Dendritic cell-mediated modulation of the immune system by endogenous danger signals

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Hunc igitur terrorem animi tenebrasque necessest
non radii solis neque lucida tela diei
discutiant, sed naturae species ratioque.

Titus Lucretius Carus

Table of contents

Chapter 1.

Introduction

1.1

Dendritic cells

Vertebrate immune system is organized in two main functional components named *innate and adaptive immunity*. Cells belonging to innate immunity are responsible of the early phase of the immune response, while adaptive immunity, also referred as acquired immunity, develops after innate response and sustain an antigen-specific and long lasting (*memory*) protection against pathogenic agents.

Dendritic cells (*DCs*) are innate cells, and play a central role in starting and regulating both innate and adaptive immune responses, through the main function of professional *antigen-presenting cells* (*APC*). This feature indicates the ability to detect the presence of infections and dangerous signals, together with uptaking and processing of antigens, in order to alert adaptive immunity, through specific activation of T lymphocytes [1] .

DCs have been initially described by Steinman and Cohn in 1973 [2], and in the last decades many different DC subtypes with specific functions have been defined [3]. However, despite phenotypic and functional heterogeneity, the common feature

shared by all DCs subtypes is the antigen presentation to adaptive immunity.

Dendritic cell function

Typically DCs are located in peripheral tissues and, after antigen encountering, they migrate through the lymphatic vessels to lymph nodes in order to encounter circulating T cells and start the adaptive response. However, recent revisions of DC classification pointed out that this model cannot be extended to all the DC subsets, as described below.

The mechanism of DC antigen presentation to T naive cells is described by the *three signal-model*. The first signal is the interaction between the major histocompatibility complex (MHC) class II plus peptide antigen, presented by DCs, with the T cell receptor (TCR) specific for the same antigen expressed by T lymphocytes. The second signal, named co-stimulation, consists in the interaction between a variety of DC co-stimulatory molecules, such as CD80, CD86, with co-receptors present on T cells. Finally different cytokines secreted by DCs play a central role in determine the specialization of the primed T cell [1].

Co-stimulation and cytokine secretion are possible only when DCs are activated by pathogen-derived molecules, which induce a marked reprogramming of DC phenotype, a process referred also

as *maturation* [4]. This phenomenon is the key event that supports the latest hypotheses about the principle of immune system activation (as widely discussed in the paragraph 1.2).

DC origin and subtypes

Hematopoietic stem cells (HSC) in the bone marrow (BM) gives rise to multi- potent myeloid, lymphoid, and erythroid progenitors, and is tightly by physiological perturbations such as infections and aging [5] (Figure 1). Recent studies have identified a short-lived progenitor in mouse BM, designated the macrophage and DC precursor (MDP), which originates from a common myeloid progenitor (CMP). The MDP is able to differentiate into conventional DC (cDC), plasmacytoid DC (pDC) and monocytes [6], and itself gives rise to a distinct progenitor cell type, called the common DC precursor (CDP) [7]. The CDP differentiates exclusively to DC and plasmacytoid DC, but not to monocytes or macrophages. DC commitment most likely takes place at the CDP stage, giving rise directly to conventional DC precursors (pre-cDC). Following their proliferation in the BM, pre-cDC migrate through the blood circulation to the spleen and lymph nodes. Here, they complete their differentiation into CD8a+ CD4 CD11b+ and CD8a CD4+ CD11b cDC, and take up their anatomic niches; namely, the marginal zone ($CD8a^{+}$) and the T cell zone ($CD8a^{-}$) [8]. These fully

differentiated DC subsets exhibit significant phenotypic and functional differences. In addition to these two spleen-resident subsets, other DC types can be recruited to the spleen in times of infection or inflammation. For example, in the case of Listeria monocytogenes infection, TNFa- and iNOS–producing (Tip)-DC help control bacterial replication and sustain the anti- bacterial immune response [9]. Tip-DC are derived not from the pre-cDC, but from the GR-1+ CCR2+ mouse monocyte subset.

Models of DCs

The main issue of studying DC development, and probably the main fascinating aspect, is that DC differentiation is not only depending on genetic commitment, but is strongly determined by inflammatory and pathogenic stimuli. Notably, TLRs are express on DCs since early differentiation stages [10] and probably contribute to the dynamic process of DC generation, upon micro-environmental influence. Therefore, DCs are a heterogeneous population, with highly variable phenotype. Because of this reason, and their low frequency in the organism, the study of DCs *in vivo* and *ex vivo* is often challenging. In the last decades, different in vitro DCs cultures have been developed. A list of the main published laboratory system to differentiate DCs is summarized in Table I. The most used DC models developed for in vitro DC studies are the human monocyte-derived DC by GM-CSF and IL-4 [11], and GM-CSF derived mouse bone marrow DCs. However, the equivalent of these cells *in vivo* have not been identified, suggesting that these subtypes could represent a transient status in inflammatory DC activation, that is not physiologically stable *in vivo*, but can be maintained in culture. Another system of DC differentiation by mouse bone marrow is the use of FLT3L to activate $FLT3^+$ bone marrow precursors. This model has been proposed as a valid alternative to GM-CSF generated DCs, as

FLT3L-differentiated DCs seems to be closer to the physiological steady state of cDCs and pDCs [12].

1.2

Regulation of the immune response: Danger model and endogenous signals

The history of the modern Immunology starts two centuries ago with the discovery and study of the *vaccine* by Jenner and Pasteur. More then one century later the concept of vaccine was related to the ability of the immune system to develop protecting antibodies against specific molecules not belonging to the host organism, referred as *antigens*, that are specifically recognize by B and T lymphocytes [13]. The concept of antigen has been crucial to define the principle of the immunization, but its efficacy in inducing a complete protection was variable, and the mechanism of immunization needed to be better defined.

The central role of the adjuvant: the INS model

In 1920 Ramon and Glenn discovered that some molecules could boost immune response induced by administration of antigens. Such molecules, called *adjuvants* were of microbial origin and

greatly improved the effectiveness of immunizations [14]. Moreover in 1960s, it was found that administration of antigen alone, as purified protein, not only was less efficient, but in some cases it could induce tolerance [15]. This observation suggested that the efficacy of the original vaccines was due to contaminations by not-protein microbial components able to improve the immunization outcome. However the mechanism driven this phenomenon remained obscure till 1989, when Janeway proposed a new hypothesis. Based on the idea that immune system cannot distinguish between all possible antigenic and not antigenic molecules (referred as self or not self molecules), he suggested that the actual discrimination was between *Infectious and Not Infectious molecules* (*INS model*), and that mammalian immune system has evolved the capacity to recognize the presence of molecules belonging to infective agents. The role of antigen presenting cells (APC) in T cell priming have been discovered just a few years before the postulation of the INS model [16]. Remarkably, without any experimental evidence of putative cellular receptors and pathways, Janeway postulated that APC, such as dendritic cells, have the property to discriminate between infectious and not-infectious agents, through specific receptors able to recognize pathogen associated molecular patterns (PAMPs), distinct from mammalian ones. This hypothesis suggested that PAMPs could represent the adjuvant part able to increase the efficacy of the vaccines. According to this model, dendritic cells

gained a central role in immune system, as keys cells able to prime and modulate the adaptive immunity.

The Danger model

The hypothesis of Janeway was greatly correct and it was confirmed in the following decades by the discovery of the receptors responsible of PAMP-recognition, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). However, the INS model was still not able to explain many physiologic and pathologic phenomenon, where the immune system is active in absence of any evident microbial infection, such as immune response to tumors, transplants and host cells infected by viruses, as well as autoimmunity and allergy. Therefore in 1994, Matzinger postulated a new theory, aimed to complete the model started by Janeway. She proposed that immune system does not discriminate between *self/not self*, or *infectious/not infectious*, but *danger/not danger (*[17]. This theory, referred as *Danger model,* postulated that even self and not-pathogen-derived molecules can signal to immune system to start a response, and immune cells as APC have evolved to recognize the presence of danger situations that can be signaled by the host cells too (Figure 2a). In parallel with the nomenclature used by Janeway to define PAMPs, the danger model postulated the presence of danger associated molecular

patterns (DAMPs), [18]. The DAMPs originally referred to danger molecules derived from any organism, including PAMPs; however the term DAMPs is often used to indicate not-microbial origin molecules in opposition to PAMPs.

The *Danger model* implicates the idea that danger signals, in the form of DAMPs, originated during to an important host process often associated with stress and damage: the *necrotic cell death*. Similarly to Janeway`s theory, the DAMP hypothesis was initially a speculation not supported by experimental observation of putative danger molecules or receptors.

The support to danger theory came later from studies on immunogenicity of dead cells. Indeed, it was showed that dead cells are able to activate DCs and have adjuvant activity increasing antigen specific response of CD4+ and CD8+ cells [19] [20]. Nonetheless, the main issue regarding the danger model is to define how the immune system can distinguish between physiological and pathological cell death and, most important, how the DAMPs get in contact with DCs only in case of danger events and not during the physiological cell-turn over. Interestingly, the adjuvant capacity is not due to newly synthesized products during necrotic process, since the treatment with protein-synthesis inhibitors did not decrease their immunogenic effect [19]. These observations suggested that danger molecules are already-existing molecules (probably with other cell functions), hidden to immune cells in intracellular stores, and released only in case of lost of

plasma membrane integrity. This event is associated with pathologic and stress events and it is related to necrotic death (*primary necrosis*) or in case of apoptosis not followed by phagocytic clearance (*secondary necrosis*). Moreover in some studies the cytosol resulted more immunogenic then nucleus [19] (Figure 2b).

Endogenous danger signals

It is still remain to be defined whether DAMPs have a definite and distinctive molecular pattern, as they PAMPs counterpart.
Interestingly, the process of necrotic cell death seems be associated with the pathogenesis of autoimmune diseases as observed in mouse model of type1 diabetes, autoimmune pericarditis, and ophthalmia [21] [22] [23]. Moreover in some cases, preventing cell death by caspase-inhibition, it is possible to block the onset of autoimmunity [21]. Similarly, injection of dead cancer cells induced immune response against the tumor [24]. Nonetheless, the observation that dead cells are immunogenic was still not supported by the identification of specific molecules and mechanisms able to mediate this process. Therefore, in the last decade, many group focused on the research of DAMPs, A defined by Rock et colleagues, DAMPs are molecules active as highly purified product, excluding possible contaminations by PAMPs, acting at patho-physiological concentrations, excluding cytokines and other immune signaling molecules [25].
Heat shock protein (HSPs) has been identified as one of the first groups of endogenous danger signals. HSPs have adjuvant activity in vivo and promote DC maturation and migration to the lymphoid organs [26] [27]. However, some of the adjuvant effect of HSPs has been suspected to be due to contaminations. Considering the high variety of HSPs in a cell, a definitive depleting experiment

able to demonstrate their crucial role in adjuvancy is difficult to design. Therefore, the function of HSPs as DAMPs is still not clear [28].

One molecule recognized as DAMP is the high-mobility group box 1 protein (HMGB1), an intracellular binding protein associated with nucleosome stabilization and transcriptional regulation. HMGB1 is released by necrotic cells and has been shown to trigger infammation [29] [30]. Interestingly, HMGB1 is one of first DAMPs investigated with a depletion experiment that demonstrated an important role in immune activation, even if a crucial function in immunization is still debated [25].

Double strand bacterial-derived unmethylated DNA have been related to immune activation of TLR9. Interestingly, also methylated double strand DNA of mammalian origin is able to induce APC activation and inflammation, suggesting a possible role as DAMP [31]. However, this observation was , not support by the observation that nuclei have very low immunogenic properties compared to cytosol [19].

Other intracellular molecules with adjuvant activity, chemotattic properties and ability to stimulate APCs are gelectins, thioredoxin, cathelicidine and defensins, even though many of them have not yet been associated with cell death [25].

Finally, two important DAMPs derived from dying cells are *adenosine triphosphate* (*ATP*) and *uric acid crystals*.

ATP physiological concentration in the cytosol is 5mM, but it can be stored in dedicated organelles at higher concentration of 100 mM and can reach 500 mM in platelets. ATP release can be triggered by physical stress, such as hydrostatic pressure, hypotonic shock and mechanical compression. ATP is involved in inflammatory conditions as asthmatic airway inflammation [32]. The concentration of ATP required to activate innate calls in vitro is higher then physio-pathological concentration and is rapidly cleared by ectonucleotidases, such as CD39, [33]. Therefore a real involvement of extracellular ATP during the onset of inflammatory pathologies is still unclear.

The role of uric acid as an endogenous danger signal will be discussed in detail in the following paragraph.

Finally, it has been reported that some extracellular endogenous molecules could act as DAMPs. These molecules are activated by cell death products and can trigger the immune response. In fact the process of cell death can activate the complement cascade and cleave the extracellular matrix, inducing the inflammatory cascade. They include hyaluronic acid, fibrinogen, fibronectin, elastin-derived peptides and laminin. Although these molecules may not have a direct interaction with innate cells nor a specific receptor, they are endogenous components that contribute to inflammation; therefore they have been suggested as part of the danger model hypothesis [25].

Uric acid as endogenous danger signal

Since centuries, it is known that uric acid, in its crystallized form, is the etiologic agent of gout is an autoinflammatory disease characterized by arthropathies, mainly due to IL-1beta release and neutrophil recruitment in the joints.

Uric acid is the end product of purine catabolism. The enzyme xanthine-oxidase metabolizes the purine-derived products, xanthine and hypoxanthine, to uric acid (Figure 3a). In birds and reptiles, uric acid is excreted in feces as a dry mass, allowing reduction of water loss, while this process in mammalian is replaced by the less complex mechanism of urea production.

Conversely, in humans and higher primates, uric acid is excreted in urine, but in the most of the other mammals it is converted to allantoin by the enzyme *uricase*, whose gene is not expressed in higher primates [34]. Uric acid acts as potent antioxidants thanks to its reducing properties [35]. It has been suggested that the loss of uricase by higher primates is the result of an evolutive advantage, that allows maintaining inside the organism antioxidant molecules, important during the aging process.

Systemic concentration of uric acid measured in the blood (named uricemia) is influenced by alimentary introduction of purinic precursors, by the balance in the process of biosyntesis and degradation of nitrogen bases, and by the urinary clearance.

The average uric acid concentration in the serum is 290μmol/L and it can be found at higher concentration in healthy cells (up to 4 mg/ml) [36]. One hypothesis in the pathogenesis of gout is that extracellular uric acid, in the presence of high levels of free sodium, forms crystals, referred as *monosodium urate* (*MSU*). MSU crystals accumulate in the joints and start a strong inflammatory process in the synovium. This process leads to the onset of gout attacks, which can involve systemic response, such as fever, and the formation in the joints of typical bunions, named tophi (Figure 3b). Interestingly, gout disease is often associated with male gender and increases during the aging process (Figure 3c). Notably, only in the form of MSU crystals, uric acid acquires the biological active structure able to exert inflammatory and adjuvant effect on immune cells [37].

Beyond gout, high level of circulating uric acid has been associated with various inflammatory diseases, such cardiovascular diseases and hypertension. Indeed uric acid could have a double-side effect as antioxidant and pro-oxidant, and it is unclear whether its involvement in some autoinflammatory diseases is a primary cause of disease or a protective response [38]. Similarly, it is not clear the significance of low concentration of uric acid observed in association with multiple sclerosis and other autoimmune diseases [39], even though there are still no evidence of a direct involvement of MSU crystals in etiology of autoimmunity [40]. However it is

likely that the pathologic effect of uric acid depends on the crystal form as well as the redox status of the tissue microenvironment.
The adjuvaticity of MSU crystals has been shown in the seminal work of Shi and colleagues, describing for the first time the role of uric acid crystals as endogenous danger signal of cell death [36]. Later MSU has been studied in animal models of diseases and recently it has been identified as key mediator of the mechanism exerted by the highly successful vaccine adjuvant alum (as discussed in detail later) [41] [42] [43, 44].

Receptor for DAMPs

Although endogenous danger molecules have been highly investigated, it is still not clear whether DAMPs are ubiquitously represented in all the tissues or whether they vary among different cell types. Moreover is not known whether they act following a common mechanism or triggering different pathways. Most probably DAMPs act through specific receptors, which have to be defined.
The biggest contribute of the Danger model to the modern Immunology is not only the definition of a new functional backbone for the immune system regulation, but mainly the innovative idea that the immune activation is not ``*tailored to the targeted pathogen*`` but ``*to the tissue in which the response occurs*` [45].

This postulation gave rise to another recent fascinating hypothesis that the sharing of receptors between PAMPs and DAMPs could be not an exception but the rule in the co-evolution process between microrganisms and mammalian immune system. In fact, pathogens and host could have evolved to interact through receptors originally designated to recognize endogenous molecules [45]. In 2005 Tschopp and colleagues added a new important finding to the Danger model saga, the discovery of a newly described class of receptors: the inflammasomes. Indeed, these intracellular protein complexes have been associated with the ability of innate cells to sense the presence of endogenous danger signals such as uric acid crystals, ATP and mammalian nucleic acids, besides the recognition of infectious agents.

1.3

The inflammasome

The ability of dendritic cells and macrophages to sense danger depends on a plethora of receptor families. The Toll-like receptors (TLRs) family is the first identified and the best characterized so far [46]. However, studies in TLR-deficient mice revealed a redundancy in the receptor pattern responsible for innate cell activation. Therefore, in the last decades many groups focused on the role of newly discovered classes of receptor different then TLRs, including NOD-like receptors (NLRs), RIG-like helicases receptors (RLR) and C-type lectin receptors [47-49].

General structure of NLRs

NLRs and TLRs families are fundamental components of innate immune system of many animal species. Evolutionary simpler species, as sea urchin, contains of 222 TLR- and 203 NLR-coding genes, mainly expressed in the gut [50]. This observation suggests that the broad repertoire of NLRs has undergone lineage specific

amplification, following the interaction with species-specific pathogens [51].

Interestingly, NLRs have not been found in other animal group, as insects, but NLR-like proteins, functionally and structurally similar to mammalian ones, have been found in plants, as a fascinating example of convergent evolution.

NLRs are proteins present in the cytosol, and upon detecting signals are able to form cytoplasmic platforms. (Martinon, 2007 #466). The core proteins of NLRs are intracellular multidomain proteins composed by a C-terminal region leucine-rich repeat (LRR), similar to plant disease-resistance genes, a central nucleotide domain (called NACHT or NOD) and an N-terminal effector domain. LLRs are composed by 20-30 residues, forming alpha helix and beta strands [52] (Figure 4).

Similarly to TLRs, LLR structures in NLRs could be important for PAMP recognition, even if a direct evidence of interaction with ligands has not been demonstrated yet for LLR of NLRs. The central domain NACHT has structural similarity with the central motif of the apoptotic mediator APAF-1, and it has been suggested to be involved in oligomerization activity. This function is, indeed, crucial for the activation of other intracellular platforms, such as APAF1-apoptosome, therefore an analogous mechanism has been proposed for the formation of NLRs platform [53, 54].

The NLR terminal domain is characterized by a death-fold domain containing a CARD or pyrin domain (PYD), originally described in

pro-apoptotic signaling pathways, and in analogy to them they usually activate caspases or NFkB pathway, inducing signal transduction. The N-terminal domains differ among NLRs, and characterize the function of every NLR. CARD and PYD typically work through the dimerization with an analogous domain, harbored by another protein that is usually strictly related to the transduction pathway and downstream effect of the specific NLR platform.

NLR family included three subfamilies classified based on molecular structures and phylogenetic distribution [55]. The family named IPAF/NAIP is evolutionarily well separated by other NLRs and is involved in the formation of the inflammasomes (as

described below). IPAF contains a CARD domain, whether NAIP include three baculovirus inhibitor domains (BIRs), probably involved in inhibition of apoptosis (Liston P, Nature 1996).
The families of NODs and NLRP will be deeply discussed in the next paragraphs.

The NOD family: the signalosomes

The first described NLR is the *nucleotide-binding oligomerization domain-containing protein (NOD) -1*. Up to now, NOD family includes five different members (NOD1-5) and CIITA, and is mainly characterized by the N-terminal CARD domain (not well defined for NOD5 and CIITA).
Upon activation NOD1 and NOD2 recruit *receptor interacting protein (RIP) -2* through homodimerization of CARD domains, leading to the activation of NFkB [56]. Moreover NOD2 can engage CARD9, which mediates the activation of *mitogen-activated protein (MAP) kinase* cascades of c-junN terminal kinase (JNK) and p38 [57].
The natural ligands of NOD1 and NOD2 are moieties derived from *peptidoglycan* (*PGN*), a polymer constituting bacteria-cell wall, more abundant in gram positive than gram negative bacteria. NOD1 detects meso-diaminopimelic acid, , while NOD2 recognizes *muramyldipeptide* (*MDP*) [58]. Since NODs and NLRs in general,

are cytosolic receptors, their ligands need to reach the internal cell compartment, usually through the entrance of the bacteria or cell-mediated phagocytosis.

NODs control the infection also on epithelial cells especially in the gastro-intestinal mucosa. Indeed, loss of function mutations in NOD2 are responsible of autoinflammatory disorders, such as Crohn`s disease, and susceptibility to bacterial infection, such as *Helicobacterium pilori* and *Listeria monocytogenes* [59-61], while gain-of-function mutations cause a systemic autoinflammatory disease named Blau syndrome [62].

NLRP family and the inflammasomes

The largest NLR-family characterized by PYD is named *NLRP* (NLR-Pyrin domain), also referred as *NALP* (Nacht Domain-, Leucine-Rich Repeat-, and PYD-Containing Protein) family. NLRP family includes 14 members in human, and their LLR region structure is very conserved [63].

The most of the NLRP protein have still to be functionally characterized. However in the last decade some members of the NLRP family has been associated to the functional platform, named *inflammasome*. The term inflammasome was coined by Tschopp in 2002 to describe the multiprotein complex that allows the activation of pro-inflammatory caspases, in analogy with the

apoptosome for apoptotic caspases [64]. The inflammatory caspases (Casp) are Casp-1, and 12 (in mouse and human), Casp-11 (only in mouse) and Casp-4,5 (only in human) [63].
A prototypical inflammasome structure includes an NLRP protein, which typically presents a N-terminal PYD domain able to interact with PYD domain of an adaptor protein, named *ASC* (apoptotic-associated speck like protein containing a caspase recruitment domain) (Figure 5a). ASC contains a second CARD domain in C-terminal, that homodimerizes with the CARD domain of a pro-inflammatory caspase. So far two NLRPs involved in inflammasome formation have been described: NLRP1, NLRP3. However, NLRP1 in human is also able to recruit directly a caspase 1 and 5 via its own CARD domain. Moreover, the NLR IPAF recruits Casp-1 directly via CARD-CARD interaction and therefore IPAF platform is referred as a third inflammasome prototype [37]. Recently, it has been suggested that different NLRs including NAIP and NOD2 could assemble forming heterocomplex-inflammasomes [65, 66].

NLRP3 inflammasome: a sensor of danger

One of the most studied inflammasome is NLPR3, which has been associated with important innate function as the sensing of endogenous danger signals, as well as detection of microbial

agents. Interestingly, NLRP3-inflammasome comprises, the NLRP3 protein (also called Cryopyrin, coded by the gene *cias1*), the adaptor protein ASC, and Casp-1 (also known as interleukin-1 beta convertase, ICE). The assembly of the inflammasome complex activates caspase-1, which then enables cleavage and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Figure 5b) [64, 67].

As for other NLR and TLR, the activation of NLRP3 inflammasome can be induced by during microbial infection (see below), but notably, NLRP3 inflammasome is the only NLR complex associated with the ability of DC and macrophages to detect and respond to endogenous danger molecules released by dying cells. Indeed, NLRP3 signals the presence of ATP, MSU crystals and calcium pyrophosphate (CPPD) crystals [68-70], and, interestingly, other large particulates of not-microbial origin (danger), such as alum, silica and asbestos [43, 71, 72], suggesting the particulate form as a common feature of NLRP3-stimuli. Moreover, NLRP3-inflammasome has been proposed as a sensor of nucleic acids [73, 74]. However, the recognition of mammalian DNA by the inflammasome is mediated by ASC but independent of NLRP3, while the role of RNA in inflammasome activation has not been completely confirmed.

NLRP3 activation mechanism

Although NLRP3 inflammasome has been widely studied, the precise mechanism of NLRP3 activation by many heterogeneous stimuli remains unclear. Indeed there is no evidence that inflammasome activating stimuli interact directly, like specific ligands, with the NLR protein.

Inflammasome activation has been investigated mainly in relation to Casp1-cleavage and IL-1β secretion. Generally it seems that inflammasome-mediated IL-1β secretion requires a two-step mechanism: pro-IL-1β transcription is obtained by TLR or NOD2 agonists, as lipopolisaccaride (LPS) or MDP, independently of the inflammasome recruitment, and secondly the inflammasome activation is induced by the NLRP3 activator [64, 70, 75]

One proposed mechanism for NLRP3 activation has been investigated in cell-free systems. Inflammasome spontaneous assembling can be obtained inducing disruption of cellular integrity, followed by the decrease of potassium below the physiological intracellular concentration of 70 mM [64] [76, 77]. Therefore it has been suggested that NLRP3 activators could act indirectly through pathways able to low potassium concentration.

This mechanism has been showed for ATP-driven inflammasome activation. Extracellular ATP is associated with inflammasome activation and IL-1β secretion mediated by ASC [70, 78], by binding a specific purino-receptor named P2X7, inducing the opening of the gap junction protein/channel pannexin-1 [79]. This process allows the potassium efflux, inducing inflammasome activation, and also ATP release triggering a second wave of signal [80].

A second putative process possibly involved in NLRP3 activation is the reactive oxygen species (ROS) induction that has been observed in macrophages stimulated by alum, silica and asbestos,

as well as ATP and MSU [43, 81, 82]. Indeed, antioxidants and NAPDH inhibitors affect inflammasome activation [82]. ROS are known to be involved in inflammation and innate cell activation, however it is not clear whether they activate NLRP3 directly either indirectly [83].

Another proposed mechanism for NLRP3 activation is the model of frustrated phagocytosis [71]. Indeed a common feature of inflammasome activators is the crystal or particulate form (MSU, alum, asbestos and silica). Interestingly, inhibition of cytoskeleton rearrangement, with colchicine and cytochalasin D, blocks IL-1β secretion. This observation suggested that the failed tentative of phagocytosis by innate cells could lead to the activation of stress-related intracellular mechanisms, that on turn could induce NLRP3 activation, possibly through generation of ROS, through NADPH [84].

Hornung and colleague showed that potassium efflux is not sufficient to trigger NLPR3, but in case of large particulates, inflammasome activation requires lysosomal swelling and leakage. Differently then the hypothesis of frustrated phagocytosis, in this model, the crystals start the phagocytic process and enter in the cell, causing to lysosomal damage and inhibition of cathepsin B, a lysosomal protease, leads to a substantial decrease in activation of the NALP3 inflammasome [44].

Although many parallel mechanisms have been proposed for inflammasome activation, we cannot exclude that some of them co-operate in a unique complex mechanism.

Inflammasome related cytokines: IL-1β and IL-18

The main outcome of inflammasome activation is the recruitment of pro-inflammatory caspases. For NLRP3 inflammasome the recruited capase-1 induces the cleavage and secretion of the pro-inflammatory cytokines IL-1β and IL-18.

IL-1β is a pleiotropic cytokine known as an endogenous pyrogen, but it has also multiple effects on central nervous system [85, 86]. The outstanding strength of IL-1β as immune-activator is counterbalanced by a unique system of regulation [87].

First of all, IL-1β production is finely controlled by two step mechanisms: one activating transcription and translation of the immature form, named pro-IL-1β, the second leading to cleavage in the mature IL-1β form by auto cleavage of Casp-1, which is recruited to NLRP3-inflammasome platform. Mature IL-1β and the cleaved-form of Casp-1 are, then, secreted through exocytose vesicles. The activation of IL-1β translation is generally obtained using TLR agonists, mainly LPS, but also R848, Pam3CSK4, CpG, or the NOD2 agonist MDP.

Notably, IL-1β and another component of IL-1 family, IL-1α, bind two common specific IL-1 receptors, named IL-1RI and IL-1RII [88]. IL-1RI forms a membrane complex with IL-1R accessory protein (IL- 1RAcP), and is responsible of IL-1 transduction pathway, through the recruitment of Myd88 adaptor protein followed by IL-1R-associated kinase/TNFR-associated factor 6 (IRAK/ TRAF6) signaling cascade [89]. Contrarily, IL-1RII does not mediate any intracellular pathway, but it has been proposed as a decoy receptor of IL-1RI, able to affect IL-1/IL-1R signaling through a competitive binding of IL-1 molecules [90, 91]. Finally, another important component in the IL-1 regulatory system is the natural antagonist of IL-1R (IL-1RA), which blocks IL-1 access to IL-1RI, without activation of the accessory protein for signaling cascade [85].

IL-1β is a potent activator of both innate and adaptive immunity and is important for host protection against infection, but it is also responsible of the pathogenesis of septic shock [85]. Also, IL-1 is produced at the tumor site and is important for the interactions between malignant cells and the immune cells, and can be crucial for tumor growth, and invasiveness [92]. However it is still not clear whether IL-1 plays a positive or negative role in anti-tumor immunity [92].

Notably, most of the pathologies involving NLRP3-inflammasome hyperactivation, such as gout and monogenic autoinflammatory diseases (see below), are strictly connect to IL-1β mediated

processes. Many pre-clinical and clinical trials are based on the blocking of IL-1R, successfully obtaining a significant reduction of the gouty inflammation. One important advantage of this approach is that IL-1R blocking can be obtained by the injection of its own natural antagonist IL-1RA [93] [94].

The autocrine and paracrine property of IL-1β can explain the strong systemic effect of this cytokine observed on immune and not-immune cells. Moreover, since IL-1R signals through Myd88, which acts also as adaptor protein of many TLR receptors, in many in vivo studies, the activation of NLRP3 resembled the same scenario observed after TLR-activation, leading to erroneous interpretations.

Beside IL-1β, many studies underlined the central role of NLPR3 in the maturational cleavage of the pro-inflammatory IL-18 [95], although, IL-18 can also be activate by other mechanisms independently of Casp-1 [96]. IL-18 is found constitutively expressed in many cells [97], however only cells expressing an active inflammasome, macrophages, DCs and Kupffer cells can cleave IL-18 to the mature form. IL-18 alone induces Th2 polarization, while in synergy with IL-12, IL-15 and IL-2 drives Th1 skewing, alternatively when coupled to IL-23, IL-18 is able to promote Th17 differentiation [67].

A novel cytokine belonging to IL-1b family is IL-33, which signals through IL-1R and is involved in Th2 response. Although direct

evidences are not yet available, IL33 has been suggested to be cleaved by inflammasome, similarly to IL-1β [98].

NLRP3 and response to infections

Despite the unique property of sensor for not-infection-derived molecules, the activation of NLRP3 inflammasome can also be triggered by many different pathogen-associated molecules, including bacterial derived-peptidoglycan [99]. However, the mechanism of NLRP3 activation by bacteria has been associated with the ability of bacteria toxin to induce ion exchange in the infected cells. Indeed, NLRP3 and ASC are activated by gram positive bacteria through pore-forming toxins, such as alpha-toxin from *Staphylococcus aureus*, aerolysin from *Aeromonas hydrophila,* and listeriolysin O from *Listeria monocytogenes* [69, 100, 101]. Recent findings underline the role of inflammasome in the recognition of viral DNA and RNA [73, 74].
Finally, NLRP3 has been associated with the control of fungi infections, such as Candida albicans [102].

NLRP3 inflammasome and autoinflammatory diseases

NLRP3 mutations resulting in inflammasome hyper-activation have been associated with a family of auto-inflammatory diseases called hereditary periodic fevers, including Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), and chronic infantile neurological, cutaneous, and articular (CINCA) [103]. These pathologies implicate recurrent episodes of fever, rush, synovities, and various degree of neurological involvement [104].

Increased secretion of IL-1β is observed in macrophages from patients with NLRP3 mutation, confirming the central role of IL-1β in the downstream effect of inflammasome activation.

It has been observed that, in 24 hours after LPS stimulation, 20% of pro-IL-1β is spontaneously cleaved and released, without adding any inflammasome activator. While adding ATP or other NLRP3 activator strongly accelerate the cleavage and release process in the range of 15 minutes, leading to a massive secretion of IL-1β [105]. Interestingly, it has been observed that monocytes from CINCA patients, as well as macrophages from a corresponding mouse model of MWS, spontaneously secrete significant amount of IL-1β only after LPS stimulation without ATP addition [106, 107]. Thus it has been suggested that NLRP3 mutations, in patients of genetic autoinflammatory diseases, could affect the conformational

and functional steady state of NLRP3 leading to a constitutive spontaneous activation [108].

However the aberrant activation of the inflammasome in diseases is not only caused by genetic mutation in NLRs, but it can also be triggered by stimuli. Indeed, NLRP3 activation has been involved in the pathogenesis of many not-infectious autoinflammatory disorders. As described above, uric acid crystals are responsible of gout disease, and NLRP3-mediated pathologies can emerge from chronic exposure to the inhalation of particulates as silica and asbestos, leading to chronic inflammation of the lung. Finally, it cannot be excluded that NLRP3 polymorphisms could influence the susceptibility to infectious diseases as observed for other TLRs, as NOD2 in Crohn's disease.

NLRP3 inflammasome in adjuvanticity and autoimmunity

The activation of NLRs is mainly related to autoinflammatory diseases, but a growing number of evidences are suggesting the possibility that NLRP3 activation by danger signal could drive adaptive immunity too.

Indeed study on alum hydroxide, already known as a potent adjuvant, showed that alum induce activation of Th2 antigen specific-adaptive immunity in a NLRP3-dependent fashion [43, 109] while another study proposed that alum causes NLRP3-

mediated Th2 activation indirectly through the induction of MSU release [110].

Accordingly to this hypothesis many groups investigated the possibility to use NLR-activators as adjuvants during antigen immunization. Indeed also silica has been found to mediate Th2-induction through inflammasome activation [111].

However, the role of IL-1b, IL-18 or eventually IL-33 in NLRP3 mediated adjuvancy is still not well defined. Interestingly Myd88-deficient mice showed differences in response to alum, in term of alum-induced recruitment of inflammatory monocytes to the draining LN, suggesting that probably IL-1R and IL-18R pathway could be crucial element for alum adjuvant properties. Nonetheless, Myd88 deficiency didn't affect alum-induced humoral immunity, a central component of alum adjuvant properties. On the other side, Li and colleagues showed that IgE induction by alum was significantly reduced in NLRP3$^{-/-}$ mice.

The relation between NLRs and autoimmunity is still unclear. Apparently NLR activation in innate cells is a central event in many autoinflammatory diseases where the adaptive response is not involved. However, the border between autoimmunity and autoinflammation is not really defined for many immune disorders [112] and the adaptive component in autoinflammation and *vice versa* could be underestimated. Moreover, a recent work describing a mouse model of NLRP3 hyperactivation, shows that

NLRP3 can mediate the pathogenic activation of specific T cell subset such as IL-17-secreting cells [107].

1.5

Th17: a new subset of T helper lymphocytes

Specificity and memory are among the most powerful characteristics of the mammalian immune system and they are achieved through a dedicated component of the immune cell repertoire, called *adaptive immunity*. *T* and *B lymphocyte* (also called *T and B cells* respectively) form the main part of the adaptive immune system and can recognize the presence of pathogen-derived antigens through a specific surface receptor called respectively T cell receptor (TCR) and B cell receptor (BCR). The process of antigen recognition leads to clonal expansion of the lymphocytes presenting an antigen-specific TCR or BCR, and in the most of the cases a part of the clonal population, referred as *memory cells*, can circulate for years, guaranteeing protection from further pathogen infections.

In the thymus, double positive T cells differentiate in two subsets $CD4^+CD8^-$ (referred as *$CD4^+$*) and $CD4^-CD8^+$ (referred as *$CD8^+$*) [113]. $CD8^+$ cells can recognize infected cells and directly induce cytotoxic cell death, while $CD4^+$ cells are functionally characterized by the ability of sustain the immune response through the

extensive production of specific cytokines, and for this reason they are called *T helper* (*Th*) .

The Th1/Th2 paradigm

The role of $CD4^+$ T helper cells remained not well defined for years, till 1986, when Mosmann and Coffman defined two functional subsets of T helper cells based on their cytokine production-profile and their effect on the activation of the downstream immune response [114].

The subset referred as Th1 is characterized by production of IFNγ and drives cell-mediated immune responses against virus and intracellular bacteria, leading to tissue damage in case of over-activation. Th2 subset, through the secretion of IL-4, IL-5 and IL-13, sustains the humoral immunity and the response against extracellular pathogens as parasites [114]. Differently from the thymic process of $CD4^+$ versus $CD8^+$ differentiation, the development of Th1 versus Th2 phenotype is not driven by a genetic commitment but it depends on the cytokine environment at the moment of T cell activation by a specific antigen, a process referred as T helper *polarization*. Indeed, the process of T cell polarization implicates the activation of a specific transcription factor that determines the polarizing profile in a one-way direction. Th1 cells expresses the transcription factor T-bet, while Th2 cells

are characterized by the transcription factor GATA-3. One of the most remarkable statement of Mosmann and Coffman model was the key idea that every T helper subset could sustain through a positive loop the activation of other cells of the same group, while dampening the activation of the opposite subset.

Th17: a revision to classical T helper model

For almost two decades the Th1 versus Th2 paradigm remained untouched, even though it became clear soon that this model failed to predict many experimental outcomes. For example, it was expected that Th1 cells could drive cell-mediated inflammatory diseases in murine models of experimental allergic/autoimmune encephalomyelitis (EAE) and experimental adjuvant arthritis, through their main effector cytokine IFNγ. Surprisingly, administration of IFNγ ameliorated EAE paralysis and arthritis severity instead [115]. Moreover, EAE worsted in IFNγ knockout mice and after injection of IFNγ-blocking antibodies [116] [117]. However, the scenario is complicated by an important observation that seemed to support the Th1 role in EAE: the typical Th1-polarizing cytokine IL-12p40 was crucial in EAE development, as in MS patients [118, 119]. The following findings showed to the immunological community how apparently unexpected and aberrant results could perfectly fit in a new model. In 2002 and

2003 two groups demonstrated that the new heterodimeric cytokine IL-23, formed by IL-12p40 together with the subunit IL-23p19 [120], was actually one of the master effectors of EAE development, while the heterodimeric cytokine IL-12p40/IL-12p70, classically referred IL-12 that drives Th1 response, was not involved [121] [122]. Afterwards, IL-23 was shown to play a crucial role in the onset of collagen arthritis and inflammatory bowel disease [123]. These observations not only helped to clarify the role of the IL-12p40 in the development of autoimmune diseases, but they also gave the opportunity to hypothesis a new T helper development to explain the pathogenesis of autoimmune phenomenon in a more appropriate way.

Indeed, IL-23 was suggested to drive the polarization *in vitro* of memory $CD4^+$ T cells towards a specific phenotype characterized by the secretion of a new defined cytokine called IL-17 [124] [125]. The IL-17-secreting T cells had been described in 2000 by Infante-Duarte, as a new independent subset of T helper cells, which later was officially defined by other groups as a new lineage distinct from Th1 and Th2, named T helper-17 (*Th17*) subset [126] [127]. Cua and colleagues defined how IL-23 could drive the development of a T cell population, producing IL-17, IL-6 and TNF, which could induce EAE when adoptively transferred in healthy recipient mice [128]. Th17 cells have been widely characterized in the last five years but many aspects of this new subset are still to be completely defined.

As for the other Th1 and Th2 subsets, Th17 differentiation starts from the combination of antigen-specific-TCR activation and cytokine-receptor signaling, that leads to the expression and activation of the steroid receptor-type nuclear orphan receptor gamma-delta (RORγt), a splice-variant of RORγ [129]. The engagement of RORγt and RORα, another recently defined Th17-specific transcriptional factor, are required for IL-17 secretion and a complete Th17 activation [130]. Also STAT3, which signals downstream to IL-6, IL-21 and IL-23 receptors, is crucial to sustain RORγt expression and the leading to transcription of IL-17 and IL-21 [131] [132].

The reciprocal interaction between Th17 and Treg

Th17 differentiation in vitro can be obtained with antibodies anti-IFNγ and anti-IL-4, by blocking Th1 and Th2 differentiation respectively. This finding suggested that the polarizing exclusion mechanism observed by Mossman and Coffman for Th1 and Th2 could be valid also for Th17 subset [127]. However this study demonstrated for the first time the potential anti-inflammatory function of Th1 cells, through the dampening the Th17-polarization. Th17 subset showed also an unexpected interplay with another subset of $CD4^+$ T lymphocytes: the T regulatory (*Treg*) cell subset. Interestingly, this finding arose from the study of differentiation

factors for Treg polarization [133]. In 2006 three independent groups showed that TGFβ drives the polarization of Treg cells, but TGFβ together with IL-6 induces Th17 differentiation of naïve T cells [134] [135] [136]. Studies on TGFβ knock-in and knock-out mice demonstrated the central role of this cytokine in Th17 and Treg differentiation versus Th1 and Th2 activation in the context of Th17-driven disease models, such as EAE [134] [137]. The crucial role of TGFβ and IL-6 as Th17-polarizing cytokines will be discussed below.

Notably, before the `Th17 revision`, TGFβ was mainly known for its tolerant function and activation of T regulatory cells. The new ambiguous role of TGFβ in the induction of highly inflammatory Th17 cells shows how close can be the relation between two T cell subsets responsible of totally opposite immune responses. This observation underlines once more the importance of fine-tuning mechanisms in the regulation of the immune system.

Defining Th17 differentiation factors

Despite the important role played by IL-23 in Th17 discovery, it became clear soon that IL-23 was not the key factor for Th17 differentiation, but for sustaining Th17 response instead. In fact, IL23 receptor is expressed only on memory Th17 cells, so IL-23 is not able to polarized naïve T cells to Th17 phenotype [135].

Starting from this observation and considering the strong inflammatory potential of the new defined Th17 subset, many groups focused on the study of the cytokine-profile able to induce Th17 differentiation from naïve T lymphocytes (Figure 6).

A role for IL-6 in Th17 polarization was already proposed in the first work describing IL-17-secreting cells [138], but the crucial function of this pleiotropic cytokine emerged later, in relation to the interplay with TGFβ. As discussed above, IL-6 and TGFβ were identified as the key combination to get Th17 differentiation. Indeed, TGFβ induces RORγt expression in Th17 but paradoxically represses RORγt function, through FoxP3 activation; moreover it can definitely inhibit Th17 polarization, at high concentration [139]. In this delicate balance IL-6 plays a crucial role. In fact, IL-6 signaling in naïve T cells cannot directly induce RORγt expression but is crucial for STAT3 activation that in turn is necessary for RORγt induction [140]. Therefore, only the synergic combination of IL-6 and TGFβ signaling brings to RORγt activation and Th17 generation. Thus, TGFβ is the key cytokine shared by Th17 and Treg determining the polarization and the outcome of the immune response towards inflammation or tolerance respectively [133]. Finally, another group pointed out that in presence of IL-6, Th17 can originate from already differentiated Treg cells [141]. Notably, Th17 seem to be resistant to suppressive action of Treg [142], differently then Th1 and Th2 cells. This observation suggested that

TGFβ could induce Th17 differentiation by another side mechanism: inhibiting Th1 and Th2 production of IFNγ and IL-4, and favoring in this way the polarization towards Th17 subset [143].

However, TGFβ results indispensable but not sufficient to get a complete Th17 differentiation and functional activation. Therefore, further studies defined the importance of other cytokines. Using IL-6 deficient mice Kuckroo and colleagues showed a redundancy in the cytokines required to block Treg differentiation and induce Th17. Indeed IL-6 knockout mice showed a normal Th17 polarization and EAE development. Upon screening for cytokines able to replace IL-6 function in Th17 differentiation, IL-21, a cytokine belonging to IL-2 family, has been identified as another inducer of Th17 subset [144]. Moreover, since Th17 cells are among the strongest producers of IL-21, probably it sustains Th17 activation through and autocrine amplification loop [145]. Interestingly, IL-17 has not autocrine effect, since it doesn't act as a differentiation factors for Th17 cells, differently than IFNγ, IL-4, and TGFβ for Th1, Th2 and Treg subsets respectively. So the discover of IL-21 role helped to define a missing mechanism in Th17 subset, compared to other known T cell lineages.

As discussed above IL-23 is not required for Th17 generation but it has an important role in sustaining Th17 function and it is crucial for the onset of many autoimmune diseases. In fact, the expression of IL-23 receptor (IL-23R) of early-differentiated Th17 is a central

point in Th17 expansion and functional activation. Recently, it has been demonstrated that IL-6 and IL-21 induce IL-23R through STAT3-dependent pathway [146].

Even though TGFβ and IL-6 seems to be necessary for Th17 generation, further studies focused on the role of other two pro-inflmammatory cytokines: IL-1β and TNFα [134] [147, 148].

IL-1β is considered not crucial for Th17 differentiation in mouse, but studies on human PBMC underlines a central role for this cytokine in driving Th17 generation. This observation emerged as one of the main differences between mouse and human Th17 subsets (as discussed below) [149]. Another component of IL-1 family, IL-18, has been described to induce Th17 skewing, but only in presence of IL-23, while the combination with other cytokines would lead to Th1 or Th2 polarization (as described above) [97].

As for IL-1β, TNFα can sustain Th17 activation especially in human, but among the Th17-polarizing cytokine it seems to be dispensable [150].

Considering all the conditions promoting Th17 differentiation, a special note goes to IL-2. This pleiotropic cytokine has been mainly related to T cell homeostasis with a minor connection to inflammation. In fact IL-2 is known to sustain T cell proliferation but also Treg activation and mice IL-2 knockout show a reduced number of Treg cells and multi-organ inflammatory disease. This phenotype is characterized by higher frequency of Th17 cells, so it

has been suggested that IL-2 has a negative effect on Th17 differentiation [151].

Finally another cytokine with inhibitory properties on Th17 development is IL-27, a heterodimeric cytokine belonging to IL-12 family. Studies on EAE models showed that IL-27 limits encephalomyelitis, but its role in human Th17 regulation is still to be defined [152, 153].

Recently, it has been shown that a well-known pro-inflammatory molecule, prostaglandin E2 (PGE2), favors Th17 differentiation. Interestingly, this molecule had been associated with inhibition of Th1 response, and the recent association with Th17-skewing helped to solve this apparent paradox [154].

Other molecules, not strictly related to immune system, have been found to promote Th17 differentiation. The aryl hydrocarbon receptor (AHR) was found highly expressed in both the Th17 and Treg and different agonists of AHR promoted or repress the expression of Th17 cells [155, 156]. Interestingly, endogenous ligands of the AHR exist, even though nothing is known about their relevance in regulating Th17 / Treg balance. Similarly, retinoic acid inhibits Th17 skewing, favoring Treg polarization, and CD103+ lamina propria DCs, but not splenic DCs, could maintain the tolerance in the gut inducing T reg through secretion of retinoic acid [157].

Effector cytokines of Th17 cells

IL-17 family includes IL-17A, IL-17B, IL-17C, IL-17C, IL-17D, IL-17-E and IL-17F [158]. The effector cytokines of Th17 subset are IL-17A (also called IL-17) and IL-17F, however their expression is common to other innate and adaptive cells such as gammadelta T cells, natural killer and natural killer T cells, neutrophils and eosinophils [159] [160]. Both IL-17A and IL-17F follow similar activation and have pro-inflammatory functions acting on many different cell types. They can induce the expression of many cytokines such as TNFα, IL-1β, IL-6, GM-CSF, G-CFS, chemokines and metallo-proteinases [161]. IL-17 production is important for neutrophils recruitment, as well as formation of germinal center of B cells [162, 163]. Generally, IL-17 mediates powerful effect on stromal cells [143]. In fact, IL-17 receptor is ubiquitously expressed in many cell types related to joint inflammation as monocytes, macrophages, chondrocytes, osteoblasts and fibroblasts, and it can mediate the onset of rheumatoid arthritis (see below)[164].

Surprisingly, Th17 can produce also IL-10, a cytokine mainly related to immune suppression [165]. This observation supported the revaluation of IL-23 in the functional development of Th17 subset. Studies on mouse model of central nervous system inflammation, support the hypothesis that TGFβ and IL-6 are crucial for initial commitment of Th17 lineage but in absence of IL-

23 Th17 cells produce IL-10 and fail to induce inflammation while IL-23 is required for the development of inflammatory phenotype [165].

Th17 subset can also produce IL-22, a cytokine related to IL-10 family. Th17 cells start to produce IL-22 only upon stimulation by IL-23, so IL-22 is associated with the terminal stage of Th17 differentiation [166]. The peculiarity of IL-22 is that IL-22R is not expressed on immune cells, so IL-22 is a tool for Th17 cells to induce functional modification on tissues and cells other then immune components. In particular IL-22 is crucial in the immune barrier function of epithelia, mediating the protection from infective agents as *Klebisiella pneumonia* [167]. However a recent work on human Th17 cells shows that, although IL-22 plays a critical role in some Th17-associated diseases, it is most closely related to IFN-γ and it is differentially regulated in respect to IL-17 [168] [169].

Finally, another novel IL-10-like cytokine named IL-26 is expressed in Th1 and Th17 cells and has not a murine homologue. Interestingly IL-26 secreting Th17 seems to be involved in gut inflammation [170].

Human and mouse Th17 express the chemokine receptor CCR6. In human $CCR6^+$ $CXCR3^+$ are Th1 or Th1/Th17 double positive, while $CCR6^+$ $CCR4^+$ are Th17 and are the main populations expressing RORc, the human ortholog of murine RORγt [171].

Interestingly, since Th17 cells express also CCL20, the ligand of CCR6, they can recruit other Th17 and Th1 cells to the inflamed tissue [172].

Comparison of Th17 cells in human and mouse

If the role of IL-1β in human Th17 development is still not clear. In general, IL-1β favors Th17 polarization, but in the mouse it seems to have a marginal effect, while in the human cell systems IL-1β would play a fundamental role during initial priming [149]. However, the case of TGFβ has been even more debated. Indeed, the ambiguous effect of this highly pleiotropic cytokine on Th17 cells gave rise to completely opposite hypotheses. Some groups claimed that in human systems TGFβ could inhibit Th17 development [142] [149] [173]. On the other side, these works where criticized starting from technical observations. For example, TGFβ is found in culture media used for human cell culture and it could appear erroneously dispensable. Human Th17 seems to be more sensitive to the TGFβ concentration.

Another important difference between human and mouse T17 subset is the presence of double positive Th1/Th17 cells (expressing both IFNγ and IL-17), a population that in the human can constitute the half of the IL-17 positive CD4 population [171]. This observation open many question about the observed antagonism between Th1 and Th17 subset. A general consideration regarding human T helper differentiation is that it is more flexible then the mouse one, as already observed in human double positive IL-4/IFNγ T helper cells. What is not clear is

whether double positive T helper origin directly from a naïve Th0 or from a single positive subset, maybe during a transitional phase.
Differently then mouse Th17 cells, RORC, is induced by IL-1β, IL-6 and IL-23 [149] [173].
However, a difference between mouse and human systems is expected first considering the differences in the cell types used as models. Moreover, naïve T cells from human peripheral blood develop in a more stimulating environment compared to mouse models housed in pathogen free facilities, and this could explain differences in polarizing capacity in presence of a certain cytokine milieu [143]. Indeed, another group showed that cord blood naïve T cells could be differentiated in Th17 cells [174]. Later Volpe and colleagues showed that a specific cytokine cocktail including TGFβ can also generate Th17 from adult peripheral blood naïve T cells too [150]. Remarkable, only the combination of TGFβ, IL-23, IL-6, IL-1β and (at less extent) TNFα could induce human Th17 differentiation, and none of these cytokines alone were sufficient neither necessary to induce Th17 cells.
While the knowledge of Th17 subset in human was initially less defined then mouse data, and often not comparable with the murine model, the human/mouse gap is narrowing with the discovering of many new feature and a better characterization of both human and mouse systems. However, this difference has to be taken in serious consideration whenever considering the

translational potential of many project started from observations in mouse models.

The role of Th17 in immune-mediated diseases

Since its discovery, Th17 subset has been associated with many autoimmune diseases and immune deregulations, that sometimes erroneously were thought to be mediated by Th1 response. As discussed before, one of the most important autoimmune disease historically associated to Th17 discover is multiple sclerosis (MS) and its mouse model experimental autoimmune/allergic encephalomyelitis (EAE).

IL-17 has been associated to multiple sclerosis years before the definition of Th17 cells as T cell subset, because of high levels of IL-17 found in blood and cerebrospinal fluid of MS patients [175].

Later studies on mouse models of EAE finally defined a role for Th17 cells and IL-23 [128]. Unfortunately, few experiments have been done to assay the functional role of IL-17 in human demyelinization disease. Recently a clinical studies showed a correlation between IL-17 levels in MS patients and spinal cord [176]. Pathogenic T cells in MS patients express IL-17, IL-22 or both and seems to have higher efficiency of migration through blood-brain barrier endothelium [177]. Double positive $IL17^{+}/IL22^{+}$ can kill in vitro neuronal cells [177].

Another important autoimmune disease related to Th17 subset is rheumatoid arthritis (RA), a chronic disease characterized by inflammation of synovial tissues in multiple joints that leads to bone and cartilage damage. IL-12p40 and IL-23p19 deficient mice are protected from collagen induced arthritis (CIA), the prototypical mouse model for RA, while neutralizing antibodies against IL-12 and IFNγ worst the disease [123, 178]. The effect of IFNγ in RA pathogenesis strongly depends on the timing of administration: in early stage it mediates pathogenesis while in late phase it results in protection [179]. As for IFNγ in late phase, IL-4 is a potent suppressor of CIA severity, probably due to the antagonism of IL-4 induced Th2 subset against Th17 one [180]. Conversely, IL-17 deficient mice alleviate joint inflammation, despite they are not completely protected from the disease development [181]. IL-17 is increased in sera and synovial fluid of RA patients and its concentration is predictive of severe joint damage progression [182]. TNFα and IL-1β can mediate Th17-activation in RA pathology and IL-17 can in turn induce TNFα and IL-1β expression in synoviocytes, supporting a local autoinflammatory loop. TNFα and more recently IL-1β are widely and successfully used as target for RA clinical trials. However, studies on mouse model suggested

that the combination of TNFα, IL-1beta and IL-17 blockade could improve RA therapy, especially considering the resistance of many patients to anti-TNFα clinical trials [183]. Indeed, the arthritic joints present a unique microenvironment where cytokine-mediated paracrine loops sustain the auto-inflammatory process. Thus, targeting at least one of these positive feedbacks may reduce significantly the inflammation in the joint without the risk of affecting systemic immune response [143].

Th17 involvement has been observed in other severe rheumatic disease as systemic sclerosis and reactive arthritis/undifferentiated spondyloarthropathy [184]. As for autoimmune encephalomyelitis, IL-12p40 has been associated with psoriasis. Initially it suggested an involvement of Th1 response, later replaced by IL-23-Th17 axis [185]. Psoriatic skin is characterized by increased expression of RORc, IL-6, IL-1β, and IL-23 and IL-22 is elevated in blood of psoriatic patients [186]. Moreover, when injected into mouse skin, IL-23 induces inflammation mediated by TNF, IL-22, IL-17 [173].

At less extent, Th17 has been associated with other autoimmune diseases as autoimmune myocarditis, endometriosis, autoimmune uveitis, and also endocrine autoimmune diseases such as type 1 diabetis and Hashimoto`s tyroiditis [143]. Other auto-inflammatory diseases emerging as associated to Th17 response are inflammatory bowel disease such as Crohn`s disease and ulcerative colitis [187]. IL23 has been related to susceptibility to gut inflammation both in human and mouse [188, 189]. However, IL-

1β, IL-6, and TNFα are important mediator of intestinal diseases [190], despite their not completely defined role in Th17 differentiation. Thus, target therapy blocking TNFα, IL-23 or IL-1β and IL-6 pathways could act on several levels, regulating both direct effect of these cytokines gut inflammation, as well as inhibiting the Th17polarization activity.

Surprisingly, Th17 have been found not only in diseases previously related to Th1 response, but also in Th2-driven pathologies as allergy. Indeed, besides autimmunity, Th17 cells are also involved in allergic diseases as asthma, atopic dermatitis and contact hypersensitivity [191-193]. Considering the involvement in psoriasis and dermatitis, it seems that Th17 cells are often associated to skin inflammation.

Th17 cells and infections

The role of Th17 in infections has been less investigated compared to the relation with autoimmune disorders. Nonetheless, the study of Th17 cells in the control of infective diseases is critical firstly to define possible manipulation in vaccine design, secondly to control potential side effects of Th17-suppression in clinical trials for autoimmune patients.

The unique role of IL17 in the protection from bacterial infection was known already before the definition of the Th17 subset. In fact

IL-17 is crucial for resistance to *Klebsiella pneumoniae* infection, not controlled by Th1 neither Th2 response [194].

A central role of Th17 in *Mycobacteria tuberculosis* control has been observed as a side effect in clinical trial for RA and inflammatory bowel diseases. In these clinical studies the inhibition of Th17 activation, aimed to control the autoimmune response, led to re-activation of mycobacteria, indicating a role for Th17 in the control of latent infections. However, the control of *Mycobacterium tuberculosis* by adaptive system seems to involve mainly Th1 response and the importance of Th17 response is not completely defined yet [195].

Th17 response is crucial for protection from other gram negative bacteria such as *Pseudomonas, Escherichia coli, Salmonella and Bordetella* species, as well as *Mycoplasma pneumoniae* and *Streptococcus pneumoniae*. Indeed Th17 are often related to airways infection and protective effect is often due to pleiotropic function of IL-17 that leads to neutrophil chemotaxis and clearance of bacteria [196]. This protective mechanism can start with TLR4 stimulation on DC by infective agents, followed by IL-23 production and T cell activation [197]. Notably, innate response plays a central role in Th17 activation by infective agents; this aspect will be broadly discussed later.

Although Th17 cells seems to be physiological programmed to protect against infections, in some cases IL-17 and IL-23 has been associated with unfavorable outcome of infective diseases. Indeed

it is know for some infections that the over-activation of immune system can be more deleterious then the spreading of the infective agents. In those cases, a therapy increasing in Th17 response could lead to tissue damage, as observed in *Heliocobacter pylori* gastric infections [198].

The deleterious role of Th17 cells in infections is probably limited to some infective diseases, including fungal infections, such as *Candida albicans* infections [199]. Indeed, Th17 subset has been also associated with the control of fungal and parasites infections [200, 201]. The relation between Th17 subset and fungal infections is even more complex then the bacterial one, especially if we consider the complexity of fungal organisms (multicellular, hyphal versus yeast) and the bigger differences in interactions with hosts (mouse versus human) compared to bacteria. Moreover, fungi often have an important balance between opportunistic and symbiontic activity. For this reason a balanced Th17 response is important to avoid fungal infections but uncontrolled activation can lead to dangerous side effects [143].

If the relation between Th17 and infective organism has been widely studies, less is know about the interaction with viruses. Paradoxixally IL-17 cytokine has been discovered in 1995, in the Herpesvirus saimiri, which carries mammalian gene homologs [125]. It is now known that other viruses can induce Th17 response [202], but a protective or pathogenic role of Th17 cells in viral infections has not been defined yet.

Th17 in cancer and transplantation

The close relation between Th17 and Treg differentiation, the antagonistic effect on Th1 response and the specificity for extra cellular pathogens, instead of intracellular ones, could probably suggest that Th17 subset is not controlling diseases such as cancer and virus infections which are mainly related to Th1 response and intracellular perturbations [203]. Indeed, despite the high immunological potential, IL-17 has been mainly considered favorable to tumor growth, maybe for its positive effect on angiogenesis. Moreover, tumor cells express IL-17 receptor, and IL-23 seems to promote tumor growth [204].

Finally IL-17 has been related to allograft rejection [205], but a central involvement in transplant control has not been demonstrated yet.

1.6

Dendritic cells and Th17 polarization

Accordingly to Janeway's model, the fate of T cell polarization is determined by DCs co-stimulatory molecules and cytokine production at the moment of antigen presentation; whereas the phenotype of the priming DC depends on the kind of pathogenic signal received. Similarly to the other T helper subsets, it is likely that patho/physiological Th17 response is initiated by DCs, as supported by studies in EAE model [206], and the activation of Th17 by specific DCs should depend on stimulation by specific danger signals.

Th17-priming DCs and infectious signals

In the last years, the polarization of Th17 subset has been widely investigated, and the cytokines responsible to induce Th17 activation in vitro have been widely defined for both mouse and human, as described previously. However, less is known about the

stimuli responsible to activate DCs towards a Th17-priming phenotype.

One example of DC-mediated Th17-priming is the study of DC activation by fungal infection. DCs can be activate by the signaling of C-type lectin receptor, *Dectin-1*, able to recognize fungal beta-glucans, such as *curdlan*. The engagement of dectin-1 in DCs recruits Syk, which activates the signaling cascade leading to NFkB and MAP-kinase activation through the *CARMA-1 related adaptor protein (CARD)-9*. This kind of activation leads to the secretion of IL-10 and IL-2, as well as pro-inflammatory cytokines TNFα, IL-6 and IL-23, but little IL-12. Notably, DCs activated by Dectin-1 agonists can prime Th17 in vitro and in vivo, and curdlan can act as a potent adjuvant in ovalbumin-antigen immunization. Interestingly in this system TGFβ, as well as TNFα and IL-23p19 were crucial for Th17 priming. Recently, a work from the same group showed a similar pathway of DC-mediated Th17 activation via Dectin-2 [49].

Although TGFβ plays a central role in Th17-priming, it is not clear whether DCs are the main source TGFβ *in vivo*, since DCs are not highly TGFβ producers. However it is know that mature DCs express the integrin αVβ8, responsible for the cleavage of inactive TGFβ to the active form, suggesting a possible indirect mechanism able to sustain Th17 activation [207].

The stimulatory conditions inducing Th17-priming APCs have been under investigation also in human cell systems. Differently of mouse, differentiation of human Th17 is more challenging, and the comparison between human and mouse experiments is not always possible, considering the different protocols and cell system used. In 2007 Acosta-Rodriguez and colleagues, pointed out how the different APC types and pathogen derived stimulatory conditions determined T cell polarization towards Th17 or Th1 [149]. As confirmed by other groups, peptidoglican promotes Th17-driving activation of APC, while LPS induces mainly Th1 subset [208]. However, in this study [149], Th17 polarization was possible only using fresh APCs, such as circulating $CD14^+$monocytes and $CD1c^+$DCs. Moreover the secretion of IL-1β was obtained only in these cells type, and was low or undetectable in IL-4-derived DCs. Contrarily, IL-23 was absent in monocytes, but it could be induced in IL-4-derived DCs in presence of CD40L costimolation.

Th17-priming DC and endogenous signals

Interestingly, a recent work pointed out that the phagocytosis of infected apoptotic cells by DC drives Th17 polarization. Indeed, inactive TGFβ is activated by BMDCs upon phacytosis of apoptotic cell, leading to Treg activation, but in presence of bacterial

stimulus, IL-6 is produced, and the combination of the two cytokines induces Th17 differentiation [209]. Moreover the central role of apoptosis in driving Th17-response to infections was showed *in vivo* [209]. This result suggests that the danger signal coming from cell death can play a crucial role for Th17 polarization, and, on the other side, the presence of apoptosis in absence of infections lead to the control of the response, through Treg activation.

Remarkably, ATP induce CD11c ^{low} cells derived from gut lamina propria to produce of IL-6, IL-23 and TGFβ, driving Th17 differentiation. The source of ATP in the gut in mainly attributed to intestinal commensal bacteria, and it could support the physiological immuno-surveillance to intestinal infections, as well as the pathological inflammatory response in case of breaking of the intestinal flora homeostasis. However, it can be suggested that this mechanism is induced in other conditions where the source of ATP is a damaged self-tissue.

Besides these interesting works, not much has been investigated regarding the role of ATP or other endogenous signals in Th17 activation by DCs [107]. However, a recent work suggested a connection between inflammasome activation and Th17 polarization. A mouse model of NLPR3-gain-of-function mutation, leading to hyperactivation of the inflammasome, shows a dominant Th17 response, characterized by skin inflammation and increased susceptibility to delayed-type hypersensitivity [107]. Macrophages

derived from these mice were more prone to IL-1β production upon TLR-stimulation, confirming a central role of IL-1β in Th17 polarization in vivo. However, this model is representative of autoinflammatory monogenic disorders associated to NLRP3 mutations, but a clear role of endogenous signals in NLRP3-mediated Th17 differentiation is still to be defined.

Scope of the thesis

Dendritic cells play a central role in the regulation of innate and adaptive immunity, and exert their sensor functions through a plethora of receptors, including the NLRP3-inflammasome. The role of NLRP3-inflammasome in sensing endogenous danger signals, such as MSU, has been deeply investigated in the last years. However the exact mechanism of MSU activation of DCs through NLPR3 is not completely defined yet. Moreover, little is known about the adjuvant role of MSU as promoter of DC-mediated priming of specific T cell subsets.

Starting from these observations, the present work aims to describe evidences of the role of endogenous danger signals in the activation of the immune system. In particular, we investigated which is the role of MSU and its putative receptor NLRP3 in the activation of DCs and how this process is involved in starting both innate and adaptive immunity.

The experimental work is articulated in two main parts, describing the study of MSU crystals in the context of innate immune activation of dendritic cells, and DC-mediated immune priming of adaptive immunity, respectively.

The first part (Chapter 2), focusing on dendritic cell activation, defines how two NLR pathways activated by MSU crystals and

MDP interplay at transcriptional level leading to a specific pro-inflammatory signature. The second part (Chapter 3) shows how dendritic cells stimulated by MSU crystals, in presence of a second inflammatory signal, can prime naïve T cells towards a Th17 polarized phenotype.

Both parts elucidate the rationale of the experimental plan, the methods used, and the resulted obtained. The overall meaning of the results and its scientific contribute is deeply discussed at the end of every chapter.

Finally (Chapter 4), the findings of the two Chapters are analyzed considering their significance in the scenario of human diseases, and their possible impacts on the translational medicine.

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Chapter 2

Synergism of NLRP3 and NOD2 promotes a unique transcriptional profile in murine dendritic cells

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Rationale and Biological question

The activation of NLRP3 in dendritic cells leads to the central event of ASC-mediated Casp-1 recruitment to inflammasome platform, and IL-1β cleavage and secretion. Initially, a two-step model was assumed, that a first stimulus (TLR or NLR-agonist) would prime DCs (or macrophages) leading transcription and translation of pro-IL-1β, while an inflammasome activator, such as ATP or MSU crystals, would induce pro-IL-1β cleavage to mature form and secretion.

However, recent findings highlight large gaps in our understanding of the two-step model. Firstly, a classical inflammasome activator as ATP seems not to activate inflammasome formation in absence of another pro-inflammatory stimulus [1-3]. According to this hypothesis, the activation NFκB by TLR- or NLR-ligands would induce not only pro-IL-1β transcription but also NLRP3 expression, allowing proper inflammasome formation and activation [4]. Secondly, ATP could be responsible for cell membrane permeabilization, allowing cytosolic access to microbial products [1]. In this model, LPS and MDP are thought to be the actual inflammasome activators [5].

Besides the issue related to the two-step model, other aspects of MSU effect on DCs are still to be defined. Indeed, apart IL-1β and

IL-18 activation, little is known about the mechanism of MSU mediated-activation of DC during inflammation. It is likely that MSU crystals promote transcription of inflammatory mediators in innate cells, eventually through ASC-mediated signaling to transcriptional factors [6].

However, in many studies, the potential transcriptomic activity of MSU was obscured by the stronger capacity of priming microbial stimuli, such as LPS, to modulate gene expression. Starting from these observations we investigated the role of NLRP3-activation by MSU in the modulation of DC transcriptional profiles, focusing on the role of the priming signal in sustaining MSU-induced pro-inflammatory phenotype.

To prime the cells for inflammasome activation the NOD2 agonist MDP was used. MDP was selected for the current study because it is considered a weak activator of innate cells when compared to TLR agonists, such as LPS or CpG [7], allowing its interplay with MSU to be revealed at the transcriptional level.

Although a direct structural interaction between MDP and NOD2 has not yet been demonstrated, NOD2-deficient macrophages cannot activate NFκB and MAP kinase pathways in response to MDP stimulation [8, 9]. Indeed, MDP acts via activation of the CARD-kinase RIP2, and is a triggering signal for both the MAP kinase pathways and the transduction cascade activating the transcription factor NFκB [10] [11].

Thus, using microarray analysis we investigated transcriptional response in murine DCs stimulated with NOD2 agonist MDP, and NLRP3 activator MSU. We identified transcriptional signatures of both MDP and MSU, and a unique signature associated with combined stimulation from MSU and MDP.

Results

MDP, MSU and MDP/MSU stimulation induce distinct transcriptional signatures in BMDC.

BMDC were stimulated with MDP (NOD2 agonist), MSU (NLRP3 activator), and a combination of MDP and MSU (MDP/MSU) for 4 hours. Cellular transcriptomes were generated using high-density mouse oligonucleotide arrays, interrogating 45,101 transcripts. Differentially expressed genes (DEG) were selected over a threshold of Log2 (Ratios) of 1.5-fold among the treatments compared to the control at the time of stimulation (time-point 0 hours). Using an unsupervised hierarchical cluster analysis, we identified the differences in gene expression among MDP, MSU, and MDP/MSU responses. As shown in Figure 1a, the response to MDP was different compared to MSU and MDP/MSU. Comparing the DEG expression profiles in the three stimulatory conditions, we observed that MDP alone induced poor transcriptional up-regulation, whereas MSU and MDP/MSU triggered a stronger up- and down-regulation of many genes (Figure 1b). Interestingly the transcriptional profile induced by MDP/MSU in BMDC was more similar to MSU than MDP indicating that MDP enhanced MSU-

mediated gene expression, but MSU was the stimulus dictating the molecular signature (Figure 2a).

In order to define the ability of MDP, MSU, and MDP/MSU to induce specific genetic reprogramming, we next compared the global gene expression changes in BMDC treated with the three stimuli. A co-response of 116 genes identified as differentially expressed overlapped among the MDP-, MSU-, and MDP/MSU-treated BMDC. MDP and MSU stimulation revealed clear differences in gene regulation. Indeed, 315 DEG were exclusively modulated in MDP-treated BMDC, whereas only 37 are exclusive for MSU-induced genes since most of them overlapped with MDP/MSU condition, emphasizing their similarities (Figure 2b). Moreover, it is noteworthy that 468 DEG were expressed exclusively after MDP/MSU co-stimulation but they were absent in MDP- or MSU-stimulated BMDC (Figure 1a right column, and Figure 2b). Functional analysis revealed that MDP/MSU-specific genes were related to inflammation, the immune response, cell motility and matrix remodeling (data not shown).

These results indicate that MSU can be considered a strong modulator of gene transcription in BMDC, and the combination MDP/MSU increases MSU-induced genes leading to the expression of unique MDP/MSU-specific genes.

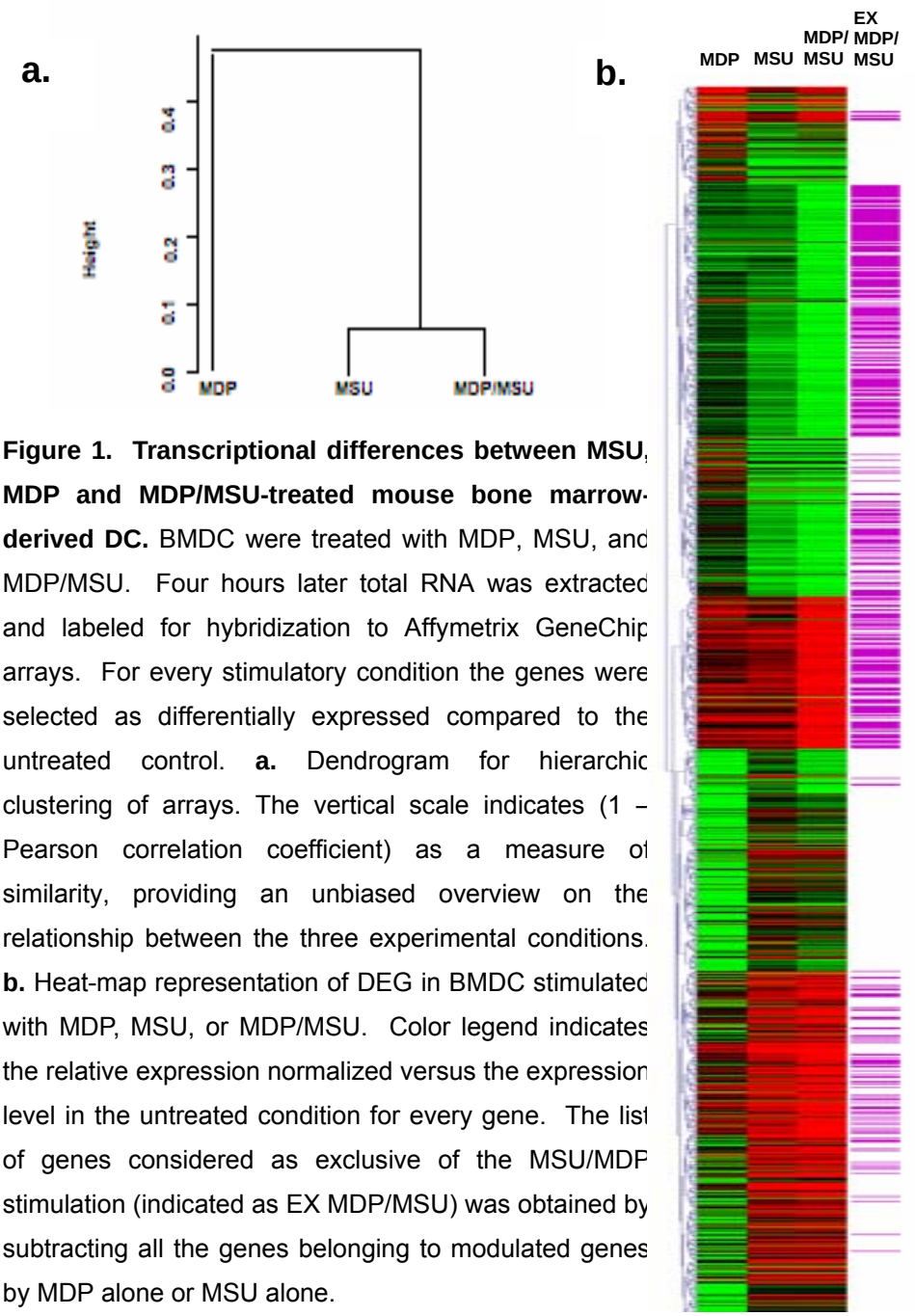

Figure 1. Transcriptional differences between MSU, MDP and MDP/MSU-treated mouse bone marrow-derived DC. BMDC were treated with MDP, MSU, and MDP/MSU. Four hours later total RNA was extracted and labeled for hybridization to Affymetrix GeneChip arrays. For every stimulatory condition the genes were selected as differentially expressed compared to the untreated control. **a.** Dendrogram for hierarchic clustering of arrays. The vertical scale indicates (1 – Pearson correlation coefficient) as a measure of similarity, providing an unbiased overview on the relationship between the three experimental conditions. **b.** Heat-map representation of DEG in BMDC stimulated with MDP, MSU, or MDP/MSU. Color legend indicates the relative expression normalized versus the expression level in the untreated condition for every gene. The list of genes considered as exclusive of the MSU/MDP stimulation (indicated as EX MDP/MSU) was obtained by subtracting all the genes belonging to modulated genes by MDP alone or MSU alone.

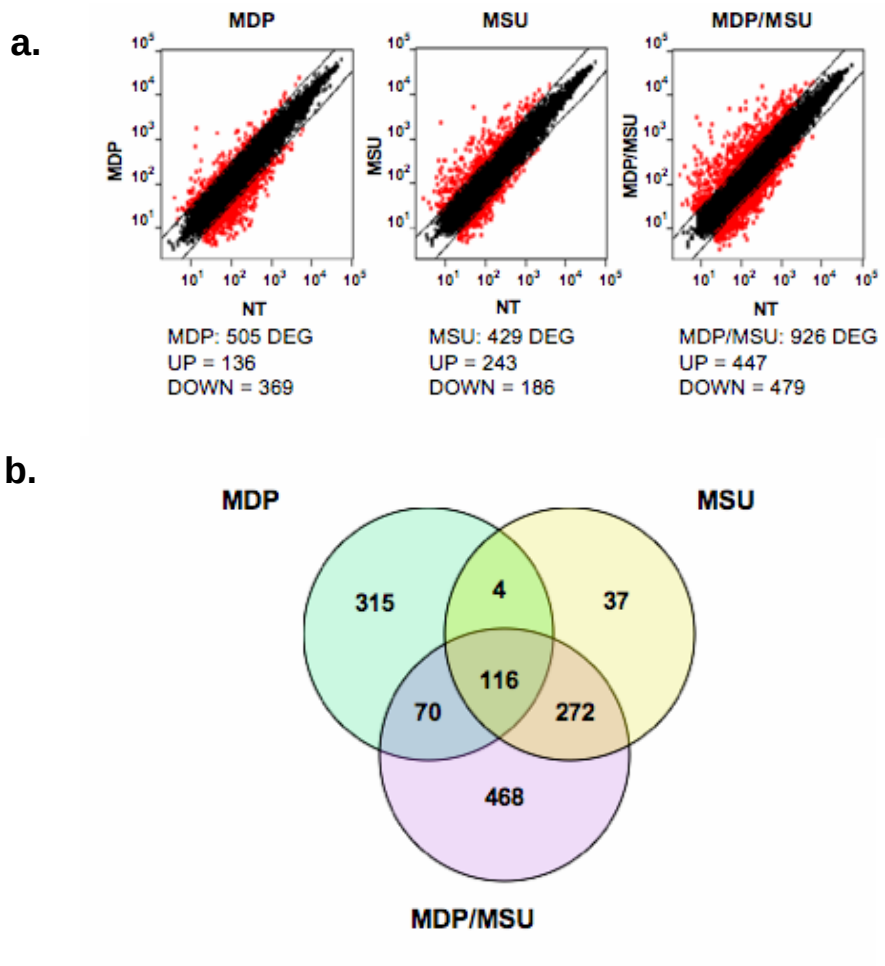

Figure 2. Transcriptional differences between MSU, MDP and MDP/MSU-treated mouse bone marrow-derived DC. a. Log scatter plots representing the DEG identified in MDP-, MSU-, and MDP/MSU-treated BMDC compared to untreated control (UT). **b**. Venn diagram showing the overlap between the 1282 genes identified as differentially expressed either in MDP, MSU or MDP/MSU-treated BMDC.

MDP/MSU stimulation induces modulation of cytokine- and chemokine-coding genes related to inflammation.

In order to characterize the role of genes associated with DC function in response to MDP, MSU, and MDP/MSU stimulation at 4 hours, we performed gene functional classification on the basis of the annotation resources provided by Gene Ontology. We identified families of cytokine and chemokine genes that were strongly up-regulated by MSU alone or MDP/MSU stimulation compared to MDP alone. However, the MDP/MSU condition induced the strongest gene up-regulation, suggesting a synergistic effect of the two stimuli (Figure 3a).

We validated the mRNA expression changes measured by the microarrays for some cytokines and chemokines. BMDC were stimulated with MDP, MSU or MDP/MSU for 2, 4, 8, 18, and 24 hours. mRNA from three independent experiments was extracted, retrotranscribed into cDNA, and used as templates for quantitative PCR. Upon MDP/MSU stimulation we observed a significant up-regulation of IL-1 family cytokine genes, including il1-α (il1a), il1-β (il1b), and to a lesser extent, il1r antagonist (il1rn), as well as il6 and tnf-α (tnf) (Figure 4). The expression of il-1β (p35) protein in response to MDP, MSU, and MDP/MSU stimulation was further validated by western immunoblot (Figure 3b). Interestingly il2, il12b

and il23a were exclusively up-regulated by MDP/MSU stimulation (Figure 2b). Lastly, the expression profile of the pro-inflammatory chemokines cxcl1 and cxcl2 was clearly enhanced by combining MDP/MSU, while ccl3 expression was up-regulated also in presence of MSU alone (Figure 4).

Our data suggests that the NOD2 and NLRP3 signaling pathways induced by MDP/MSU may synergize, leading to a unique transcriptional regulation of cytokine and chemokine genes.

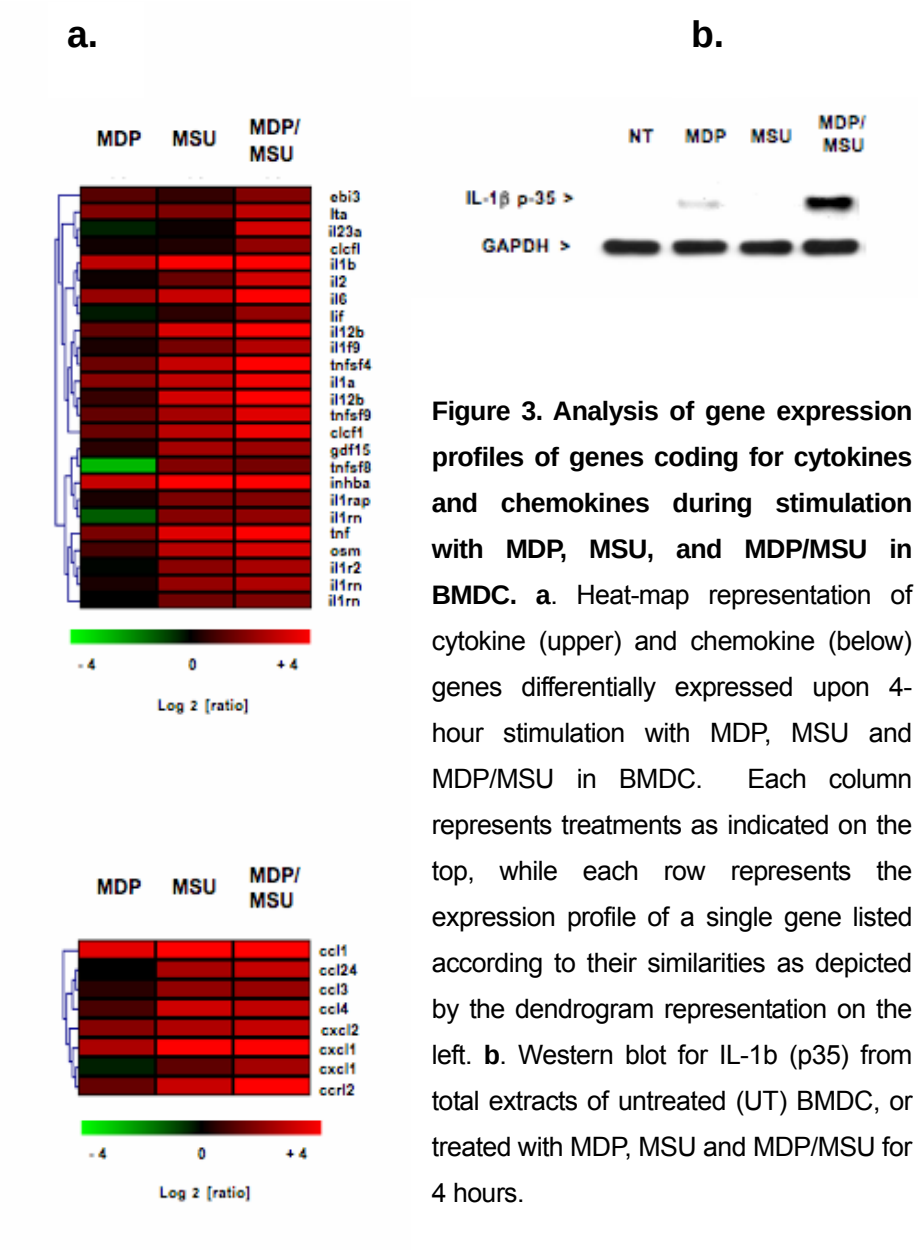

Figure 3. Analysis of gene expression profiles of genes coding for cytokines and chemokines during stimulation with MDP, MSU, and MDP/MSU in BMDC. a. Heat-map representation of cytokine (upper) and chemokine (below) genes differentially expressed upon 4-hour stimulation with MDP, MSU and MDP/MSU in BMDC. Each column represents treatments as indicated on the top, while each row represents the expression profile of a single gene listed according to their similarities as depicted by the dendrogram representation on the left. **b**. Western blot for IL-1b (p35) from total extracts of untreated (UT) BMDC, or treated with MDP, MSU and MDP/MSU for 4 hours.

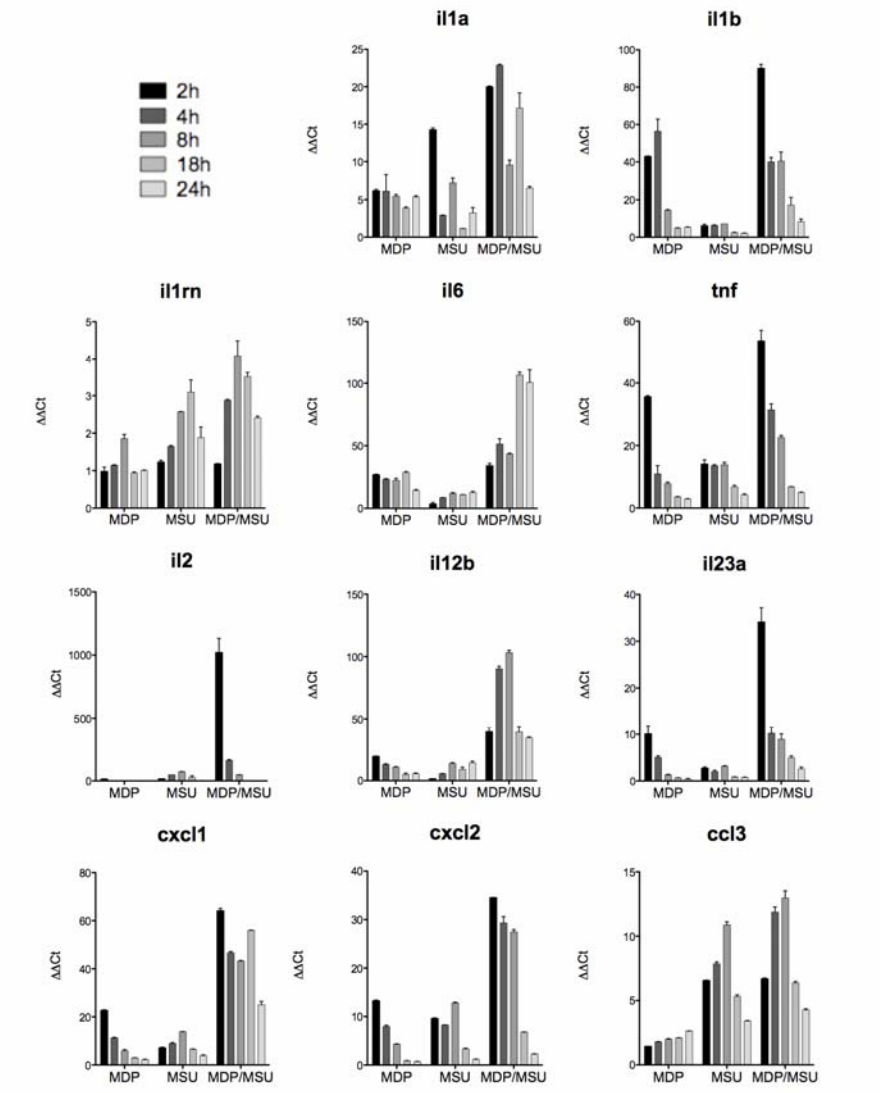

Figure 4. MSU synergizes with MDP in the up-regulation of cytokine and chemokine expression. Profiles of il-1b (il1b), il-1a (il1a), il-1r antagonist (il1rn), il6, tnf a (tnf), il12b, il2, and il23a, cxcl1, cxcl2, ccl3 genes in BMDC stimulated with MDP, MSU or MDP/MSU for the indicated time points as assessed by quantitative RT-PCR analysis. ΔΔCt, difference with GAPDH Ct and untreated Ct.

BMDC activated by MDP/MSU exhibited an inflammatory phenotype characterized by up-regulation of integrins and co-stimulatory molecules.

Upon activation BMDC initiate genetic reprogramming leading to maturation, which is associated with up-regulation of costimulatory molecules required for T-cell help, antigen presentation, and adhesion. Thus, we studied the transcriptional profile of integrins and maturation markers induced by MDP, MSU, and MDP/MSU stimulation in BMDC. We identified the integrin-coding genes itgav, itgb3, and itga5 as up-regulated in response to MDP/MSU, suggesting increased adhesion properties and cell-cell interactions in BMDC at early time points (Figure 5). Interestingly, some integrins, such as CD47, have been recently shown in DC to be involved in signal transduction, as well as phagocytic processes, suggesting a possible involvement of integrins in the mechanism of MSU interaction with DC [12, 13].

Moreover, flow cytometric analysis revealed increased expression of co-stimulatory and antigen-presenting molecules, such as CD80 (B7.1), CD86 (B7.2) and MHC class II, after 24 hours stimulation with MDP/MSU (Figure 5a and 5b). In accordance with the transcriptome data CD40 expression was up-regulated mainly by MDP stimulation alone.

a. **b.**

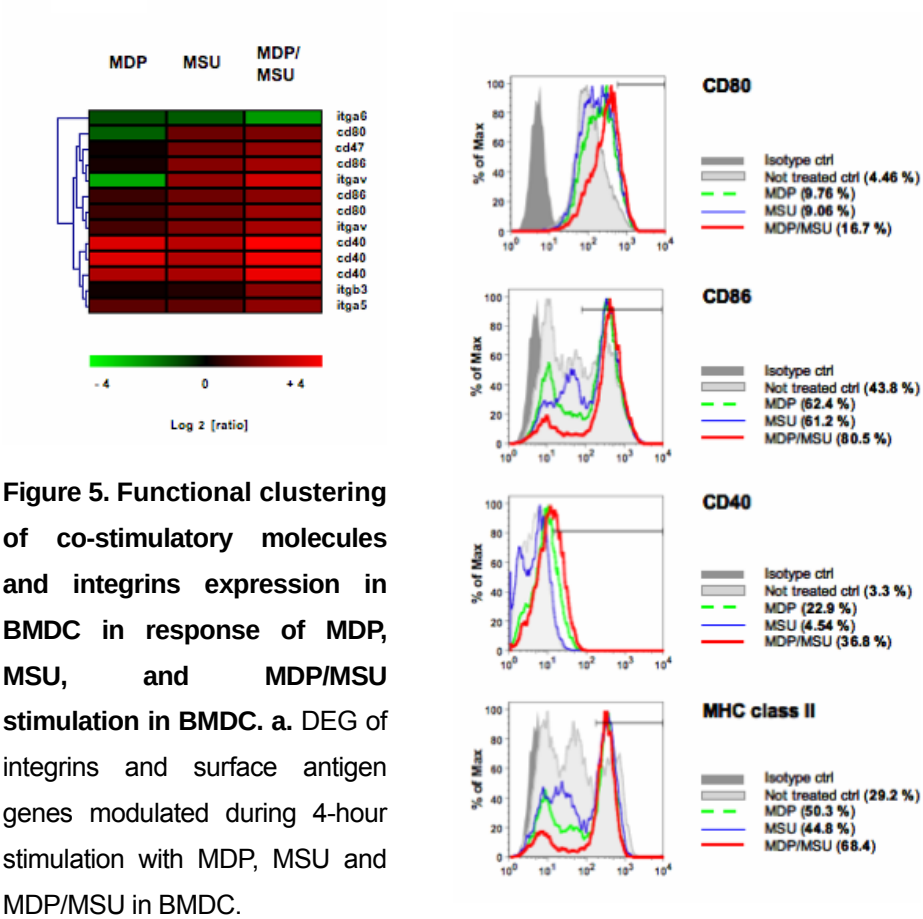

Figure 5. Functional clustering of co-stimulatory molecules and integrins expression in BMDC in response of MDP, MSU, and MDP/MSU stimulation in BMDC. a. DEG of integrins and surface antigen genes modulated during 4-hour stimulation with MDP, MSU and MDP/MSU in BMDC.

b. Surface expression of BMDC maturation markers following treatment with MDP, MSU, and MDP/MSU performed at 24 hours after stimulation. Each plot shows superimposed histograms representing the surface expression of the CD80, CD86, CD40, and MHC class II, as assessed by flow cytometry. Different line styles correspond to distinct BMDC treatments, the isotype and untreated controls, according to the legend.

Synergism of MDP/MSU in DC activation involves mitogen-activated protein (MAP) kinase signaling pathways.

The synergistic effects induced by MDP/MSU treatment of BMDC may be the result of the activation of additional signaling pathways as a consequence of concurrent NOD2/NLRP3 stimulation. Thus, we selected DEG coding for transcription factors from microarray data by using Gene Ontology. We found that most of the genes coding for transcriptional factors were up-regulated in response to MDP/MSU stimulation (Figure 6a). Interestingly, transcription factors pivotal for DC activation, such as stat1, stat5a, irf4, nf-κb family members, junB, and fosb genes, were strongly induced by MDP/MSU. It has been shown that the expression of genes coding for transcription factors regulated by post-transductional modifications is informative concerning their involvement in signal transduction. Therefore, to determine the contribution of MAP kinase signaling pathways to the activation of BMDC in response to MDP, MSU, and MDP/MSU, we investigated the phosphorylation of cJUN (Ser^{73}), ERK1/2 (Thr^{183} and Tyr^{185}), and p38 (Thr^{180}, Tyr^{182}), at early time points (15, 30, 60, 120 minutes) by western blot. GAPDH was included as a loading control. MDP, MSU, and MDP/MSU all induced ERK1/2 and p38 phosphorylation (Figure 6b). Of note, c-Jun (Ser^{73}) was strongly phosphorylated by

MDP/MSU compared to MDP or MSU alone (Figure 6b), and this was further confirmed by phosphoprotein assay (Figure 6c).

Collectively these data suggest that the presence of MSU might sustain the capacity of MDP to activate the MAP kinase pathway.

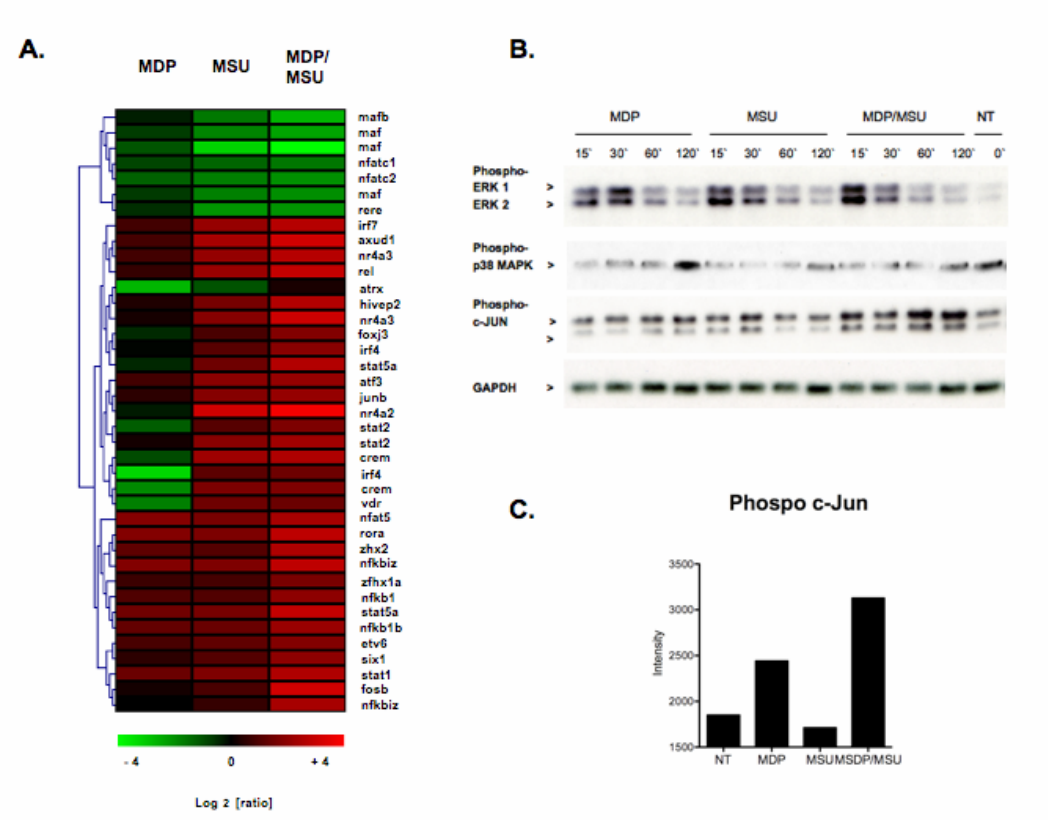

Figure 6. Specific expression profile and activation of transcription modulators by MDP/MSU activation in BMDC. a. Microarray data representation of genes coding for transcription factors induced by MDP, MSU and MDP/MSU stimulation in BMDC. **b.** Western Blot analysis to assess phosphorylation of c-JUN (Ser^{73}), p44/p42 ERK 1/2 (Thr^{183}, Tyr^{185} on ERK2), and p38 MAP kinase (Thr^{180}, Tyr^{182}) compared with GAPDH expression as housekeeping, in BMDC stimulated for 15, 30, 60 or 120 minutes in response to MDP, MSU, and MDP/MSU stimulation, or untreated control (UT). **c.** Phosphoprotein assay for detection of phosphorylated c-JUN (Ser^{73}) from BMDC stimulated with MDP, MSU and MDP/MSU for 2 hours.

MDP/MSU stimulation induces exclusive up-regulation of genes related to inflammation and intracellular transport.

In order to dissect the specific transcriptional signature of the interaction between MDP and MSU, we ranked the top 30 up-regulated genes belonging to the group of DEG exclusively expressed following MDP/MSU stimulation (Table I). The ranking was based on the value of fold change expression of every gene, normalized versus the untreated condition. We observed that the cytokine-coding genes il2, il23a, il1f9, already found as exclusive in previous functional analysis, ranked in the first positions, as well as other inflammation-related genes such as the transcriptional factor fosb, integrin β 8 (Itgb8), tumor necrosis factor receptor superfamily, member 13c (Tnfrsf13c), and the immune semaphorin 7a (Sema7a) (Table I). Interestingly, we identified genes coding for cell plasma membrane-mediated processes, such as endocytosis, (caveolin 1, Cav1; Rab11 GTPase Fip1, Rab11Fip1), ion exchange (Gap junction 43 kDa heart protein, Gja1; solute carrier family 22 -organic cation transporter member 4, Slc22a4; solute carrier family 15 H+/peptide transporter- member 2, Slc15a2) and transmembrane molecules involved in signaling pathways, including the G protein-coupled receptor, family C, group 5, member A (Gprc5a) (Table I). Overall it seems that genes

related to inflammation and to plasma membrane activities where the most up-regulated DEG in response to double stimulation by MDP/MSU.

Table I. Top 30 genes exclusively induced by MDP/MSU stimulation in DC

Gene Name	Description	FC[(a)]	Log2 (R)[(b)]
Cav1	caveolin, caveolae protein 1	9.70	3.27
Il2	interleukin 2	9.26	3.21
Il23a	interleukin 23, α subunit p19	9.20	3.20
Fosb	FBJ osteosarcoma oncogene B	9.00	3.16
Rab11fip 1	RAB11 family interacting protein 1 (class I)	7.53	2.91
Gja1	gap junction membrane channel protein α 1	7.40	2.89
Pla1a	phospholipase A1 member A	7.32	2.87
Il1f9	interleukin 1 family, member 9	6.20	2.63
Ets1	E26 avian leukemia oncogene 1, 5' domain	6.11	2.61
Per1	period homolog 1 (Drosophila)	5.86	2.55
Zhx2	zinc fingers and homeoboxes protein 2	5.81	2.54
Btbd4	BTB (POZ) domain containing 4	5.76	2.52
Gprc5a	G protein-coupled receptor, family C, group 5, member A	5.70	2.51
Hdc	histidine decarboxylase	5.70	2.51
Siglecg	sialic acid binding Ig-like lectin G	5.65	2.50
Col27a1	procollagen, type XXVII, α 1	5.64	2.50
Tmtc2	transmembrane and tetratricopeptide repeat containing 2	5.52	2.46
Slc22a4	solute carrier family 22 (organic cation transporter), member 4	5.49	2.45
Rtn4rl2	reticulon 4 receptor-like 2	5.19	2.37
Zwint	ZW10 interactor	5.14	2.36
Hectd2	HECT domain containing 2	5.05	2.33
Itgb8	integrin β 8	5.04	2.33
Pank2	pantothenate kinase 2 (Hallervorden-Spatz syndrome)	5.02	2.33
Tnfrsf13c	tumor necrosis factor receptor superfamily, member 13c	5.02	2.33
Slc15a2	solute carrier family 15 (H+/peptide transporter), member 2	5.00	2.32
Htra1	HtrA serine peptidase 1	4.99	2.31
Rusc2	RUN and SH3 domain containing 2	4.97	2.31
Sema7a	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	4.93	2.30
Tomm70 a	translocase of outer mitochondrial membrane 70 homolog A (yeast)	4.92	2.30
Bcl2a1b	B-cell leukemia/lymphoma 2 related protein A1b	4.86	2.28

(a) FC = fold change corresponding to ratio between gene expression in MDP/MSU-stimulated DC and gene expression in not-treated DC.
(b) Log2(R) = base 2 logarithm of ratio between gene expression in MDP/MSU-stimulated DC and gene expression in not-treated DC.

Discussion

The precise mechanism promoting NLRP3 signaling is poorly defined [14], although many different molecules have been identified as activators of NLRP3. Similarly to other NLRP3 putative activators, the role of MSU in inflammasome activation has been studied mainly in respect to IL-1β secretion, a process that first requires a priming signal delivered by another stimulus. In the present work we focused on the analysis at transcriptional level of the synergism of MSU with the priming stimulus MDP. Analysis of the transcriptome of BMDCs stimulated with MDP, MSU or MDP/MSU confirmed the low capacity of MDP to induce transcription of pro-inflammatory genes, however MDP did induce a significant down-regulation of many genes. MSU application in contrast caused a strong up-regulation of inflammatory-related genes, whether applied to the cells alone or in combination with MDP. Interestingly, we found that MDP/MSU stimulation greatly increased the pro-inflammatory activity of MDP, but also induced the expression of genes uniquely modulated by MDP/MSU. The synergistic combination of MDP/MSU induced up-regulation of many cytokine genes including tnf, il6, il1α and il1β, clearly indicating DC activation towards a pro-inflammatory phenotype. We also found increased expression of chemokine genes, such as

cxcl1 and cxcl2, which are involved in neutrophil recruitment [15-17] an important process in the pathogenesis of autoinflammatory diseases including gout. Notably integrin expression was also enhanced by the combination of MDP/MSU, including CD47 that was recently described as crucial for DC migration to mesenteric lymph nodes in a model of experimental colitis [12]. Both genes itgb3 and itgav were up-regulated upon MDP/MSU stimulation, suggesting enhanced expression of the integrin heterodimer Itgβ3/ItgαV, receptor for matrix metalloproteases and coagulation factors, a mechanism that could be involved during the inflammatory process.

The synergic effect of MDP/MSU was most obvious for the expression of cytokine genes, such as il2, il23a and il12b. This is the first time that transcription of genes encoding IL-2 and the IL-23 heterodimer have been associated with MSU activation of innate cells. It is known that IL-2 plays a crucial role in modulating T cell homeostasis and that DCs can regulate the balance between tolerance and immune activation through IL-2 secretion [18-20]. Moreover IL-2 and IL-23 characterize the inflammatory profile of DC activated by fungi. Indeed, curdlan, a fungi-derived stimulus, activates dectin-1, leading to downstream signaling through Syk, CARD9 and the MAP kinase cascade [21], which on turn induce IL-2 and IL-23 gene expression, mediated by NFκB and AP-1 transcriptional factors [22-24]. Notably, Syk is the molecule signaling the interaction of MSU crystals with the plasma

membrane [25, 26], therefore this mechanism might be responsible for activation of IL-2 and IL-23 expression by MSU/MDP synergism observed in our study. Supporting this hypothesis, our data show that MDP/MSU stimulation induced up-regulation of the expression of the transcriptional factor component of AP-1, Fosb. Since it is known that MAP kinase activation promotes Fosb transcription, we suggest that NLRP3 and NOD2 could synergize at level of MAP kinase signaling pathways [27-29]. Confirming our initial hypothesis, analysis of phosphorilation levels showed that phosphorylation of c-Jun transcription factor was increased in MDP/MSU stimulation, suggesting elevated AP-1 recruitment. Interestingly, MSU has already been investigated for activation of MAP kinase cascade in a monocytic cell line [30]. Moreover, a recent work found that ASC activates AP-1 [6]. Therefore, our data confirm the involvement of MSU in MAP kinase-mediated activation of AP-1 in BMDC, but in addition, we suggest that the synergism of NOD2 could contribute to promote activation of AP-1 at levels necessary for the transcription of il2 and il23a genes.

In support of our data, in a murine air pouch model designed to resemble the human joint synovium MSU-mediated up-regulation of a specific pro-inflammatory transcript profile was also observed [31]. Interestingly, many genes identified in that study as differentially regulated after in vivo MSU injection (il6, tnf and il1β) have been found to exhibit a similar kinetic profile in our transcriptomic analysis.

Our observations support the idea that the interaction between NLRs should be studied at different levels, and demonstrate for the first time that NLRP3 and NOD2 synergism can be investigated not only in regards of IL-1β secretion but also of transcriptional regulation.

Despite its weak activity in vitro, MDP is considered a strong adjuvant in vivo [32]. In view of our data, we can speculate that adjuvant properties of MDP may be sustained in vivo by MSU, or other danger molecules released by stressed or dying cells, leading to a stronger activation of innate cells than in vitro. Moreover, the unique effects of combining an endogenous molecule (MSU) with an exogenous stimulus (MDP) may lead to speculation on the etiology of auto-inflammatory diseases in vivo, such as gout or arthritis. Indeed, our data support the theory that infections may play a role in breaking tolerance, ultimately leading to development of autoinflammatory and autoimmune diseases [33-36].

Finally, we ranked the top 30 highly expressed genes within the group of DEGs uniquely expressed upon combined MDP/MSU stimulation. This analysis revealed the upregulation of many genes related to plasma membrane-mediated functional properties, such as Cav1 and Rab11Fip1 genes, encoding proteins related to endocytosis, and Slc22a4, Slc15a2 and Gja1, involved in cation exchange. Notably, these cellular processes have been studied as putative mechanisms involved in NLRP3 activation [1, 37, 38], but

a direct involvement of the genes observed in our analysis has not been shown yet. Therefore, we suggest that activation of DC by MDP/MSU could lead to a modification in the plasma membrane activities promoting crystal uptake and increasing the effect exerted by MSU. Notably, Slc22a4 polymorphisms have been associated with susceptibility to rheumatoid arthritis and Crohn`s disease [39, 40]. Since it is well known that NLRP3 and NOD2 are involved in gout arthritis [41] and Crohn`s disease, respectively [42], we speculated that synergic effect of NLRP3 and NOD2 stimulation could be involved in the pathogenesis of autoinflammatory and autoimmune diseases, through the activation of specific pathways including the regulation of ione-exchange. Moreover these data indicate that the transcriptomic study of NLR-activation in innate cells is a valuable approach to define new markers of auto-inflammatory diseases and to investigate potential therapeutic targets. Our data suggest that the mechanisms modulating DC activation by MSU and MDP could also involve a fine-tuning mechanism at the transcriptional level, able to promote a unique reprogramming of DC towards an inflammatory phenotype. Moreover the observed synergism between an endogenous and an exogenous signal suggests interesting points of speculation regarding possible involvement of this mechanism in the onset of autoinflammatory and autoimmunity diseases, as well as in the host defense against infections. Further investigations are needed to define the mechanism and the relevance of the NLR-

synergism in relation to autoinflammatory disease as well as pathogen-mediated infections.

Materials and Methods

Reagents

MSU crystals (250 μg/ml) were prepared as previously described [43]. MDP (Invivogen, Carlsbad, CA) was used at 10 μg/ml.

Mice

C57BL/6 mice were from the Biological Resource Center colony (A*STAR, Singapore) and housed under specific pathogen-free conditions. All experiments were performed under Institutional Animal Care and Use Committee approval in accordance with the local Law and Guidelines for Animal Experiments of the Biological Resource Center, Singapore.

BMDC preparation

Bone marrow (BM) cells from C57BL/6 mice were cultured in Iscove's modified Dulbecco's medium (IMDM, GIBCO-BRL, Gaithersburg, MD), supplemented with 10% Euroclone fetal calf serum, 2 mM L-glutamine (GIBCO-BRL, Gaithersburg, MD), 50 μm 2-mercaptoethanol, 100 U/ml penicillin (GIBCO), 100 μg/ml streptomycin sulfate (GIBCO-BRL, Gaithersburg, MD) (IMDM complete medium), and 10% supernatant of GM-CSF-transduced B16 tumor cells [44]. After 7–10 days of culture, cells were analyzed for CD11c expression and used in assays when >90%

were CD11c positive. BMDC were plated in suspension dishes (Corning, NY) at $1x10^6$/ml in the presence of MSU, MDP, or a combination of MSU and MDP for the indicated time.

Total RNA extraction

Total RNA was isolated following the double extraction protocol using acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol®, Invitrogen, Carlsbad, CA), followed by Qiagen RNeasy clean-up procedure (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA purity was assessed by spectrophotometer to evaluate 260/280 ratios. If the 260/280 ratio was <1.9, RNA samples were ethanol precipitated. RNA integrity was assessed with Agilent Bioanalyser (Agilent Technologies, Santa Clara, CA). Only high-quality RNA preparations with RIN greater than 8.5 were used for microarray analysis.

Array hybridization

Eight micrograms of total RNA were used for cRNA target preparation according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA), utilizing the one-cycle target labeling kit according to the manufacturer's instructions. Fifteen micrograms of biotinylated cRNA was hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 arrays (Santa Clara, CA). Bioconductor32 was used for most data handling. The Guanine Cytosine Robust Multi-array Analysis

GCRMA33 method was used to calculate probe set intensity. Quantile normalization methods were applied.

Microarray analysis

Microarray analysis was performed using R language and Bioconductor packages [45]. Probeset signals were generated using a GC Robust Multi-array Average (GCRMA) background adjustment [46], and normalized with a quantile method [47]. The data were filtered, discarding the probesets showing an absent call in all the conditions and signal intensity lower than the 95th percentile of the overall absent call distribution. The differentially expressed genes were selected over a threshold of Log2 (Ratios) of 1.5-fold among the treatments at 4 hours versus the control at 0 hours. To address a biological framework involved in the stimulation processes, we focused on different gene families extracted from the Database for Annotation, Visualization and Integrated Discovery (DAVID) [48, 49]. The changes in expression levels for each gene in each family were represented using MeV software version 4.4 [50] generating hierarchical clustering [51] of Log2(Ratios) signals and setting a Pearson correlation as metric measure and an average linkage as linkage method.

Quantitative real-time RT-PCR

Genomic DNA was removed from total RNA by using Turbo DNA-free kit (Ambion, Austin, TX) and RNA was copied into cDNA by

High-capacity cDNA Reverse Transcription Kits (Applied Biosystem, Foster City, CA). Quantitative real-time RT-PCR was performed using Taqman primers (Applied Biosystem, Foster City, CA) for the following genes: il1b (Mm01336189_m1), il6 (Mm00446190_m1), tnf (Mm00443258_m1), il2 (Mm00434256_m1), il23a (Mm00518984_m1), gapdh (RefSeq: NM_008084.2). For iQ SYBER green real-time PCR (BioRad Laboratories, Hercules, CA) the following primer pairs were used: il1a forward 5'-CCAGAAGAAAATGAGGTCGG-3', reverse 5'-AGCGCTCAAGGAGAAGACC-3'; il1rn forward 5'-TTGTGCCAAGTCTGGAGATG-3', reverse 5'-CAGCTGACTCAAAGCTGGTG-3'; il12p40 forward 5'-CCAATTACTCCGGACGGTTC-3', reverse 5'-AGTCCCTTTGGTCCAGTGTG-3'; cxcl1 forward 5'-TCTCCGTTACTTGGGGACAC-3', reverse 5'-CCACACTCAAGAATGGTCGC-3'; cxcl2 forward 5'-TCCAGGTCAGTTAGCCTTGC-3', reverse 5'-CGGTCAAAAAGTTTGCCTTG-3'; ccl3 forward 5'-CCAGGTGTCATTTTCCTGACTAA-3', reverse 5'-CTGCCTCCAAGACTCTCAGG-3'; hprt forward 5'-CAGGCCAGACTTTGTTGGAT-3', reverse 5'-GGCTTTGTATTTGGCTTTTCC-3'. Quantitative amplification was performed using Applied Biosystems 7500 real time PCR system (Applied Biosystem, Foster City, CA). The amount of target genes was normalized for the housekeeping GAPDH and HPRT genes,

and relative to untreated control as calibrator, given by 2(-ΔΔCt) formula.

Cytofluorimetry

Maturation marker expression was analyzed by cytofluorimetry. BMDC stimulated for 24 hours were collected and stained with the following antibodies: phycoerythrin-conjugated hamster anti-mouse CD80 (16-10A1, BD Pharmingen, San Diego, CA), rat anti-mouse CD86 (GL1, BD Pharmingen, San Diego, CA), rat anti-mouse CD40 (3/23, BD Pharmingen, San Diego, CA), anti-mouse I-Ab MHC Class II (AF6-120.1, BD Pharmingen, San Diego, CA), and allophycocyanin-conjugated anti-mouse CD11c (N418, Biolegend, San Diego, CA), and analyzed with FACS Calibur (BD Biosciences, San Jose, CA). Data were analyzed with FlowJo software (Three Star, Ashland, OR).

Western blot

BMDC were stimulated with MDP, MSU or MSU/MDP for the indicated times. Cells were lyzed and stored in the following lysis buffer: 50 mM TRIS pH 8.0, 1 mM EDTA, 0.1% NP-40, 0.25 M NaCl, 10% Glycerol, 50 mM NaF, 1 mM NaOrtovanadate, 1 mM PMSF, protease inhibitory cocktails (Sigma-Aldrich, St Louis, MO) and phosphatase inhibitory cocktail (Sigma-Aldrich, St Louis, MO). Proteins were separated under denaturating conditions by SDS-PAGE, and transferred to PDVF membrane by electroblot.

Membranes were immunoblotted with goat polyclonal anti-IL-1β (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-phospho c-JUN (Ser^{73}) (Cell Signaling, Danvers, MA), purified mouse immunoglobulin anti-p44/p42 ERK1/2 (Thr^{183}, Tyr^{185} on ERK2) (Clone MAPK-YT) (Sigma-Aldrich, St Louis, MO), rabbit monoclonal antibody anti-p38 mitogen-activated protein (MAP) kinase (Thr^{180}, Tyr^{182}) (clone 3D7) (Cell Signaling, Danvers, MA) and mouse monoclonal antibody anti-GAPDH (clone 6C5) (Millipore, Billerica, MA).

Phosphoprotein detection

BMDC were stimulated for 2 hours with MDP, MSU, and MDP/MSU. Cell lysates were prepared using the Bio-plex cell lysis kit (BioRad Laboratories, Hercules,CA) and Bio-plex Phospho-c-jun (Ser^{73}) Assay (BioRad Laboratories, Hercules,CA) according to the manufacturer's instructions.

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Chapter 3.

Monosodium urate crystals program dendritic cell-mediated Th17 differentiation through a NLRP3-dependent pathway

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In preparation

Rationale and biological question

The pro-inflammatory effect of uric acid crystals is known since decades from the study of the gout. However, the capacity of MSU to stimulate DCs, through NLRP3 activation, and to mediate systemic inflammation has been recently investigated, since MSU has been identified as one of the first endogenous danger signals [1].

In the Chapter 2, we described how MSU-mediated activation of NLRP3 could synergize with the NOD2-agonist MDP, leading to a marked reprogramming of DCs towards a specific-proinflammatory phenotype. Starting from this observation, we decided to investigate whether such peculiar activation could mediate specific effects on the DC-mediated immune response, particularly the ability of DCs to prime T cells, leading to activation of adaptive immune response. Thus, we performed experiments in order to define the phenotype of T cells activated by DCs upon MSU stimulation.

According to the theory of the danger model, the activation of immune system by DAMPs is crucial to determine the outcome of the response; nonetheless only a few groups have described a connection between endogenous signals and adaptive immunity [1-3]. Therefore, the main contribution of our investigation is not

only to define a possible immunogenic mechanism of MSU crystals, but in general to answer the question whether an endogenous signal can mediate specific activation of adaptive immunity, beside the induction of innate response. Moreover, starting from the observation that MSU synergism with other stimuli can induce a unique pro-inflammatory phenotype, for the first time we specifically investigated the synergism between endogenous and exogenous signals in the context of activation of adaptive immunity.

Results

Splenic DC cell line stimulated with MSU and MDP polarize naïve T cells towards Th17 phenotype.

In order to define the activation of T cells by DCs, upon MSU stimulation, we firstly study the activation of a murine splenic DC line, named D1 [4]. D1 cells where activated with MSU crystals, coupled to MDP, and cytokine and secretion at 24 hours have been measured (Figure 1a). Interestingly, D1 cells produced IL-1β only in presence of MSU coupled to MDP, and the secretion upon MDP alone, or TLR-agonist, LPS, was not detectable. Moreover, we tested the combination of two exogenous signals, LPS and MDP (LPS/MDP), which are agonists of TLR4 and NOD2 respectively. The synergism of NOD2 with TLRs in induction of Th17 has been recently shown [5], therefore we used LPS/MDP stimulation as positive control [6]. Notably, we found that in our system LPS/MDP was not able to induce IL-1β secretion, contrarily to MDP/MSU condition. However, IL-6, TNFα, IL-2 and IL-23 were secreted in higher amount upon both MDP/MSU, and LPS/MDP stimulation, compared to the untreated and the single stimuli alone.

To test the effect of MSU on the priming capacity of DCs, D1 cells were used to activate polyclonal naïve $CD4^{+}$T cell population. Thus, D1 cells were stimulated with MSU, MDP, MSU/MDP, LPS, the positive control LPS/MDP, or medium as control, and two hours later naïve CD4+T cells ($CD4^{+}CD62L^{high}CD44^{low}$), purified from C57/B6 mice splenocytes were added to the culture. At 5 days, supernatants of the co-culture were collected and analyzed by ELISA (Figure 1b). We observed that stimulation of D1 cells with MDP/MSU induced high level of IL-17 compared to single stimuli MDP, MSU and LPS. Interestingly, we confirmed that LPS/MDP stimulation was able to induce IL-17 secreting T cell as well. The secretion of IL-4 was at low or undetectable levels (data not shown).

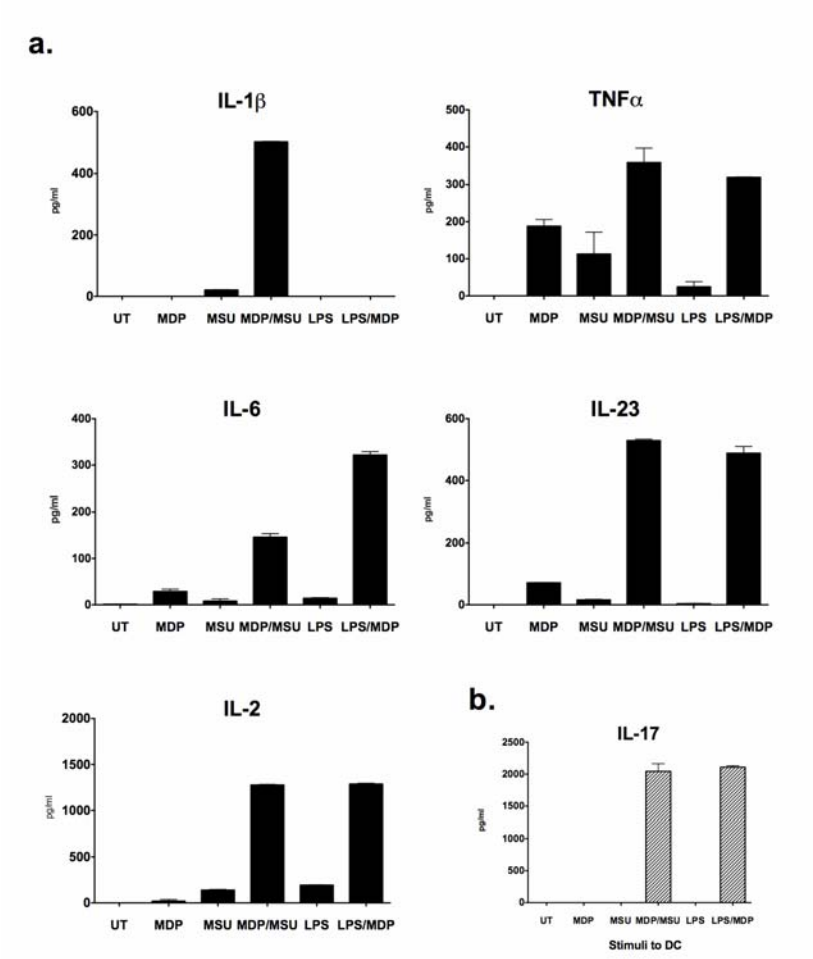

Figure 1. Splenic DC upon MDP/MSU stimulation secrete Th17-polarizing cytokines and prime naïve CD4$^+$ T cells to IL-17 secretion. D1 cells were stimulated with MSU, MDP, LPS or the combinations MDP/LPS, MDP/MSU. **a**. Culture supernatants were collected at 24 hours and analyzed by ELISA. **b**. D1 cells where stimulated as shown and two hours later, naïve CD4$^+$T cells (CD4$^+$CD62L$^+$CD44^{low}) were added to the culture, (DC : T ratio 1:1) in presence of anti-CD3 antibody . Five days later, culture supernatants were collected and analyzed by ELISA for IL-17 secretion.

Th17 polarization by MDP/MSU-stimulated spleen-DC is dependent on IL-1/IL-1R feedback loop

To test the polarization of naïve CD4+T cells in the presence of MSU-stimulated DC (D1 cell line), $CD4^{+}CD62L^{high}CD44^{low}$ lymphocyte were purified and added at the culture as previously described. At 5 days, supernatants were collected and cells were re-stimulated with PMA/ionomycin plus BFA. T helper cytokines IFNγ and IL-17 were stained intracellularly, and the cells were analyzed by cytofluorimetry (Figure 2a). We observed that MDP/MSU induced a marked polarization towards IL-17+IFNγ- cells, that we referred as Th17 cells, while single stimuli MDP, MSU and LPS, polarized towards a Th1 response. Interestingly, we confirmed that LPS/MDP stimulation was able to induce Th17 polarization as well. No significant numbers of double positive cells were detected in any condition.

Since, the main effect of MSU on innate cells is the release of IL-1β, we assumed that IL-1β secretion, observed by MDP/MSU stimulated DCs played a central role in Th17 activation. To test our hypothesis, we performed the co-stimulatory experiment in presence of IL-1 receptor antagonist (IL-1Ra), in order to block the effect of IL-1β (Figure 2b). Interestingly we observed that upon MDP/MSU stimulation, in the presence of IL-1Ra, the frequency of Th17 cells drastically decreased compared to the condition without

IL-1Ra. Conversely, Th1 frequency increased. These data indicate that in this system IL-1β plays a central role in maintaining Th17 polarization, and the block of IL-1R signaling determines a skewing to Th1 phenotype.

However, since IL-1R is expressed by both T cells and DCs, it was not clear whether the mechanism of blocking of Th17 polarization by IL-1Ra depended on the blocking of IL-1R on T cell either on DCs. Secondly, we measured Th17 polarization also upon LPS/MDP condition, where IL-1β was not produced, indicating a different mechanism of Th17 polarization, probably due to other cytokines as IL-6. Therefore, in order to better define the role of IL-1β-mediated Th17 polarization in our system, we investigated the possibility that IL-1β secreted by D1 cells induce the transcription of other cytokines by the same cells through an autocrine loop. D1 cells were activated as described, in presence of IL-1Ra or blocking antibodies for IL-1β, IL-6 and IL-23p19, alone or in combination (Figure 3). Interestingly, IL-1β was reduced of half of its amount, indicating that the autocrine loop of IL-1β sustain a second wave of expression of IL-1β itself. Moreover, confirming our hypothesis, the block of IL-1β signaling by IL-1Ra drastically inhibited the secretion of IL-6 and IL-23, upon MDP/MSU stimulation. Conversely, IL-6 and IL-23 secretion were not affected by the presence of IL-1Ra, upon LPS/MDP, indicating that IL-6 and

IL-23 were directly induced by LPS/MDP stimulation, independently of IL-1β secretion.

Anti-IL-1β blocking antibody only partially affected IL-6 secretion with no effect of IL-23 production. This result could be explained by the presence of another agonist of IL-1R, the cytokine IL-1α, which could mediate part of the autocrine loop on IL-1R. However, IL-1α was not detectable in the culture and adding of IL-1α -blocking antibodies did not affect IL-6 secretion (data not shown). Finally, we could not exclude a partial inhibition by using blocking antibodies compared to physiological IL1-Ra.

Anti IL-6-blocking antibody did not show any loop effect on cytokine secretion. Instead, blocking IL-23p19 increased IL-6 and IL-1β production. We cannot exclude that the depletion of IL-23p19 could increase the concentration of IL-12p40 free molecules and favor IL-12 formation, leading to a loop of cytokine secretion.

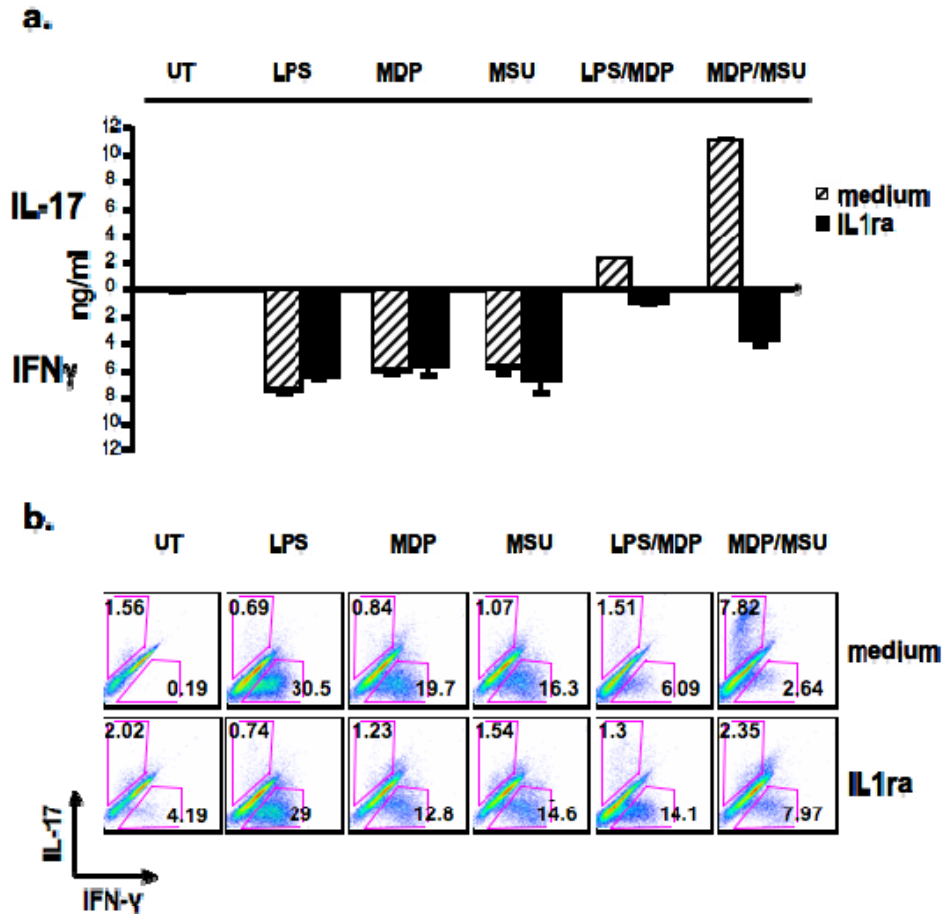

Figure 2. MDP/MSU stimulation drives Th17 skewing in a IL-1R dependent fashion. D1 cells where cultured and stimulated as described, in presence or absence of IL1ra. Two hours later, $CD4^{+}CD62L^{+}CD44^{low}$ naïve $CD4^{+}$T cells were added to the culture, (DC:T ratio 1:1). **a.** After 5 days, supernatants were collected and analyzed for IFNγ and IL-17 production. **b.** Cells were stimulated with PMA-Ionomycin for 6 hrs, and (5 μg/ml) Brefeldine A for the last 4 hours, followed by surface staining of CD4 and intracellular-staining for IFN-γ and IL-17. Gated on live and CD4+ cells. Representative of 3 experiments.

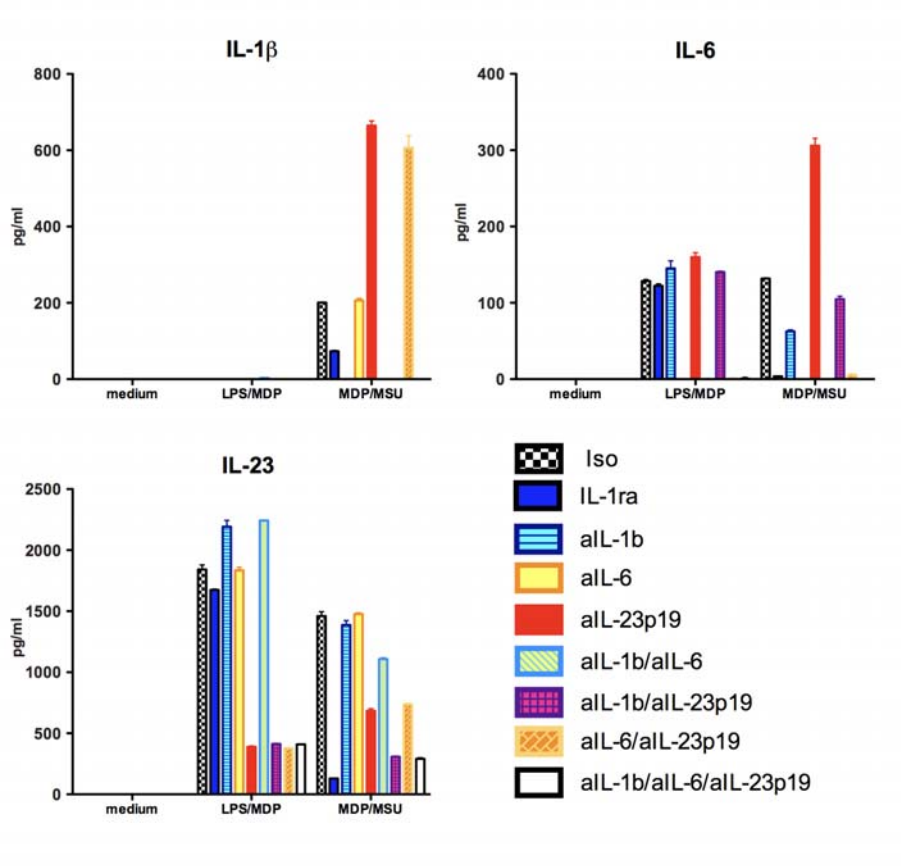

Figure 3. Blocking of IL-1β autocrine loop inhibits IL-6 and IL-23 secretion, upon MDP/MSU stimulation. D1 cells were stimulated with MDP/LPS, MDP/MSU or medium as control, in presence of IL-1Ra (IL-1ra) or blocking antibodies against IL-1β, IL-6, IL-23p19, or isotype control (Iso), in all the combinations. Cell supernatant was collect after 20 hours and analyzed by ELISA for IL-1β, IL-6, and IL-23 production. Representative of 3 experiments.

BM-DC activated by MSU/MDP prime naïve T cell towards a Th17 phenotype

Starting from the observation that splenic DC line stimulated with MDP/MSU stimulation drives Th17 polarization, we decided to investigate whether the same phenomenon could be extended to another experimental system of DC, BMDC. The stimulatory capacity of MDP/MSU in the activation of BMDC has been widely described in the previous Chapter. Notably, BMDC mainly differ from D1 cells for the capacity to secrete IL-1β and IL-6 upon LPS stimulation, suggesting a more inflammatory phenotype as suggested recently [7]. However, the activation upon MSU crystal stimulation and the synergic effect between MDP/MSU was largely confirmed in both the cell types.

MSU synergic activity was investigated in presence of other stimuli then MDP, such as LPS, CpG, CD40 ligand (CD40L) and GM-CSF, and we observed that MSU induced activation of pro-inflammatory cytokines IL-1β, IL-6, TNFα, IL-23p40 in presence of a priming signals (data not shown).

To test whether such a cytokine profile could induce specific T helper polarization, as observed with D1 splenic DCs, we stimulated BMDC with MSU alone or in combination with other priming signals. We added purified naïve T cells as previously described, and, five days later, we detected a marked increased in

Th17 polarization in the condition where MSU was coupled to another stimulatory co-signal, with the only exception of LPS (Figure 4). Conversely, single stimulations, including MSU alone, induced mainly Th1 response. LPS activation showed a strong apoptotic effect on BMDC [8] (data not shown), leading to a weak T cell activation. Therefore, the activation of naïve T cells by LPS/MSU co-stimulation resulted barely reproducible. Remarkably, we observed a strong enhancement in Th17 frequencies when MSU was coupled to GM-CSF and CD40L suggesting that pre-existing inflammatory conditions could favor MSU activity in inducing Th17 polarization.

When the naive T cell population was not depleted from $CD25^+$ we observed a basal frequency of $FoxP3^+$ T cell around 15% of CD4+ cells. Interestingly $FoxP3^+$ T cell population decreased upon stimulation of DCs by MDP and MSU alone (13% and 11%, respectively), with a further drop to 8.4% upon MDP/MSU (data not shown). This could be due to the increased amount of IL-6 observed in MDP/MSU condition, confirming published data about the competition between Th17 and Treg subtypes during differentiation [9].

Finally, we test MDP/MSU activation in an antigen specific system. BMDCs from balb/c mice were pulsed with OVA protein together with stimuli, and put in culture with naïve T cells purified from DO11.10 mice transgenic mice. Accordingly to the data on polyclonal stimulation by anti-CD3 antibodies, also antigen specific

activation induced Th17 polarization upon MDP/MSU condition (Figure 5). Moreover we confirmed that observed phenomenon was reproducible on cells deriving from a different mouse strain.

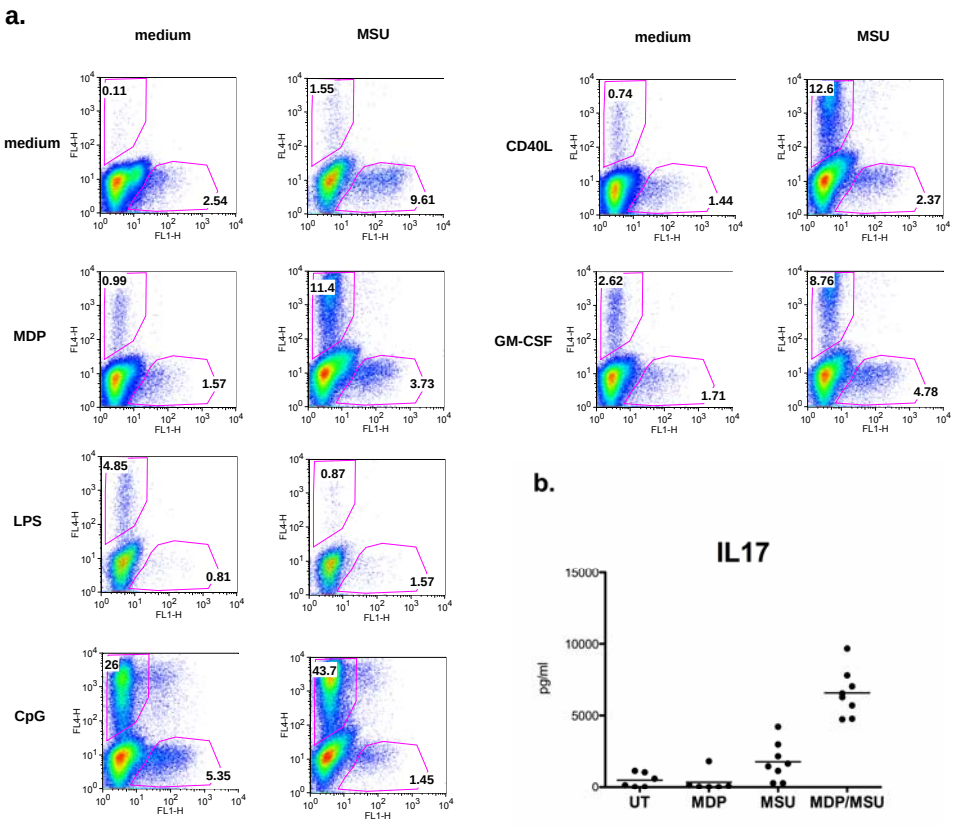

Figure 4. MSU synergizes with exogenous stimuli and endogenous inflammatory molecules, leading to Th17 priming. BMDC were stimulated by MSU alone or in presence of MDP, LPS, CpG, CD40L, or GM-CSF. After 2 hours, naïve $CD4^+$ T cells were added to the culture. **a.** Five days later the cells were collected, re-stimulated, stained for CD4, IFNγ, IL-17 and analyzed by FACS. Representative of 2 experiments. **b.** Alternatively, IL-17 was measured in the supernatant. Mean of 6 to 8 experiments.

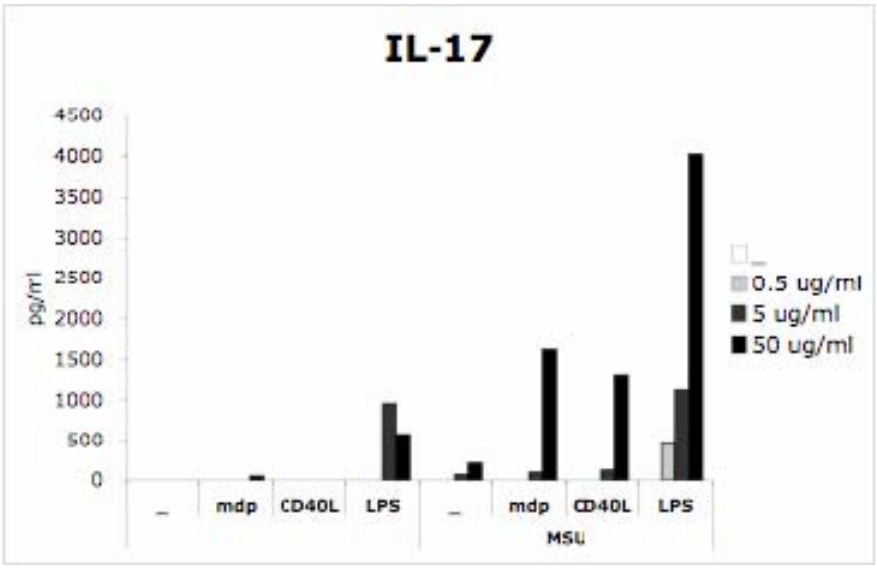

Figure 5. Antigen specific activation of Th17 cells by BMDC stimulated by MSU coupled to a second signal. Balb/c BMDCs were stimulated with different stimuli in the presence or absence of MSU, plus ovalbumine protein. Naïve T cells were added and five days later concentration of IL-17 in the co-culture was measured by ELISA.

MSU/MDP induce BMDC to activate Th17 cells in an NLRP3-dependent fashion.

To investigate the role of IL-1β in Th17 priming by BMDCs, upon MSU activation, we used BMDCs differentiated from *nlrp3*$^{-/-}$ mice. Indeed, *nlrp3*$^{-/-}$ BMDCs failed to secrete IL-1β upon activation by NLRP3 signals, such as MSU crystals [10]. BMDC from *wt* and *nlrp3*$^{-/-}$ C57/B6 mice were used. As already described, *wt* naïve CD4^{+} T cells were added at the BMDCs culture activated with MSU/MDP (or other co-stimuli, data not shown). After 5 days, T cells primed by *wt* MDP/MSU-stimulated BMDC polarized towards Th17 phenotype, while T cells primed by MDP/MSU-stimulated *nlrp3*$^{-/-}$ BMDCs failed to develop Th17 polarization, showing a skewing towards Th1 phenotype (Figure 6). Interestingly, the lack of NLRP3 in MDP/MSU condition induced an increase in Th1 production, but did not affect Th2 secretion. Conversely, stimulation by MSU alone induced Th1 polarization by wt BMDCs, whereas nlrp3$^{-/-}$ BMDCs skewed Th2 cells. This data indicate a central role for NLRP3 in DCs important for Th17 polarization.

This result suggested us that nlrp3$^{-/-}$ could play the essential role to induce IL-1β secretion and promote Th17 polarization. From previous results using a splenic DC line, we knew that that IL-1β was dispensable for Th17 skewing of mouse naïve cells, but it was crucial to promote autocrine loop on DCs inducing secretion of IL-6

and IL-23, responsible of Th17 polarization. Therefore we tested whether this system was reproduced in BMDC-mediated stimulation. Surprisingly, nlrp3$^{-/-}$ BMDCs stimulated by MDP/MSU expressed and secreted IL-6 without significant differences from wt BMDCs, and blocking IL-1R with IL-1Ra didn't affect IL-6 and IL-23 production (data not shown), indicating that IL-6 and IL-23 induced Th17 polarization independently of IL-1β autocrine loop on DCs, therefore in BMDCs IL-6 and IL-23 production was not dependent on NLRP3/IL-1β mediation. This observation suggested us that a possible alternative mechanism could mediate the role of NLRP3 in the process of Th17 induction.

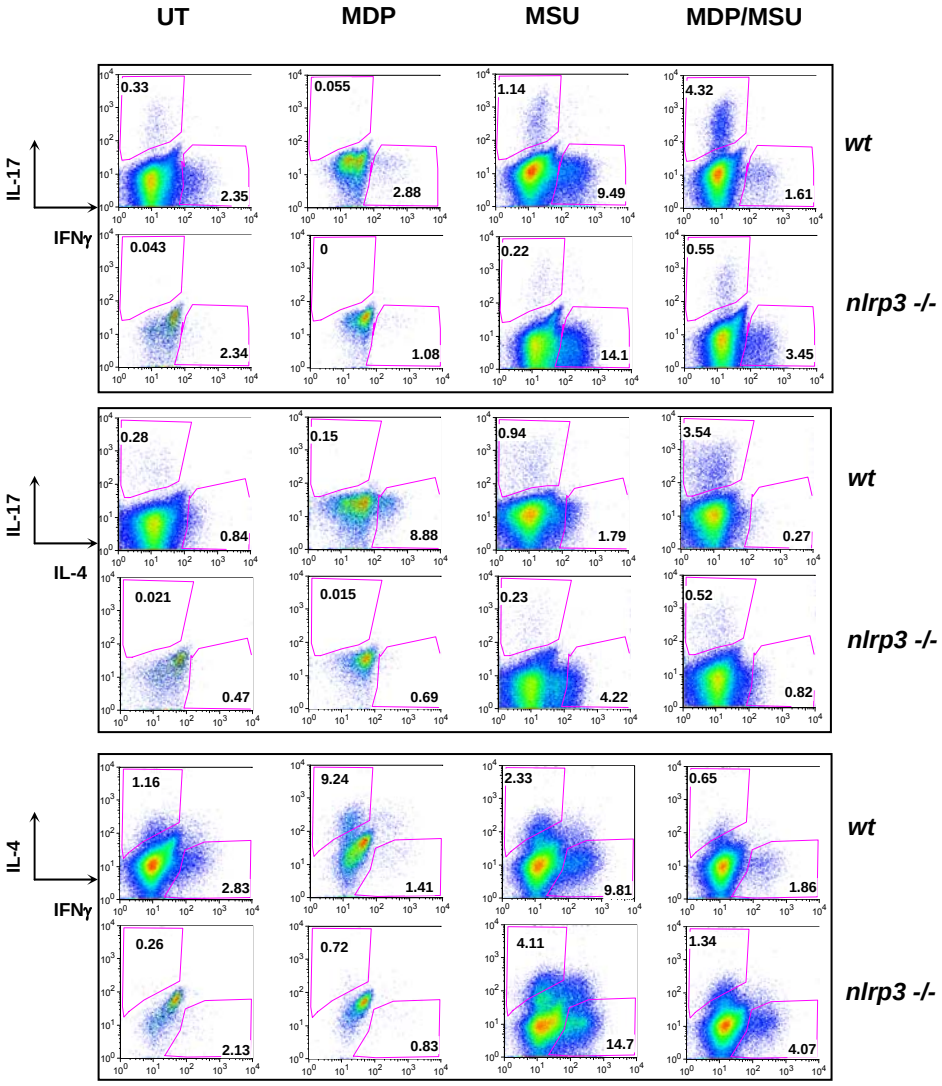

Figure 6. NLRP3 is crucial for Th17 skewing by MDP/MSU. Naïve $CD4^+$ T cells were primed by wt and nalp3-/- BMDCs stimulated with MDP, MSU or MDP/MSU. (**a.)** Five days later cells were analyzed by FACS for IFNγ, IL-17 and IL-4 production.

MSU induces recruitment of neutrophils and DC, and secretion of IL-23p19 by CD11c^{+} cells.

In order to define the in vivo role of MSU, we injected MSU intraperitoneally in C57/B6 mice, and at 6, 24 and 48 hours, peritoneal lavage was collected and cells were analyzed to define the composition of the peritoneal population. Neutrophils (CD11b^{+}Ly6G^{high}F4/80^{-}) were recruited in the first 6 hours after injection, whereas macrophages (SSC^{high}CD11b^{high}F4/80^{high}), and CD4 T cells (CD3^{+}CD4^{+}) left the peritoneal cavity (Figsure 7a). Alternatively, MSU or saline control was injected intraperitoneally in C57/B6 mice pre-treated with FLT3L tumor cells. At 24h cells of the peritoneal lavage were collected, stained for DC markers and analyzed by cytofluorimetry. MSU-injection induced a significant increase of DCs. (CD11c^{+}MHCII^{high}) in the peritoneum (Figure 7b). The absolute numbers of DCs were calculated starting from the percentage of CD11c^{+}MHCII^{high} cells, and the absolute number of cells collected during the lavage. Alternatively, peritoneal cells were stimulated for 3 hours with PMA and ionomycin in presence of brefeldin A, and stained for DC surface marker and intracellular IL-23p19, followed by fluorescence-microscopy analysis. CD11c^{+} from mice injected with MSU showed an increased expression of IL-23p19 (Figure 7c).

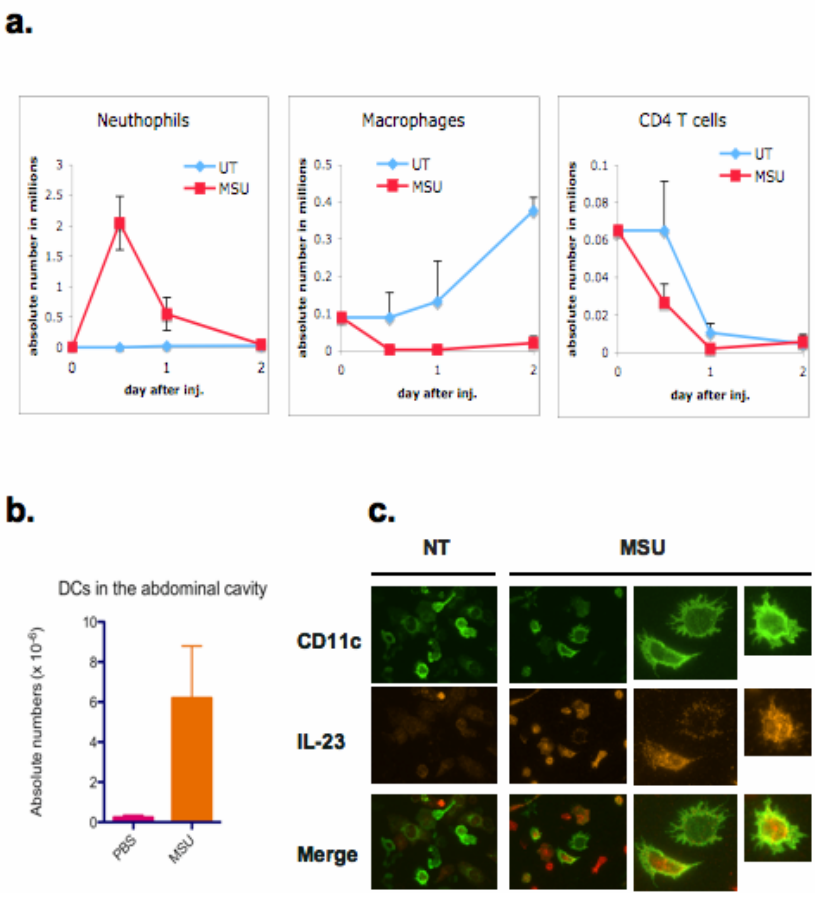

Figure 7. Intraperitoneal injection of MSU induced recruitment of innate immune cells and DCs expressing IL-23p19. Mice were injected intra-peritoneally with 2 mg MSU in 500 µl saline solution (five mice) or saline alone (three mice). After 6hrs, 24hrs, 48hrs cells of peritoneal cavity were collected and analyzed to characterize the composition of the population (a). Alternatively mice pre-treated with FLT3L tumor cells, were injected with MSU (five mice) or saline (three mice). Cells from peritoneal washing were counted and stained for DC markers and IL-23p19.

Analysis of synovium and serum of RA patient reveals high concentration of MSU.

Synovial fluid and serum from peripheral blood were collected from RA, gout, osteoarthritic patients, and healthy controls. Synovial and serum samples were collected and analyzed for uric acid concentration (Figure 8). MSU resulted increased in synovium of RA patient compared to physiological concentration, but lower then the values in gouty patients. Uricemia of RA patients was over 6 mg/dL, which is the clinical threshold of hyperuricemia in gouty patients.

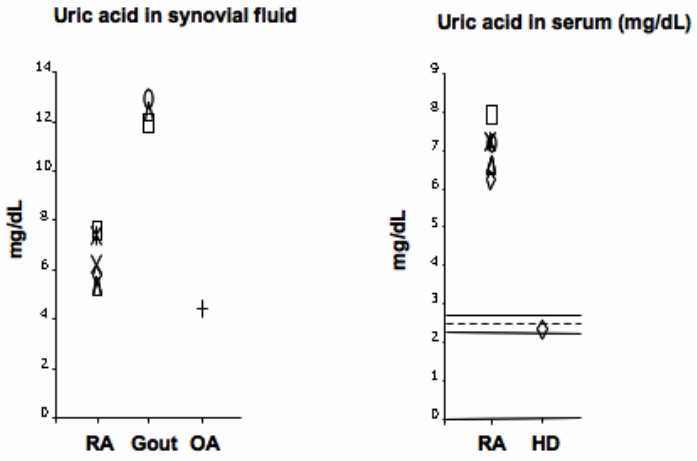

Figure 8. RA patients present high MSU concentration in synovial fluid and serum. OA osteoarthritis, HD healthy donor

Discussion

In the presented work, we investigated the capacity of MSU to promote a unique dendritic cell phenotype able to exert a specific T cell polarization. As described, the synergism of MSU with another signal as MDP induced a specific pattern of gene regulation characterized by increased pro-inflammatory cytokine expression, including IL-1β, IL-6, IL-23 and TNFα. Considering recent findings regarding Th17 polarization, we supposed that the DC phenotype driven by MDP/MSU could induce the skewing of naïve T cells toward a Th17 polarization. Indeed, stimulation of a splenic derived DC line or BMDC, with MDP/MSU drove naïve T cells to secrete high level of IL-17 and low amount of IFNγ. Moreover, IL-17^{+}IFNγ^{-} cells resulted predominant in co-culture with MDP/MSU-stimulated DCs, compared to MDP or MSU, confirming our hypothesis of Th17 differentiation. Remarkably, MSU alone as well as MDP alone induced Th1 activation, assessed as IL-17^{-}IFNγ^{+} cells. However we observed a very low production of cytokines by DCs upon MSU stimulation alone. Further investigations need to be performed to understand which factors are responsible of Th1 polarization by MSU alone. For instance, MSU alone could induce Th1 polarizing cytokines as IL-12p70. Interestingly, MSU could polarize Th17 cells also when coupled to stimuli other then MDP.

Indeed, using TLR agonists, such as CpG, the frequency of Th17 cells increased significantly in presence of MSU compared with TLR agonist alone. However, TLR agonists could induce DC death. The main known effect exerted by MSU crystals on innate cells is the secretion of IL-1β, thus, we first tested whether IL-1β could play a major role in Th17 activation. Using a splenic derived DC line, we primed naïve T cells in presence of IL-1Ra, and we found that Th17 population induced by MDP/MSU decreased drastically in presence of IL-1Ra, indicating that IL-1 signaling pathway was crucial for Th17 activation. However, we knew that other stimulatory conditions (such as LPS/MDP) were able to induce Th17 polarization without any detectable IL-1β production. Therefore, we tested the possibility that IL-1 could act on T cells indirectly, through an autocrine loop on DCs. As expected, IL-1Ra blocked the production of IL-6 and IL-23 by DCs upon MDP/MSU stimulation, suggesting that IL-1β indirectly induced Th17 polarization increasing the secretion Th17-polarizing cytokines by DCs. Moreover, we observed that LPS-mediated activation of DC on Th17 polarization was totally independent of IL-1β and consequently not affected by IL-1Ra.

To confirm our observation about the central role of IL-1β in our system, we tested the same stimulatory conditions on BMDCs comparing *wt* with *nlrp3*$^{-/-}$ cells, which are impaired in IL-1β and IL-18 secretion upon MSU activation. BMDC from *nlrp3*$^{-/-}$ cells failed

to induce Th17 polarization upon MDP/MSU stimulation and, interestingly skewed T cells towards a Th1 phenotype. Probably, the absence of Th17 polarization spontaneously favored Th1 development. However, IL-1β seemed to be not crucial in Th17 polarization by MDP/MSU on BMDC, since *nlrp3-/-* BMDC did not show decreasing in Th17 polarizing cytokines.

We are currently investigating whether IL-18 could have a role in this process. Preliminary results indicated that Th17 polarization upon MDP/MSU in both IL-18R$^{-/-}$ T cells and IL-1R$^{-/-}$ T cells is halved, but none of the two receptors is completely responsible of Th17 skewing (data not shown). However, we cannot exclude that the absence of the two signaling pathways together could lead to a complete blocking of Th17. From our results on splenic DCs we showed that under certain stimulatory conditions, such as LPS/MDP, mouse Th17 polarization can be obtained independently of IL-1β, but we assume that in the system of BMDC stimulated by MDP/MSU the cytokine cocktail able to induce Th17 required IL-1β signaling on naïve T cells.

It is known that Th17 polarization depends on the presence of several cytokines, which interplay in different combinations [11]. Therefore, a single cytokine, as IL-1β, could be not sufficient and not necessary alone to induce Th17 skewing, but it could play a major role when in combination with other polarizing cytokines. We know from transcriptomic data on BMDCs that other cytokines and putative polarizing factors could be involved in this process. Some

examples are IL-6, IL-23 and TNFα. Interestingly we observed that also another inflammatory cytokine, IL-1alpha, was increased by MDP/MSU stimulation. Preliminary results indicated that the blocking of IL-12p40, IL-6, or IL-1alpha drastically reduced Th17 polarization upon MSP/MSU, while IL-1β blocking antibodies induced an unexpected increased in IL-17 production (data not shown). However, the use of blocking antibodies could give misleading results, especially when used on a long kinetic and in mixed culture. Therefore, in order to better define which cytokine predominantly drive the mechanism involved in Th17 polarization by MDP/MSU, further experiments will be performed using BMDCs and T cells derived from mice deficient in cytokines and cytokine receptors, such as IL-1R, IL-18R, IL-12β1 and IL-23R.

Another important observation regards the crucial role of TGFβ in Th17 polarization. Inactive TGFβ is present at high concentration in the serum [12]. Thus, we tested several sera and media preparations (RPMI, IMDM, X-VIVO), and we found that in our system the culture medium plays a marginal role in the polarization of Th17 cells, since several preparations presented comparable pattern of Th17 polarization, including media at low serum concentration (data not shown). However, we cannot exclude that residual inactive TGFβ content present in the medium could be an essential component for Th17 activation by BMDC upon MDP/MSU stimulation. DCs do not secrete high levels of of TGFβ but they can

induce activation of inactive TGFβ through expression of integrins, such as αV, β6 and β3 containing integrins [13], and this mechanism can be involved in Th17 activation [14]. Notably, from transcriptomic analysis we found an increased expression of αV and β3 integrins upon MDP/MSU stimulation (shown in Chapter 2), suggesting a possible involvement in Th17 polarization by MDP/MSU.

Our microarray analysis of BMDCs upon MDP/MSU stimulation revealed many cytokines and polarizing factors possibly involved in Th17 polarization. However whether these factors depend on the presence of NLRP3 in the cell is still to be defined. A comparative microarray analysis between wt and nlrp3 $^{-/-}$ BMDCs will help us to define how NLRP3 can affect Th17-polarizing phenotype in DCs.

Our data indicate that different DCs subtypes could be affected in different ways by MSU stimulation. BMDCs are the most common model of DCs currently used by many groups, while we cannot predict whether the phenotype observed in D1 cells resembles splenic DCs or is a specific feature of our cell line. However the comparison between these two DCs models could help in the definition of the mechanism of MSU on DCs. Indeed, it can be assumed that GM-CSF-derived BMDCs show a phenotype more prone to inflammatory activation then D1 cells. Accordingly to other groups, we observed that LPS induced a spontaneous IL-1β secretion at 24 hours in absence of inflammasome activators,

through a mechanism that is still to be defined [15, 16]. Therefore we can assume that the pro-inflammatory phenotype of GM-CSF-derived BMDC favor a constitutive weak activation of the inflammasome. Conversely, D1 cells secrete IL-1β exclusively when LPS stimulation is coupled to an inflammasome activator, such as MSU, without any IL-1β secretion by LPS alone. This data suggest that D1 cell can be a very useful model to study specific mechanism of inflammasome activation. Moreover, we cannot exclude that D1 resemble in vivo steady state DCs more then BMDC do. Further investigations needs to be performed to test how much D1 phenotype represents the one of freshly derived splenic DCs.

We wish to emphasize that most of the studies on inflammasome has been performed on macrophages or monocytic cell lines, THP1, while little is know about the mechanism of inflammasome activation in DCs. Often many observations on the mechanism of NLRP3 function are translated among different innate cell types. However, considering our observations about the central role of MSU in activating DCs in their unique role of professional antigen presenting cells, we pointed out that further studies on adjuvant properties of inflammasome should be performed specifically on different DC subtypes.

Recently, Shi and colleagues proposed a different mechanism for MSU activation involving the adaptor protein Syk. Accordingly to them, MSU interacts with DC surface in a receptor-independent

system, leading to the aggregation of membrane cholesterol and engagement of Syk. Interestingly, Syk has been associated with *Candida albicans*-mediated MAP kinase activation in DC leading to Th17 polarization [17], but also with NLRP3-mediated protection from candidosis [18]. All these observations fit together in a complete scenario where MSU could induce Syk-mediate activation of DC specific transductional pathways, as MAP kinases, leading to a unique phenotype able to induce Th17 activation. Further investigations needs to be performed to check whether this mechanism account for the Th17 polarization observed in our experimental system.

In order to test the inflammatory effect of MSU in vivo, we injected MSU crystals intraperitoneally in mice and at different time points we analysed the frequency of different cell populations in the peritoneal lavage. We observed recruitment of innate cells including neutrophils and dendritic cells. Remarkably, kinetic profile observed for many cell types was comparable with the profiles of innate recruitment by alum, which is supposed to work through induction of MSU release [2]. Interestingly, analysis of peritoneal DCs after MSU injection revealed production of IL-23, confirming our in vitro data. Further experiments are currently ongoing to define the adjuvant role of MSU/MDP in activation of Th17 cells in vivo.

In conclusion, in the present study we show that MSU crystals in presence of a priming signal promote a reprogramming of DC

phenotype, leading to the activation of naïve T cells toward Th17 polarization, through a NLRP3 dependent mechanism. Our data show for the first time that an endogenous danger signal, such as MSU, can orchestrate the activation of innate and adaptive immunity, promoting specific immune responses, which could be involved in the onset of immune-mediated diseases as well as in the host protection against danger not-microbial agents, such as tumors and toxic particulates (silica and asbestos). Moreover our finding could help to explain the working mechanism of adjuvants, such as alum. Further investigations will better elucidate the mechanism of MSU mediated Th17 differentiation in vitro and in vivo.

Materials and methods

Dendritic cell culture. Homogeneous, spleen-derived growth factor–dependent DC population, referred as D1 cells [4], were used in the experiments of dendritic cell activation by endogenous stimuli and to perform assay of antigen presentation to T lymphocytes. Culture medium for generation and expansion of D1 was complete IMDM (10% fetal bovin serum-Australia, Gibco) supplemented with 20-30% NIH/3T3 conditioned medium containing 10–20 ng/ml mouse GM-CSF, referred as R1 medium. Cultures were fed with fresh R1 medium every 2-3 days. During passages, both suspended and weakly adherent cells were detached using PBS with 2 mM EDTA and expanded. Experiments on D1 cells were performed not over 6-7 passages.

Bone marrow-derived DCs were obtained flushing femuri of C57BL/6 mice 8-12 weeks and culturing bone marrow cells in IMDM complete (10% Fetal bovine serum, Euroclone) supplemented with 10% B16 conditioned medium containing 10–20 ng/ml mouse GM-CSF. After 10 days the CD11c+ expression of the population was analyzed to test the differentiation of DCs. For the experiments of DC activation, DCs were plated in suspension dishes, (D1: 0.3 10^6 cells/ml, BM-DC: 1 10^6 cells/ml) in presence of stimuli or control medium. Stimuli for DC maturation were used in the following concentrations: 1 μg/ml LPS (Alexis), 10 μg/ml MDP

(Invivogen), 250 µg/ml MSU (Alexis) or from laboratory preparations, tested for functionality and purity (LAL test). At different time points from stimulation, activation markers were analyzed by cytofluorimetry (FACSCalibur, Becton Dickinson) and inflammatory cytokines were measured in the supernatant by ELISA (BD purified and biotin-conjugated antibodies for IL-2, R&D ELISA Duoset kit for IL-1α, IL-1β, IL-6, TNF-α and e-Bioscience ELISA-kit for IL-23p19, IL-12totp40, IL-12p70). Blocking antibodies anti-IL-1a, IL-1b, IL-23p19, IL-6 (eBioscience) have been titrated and used with corresponding isotypes control (eBioscience). IL-1Ra (R&D) has been used at 1-5 µg/ml, and added to the culture 30 minutes before the stimuli.

T lymphocyte differentiation. CD4+ T cells were isolated from spleen cell suspension with a MACS (Miltenyi) sorting for CD4+ cells (PE-CD4 and beads-conjugated anti-PE, Biolegend), followed by cytofluorimetry sorting of naive T cells population $CD4^+$ $CD62L^+$ $CD44^{low}$ $CD25^-$ (FACSAria\, Becton Dickinson) stained with fluorochrome conjugated antibodies PE-CD4, FITC-CD62L and PE-Cy7-CD44, (Biolegend). DCs were activated with stimuli (0.2 10^6 cells/ml). After 2 hours, T cells were added to the culture (DC:T ratio, 1:1) in presence of purified antibody anti-CD3 (1 µg/ml) and maintained for 5 days. Alternatively, Balb/c derived BMDC pulsed with OVA protein at different concentration and used to stimulate naïve T cells derived from DO11.10 mice.

Supernatants were collect and analyzed for IFN-γ and IL-17 (R&D ELISA Duoset). Cells were stimulated with PMA-Ionomycin for 6 hrs, with addition of (5 μg/ml) Brefeldin A (Sigma) after 2 hours, following by surface staining for APC-CD4 (BD Pharmingen), fixation, permeabilization and intracellular-staining for IFN-γ, IL-17, IL-4 (Biolegend).

In vivo experiments. Female mice 8 weeks old, were injected intra-peritoneally with 2 mg Monosodium Urate Crystals suspension in 500 μl saline solution. Controls mice where injected with saline alone. After 6hrs, 24hrs, 48hrs, mice where euthanized and 3 ml of PBS-EDTA were injected in the peritoneal cavity and collected soon. Cells from peritoneal washing where counted and stained to characterize the composition of the population recruited to the peritoneal cavity, during the MSU-induced peritonitis. Briefly, peritoneal cells were treated with brefeldin A and stained for surface DC marker CD11c and intracytoplasmic cytokines (IL-23p19) followed by fluorescence-microscopy analysis.

Flow cytometry. Flow cytometry analysis was performed with a FACScalibur (Becton Dickinson) followed by FlowJo software analysis.

Mice. C57BL/6 mice were purchased from Biological Resource Center (A-STAR, Singapore) and kept under SPF conditions. All the experiments were performed under IACUC approval in accordance with the Guidelines for Animal Experiments of Biological Resource Center.

Patient samples. Five RA, three gout and one osteoarthritic patients were included in this study. Synovial and serum samples were collected and analyzed for uric acid by Uric Acid Assaykit QuantiChrom^{TM}. All patients gave informed consent and the study protocol was approved by IRB, Istitutional Review Board, Singapore.

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Chapter 4

Conclusion

Summary

The ability of uric acid crystals to induce DC maturation and to promote the secretion of the pro-inflammatory cytokine IL-1β is well known, but a complete analysis of DC phenotype upon MSU stimulation was still missing. As described in the chapter 2, we uncovered new features of MSU activity on DCs, through a transcriptomic analysis, and we defined a peculiar synergism between MSU and another NLR activator, MDP. Characterizing DC phenotype upon MDP/MSU we found specific markers of inflammatory activation, included Th17 polarizing cytokines, IL-6 and IL-23. This finding suggested us to investigate the possible role of MSU as adjuvant able to polarize Th17 cells through specific DC stimulation.

MSU crystals are among the first endogenous danger signals identified. Notably, the first seminal work describing immunostimolatory properties of MSU clearly illustrated the adjuvant potential of MSU crystals. However, in the following years a few groups focused on the characterization of the adjuvant mechanisms exerted by MSU [1-3]. Notably we observed that upon MDP/MSU stimulation DCs prime T cells towards Th17 polarization, and this process was completely dependent on NLRP3.

Whether MSU can exert Th17 polarization in vivo is currently under investigation, however interesting parallels can be proposed to relate our observations to the mechanisms involved in the pathogenesis of autoinflammatory and autoimmune diseases.

Synergism between endogenous and exogenous signals

Considering the importance of MDP/MSU synergism in our system, the first possible speculation regards the biological meaning of these two stimuli, in the context of physio/pathological immune activation. Accordingly to the danger model hypothesis, events of tissue damage can be sensed by the innate immune system and activate the innate response, eventually followed by the alert of adaptive immunity. In this system, however, it is not defined whether immune response can be activated by endogenous danger signals even in absence of any microbial infection, or whether microbial-derived stimuli together with endogenous signals could co-operate in initiating the immune response. Interestingly, the two-steps mechanism of activation of NLRP3 inflammasome, requiring endogenous and exogenous signals together, could represent in a simple system a general mechanism of immune-regulation aimed to maintain a fine-tuning of the immune activation. Accordingly to our data, the interplay between an endogenous danger signal and a microbial stimulus has been observed also for DC-mediated activation of Th17 cells. Phagocytosis of apoptotic cells by DCs in presence of LPS stimulation induced Th17 polarization [4]. In this system, TGFβ was activated by BMDCs

upon phagocytosis of apoptotic cell, while LPS induced IL-6 secretion. Interestingly, the absence of LPS leaded to Treg activation, instead, suggesting a protective mechanism aimed to avoid self-activation by danger signals [4]. These data together with our observation suggest that endogenous danger molecules could require a second exogenous signal for a complete activation of immune response. This mechanism could guarantee the control of unsought response against self. Alternatively, our data on synergism between MSU and CD40L in Th17 skewing suggest that danger signals could activate adaptive immunity when a pre-existing inflammatory condition exists. Therefore we could speculate that endogenous danger signals during homeostasis require an exogenous activation to start a complete Th17 response, while pre-existing pro-inflammatory events, even in sterile conditions, could support endogenous signal-mediate response in absence of microbial infection. This second scenario could describe aberrant Th17 activation during autoimmune diseases.

Synergism between endogenous and exogenous signals could drive immune response in both physiological and pathological conditions. Accordingly to our hypothesis, it has been proposed that microbial and viral infections could play an important role in the pathogenesis of autoimmune diseases, or at least in favoring the onset of the autoimmune response in susceptible individuals [5, 6]. In this scenario, the release of danger signals by infected

necrotic cells could induce an aberrant activation of the adaptive immune response against self antigen, deriving from the infected dying cells [7]. Therefore we could assume that microbial-derived stimuli, in presence of significant amount of danger signals from tissue damage, could induce the activation of T cells against self antigens and start the adaptive autoimmune response. Supporting this hypothesis, it has been observed that in the context of an autoimmune disease as multiple sclerosis (MS) dendritic cells from patients with an active bacterial infection were more potent stimulators of Th17 cells compared to DCs from non infected MS patient and non-MS subjects infected by bacterial meningitis [8]. Even though the role of infections in the pathogenesis of autoimmunity is a commonly supported hypothesis [7], it is difficult to clinically discriminate whether infections are primary or secondary to the onset of autoimmunity.

The synergism between endogenous and exogenous signals, and the downstream immune response, could also depend on the kind of microbial mediated-stimulation. In the present work we observed that the microbial-derived stimulus MDP induce a weaker DC reprogramming, compared to TLR ligands, such as LPS and CpG, and only in synergism with MSU induced a marked up-regulation of pro-inflammatory genes. Therefore, we speculated that synergism between NLRs could have a different biological role then the synergism between TLRs and inflammasome, described in many works [9-11]. Recently it has been suggested that NLRs own a

property not present in TLRs: the control of cytosolic access [12]. For this reason NLRs controls different pattern of PAMPs and DAMPs, eventually signaling cell stress due to rupture of endosomal compartment. So, it is not surprising that NLRs-mediated pathways are programmed to mediate different innate and adaptive response then TLRs.

It is known that MDP is a weak cell activator *in vitro*, while it exerts strong immuno-stimolatory properties *in vivo* [13]. It can be suggest that endogenous danger signals mediate immune activation by MDP *in vivo*. In this scenario, danger signals, as MSU, could induce the access to cytosolic compartment to other microbial stimuli, playing an indispensable role in the initiation of immune response. Therefore, the synergism between NLRs in the control of cytosol access could be a crucial checkpoint in the initiation of specific innate and adaptive immune responses.

Supporting IL-1Ra-based therapy

The activation of the inflammasome by danger signals leads to IL-1β and IL-18 secretion. It has been widely shown that this event can play a central role in the initiation of many immune mediated diseases, since IL-1β and possibly also IL-18 [14] exert a potent systemic pro-inflammatory effect on the organism. Autoinflammatory diseases related to mutation in nlrp3 genes are the best examples of IL-1 mediated immuno-disorders [15, 16]. However, an efficient physiological system controls the activation and the signaling of IL-1 family cytokines (as described in the introduction). One important component of this control system is the specific IL-1R antagonist, IL-1Ra. Notably, IL-1Ra seems to be impaired in rheumatoid arthritis (RA) patients, suggesting that dysfunctions in the IL-1 control system could be central in the pathogenesis of both autoinflammatory and autoimmune diseases [17].

From experiment using splenic DC line, we observed that MDP/MSU stimulation induced IL-1β mediated activation of Th17 cells while microbial derived stimuli exerted Th17 activation independently of IL-1β. Interestingly, blocking IL-1β via-IL-1Ra completely blocked Th17 skewing in MDP/MSU condition, but didn’t affect Th17 polarization by microbial stimuli. We cannot

exclude that danger signals, as MSU, could exert also *in vivo* a mechanism totally dependent on IL-1β, as the one that we observed in our *in vitro* system. In this case the central role of IL-β in orchestrating DC phenotype and T cell polarization could explain the high efficacy of IL-1 blocking therapy in improving Th17-mediated autoimmune diseases such as rheumatoid arthritis [18]. Moreover, we can speculate that *in vivo* IL-1Ra subministration, could target exclusively danger signal mediated-responses without affecting normal host response to microbial stimuli. Indeed, accordingly to many clinical trials on rheumatoid arthritis patients, the safety of IL-1Ra treatment in term of risk of infections or malignancies is very low compared to the most common anti-TNFα therapy [18]. In vivo studies need to be performed to test whether the mechanism observed in our system represents a real condition occurring *in vivo* during inflammatory and autoimmune responses.

Autoimmunity and autoinflammation: the immunological disease continuum

Janaway model proposed for the first time the central role of innate immunity in determining the fate of the adaptive immune response, while later the danger model mainly focused on the independence of innate immunity from adaptive immunity in the initiation of immune mediated responses, postulating the existence of autoinflammatory diseases.

In the present work, we show that the activation of innate cells by endogenous danger signals can induce both innate and adaptive immunity. Thus, we suggest a possible involvement of a specific adaptive immunity in the onset and development of auto-inflammatory diseases that are mediated by endogenous danger signals or hyper-activation of NLRP3-inflammasome. Presently, there is no evidence of an involvement of adaptive immunity in the pathogenesis of NLRP3-mediated autoinflammatory diseases. However, a recent work uncovered an increased Th17 activation in mice that carry a specific gain of function mutation in NLRP3, resembling the mutation of MWS patients. Moreover, polymorphisms of NLRP1-inflammasome has been related to development of autoimmune vitiligo [19].

The role of uric acid crystals in autoimmunity is still unclear. Hyperuricemia has been related to a number of inflammatory diseases including hypertension and cardiovascular pathologies [20]. However, uric acid, not in crystal form, is considered an important antioxidant, and low levels of uric acid are associated with MS [21]. Thus, it is likely that crystallization process of uric acid molecules plays a crucial role in determining immune stimulatory-properties versus antioxidant protection.

Considering the possible correlation between uric acid and Th17 activation, an emblematic example comes from the comparison between two arthritic diseases: gout and rheumatoid arthritis (RA). Gouty arthritis is an auto-inflammatory disease depending on uric acid accumulation, while RA is an autoimmune disorder that involves Th17–mediated response [22]. Interestingly, IL-1β plays a central role in these pathologies, and IL-1Ra based-therapies are extremely efficient in decreasing the severity of both the diseases [18]. However, despite very similar clinical features, these two arthropathies are differentially diagnosed. Starting from the observation that IL-1-mediated Th17 response is central in RA pathogenesis, we speculated that the accumulation of the danger signal uric acid could play a major role in the etiogenesis of autoimmune response in RA patients. Analyzing synovial fluid and serum samples from RA patients we found that the levels of uric acid was increased compared to healthy controls, and similar or comparable to concentrations observed in gouty patients. As far as

we know, a direct correlation between RA disease and uric acid accumulation in the synovium and serum of RA patients has not been clearly investigated yet. However supporting our hypothesis, the concentration of hypoxantine (the precursor of uric acid) is increased in the synovium of both RA and gouty patients, and correlates with the grade of joint destruction. Moreover methotrexate, an inhibitor of purine biosynthesis, is prescribed as a well-known anti-rheumatic agent [23].

As for many autoimmune diseases, the etiogenesis of RA is still not defined, but is probably related to a polygenic susceptibility [24, 25]. However, it has been proposed that autoinflammation could act as a secondary mechanism contributing to the clinical expression of RA [26]. This observations suggested that a clear classification as autoimmune or autoinflammatory could be not possible for many immune related diseases. This hypothesis is supported by a recent proposed revision in the classification of the immune-related pathologies [27]. Accordingly to this model an overlapping of autoinflammatory and autoimmune features at different ratios characterizes the immune-mediated disorders. The etiogenesis of monogenic autoimmune diseases, affecting the central and peripheral adaptive system, results totally determined by the autoimmune response. Similarly, monogenic autoinflammatory diseases, such as nlrp3 mutations, are dominated by the autoinflammatory part. Instead, polygenic diseases, as gout and RA, can present different combinations of

autoinflammatory and autoimmune features, which variably determine the outcome of the disease.

The proposed `continuum` in immune-mediated pathologies could help to define the role played by the endogenous danger signals in the initiation of inflammatory as well as autoimmune diseases.

In conclusion, the endogenous danger signal, uric acid, could have an important association with the pathogenesis of both autoimmune and autoinflammatory diseases, but it could also have possible applications in the design of new adjuvants to improve host response to infections, and eventually malignancies. Thus, the present work underlines that the study of endogenous danger signals in the modulation of immune response could give a noteworthy contribution to the translational medicine.

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Contribution to International publications

Alessandra Mortellaro, Cristina Conforti-Andreoni, Jan Fric, Paola Ricciardi-Castagnoli **Dendritic cells as sensors of environmental perturbations** Microbes Infect 2008 vol. 10 (9) pp. 990-4

Under revision at Journal of Leukocyte Biology:

Cristina Conforti-Andreoni, Ottavio Beretta, Matteo Urbano, Ginevra Licandro, Federico Vitulli, Paola Ricciardi-Castagnoli, and Alessandra Mortellaro. **Synergism of NLRP3 and NOD2 promotes a unique transcriptional profile in dendritic cells**

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