells by GM-CSF and IL-4 treatment also suggest that NLRP12 transcription is regulated differently during different steps of myeloid cell differentiation. In conclusion, the data show a correlation between NLRP12 expression and monocyte differentiation. However, it does not tell us whether this protein can eventually play a direct role in the process. To address this question, NLRP12 was over-expressed in U937 cells. There was not apparent difference in the expression of CD11b utilized as macrophage differentiation marker following PMA treatment between Mock-transfected and NLRP12 over-expressing cells. Since PMA treatment of U937 induces G1 cell cycle arrest during differentiation, we also analyzed cell cycle progression after PMA

treatment of NLRP12 over-expressing and Mock-transfected cells. Surprisingly, G2/M phase accumulation was observed in the cells over-expressing NLRP12. Cyclin B1 staining revealed that even the mitotic indexes, a measure for the proliferation status of a cell population, were comparable between Mock-transfected and NLRP12 over-expressing cells. However, the percentage of the positively stained interphase cells was higher in NLRP12 over-expressing cells. This observation was confirmed by cyclin B1 staining together with DNA content analysis which showed increased and unscheduled expression of cyclin B1 in NLRP12 over-expressing cells: cyclin B1 expression was detected also in G1 and S phases of the cell cycle. Increased expression of cyclin B1 was also detected by western blot analysis of U937 cells transfected with different plasmid constructs. While cyclin A, the other G2/M cyclin, was also up-regulated in NLRP12 over expressing cells, the expression of cyclin D1, a G1 phase cyclin, remained unchanged. These findings suggest that the increased expression of NLRP12 induces an up-regulation of the G2/M phase specific cyclins. PMA treatment, which causes cell cycle arrest, probably leads to transfer of cytoplasmic G2/M cyclins into the nucleus and results in a higher amount of cells accumulated in G2/M.

To understand the mechanisms behind the increased levels of G2/M cyclins, we asked whether it is related with the proteasome function, since polyubiquitination and degradation of the cell cycle phase specific cyclins by the proteasome is

important for the proper progression of the cell cycle[86]. When we used MG-132 proteasome inhibitor, which effectively blocks the proteolytic activity of the proteasome complex, we observed that the accumulation rate of cyclin B1 and cyclin A were similar between NLRP12 over-expressing and Mock-transfected cells, even though NLRP12 over-expressing cells have a higher expression of both cyclin B1 and cyclin A. The fact that the proteasome inhibition rate was similar for NLRP12 over-expressing and Mock-transfected cells seems to suggest that NLRP12 does not interfere with proteasome activity.

As mentioned before, among the members of NLRs, CIITA associates and cooperates with transcription factors in the MHC class II promoter so to regulate MHC class II gene expression[87]. Recently, NLRC5 was shown as a transcriptional regulator of MHC class I genes[42]. CIITA shuttles between the nucleus and the cytosol via importin-α-mediated nuclear import and CRM1-dependent nuclear export. Similar to CIITA, NLRC5 was shown trapped in the nucleus upon treatment with the CRM1 inhibitor leptomycin B (LMB), indicating that NLRC5 also shuttles between the cytosol and nucleus, although it localizes in the cytoplasm upon over-expresion[42, 87]. Based on these findings, one can suggest that the increased level of G2/M cyclins can be caused by the transcriptional regulator activity of NLRP12. Further investigation is necessary to determine the cellular localization of NLRP12.

Although we do not know whether NLRP12 has an effect on G2/M cyclins in physiological conditions and/or leukemia, there are findings about their roles as tumor antigens. It has been shown that the peptides derived from cyclin B1 can be displayed by HLA class I molecules on tumor cells, then are recognized by the cancer patient's immune system as tumor specific antigens. Interestingly, cyclin B1-specific antibody and memory CD4^{+} and CD8^{+} T cells have been also found in many healthy individuals who have no history of cancer, suggesting a role for cyclin B1 in immunity[88]. Furthermore, inactivation of the tumor suppressor gene p53 has been shown to be correlated with an abnormal expression of cyclin B1 which serves as tumor antigen[89]. It is important to highlight that p53 is defective in U937 cells. Likewise cyclin B, cyclin A epitope specific CD8^{+} T cells were shown to target AML cells[90]. In this regard, the role of NLRP12 in the cyclin related immunity is worthwhile to investigate, since there is strong evidence indicating a role for NLRP12 in colon inflammation and tumorigenesis[69, 70].

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