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Inhibitor of apoptosis proteins (IAPs) expression in monocyte to macrophage differentiation and M1/M2 polarization

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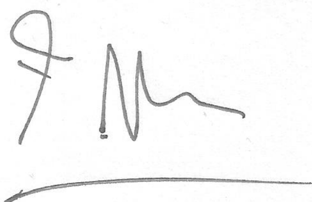

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A mis hermanos

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Rosalind Franklin.

Never interrupt someone doing something you said couldn't be done.

Amelia Earhart.

Summary

Monocytes and macrophages constitute the first line of defense of the immune system against external pathogens and fulfill other essential roles in tissue homeostasis. The ability of monocytes to differentiate into macrophages constitutes an important asset in inflammatory situations because it allows for the quick recruitment of immune cells from the bloodstream to the site of infection. Furthermore, macrophages are highly plastic and can respond differently to changes in their environment and acquire a more cytotoxic and pro-inflammatory phenotype (M1) or a more healing and anti-inflammatory one (M2) and perform different roles during inflammation and resolution of inflammation. This polarization capacity enables the innate immune system to quickly respond to threatening situations faster than tissue specific differentiation or adaptive immune responses. Both processes, differentiation and polarization of macrophages, are important in inflammation resolution and their deregulation promotes the development of several pathologies, including cancer. On the other hand, macrophage activation syndrome (MAS), is a life-threatening complication of some rheumatic diseases, it is caused by an excessive activation and expansion of macrophages and T-lymphocytes. The inhibitor of apoptosis protein family (IAPs) has important roles in the regulation of several cellular processes and in innate and adaptive immune responses, specifically in many macrophage functions and almost every signaling pathway leading to NF-κB activation, which is implicated in pro-inflammatory cytokine production.

In this thesis we wanted to characterize the expression profile of IAPs in the differentiation of monocytes to macrophages and in polarization into M1/M2 states, study the effect of IAPs pharmacological inhibition in macrophage differentiation and polarization and analyze the possibility of IAPs targeting as a therapeutic approach for macrophage activation syndrome.

We first set and optimized the *in vitro* macrophage differentiation and polarization models for the human monocytic cell lines THP-1 and U937 or primary monocytes from human peripheral blood mononuclear cells. We exposed them to different treatments (PMA for macrophage differentiation, LPS and IFN-γ for M1 and IL-4 for M2 macrophages) and, once we established the models, we analyzed the characteristic expression pattern of some members of the IAP family, namely, NAIP, cIAP1 and cIAP2 during monocyte to macrophage differentiation and in macrophage polarization. We have also evaluated the impact of pharmacological inhibition of IAPs in macrophage differentiation and polarization using a SMAC mimetic compound (SMC), SMC-LC161, an IAP antagonist currently used in cancer clinical trials. We also used SMC-LC161 to analyze the basal expression of cIAP1 and cIAP2 in MAS and the effect of IAP antagonism in a MAS-like mouse model.

We have seen that NAIP decreases its expression in the monocyte to macrophage differentiation process but increases when macrophages are polarized to M2, which we hypothesized is related to the implication of NAIP in cell proliferation. cIAP1 and cIAP2 present an inverse pattern of expression in polarized macrophages, with especially elevated levels of cIAP2 in M1 macrophages. Interestingly, the SMAC mimetic treatment leaded to an upregulation of NAIP, principally in M2, at the same time promoted the reduction of cIAP1 in M1 and M2 and a substantial increase of cIAP2 in M1. In the MAS-like mouse model we found that the treatment with SMC-LCL161 is not a valid approach to treating the disease, as the intended therapy did not revert the characteristic MAS features. In addition, SMC treatment of monocyte derived macrophages from MAS patients did not show a significant alteration in cIAP1 or cIAP2 expression after treatment.

These findings may lead to a better understanding of macrophage function and may contribute to the development of therapies in inflammatory conditions based on polarization switching.

Resumen

Monocitos y macrófagos constituyen la primera línea de defensa del sistema inmunitario innato frente a patógenos externos además de cumplir otros importantes papeles en la homeostasis tisular. La capacidad de los monocitos de diferenciarse a macrófagos es esencial en situaciones de inflamación, ya que esto facilita que el tejido afectado reclute monocitos de sangre periférica rápidamente. Además, los macrófagos son células muy plásticas y pueden responder de forma diferente a variedad de estímulos, así pueden adoptar un fenotipo más citotóxico y proinflamatorio (M1) o uno más protector antiinflamatorio (M2). Esto les permite cumplir varios papeles durante la inflamación y la reconstrucción del tejido dañado. La capacidad de polarización habilita al sistema inmunitario innato para responder rápidamente a situaciones de amenaza, en menos tiempo de lo que conllevaría una diferenciación específica o una adecuada respuesta immunitaria adaptativa. Ambos procesos, el de diferenciación y polarización de macrófagos están fuertemente regulados dada su importancia en los procesos de inflamación y fallos en su control están implicados en el desarrollo de varias patologías, incluído el cáncer. El síndrome de activación macrofágica puede ser una de estas patologías. Este síndrome es una enfermedad grave y potencialmente letal, generalmente se da como complicación de algunas enfermedades reumáticas y está causada por una proliferación y activación excesiva de macrófagos y linfocitos T. Las proteínas inhibidoras de la apoptosis (IAPs) constituyen una familia de proteínas con importantes papeles en la regulación de varios procesos celulares y en las respuestas inmunitarias innatas y adaptativas y especialmente en la función macrofágica y en las vías de señalización que llevan a la activación del NF-κB, que a su vez está implicado en la producción de citokinas proinflamatorias. En esta tesis queríamos evaluar la implicación de los IAPs en la diferenciación y polarización de macrófagos y estudiar la posibilidad de usar los IAPs como

dianas terapéuticas en el tratamiento del síndrome de activación macrofágica.

Empezamos optimizando modelos *in vitro* de diferenciación macrofágica y polarización con las líneas celulares THP-1 y U937 y con monocitos extraídos de sangre periférica. Estas células fueron expuestas a diferentes tratamientos, PMA para la diferenciación macrofágica, LPS e IFN-γ para la polarización a M1 y IL-4 para M2. Una vez que estudiamos marcadores de diferenciación y polarización y establecimos los modelos, estudiamos el patrón de expresión de algunos miembros de la familia de los IAPs como NAIP, cIAP1 y cIAP2. Para analizar la implicación de los IAPs, cIAP1, cIAP2 y XIAP en la polarización utilizamos un compuesto mimético de SMAC (SMC), LCL161, un compuesto que antagoniza dichos IAPs y que actualmente se usa como tratamiento en algunos tipos de cáncer. También utilizamos este compuesto para analizar el efecto de la inhibición de los IAPs en un modelo murino de síndrome de activación macrofágica. Además, en monocitos de pacientes afectados pudimos estudiar el patrón de expresión de cIAPs.

Hemos visto que la expresión de NAIP disminuye como consecuencia del proceso de diferenciación macrofágica y aumenta de forma considerable al polarizarse a M2. Pensamos que estas diferencias en la expresión de NAIP están relacionadas con la capacidad proliferativa de la célula, dada la implicación de NAIP en el proceso de citocinesis en división celular. También hemos advertido que cIAP1 y cIAP2 presentan un patrón inverso en la polarización macrofágica, en la que cIAP2 se encuentra especialmente elevado en macrófagos M1. Curiosamente, la exposición al compuesto LCL161 provocó una reducción de cIAP1 en M1 y M2 pero no así de cIAP2 y además previno la completa polarización de los macrófagos. Esto nos lleva a pensar que cIAP1 y cIAP2 están involucrados en la polarización.

En el modelo murino del síndrome de activación macrofágica hemos podido comprobar que el antagonismo de IAPs con miméticos de SMAC no es una buena aproximación al tratamiento de esta enfermedad, dado que la exposición a LCL161 no revirtió en ningún caso los síntomas característicos

del síndrome.

Nuestros hallazgos contribuyen a entender un poco más la función macrofágica y pueden servir para el desarrollo de terapias en enfermedades inflamatorias en las que exista un desequilibrio en la polarización.

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List of abbreviations

ACK .... Ammonium-chloride-potassium

BIR .... Baculovirus IAP repeat

BIRC .... Baculovirus IAP repeat containing

CARD .... Caspase activation and recruitment domain

cDNA .... Complementary DNA

cIAP1/2 ..... Cellular inhibitor of apoptosis 1 and 2

CPC .... Chromosomal passenger complex

DAMP .... Damage-associated molecular pattern

DIABLO ..... Direct IAP binding protein with low PI

HLH .... Hemophagocytic lymphohistiocytosis

IAP .... Inhibitor of apoptosis protein

IBM .... IAP-binding motif

IL .... Interleukin

INF .... Interferon

IRF .... Interferon Regulator Factor

GFP .... Green fluorescent protein

LPS .... Lipopolysaccharide

LRR .... Leucine rich repeat

MAMP .... Microbial associated molecular pattern

MAS .... Macrophage activation syndrome

MCSF .... Macrophage colony-stimulating factor

MyD88 .... Myeloid differentiation primary response protein 88

NAIP .... Neuronal apoptosis inhibitory protein

NF-κB .... Nuclear factor kappa-light-chain-enhancer of activated B cells

NK .... Natural killer cells

NOD .... Nucleotide-binding and oligomerization domain.

NT .... Non targeting

PAMP .... Pathogen-associated molecular pattern

PBMC .... Peripheral blood mononuclear cell

PMA Phorbol-12-myristate-13-acetate

PRR .... Pathogen Recognition Receptor

RING .... Really interesting new gene

siRNA .... small-interference RNA

sJIA .... Systemic juvenile idiopathic arthritis (sJIA)

SMAC .... Second mitochondrial activator of caspases

SMC .... Smac mimetic compound

TAM .... Tumor associated macrophage

TGF-β .... Transforming growth factor-β

TLR .... Toll like receptors

TNF .... Tumor necrosis factor

TNFR .... Tumor necrosis factor receptor

XIAP .... X-linked inhibitor of apoptosis

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CHAPTER 1

Introduction

1.1 Macrophages

The innate immune system is the first line of defense against external pathogens. Its response is nonspecific to a particular pathogen and depends on macrophage activity. Macrophages are a heterogeneous cell population involved in several processes that maintain tissular homeostasis, such as removal of apoptotic cells and cellular debris, and remodeling and repairing damaged tissue. As a reaction to certain changes in their environment they can modify their phenotype, polarize in order to give a more appropriate response, and acquire a more pro-inflammatory state (M1 macrophages) or more anti-inflammatory and healing state (M2 macrophages).

1.1.1 Innate immune system and inflammation

Vertebrates are the only organisms with the ability to develop an adaptive immune system; thus, for most organisms, the innate immune system is the first and only line of defense against external pathogens [1, 2]. Any innate immune system must be able to at least recognize pathogens and kill them [2]. In order to recognize microbes and protect the host against external threats, cells of the innate immune system express several germ-line encoded pattern recognition receptors (PRR) that are able to recognize pathogen-associated molecular patterns (PAMPs) [1] and microbe-associated molecular patterns (MAMPs) from invader pathogens. They can also recognize microbial nucleic acids, lipoproteins, and any other molecule expressed in pathogens but not in the host [3–5]. PRR can also recognize endogenous molecules released during cellular or tissue injury, known as damage-associated molecular patterns (DAMPs) [6]. PRR are classified according to their subcellular localization, structure, and ligand repertory, resulting in diverse PRR families such as Toll-like receptors (TLR), NOD-like receptors (NLR), and RIG-I-like receptors (RLRs) [5, 7]. The detection of pathogens by the PRRs initiates different signaling pathways, depending on the pathogen and specific PRR activated. This results in the activation of nuclear factors such as NF-κB, IRF3 and IRF7 [8–11]. The activation of these nuclear factors induces the production

and release of antimicrobial and antiviral peptides, chemokines, cytokines, and type I interferons, all of which are pro-inflammatory molecules that promote stimulation and recruitment of lymphocytes and monocytes to the inflamed site [3,11,12]. Many PRRs can build high-molecular-weight structures, called inflammasomes, which activate caspase-1 and promote the maturation of IL-1β and IL-18 [11, 13, 14]. Each signaling pathway involved in the inflammation process requires precise regulation, as an excessive inflammatory response can induce harmful immune-related disorders [14, 15].

1.1.2 Macrophages origin

Monocytes comprise approximately 10% of circulating leukocytes in human peripheral blood [16]. They are derived from progenitors in the bone marrow, then circulate in the bloodstream and migrate to peripheral tissues where they further differentiate into macrophages or dendritic cells [17, 18]. Monocytes are effector cells of the innate immune system that express surface receptors and adhesion molecules necessary during tissue recruitment of monocytes [17]. In an infection state, bloodstream monocytes are recruited to the infected tissue; upon arrival, they respond to pro-inflamatory mediators and differentiate to macrophages [19]. Monocyte to macrophage differentiation is promoted by MCSF (Macrophage colony-stimulating factor) and involves an extreme transformation by the cells as they increase their number of lysosomes and mitochondria [20]

Macrophages are highly specialized phagocytes with a very high catabolic and proteolytic metabolism [17, 19]. Phagocytes kill microbes and, through the production and release of chemokines, are able to recruit other myeloid cells to the site of inflammation. Prokaryotic cells have an extremely high cell division rate compared to leukocytes, leaving little time for the host's adaptive immune system to take over; thus, it is critical that macrophages are able to kill the invading microbes quickly [21]. As macrophages have proliferative capacity, they do not need to continue relying on monocytes to maintain their population once they are fully differentiated [18, 20, 22].

Historically, tissue resident macrophages were thought to belong to the mononuclear phagocyte system (or network). This mononuclear phagocyte system comprised macrophages, monocyte precursors, bone marrow precursors, and other cells derived from this lineage. The population in the different tissues were thought to be continually supplied and maintained by the blood circulating monocytes; the possibility of self-maintaining populations of tissue-resident macrophages was not considered. Although this system guided research for over 30 years, it was recently proved inaccurate. New research shows the existence of two main groups of tissue-resident macrophages according to their developmental history and mechanisms of maintenance: the first is bone marrow originated macrophages, which are short-lived monocyte-derived macrophages that require constant supply from circulating blood monocytes [23]; the other is tissue-resident macrophages with embryonic origin, the progenitors of which emerge from the yolk-sac and colonize different tissues during embryogenesis, prior to the development of definitive hematopoiesis, and form a specific long-term population of macrophages that can proliferate and self-maintain and are independent of circulating replacements [24]. These macrophage colonizers originate from yolk-sac (as early as day 8.5 of development) and from the fetal liver after gastrulation, so they migrate in at least three waves during embryonic development [22, 25].

1.1.2.1 Macrophage heterogeneity

Macrophage phenotype is influenced by tissue environment, allowing them to act independently of their origin and perform tissue-specific tasks [25, 26]. Therefore,several distinct macrophage populations can be found in different tissues [2]:

- Adipose tissue
  - Adipose-associated macrophages: These comprise 10% of cells in adipose tissue in lean humans, and up to 50% in obese humans, and are tightly linked to obesity-related metabolic diseases [27,28].
- Blood
  - Perivascular macrophages: Monocytes/macrophages protect the inner surface of endothelial walls and contribute to vessel barrier integrity [29]
- Bone
  - Osteoclasts: These large, multinucleated cells, formed by fusion of myeloid precursors, are not macrophages but members of the phagocityc system. They are involved in the bone remodeling cycle by removing old or damage bone matrix. They are also implicated in the regulation of calcium signaling by releasing calcium from the bone [30, 31]
  - Osteal macrophages: These macrophages, located adjacent to osteoblasts, help regulate bone formation in the bone remodeling cycle. They have diverse roles in skeletal homeostasis. [32, 33]
  - Bone marrow macrophages: There is a large population of macrophages in the bone marrow that contribute to the maintenance of the hematopoietic cell population and are involved in erythropoiesis [31, 32]

- Central nervous system
  - Microglia: A subpopulation of resident macrophages of the central nervous system (CNS), microglia are in direct contact with neurons, synapses and other glial cells. Microglia act in immunosurveillance and support and monitor synaptic function [34–37]
  - Perivascular macrophages: Found next to endothelial cells, these are antigen-presenting cells and participate in immunosurveillance of the CNS. [38, 39]
  - Meningeal macrophages: Located close to meningeal fibroblasts, meningeal macrophages have similar functions to perivascular macrophages. [40]
- Liver
  - Kupffer cells: A large population of macrophages in the liver, Kupffer cells are located along the sinusoidal endothelial cells. They act as scavenger cells, clearing bacterial products from the blood as well as damaged erythrocytes, and are also involved in iron and bilirubin metabolism. Kupffer cells have self-renewal capability. [41, 42]
- Lung
  - Alveolar macrophages: Located on the alveolar epithelial surface, these cells have a role in the removal of airborne pollutants and microbes. They also participates in lung homeostasis by clearance of surfactant [43, 44]
  - Interstitial macrophages: These cells are located in alveolar walls. Their main function is to prevent the development of allergic reactions in airways by modulating dendritic cell function. They are also better antigen presenting cells than alveolar macrophages. [43, 44]

- Skin
  - Dermal macrophages: Immunological sentinels involved in wound healing and early sensing of invading microorganisms. [45, 46]
  - Langerhans macrophages: These cells are classified as dendritic cells by some authors [46] and as macrophages by others [44, 47]. They are located in the basal and suprabasal epidermal layers, constitute the first line of defense against external pathogens, and have the ability to move to lymph nodes. They also regulate the T-cell response. [47]
- Spleen
  - Marginal zone macrophages and metallophilic macrophages: It is known that these two subpopulations of macrophages are present in the marginal zone of the spleen. Both have the ability to eradicate bacteria and are involved in the degradation and clearance of viruses. [48]
  - Red pulp macrophages: Located in the red pulp area of the spleen, usually in association with arterioles. These macrophages are critical to iron recycling metabolism and the removal of bacteria and senescent erythrocytes from the blood. [49, 50]

1.1.3 Macrophage polarization

As mentioned previously, macrophages are highly plastic and can respond to changes in their environment by modifying their phenotype and metabolism. This process of phenotypical adaptation is known as polarization and allows macrophages to direct their function to what is required by the organism. Similar to Th-1 (pro-inflammatory) and Th-2 (anti-inflammatory) nomenclature, macrophages may be classified as one of two polarization states, which correspond to extremes on either end of a broad spectrum of polarization states [51]: M1 macrophages, or classically activated macrophages, and M2 macrophages, or alternatively activated macrophages [52]. The M1 state

is a pro-inflammatory state characterized by a high production of effector molecules (reactive oxygen and nitrogen intermediates) and immunostimulatory cytokines (TNFα, IL-1β and IL-16). M1 macrophages are found largely during cell-mediated immune responses and have microbicidal and tumoricidal properties as they have cytotoxic activity. M2 macrophages are characterized by high levels of scavenger, mannose- and galactose-type receptors. M2 macrophages are involved in wound healing and have an important role during allergic responses and helminth-driven inflammatory reactions [53,54]. Polarization of macrophages is present under physiological conditions, but several studies implicate them in various pathologies [55] such as diabetes [56,57], cancer [58,59], atherosclerosis [60,61], myocardial infarction [62], and asthma [63].

1.1.3.1 M1/M2 macrophage polarization

- M1 macrophages: Also known as classically activated macrophages, they are polarized when macrophages are stimulated by microbial or pro-inflammatory cytokines such as LPS, INF-γ, TNF, TLR ligands and GM-CSF. This triggers several signaling pathways in the cell that lead to an activation state characterized by high antigen presentation, high production of pro-inflammatory cytokines such as IL-12 and IL-23 [64], and chemotactic molecules (such as CXCL9 and CXCL10) that help in the recruitment of leukocytes and macrophages. The main function of M1 macrophages is the killing of pathogens and protection against dangerous stimuli. M1 macrophages are highly reactive, and their cytotoxic activity is supported by a strong production of reactive oxygen species and nitrogen intermediates. A pronounced difference between murine and human macrophages has been observed: murine macrophages have the capability of NO production, whereas human macrophages lack this ability [55]. Because M1 macrophages promote a Th1 immune response, their regulation is very important. Among other possible outcomes, long-term unregulated inflammatory stimuli can have a tumorigenic effect.

- M2 macrophages: Also known as alternatively activated macrophages (or wound-healing macrophages by some authors), M2 macrophages constitute a heterogeneous group with several subsets of macrophages, later named M2a, M2b, and M2c. M2 macrophages are considered resting macrophages; tissue-resident macrophages are often recognized as M2. This state can be further improved by certain cytokines as M-CSF, IL-4, IL-13, IL-10 and TGF-β. M2 macrophages are characterized by the expression of Dectin-1, mannose receptor, scavenger receptor A, CCR2, and CXCR2 (among others) [65] and the production of ornithine and polyamines that participate in the reparative function of alternatively activated macrophages [64]. They are highly involved in host defense against helminths and nematodes, where they are able to participate in the clearance of parasites, confinement of granulomas, and repair of tissue damage by parasites [55]. M2 macrophages are poor antigen-presenting cells; they are key effectors of Th2 response and counteract Th1 response. Their presence is linked to the resolution of inflammation situation, scavenging of debris, and tissue homeostasis maintenance.

The aforementioned M1 and M2 polarization states correspond to two extremes of a wide spectrum of states, and it is common to find intermediate subsets that present mixed characteristics. Thus, the use of M1 and M2 classification is an oversimplified model.

It is well documented that a population of macrophages can change its phenotype to adapt to the needs of the host, although whether the same macrophages can undergo subsequent phenotypic changes to further adapt to changing environments is unclear. Some studies have shown the ability of M2 polarized macrophages to respond to the presence of certain agents by switching to a killing phenotype (M1-like). Specifically, this has been shown to occur in the case of a helminth infection and in the presence of LPS/INF-γ [66]. In their review, Zen, K. and colleagues [64] [67] hypothesized that some

miRNAs[1] can trigger and regulate the switching of the polarization state from M1 to M2. The capacity to switch phenotypes has been explored in many studies as a possible target in the treatment of some diseases where the balance, or lack of it, between M1 and M2 macrophages is involved in the pathology.

There is a complex network of transcription factors, electric [68] and other signaling pathways, post-transcriptional regulators, and epigenetic mechanisms orchestrating the different activation pathways of macrophages. Usually, a TLR ligand will promote the MyD88-dependent production of TNF, that will cooperate with INF-γ in an autocrine manner, enhancing the activation of the macrophage population [51]. Other members of the interferon and TLR antagonist family induce the activation of STAT1 via activation of the transcription factor IFN regulatory factor 3 (IRF-3), which leads to the expression of IFN-β and activation of the canonical NF-κB signaling pathway. This activation promotes the upregulation of pro-inflammatory genes, resulting macrophages increasing the expression of pro-inflammatory cytokines such as IL-12, IL-23 and CXCL10 [69]. Interestingly, M2 polarization inducers (IL-4, IL-10 or IL-13) usually lead to an inhibition of M1 chemokine expression [70] by downregulating STAT1 and NF-κB, leading to a control of the inflammation (the inhibition that M1 polarization signaling produces on the M2, and vice versa, is very well documented in *Sica, A. and Mantovani, A.* [69]). In the case of IL-4 stimulation used in current polarization studies, IL-4 receptors activate STAT6, which requires STAT1 and NF-κB coactivators. Once STAT6 is activated, it migrates to the nucleus where it promotes the transcription of typically M2 genes, such as mannose receptor (also known as CD206), PPARγ and arginase-1[2]. The signaling pathways triggered by different ligands leading to macrophage polarization are summarize in Figure 1.

[1]microRNAs: small non-coding RNA composed of 19-24 nucleotides that function in gene silencing at a posttranscriptional level.

[2]Expression of ARG1 is increased specially in mice M2 macrophages but not in humans.

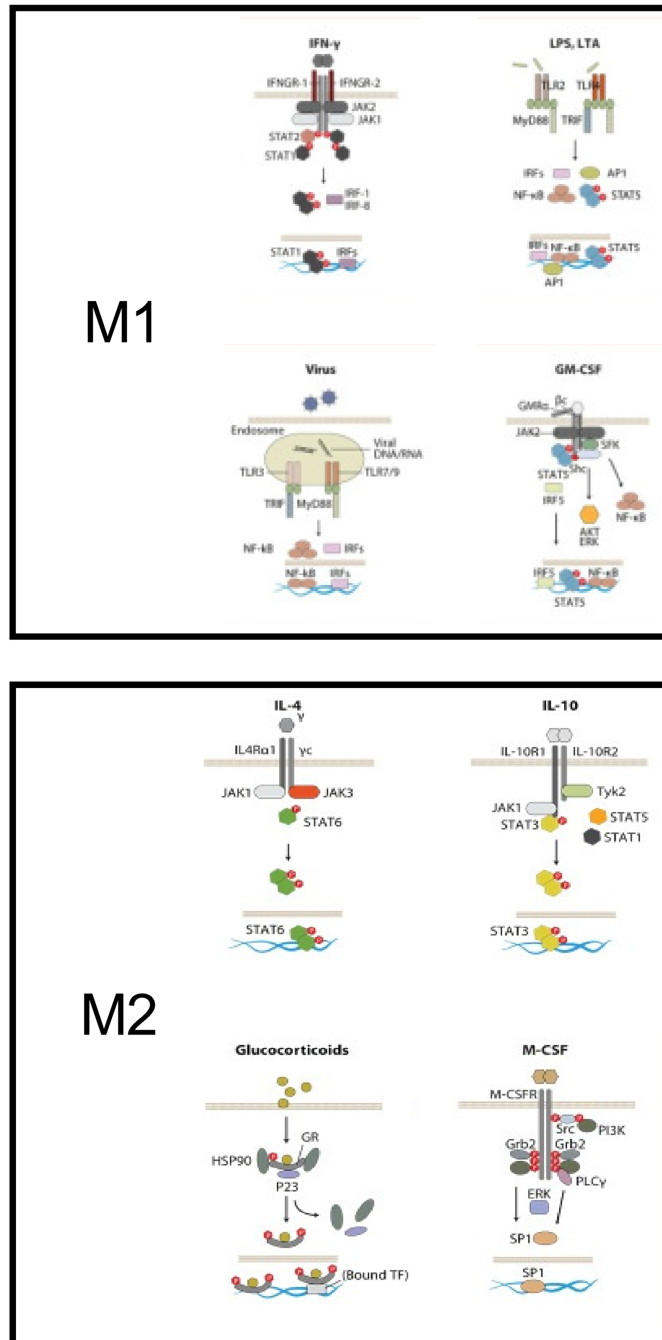


Figure 1. Molecular pathways involved in macrophage polarization: Figure taken and modified from *Martinez, O. and Gordon S.* [71].

1.1.3.2 Macrophage polarization under physiological conditions

Macrophage polarization balance is critical to maintaining homeostasis and in the regulation of some physiological situations. For example, macrophages constitute an essential player during pregnancy, a biological process requiring dynamic immunologic control. Large numbers of macrophages are needed to facilitate the implantation of the zygote after fecundation; during this step they are polarized towards M1. Following this, the population of macrophages present in the surrounding area display a mixed M1/M2 profile [72]. After the first trimester, when placentation is complete, macrophages located in the interface between mother and fetus shift to an immunosuppresive state, described as M2-like macrophages [73]. They maintain this phenotype until the end of the pregnancy, when M1 macrophages accumulate in the uterus and birth occurs. Several studies conducted in mice have found that fetal macrophages display an M2 phenotype and participate in extracellular matrix remodeling, angiogenesis, organogenesis, and proliferation of epithelial cells [55, 74].

Polarization balance is also important in wound healing. After an injury, both macrophage polarization states are needed during different phases of tissue repair. In the first phase of wound healing, inflammation takes place to promote defense of the host from pathogen colonization. During this stage, macrophages are classically activated, producing pro-inflammatory mediators and cytokines and participating in recruitment of neutrophils and other leukocytes. Next, during the resolution phase, a shift from M1 towards M2 begins to occur, resulting in macrophages that produce anti-inflammatory cytokines, such as TGF-β and IL-10, and downregulate inflammatory mediators. M2 macrophages also get rid of apoptotic neutrophils and damaged cells. During the next stage of wound healing, known as the proliferation phase, M2 macrophages produce growth factors (EGF, FGF and VEGF) promoting the expansion of cells involved in tissue repair. M2 macrophages also play a key role in the final phase, known as remodeling, where they help in the maturation of new tissue, vascularization, and in the reorganization of

the extracellular matrix [55, 69, 75].

Macrophage polarization is important in other situations: M2 macrophages promote angiogenesis, a process required during embryogenesis and in wound healing [76]; In the heart, tissue resident macrophages have an M2-like phenotype. In the case of myocardial infarction, macrophages in the heart act similarly to those involved in wound healing: both polarized populations are involved in the resolution of damaged tissue [77]. Polarized macrophages also constitute an important regulator of metabolism. For instance, in iron metabolism, M1 macrophages that express proteins involved in iron storage, such as ferritin, sequester iron, whereas M2 macrophages present high levels of iron exporters, such as ferroportin, and favor iron release [78, 79]. Alternatively, activated macrophages have been found to act as a source of warmth in adipose tissue in a process known as thermogenesis [80]. In lean individuals, adipose tissue macrophages present an M2-like phenotype and help maintain tissue homeostasis, protecting against inflammation and contributing to lipolysis and insulin sensitivity [28, 81].

1.1.3.3 Macrophage polarization in disease

Macrophage polarization is involved in many homeostatic mechanisms and its regulation is essential. However, there are some cases in which polarization is associated with pathologies. As mentioned previously, the polarization of macrophages plays an important role during pregnancy. Incorrect patterns of macrophage polarization can result in several problems during pregnancy, such as preterm labor, preeclampsia, fetal intrauterine growth restriction, and even miscarriage [72]. During the resolution phase of wound healing, an excessive accumulation of M2 macrophages can lead to an excess in collagen production that can evolve to fibrosis [82]. This macrophage-associated fibrotic effect has also been seen in fibrosis linked with Duchenne muscular dystrophy, as well as in lung and liver fibrosis [55, 83].

Adipose resident macrophages maintain an M2 phenotype in lean individ-

uals. In the case of obesity, adipocytes release inflammatory mediators that promote the accumulation of macrophages in the tissue and the switch to an M1 state, producing pro-inflammatory cytokines and shifting to a state of chronic inflammation that can lead to the development of related diseases such as insulin resistance, type 2 diabetes, or metabolic syndrome. Additionally, adipose tissue of obese individuals often contains necrotic cells that increase inflammation by releasing cytokines and chemokines that recruit additional macrophages to the tissue [51, 55, 84]. There are some theories linking the development of atherosclerosis with macrophage polarization switching. It is well documented that macrophages uptake oxidized fats that lead to the formation of foam cells; the first step in the pathogenesis of atherosclerosis. Some murine studies show a dynamic balance of M1/M2 populations in and around arterial plaque, with M1 macrophages more predominant when the disease is advancing [55, 85].

In allergies and allergic asthma, there is a strong correlation between M2 polarized macrophages and severity of the disease. Allergy pathogenesis depends on M2 cytokines such as IL-4, IL-9, IL-13 [65]. In necrotizing enterocolitis, M1 macrophages have been found to promote the disease by damaging the intestinal epithelium [86]. There is even evidence that associates macrophage polarization imbalance with mental health disorders, such as depression [87] and bipolar disorder, in which microglia are polarized towards a pro-inflammatory phenotype [88].

Other indications of polarization involvement in pathogenesis are depicted in the following image.

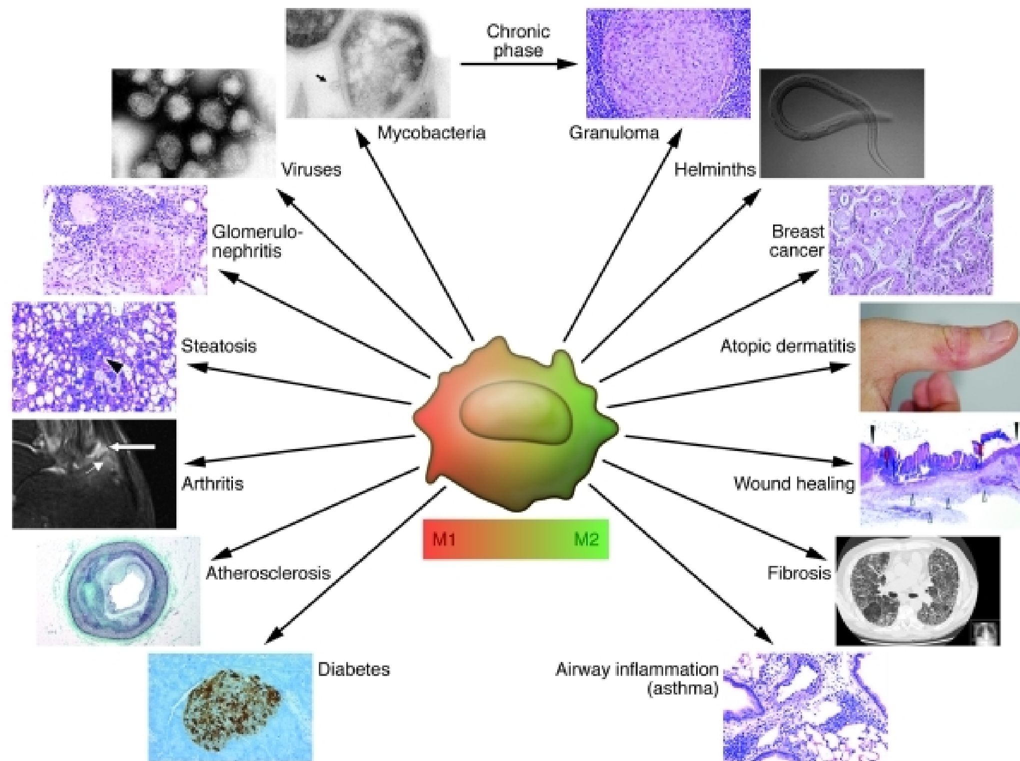


Figure 2. Representation of macrophage polarization involvement in some disease pathologies: Figure taken from *Sica, A. and Mantovani, A.* [69].

Cancer

During chronic inflammation, the continuous presence of pro-inflammatory cytokines, reactive oxygen species and other inflammatory mediators, predispose surrounding area to the appearance of neoplastic tissue [89]. Although M1 macrophages have been described as tumoricidal, they also can play a role in the first steps of tumor formation. Thus, a prolonged inflammatory state can be a cause of cancer and may also be present in the surrounding environment of growing tumors. Tumor cells express pro-inflammatory cytokines and other chemoattractants that work in agreement with adhesion molecules in order to recruit macrophages to the tumor area [90], where they will change their phenotype and become tumor associated macrophages (TAM), with an immunosupressive phenotype that caused them to be described

as M2-like macrophages in early studies.

TAMs are a major component of the immune cell population in the tumor microenvironment. In most cases, these macrophages present an M2-like polarized phenotype. NF-κB has emerged as one pathway involved in the initiation of some types of cancer and is activated through a MyD88 dependent-pathway in some TAMs [51]. Although TAMs have been commonly characterized as M2 macrophages, they maintain some M1 signaling pathways such as STAT1/IRF-3 [65] and MyD88/NF-κB. These pathways are typical of M1 macrophages and produce some pro-inflammatory cytokines such as IL-6, which is important in the upregulation of some genes needed for the survival of tumor cells and the inhibition of apoptosis [91].

The role of TAMs is diverse: they participate in tumor evasion of the immune system by presenting tumor-associated antigens to T cells and avoid anti-tumor functions of NK and T cells. They also help propagation of the tumor. In hypoxic conditions, which naturally occur in some areas of growing tumors, TAMs are recruited to the hypoxic regions that induce the expression of angiogenesis and metastasis related genes [79]. TAMs can promote tumor expansion by releasing proteases to degrade extracellular matrix and growth factors for proliferation of endothelial cells and formation of microvessels, vascularizing the area to promote tumor growth. Additionally, hypoxic TAMs also improve the ability of tumor cells to invade and metastasize to other organs [59]. Furthermore, the presence of M1 macrophages within the tumor microenvironment is associated with the elimination of tumors following combinatorial anticancer therapy strategies [92,93]. Clearly, the investigation and further understanding of the mechanisms leading to macrophage polarization and the switch between M1/M2 states are of current interest and might contribute to novel therapeutic approaches.

1.2 Inhibitor of apoptosis, IAP, protein family

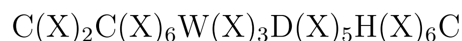
Inhibitor of apoptosis (IAP) proteins constitute an evolutionarily conserved family of intracellular proteins characterized by the presence of one to three Baculovirus Inhibitor of Apoptosis (BIR) repeats in their structure [94, 95]. IAPs or cellular homologs have been identified in different organism from yeast to higher vertebrates, and a paralog family protein has been described in plants [96]. In humans, as in all mammals, this family is formed by eight members. Early studies of this family were conducted with the notion that IAPs inhibit apoptosis by inhibiting caspases. This was proved to be only partially true, as some IAPs, such as XIAP, cIAP1 and cIAP2 can, through their BIR domain, act over caspases and block cellular apoptosis [97]. However, inhibition of apoptosis is not their only function and, in recent years, it has become more apparent that this family is involved in several cellular processes beyond caspase and cell death inhibition such as cytokinesis, proliferation, differentiation, signal transduction and heavy metal homeostasis [94,98]. IAPs also regulate innate and adaptive immune responses by modulating functional properties of immune cells; in fact, XIAP, cIAP1, cIAP2 and NAIP regulate monocytes, macrophages, lymphocytes and NK cell signaling pathways [94, 98]. During an innate immune response, cIAP1, cIAP2 and XIAP regulate NOD and TLR signaling through NF-κB and MAPK signaling pathways. NAIP acts as a microbial sensor and can activate the inflammasome due to its relation with NLRC4 [99, 100], which interacts with caspase-1 and promotes a pro-inflammatory response.

1.2.1 History

The first IAP protein family member was described more than 20 years ago in baculovirus infected insect cells, where it is involved in inhibition of host cell apoptosis so the virus can continue replicating itself [101]. Since then, IAPs have been identified in other organisms, such as Drosophila, yeasts, and most of vertebrate species [102]. The principal characteristic of the IAP family is the presence of at least one BIR domain. Although not all IAP members are able to inhibit apoptosis, the name Inhibitor of Apoptosis family has been maintained. The first non-viral IAP discovered in mammals was NAIP (Neuronal Apoptosis Inhibitory Protein) during an investigation of the development of spinal muscular atrophy [103]. In mammals, eight different IAPs have been discovered in the past two decades: NAIP, cellular IAP 1 (cIAP1), cellular IAP 2 (cIAP2), X linked IAP (XIAP), Survivin, BIR Repeat-containing Ubiquitin-Conjugating Enzyme (BRUCE/Apollon), Melanoma IAP (ML-IAP) and Inhibitor of apoptosis-Like Protein-2 (ILP2) [104].

1.2.2 Structure and function

Members of the IAP family have very diverse structures, although they all share the presence of one to three tandem copies of **BIR domain**. BIR domains are present in more than 80 different eukaryotic proteins. These domains contain 70-80 amino acids and they are rich in histidine (H) and cysteine (C), which allow the domain to capture zinc and adopt a globular configuration with four α-helices and a variable number of anti-parallel β-sheets. BIR domains display a nucleus with a consensus sequence of amino acids:



$$\mathrm{C(X)_2C(X)_6W(X)_3D(X)_5H(X)_6C}$$

where X represents any amino acid. BIR domain enables protein-protein interactions and, in the case of some IAPs, is necessary for interaction with proapoptotic factors that allow IAPs to directly interact with caspases to inhibit their function [105].

In addition to BIR domain, IAPs, whose structure is represented in Fig 3, also exhibit other domains such as **RING** (acronym for “really interesting new gene”) present at the C terminal position in XIAP, cIAP1, cIAP2 and ILP2. The RING domain confers an E3 ubiquitin-ligase activity to the protein. This ubiquitin-ligase function has several roles in auto- and para-ubiquitination essential in proteasomal degradation or ubiquitin targeting needed in signal transduction of some central cellular signaling pathways [106]. Another domain necessary for ubiquitination function and present in some IAPs is a ubiquitin-associated domain (UBA), which allows the protein to bind the ubiquitin chain.

cIAP1 and cIAP2 also have a CARD (caspase activation and recruitment domain) in their structure. Although CARD are protein-protein interaction domains, their function in these two proteins is unknown [107, 108].

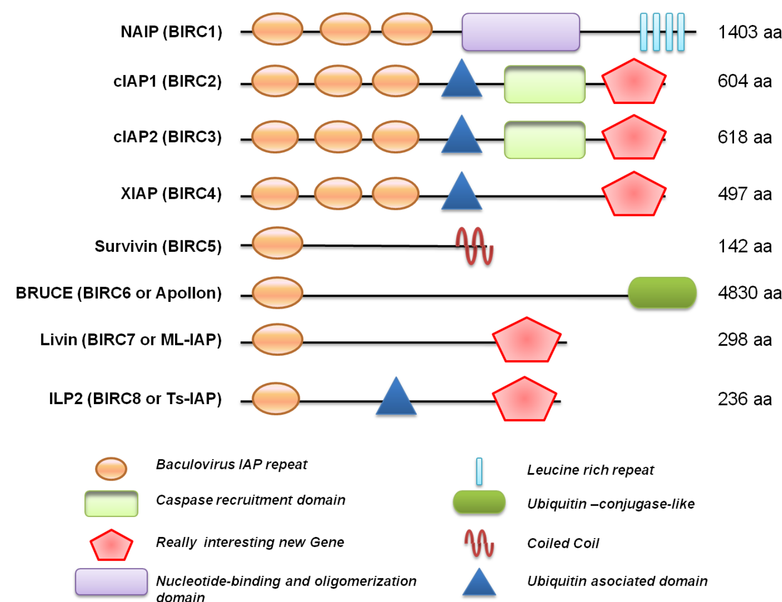


Figure 3. The human IAP family members. A total of eight different human IAPs have been described. Functional domains and number of residues of each member are shown.

Other IAPs exhibit additional domains. Survivin, the smallest of mammal IAP members, possesses a coiled coil domain at its C-terminus. NAIP has a characteristic structure, with a central nucleotide- binding and oligomerization domain (NOD) and a leucine rich repeat (LRR) region that makes it belong not just to the IAP, but also to the NLR family. The LRR region acts as PRR, while NOD acts as a scaffold where protein signaling complexes can assemble in order to continue the response against pathogens.

1.2.3 Apoptosis

Apoptosis is a cellular process defined by Fulda et al. as "an evolutionary conserved, intrinsic program of cell death that occurs in various physiological and pathological situations and is characterized by typical morphological and biochemical hallmarks, including cell shrinkage, nuclear DNA fragmentation and membrane blebbing" [109]. There are two main apoptosis induction pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. Both are summarized in Fig 4:

1. Intrinsic pathways, that involves an initial disturbance of the cell, which could be cellular stress or cytotoxic damage, that drive to opening of the mitochondrial outer membrane and the release of some proapoptotic molecules that lead to activation of the caspase cascade through caspase-9 activation [110–112].

2. Extrinsic apoptosis pathway is triggered by external ligands that bind to members of the TNF family death receptors (as TNFR1, Fas/CD95 and TRAIL-Rs), induce their activation. They then recruit cytoplasmic adapter proteins which drive to procaspase-8 activation and therefore the caspase cascade [111–113] and execution of apoptosis.

Both the extrinsic and intrinsic apoptotic pathways converge on the signaling of the effector caspases -3 and -7. IAPs exert control of both pathways so cells cannot undergo apoptosis except under specific conditions. IAPs prevent apoptosis through different mechanisms:

- **Extrinsic precursors interaction**: The interaction of TNF-α with its receptor, TNFR1, provokes the assembly of a multiprotein complex involving TRADD, RIPK1 and TRAF2 [114]. In this structure, cIAP1 and cIAP2 mediate ubiquitination that leads, ultimately, to the activation of NF-κB [115]. This induces survival signal transduction [116], thus avoiding the apoptotic process triggered by the death signal.

- **Inhibition of IAPs antagonists:** BRUCE is a potent anti-apoptotic protein and through its UBC domain ubiquinates IAP antagonists (some released by the mitochondria) and promotes their proteasomal degradation [117,118]. As its degradation triggers the intrinsic apoptosis pathway itself, BRUCE constitutes an essential IAP [119]. Livin (ML-IAP) is expressed in only a few situations, where it confers resistance to apoptosis in the same way BRUCE does, by ubiquitination of pro-apoptotic antagonists of IAPs [120, 121].

- **Caspase inhibition:** XIAP is the only IAP capable of inhibiting caspases by direct contact with them. It has been shown that cIAP1 and cIAP2 are able to bind to caspases but the physiologic consequences of this remain unknown [122]. XIAP binds to caspase-9 (starter) through its BIR3 domain, [123], and can also bind to caspases 3 and 7 by a sequence situated in the BIR2 domain. This binding induces conformational changes in the caspases that hide the active site [116]. Both the changes and the inhibition are reversible [124]. Survivin also inhibits the activation of caspase-9; this is not through direct binding, but through the apoptosome [125]. The apoptosome is a protein complex involved in the apoptosis intrinsic pathway that activates caspase-9 [126]. BRUCE is also able to inhibit the caspase cascade, which is thought to happen through binding an inactivation of caspase-3 [127]. NAIP, the founding member of the mammalian IAP family, has been shown to inhibit the executioner caspase-3 and -7 and the initiator pro-caspase 9 through one of its BIR domain [128–130].

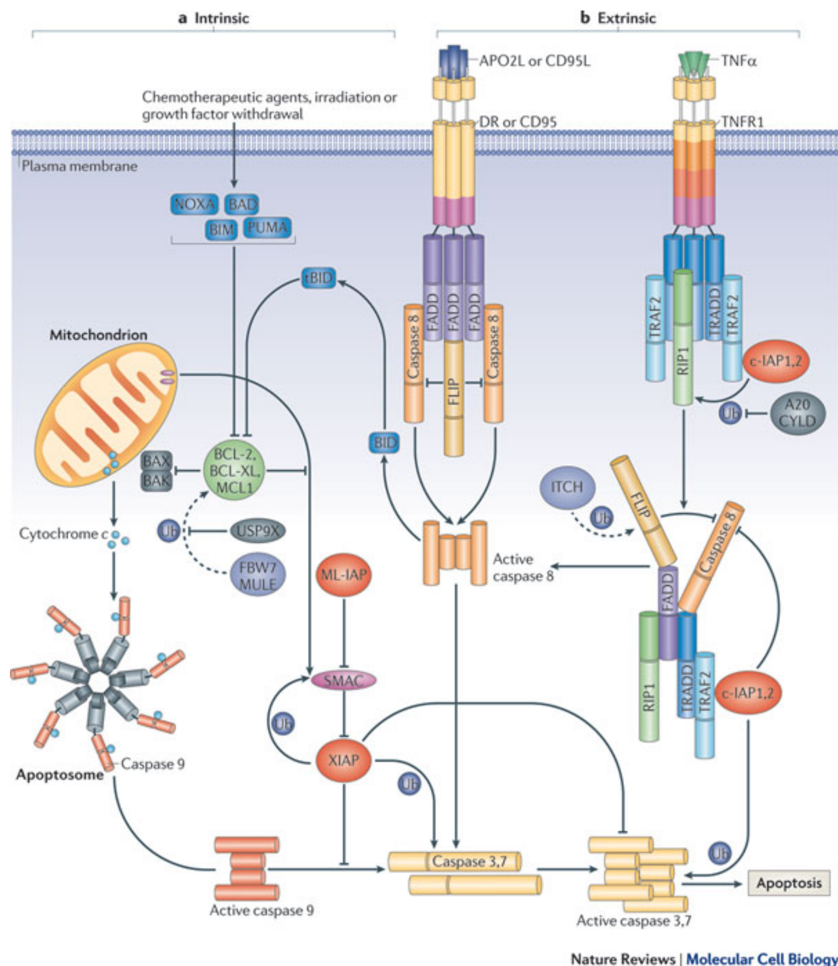


Figure 4. Apopsosis pathways. Intrinsic and extrinsic apoptosis pathways including IAP (red tabs) involvement. Taken from Vucic et al. 2011 [113]

1.2.4 Non-apoptotic functions

When they were first discovered, IAPs were thought to be inhibitors of apoptosis due to their ability to bind and inhibit caspases. Later, this was found to be true as some IAPs can accomplish that role both in early and in later stages of apoptotic signaling. However, in addition to this antiapoptotic role, IAPs have other non-apoptotic roles such as transduction intermediates in different signaling cascades associated with essential cell functions; these include heavy metal metabolism, cellular migration control, and regulation of cell division. In fact, some IAPs are essential for the survival of certain cell

populations like neurons [131], cardiomyocytes [132] and macrophages [133], and the differentiation of several cell types like muscle fibers, neurons, and macrophages. The differentiation and polarization of macrophages will be a central topic of this thesis.

1.2.4.1 IAPs as signal transducers.

TNFRs: TNF receptors are localized to the cell membrane and, when antagonized, they recruit proteins in the cytoplasm and build a receptor complex able to induce multiple signaling cascades. There are several members of the family, and each one promotes a different signaling cascade, resulting in different cell fates depending on the receptor and on the presence of cIAPs [106].

- TNFR1: Upon ligand binding, TNFR1 recruits TNFR-1-associated death receptor (TRADD), TNFR associated factor-2 (TRAF2), RIP1, cIAP1 and cIAP2. In the absence of cIAP1/2, RIP1 forms a cytosolic complex that activates caspase-8 and leads to apoptosis. Instead of this, cIAP1/2 ubiquitinates RIP1 which allows the recruitment and activation of further factors and ultimately activates NF-κB [94, 106].
- CD40 and other TNF receptors: Previous receptor activation of cIAP1 and cIAP2 results in an independent complex with TRAF2 and TRAF3. This complex is able to bind NIK (NF-κB-inducing kinase) so cIAP can mediate its ubiquitination and degradation. The agonist-binding to the receptor leads to degradation of cIAPs and TRAF2, leading to NIK stabilization, which activates NF-κB (alternative pathway) [94, 106].

TGF-β: TGF is a superfamily of polypeptide growth factors that regulates immune system interactions and is also involved in proliferation, morphogenesis and differentiation [134]. These cytokines bind to membrane receptors and trigger a signaling pathway cascade to the nucleus. This signaling happens in two different branches, always resulting in NF-κB activation and, in both cases, XIAP participates as a positive regulator [135, 136] and avoids TGF-β apoptosis induction [137].

NF-κB signaling pathways: NF-κB is a family of dimeric transcription factors. In mammals, five members are present: RelA (p65), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2). These transcription factors play essential roles in several diverse cellular mechanisms, such as cell survival, differentiation and proliferation, and regulation of specific gene expression [138]. NF-κB signaling pathways have been classified into two categories: canonical (also known as classical) and non-canonical (also known as alternative). cIAP1 and cIAP2 are key effectors of both classical and alternative signaling pathways. Through their E3 ligase domain they mediate ubiquitination of specific targets.

- Classical NF-κB pathway: This pathway depends on the presence of a death ligand, such as TNF-α, in which cIAPs positively regulate NF-κB. NF-κB's signal moves into the nucleus where it induces several pro-survival genes, including cIAP2 and iNOS, among others [139].

- Non-classical NF-κB pathway: One of the main differences with the classical pathway is speed; the non-classical pathway is much slower as it requires new protein synthesis [120]. Target genes of this pathway are involved in several processes like dendritic cell activation or B cell maturation [140]. Besides the activation of the canonical pathway, some TNFRs, such as CD40, are able to trigger the non-classical NF-κB pathway. Additionally, IAP antagonists can also stimulate this pathway. In this pathway cIAP1/2 are degraded, and thus NIK is not ubiquitinated. NIK then accumulates until there is enough of it to trigger a downstream cascade of phosphorylations that lead to the activation (or inhibition) of the target genes [94].

1.2.4.2 IAPs in cell proliferation

Besides their protective role, IAPs have other roles in cell homeostasis such as mitotic control. **Survivin**, the smallest IAP, has cell cycle regulated expression and has been described as part of the chromosomal passenger complex (CPC) involved in chromosome alignment and segregation during mitosis [115]. NAIP has been recently described by our lab as involved in

the cytokinetic process, co-localizing with the CPC throughout all stages of cytokinesis [141]. Like survivin, **cIAP1** and **cIAP2** expression is cell cycle regulated, peaking at the G2/M phase of the cell cycle [142]. In addition to that, **cIAP1** localizes almost exclusively in the nucleus where is suspected to exert cell cycle control [143]. **BRUCE** is important in the resolution of cytokinesis, and is involved in cytokinetic abscission. It is thought to be a platform mediating the delivery of membrane vesicles to the intercellular bridge abscission point and also coordinates multiple steps in the formation and resolution of the midbody [144].

1.2.4.3 IAPs in development and differentiation

In development, IAPs play an important role in controlling programmed cell death, cellular migration, and differentiation. These processes must be well coordinated to avoid mistakes in embryogenesis and morphogenesis that would lead to the death of the organism.

IAPs exert positive and negative control of cellular migration [105], which is essential to proper development. It is well-known that XIAP and cIAP1 participate in actin polymerization, which is necessary for migration. These IAPs restrain migration through interaction (ubiquitination and posterior depletion) with actin cytoskeleton regulators [145, 146]. Studies have also demonstrated that XIAP is able to monitor Rho-GDI activity, which increases actin polymerization and enhances migratory ability of the cells [147, 148].

Survivin expression is restricted to embryonary apoptosis-regulated tissues like thymic medulla, stem cell layer of stratified epithelia and endocrine pancreas [149]. In addition to its role in proliferation, survivin is also involved in some differentiation processes. In fact, decrease of survivin levels is required for correct differentiation of polyploid cells (Megakaryocytes) [150, 151], and smooth muscle vascular cells [152]. Survivin expression is necessary to an accurate development of the lens so lens epithelial cells can correctly differentiate to lens fiber cells [153]. XIAP and cIAP1 have also been shown to be involved

in lens fiber differentiation [154, 155].

Muscular differentiation: Skeletal muscle is composed by multinucleated contractile muscle cells (myofibers) formed by the fusion of multinucleated progenitor cells (myoblasts). In adult tissue, there is a population of stem cells in the muscle called satellite cells that, in case of muscle injury, have the ability of re-enter (from a quiescent state) the cell cycle and rapidly proliferate to produce a high number of myoblasts that will fuse to repair the damaged tissue. During these proliferation and differentiation processes, both NF-κB signaling pathways (canonical and non-canonical) remain active. Their role in this situation is complimentary. The canonical pathway is important to myoblast proliferation, while non-canonical has a role in the myoblast fusion step. TNF-α mediated NF-κB canonical signaling pathway requires cIAP1 and cIAP2 positive regulation at the same time that they act as negative regulators of the non-canonical NF-κB pathway [156–158].

Neuronal Differentiation: During central nervous system development there are a series of critical events, such as migration and differentiation of neuronal cell populations. In these processes, neurogenic precursor cells are created in excess and removed in a controlled manner throughout development, making apoptosis a highly important process during central nervous system development [159]. Afterwards, neuron conservation is crucial for good performance of the body [160]. Survivin is highly expressed in the adult brain, the cerebellum, the retina, spinal cord, and specially in neural precursor cells [161], in which this protein is essential to survival and proliferation [162]. In some studies with PC12 cells, NAIP has been seen to interfere with neural precursor cell differentiation and restrict neurite growth in the presence of NGF (Nerve Growth Factor). Once cells are differentiated, NAIP has a protective role against apoptosis [163], effect also exerted by other IAPs [126, 160, 164], like cIAP2 [165] and XIAP [166].

1.2.5 IAPs in health and disease.

IAPs are involved in several cellular processes, such as migration, proliferation and differentiation of some cell types and especially in control/restrain of apoptosis. Thus, they are implicated in a considerable number of pathological mechanisms.

The founding member of IAPs in mammals, NAIP, was first described as an anti-apoptotic protein associated with spinal muscular atrophy [103], where the deletion of NAIP is present in two thirds of the most severe cases of the disease [167–169]. NAIP has also been related to other neurodegenerative diseases, such as Alzheimer's [170] and Parkinson's [171], in which survivin is also involved. NAIP is also associated with Down's Syndrome [172] and some autoimmune diseases like multiple sclerosis [173, 174]. Additionally, it has been reported to be overexpresed in obesity [175]. cIAP1, cIAP2, XIAP and survivin have been found to be overexpressed in endometriosis [176].

1.2.5.1 IAPs as immune response modulators

Inflammation is the first response of the body against an infection or an injury that elicits a stress signal that is identified by the innate immune system. IAPs regulate the function of several immune cell types, like macrophages, dendritic cells, NK, T and B lymphocytes [94, 177–179]. The main function of inflammation is to eliminate the initial cause of injury while initiating a series of events in order to reconstitute the damaged tissue. The first job of the innate immune system is to recognize danger; this is done by the PRR family, which is able to recognize and respond to a wide variety of antagonists. When these receptors are activated, they perform their protective role through two different pathways: by modulating NF-κB transcription factor activation, which in this case will act as a pro-inflammatory response regulator that promote cytokines and production of co-stimulator factors [8]; and by formation of the inflammasome, a multiprotein complex that activates Caspase-1, which regulates IL-1β and IL-18 pro-inflammatory cytokines. Caspase-1 can also induce pyroptosis [8, 180, 181]. IAPs are involved in:

1. **Pathogen recognition:**

    - NOD1 and NOD2: NOD1 and NOD2 are two cytosolic PRRs of the NLR family capable of recognizing bacterial peptidoglycan and triggering an immune response. In this pathway, cIAP1 and cIAP2 are necessary for ubiquitination of RIP2, which is necessary for the completion of the pathway [182, 183]. XIAP is required in this pathway in the presence of some bacterial infections, such as *Listeria*, and it mediates the ubiquitination of RIP2 [184, 185]. A mutation in XIAP interferes in this pathway and causes X linked lymphoproliferative syndrome type 2 [186].

    - TLR: TLR is a family of membrane PRRs capable of detecting and triggering an immune response against a wide range of PAMPs and DAMPs. TLR signaling cascade can be mediated via recruitment of MyD88 or recruitment of TRIF, or in the case of TLR4 by both [187]. MyD88 leads to NF-κB induction via MAPK activation. For this to happen, cIAP must ubiquitinate TRAF6 [104]. Alternatively, TRIF can promote the production of type I IFNs. In this process, the ubiquitination of TRAF3 and TRAF6 by the cIAPs is also needed [104, 120].

2. **Inflammasome formation:** Inflammasomes are multiprotein complexes formed in response to certain DAMPs and PAMPs that promote the activation of the zymogen of caspase-1 (pro-caspase-1), which in turn controls the production of other important pro-inflammatory cytokines like IL-1β and IL-18 [188]. NAIP, in addition to the IAP family, belongs to the NLR family; as such it is involved in intracellular recognition of flagellin (the main structural component of the bacterium flagellum) and the inner rod protein of type III secretion systems of diverse motile bacterial species like *Salmonella* or *Legionella* [99, 100]. NAIP recruits other proteins, such as NLRC4, to constitute the NLRC4 inflammasome [189]. Besides NAIP, XIAP, cIAP1 and cIAP2 have been described as negative modulators of NLRP3 inflammasome formation [104, 190].

The dysregulation of inflammatory processes in which IAPs are involved, like NOD2 or TLR signaling pathways, leads to inflammation and support the pathogenesis of several diseases like Crohn's disease, inflammatory bowel disease, asthma, and multiple sclerosis, among others [174, 191, 192].

1.2.5.2 IAPs and cancer

Cancer is caused by a mutated cell with an uncontrolled proliferation rate invading its own tissue and eventually other tissues. It is a very heterogeneous disease with characteristics that vary from one tumor to another and from one person to another, but the main characteristic of tumorigenic cells is their ability to escape cell-death. IAPs have been found to be dysregulated in many cancer cell lines, either because their level of mRNA or protein are increased or because there is a decrease in the level of inner IAP antagonists [193–195]. In general, cIAP1, cIAP2 and XIAP facilitate growth and spread of the tumor and confer protection against TNF-α-induced cell death [196]

XIAP is found to be overexpressed in breast cancers, melanomas and clear-cell renal carcinoma [197]; cIAP1 is involved in the development of esophageal squamous cell carcinoma [198]; the expression of cIAP1 and cIAP2 is increased in some tumors such as medulloblastomas, glioblastomas, non-small cell lung carcinomas and gastric carcinomas [199]. A gene translocation cIAP2 has a basic role in the development of MALT lymphomas [200, 201]. Livin is usually found in melanoma, and sometimes in non-small cell lung cancer (hardly in any other tissue) and is normally related to poor prognosis.

In addition to their ability to inhibit apoptosis, IAPs also participate in cancer due to their role as signaling transducers and positive regulators of the NF-κB pro-survival pathway [120, 195]. IAPs can also contribute to radiation or chemotherapy resistance and, in some cases, their expression increases after those treatments [202], often leading to a poor prognosis.

1.2.6 IAP antagonism

Given the relevance of IAPs in the pathogenesis of several diseases, including some inflammatory diseases and, as noted previously, in cancer, the idea of using them as therapeutic targets has became popular. Multiple strategies have been studied and one of the most attractive is the development of small compounds that copy the inhibition mechanisms of natural IAP-inhibitors molecules released by the mitochondria during the intrinsic apoptotic pathway. The IAP antagonists are: smac/DIABLO, Omi/HtrA2 and GSPT1 /eRF3 [203,204]. These neutralize IAP activity by binding to the IBM-sites, localizing to the BIR domains, replacing caspases in the unified sites and, in some cases, promoting auto-ubiquitination and degradation [127, 205]. Several compounds have been developed to mimic the effect of those molecules able to successfully antagonize IAPs. One of the most clinically advanced approaches in cancer treatment is the use of Smac mimetic compounds (SMC) that mimic the N-terminus of the endogenous smac. These SMC bind and inhibit XIAP and promote the degradation of cIAPs. The effect of IAP inhibition results in conversion of the NF-κB survival pathway into an apoptotic response [194, 206].

Based on structure, there are two kinds of SMC: monovalent and bivalent. The first one, monovalent, consists of one single smac-mimicking unit; while bivalent comprises two smac-mimicking motifs which enable them to bind and downregulate XIAP more effectively [120, 206]. Treatment with SMC usually induces cIAP1 and cIAP2 degradation very quickly. This sensitizes cells to death elicited by another anti-cancer agent (as cells usually require a death signal to trigger the apoptosis pathway) as shown in Fig 5. There are currently several SMC under study in clinical trials, mostly monovalent, as monotherapy or in combination with other therapies [207, 208]. LCL161 (Fig 6) is a monovalent SMC developed by Novartis that can be delivered orally, currently under study in a few clinical trials [207] and widely studied in pre-clinical trials in several tumor cell lines. LCL161 is perhaps the most advanced in clinical trial evaluation and is one of the SMCs that we use in our studies.

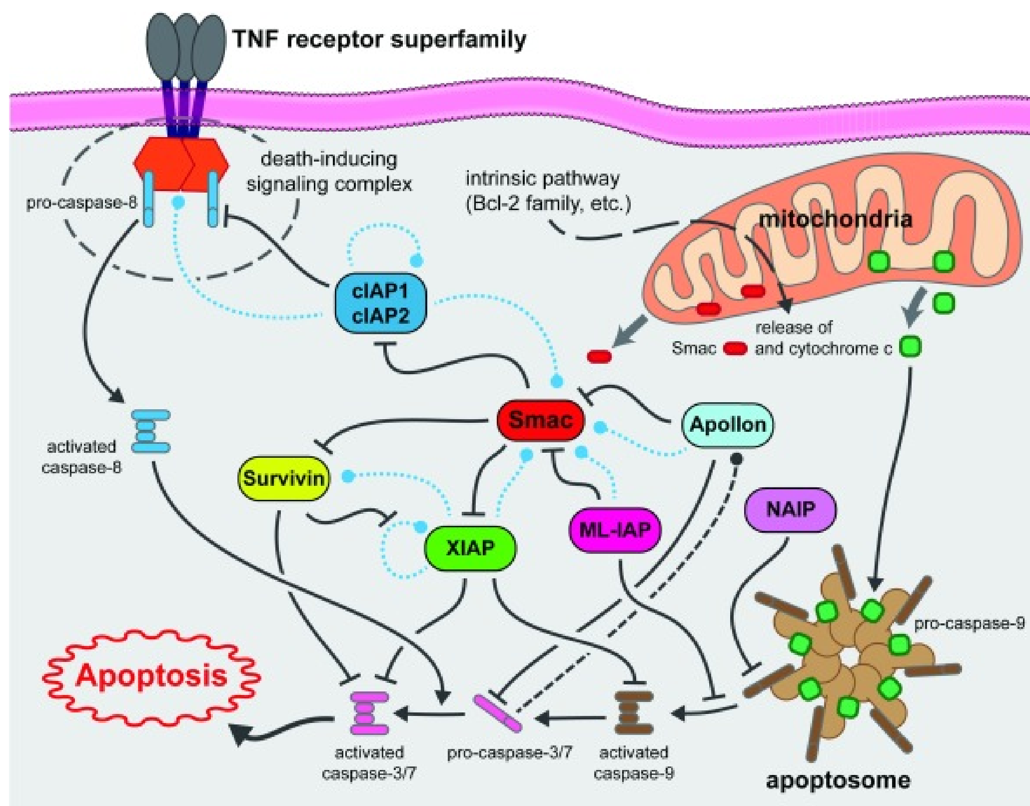


Figure 5. Schematic of pertinent inhibitor of apoptosis signaling pathways and smac interaction. Dashed lines indicate potential degradative events (blue = ubiquitin-mediated, black = caspase-mediated). Taken from Finlay D, Teriete P, Vamos M et al. 2017 [207]

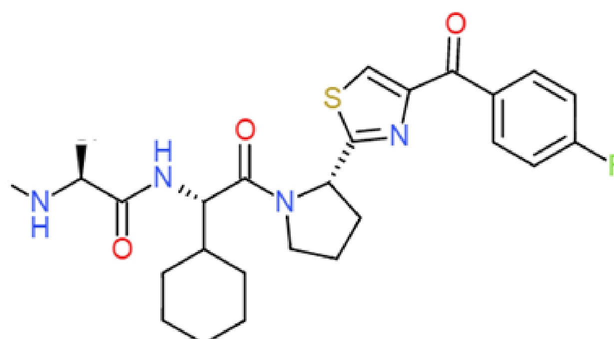


Figure 6. Chemical structure of LCL161

1.3 Macrophage activation syndrome

Macrophage activation syndrome (MAS), also known as reactive hemophagocytic lymphohistiocytosis, is a serious, potentially life threatening complication of some rheumatic diseases, especially systemic-onset juvenile idiopathic arthritis (sJIA). MAS is a very serious condition with a reported mortality rate between 8% [209] and 40% [210]. It is characterized by a set of clinical symptoms, including but not limited to prolonged fever, peripheral cytopenias, and organ dysfunction, all of which are caused by an excessive activation and proliferation of well differentiated T lymphocytes and macrophages that exhibit hemophagocytic activity. Due to their activated state, these cells secrete excessive amounts of cytokines, such as IL-1, IL-6, IL-18 and TNF-α. This event, known as a cytokine storm, leads to a hyperinflammation state in the body [210]. The clinical features are very similar to those presented in hemophagocytic lymphohistiocytosis (HLH) and MAS is now classified as secondary HLH [211].

1.3.1 Epidemiology

MAS is mostly seen in the context of sJIA and in its adult version, adult-onset Still disease; albeit more and more cases are being reported in other disorders like Kawasaki disease, also known as juvenile systemic lupus erythematosus [212–214]. MAS occurring in the context of sJIA is the most studied and its incidence is estimated to be around 10% in patients with sJIA, although some studies suggest even higher percentages [215, 216]. MAS can occur at any time with no distinction of age, is slightly more predominant in girls than in boys (6:4 female/male ratio) [209], and can be developed at any stage of the active underlying disease, including remission [217].

1.3.2 Diagnosis/Clinical features

MAS mainly manifests as high and recurrent fever episodes. When the fevers occurs in a patient with an active rheumatic disease along with a

fall in platelet count and erythrocyte sedimentation rate, the possibilities of MAS increase remarkably [218]. In order to be identified as MAS, diagnosis requires the presence of at least two laboratory/clinical criteria:

- Laboratory criteria: Cytopenia, abnormal liver function test, coagulopathy, decreased eythrocyte sedimentation rate, hypofibrinogenemia, hyponatremia, hypoalbuminemia and hyperferritinemia (extremely marked).

- Clinical criteria: Non-remitting high fevers, hepatomegaly, splenomegaly, lymphadenopathy, hemorrhagic manifestations and central nervous system dysfunction. [212]

The presence of highly activated histiocytes in the bone marrow, with anti-CD163 staining, confirms the diagnosis when in doubt [210].

1.3.3 Pathophysiology

Different rheumatic conditions exploit different pathways to arrive at the MAS phenotype. Patients with sJIA harboring polymorphisms associated with familiar HLH genes have an increased risk of developing MAS [212, 219]. Due to an exacerbated activation and expansion of macrophages and T lymphocytes, the body is under a hyperinflammation state marked by a large amount of cytokines, both, pro-inflammatory and anti-inflammatory. MAS has a broad number of triggers, including bacterial infections (*Enterococcus spp.*), viral infections (Epstein Barr), drug treatments and stem cell transplantation [220]. Yet, although many triggers have been described, the actual underlying mechanism that allows MAS to develop is unknown. Some authors propose models related to those found in primary HLH, such as failure to induce apoptosis due to cytotoxic dysfunction caused by mutations leading to impaired NK and CD8 T-cell cytotoxicity [210, 221].

1.3.4 Therapy

There are no controlled studies on the treatment of MAS, so the treatment is empiric and based on anecdotal evidence. MAS is a difficult disease to control and often requires intense immunosuppresion. Patients are usually treated with corticosteroids, prednilosone, cyclosporin, intravenous immunoglobulin (to prevent sepsis), and, in some cases, etoposide [209,212]. Recently, directed therapies against pro-inflammatory cytokines present in the MAS cytokine storm (especially IL-1 and IL-6) have been under examination, but require further study [218, 221].

CHAPTER 2

Rationale

Rationale

Macrophages show great functional diversity. They have roles in immunity, reparative and homeostatic functions, and development. They control normal physiology and have been implicated in diverse pathologies and novel therapeutic approaches. Consequently, in recent years there has been a growing interest in macrophage biology and in the understanding of the regulation of M1/M2 polarization and their switch in phenotype.

IAPs are important inflammatory modulators. They participate in ubiquitin dependent signalling events that regulate activation of NF-κB transcription factors in order to drive the expression of genes important for immunity and inflammation [196]. In this regard, cIAP1, cIAP2 and XIAP have been implicated in the modulation of NF-κB activation and inflammatory signaling [222]. In turn, NF-κB induces its activation in a positive feedback loop upregulating the expression of cIAP1, cIAP2 and XIAP. Furthermore, cIAP1 and cIAP2 have been shown to be particularly relevant in the regulation of canonical and non-canonical NF-κB signaling [139, 223]. Apart from their intervention in immunity and inflammation, IAPs are implicated in several other cellular processes, such as cell proliferation, cell migration and cell survival [98, 141], which are frequently altered in cancer and contribute to tumor maintenance, metastasis and disease progression [196]. Consequently, IAPs have emerged as a promising therapeutic target for a plethora of disorders, and the development of pharmacological inhibitors of IAPs has attracted the attention of industry and researchers.

Monocytes constitute approximately 10% of the white blood cell count in humans [16]. When monocytes differentiate to macrophages their lysosomal content and the amount of hydrolytic enzymes are increased, they show a higher metabolic rate than monocytes and distinctive transcriptional profiles. Monocyte to macrophage differentiation occurs in response to MCSF and involves substantial changes in cell physiology, notably at the transcriptional level with the upregulation of *PU.1* and *SCL* transcription factor genes [20, 224, 225]. TNF-α and other pro-inflammatory cytokines can trigger macrophage differentiation. As mentioned in the introduction chapter, TNF-α

binds to the receptor TNFR1 which elicits the assembly of a multiprotein complex containing TRAF2. In monocytes, TNF-α binding and the presence of TRAF2 in the complex lead to macrophage differentiation, which requires the removal of TRAF2 to be completed. During the differentiation process, cIAP1 translocates from the nucleus to the Golgi apparatus, [179] where cIAP1 participates in TRAF2 ubiquitination, facilitating its removal by degradation [178, 226]. Once macrophages are fully differentiated, XIAP, cIAP1 and cIAP2 monitor pro-inflammatory cytokine production [227]. In addition, cIAP1 and cIAP2 prevent macrophage necroptosis during the immune response against pathogens [227,228]. Previous studies have shown XIAP upregulation during monocyte to macrophage differentiation [229,230], although its expression during polarization remains to be investigated.

The expression profiles of the IAPs during the differentiation of monocytes to macrophages and in the polarization into M1/M2 states is unknown. Moreover, the functional roles of the IAPs in modulating macrophage differentiation and polarization remains unexplored. The aim of this study is to examine the differential expression of the IAPs, namely, NAIP, cIAP1 and cIAP2, during monocyte-to-macrophage differentiation and M1/M2 polarization process. In view of the multiple roles played by the IAPs, we hypothesize that monocytes, M0, M1 and M2 macrophages, might show a distinctive IAP expression profile, accounting for the characteristic properties of each one of the different macrophage activation states described.

Although M1/M2 macrophage polarization models correspond to an oversimplification of the more intricate situation, *in vitro* polarization models have proven to be adequate in M1/M2 macrophage research [86,231,232] and in the analysis of the switch between the M1/M2 states [92, 93]. We have chosen to work with monocytic human cell lines whose derived-macrophage like cells present features similar to primary macrophages [233], and monocytes from peripheral blood mononuclear cells in the establishment of the differentiation and polarization models.

Macrophage dysregulation and the alteration of M1/M2 polarization balance have been implicated in pathologies such as cancer [58,59,92,93], diabetes [56,57], obesity [234], atherosclerosis [60,61], myocardial infarction [62] and asthma [63]. There is therefore a growing interest in understanding the regulation of M1/M2 polarization and the M1/M2 phenotype switch, both because of implications with diverse pathologies but also the formulation of novel therapeutic strategies. We believe for these reasons that the investigation of the IAP expression profile in the different macrophage types will contribute to a better understanding of macrophage differentiation and M1/M2 polarization and also in establishing new approaches for investigation leading to a greater insight into the different macrophage types functional roles.

2.1 Objectives

1. **Analyze NAIP protein and gene expression in the monocyte to macrophage differentiation.**

   Preliminary observations of our group unexpectedly indicated a reduction in NAIP expression in the monocyte to macrophage transition. We wanted to investigate this finding in depth in well established *in vitro* monocyte to macrophage differentiation models.

2. **Determine the differential protein and gene expression of NAIP, cIAP1 and cIAP2 in the monocyte to macrophage differentiation and M1/M2 polarization process.**

   The well known IAPs implication in the regulation of immunity and inflammation made us think that monocytes, M0, M1 and M2 macrophages, might exhibit a differential IAP expression profile characteristic of each macrophage type. With this purpose, *in vitro* macrophage polarization models were established for monocytic cells lines and primary human PBMCs monocytes from healthy donors.

3. **Evaluate the impact of pharmacological inhibition of IAPs in macrophage differentiation and polarization.**

   The development of small IAP antagonists, already in use in clinical trials, and the emergence of macrophages as promising therapeutic targets in different conditions, prompted us to asses the response to IAP antagonism in macrophage differentiation and polarization with the aim of generating helpful information to be considered in such approaches.

4. **Determine the effect of IAP targeting in a macrophage medied disease.**

   A mouse CpG-oligodeoxynucleotides macrophage activation syndrome (MAS) model and MAS patient samples were treated with the SMAC mimetic compound LCL161 as an initial approximation in the potential treatment of MAS with IAP inhibitors.

CHAPTER 3

Materials and methods

3.1 Cell culture and treatments

3.1.1 Cell lines

3.1.1.1 Monocyte to macrophage differentiation

Human myeloid leukemia THP-1 cells and human myeloid lymphoma U937 cell line were obtained from the *Centro de Instrumentación Científica*, University of Granada. Both cell lines were grown in RPMI 1640 (Lonza, Allendale, NJ) supplemented with 10% of heat inactivated fetal calf serum (GIBKO, California, USA), 1mM of L-glutamine (PAA) and 1% of penicillin-streptomycin (Cambrex, Bio Science) in standard conditions (37ºC in 5% CO2 humidified atmosphere). THP-1 monocytes were differentiated into macrophages by 24h incubation with 10ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) followed by 24h of culture in fresh complete RPMI 1640 media.

3.1.1.2 Cell cycle and analysis

HeLa cells (CCL2; American type culture collections, Manassas, Va) were maintained in standard conditions (37ºC in 5% CO2 humidified atmosphere) in Dulbecco's modified Eagle's medium (HycClone DMEM Ca#SH30022.01) supplemented with 10% fetal calf serum and 1% antibiotics (100 U/mL penicillin-streptomycin). For synchronization, HeLa cells were treated for 16h at 37º with 400μM L-mimosine (Sigma-Aldrich) to arrest cells in G1, 2μM thymidine (Sigma-Aldrich) to arrest cells in S, or 0,4μg/mL nocodazole (Sigma-Aldrich) to arrest cells in G2/M.

3.1.1.3 Macrophage polarization

THP-1 cells were seeded, further differentiated to what it has been called M0 macrophage, (24h of PMA followed by 24h of fresh media). And then exposed to different stimuli for 48h in order to reach polarization.

- M1 polarization: M0 macrophages were exposed to 100ng/mL of LPS and 20ng/mL of human recombinant INF-γ (life technologies PHC4031).
- M2 polarization: M0 macrophages were incubated in the presence of 20ng/mL of human recombinant IL4 (life technologies 11846-HNAE)

3.1.2 PBMCs

3.1.2.1 Monocyte isolation

Human peripheral blood mononuclear cells were isolated from Buffy Coat of healthy donors by density-gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO) followed by immunomagnetic separation with the Dynabeads® Untouched™ Human Monocytes Kit (Invitrogen). The purity of the separation was confirmed measuring CD14 positive cells by flow citometry, been always superior to 95%.

3.1.2.2 Macrophage differentiation and polarization

Blood monocytes differentiation in resting macrophages occurred after 7 days of culture in RPMI 1640 supplemented with 10% of heat inactivated fetal calf serum, 1mM of L-glutamine and 1% of penicillin-streptomycin in standard conditions.

- M1 polarization: After isolation, monocytes were cultured in their media for five days, with a change of media on day 4, and then exposed to 20ng/mL of INF-γ and 1 hour later 100ng/mL of LPS was added to their media. This culture condition was maintained for 48h.
- M2 polarization: After isolation, monocytes were maintained in culture for 6 days, with change of media on day 4, on the conditions previously described. Cells were exposed to 20ng/mL of IL-4 for 24h.

3.1.2.3 Macrophage activation syndrome patient samples

Human blood was obtained from healthy volunteers and from 3 MAS patients. Institutional research ethics board approval was obtained (Children's Hospital

of Eastern Ontario) and the patients and family members were enrolled with informed consent. Peripheral blood mononuclear cells (PBMCs) were extracted using Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) to create a density gradient following centrifugation of whole blood at 1600 RPM for 45 min at 4°C in Allegra X-12R centrifuge (Beckman Coulter, Brea, California). The cell layer containing mainly PBMCs was collected and counted. To generate MDMs, monocytes were isolated via the adherence method. PBMCs were resuspended at 4 x 10^6 cells/mL in Iscove's Modified DMEM 1X media (Sigma-Aldrich). The cells were seeded at 1 mL/well in 12 well polystyrene plates (Thermoscientific, Rochester, New York), monocytes were allowed to adhere to the plate for 3h in a humidified environment at 37°C, 6% CO2/air mixture. Non-adherent cells were washed off and adherent cells were cultured for six days with complete media containing 100 units/mL penicillin and 100 μg/mL gentamicin (Sigma-Aldrich) with 10% v/v fetal bovine serum (GE Healthcare) and 10 ng/mL recombinant human macrophage colony stimulating factor (M-CSF).

3.2 Animals

Female C57BL/6 mice were purchased from Charles Rivers Laboratories (Montreal, QC, Canada). Animals were housed in the University of Ottawa Animal Care and Veterinary Services facility in accordance with the Institutional Animal Care and Use Guidelines. Mice we given free access to food and water. The study as presented was approved by the local Committee for Care and Use of Laboratory Animals.

3.2.1 CpG animal model treatments

CpG 1826 oligonucleotide was synthesized by IDT with sequence 5' ⇒ 3' T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T (asterisks mean that bases are phosphorothioated [1]). In order to induce MAS-like syndrome mice

[1] phosphorithioate bond: one of the non-bridging oxygen is replaced for a sulfur atom, so internucleotide linkage is more resistant.

were injected with CpG with a dose of 2.5mg/kg with a final concentration of 0.25 μg/μL diluted in Dulbecco's Phosphate Buffered Saline (DPBS) or just with DPBS in the control groups, every two days for 8 days. 50 mg/kg LCL161 diluted in acid buffer (30% 0.1N HCl, 70% 100mM NaAcetate at pH 4.63), or just the corresponding amount of acid buffer alone to the control groups, was delivered by oral gavage.

3.2.2 Collection of tissue

- Hepatocytes isolation: Livers were isolated, photographed and weighted. Then, they were homogeneized with surgical scissors and passed through a 70μm nylon mesh. Red blood cells were removed from the homogenate using ammonium-chloride-potassium (ACK) lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) for 2 min.

- Spleenocytes isolation: Spleens were isolated, photographed and weighted. Then, they were homogeneized with surgical scissors and passed through a 70μm nylon mesh. Red blood cells were removed from the homogenate using ammonium-chloride-potassium (ACK) lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) for 2 min.

3.3 Flow cytometry analysis

3.3.1 Human samples

THP-1, U937 and blood monocytes/macrophages were washed 2 times with cold PBS, scraped, collected by centrifugation and resuspended in FACS buffer (PBS supplemented with 0,5% bovine serum albumin and 5mM of EDTA). Cells were stained with the antibodies shown in Table 3.1 or their isotype controls.

Immunostained cells were analyzed on a BD LSRFortessa X-20 Cell Analyzer (BD Bioscience) with post-processing in FlowJo software (Tree star Inc). Cell populations were gated on forward and side scatter to select intact single cells.

Target	Clone	Conjugated fluorophore
anti human CD11b	ICRF44	FITC
anti human CD14	M5e2	APC
anti human CD206	15-2	APC/Cy7
anti human CD86	IT2.2	Alexa Fluor 488
anti human CD163	RM3/1	Alexa Fluor 647

Table 3.1. Human antibodies used in flow cytometry, all purchased from Biolegend

3.3.2 Murine samples

Cells obtained from liver and spleen of the individuals of the CpG model were washed with cold PBS, collected by centrifugation and resuspended in FACS buffer (PBS supplemented with 0,5% bovine serum albumin). Cells were stained with the following antibodies (or their isotype control):

Target	Clone	Conjugated fluorophore
Anti mouse CD4	GK1.5	APC/Cy7
Anti mouse CD8a	53-6.7	PE/Cy7
Anti mouse CD3	17A2	Pacific blue
Anti mouse F4/80	BM8	Brilliant violet 605
Anti mouse Cd206	CO68C2	APC
Anti mouse I-a/1-e	M5-114.15.2	Alexaflour 700
Anti mouse NK1.1	PK136	PerCP/Cy5.5
Anti mouse CD11b	M1/70	PE/Cy5

Table 3.2. Mouse antibodies used in flow cytometry, all purchased from Biolegend

Immunostained cells were analyzed on a BD LSRFortessa X-20 Cell Analyzer (BD Bioscience) with or without the High Throughput System (BD Bioscience)

with post-processing in FlowJo software (Tree star Inc). Cell populations were gated on forward and side scatter to select intact single cells.

3.3.3 Cell cycle analysis by intracellular propidium iodide (PI) staining

Cells were harvested and washed with 1X PBS and fixed with methanol at 4ºC for 15 min. Cells were washed again with PBS and treated with 25 μL of 10 μg/mL RNase A, followed by staining with 25 μL of 1 mg/mL PI solution (Sigma-Aldrich) at 4ºC for 1h. The amount of DNA present was analyzed using a FACSCanto flow cytometer (BD Biosciences) and the FACSDiva software.

3.4 RNA isolation and RNA expression analysis

Total RNA was extracted from cells with Trizol reagent (Invitrogen) as recommended by the supplier when experiments were performed in the University of Granada. When experiments were executed in Canada (IAP expression analysis in M1/M2 polarization) RNA was extracted using RNeasy mini-kit (QIAGEN, Mississauga, Ontario, Canada). cDNA was obtained using the Promega Reverse Transcription System kit according to the instructions of the manufacturer. The synthesized cDNA was then used for semi-quantitative PCR or real-time quantitative PCR. For the semi-quantitative PCR, PCR mastermix of Promega (M7501) was employed. For the real-time PCR, we employed the SsoAdvanced SYBR Green supermix (Biorad) on a Mastercycler RealPlex2 (Eppendorf) using the Realplex software. Real-time PCRs were done using the following primers:

Primers	**5'⇒ 3'**
hNAIP exon 4-forward	GCTCATGGATACCACAGGAGA
hNAIP exon 4-reverse	CTCTCAGCCTGCTCTTCAGAT
hNAIP exon 16-17-forward	GAATTTATCGAGTGGCCAAAC
hNAIP exon 16-17-reverse	TCAAAGACTTGACTGTTGTGG
hCXCL10-forward	GAAAGCAGTTAGCAAGGAAAGGTC
hCXCL10-reverse	ATGTAGGGAAGTGATGGGAGAGG
hCD14-forward	ACAGGTGCCTAAAGGACTGC
hCD14-reverse	GATTCCCGTCCAGTGTCAGG
hCD18-forward	CAGCTCACTCTGACCACTTCT
hCD18-reverse	TCTGCCAGGAGGTATAGACGA
hCD163-forward	GTCGCTCATCCCGTCAGTCATC
hCD163-reverse	GCCGCTGTCTCTGTCTTCGC
hCD206-forward	ACCTCACAAGTATCCACACCATC
hCD206-reverse	CTTTCATCACCACACAATCCTC
hActin-forward	TGACGGGGTCACCCACACTGTGCCCATCTA
hActin-reverse	CTAGAAGCATTTGCGGTGGACGATGGAGGG
hHPRT1-forward	TGACACTGGCAAAACAATGCA
hHPRT1-reverse	GGTCCTTTTCACCAGCAAGCT
hGAPDH-forward	TGCACCACCAACTGCTTAGC
hGAPDH-reverse	GGCATGGACTGTGGTCATGAG

Table 3.3. Primers used to determine mRNA
An n=3 of biological replicates was used to determine statistical measures (mean, standard deviation).

3.5 Western blot analysis

- Whole extracts: Cells at a minimum amount of 0.5x10^6 were washed 2 times with cold PBS and scraped and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Roche) for 30 min at 4°C, followed by centrifugation at 13,000g for 15 min. Supernatant was collected.

- Cytoplasmic protein extraction: At least 1x10^6 cells were washed 2 times with cold PBS, then incubated for 15 min at 4°C with lysis buffer A (HEPES 100mM, KCl 10mM, EDTA 0.1mM, EGTA 0.1mM, all supplemented in the moment with DTT 1M, PMSF 100mM, Sodium Molybdate 1M, and protease inhibitor cocktail). After the incubation period, NP-40 at 10% was added and vortexed strongly for 10 s and centrifuge for 30 s at max speed. Supernatant was collected.

- Nuclear fraction extraction: The pellet left in the cytoplasmic extraction, was incubated in Buffer C (HEPES 20mM, NaCl 400mM, EDTA 0.1mM, EGTA 0.1mM. All supplemented in the moment with DTT 1M, PMSF 100mM, Sodium Molybdate 1M, and protease inhibitor cocktail.) for 30 min at 4°C. Following the incubation period, samples were centrifuge for 5 min at 13000g. Supernatant was collected.

Supernatants were collected and kept frozen at -20°C. Total amount of protein were determined using a Bio-Rad protein assay kit. Equal amounts of soluble protein were separated on polyacrylamide gels (7-10%) followed by transfer to nitrocellulose membranes. Individual proteins were detected by Western blotting using the following antibodies: NAIP-J2 rabbit polyclonal antibody; rabbit anti-rat IAP1 and IAP3 polyclonal antibodies were used to detect human cIAP1/2 and XIAP, respectively [139, 235], HSC-70 (mouse monoclonal, Santa Cruz biotechnology, sc-7298), anti-β-Actin (mouse monoclonal, ABCAM, AB8245), anti-TATA binding protein (Mouse monoclonal, ABCAM, AB818). In some membranes AlexaFluor680 (Invitrogen) or IRDye 800 (Li-Cor) were used to detect the primary antibodies, and infrared fluores-

cent signals were detected using the Odyssey Infrared Imaging System (Li-Cor). The rest of the membranes were incubated with a secondary antibody (anti -rabbit or -mouse, Cell signaling) for 1 h at room temperature at the dilution suggested by the manufacturer and Blots were developed with the chemiluminescent ECL Plus detection system (GE Healthcare) and signals were detected using a luminescent image analyzer (LAS-4000 Mini, Fujifilm). Quantification was performed by densitometry analysis using the ImageJ software (National Institutes of Health, USA).

3.6 Immunostaining and microscopy

Human peripheral blood monocytes isolated and treated for M1/M2 polarization as previously described, were grown in 2-well glass chambered coverslip for confocal microscopy ibidi Ca#80286. The cells were fixed for 10 min in ice-cold 2% paraformaldehyde in PBS, briefly rinsed in PBS and permeabilized with 0.2% Triton X-100/PBS for 10 min. Permeabilization solution was next aspirated and the chambered coverslips were then incubated overnight at 4°C with the primary antibody diluted in PBS abcam ab98020, epitope mapping to the first 1-100 residues of human NAIP (1:200); and abcam ab25968, epitope mapping to 13 C-terminal residues of human NAIP (1.4:1000). Chambered coverslips were then rinsed 3 times for 5 min with PBS and incubated for 50 min at room temperature with the secondary antibody, goat anti-mouse Alexa Fluor 488 (Invitrogen, A-11070) or goat anti-rabbit Alexa Fluor 568 (Invitrogen, A-11036) diluted at 1:1000 in PBS. The slides were then rinsed 3 times for 5 min with PBS and mounted with ProLong Gold (Invitrogen). During the second wash, the slides were counterstained for 5 min with Hoechst 33342 (Invitrogen) diluted at 10μg/mL in PBS. Confocal microscopy was performed with a Nikon Eclipse Ti-E microscope. Mean fluorescence intensity per pixel per cell in resting, M1 and M2 immunostained macrophages was determined using the *ImageJ* software (National Institutes of Health, USA).

3.6.1 Viability assays

3.6.1.1 YOYO-1 dye

100 nM of YOYO-1 Iodide (Life Technologies). This membrane-integrity based viability assay measures the uptake of the fluorescence dye, normally excluded from intact, viable cells. Measurements of cell death, indicated by YOYO-1 fluorescence were performed over 72 h using the IncuCyte ZOOM Live Cell Imaging System (Essen Bioscience, Ann Arbor,MI, USA). 4 phase-contrast and green fluorescent images of the same optic field were automatically taken every two hours in each well over at least 48 h. For end point normalization, YOYO-1- positive cell counts were expressed as a fraction of the total number of cells using the Incucyte image analysis tools software (Essen Bioscience)

3.6.1.2 Alamar blue

Media was removed and replaced with 1% Alamar Blue (Resazurin sodium salt; Sigma) in media. Cells were incubated for at least 4h. Cell viability was then read using the Gen5 Data Analysis Software (BioTek Instruments Inc., Winooski, Vermont, USA) Data for all Alamar Blue assays were normalized to vehicle-treated cells.

3.7 Lentiviral transduction

The lentiviral vector system used and NAIP transfer vectors were constructed as previously described [236]. NAIP transfer vectors were constructed by standard cloning techniques; BamHI-AsiSI restriction enzymes were used to replace the p27 cDNA in the Cp27WP plasmid (a SIN-LV plasmid expressing p27 through the CMVTetO promoter, F. Martin's lab, unpublished) with the NAIP insert obtained by PCR from OriGene SC303496 cDNA clone, the resulting plasmid was called cNAIP-WP. To generate cNAIP + Neo-WP and cNAIP + GFP-WP, a pGEMT/XhoI-neo insert (expressing the Neo resistance gene through the PGK promoter) and a blunted PstI 1.2Kb frag-

ment (expressing eGFP through the Spleen Fcus Forming Virus (SFFV) LTR promoter) from the CTetOVSEWP plasmid (F. Martin's lab, unpublished) were ligated to the opened XhoI and XhoI-blunted cNAIP-WP respectively (vector maps in Supplementary information). The resulting vectors were sequenced and confirmed to be error free and inframe as designed.

Lentiviral particles production: Briefly, 293 T cells cultured in 10 cm dishes were transfected with 8 μg of transfer, packaging and envelope vectors (plasmid proportion 4:3:1) diluted in 1.5 mL of Opti-MEM (GIBCO) and mixed with 20 μl of X-tremeGene HP (Roche). Culture media with the DNA mix was replaced with fresh media 24 hours after transfection, the supernatant with lentiviral particles was harvested and filtered (0.45 μm pore size) 48 h after transfection started.

Transduction: 1 mL of readily collected lentiviral particles was added to THP-1 cells grown in 6-well plates and replaced with fresh media 24 h after transduction was initiated.

Figure 7. NAIP transfer vector maps

3.8 siRNA transfection.

NAIP siRNA silencing was performed in 6-well plates. NAIP siRNA duplexes were transfected at a final concentration of 50μl of 80nM siRNA and 50μl of 2X/Opti-MEM (GIBCO) with 0,25μl of Lipofectamine RNAiMAX (Invitrogen)

were plated per well and left to mix for 20 min, 800μL of media with THP-1 cells (0,625x10^6 cells per mL). 72hours after the siRNA transfection.

NAIP siRNA duplexes used:

- Exon 1; 5'-GAUUAGAGGUCUGGGAUUUUU-3'
  and 5'-CCACAGGUUUGGAGAGCUUUU-3'
- Exon 6; 5'-GUGAAUUUCUUCGGAGUAAUU-3'
- Exon 10; 5'-GGAAGUGACUCCAGACCUUUU-3'
- Non-targeting: QIAGEN Negative Control siRNA.

3.9 Statistical analysis

Unpaired 2-tailed Student's t-test was used to compare data sets consisting of 2 treatment groups. One-way ANOVA or Two-way ANOVA were used to calculate the levels of significance between multiple groups. Statistical analysis was performed with Graphpad Prism 5 software with a threshold significance level set in 0,05.

Symbol	**P value**
ns	$\geqslant$ 0.05
*	0.01 to 0.05
**	0.001 to 0.01
***	0.0001 to 0.001
****	<0.0001

Table 3.4. Significance levels with a setted threshold significance level of 0.05

CHAPTER 4

Results

4.1 NAIP expression in monocyte to macrophage differentiation

As mentioned in the Rationale chapter, based on preliminary work by our group, we aimed to investigate whether NAIP could be involved in the differentiation of monocytes to macrophages. For this, we chose two human monocytic cell lines, THP-1 and U937, which are commonly used in macrophage differentiation research; and we also investigated a model of primary monocytes obtained from peripheral blood mononuclear cells from healthy donors.

4.1.1 THP-1 and U937 differentiation models

For the purpose of studying NAIP expression throughout the differentiation pathway from monocytes to macrophage, we selected two well-established models to work with. In monocyte to macrophage differentiation studies, THP-1 and U937, both of which are human monocytic cell lines, have been described as good models in macrophage differentiation when exposed to PMA. To validate PMA-induced differentiation, we tested the expression of CD11b, a typical surface marker of both monocytes and macrophages, but with an increased expression in differentiated macrophages [237]. THP-1 and U937 cells were treated with 10ng/mL of PMA for 24hrs and then incubated with fresh media for another 24hrs; following the incubation periods, the cells were sorted by flow cytometry as seen in Fig 8 and the proportion of CD11b-positive cells in a PMA-treated population were compared to an untreated monocyte culture. For both THP-1 and U937 cell lines, cells treated with PMA had increased CD11b expression at the surface and altered morphology, becoming adherent and acquiring a typical, more flat and amoeboid-like shape of macrophages. The increase of CD11b expression and the aspect changes validate the differentiation capacity in both cell lines.

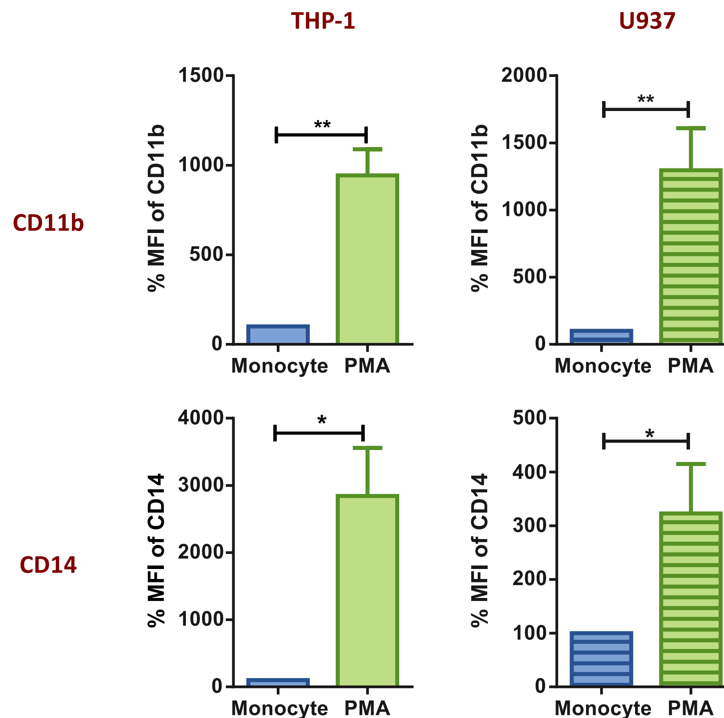


Figure 8. CD11b is increased in THP-1 and U937 cells exposed to PMA. THP-1 and U937 cells (0.5x10^6cells/well) were seeded in 6 well plates and treated with 10ng/mL of PMA for 24 h followed by 24 h incubation in regular media. Cells were then scraped and stained with CD11b and CD14 antibodies, analyzed by flow cytometry and compared to PMA-untreated cells. MFI of three independent experiments are summarized. Student t test was performed.

Once we determined PMA-exposed THP-1 and U937 macrophage differentiation models were suitable for our purpose, we analyzed the expression of NAIP in THP-1 and U937 resting monocytes and in PMA induced macrophages. First, we assessed the level of NAIP protein in cellular and nuclear fractions of the cells as well as in total protein extract by western blot. We found NAIP protein level decreases significantly in both THP-1 and U937, Fig 9. NAIP expression decreases in macrophages with respect to that in monocytes, although this decrease is greater and more significant in THP-1 than in U937 cells, and the distribution of NAIP is more homogeneous in THP-1 than in U937 where we see a greater amount of NAIP in the nucleus.

We wanted to see if the decrease of the protein was promoted by gene regulation. In order to do so, NAIP gene expression was measured by RT-qPCR. We assessed NAIP mRNA levels in both THP-1 and U937 (Fig 10), and found that the expression of NAIP in both models was significantly decreased when the cells were differentiated to macrophages.

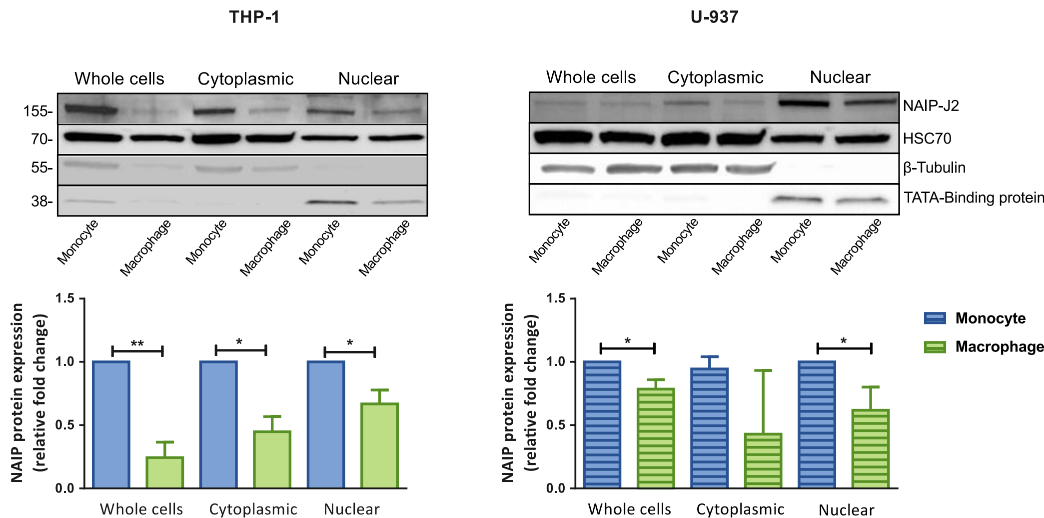


Figure 9. NAIP protein decreases in THP-1 and U937 monocytes differentiated to macrophages. THP-1 and U937 cells (0.5x10^6cells/cell) were seeded in 6 well plates and treated with 10ng/mL of PMA for 24 h followed by 24 h incubation in regular media. Whole cell, cytoplasmic and nuclear lysates were assessed by western blotting to analyze the NAIP presence using the NAIP-J2 antibody. HSC-70 was used as control, TATA-BP antibody was used as purity control of the nuclear fraction and β-Tubulin was used as cytoplasmic purity control. Representative western blot from one of three experiments with similar results. Graph represents the quantification of NAIP abundance normalized by the corresponding HSC-70 in three independent experiments. One-way ANOVA with Bonferroni post hoc was performed.

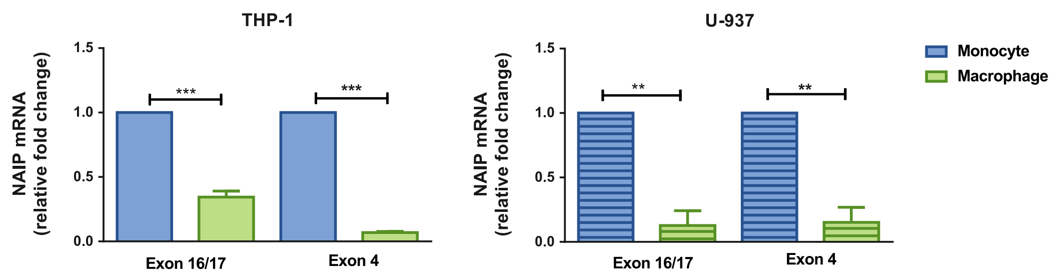


Figure 10. NAIP gene expression decreases in THP-1 and U937 monocytes differentiated to macrophages. THP-1 and U937 ($0.5x10^6$cells/well) cells were seeded in 6 well plates and treated with 10ng/mL of PMA for 24 h followed by 24 h incubation in regular media. Total RNA was extracted using RNeasy mini-kit, reverse transcribed and NAIP mRNA was analyzed by RTq-PCR. Values are normalized to internal controls (HPRT1 and GAPDH) and presented as mean values ± S.D. of fold-change of three independent experiments. One-way ANOVA with Bonferroni post hoc was performed.

4.1.2 PBMCs differentiation model

In order to confirm our results in a primary human monocyte to macrophage differentiation model, the expression of NAIP was analyzed in differentiated monocytes from peripheral blood mononuclear cells, PBMCs, from healthy donors. Blood was extracted, monocytes were isolated by gradient centrifugation and negative selection beads (explained in the monocyte isolation section in the Material and Methods chapter), and maintained in culture for a week in order to obtain macrophages. NAIP expression was measured by qPCR and as shown in Fig 11, NAIP expression in monocytes and macrophages from PBMCs is consistent with the results previously obtained in THP-1 and U937, NAIP gene expression decreases when monocytes undergo differentiation to macrophages.

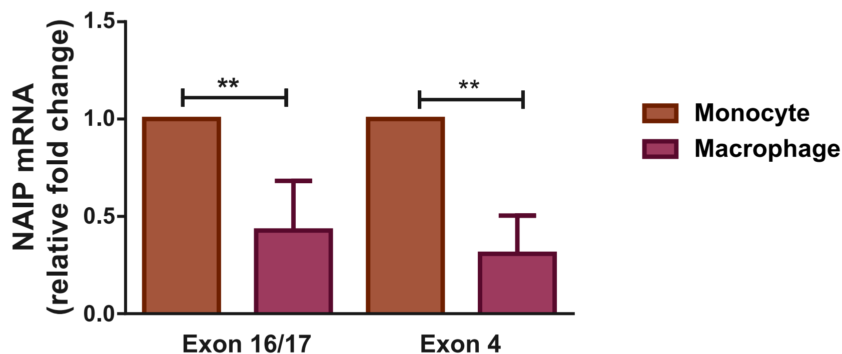


Figure 11. NAIP gene expression decreases in human monocyte-derived macrophages. Approximately 0.5x10^6 monocytes from PBMCs from 4 healthy donors were cultured for 3 h (monocytes) or maintained in culture for a week in order to force differentiation (macrophage). Total RNA was extracted using the Trizol protocol, reverse transcribed and NAIP mRNA was analyzed by semiquantitative-PCR. Values are normalized to internal controls (Actine) and presented as mean values $\pm$ S.D. of 4 independent experiments with samples from 4 different healthy patients. Student t test was performed.

The level of NAIP protein was measured by western blot (Fig 12), and the distribution of NAIP in monocytes and macrophages was further characterized by immunolocalization studies using confocal microscopy (Fig 13). As we expected, the amount of NAIP protein shown by western blot was significantly higher in monocytes than in macrophages and confirmed our previous results in the THP-1 and U937 differentiation models. Confocal microscopy analysis of NAIP immunofluorescence was also consistent with the previously obtained results and showed a significant decrease in NAIP after differentiation, although determination of a specific localization of the protein in the cell was not possible and we were not able to discriminate any distribution patterns in the cells before and after differentiation.

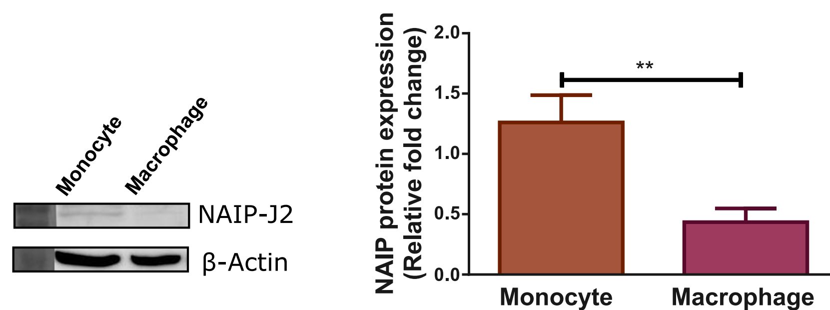


Figure 12. NAIP protein expression declines in human monocyte-derived macrophages. Approximately 1x10^6 monocytes from PBMCs from 4 healthy donors were cultured for 3 hours (monocytes) or maintained in culture for a week in order to force differentiation (macrophage). Cell proteins were extracted and NAIP protein abundance was assessed by western blotting, β-Actin was used as control. Representative western blot from one of the patient samples. Graph represents the quantification of NAIP abundance normalized by the corresponding β-Actin, presented as mean values $\pm$ S.D. of 4 independent experiments with samples from 4 different healthy patients. Student t test.

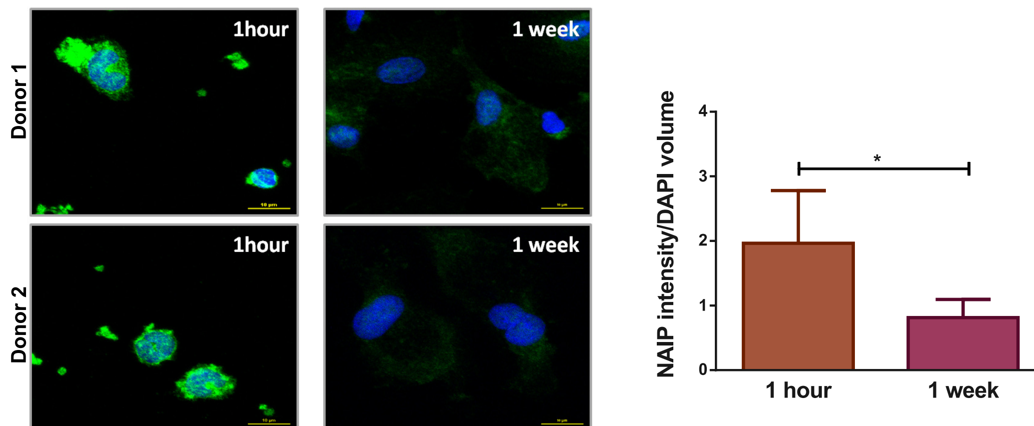


Figure 13. NAIP immunostaining analysis in human monocyte-derived macrophages. Left panel: Representative confocal microscopy of PBMCs monocytes and monocyte-derived macrophages from two volunteers. Right panel: Ratio of NAIP mean fluorescence intensity per pixel per cell and DAPI volume, in monocytes and macrophages from two healthy donors. 10 randomly selected cells in each case were analyzed for their mean fluorescence intensity per cell area using the *ImageJ* software. Bar=10 μm. Student t test was performed.

4.1.3 NAIP overexpression and macrophage differentiation

In an effort to elucidate whether or not NAIP has a key role in monocyte to macrophage differentiation process (as seen before in the case of another IAP [178]), the impact of NAIP over-expression in THP-1 and U937 cells was studied. Cells were transduced with a bicistronic lentiviral vector system expressing the open reading frame of NAIP and the neomycin-resistance (neo) genes in the case of THP-1 cells and NAIP and green fluorescent protein (GFP) genes in the case of U937 cells. The over-expression of NAIP was confirmed (Fig 14). In THP-1 cells, NAIP protein levels were more than 4 times higher in the neomycin-resistant cells, a difference that was higher in the U937 GFP+ sorted cells with a 6 fold increase in NAIP expression.

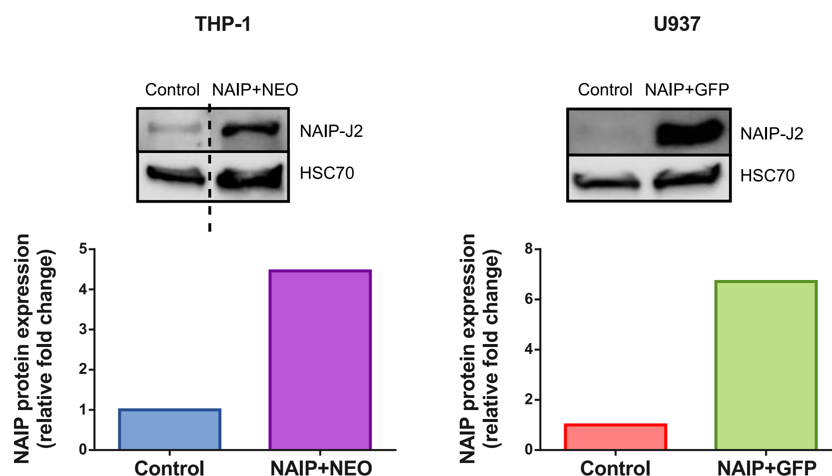


Figure 14. NAIP overexpression in THP-1 and U937 cells. THP-1 and U937 cells were seeded in 6 well plates ($0.2x10^6$ cells/well) and incubated with 1mL of media containing lentiviral particles and replaced with regular media 24 h after transduction started. THP-1 cells transduced with NAIP+neo particles were selected as geneticin (400μg/mL) resistant. U937 cells transduced with "NAIP+GFP" particles were selected by flow-sorting. The amount of NAIP protein was assessed by western blotting. Densitometry of transformed and non-transformed THP-1 (left graph) and U937 (right graph) of NAIP protein expression normalized to HSC70 expression. n=1

Once transduction and overexpression of the transgenes were confirmed, we examined the effect of supraphysiological NAIP levels in the differentiation state of monocytes and PMA differentiated macrophages by analyzing the expression of CD11b and CD14 macrophage markers, which have been shown to be over-expressed in differentiated THP-1 and U937 [238, 239] by flow cytometry. In THP-1 cells, there were no significant differences in CD11b expression between transduced and non-transduced cells, while in U937 cells we found a highly significant increase in CD11b in cells which overexpressed NAIP and were exposed to PMA differentiation treatment, Fig 15. In the case of CD14 expression, the results were more consistent between the two cell models, THP-1 and U937, in that there was a significant decrease in CD14 levels in NAIP overexpressing cells treated with PMA.

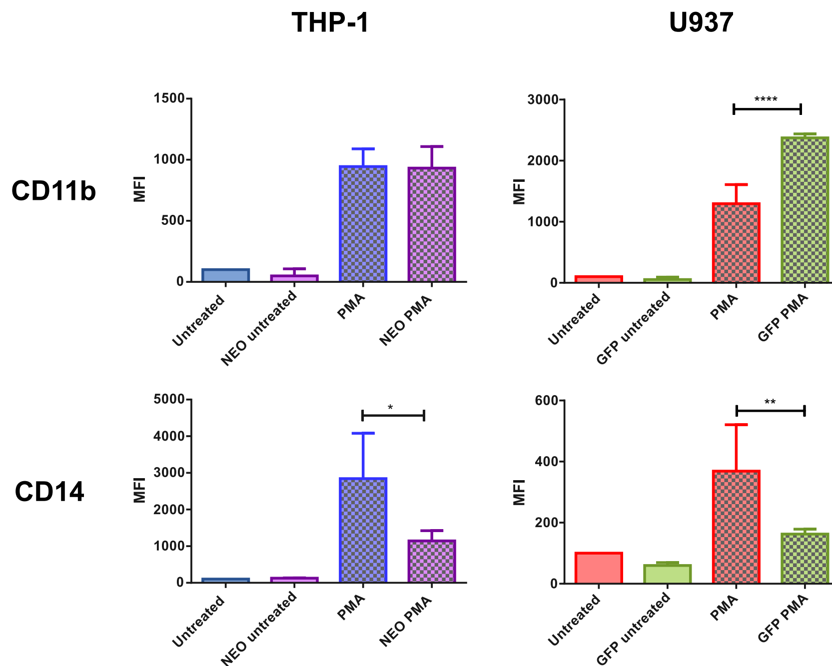


Figure 15. Supraphysiological NAIP causes a lesser induction of CD14 in PMA differentiated macrophages. THP-1 and U937 NAIP-overexpressing cells (here called NEO and GFP, respectively) and normal THP-1 and U937 cells were plated ($1x10^6$ cells/well) in 6 well plates, treated with PMA (10ng/mL) for 24 h, followed by 24 h incubation in regular media and then the expression of CD11b and CD14 was analyzed by flow cytometry. Presented as mean values ± S.D. of MFI of three independent experiments. One way ANOVA with Tukey post hoc was performed

IAPs, such as survivin, cIAP, and NAIP, have been shown to be involved in cell cycle regulation. We performed a cell cycle analysis in order to determine if the supraphysiologic levels of NAIP could be modifying not only the differentiation process, but the cell cycle progression too. We analyzed the cell cycle phases in THP-1 cells by propidium iodide staining FACS analysis (Fig 16). In both untreated monocytic cells and PMA-treated cells, we found no differences in the distribution of the cell cycle stages in cells overexpressing NAIP compared to their untransduced control counterparts.

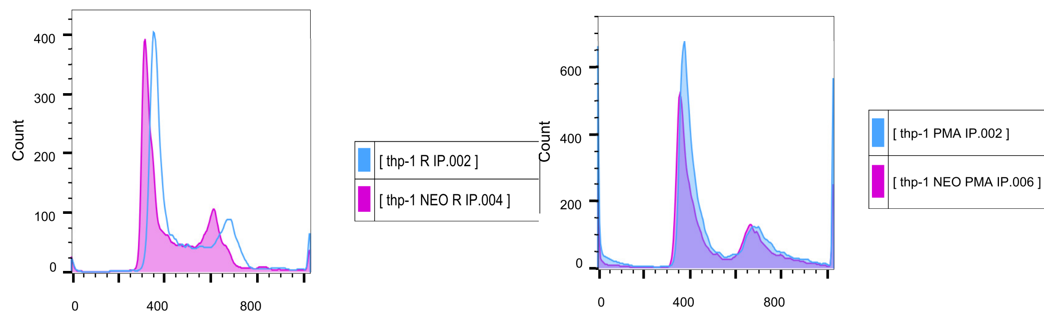


Figure 16. Cell cycle analysis in NAIP-overexpressing THP-1 cells. NEO-THP-1 and normal THP-1 cells undifferentiated (right graph) and differentiated with PMA (left graph) cell cycle analysis with intracellular PI staining by flow cytometry.

4.1.4 NAIP silencing and macrophage differentiation

NAIP gene silencing, with the purpose of determining if the presence of NAIP was mandatory to accomplish macrophage differentiation, was also studied by small-interference RNA (siRNA) technology in THP-1 cells. First, we tried two different concentrations for two different siRNA duplexes combinations and demonstrated that NAIP was being silenced by mRNA analysis using RT-qPCR, Fig 17. NAIP gene expression was 70% reduced in cells transfected with siRNA duplexes directed at NAIP-mRNA exons 6 and 10 and 40% reduced in cells transfected with duplexes directed at NAIP-mRNA exon 1, regardless of the concentration.

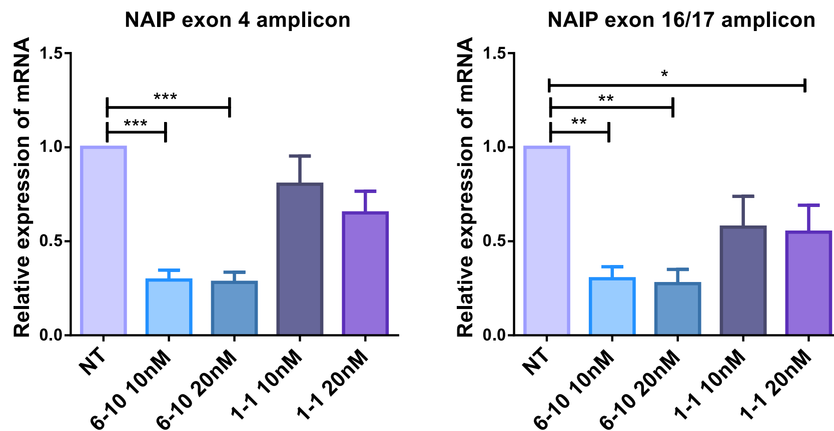


Figure 17. NAIP gene expression after NAIP-siRNA transfection. THP-1 cells (0.5x10^6 cells/well in 6 well plates) were transfected with non-targeting (10nM) or with two different combinations of NAIP siRNA duplexes targeting the indicated NAIP-mRNA exons at 10nM or 20nM for 72 h. Total RNA was extracted using RNAeasy minikit, reverse transcribed and NAIP mRNA was analyzed by RTq-PCR in order to evaluate the efficacy of the transfection. Values are normalized to internal controls (HPRT1 and GAPDH) and presented as mean values $\pm$ S.D. of three independent experiments. Anova with Bonferroni post hoc was performed.

We studied the effect of the infra-physiologic level of NAIP in monocyte differentiation, first in monocytic THP-1 cells, where we saw no effect in CD11b, CD14 expression in normal conditions (data not shown). We aimed to check if the role of NAIP in the differentiation process was similar to that of cIAP1, which is necessary at the beginning of the differentiation process and its degradation is required to successfully finish differentiation. We tested if NAIP silencing could lead to prevention of monocytes to differentiate. The expression of CD11b and CD14 was measured by flow cytometry in NAIP-silenced cells challenged with PMA (Fig. 18) and the results were compared to PMA differentiated THP-1 cells without NAIP silencing. As a result of NAIP silencing, CD11b is upregulated, showing statistical significance in one of the cases when compared to non silenced PMA-differentiated cells. As for CD14 levels, there is a statistical significant upregulation in all conditions studied when compared to control conditions.

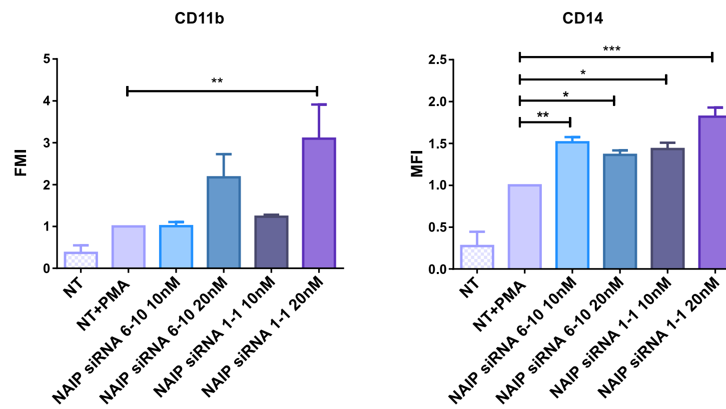


Figure 18. Infraphysiologic NAIP levels cause an increased induction of CD14 and CD11b in PMA differentiated macrophages. NAIP siRNA transfected THP-1 cells ($0.2x10^6$ cells/well in 6 well plates) were exposed 24 h to 10ng/mL of PMA followed by other 24 h incubation in regular media. the expression of CD11b and CD14 was analyzed by FACS. Presented as mean values ± S.D. of MFI of three independent experiments. One way ANOVA with Tukey post hoc was performed.

4.2 NAIP involvement in cell proliferation

A study of NAIP distribution in human tissues [240] showed an intense staining in the small intestine epithelium. Based on these results, the further characterization of NAIP cytoplasmic localization by confocal microscopy in the human epithelial carcinoma cell line HeLa, was undertaken and unexpectedly demonstrated NAIP intervention in cytokinesis. A representative view of NAIP dynamics during the whole cytokinetic process in HeLa cells is shown an explained in Fig. 19.

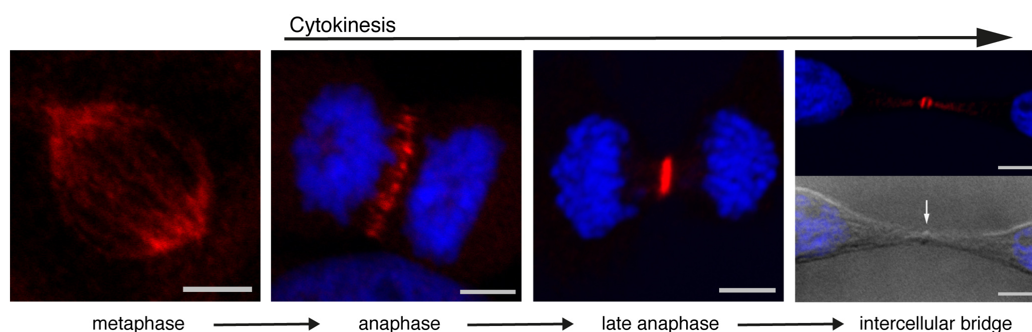


Figure 19. NAIP dynamics during cytokinesis. Confocal differential interference contrast, nuclear Hoechst staining and NAIP immunofluorescence channels merged accordingly of HeLa cells in mitosis. In the metaphase image the Hoechst channel has been omitted for clarity. NAIP accumulates in the spindle poles in metaphase and is also shown in spindle microtubules, later, once cytokinesis has started, NAIP is restricted to the middle of the central spindle gradually concentrating along the cell division plane as the cleavage furrow progressively constricts the dividing cell. Near cytokinesis completion, NAIP is present in the outflanking regions of the stem body (arrow) in the center of the intercellular bridge. NAIP immunostaing was performed with an antibody mapping to 13 C-terminal amino acids of human NAIP (abcam, ab25968). Bar=5μm.

In view of these results demonstrating the participation of NAIP in the cytokinetic process, we decided to determine whether NAIP is differentially expressed along the cell cycle. NAIP mRNA or protein expression was measured in HeLa cells chemically arrested in G1 with L-mimosine (75%), in S with thymidine (65%) and in G2/M with nocodazole (60%) as determined by DNA-content analysis. NAIP mRNA in G2/M was 2.7 fold that of NAIP

mRNA in G1, this increase was reflected at the protein level with NAIP two fold higher in G2/M compared to G1 (Fig. 20).

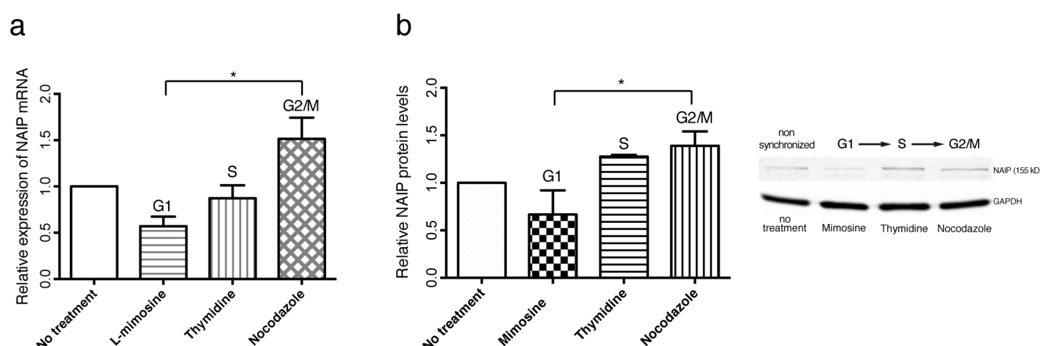


Figure 20. NAIP expression increases with cell cycle progression. **(a)** NAIP mRNA relative expression in exponentially growing (no treatment) or drug synchronized HeLa cells (400μM L-mimosine, G1; 2mM thymidine, S; 0,4μg/mL nocodazole, G2/M). **(b)** NAIP protein levels and representative western blot of non-treated, G1, S and G2/M synchronized HeLa cells. Data are the mean values ± S.D. of three independent determinations.

At the same time that NAIP positioning along cytokinesis was detailed, the effect of supraphysiologic NAIP in HeLa cells was examined. Cells transduced with a bicistronic lentiviral vector system expressing the open reading frame of NAIP and either the green fluorescent protein (GFP) or the neomycin-resistance (neo) gene, promptly produced a multinuclear phenotype that was evident three days after transduction, Fig 21(a). The multinuclear phenotype was precisely observed in those cells expressing the highest levels of NAIP as determined by immunostaining. The proportion of HeLa cells with three or more nuclei increased over time with approximately a 30 fold increase observed after a week of NAIP exposure (Fig 21b).

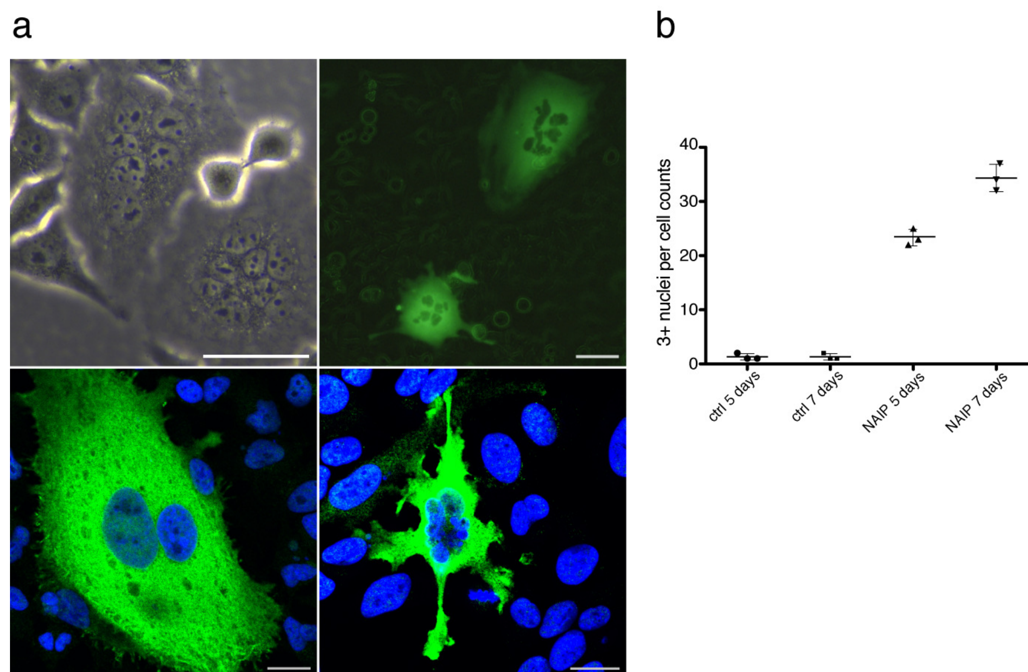


Figure 21. NAIP overexpression in HeLa cells generates a multinuclear phenotype. **(a)** Upper panel: Phase-contrast and fluorescence live microscopy images of HeLa cells 7 days after transduction with '*NAIP+neo*' or '*NAIP+GFP*' lentiviral particles respectively. Non transduced cells surrounding the multinuclear fluorescent '*NAIP+GFP*' transduced cells show a faint green glow profile. Bar=50μm. Lower panel: NAIP immunostaining with abcam ab25968 of HeLa cells (Alexa Fluor-488) 7 days after transduction with '*NAIP+neo*' lentiviral particles demonstrating that the multinuclear phenotype is observed in cells overexpressing NAIP. Bar, 20μm. **(b)** Total visual counts of HeLa cells with three or more nuclei in four randomly selected 10X optical fields from control (non-transduced) and '*NAIP+neo*' transduced HeLa cells 5 and 7 days after transduction. Data are the mean values $\pm$ S.D. of three independent transductions.

On the other hand, NAIP expression silencing by siRNA transfection resulted in a lethal apoptotic phenotype for HeLa cells (Fig. 22). Apoptosis in dying siRNA transfected cells was demonstrated by monitoring caspase-3 and caspase-7 activity over 48 h during the second and third day following transfection (Fig. 22a). NAIP expression silencing was performed using 8 different siRNA duplexes combined in 4 duplexes-pairs altogether targeting at a total of 6 different NAIP mRNA exons; similar cell death was seen with

all combinations (Fig. 22b).

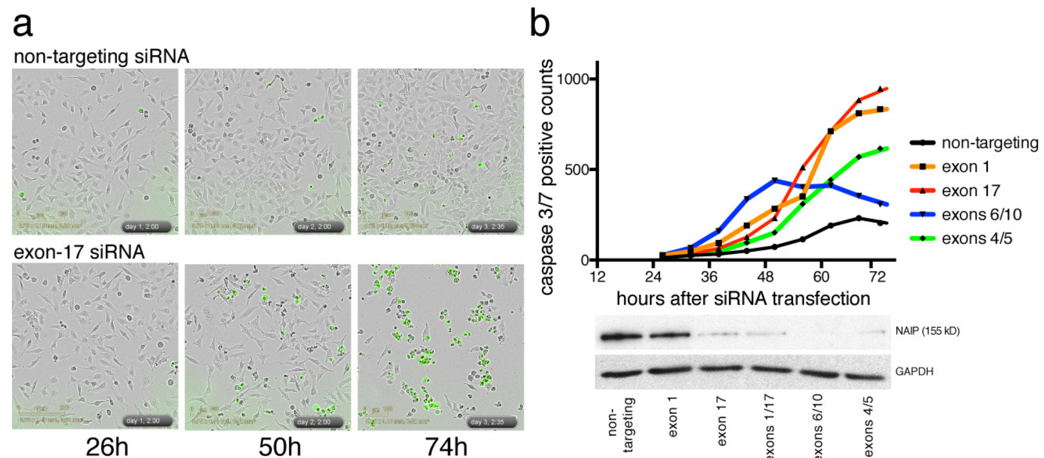


Figure 22. NAIP silencing in HeLa cells produces a lethal apoptotic phenotype. **(a)** Phase-contrast and green-fluorescence IncuCyte ZOOM merged images of non-targeting and NAIP exon17-siRNA transfections taken 26, 50 and 74 h after siRNA transfection. Fluorescent cells show active caspase-3/7, a reflection of the processing of a non-fluorescent caspase-3/7 substrate releasing a green fluorescent dye. **(b)** Apoptosis cell counts 26 to 74 h after NAIP siRNA transfection. Each point represents the mean of the caspase-3/7 counts for 4 different optic fields analyzed per well and treatment at a given time point. The graph is representative of one out of four replicas of two independent siRNA transfections. Western blot showing the corresponding efficacy in NAIP silencing.

4.3 IAPs expression in macrophage polarization

In recent decades, there has been an increased tendency to study macrophage activation from a functional point of view. Macrophages constitute a heterogeneous population with the ability to respond differently to various microenvironmental stimuli. They are able to change their morphology and function to acquire a pro-inflammatory phenotype (M1, classically activated macrophages) or a more healing phenotype (M2, alternatively activated macrophages). In the context of phenotypic change, also known as polarization, the upregulation of certain IAP genes has been noted but never studied in depth. Here, we present a study of several IAPs (NAIP, cIAP1 and cIAP2) during macrophage polarization.

4.3.1 Establishment of M1/M2 polarization models

4.3.1.1 THP-1 polarization model

First, based on previous reports in the literature [86,231,232], we optimized a protocol to obtain M1 and M2 polarized macrophages from THP-1 cells. We started with THP-1 monocytes, which were treated with PMA to differentiate the cells to a "neutral" macrophage state called M0. M0 macrophages were subjected to treatments at different times and concentrations in order to find the best time/dose combination to successfully polarize the macrophages. Exposing M0-THP-1 cells for 48 h to LPS and IFN-γ yielded M1 polarized macrophages, and 48 h exposure to IL-4 yielded M2 polarized macrophages. As a result of the incubation methods, cells presented typical morphological changes (Fig 23). Undifferentiated THP-1 cells are non-adherent and have a round shape, while PMA-treated cells become adherent with a flat and amoeboid shape. LPS and IFN-γ (M1) and IL-4 (M2) incubated THP-1 cells presented typical cellular protrusions of an activation state, including lamellipodia and filopodia (Fig 20) [241].

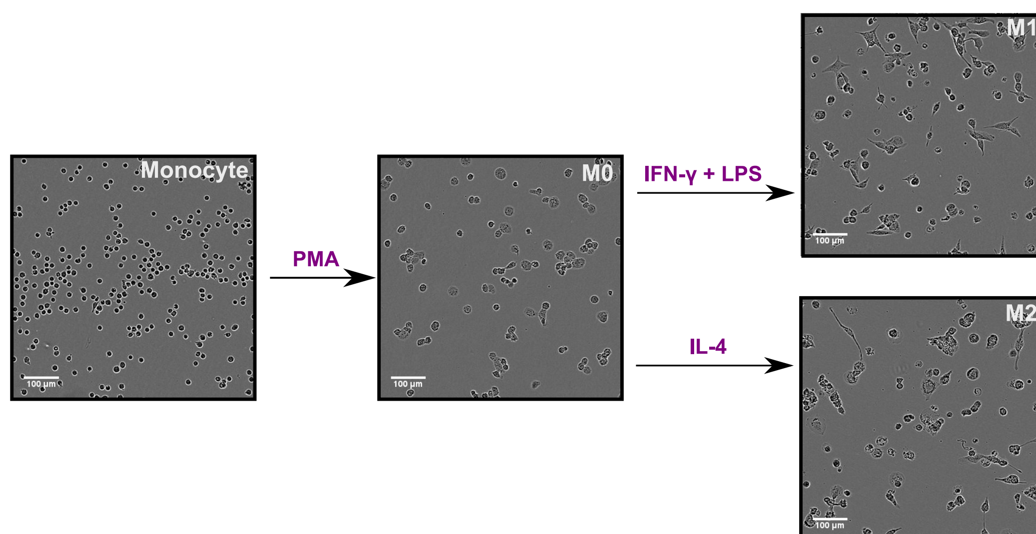


Figure 23. THP-1 M1/M2 polarization model. M0 macrophages were obtained by exposing THP-1 cells (1x10^6 cells/dish in 60mm culture dishes) to PMA (10ng/mL) for 24 h followed by 24 h incubation in regular media. M0 THP-1 macrophages were then incubated for 48 h with LPS (100ng/mL) and IFN-γ (20ng/mL) in order to obtain M1 macrophages, or with IL4 (20ng/mL) in order to obtain M2 macrophages. Phase contrast microscopy images were taken using the Incucyte Zoom. Images are representative of 3 independent experiments.

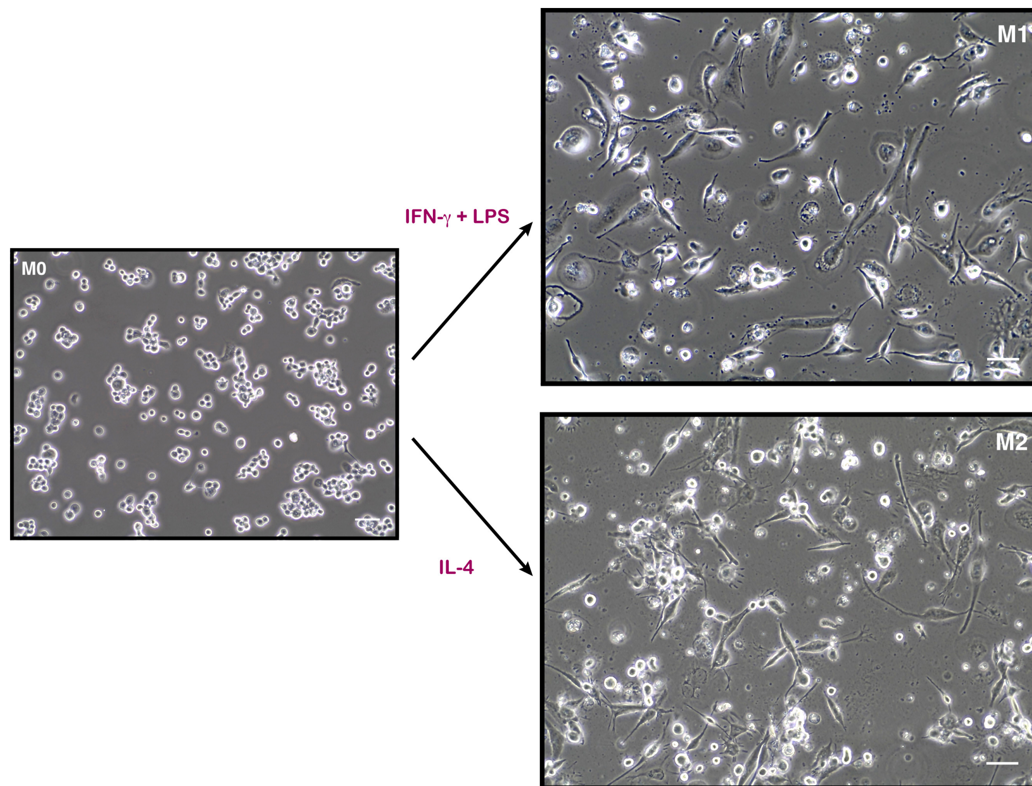


Figure 24. THP-1 cell morphology after M1/M2 polarization stimuli. Phase contrast microscopy images representative of M0-, M1- and M2-macrophages. Polarized macrophages show typical activation state macrophage differentiations like lamellipodia and filopodia. Bar, 50 μm.

As a consequence of polarization induction, the expression of surface markers is modified. Analysis by flow cytometry (Fig. 25) and qPCR (Fig. 26) showed upregulation of CD206, a mannose receptor that has been previously reported to be present in M2 polarized macrophages, in cells treated with IL-4 [75]. In the group incubated with LPS and IFN-γ, an increase in the level of CD86 and a higher expression of CXCL10 were observed, both of which are usually associated with M1 macrophages.

Altogether, the study of surface markers and the differences at the mRNA level indicated that the protocol followed to obtain M1 and M2 macrophages from THP-1 M0 macrophages (24 h PMA treatment followed by 24 h incubation in regular media) was successful. All further experiments therefore used this protocol (see p. 45) to obtain polarized macrophages.

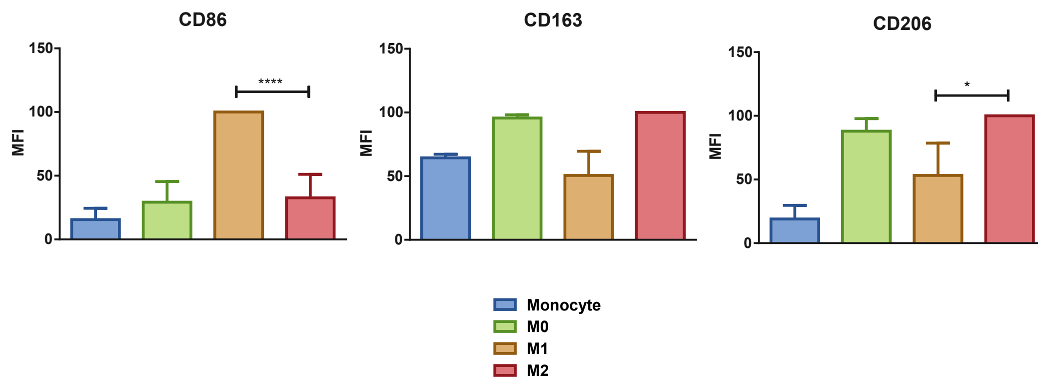


Figure 25. Polarization markers in THP-1 cells are increased after M1/M2 stimuli. M0 and M1, M2 polarized macrophages and monocytic THP-1 cells (initial 1x10^6cells seeded) were harvested and stained with CD86, CD206 and CD163 antibodies and analyzed by flow cytometry. Presented as mean values ± S.D. of MFI of three independent experiments. One way ANOVA with Tukey post hoc was performed.

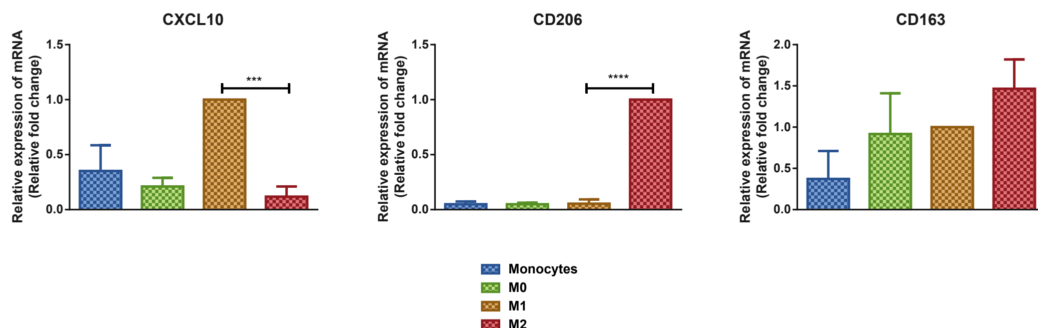


Figure 26. Gene expression of polarization markers in THP-1 is increased after M1/M2 stimuli. M0 and M1, M2 polarized macrophages and monocytic THP-1 cells (initial 0.5x10^6cells seeded) were harvested and total RNA was extracted using RNAeasy minikit, reverse transcribed and the expression of CXCL10 (M1 marker), CD206 (M2 marker) and CD163 (generally considered as an M2 marker) genes were examined by RT-qPCR. Values are normalized to internal controls (HPRT1 and GAPDH) and presented as mean values ± S.D. of three independent experiments. ANOVA with Tuckey test post hoc was performed.

4.3.1.2 PBMCs polarization model

To obtain polarized macrophages from M0 primary human macrophages, PBMCs from healthy donors were cultured (explained in p. 46). In the case of THP-1 cells, the expression of polarization markers CD206, CXCL10, CCL18 and CD163 was analyzed (Fig. 27). As expected, after IL-4 treatments the expression of M2 markers, such as CD206, CCL18 and CD163, were upregulated in contrast to the expression of CXCL10 (an M1 marker which was only found in those macrophages incubated with LPS/INF-γ). Based on these gene expression observations, our protocol to obtain M1-like and M2-like macrophages from peripheral blood monocytes is effective.

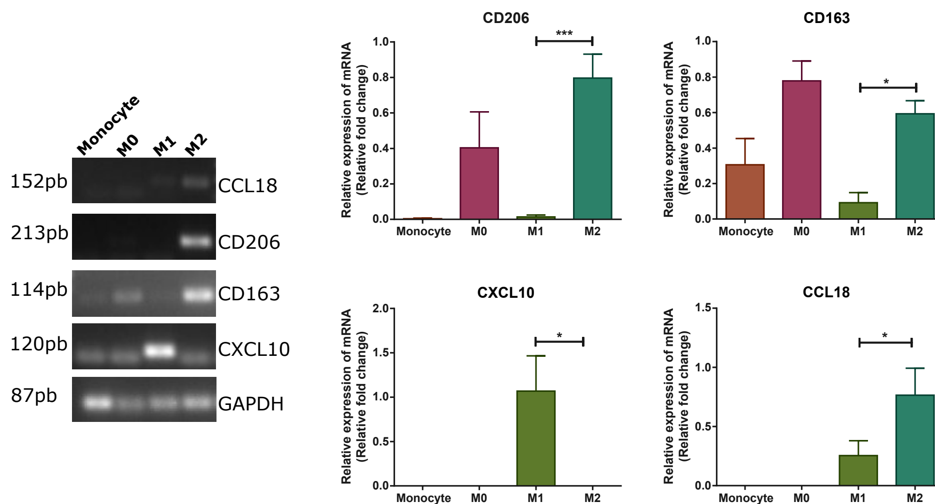


Figure 27. Gene expression of polarization markers in human monocyte-derived macrophages is increased after M1/M2 stimuli. Peripheral blood monocytes, M0 macrophages, and LPS/INF-γ-incubated macrophages (M1) and IL-4-incubated macrophages (M2) were harvested and RNA extracted using the Trizol protocol. mRNA expression of CXCL10, CD206, CCL18 and CD163 was analyzed by semi quantitative-PCR (left). The expression of each marker was normalized to GAPDH (right). Data represents the mean $\pm$ S.D. of four independent experiments with samples from four different healthy patients. ANOVA with Tuckey test post hoc was performed.

4.3.2 IAPs expression in M1/M2 macrophages

IAPs have important roles in the regulation of several cellular processes, including innate and adaptive immunity, and have been targeted in many cancer therapies. However, the pattern of expression of certain IAPs during macrophage polarization remains unclear, as does the effect of inhibition of these proteins in polarized macrophages. Inhibition of such proteins is relevant in several disease models where macrophage polarization is implicated.

4.3.2.1 NAIP expression analysis

In polarized THP-1 macrophages:

NAIP expression profile was characterized in polarized THP-1 macrophages by Western blot and by PCR. Fig 28 shows that NAIP protein is significantly higher in M2 macrophages than in both M1 and M0 macrophages. NAIP is also upregulated at the transcript level in M2 macrophages; M2 showed more than two fold levels of mRNA compared to M1. NAIP gene expression is higher in M1 than in M0, and comparable to that found in monocytes.

In polarized PBMCs macrophages:

Once NAIP expression pattern in polarized THP-1 macrophages was determined, we wanted to assess whether that pattern was consistent in primary human polarized macrophages. We studied the expression of NAIP in polarized PBMCs and analyzed it against that of monocytes. We found similar results in polarized PBMCs to those obtained in our THP-1 model. NAIP protein was more abundant in M2 macrophages than in M1, although this difference was not significant in western blots, at the transcript level NAIP was significantly upregulated in M2 compared to M1, while both M1 and M2 have reduced expression when compared to peripheral blood monocytes (Fig. 29).

To further study NAIP protein expression, immunofluorescence confocal microscopy analysis was performed in human peripheral blood monocytes

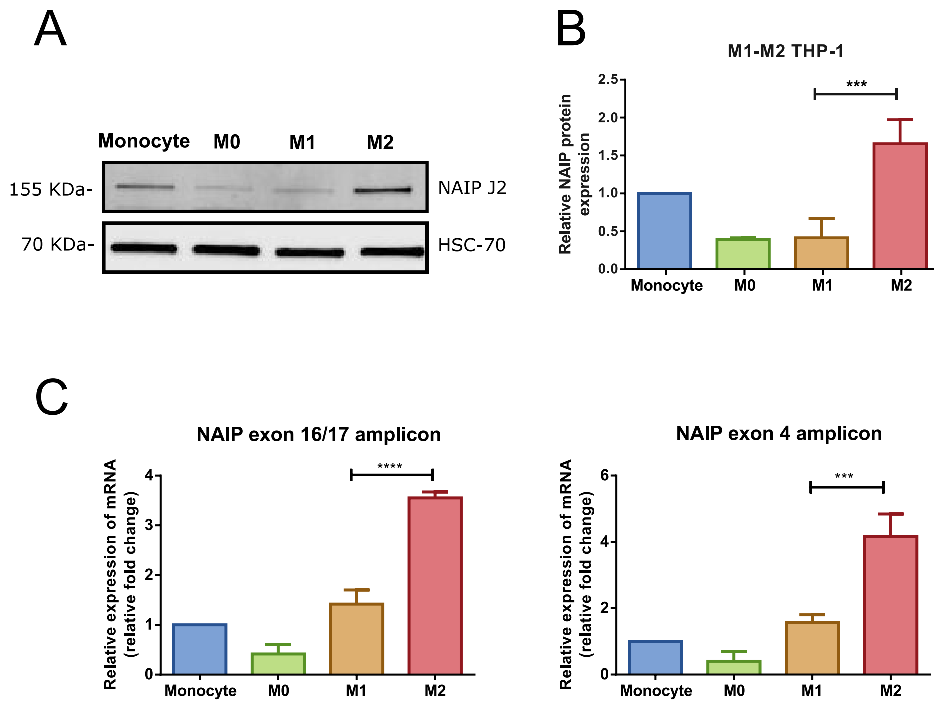


Figure 28. NAIP expression is increased in M2 THP-1 macrophages. Upper graphs: NAIP protein level was assessed by Western blotting of cell extracts from monocytes, M0, M1 and M2 THP-1 cells using the NAIP-J2 antibody. HSC-70 was used as loading control. Representative Western blot of one of three experiments with similar results is shown. Graph represents quantification of NAIP protein normalized to HSC-70. Cells were harvested and RNA was extracted using the Qiagen RNeasy mini-kit. NAIP mRNA expression was analyzed by RT-qPCR. Values are normalized to internal controls (GAPDH and HPRT1). All graphs are presented as mean values $\pm$ S.D. of three independent experiments. ANOVA with Tukey test post hoc

and monocyte-derived M1/M2 macrophages from two healthy donors (Fig 30). Primary monocyte-derived M1/M2 macrophages showed after polarization stimuli typical macrophage morphology and cellular differentiation features like lamellipodia and filopodia protrusions. In general, M1 and M2 *in vitro* polarized macrophage morphology corresponded to that of an amoeboid macrophage (Fig 31), but sometimes, big rounded cells were obtained after polarization treatment. Future studies will elucidate whether these two phenotypes, big-rounded and amoeboid, correspond to distinct macrophage activation states.

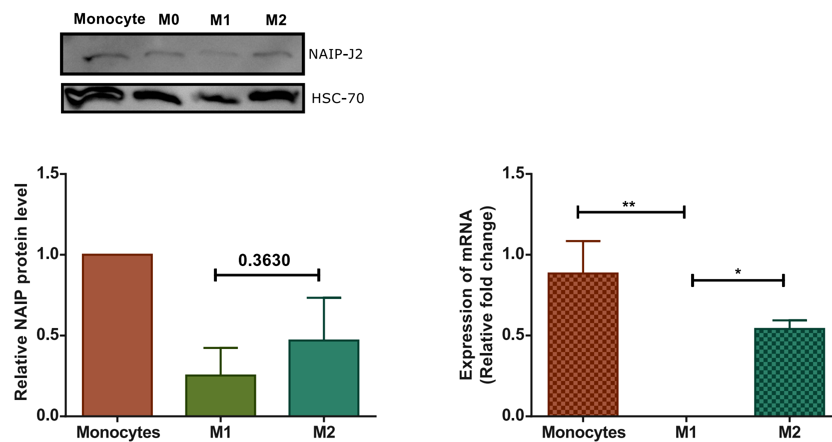


Figure 29. NAIP expression is increased in primary human monocyte-derived M2 macrophages with respect to M1. Peripheral blood monocytes and LPS/INF-γ- induced M1 macrophages and IL-4 induced M2 macrophages, were harvested and protein extracted. Left graph, protein level of NAIP was determined by western blot, using the NAIP-J2 antibody and normalized to HSC-70 antibody. Cells were also harvested and RNA extracted by TRIZOL protocol, NAIP mRNA was analyzed by RT-qPCR using the pair of primers targeting exon 4 of NAIP and normalized to internal controls (GAPDH). Graphs are presented as mean $\pm$ S.D. from four experiments from 4 different healthy donors. ANOVA with Tuckey test post hoc.

NAIP immunostaining confocal microscopy quantification (10 randomly selected cells were analyzed in each case) was consistent with what was previously seen in THP-1 cells; Fluorescence intensity per pixel per cell showed in M2 macrophages, approximately three times the level of NAIP in monocytes and twice that of M1, (Fig. 32).

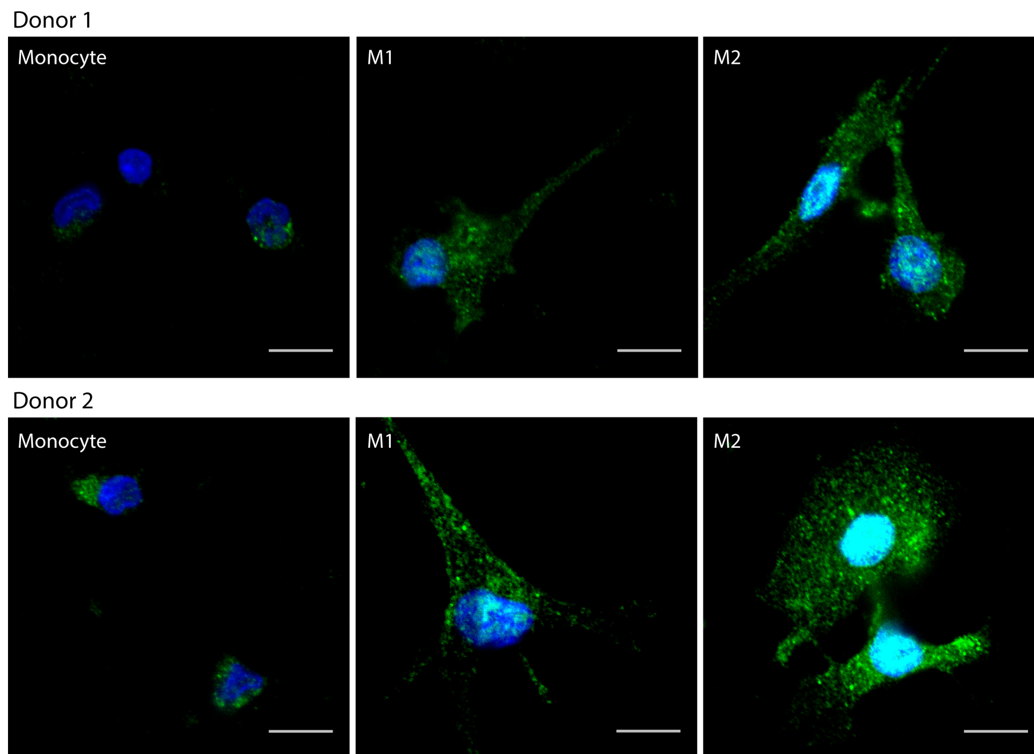


Figure 30. NAIP immunostaining in polarized primary human monocyte-derived macrophages. Human peripheral blood monocytes and M1/M2 polarized macrophages were stained with NAIP-ab98020 antibody and counterstained with Hoechst. Following polarization treatment, M1 and M2 cells showed proper macrophage morphology and differentiation characteristics like lamellipodia and filopodia. Representative micrographs of donor 1 and 2. Bar = 10μm.

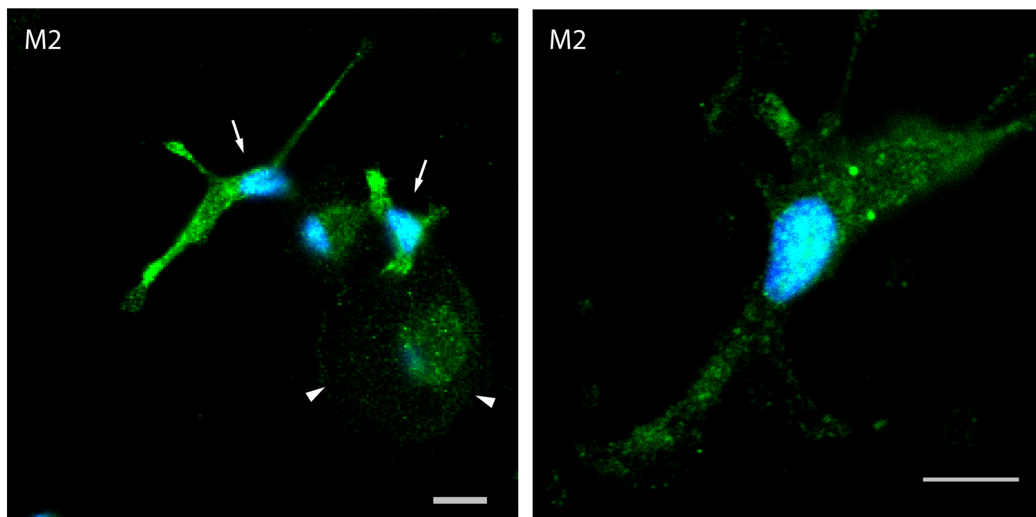


Figure 31. Primary human monocyte-derived polarized macrophages show an amoeboid cell shape. Representative micrographs of NAIP-ab98020 immunostained M2 macrophages showing a classic amoeboid shape (arrows) and a large rounded cell (arrowheads). Bar = 10μm

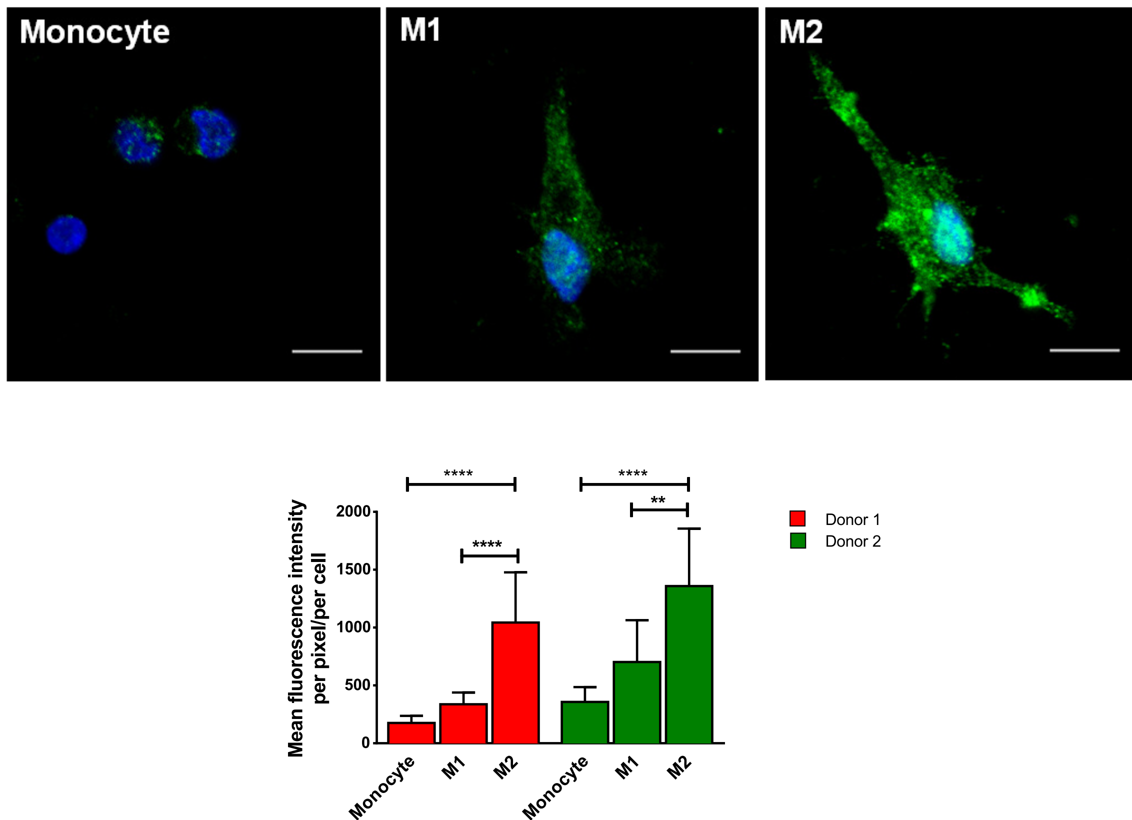


Figure 32. Immunostaining quantification shows the highest NAIP levels in M2 macrophages. Peripheral blood monocytes and LPS/INF-γ induced M1 macrophages and IL-4 induced M2 macrophages, were fixed and stained with NAIP-ab98020 antibody and counterstained with Hoechst. Representative confocal microscopy images from donor 1. Bar = 10μm. Graph represent mean fluorescence intensity in cells from donor 1 and 2. Selected cells were analyzed for their mean fluorescence per cell area using the *ImageJ* program. Values are presented as mean values $\pm$ S.D. ANOVA with Bonferroni post hoc.

4.3.2.2 cIAP1 and cIAP2 expression analysis

The essential role of cIAP1 during differentiation of monocytes into macrophages [178] and the protective function of cIAP2 in macrophages under pro-inflammatory conditions [133] is well documented. We analyzed the expression of cIAP1 and cIAP2 in polarized THP-1 cells, shown in Fig. 32. The amount of cIAP1 present in macrophages was increased following PMA-differentiation of monocytes to M0. Expression levels were maintained after incubation under M0 to M2 differentiation conditions, but not under M0 to M1 differentiation conditions, in which case there was a slight decrease in the amount of cIAP1 found in the cell. However, this decrease in protein is not due to gene downregulation, as the presence of cIAP1 transcript is higher in M1 than in any other polarization state. In regards to cIAP2 expression, we have observed that its transcript level correlates with its protein level in all macrophage states, with cIAP2 being more abundant in M1 than in any other activation state.

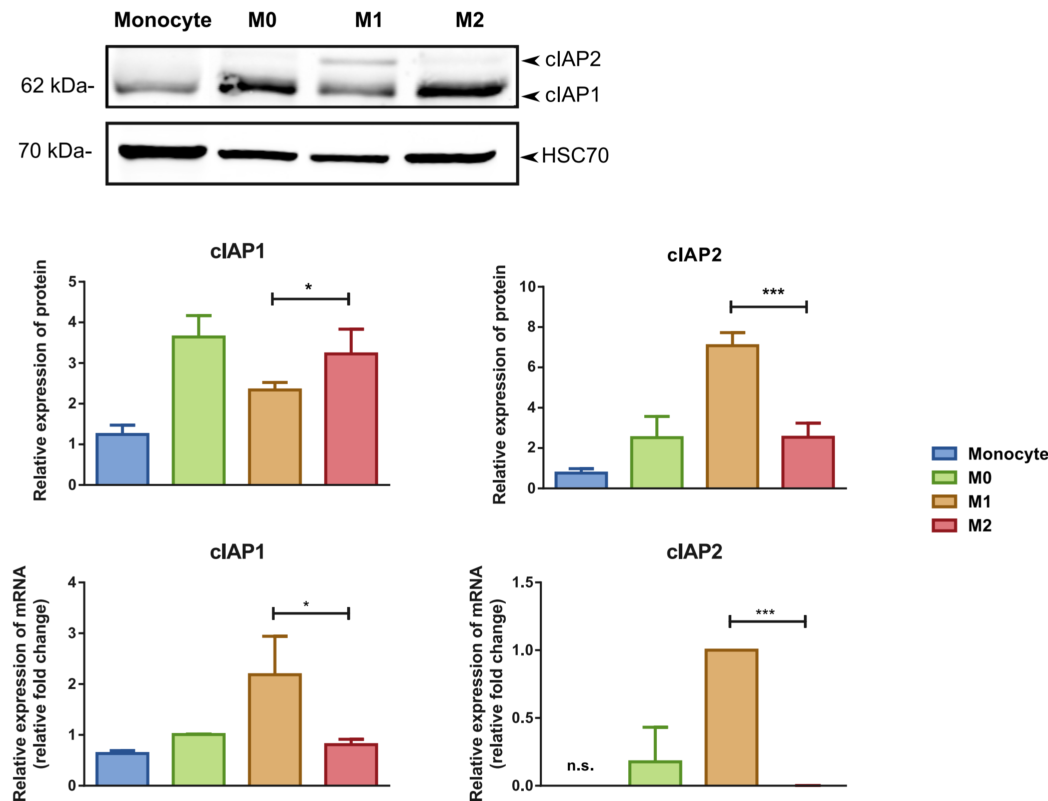


Figure 33. cIAP1 and cIAP2 show an inverse pattern of protein expression in polarized macrophages. Monocytes, M0, M1 and M2 THP-1 cells (initial 0,5x10^6cells/well in 6 well plates) cIAP1 and cIAP2 expression were analyzed by western blotting (upper graphs) or by RT-qPCR (lower graphs). Representative western blot of one of three experiments with similar results is shown. cIAP1/2 RIAP1 antibody and HSC-70 antibody as loading control were used. Graphs represent quantification of cIAP1 or cIAP2 proteins normalized to HSC-70. Cells were also harvested and RNA extracted using the RNaeasy mini-kit. cIAP1 and cIAP2 mRNA expression was analyzed by RT-qPCR. Values are normalized to internal controls (GAPDH and HPRT1). All graphs are presented as mean values $\pm$ S.D. of three independent experiments. ANOVA with Tukey test post hoc was performed.

4.3.2.3 XIAP expression analysis

Along with cIAP1 and cIAP2, XIAP is one of the most studied IAPs, due to its important anti-apoptotic role. It is the only IAP able to inhibit caspase by binding them. In previous studies, it has been seen to be increased during monocyte to macrophage differentiation [229] and it has been related to production of nitric oxide in response to LPS [242]. But just as happens in cIAP1, the actual pattern of expression in polarized macrophages remained unknown. So we studied the expression during macrophage polarization in THP-1 cells and found that protein level of XIAP was constant between the different polarization conditions. At a transcript level, XIAP was significantly increased in M1 compared to M2, but that upregulation of XIAP genes was not translated to increase in protein levels.

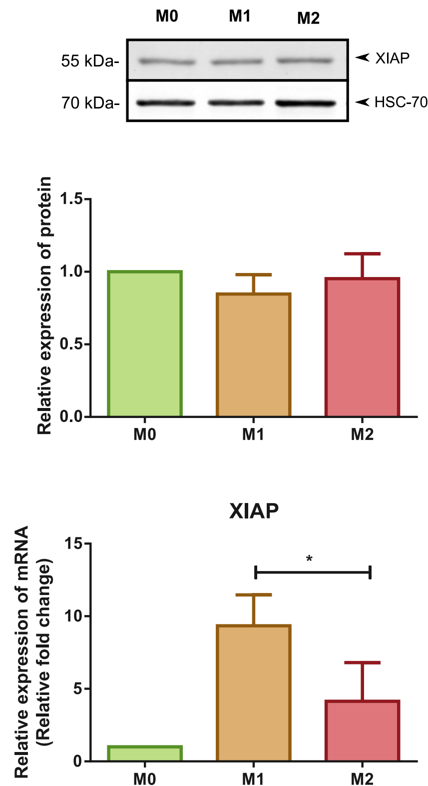


Figure 34. XIAP protein level is maintained in THP-1 polarized macrophages. M0, M1 and M2 THP-1 cells (0,5x10^6cells/well in 6 well plates) XIAP expression was analyzed by western blotting (upper graph) and RT-qPCR (lower graph). Representative western blot of one of three experiments with similar results is shown, cIAP1/2 RIAP3 antibody and HSC-70 antibody as loading control were used. Graphs represent quantification of XIAP protein normalized to HSC-70. Cells were also harvested and RNA extracted using the RNaeasy mini-kit. XIAP mRNA expression was analyzed by RT-qPCR. Values are normalized to internal controls (GAPDH and HPRT1). Both graphs are presented as mean values $\pm$ S.D. of three independent experiments. ANOVA with Tukey test post hoc was performed.

4.3.3 Pharmacological inhibition of IAPs and macrophage polarization

SMAC mimetic compounds (SMCs) are a class of small molecules that mimic the structure of the endogenous second mitochondrial activator of caspases (SMAC). SMCs are IAP antagonists, whereby they inhibit the function of cIAP1, cIAP2 and XIAP [194]. The SMC, LCL161 [243, 244], was used to determine whether IAP antagonism can modulate polarization of macrophages into M1 and M2 phenotypes.

4.3.3.1 M0, M1 and M2 macrophages response to SMAC mimetic compound LCL161

Macrophage viability response to LCL161 and TNF-α:

As SMC has been known to sensitize cells to apoptosis in the presence of some death ligands (including TNF-α), we first studied the susceptibility of polarized THP-1 macrophages to smac mimetic mediated cell death, alone and in combination with TNF-α [245–247]. To do so, we used two different tests: one with YOYO-1 dye (Fig. 35), measuring cell death by the ability of YOYO-1 to penetrate the cell when its plasma membrane is compromised or damaged (a sign of irreversible cell damage), and another with alamar blue (resazurin sodium salt) (Fig. 36), which functions as a metabolic and cell health indicator. We did a previous test measuring the toxicity of different concentrations of LCL161, the SMC used in this study (data not shown), and chose 1μM as the working dosage. The viability assay using YOYO-1 dye showed that SMC treatment of M0 macrophages in the presence of TNF-α promoted cell death in the hours following treatment, but then reached a plateau. This was also found in M2 macrophages in the presence of TNF-α with or without LCL161 further treatment. But, after 48 h no significant differences between groups of treatment were found.

We found similar results when analyzing metabolic rates, in Fig 36. There is a slight decrease in metabolic capacity of M0 and M2 macrophages in the presence of both TNF-α and SMC, but no significant differences were found among all groups of treatments. So, we found no actual TNF-α cell death sensitization in THP-1 polarized macrophages when exposed to LCL161.

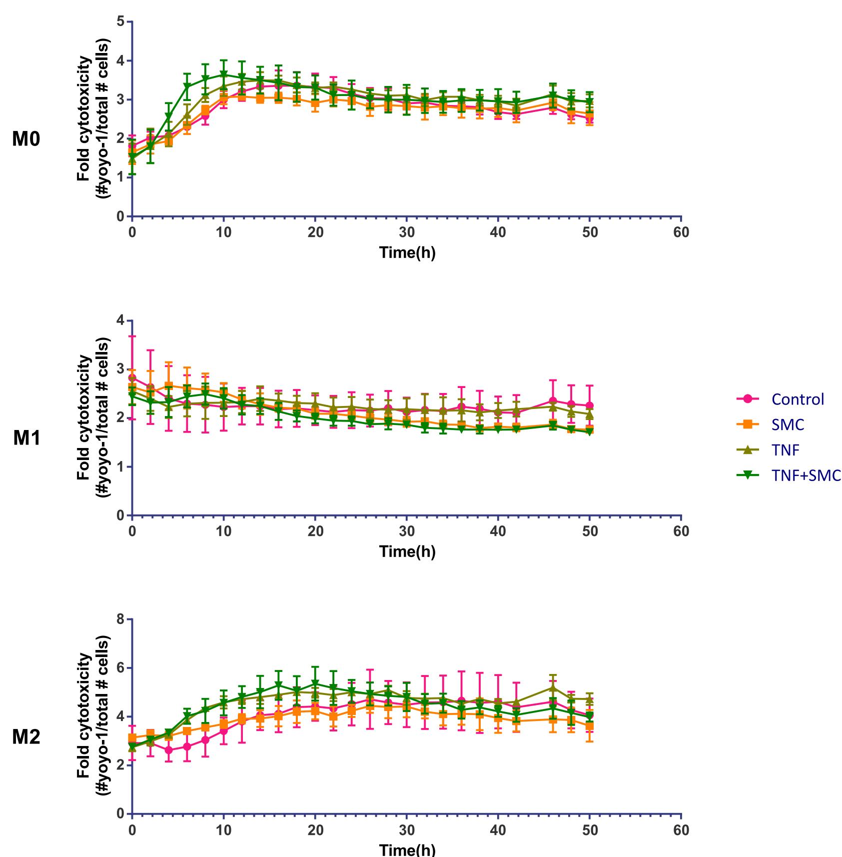


Figure 35. Cytotoxicity analisis of polarized THP-1 macrophages in the presence of SMC and TNF-α. M0, M1, and M2 THP-1 macrophages (initial 0,2x10^5 cells/well seeded in 96 well plates, in the same plate that polarization was performed) were treated with 1μM LCL161 and/or 10ng/mL of TNF-α at the same time, YOYO-1 dye (2,5 μM) was added. Incorporation of YOYO-1 to the cells was monitored over 48 h using the INCUCYTE^{TM} Live-Cell Imaging System.

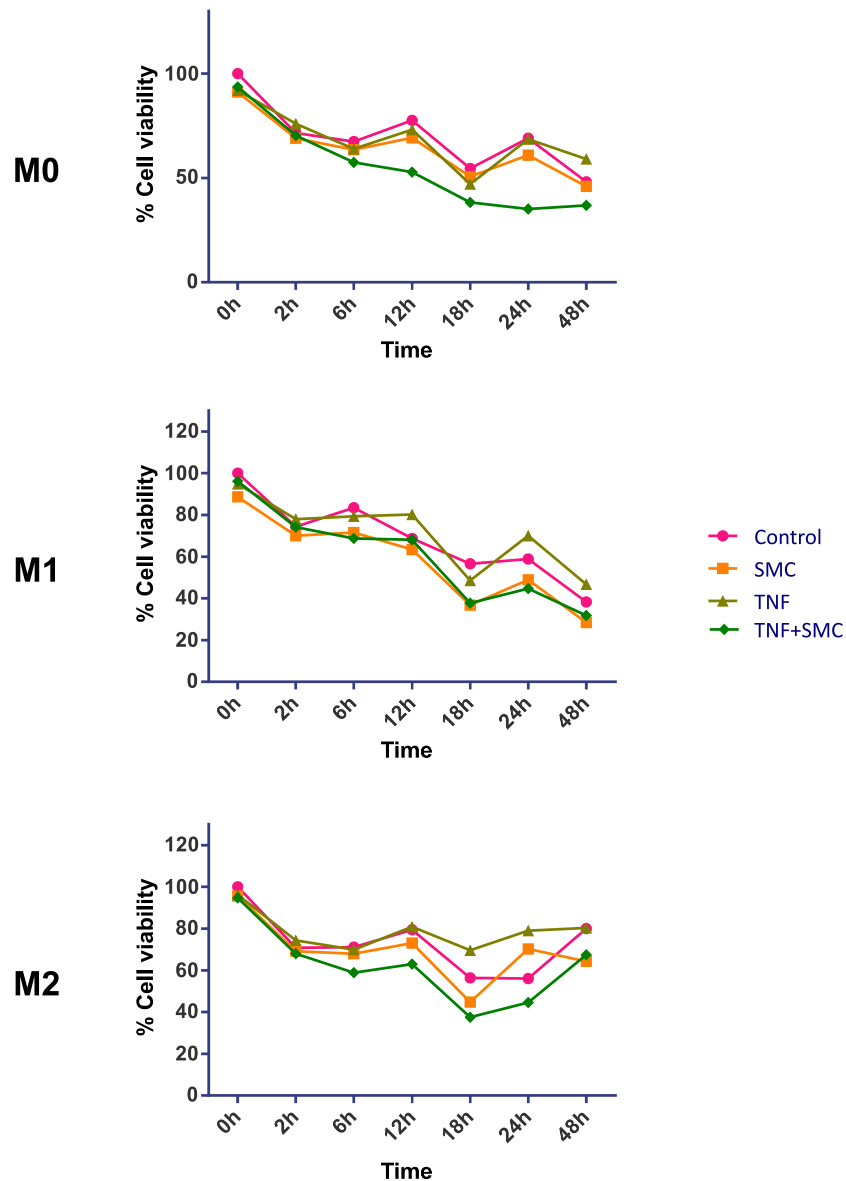


Figure 36. Viability variation of polarized THP-1 in the presence of SMC and TNFα. M0, M1, and M2 THP-1 macrophages (initial 0,25x10^6 cells/well seeded in 24 well plates) were treated with 1μM LCL161 and/or 10ng/mL of TNF-α and cell viability was determined by Alamar blue assay at the specified time points over a 48 h period. Each point of the graph represents the mean of three independent experiments.

LCL161 and TNF-α effect in polarization markers expression:

We wondered whether the polarization state would be maintained if polarized macrophages were to be challenged with SMC or/and TNF-α, which is a situation driven by combinatorial therapies in cancer [248]. The expression of some markers of polarization was determined by flow cytometry and by qPCR (Fig 37). In CD206 (both, flow and qPCR) we set the expression of the control M2 as a base line, and the control M1 was used as a base line in the expression of CXCL10 and CD86. As shown in Fig 37, cells respond to SMC treatment by increasing their expression of CD206, even more so in the case of already polarized M2 THP-1 cells. There was also a decrease in M1 markers, both CD86 and CXCL10. In the presence of TNF-α, the situation reverses, decreasing the expression of CD206 and not having an effect on the M1 marker expression under study.

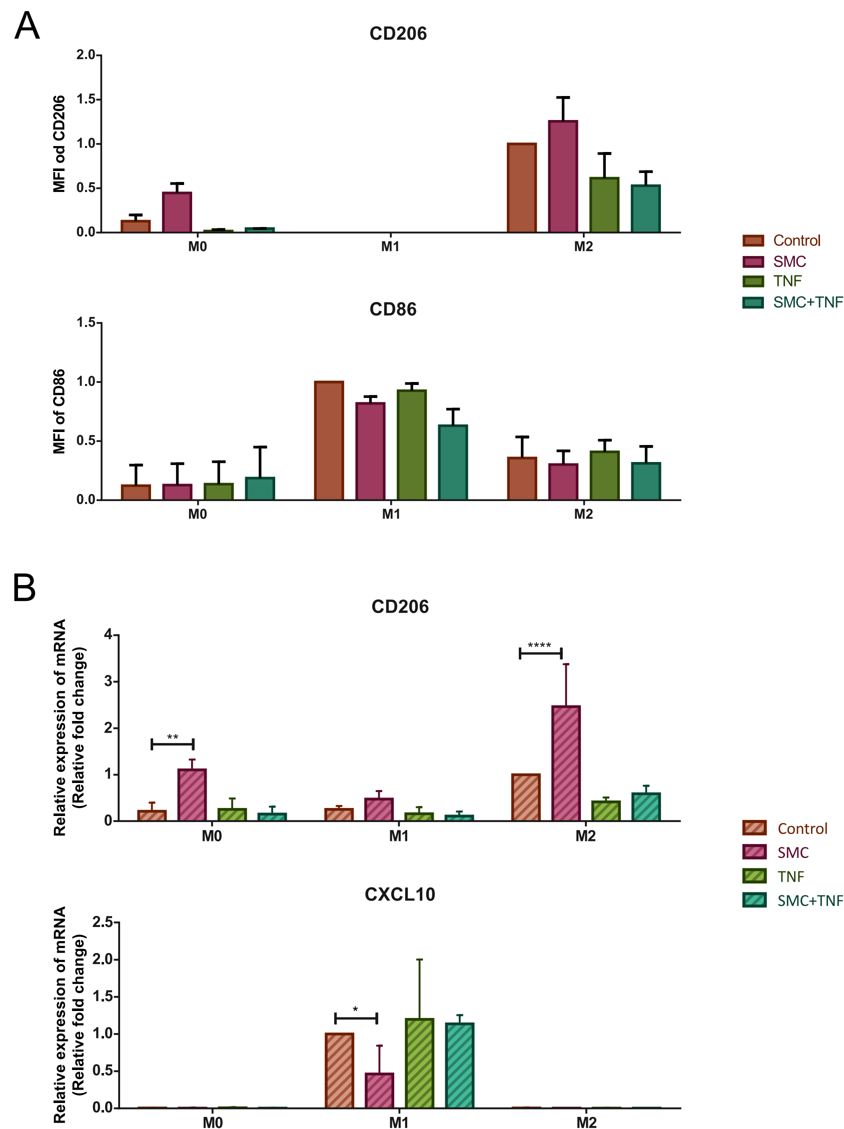


Figure 37. SMC upregulates CD206 and downregulates CXCL10 gene expression. Monocytes, M0, M1, and M2 THP-1 cells (initial 1x10^6 cells/well seeded in 6 well plates), were exposed to 1μM LCL161 and/or 10ng/mL of TNF-α for 48 h. Then the effect on polarization was analyzed by flow cytometry (a) and qPCR (b). Cells were stained with CD86 and CD206 antibodies and fluoresce was measured by flow cytometry. MFI values were obtained with Flowing Software. Cells were harvested and total RNA was extracted using RNAeasy minikit, reverse transcribed and the expression of CXCL10 and CD206 genes were examined by RT-qPCR. Values are normalized to internal controls (HPRT1 and GAPDH). Graphs are presented as mean values $\pm$ S.D. of three independent experiments. Two-way ANOVA with Dunnett's multiple comparison test was performed.

LCL161 and TNF-α effect in polarized macrophages IAP expression:

The effect of SMC on the amount of IAPs available in the cell is well documented: it promotes cIAP1 degradation, cIAP1-dependent cIAP2 degradation and inhibits XIAP anti-caspase function. Usually, when cIAP1 is present, TNF-α activates NF-κB signaling pathway, inducing an upregulation of cIAP genes. This was consistent with our findings when we analyzed the IAP expression pattern of macrophages exposed to SMC and TNF-α (Fig 38.). The amount of cIAP1 protein in polarized and unpolarized macrophages exposed to TNF-α is similar to that found in those unexposed. As expected, the level of cIAP1 is significantly decreased in the presence of SMC. The expression of cIAP2 is usually low in M0 and M2 macrophages, but exposure to TNF-α promotes the induction of cIAP2. The increase is partially maintained even in the presence of SMC, which is supposed to trigger cIAP2 degradation; however, we saw that the level of cIAP2 in the presence of SMC does not vary significantly, even in the case of M1 where its presence is already high. SMC, TNF-α, nor a combination of both have any significant effect on levels of XIAP protein in the cell, which is consistent among the different polarization states and treatments.

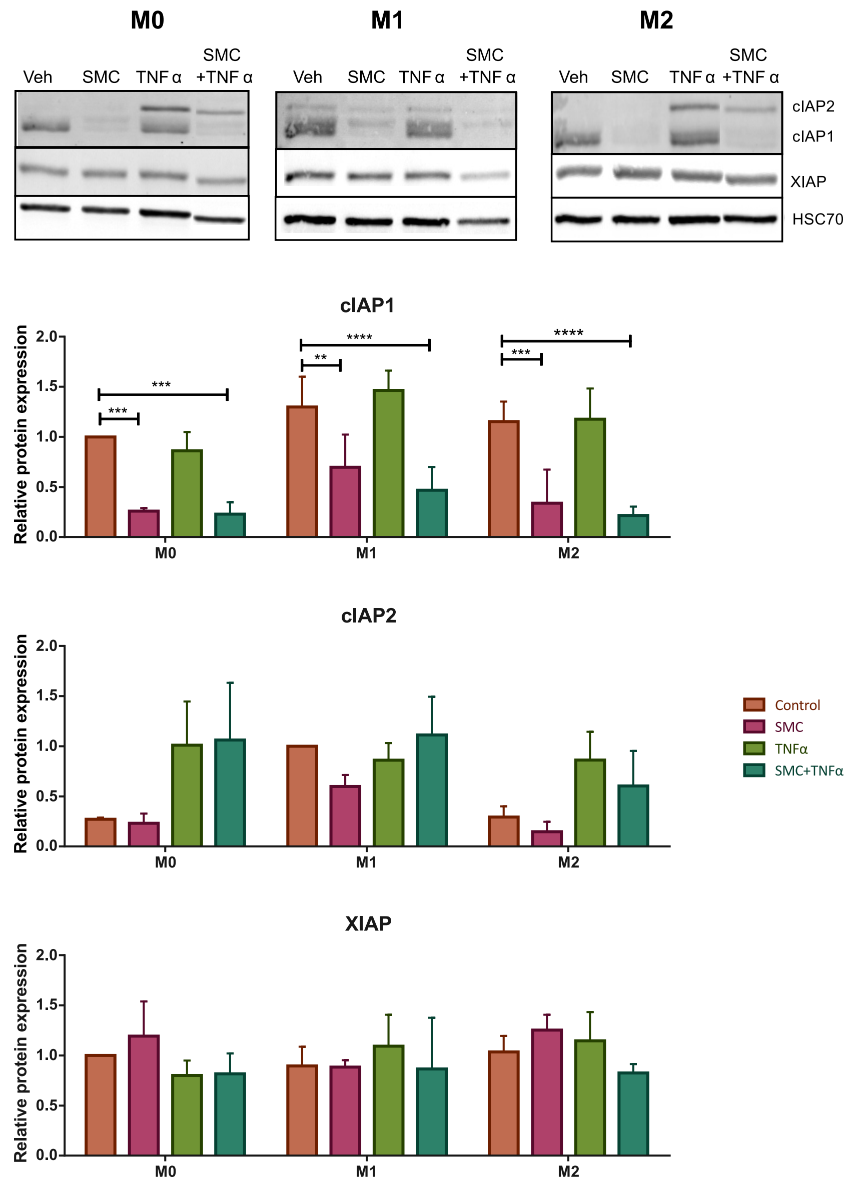


Figure 38. cIAPs and XIAP expression in SMC and TNFα treated polarized THP-1 macrophages. M0, M1, and M2 THP-1 cells (initial 1x10^6 cells/well seeded in 6 well plates), were exposed to 1μM LCL161 and/or 10ng/mL of TNF-α for 48 h. Cell proteins were extracted and cIAP1, cIAP2 and XIAP abundance was assessed by western blotting using the RIAP1 and RIAP3 antibody. HSC-70 was used as loading control. Representative western blot of one of three experiments with similar results. Quantification of cIAP1, cIAP2 and XIAP protein abundance in western blots normalized to HSC-70 and presented as mean values $\pm$ S.D. of three independent experiments. Two-way ANOVA with Tukey's multiple comparison test was performed.

4.3.3.2 IAP antagonism effect in M1/M2 macrophage polarization

We examined whether IAP antagonism through the use of SMC can modulate macrophage polarization. To do so, we exposed M0 THP-1 to SMC and then incubated under regular polarization conditions (M1 polarization: 100ng/mL of LPS + 20ng/mL of INF-γ for 48 h; M2 polarization: 20ng/mL of IL-4 for 48 h.). First, we examined whether the functional blockage of IAPs could prevent macrophages from polarization when exposed to the appropriate stimuli. To do so, we studied the expression of cell surface molecules by flow cytometry analyses (Fig. 39) and the differences in polarization gene regulation (Fig. 40). M1 expression was set as base line in the analysis of CD86, CD163 and CD14. M2 expression was set as base line in CD206 and Cd11b.

The expression of typical macrophage polarization markers is attenuated as a result of previous exposure of macrophages to SMC before the induction of polarization. We observed a decreased expression profile of the M1 marker CD86 within THP-1 macrophages stimulated into the M1 state (LPS and INF-γ), and saw concomitant upregulation under conditions that induce polarization into M2. Consistent with these results, we observed a downregulation of CXCL10, a chemokine commonly associated with M1, in THP-1 macrophages stimulated into the M1 state. By contrast, we observed complete ablation of CD206 transcripts in SMC-treated M2 macrophages, which translated to a slight reduction of the expression of CD206 at the cell surface when analyzed by flow cytometry.

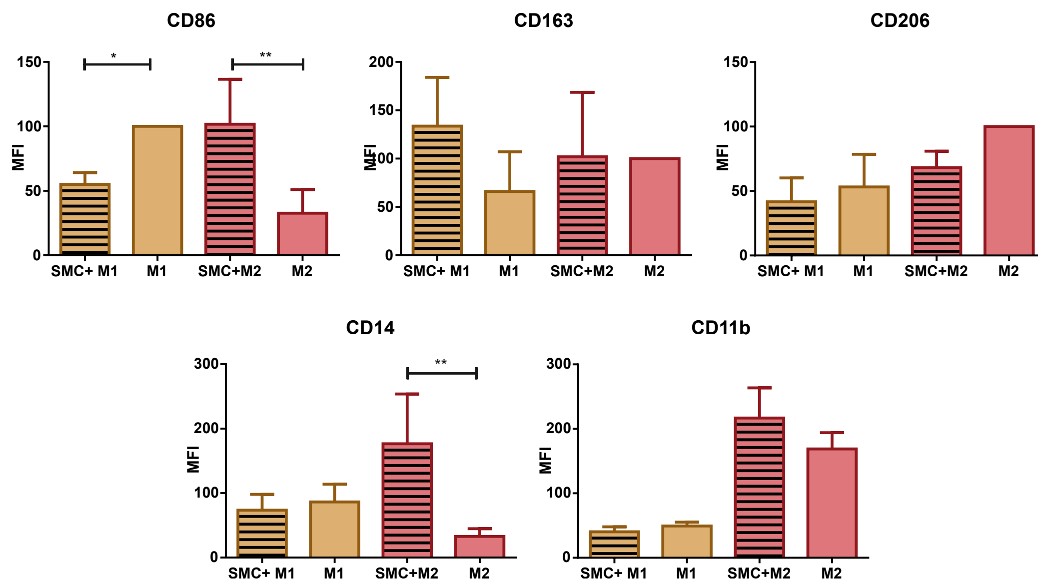


Figure 39. M1/M2 markers expression in SMC pre-exposed polarized THP-1 macrophages. M0 THP-1 macrophages (initial 0.5x10^6 cells/well seeded in 6 well plates) were exposed to 1μM LCL161 for 24 h, then they were submitted to the polarization stimuli, LPS (100ng/mL) and IFN-γ (20ng/mL) to obtain M1 and IL4 (20ng/mL) to obtain M2 for 48 h. Cells were then stained with CD86, CD163, CD206, CD14 and CD11b antibodies and analyzed by flow cytometry. Graph represent Median Fluoresce Intensity $\pm$ S.D. of three independent experiments. One-way ANOVA with Tukey post hoc was performed.

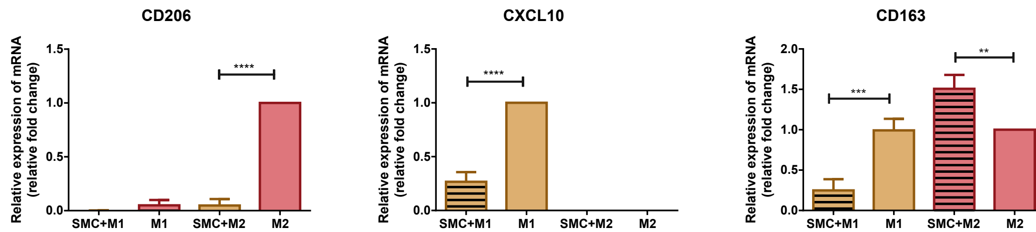


Figure 40. M1/M2 markers gene expression in SMC pre-exposed polarized THP-1 macrophages. M0 THP-1 macrophages (initial 0.5x10^6 cells/well in 6 well plates), were exposed to 1μM LCL161 for 24 h, then they were submitted to the polarization stimuli, LPS (100ng/mL) and IFN-γ (20ng/mL) to M1 and IL4 (20ng/mL) to M2 for 48 h. mRNA was extracted with RNAeasy mini-kit and CD163, CD206 and CXCL10 expression was analyzed by RTq-PCR. Values are normalized to internal controls (HPRT1 and GAPDH) and presented as mean values $\pm$ S.D. of three independent experiments. One-way ANOVA with Tukey post hoc was performed.

We exposed M0 THP-1 cells to LCL161 before the induction of M1/M2 polarization and then examined the expression of cIAP1, cIAP2 and NAIP by Western blot as shown in Fig 41. Interestingly, we found that pre-polarization exposure to SMC enhances NAIP expression in macrophages previously incubated in polarization conditions. This enhancement is even more noticeable in the case of the macrophages cultured under M2 polarization conditions, where NAIP is already increased compared to M1 macrophages. When cells are pre-exposed to SMC, levels of NAIP increased significantly. As expected, when we analyzed cIAP1 and cIAP2, we found that cIAP1 protein is clearly and significantly reduced as a consequence of SMC pre-treatment. This is consistent with SMC leading to degradation of cIAP1. We did not see this with cIAP2 in cells that were pre-exposed to SMC and cultured in M1 conditions, where cIAP2 levels were almost three times higher than in non LCL161-primed M1 macrophages. This large difference was not found in M2 macrophages, were cIAP2 levels were almost the same in SMC treated macrophages, and non-treated were even lower in the case of those with the SMC treatment.

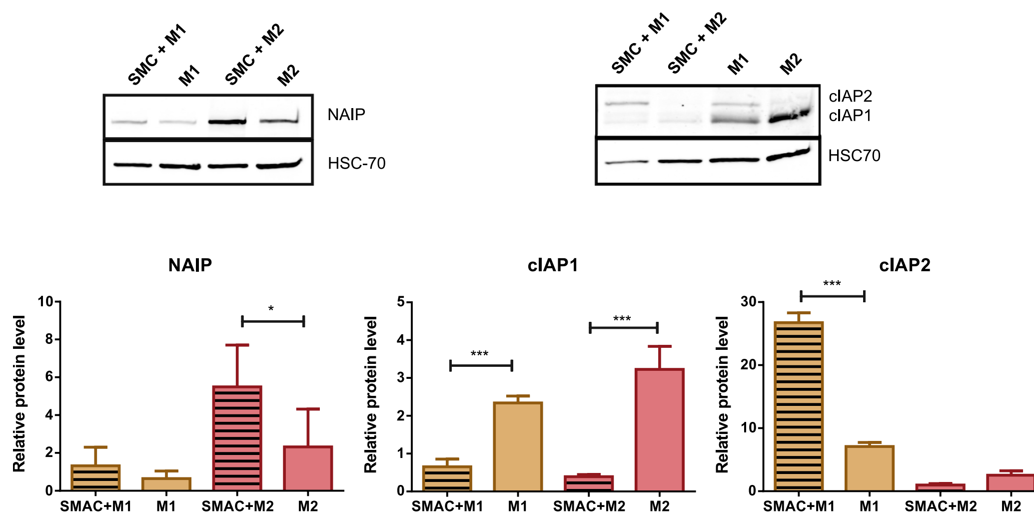


Figure 41. NAIP and cIAP2 upregulation in M2 and M1, respectively, SMC pre-exposed polarized THP-1 macrophages. M0 THP-1 macrophages (initial 0.5x10^6 cells/well in 6 well plates), were exposed to 1μM LCL161 for 24 h, then they were submitted to the polarization stimuli, LPS (100ng/mL) and IFN-γ (20ng/mL) to M1 and IL4 (20ng/mL) to M2 for 48 h. Cell proteins were extracted and NAIP, cIAP1 and cIAP2 abundance were assessed by western blotting using the NAIP-J2 and RIAP1 cIAP1/2 antibodies. HSC-70 was used as loading control. Representative western blot of one of three experiments with similar results. Graphs represent quantification of NAIP, cIAP1 and cIAP2 protein normalized to HSC-70 and are presented as mean values $\pm$ S.D. of three independent experiments. One-way ANOVA with Tukey's multiple comparison test was performed.

Next, we sought to determine if the differences in NAIP observed between untreated cells and cells treated with SMAC were due to differential regulation of NAIP degradation machinery, or by regulation of gene expression. We examined the latter by qPCR (Fig 42) and found an upregulation trend when macrophages were pre-exposed to SMC before incubating them in M1/M2 polarization media. This tendency is found under M1 condition as well as under M2, which suggests that NAIP protein levels are a consequence of differences in gene regulation.

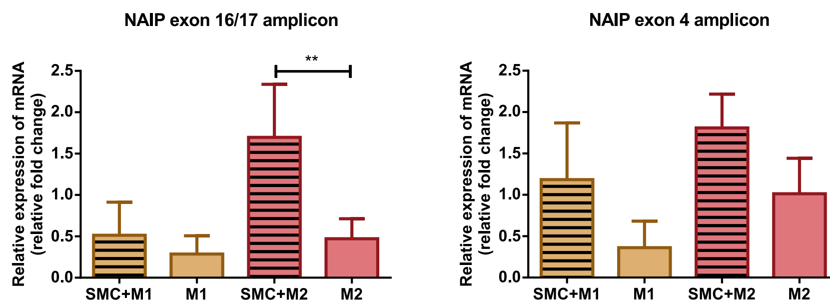


Figure 42. NAIP mRNA upregulation in M2 SMC pre-exposed polarized THP-1 macrophages. M0 THP-1 macrophages (initial $0.5\text{x}10^6$ cells/well in 6 well plates) were exposed to 1μM LCL161 for 24 h, then they were submitted to the polarization stimuli, LPS (100ng/mL) and IFN-γ (20ng/mL) to M1 and IL4 (20ng/mL) to M2 for 48 h. mRNA was extracted with RNAeasy mini-kit, reverse transcribed and hNAIP mRNA was analyzed by RTq-PCR. Values are normalized to internal controls (HPRT1 and GAPDH) and presented as mean values $\pm$ S.D. of three independent experiments. One-way ANOVA with Tukey's multiple comparison test was performed.

4.4 Macrophage Activation Syndrome

Based on previous works, we hypothesized that targeting IAPs could be an interesting approach to inflammatory diseases; specifically, to MAS.

4.4.1 MAS mouse model

As access to patient samples was very limited, we opted to work with a murine model. This is the only mouse model available to date. In this model, mice are treated with CpG ODN, a single stranded DNA with some CG motifs that are recognize by the TLR9. The stimulation is done every two days for one week or more, which causes a MAS-like phenotype in the mice (spleenomegaly, ferretinemia, cytopenia and hepatomegaly) [215, 249].

To study the possibility of IAPs as therapeutic targets in MAS, we established four groups of study:

1. Control group: Treated with DPBS (instead of CpG) and with vehicle (instead of LCL161)
2. CpG group: Treated with CpG and vehicle. This serves as a MAS-like induced control group.
3. SMC group: Treated with DPBS and LCL161. Serves as SMC control group.
4. CpG+SMC group: Treated with CpG and LCL161. This group will show the effect of SMC as MAS treatment.

Mice were administer CpG at day 1 and every two days in order to develop MAS-like status. We started treating mice with LCL161 (the SMC) on day 3 and every other day during the course of the experiment. The model was established at day 8. The weight of the mice was stable in all the treatment groups, except for the group of MAS-like induced mice treated with SMAC mimetic that presented a significant weight loss.

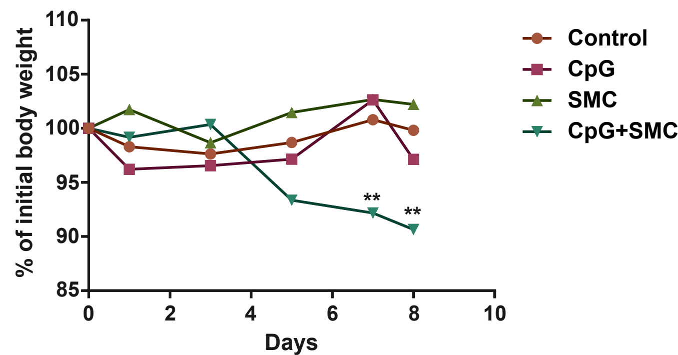


Figure 43. Body weight change in MAS-like mice. Percent of initial body weight change in MAS-like mice treated and untreated with SMC. Weight was measured on days 1, 3, 5, 7 and 8 and expressed as the percentage of weight change compared to day 0. The means from 4 mice is shown in each group. Two ways ANOVA with Dunnett's analysis.

Spleen and liver sizes are increased in MAS patients, and this features is mimicked in the CpG mouse model. We decided to analyze the size of these two organs in every group. When seen with the naked eye, as in Fig 44, we noticed an obvious and expected increase in both spleen and liver in those MAS-like induced mice. Spleen size was also bigger in both groups of mice treated with SMAC.

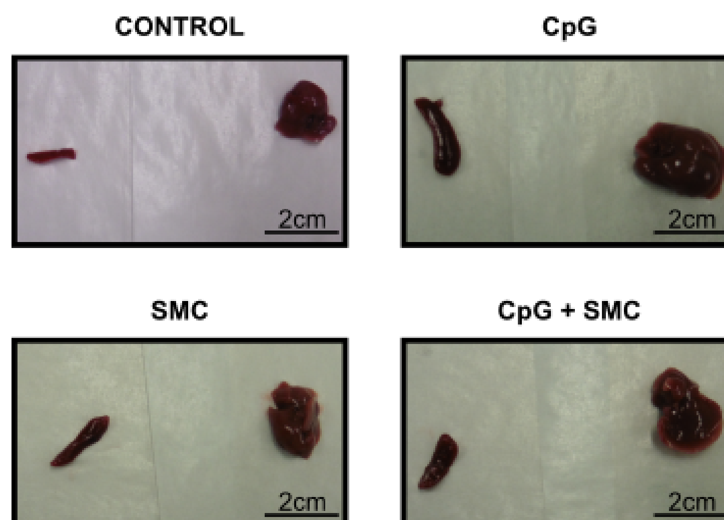


Figure 44. Macroscopic comparison of spleen and liver in MAS-like mice. Mice were sacrificed and spleen, left organ, and liver, right organ, were collected, weighed and photographed.

When we compared the organ:body weight ratio for liver and spleen, (Fig 44) we saw that the difference between a healthy organ and a MAS-like affected one is, as previously observed, highly significant. The effect of SMC treatment in MAS-like model mice was not promising. There was a visible reduction in splenic size, but the size was still larger than the control and slightly larger than the SMC treatment alone, which also produced an augmentation of the splenic size.

With respect to the liver, the ratio was similar in the CpG and the CpG+SMC groups, with the the latter being slightly increased. Both groups were significantly bigger than the control group. No differences were found between the control and SMC group.

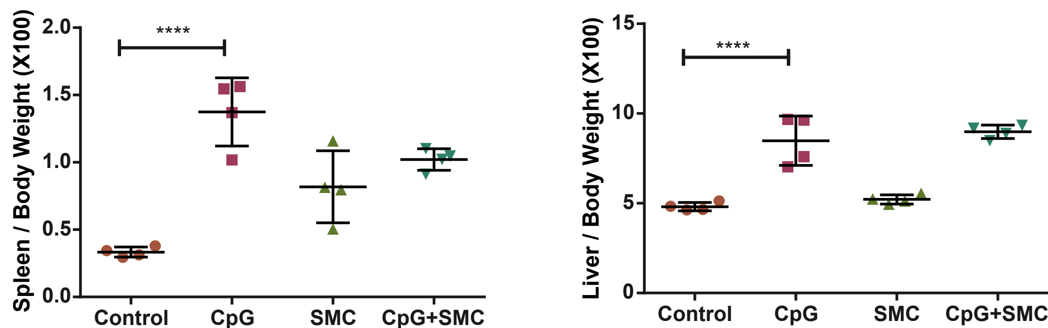


Figure 45. Spleen and liver hypertrophy in MAS-like mice. Liver and spleen sizes were measured at sacrifice. The graphs represent the relative to the body weight mass of the spleen (left graph) and liver (right graph) per animal within each group of study. Mean values ± S.D. ANOVA with Tukey post hoc was performed.

Infiltration of leukocytes in spleen, bone marrow, and lymph nodes has been described as a main feature in the acute phase of the macrophage activation syndrome [250] Therefore, we decided to take a closer look at the different leukocyte populations in the liver and the spleen of the different groups. In order to do so, liver and spleen cell populations were analyzed by flow cytometry as shown in Fig. 46.

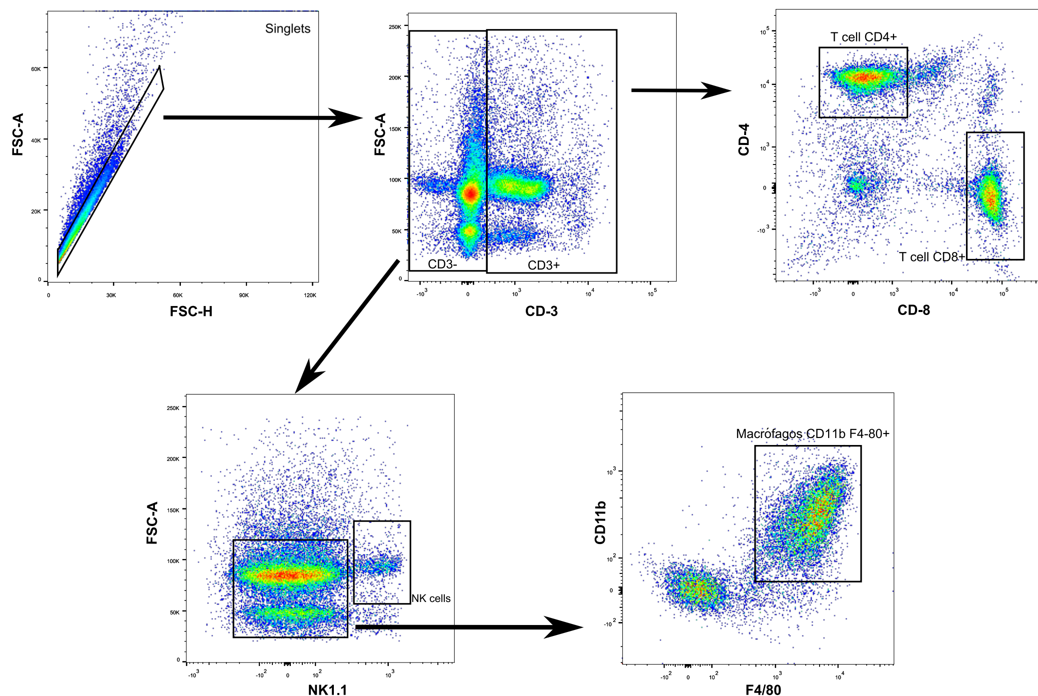


Figure 46. Gating of spleen and liver cell populations. Livers and spleens were homogenized and dissociated to a single cell suspension using 70 μm mesh. Red blood cells were lysed using ammonium-chloride-potassium (ACK) buffer. Cells were then stained with CD3, CD4, CD8, NK1.1, F4/80, CD11b, MHC-II, and CD206 antibodies and analyzed by flow cytometry. The graphs represent the gating system followed. In first place, and after the discrimination of cell aggregates, cells were separated, according to their expression of CD3, between CD3+ and CD3-. CD3+ were described as T lymphocytes, which were further analyzed to evaluate the specific populations of CD4+ and CD8+ T cells. CD3- cells were additionally gated, NK cells were identified as NK1.1+ and macrophages were classified according to their expression of F4/80 marker and CD11b. Among macrophages those classically activated were identified by the presence of MHC-II in their surface and alternatively activated macrophages, with CD206 as marker.

The distribution of lymphocytes, T cells, NK cells and macrophages in the spleen and liver is presented in Fig. 47. The frequency of the different populations in the spleen does not change as a consequence of any of the treatments; they remain more or less the same in the four groups of mice. This was in contrast to the liver, where the only cell population that remained unchanged were the NK cells. There was an increase in the number of lymphocytes and T cells in the groups where the syndrome was induced, and a decrease in the population of macrophages.

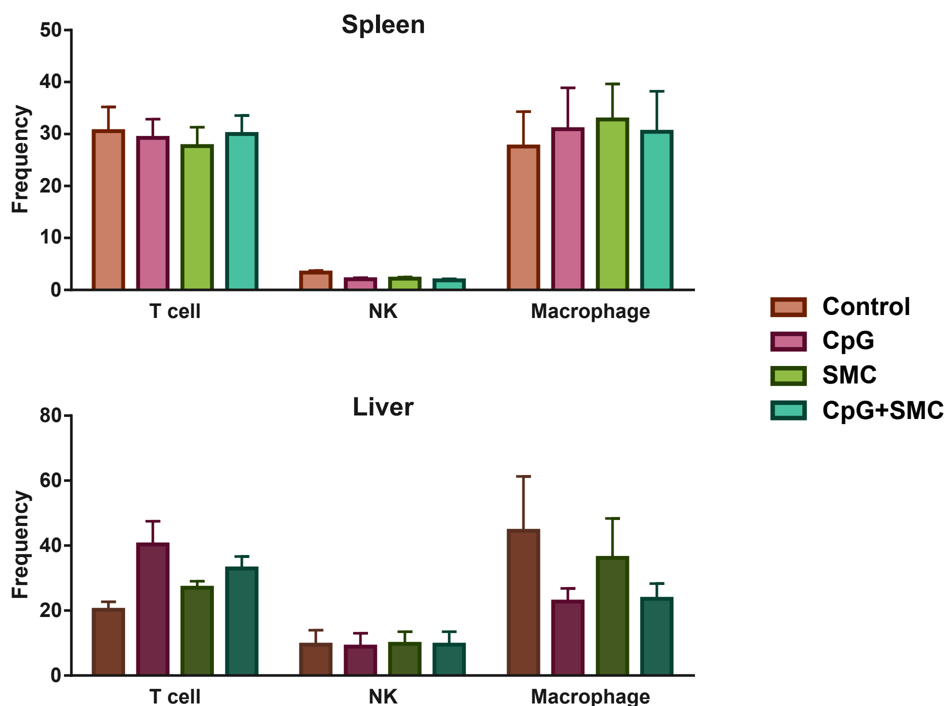


Figure 47. Spleen and liver cell populations. Flow cytometry analysis showing the frequency of T cell, NK cell and macrophage populations in the spleen (top) and the liver (bottom). Gating methods were explained in the previous figure. Graphs display the mean values ± S.D. of the frequency for the four individuals in each group of study. Two-way ANOVA with Tukey post hoc was performed.

We focused on the changes among the different populations of T cells and macrophages; that is, in CD4+ and CD8+ T cells and in M1 and M2 like macrophages. We compared the ratios of these cell populations between the different treatment groups as shown in Fig. 48. We observed that the ratios

are similar, with no significant differences between MAS-like induced mice and those treated with SMC in the spleen. But, again, as with leukocyte populations, a different trend was observed in the liver. There, CD4/CD8 T cell ratio was significantly decrease in CpG-treated mice, with or without further SMC treatment. The reverse was observed in macrophages, where the M1/M2 ratio was significantly increased in those groups. With regard to splenomegaly and hepatomegaly, we did not find any evidence to suggest that SMAC mimetics are a good method of treatment for macrophage activation syndrome in the spleen or liver.

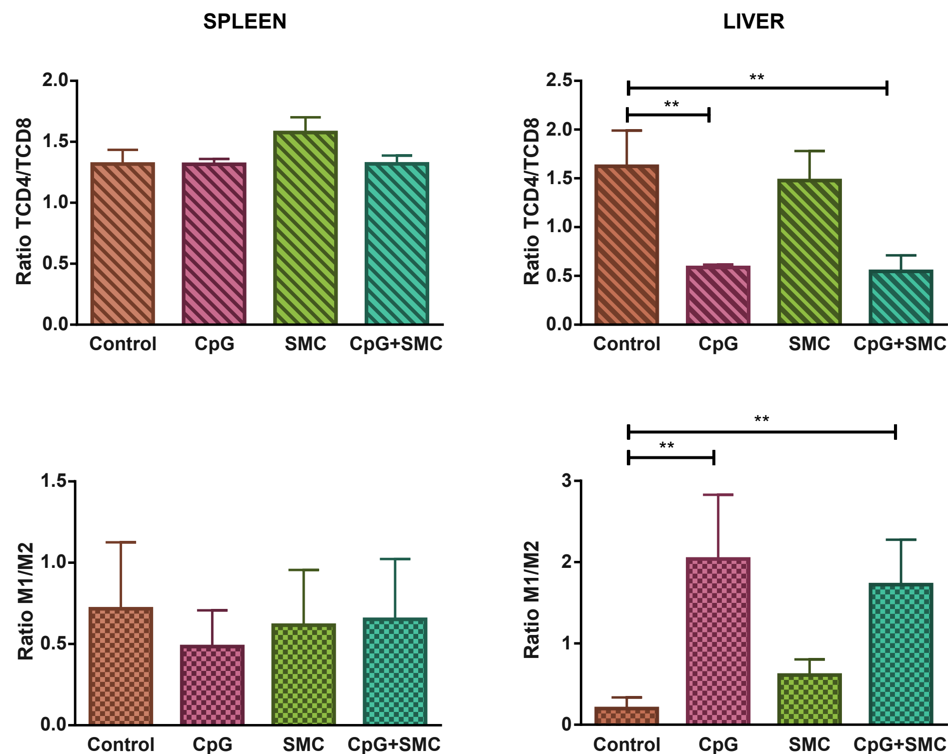


Figure 48. Spleen and liver T cell and macrophage ratios. From the populations gated previously in the spleen (top) and liver (bottom), CD4/CD8 T cells (left) and M1/M2 macrophage (right) ratios were calculated. Graphs presented as mean values ± S.D. One way ANOVA with Tukey post hoc analysis was performed.

4.4.2 MAS patient samples

The procurement of MAS patient samples was very limited. We had access only to blood samples from three MAS patients:

- Patient 1: Low ferritin levels at the time of blood draw, MAS developed from sJIA, under control.
- Patient 2: Macrophage activation syndrome of unknown origin. Low ferritin levels at the time of blood draw.
- Patient 3: MAS developed from sJIA. Very high levels of ferritin at the time of blood draw.

Having these samples allowed us to do a first approach to the study of their macrophages, and their susceptibility to SMAC mimetics. Using Alamar Blue, we analyzed the viability of MAS macrophages after treatment with SMC and exposure to a bacterial ligand, LPS (Fig. 49). We found that susceptibility to SMC was similar in control and macrophage activation syndrome samples.

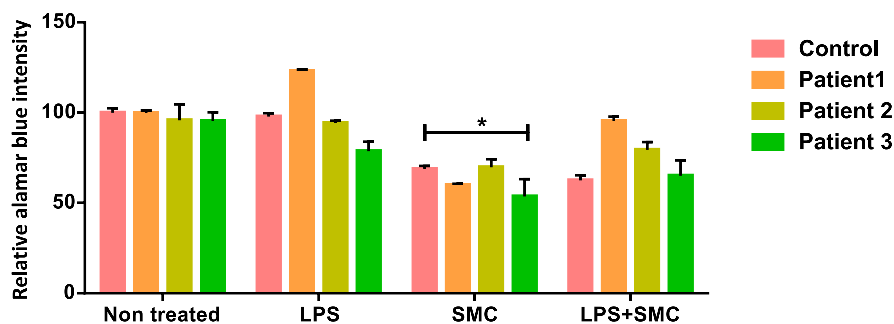


Figure 49. Viability assay of MAS macrophages. Macrophages cultured for 6 days in IMDM were exposed to SMC-LCL161 at 1μM and/or LPS at 100ng/mL for 24 h, viability was then determined by Alamar blue assay. Two-way ANOVA with Tukey post hoc analysis was performed.

We studied the ability of patient macrophages to respond to bacterial ligands in the context of cIAP expression. We investigated whether there were any differences in the amount of cIAPs expressed, to determine if they could be interfering with the normal, apoptotic-non apoptotic resolution of the inflammation in the macrophages. To do so, we studied the expression of cIAP1 and cIAP2 in monocyte-derived macrophages from MAS patients and compared them to those from healthy patients (Fig. 50). Interestingly, we found that in patient 1, the expression of cIAP1 was decreased compared to the controls and the other two patients, but the amount of cIAP2 was comparable to the controls. Exactly the opposite was found in macrophages from patients 2 and 3, where expression of cIAP1 was normal, but, surprisingly, cIAP2 expression was elevated under all conditions.

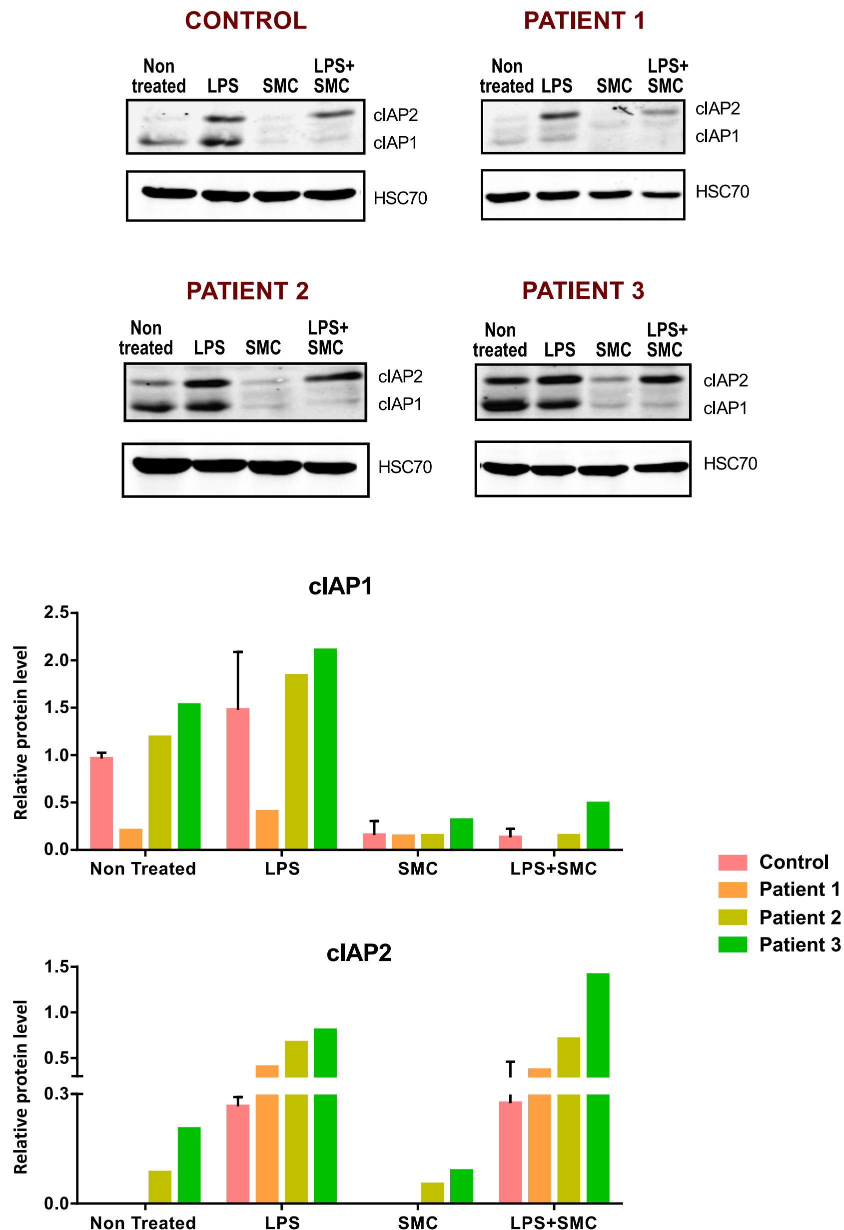


Figure 50. cIAP protein expression in MAS patient macrophages. Macrophages cultured for 6 days in IMDM were exposed to SMC-LCL161 at 1μM and/or LPS at 100ng/mL for 24 h. Protein was extracted and analyzed by western blotting against with RIAP1 antibody, using HSC70 as control. Representative western blot from samples from one of the healthy patients is presented besides the ones from the three patients. Graph represents the quantification of cIAP1 and cIAP2 abundance normalized by the corresponding HSC-70. Control patient bars (pink) presented as mean values $\pm$ S.D. from samples from 4 different healthy patients.

CHAPTER 5

Discussion

5.1 NAIP expression in macrophage differentiation and polarization

There are two different groups of tissue resident macrophages according to their origin. The first one corresponds to macrophages originated during the early embryogenesis from myeloid precursors. The second group is formed by monocyte derived macrophages sustained by continuous supply of blood monocytes. This last group constitutes an important asset in inflammation situations, and the study of the differentiation process could shed light on how to improve the resolution of some infectious diseases and other illnesses in which uncontrolled migration and differentiation can lead to tissue damage and chronic inflammation [251].

On the other hand, polarization of macrophages appears to be a necessary process in tissue homeostasis. Under certain circumstances, macrophages respond to micro-environmental signals by changing their phenotype into a pro-inflammatory (M1) state that can act as a defense against external pathogens or, if the environment requires it, by changing into a healing phenotype (M2) that is able to repair damaged tissue. This polarization process is more flexible than tissue specific differentiation, given the broad spectrum of polarization states, and is developed to quickly respond to environmental changes. There are some cases in which this phenotypic change of macrophages is associated to pathologies [51,55]. In cancer, tumor-associated macrophages usually presents as an M2 phenotype and help tumor progression and the dissemination of the cancer cells [90, 252]. M2 are also related to the pathogenesis of tuberculosis [253,254] and to induction of type 2 diabetes caused by obesity [81]. Additionally, M1 polarization has been connected to bipolar disorder [88] and the development of nonalcoholic fatty liver disease [255, 256]. This shows the importance of the investigation and further understanding of macrophage polarization, as switching from one polarization state to the other could be used as a therapeutic treatment for many diseases.

5.1.1 Optimization of differentiation and macrophage polarization models.

THP-1 and U937 cell lines are monocytic cell lines typically used in macrophage differentiation mechanistic studies, and polarization studies in the case of THP-1. THP-1 and U937 present several advantages compared to primary human cells, such as their homogeneous genetic background, so results obtained with these models are more uniform. These two cell lines have been described as able to differentiate into macrophages in response to PMA at concentrations as low as 100ng/mL for 48 h plus 24 h of resting (72 h total) [257]. One of our objectives in this study was to optimize this protocol. We managed to decrease the amount of PMA and the time needed for the completion of differentiation to 10ng/mL stimulation for 24 h followed by 24 h of resting (48 h total). The characteristics of THP-1 and U937 cells after our treatment with PMA, resulted in the acquisition of macrophage features (Fig. 8), so the optimization of THP-1 and U937 differentiation protocol was accomplished.

The amount of different protocols available to obtain polarized macrophages from THP-1 cells is immense [231, 232, 258], and even more from monocyte derived macrophages [231, 259, 260], therefore the need to refine them. Regarding THP-1 cells, we managed to successfully induce macrophage polarization by treating the previously obtained THP-1 macrophages with specific concentrations of LPS and INF-γ and with IL-4, this is shown by the induction and expression of characteristic markers and the differences in the look and shape of the cells, seen in Figures 23, 25 and 26. The novelty of our protocol lies on the time and the amount of PMA used to obtain THP-1 macrophages, which are the same cells used as the foundation for our differentiation studies.

Even though, THP-1 and U937 cells constitute a good, easy and reliable source of information in macrophage differentiation and polarization, it is easy to forget that these are cancer cell lines and come with a high malignant background that can induce biased results. That is why, when possible, we also like to work with human primary monocytes and monocyte-derived

macrophages [257]. Hence, we have undertaken the task of setting a polarization model for human peripheral blood monocytes and succeeded, as shown by the morphology changes and differential gene expression .

5.1.2 IAPs in macrophage differentiation and polarization

The IAPs have been characterized to be involved in several immune system processes, and specifically in many macrophage functions, including the response to some PAMPs and DAMPs like flagellin and PrgJ, in the case of NAIP [99, 100], and CD40 ligand in the case of cIAP1 [178].

5.1.2.1 NAIP in macrophage differentiation and polarization

NAIP expression decreases in macrophage differentiation

Preliminary work by our team on monocyte differentiation showed the decrease in NAIP after the differentiation into macrophages was complete. This result was unexpected since, as mentioned before, NAIP has a key role in bacterial recognition and inflammasome formation in macrophages as a response to certain bacterial ligands. We were therefore predicting to find an increase of NAIP, but instead we found a decrease of NAIP level, as we can see in Figures 9 and 10 and even in monocyte derived macrophages obtained from healthy donors, showed in Figures 14 and 15. The downregulation of NAIP was even higher at the transcriptional level, where NAIP mRNA levels decreased more significantly than at the protein level. This could be due to the maintenance of a residual amount of NAIP in differentiated macrophages so it can execute its surveillance role.

Our team has recently discovered the regulatory role of NAIP during mitosis [141], which we will discuss afterward, being indispensable for the accurate completion of cytokinesis. The differentiation of monocytes into macrophages entails a decrease in the mitotic rate of the cell. The fact that NAIP decreases might be related to that fact.

Experiments of supra-physiologic expression of NAIP in monocytes and macrophages did not clear up this matter, since the cell cycle did not change (Fig. 16) and the response to a differentiation stimulus was normal, except for the expression of CD14 that we will discussed below. On the other hand, NAIP gene silencing did not prevent differentiation, or trigger it just by itself, although the expression of macrophage markers was increased when differentiated with PMA. These results do not, however, clarify if NAIP is involved in the monocyte cell cycle or that its decrease when differentiating into macrophages corresponds with a reduction in their ability to proliferate.

NAIP expression increases in M2 polarized macrophages

When analyzing polarized macrophages, we have seen a characteristic pattern of NAIP expression, being lower in classically activated macrophages than in alternatively activated macrophages (Figs. 28, 29 and 32). This was unexpected from the current view of NAIP as NLR and inductor of the inflammasome [100], in which case we would expect to see an increase in the more cytotoxic M1 macrophages. Contrary to what we anticipated, NAIP expression was increased in M2 macrophages. This can be explained by the fact that, as mentioned above, NAIP is actively involved in the mitotic process [141] and M2-like tissue resident macrophages have self-renewal capacity [261]. In fact, IL-4 contributes to the increment of the polarization [262]. Hence, the enhanced expression of NAIP in M2 macrophages might account for the potential macrophage polarization.

Interestingly, it has been seen that NAIP is also increased during adipogenesis [263] and the M2 state has been described as closest to the phenotype found in adipose tissue macrophages which clearly present local proliferation [56, 81, 264]. An interesting approach that should follow would be, doing what has already been done in differentiation experiments: silencing and over-expressing NAIP to study the effects in polarization and on the cell cycle of these cells.

NAIP involvement in cell proliferation

Our finding exhibiting NAIP intervention in cytokinesis, whose dynamics are shown in Fig. 19, prompted us to analyze NAIP expression along the cell cycle. Proteins required for mitosis advancement are commonly expressed in the G2 phase of the cell cycle. Consistent with a role for NAIP during the M phase, we have found a sustained expression of the NAIP gene (Fig. 20) as the cell cycle progresses with a 2.7 fold increase observed in G2 versus G1. The acquisition of a multinuclear phenotype in HeLa cells overexpressing NAIP is consistent with a functional role for NAIP in cytokinesis, it may be that NAIP overexpression results in deficient definition of the division plane; repeated cell cycling combined with a loss of cytokinesis completion resulting in large multinucleated cells (Fig. 21). In contrast, the lethal apoptotic phenotype observed after NAIP siRNA gene silencing (Fig. 22) might be due to a multiple loss of function impact. Previous studies have shown the anti-apoptotic effect of NAIP overexpression in cultured cells [128]; it may be, conversely, that the loss of NAIP confers some degree of apoptotic susceptibility in HeLa interphase cells.

Our analysis of NAIP participation in cell division [141] make us think that NAIP could be involved in broader functions, have a role in mitosis and possibly in cell cycle progression; now under investigation in our group, these considerations help to interpret the NAIP expression profile here described in the monocyte to macrophage and polarization process.

5.1.2.2 cIAPs and XIAP in macrophage polarization

cIAP2 is preferentially expressed in M1 macrophages

When analyzing the expression of cIAP1 and cIAP2 in polarized macrophages (Fig. 33), we observed an increase of cIAP2 in M1 macrophages. This finding is consistent with the premise that IFN-γ [265] and LPS treatments induce the upregulation of cIAP2 [133,266]. Similarly, it is known that LPS is

a weak activator of the alternative NF-κB pathway [140], and is also reported to upregulate cIAP2. Additionally, cIAP2 transcript has already been seen to be upregulated in M1 [267,268], although differences at the protein level have never been described before. According to previous studies the induction of cIAP2 in response to LPS, would provide protection in an inflammation situation in which cytotoxic factors promote apoptosis [133], so in this case, that induction allows M1 macrophages to develop their pro-inflammatory role without dying.

cIAP1 mRNA does not correlate with protein expression in polarized macrophages

In our analysis of the expression of cIAP1, we see that its transcription levels do not correlate with the protein levels (Fig. 33). This has already been described in some cancer cells [269], which suggests that there are post-transcriptional regulations. Here, cIAP1 mRNA is more expressed in M1, but protein levels are higher in M2 and M0. M1 stimuli involves TLR signaling through MyD88 [51] and a new study exposed that when TLR-MyD88 is engaged, this triggers the degradation of cIAP1 [270]. This could explain why there are discrepancies between the mRNA and protein levels of cIAP1.

Another reason for this could be that perhaps cIAP1 is engaged in a protein complex and we are not able to detect it in the usual manner because the epitope that is recognized by the antibody is blocked. To our knowledge, and contrary to what happens in cIAP2, there have not been any studies that report the induction of cIAP1 during treatment of macrophages with LPS or IFN-γ. A further consequence of cIAP1 decrease in M1 macrophages is the resulting increase of cIAP2 stability, since the degradation of cIAP1 promotes non-canonical activation of NF-κB that leads to the upregulation of the cIAP2 genes [271].

To summarize briefly, cIAP1 and cIAP2 show an inverse pattern of protein expression in polarized macrophages.

XIAP expression is maintained during macrophage polarization

XIAP is the only IAP able to inactivate caspases by direct binding to them. Previous studies have described an increase in XIAP expression during macrophage differentiation [229] This fact has been hypothesized to be more related to the XIAP anti-apoptotic role than to the fulfilling of a functional role in differentiated macrophages [272]. At the same time, activation of NF-κB had been described to induce XIAP expression [230, 273] which is involved in LPS-induced production of nitric oxide (NO) [242]. This production is typical of M1 macrophages [274]. This made us think that when analyzing XIAP in polarized macrophages we would see an increase in M1. The results in Fig. 34 show that XIAP expression was not consistent at the protein level with our presumption, as we found a constant level of XIAP on the different polarization states. Nevertheless at the transcript level, as we expected, we do see an upregulation of XIAP expression in classically activated macrophages. As in the case of cIAP1, this discrepancy has been described before [269], further studies are necessary to elucidate the causes of this event.

5.1.3 IAPs antagonism in macrophage polarization

SMCs (SMAC mimetic compounds) constitute a group of small molecules that are able to target IAPs. They bind and block XIAP and promote the degradation of cIAP1 and cIAP2 [194, 271]. SMCs are currently used in clinical trials for the treatment of cancer, either alone or in combination with other therapeutic agents [185, 206, 207]. Also, polarized macrophages are tightly involve in different steps of tumorigenesis and constitute a major component present in the tumor microenvironment [59,91]. There is therefore, a great chance for SMC treatment to affect the tumor microenvironment macrophage population.

5.1.3.1 THP-1 polarized macrophages are resistant to SMC sensitization

Some cancer treatments are focusing on the synergistic effect of SMCs and other cancer therapeutic agents to promote the death of tumor cells. One of the options currently under study is the combination of SMCs with innate immune stimuli [244]. This is why we focused our attention on the effects of LCL161 in the presence of TNF-α on already polarized macrophages. First we studied the survival rate of the cells and found that there were no differences between the treatment groups, and in the case of M0 exposed to both stimuli, TNF-α and LCL161, we saw a slight increase in mortality. The resistance to apoptosis induced by SMC in macrophages was already described [275] but not specifically in polarized macrophages.

One aspect to consider in respect to macrophages, is their ability to switch their polarization state [66]. This, and the fact that polarized macrophages have an specific IAP expression pattern, made us think of the possibility that SMC treatment could promote a phenotypic change in them. We studied the expression of some polarization markers (figure 37), and saw no significant change in surface markers, although gene expression of CD206 (M2 marker) and CXCL10 (M1 marker) were upregulated and downregulated respectively in the presence of SMC alone. This may not have a functional effect on the macrophage but in order to clarify that, further research is needed, especially to study the cytokine production profile.

Looking at IAPs expression, we found that the levels of cIAP1 and cIAP2 decreased after the exposure to LCL161 and increased or remained higher in the presence of TNF-α while XIAP expression remained constant. This was expected, due to the ability of SMCs to promote cIAP1 and cIAP2 degradation and bind and inhibit XIAP, and since the TNF-α signaling pathway (when cIAPs are available) activates NF-κB and promote survival genes, including cIAP2 [194]. We also saw that TNF-α induces expression of cIAP2, and that induction is maintained even in the presence of SMC. This could be explained by the fact that both treatments were done at the same time, so there was enough cIAPs available to direct TNF-α signaling into

upregulation of cIAP2. An interesting approach to analyze this could be to pre-expose those macrophages to SMC, so the level of available cIAPs is low, and then treat the cells with TNF-α to see if cIAP2 is still being upregulated or, on the contrary, if the cells are dying.

5.1.3.2 IAP antagonism influences macrophage polarization

We also studied the effect of IAP antagonism during *in vitro* macrophage polarization. As different macrophage polarization states are reported to play different roles in tumorigenesis [59, 91], it is possible that the ability of SMCs to modulate macrophage polarization within tumors can be exploited in cancer therapeutics. Indeed, it has been seen by other groups that modulation of NF-κB, with [276] or without [277] SMC could lead to a phenotypic switch in macrophages. Here (Figs. 39 and 40) we see that when THP-1 macrophages are treated with SMCs prior to M1/M2 polarization induction, the expression of the specific polarization markers is affected, not achieving the complete polarized condition, or leading towards a more M1-like phenotype. For us to be able to properly say that, however, we need to collect more data about the cytokine production and the phagocytic activity of these cells. This would imply that either cIAP1, cIAP2, or even XIAP, have a role in the polarization process.

5.1.3.3 IAP antagonism reinforces IAPs expression profile signature in macrophage polarization

The treatment of "neutral" macrophages (M0) with LCL161 lead to a decrease in cIAP1 level after the exposure to M1 and M2 stimuli, but we found that there is a significant increase of cIAP2 as a consequence of the SMC pre-treatment and the exposure to M1 stimuli (Fig. 41). The induction of cIAP2 could be caused by the LPS-induced engagement of the alternative NF-κB pathway, triggered by the loss of cIAPs, which promotes the expression of cIAP2 [133, 266]. Alternatively, SMCs promote the degradation of cIAP1 and 2, but cIAP2 degradation is cIAP1-dependent, so the absence of cIAP1 leads to less ubiquitination of cIAP2 and consequently to less degradation

[271]. Or maybe, it is the result of these two theories combined. Both cIAP1 and cIAP2 possess some redundant functions in NF-κB regulation, and in some cases it has been seen as a strong compensatory mechanism by which a reduction in cIAP1 leads to an important increase of cIAP2 [278, 279]. In this case, this could be happening in order to protect the macrophage.

SMCs interaction with NAIP has been studied before, always with similar results. That is, SMC does not antagonize NAIP or its ability to inhibit caspase-9 [130, 280]. When we studied the expression of NAIP during macrophage polarization after SMC exposure, we were therefore expecting no change at all. Instead, we have seen an increase of NAIP when macrophages were pre-exposed to SMC, which is especially evident when they are then polarized to M2, as seen in Fig. 41. NAIP expression has been seen to be downregulated when cells are treated with NF-κB inhibitors [281]. In this case, we obtained the opposite reaction. Although NAIP mRNA differential expression is not significant enough, we see a trend of upregulation when cells are treated with SMAC. We believe that this happens because somehow the mitogenic activity of IL-4 [262] is boosted by the SMC treatment. This lead to increased cell proliferation rate and NAIP is overexpressed to be able to face the cytokinetic demand, but this requires additional investigation.

5.1.3.4 Correlation between NAIP and CD14

CD14 is a membrane PRR protein present on the surface of monocytes and macrophages, usually used as a THP-1 (sometimes in U937) differentiation marker and increases its expression when differentiated into macrophages [282]. CD14 participates in the recognition and response against external pathogens and facilitates the response of some TLRs [283]. In our study we have found an inverse relation between the expression of NAIP and the expression of CD14: when NAIP is overexpressed, CD14 is downregulated (Fig 15), conversely, NAIP-siRNA silencing induces CD14 upregulation (Fig 18). The results we found do not correlate with what has been reported in other studies, in which the induction of CD14 leads to an induction of

NAIP and other anti-apoptotic proteins [284]. This is actually what we found previously when analyzing CD14 expression in polarized macrophages pre-exposed to SMAC, (Fig. 39). In light of these results, we would think that NAIP and CD14, although not functionally related, concur at some point in their regu- lation. The expression of CD14 is known to be directly affected by the non-canonical NF-κB pathway [284], in which cIAPs have a negative regulatory role, so when these proteins are antagonized the level of CD14 is increased.

5.2 Macrophage activation syndrome

5.2.1 SMC treatment does not prevent MAS-like induction in mice.

Macrophage activation syndrome (MAS) is frequently found as a complication of other diseases, usually rheumatic, and its study is still very precarious. This partially because MAS is very difficult to diagnose since its features can be masked by the other disease characteristics, but also because of the lack of animal models. MAS is a inflammatory condition caused by an an excessive activation and proliferation of well differentiated T lymphocytes and well differentiated macrophages that exhibit hemophagocytic activity and induce a cytokine storm in the body that leads to an hyperinflammation state [210]. In the study of similar diseases belonging to the hemophagocytic lymphohistiocytosis group of illnesses, the implication of the protein MyD88 in the pathogenesis and development of these kind of diseases has been noted [285]. We hypothesized that the antagonism of the cIAPs, so certain pro-inflammatory NF-κB pathways are disrupted [183], will improve MAS conditions.

For the time being, there is just one MAS animal model described and although it mimics some features of the syndrome, it lacks enough similarities in the macrophage activation aspects [215]. Taking all of this into account, we started working with the aforementioned mice model. This consisted

of constant stimulation of the TLR9 with CpG ODN, a single stranded oligodeoxynucleotide that contains a nonmethilated cytosyne-guanine motif. This compound is recognized by the TLR9 as a danger signal and triggers an inflammatory response in the animal that promote the develop of some of the main features of MAS, such as an increase in the size of the spleen (splenomegaly) and of the liver (hepatomegaly), as we have confirmed in Fig. 44. We were expecting to see a complete reduction of the spleen and liver to normal sizes in those mice treated with SMCs, but instead, we found a partial recovery of the spleen and no change in the liver. On the other hand, mice exposed to SMCs with no MAS-like symptoms induced also had an increased spleen size. Evidently, the SMCs themselves were promoting splenomegaly, (Fig. 45).

After seeing no important differences amongst the frequency of different immune populations in the spleen and liver (Fig. 47), we focused our attention on the inflammatory ratios of the T lymphocyte and macrophage populations in the liver and spleen, as an increase in the presence of T CD8 positive lymphocytes has been documented in liver biopsies from MAS patients [250]. We saw that the CD4/CD8 ratios are similar (Fig. 48), with no significant differences between MAS-like induced mice and those treated with SMC. Both groups presented an increase of cytotoxic, pro-inflammatory T cells (CD8) and macrophages (M1).

According to the data collected from the experiments performed in the mouse model, SMCs are not a good option for treatment in the case of MAS. As we saw, the effects caused by the CpG were not in any case reverted by the treatment with SMAC.

cIAP2 is highly expressed in MAS macrophages

We were able to have access to blood samples from three different patients and analyzed the response of their blood monocytes to different stimuli, including SMC. Also, we were able to evaluate if SMC treatment induces cell death in their macrophages and found no differences in the viability of their cells when compared to the controls (Fig. 49). Additionally, we studied

the expression of cIAP1 and cIAP2 in response to a bacterial ligand, LPS, to see if the affected macrophages had a normal response. Interestingly, we found that cIAP2 in two of the patients (numbers 2 and 3) was highly expressed compared to Patient 1 and the control samples. Samples from Patient 1 were later sent for whole-exome sequencing analysis, looking for mutations to explain the patient's situation. Among all of the genes tested no deletions or duplications were found in any "normal" gene except for the NLRC4 gene. These proteins interact with NAIP to form the NAIP/NLRC4 inflammasome [100, 189], so one option in this specific case of MAS is that the mutation of NLRC4 overactivates it, so the inflammasome is constantly active and inducing IL-1β and other pro-inflammatory cytokine activation, thus leading to MAS.

Seeing the results of the expression of IAPs on the MAS patient samples, we are inclined to think that unless there is any underlying genetic reason for the pathophysiology of the syndrome, cIAP2 will be found in increased amounts in macrophages. This could be a cause or consequence of an M1 polarization state of the macrophage that contributes to the cytokine storm and, if this is confirmed by future studies, it might be used as an indicative syndrome at the time of diagnosis.

CHAPTER 6

Conclusions/Conclusiones

Conclusions

1. NAIP expression decreases in monocytes undergoing differentiation to macrophages.

2. NAIP expression is cell cycle dependent and localizes with the cytokinetic machinery during mitosis.

3. There is an IAP expression pattern in polarized macrophages in which we find:

    - NAIP is highly increased in M2 macrophages
    - cIAP1 and cIAP2 present an inverse pattern of expression in polarized macrophages, cIAP2 being preferentially expressed in M1 macrophages, and cIAP1 being slightly increased in M2.

4. IAP antagonism by SMAC mimetic compounds prevents complete macrophage polarization and causes changes in the IAP expression pattern, upregulating NAIP remarkably.

5. SMAC mimetic compounds are not a suitable treatment for macrophage activation syndrome.

Conclusiones

1. La expresión de NAIP disminuye durante la diferenciación de monocito a macrófago.

2. La expresión de NAIP depende del ciclo celular y se localiza con la maquinaria citocinética durante la mitosis.

3. Existe un típico perfil de expresión de IAPs en la polarización de macrófagos, en la que encontramos:

   - NAIP es mucho más abundante en macrófagos M2.
   - cIAP1 y cIAP2 presentan un patrón inverso de expresión en macrófagos polarizados, siendo cIAP2 expresado preferentemente en macrófagos M1 y la expresión de cIAP1 ligeramente superior en M2.

4. El antagonismo de IAP previene la completa polarización de macrófagos y provoca cambios en el patrón de expresión de los IAPs, incluyendo un notable aumento de NAIP.

5. SMAC mimetics compounds no constituyen un tratamiento adecuado para el síndrome de activación macrofágica.

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