Ph.D. PROGRAM IN
MOLECULAR AND TRANSLATIONAL MEDICINE
DIMET

UNIVERSITY OF MILANO-BICOCCA
SCHOOL OF MEDICINE AND FACULTY OF SCIENCE

Characterization of the intracellular pathways involved in IL-22 production by dendritic cells

COORDINATOR: Prof. Andrea Biondi
TUTOR: Dr. Maria Foti

Dr. Silvia Fumagalli
Matricola: 041086

XXIV CYCLE
ACADEMIC YEAR
2010-2011

A Michele e
alla mia famiglia

TABLE OF CONTENTS

Chapter 1: INTRODUCTION 1

Chapter 1: INTRODUCTION

1.1 The immune system

The immune system can be broadly divided into innate and adaptive components, with extensive crosstalk between the two. The innate response includes soluble factors, such as complement proteins, and several cellular effectors, including granulocytes, mast cells, macrophages, dendritic cells (DCs) and natural killer (NK) cells. Innate immunity serves as the first line of defense against infection, as germ-line-encoded pattern-recognition receptors and other cell-surface molecules quickly detect microbial constituents, thereby orchestrating inflammatory reactions (Janeway CA 2001). By contrast, adaptive immunity, mediated by antibodies and $CD4^+$ and $CD8^+$ T cells, is slower to develop. This reflects the requirement for the expansion of rare lymphocytes that harbour somatically rearranged immunoglobulin molecules, or T-cell receptors that are specific for either microbial-derived proteins or processed peptides that are presented by major histocompatibility complex (MHC) molecules. NKT cells and $\gamma\delta$ Tcells are cytotoxic T lymphocytes that function at the intersection of innate and adaptive immunity.

1.1.1 DENDRITIC CELLS (DCs)

In 1973, Ralph Steinman and Zanvil Cohn reported the identification of a novel cell type in the peripheral lymphoid organs of mice. Because of their morphology these cells were named “dendritic” cells. DCs represent a special type of leukocytes which sense the peripheral environment for the presence of micro-organisms. DCs are able to capture and internalize pathogens and, subsequently, to process and present antigens on MHC class I and class II molecules to CD8+ and CD4+ T cells in the T cell-area of lymph nodes. DCs can be subvided into several sub-populations according to surface expression of particular markers and tissue distribution (Shortman K and Yong-jun Lin 2002).

DCs types and subtypes

Distinct subtypes were initially more evident among mouse DCs than among human DCs, because of the ready availability of different murine lymphoid tissues and the expression on mouse DCs of markers not present on human DCs. Mouse DCs that are classified as 'mature' express CD11c (the integrin-α chain) and the co-stimulator molecules CD80, CD86 and CD40, and have moderate to high surface levels of MHC II, although the levels of all of these can be further elevated on activation. These features always correlate with a striking ability to induce the proliferation of allogeneic T cells (Iwasaki

A et al. 2000; Henri S et al. 2001). Surprisingly, the T-cell markers CD4 and CD8 are expressed on mouse DCs and are useful for segregating the different subtypes. Other markers that are useful for segregating mouse DC subtypes include CD11b (the integrin α_M chain of Mac-1) and the interdigitating DC marker CD205 (the multilectin domain molecule DEC205, originally known as NLDC-145). Using these surface markers, five DC subtypes are consistently found in the lymphoid tissues of mice. Spleen contains three of these: the $CD4^-$ $CD8^+$, the $CD4^+CD8^-$ and the $CD4^-CD8^-$ DCs (Vremec D et al. 2000). The $CD8^+$ DCs are concentrated in the T-cell areas and the $CD8^-$ DCs in the marginal zones, but the $CD8^-$ DCs migrate into the T-cell zones on stimulation with microbial products (Reis e Sousa C et al. 1997). The $CD4^-CD8^+$ DC subtype, which is $CD205^+CD11b^-$, is also found at moderate levels in LNs, but is the dominant subtype among thymic DCs. LNs contain two extra DC subtypes that are not normally found in spleen, which have apparently arrived in the LNs through the lymphatic system. $CD4^-CD8^-CD11b^+$ DCs express moderate levels of CD205, in contrast to spleen $CD8^-$ DCs. This LN DC subtype is believed to be the mature form of tissue interstitial DCs. Another distinctive DC subtype, found only in skin-draining LNs, expresses high levels of langerin, a characteristic marker of epidermal Langerhans cells, and is believed to be the mature form of this Langerhans cell (Henri S et al. 2001). It also expresses a range of myeloid markers, including CD11b,

stains at low levels for CD8α and has high surface levels of CD205, as high as on the $CD8^{high}$ DC subtype. These Langerhans DCs in LNs are also distinguished by their high surface levels of MHC II and high expression of CD40, CD80 and CD86 (showing many characteristics of fully activated DCs). Mouse plasmacytoid DC precursor has recently been isolated from mouse lymphoid tissues and mouse blood. As with the human plasmacytoid population, it is a potent producer of both interferon-α (IFN-α) and IFN-β when stimulated by bacterial CpG or by viral infection. This population also expresses B220 (CD45)(Shortman K and Yong-jun Lin 2002).

DCs functional specialization

The different subtypes of mouse DC share a common capacity to present antigens to T cells and to promote cell-cycle progression. However, they differ in the other aspects of DC–T-cell signalling that determine the subsequent fate of the T cells that they activate. The most striking biological difference so far discovered is the ability of the $CD8^+$ DCs to induce a T_H1-biased cytokine response in reactive $CD4^+$ T cells, whereas $CD8^-$ DCs tend to induce a T_H2-biased response (Moser M et al. 2000; Maldonado-Lopez R et al. 1999). The main factor in this difference is the much higher level of IL-12 p70 produced by $CD8^+$ DCs under the experimental conditions, but other factors might be involved, and there might be a specific signal for

inducing the T_H2 cytokine pattern. $CD4^-CD8^-$ splenic DCs can also produce some IL-12 p70 and this might be sufficient to induce T_H1 cytokines during a close DC–T-cell interaction. The production of IL-12 p70 is regulated by many factors. Although the appropriately activated $CD8^+$ DCs have the greatest capacity for IL-12 production, different stimuli can change the balance. The three DC subtypes differ in their capacity to produce cytokines when stimulated under conditions designed to induce a maximal response (Hochrein H et al. 2001). The $CD8^+$ DCs can produce a small amount of IFN-α and IFN-β, as well as producing the highest levels of IL-12. By contrast, $CD4^-CD8^-$ DCs produce the most IFN-γ in the presence of exogenous IL-12 and IL-18. The $CD4^+CD8^-$ DCs seem to be poor producers of all three cytokines. In addition to their effects on the type of cytokine produced by the activated T cells, the DC subtype can influence the amount of cytokine these T cells secrete. $CD4^-CD8^-$ DCs induce more IL-2, IFN-γ, GM-CSF and IL-3 from $CD8^+$ and $CD4^+$ T cells than do $CD4^-CD8^+$ DCs (Kronin V et al. 2001). An intriguing point is that the enhanced cytokine production on stimulation with $CD4^-CD8^-$ DCs compared with $CD4^-CD8^+$ DCs is obtained even when the T-cell proliferation is equivalent, indicating that there are signals from the DCs that govern T-cell cytokine output separately from T-cell proliferation.

1.1.2 MACROPHAGES (Mϕ)

Macrophages are strategically located throughout the body tissues, where they ingest and process foreign materials, dead cells and debris and recruit additional macrophages in response to inflammatory signals. They are highly heterogeneous cells that can rapidly change their function in response to local microenvironmental signals. Macrophages are characterized by expression surface markers such as F4/80, CD11b and CD18 (also known as MAC1), CD68 and Fc receptors (Murray PJ and Wynn T 2011).

Tissue distribution of macrophages

Macrophages are divided into subpopulations based on their anatomical location and functional phenotype (Gordon S and Taylor PR 2005). Specialized tissue-resident macrophages include osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and Kupffer cells (liver). The gut is populated with multiple types of macrophages and DCs, which have distinct phenotypes and functions, but work together to maintain tolerance to the gut flora and food. Secondary lymphoid organs also have distinct populations of macrophages that perform unique functions, including marginal zone macrophages in the spleen, which suppress innate and adaptive immunity to apoptotic cells (McGaha TL

et al. 2011), and subcapsular sinus macrophages of lymph nodes (LNs), which clear viruses from the lymph and initiate antiviral humoral immune responses (Iannacone M et al. 2010).

Phenotype and function of macrophages subsets

Because there is great overlap in surface marker expression between the different macrophage subsets, a useful characterization approach has been to quantify specific gene expression profiles after cytokine or microbial stimulation (Cros et al. 2010). Several macrophage subsets with distinct functions have been described. Classically activated macrophages (M1 macrophages) mediate defence of the host from a variety of bacteria, protozoa and viruses, and have roles in antitumour immunity. M1 macrophages subset is activated by TLR ligands and IFN–γ. They express pro-inflammatory cytokines and inducible nitric-oxide synthase. Alternatively activated macrophages (M2 macrophages) have anti-inflammatory function and regulate wound healing. M2 macrophages are stimulated by IL-4 or IL-13. They express arginase1, the mannose receptor CD206 and the IL-4 receptor α chain. 'Regulatory' macrophages can secrete large amounts of interleukin-10 (IL-10) in response to Fc receptor-γ ligation. Tumour-associated macrophages (TAMs) suppress antitumour immunity, and myeloid-derived suppressor cells (MDSCs) are linked to TAMs and may be their precursors (Mosser D et al.

2008). Although there are obvious differences among the M2 macrophage, regulatory macrophage, TAM and MDSC subsets, they all exhibit immune suppressive activity (Murray PJ et al. 2011). Consequently, when stimulated, macrophages adopt context-dependent phenotypes that either promote or inhibit host antimicrobial defence, antitumour immunity and inflammatory responses. It is generally believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations. Indeed, numerous studies have documented flexibility in their programming, with macrophages switching from one functional phenotype to another in response to the variable microenvironmental signals of the local milieu (Rutshman R et al. 2001).

1.1.3 Macrophages and DCs express several PRRs .

Both DCs and macrophages express several pattern recognition receptor (PRRs) on their surface. These receptors help APCs to exert their function by recognizing pathogen associated molecular patterns (PAMPs). Because normal cells of the body must not be mistakenly removed or compromised, macrophages are selective of the material that they phagocytose. During and following phagocytosis, PRRs (including TLRs, C-type lectin receptors (CLRs), scavenger receptors, retinoic acid-inducible gene 1 (RIG1)-like helicase receptors (RLRs)

and NOD-like receptors (NLRs)) recognize signals associated with invading pathogens, foreign substances (for example, silica or asbestos) and dead or dying cells (Geissmann F et al. 2010; Gordon S et al. 2005). Some PRRs (such as the mannose receptor, DC-specific ICAM3-grabbing non-integrin (DC-SIGN) and macrophage receptor with collagenous structure (MARCO)) function in pathogen binding and phagocytosis, whereas signalling PRRs (which include the TLRs, NLRs and RLRs) sense microbial products and aberrant self on the cell surface or in the cytoplasm of cells and activate transcriptional mechanisms that lead to phagocytosis, cellular activation and the release of cytokines, chemokines and growth factors (Kawai T and Akira S 2011; Osorio F et al. 2011). Macrophages also express numerous secreted molecules, including complement and Fc receptors that bind opsonin molecules, C3b and antibodies, which activate the complement cascade and enhance the process of phagocytosis by tagging the pathogen surface. Thus, macrophages use various surface receptors and secreted molecules to monitor and respond to changes in their environment. Also DCs express several pattern recognition receptors (PRRs) that induce chemokines and cytokines expression upon stimulation. On the contrary of macrophages, triggering of several PRRs simultaneously in DCs, can induce diverse innate responses, which provides the diversity that is required to shape an effective adaptive immune response. PRRs in

DCs can be associated with cellular membrane, such as toll-like receptor (TLR) and C-type lectin receptor (CLRs) or present in the citosol such as RIG-like helicase (RIG-1), NOD-like receptors and DNA sensors (RAGE).

1.2 Toll-like RECEPTORS (TLRs)

Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos (Hashimoto C et al. 1998). In 1996, Hoffmann and colleagues demonstrated that Toll-mutant flies were highly susceptible to fungal infection (Lemaitre B et al. 1996). Subsequently, a mammalian homologue of *Drosophila* Toll receptor (now termed TLR4) was shown to induce the expression of genes involved in inflammatory responses (Medzhitov et al. 1997). In addition, a mutation in the *Tlr4* gene was identified in mouse strains that were hyporesponsive to lipopolysaccharide (Poltorak A et al. 1998). Since then, Toll receptors in mammals have been a major focus in the immunology field. First, several proteins that are structurally similar to TLR4 were identified and named TLRs (Rock FL et al. 1998). The cytoplasmic portion of TLRs shows high similarity to that of the interleukin (IL)-1 receptor family, and is now called the Toll/IL-1 receptor (TIR) domain. Despite of this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-

1 receptors possess an Ig-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain. Genetic approaches have mainly been conducted to analyze the physiological function of TLRs, and have revealed essential roles for TLRs in the recognition of pathogens. Each TLR has been shown to recognize specific components of pathogens, thus demonstrating that the mammalian immune system detects invasion by pathogens via the recognition of microbial components by TLRs (**fig.1**).

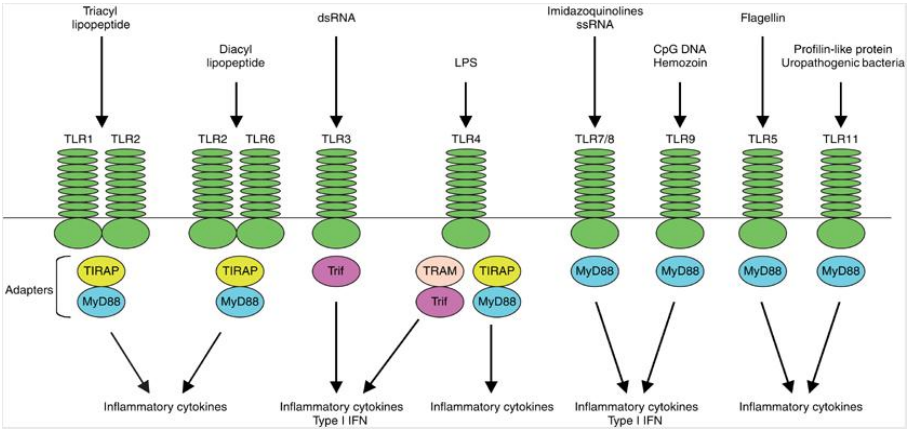


Fig.1 : TLRs and their ligands

To date, 10 members of Toll-like receptors (TLRs) have been identified in human, and 13 in mice, and they are responsible for recognizing pathogens as diverse as Gram-positive and Gram-negative bacteria, viruses, and fungi as well as protozoa. LPS of Gram-negative bacteria is recognized by TLR4. TLR2, in concert with TLR1 or

TLR6, recognizes various bacterial components, including peptidoglycan, lipopeptide and lipoprotein of Gram-positive bacteria and mycoplasma lipopeptide. In particular, TLR1/2 and TLR2/6 discriminate triacyl lipopeptide and diacyl lipopeptide, respectively. TLR3 recognizes double-stranded RNA (dsRNA) that is produced from many viruses during replication (Alexopoulou et al. 2001).TLR5 recognizes bacterial flagellin. Mouse TLR11 recognizes yet unknown components of uropathogenic bacteria, and a profilin-like molecule of the protozoan parasite *Toxoplasma gondii.* TLR7 recognizes synthetic imidazoquinoline-like molecules, guanosine analogs such as loxoribine, single-stranded RNA (ssRNA) derived from human immunodeficiency virus type I (HIV-1), vesicular stomatitis virus (VSV) and influenza virus, and certain siRNAs. While mouse TLR8, which shows the highest homology to TLR7, is thought to be nonfunctional, human TLR8 mediates the recognition of imidazoquinolines and ssRNA. TLR9 recognizes bacterial and viral CpG DNA motifs and malaria pigment hemozoin. After recognition of microbial pathogens, TLRs trigger intracellular signaling pathways that result in the induction of inflammatory cytokines, type I interferon (IFN) and chemokines. Importantly, TLRs activate a common signaling pathway that culminates in the induction of inflammatory cytokines such as tumor necrosis factor (TNF)α, IL-6, IL-1β and IL-12, as well as alternative pathways that induce appropriate and effecter responses

against different types of pathogens (Akira S and Takeda K 2004). In particular, TLR3, TLR4, TLR7, TLR8 and TLR9 induce antiviral responses by inducing type I IFN (IFNβ and multiple IFNα).

1.2.1 TLRs signalling pathways

Each TLR is able to induce a unique signalling pathway which lead to the production of several cytokines and kemokines.

***Activation of NF-kB and AP-1 by the MyD88-dependent pathway*.**

TLRs contain extracellular leucine-rich repeats responsible for the recognition of pathogens, and the transmembrane and cytoplasmic Toll/interleukin-1 receptor (TIR) domains required for initiating intracellular signaling. All TLRs elicit conserved inflammatory pathways, culminating in the activation of nuclear factor (NF)-*k*B and activating protein-1 (AP-1). NF-*k*B is a dimeric transcription factor that belongs to the Rel-homology domain-containing protein family, which includes p65/RelA, p50/NF-*k*B1, p52/NF-*k*B2, RelB and c-Rel (Karin M and Greten FR 2005). The prototypical NF-*k*B is thought to be a heterodimer composed of the p65 and p50 subunits in most types of cells. In unstimulated cells, NF-*k*B is sequestered in the cytoplasm as an inactive form by interacting with inhibitor of NF-*k*B (I*k*B) proteins. Upon stimulation with various TLR ligands, I*k*Bs are phosphorylated at serine residues by an IKK complex consisting of

IKKα and IKKβ protein kinases and a regulatory molecule, IKKγ/Nemo. The phosphorylation targets I*k*Bs for ubiquitination and degradation by the 26S proteasome, allowing NF-*k*B to be released into the nucleus and to bind to the *k*B site. AP-1 is a dimeric basic region leucine zipper (bZIP) protein composed of members of Jun, Fos, activating transcription factor (ATF) and the Maf subfamily, which bind TPA-response elements or cAMP-response elements. Among the AP-1 family proteins, c-Jun is thought to play central roles in inflammatory responses. AP-1 activation in TLR signaling is mostly mediated by MAP kinases such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK). Many TLR ligands activate these MAP kinases in similar kinetics. The proximal events of TLR-mediated intracellular signaling are initiated by TIR-domain-dependent heterophilic interactions with TIR-domain-containing cytosolic adapters such as myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adapter protein (TIRAP)/Mal, TIR domain-containing adapter inducing IFNβ (Trif) (also known as TICAM1) and Trif-related adapter molecule (TRAM) (also known as TICAM2) (Takeda K and Akira S 2005; Akira S and Takeda K 2004). MyD88 is a central adapter shared by almost all TLRs. The association of TLRs and MyD88 recruits members of the interleukin-1 receptor-associated kinase (IRAK) family (**fig.2**).

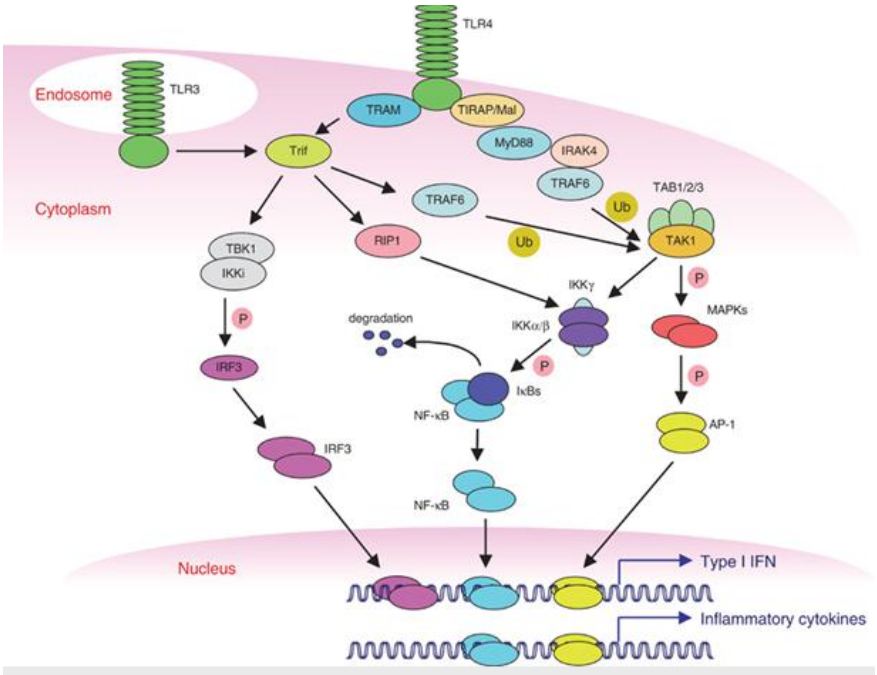


Fig.2: TLRs signalling: MyD88 dependent and MyD88 independent pathways

So far, four IRAKs are identified: IRAK1, IRAK2, IRAK4 and IRAK-M. While IRAK1 and IRAK4 possess intrinsic serine/threonine protein kinase activities, IRAK2 and IRAK-M lack this activity, suggesting that they negatively regulate TLR-mediated signaling. In response to stimuli, IRAK4 and IRAK1 are sequentially phosphorylated and dissociated from MyD88, which results in activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), which contains an N-terminal RING domain that is found in a number of E3 ubiquitin ligases, and forms a complex with Ubc13 and Uev1A, serving as the ubiquitin E3 ligase to promote synthesis of lysine 63-linked

polyubiquitin chains. TRAF6 in turn activates transforming growth factor-β-activated protein kinase 1 (TAK1), a member of the MAP kinase kinase kinase (MAP3K) family, in a ubiquitin-dependent manner (TAK1 forms a complex with TAB1, TAB2 and TAB3 (Chen ZJ et al. 2005). TAK1 activates the IKK complex that leads to NF-*k*B activation. However, a direct substrate of TAK1 that is responsible for the IKK activation in TLR signaling remains unknown. TAK1 simultaneously phosphorylates two members of the MAP kinase kinase family, MKK3 and MKK6, which subsequently activate JNK and p38. ERK is also activated in response to TLR ligands through the activation of MEK1 and MEK2, although an upstream kinase activating MEK1 and MEK2 in TLR signaling remains unknown. Other members of MAP3K, MEKK3 and Tpl2 (also known as Cot1) are also implicated in the activation of MAP kinase activation in TLR4 signaling. JNK and p38 activation and IL-6 production in response to LPS are impaired in MEKK3-deficient cells (Huang Q et al. 2004). While JNK and p38 activation in response to LPS is intact in Tpl2-deficient macrophages, ERK activation and TNFα production are severely impaired (Sugimoto K et al. 2004). The signaling pathway from MyD88 to the activation of NF-*k*B and AP-1 is utilized by almost all TLRs, and this MyD88-dependent pathway controls inflammatory responses.

Activation of NF-kB and AP-1 by the Trif-dependent pathway

Macrophages and DCs isolated from MyD88-deficient mice fail to activate NF-*k*B and MAP kinases and produce inflammatory cytokines in response to various TLR ligands, including TLR2, TLR5, TLR7 and TLR9, indicating that MyD88 is the sole adapter for these TLRs (Zhang D et al. 2004; Hayashi F et al. 2001; Hemmi H et al. 2002). MyD88-deficient cells also fail to produce inflammatory cytokines in response to LPS. However, LPS is still capable of activating NF-*k*B and MAP kinases in MyD88-deficient mice; moreover, this activation in MyD88-deficient cells is delayed in reaching a peak in comparison with wild-type cells (Kawai T et al. 1999). Importantly, LPS can induce IFNβ production by macrophages and DCs in a manner independent of MyD88 (Kawai T et al. 2001). Type I IFN, which are produced in response to virus infections or LPS, are key cytokines that induce an antiviral state in target cells, as well as facilitate antiviral adaptive immune responses. Together, these observations suggest that other adapters mediate late activation of NF-*k*B and MAP kinases which is associated with type I IFN induction in TLR4 signaling. In such contexts, Trif was identified to be involved in the MyD88-independent pathway, and importantly has the ability to activate NF-*k*B, MAP kinases and IFNβ promoter, in contrast to MyD88 and TIRAP/MyD88-adapter-like (MAL), which have no ability to activate IFNβ promoter (Oshiumi H et al. 2003). Trif-deficient mice show

defective type I IFN in response to LPS. Early activation of NF-*k*B and MAP kinases in response to LPS is normally observed in Trif-deficient mice; thus, Trif plays a critical role in type I IFN induction in TLR4 signaling (Yamamoto M et al. 2003). However, production of inflammatory cytokines was reduced in Trif-deficient mice, suggesting that both MyD88- and Trif-dependent pathways are required for maximal induction of inflammatory cytokines in response to LPS, although it is unclear how a Trif-dependent pathway facilitates inflammatory responses. While Trif appears to be utilized by TLR4, TLR4 does not directly interact with Trif. The fourth adapter, TRAM, is selectively involved in the TLR4-mediated pathway. In contrast to TIRAP/MAL, which mediates activation of the MyD88-dependent pathway, TRAM is required for the activation of the Trif-dependent pathway, indicating that TRAM is an adapter linking TLR4 to Trif (Yamamoto M et al. 2003). Responses to poly IC, a TLR3 ligand, are relatively normal in MyD88-deficient cells. It has been shown that TLR3 does not interact with MyD88; thus, MyD88 is not utilized by TLR3 signaling. All responses to poly IC, including inflammatory cytokine and type I IFN induction and activation of NF-*k*B and MAP kinases, are ablated in Trif-deficient mice, whereas TRAM-deficient cells normally respond to poly IC. Collectively, Trif is the sole adapter utilized by TLR3.

Activation of IRFs by TLR3 and TLR4

As mentioned above, TLR3 and TLR4 activate the Trif-dependent pathway, which leads to induction of type I IFN, particularly IFNβ. Transcription of IFNβ gene is tightly controlled by cooperative activation of several transcription factors, including NF-*k*B, ATF2/c-Jun, interferon regulatory factor (IRF)3 and IRF7 (Theofilopoulus AN et al. 2005). While NF-*k*B and ATF2/c-Jun are activated by numerous stimuli such as TLR ligands, IL-1β, TNFα and DNA damage, IRF3 and IRF7 are activated when cells are exposed to LPS, poly IC and virus infection, and mainly control type I IFN. IRF3 and IRF7 are structurally related proteins present in the cytoplasm in unstimulated conditions. Upon stimulation, these proteins become phosphorylated by noncanonical IKKs, TANK-binding kinase 1 (TBK)1 (also known as T2K or NAK) and IKKi (also known as IKKε), translocating into the nuclei to regulate target gene expression (Sharma S et al. 2003; Fitzgerald KA et al. 2003). While IRF3 is constitutively expressed, expression of IRF7 is weak in unstimulated cells and dramatically induced by stimulation with LPS, type I IFN or virus infections. Thus, initial induction of IFNβ is largely dependent on IRF3 activation. Secreted IFNβ acts on neighboring cells and activates the JAK–STAT pathway via type I IFN receptor to induce IRF7 expression, which results in the amplification of type I IFN induction through a positive feedback mechanism. While

MyD88-deficient cells show normal IRF3 activation in response to LPS and poly IC, Trif-deficient cells fail to activate IRF3.

Activation of IRFs by TLR7/8 and TLR9

It is known that TLR7/8 and TLR9 ligands also trigger type I IFN induction by plasmacytoid DCs (pDC), a subset of DCs that are specialized to produce large amounts of type I IFN in response to TLR7/8 and TLR9 ligands and virus infections (Liu YJ et al. 2005). Treatment of pDCs with TLR9 ligands causes nuclear translocation of IRF7, and pDCs derived from IRF7-deficient mice are incapable of producing type I IFN in response to TLR7/8 and TLR9 ligands. Thus, IRF7 is an essential transcription factor that regulates type I IFN induction in pDCs. Importantly, IRF7 interacts with MyD88, IRAK1 and TRAF6 to form a signaling complex **fig.3** (Kawai T et al. 2011).

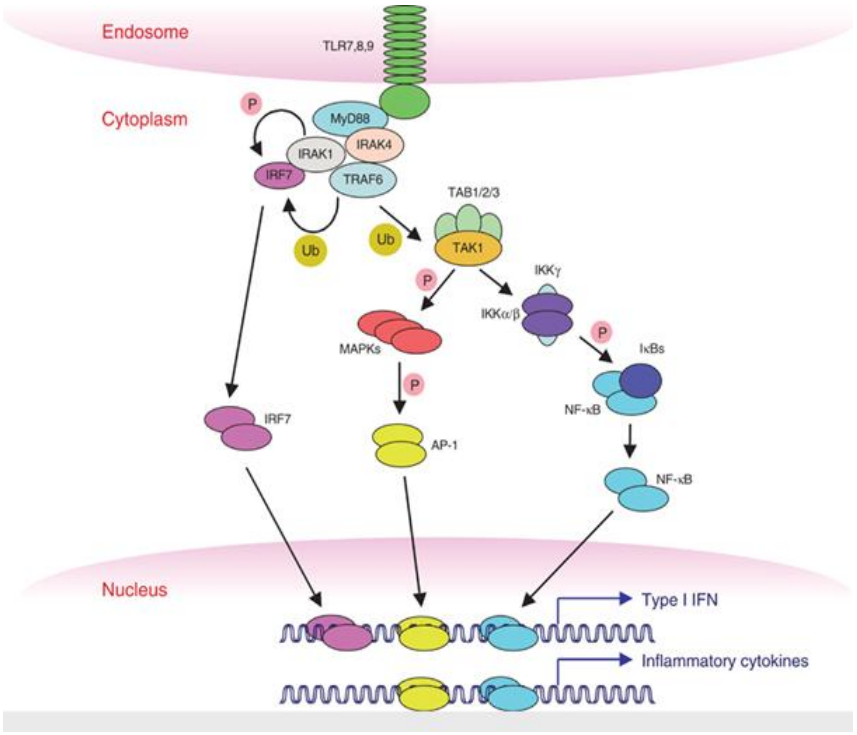


Fig.3: TLRs signalling downstream TLR7, TLR8 and TLR9

It is also reported that IRF5 interacts with MyD88. Unexpectedly, IRF5 is essential for inflammatory cytokine induction rather than type I IFN induction by TLR4, TLR7, TLR9 and TLR3 in mice. IRF5 translocates into the nucleus in response to ligand stimulation. In the nucleus, IRF5 binds ISRE motifs present in the promoter region of inflammatory cytokine genes and causes their expression, probably through the coordinated activation with NF-*k*B (Takaoka A et al. 2005). IRF8 is implicated in TLR9-mediated responses. In IRF8-deficient pDCs, both type I IFN and inflammatory cytokines induced

by TLR9 ligand are abolished. In these cells, the NF-*k*B DNA-binding activity in response to TLR9 ligand was severely impaired, suggesting that IRF8 activates NF-*k*B in pDCs (Tsujimura H et al. 2004).

1.3 C-type lectin receptors (CLRs)

The term C-type lectin was introduced to distinguish between Ca^{2+}-dependent and Ca^{2+}-independent carbohydrate-binding lectins. CLRs share at least one carbohydrate recognition domain, which is a compact structural module that contains conserved residue motifs and determines the carbohydrate specificity of the CLR. The CLR family now includes proteins that have one or more domains that are homologous to carbohydrate recognition domains but do not always bind carbohydrate structures (Zelensky A et al. 2005). CLRs exist both as soluble and transmembrane proteins. The CLRs can be divided into two groups: group I CLRs belong to the mannose receptor family and group II CLRs belong to the asialoglycoprotein receptor family and include the DC-associated C-type lectin 1 (dectin-1; also known as CLEC7A) and DC immunoreceptor (DCIR; also known as CLEC4A) subfamilies CLRs expressed by DCs interact with pathogens primarily through the recognition of mannose, fucose and glucan carbohydrate structures. Together, these CLRs recognize most classes of human pathogens; mannose specificity allows the recognition of viruses, fungi and mycobacteria, fucose structures are more specifically

expressed by certain bacteria and helminths and glucan structures are present on mycobacteria and fungi (Rothfuchs A et al. 2007; Van Kooyk Y et al. 2008). Recognition by CLRs leads to the internalization of the pathogen, its degradation and subsequent antigen presentation. CLR triggering by different pathogens can induce diverse immune responses. The underlying signalling processes are complex and depend on crosstalk with other PRRs, the ligand- or carbohydrate-specific signalling pathway and the DC subset. Recent studies suggest that there are two general ways by which CLRs induce signalling pathways. CLRs, such as macrophage-inducible C-type lectin (mincle; also known as CLEC4E), dectin 2 (also known as CLEC6A), blood DC antigen 2 protein (BDCA; also known as CLEC4C) and C-type lectin domain family 5, member A (CLEC5A), are associated with and induce signalling pathways through immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules, such as Fc receptor γ-chain (FcRγ) or DAP12 (Sato K et al. 2006; Cao et al. 2007). Other CLRs, such as dectin 1, DC-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209), DCIR and myeloid C-type lectin-like receptor (MICL; also known as CLEC12A) induce signalling pathways through the activation of protein kinases or phosphatases that either directly or indirectly interact with their cytoplasmic domains (Gringhuis S et al. 2007; Rogers N et al. 2005). Several CLRs, such as DC-SIGN, BDCA2, DCIR

and MICL, induce signalling pathways that modulate TLR-induced gene expression at the transcriptional or post-transcriptional level. However, to date these receptors have not been shown to induce gene expression in the absence of other PRR signalling (Gringhuis S et al 2007; Cao et al. 2007). By contrast, other CLRs, such as dectin 1, dectin 2 and mincle, induce gene expression following carbohydrate recognition independently of other PRRs (Rogers N et al. 2005; Sato K et al. 2006). The transcription factor nuclear factor-*k*B (NF-*k*B) is a key mediator of inducible gene expression in the immune system, although many other transcription factors have an equally essential role. Indeed, the activation of the transcription factors activator protein 1 and members of the IFN regulatory factor family by diverse PRRs provides flexibility and variability to the regulation of cytokine gene expression, which is needed to combat different pathogens (Akira S et al. 2006; Goriely S et al. 2008). CLR-induced signal transduction seems to mainly activate or modulate NF-*k*B functions, and the regulation of other transcription factors by CLRs has received little attention to date.

1.3.1 Dectin-1 signaling pathway

Dectin 1 induces a gene expression profile independently of other PRRs. Specifically, dectin 1 activates gene expression through the recognition of β-1,3-glucan PAMPs expressed by a broad range of

fungal pathogens, including *C. albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii*, which then leads to the activation of NF-kB (Gross O et al. 2006; Saijo S et al. 2007) (**fig.4**). Recruitment of SYK to the phosphorylated YxxL motif in the cytoplasmic tails of two dectin 1 molecules (Rogers N et al. 2005; Underhill DM et al. 2005) is required for the assembly of a scaffold complex that consists of CARD9, BCL-10 and MALT1). Dectin 1-induced NF-*k*B activation and cytokine production by DCs from mice deficient in CARD9, BCL-10 or MALT1 is severely defective, and the survival of CARD9-deficient mice following infection with *C. albicans* is greatly impaired (Gross O et al. 2006).

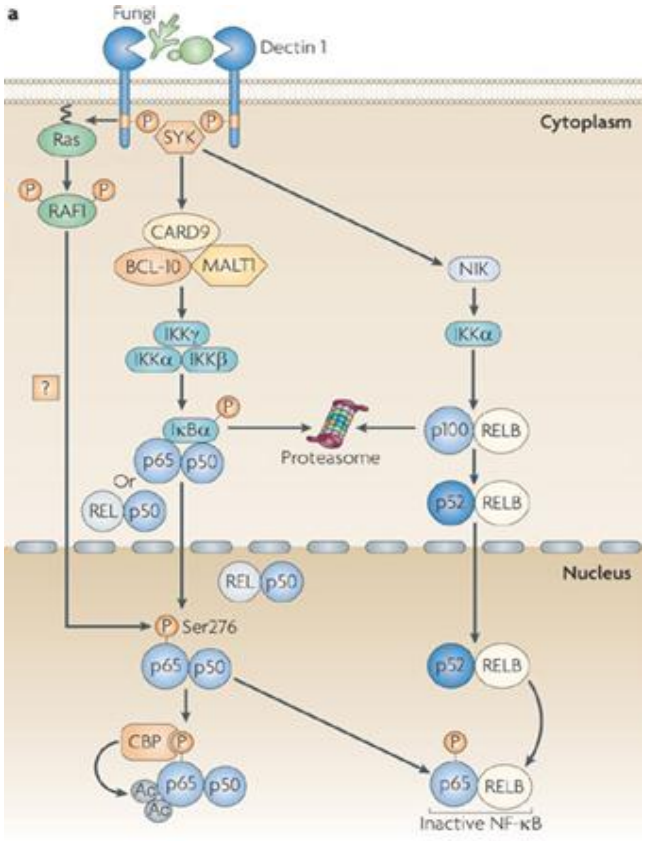


Fig.4: Dectin-1 signalling through SYK and RAF1 directs NF-kB-mediated cytokine expression.

It remains to be established how the CARD9–BCL-10–MALT1 complex relays the activation signals for NF-*k*B, but it seems possible that this involves the recruitment and activation of the TNF receptor-associated factor 2 (TRAF2)–TRAF6 complex in an analogous manner to how lymphocyte antigen receptors use the CARMA1–BCL-10–

MALT1 complex to activate NF-*k*B (Rawlings DJ et al. 2006). Dectin 1 triggering of the SYK–CARD9 pathway results in the activation of the NF-*k*B subunits p65 and REL, which are part of the the canonical NF-*k*B pathway (Gross O et al. 2006). NF-*k*B subunits, a recent study has shown that the activation of the SYK pathway by dectin 1 leads to the induction of the non-canonical NF-*k*B pathway, which mediates the nuclear translocation of RELB–p52 dimers through the successive activation of NF-*k*B-inducing kinase (NIK) and I*k*B kinase-α (IKKα). It is unclear how SYK activation by dectin 1 results in the induction of the non-canonical NF-*k*B pathway. Finally, a recent study in mice has proposed a role for the SYK–CARD9-dependent pathway that is activated in response to *C. albicans* infection in the activation of the NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3) inflammasome, which is required for the processing of pro-IL-1β to active IL-1β by caspase 1 through the generation of reactive oxygen species (Gross O et al. 2009). Together, the data suggest that SYK activation downstream of dectin 1 activates different signalling pathways that lead to the activation of the canonical and non-canonical NF-*k*B pathways, as well as the activation of the NLRP3 inflammasome. In addition to SYK activation, dectin 1 induces a second signalling pathway that leads to RAF1 activation. Although RAF1 does not depend on SYK signalling for its activation, the two pathways converge at the level of NF-*k*B activation, as RAF1

activation leads to the phosphorylation of SYK-induced p65 at Ser276 (Gringhuis S et al. 2009).

1.4 Interleukin 22 (IL-22)

In 2000, the Renauld group described a secreted, a-helical protein that they discovered during a search for differentially expressed genes in IL-9 stimulated murine T lymphoma cells (Dumoutier L, Louahed J et al. 2000). Mouse IL-22 shares structural similarities and 22% homology with mouse IL-10 and was originally called IL-TIF for IL-10-related T cell-derived inducible factor. Few times later the human protein was identified (Moore KW, de Waal MR et al. 2001; Schulze-Tanzil G, Sabat R et al. 2009). Human IL-22 shares 79% homology with mouse IL-22 and 25% identity with human IL-10 (Dumoutier et al 2000). IL-22 was further classified as a class 2 a-helical cytokine of the IL-10 family cytokines (Pestka S et al. 2004). IL-10 cytokine family comprises IL-10, IL-19, IL-20, IL-24, IL-26, IL-28a, IL-28b and IL-29. The assembly of these cytokines to a family is based on their similarities in terms of the structure of their encoding genes, their encoding genes, their protein structure, and their receptor use. These cytokines elicit diverse host defence mechanisms, especially from epithelial cells, during various infections and are essential for maintaining the integrity and homeostasis of tissue epithelial layers (Ouyang W, Rutz S et al. 2011). The IL-10 family members exert their

biological effects via heterodimeric, transmembrane receptor complexes composed of a type 1 receptor chain (R1) and type 2 receptor chain (R2). R1 chains have the longer intracellular moiety able to bind signal transducer and activator of transcription (STAT) molecules.IL-10 family members share receptor chains, in fact, the family comprises nine cytokines but there are only four R1 chains and two R2 chains (Wolk K and al. 2009) (**fig.5**)

	IL-10R1	**IL-20R1**	**IL-22R1**	**IL-28R1**
IL-10R2	IL-10	IL-26	IL-22	IL-28α,β IL-29
IL-20R2	?	IL-19 IL-20 IL-24	IL-20 IL-24	?

Fig5: Combination of receptor chains in receptor complexes enabling the effects of the IL-10 family members

Despite these numeral similarities the IL-10 family members exhibit different biological effects (Wolk K and Sabat R 2006).

1.4.1 The expression of IL-22 in cells of the immune system

IL-22 is expressed by many different types of lymphocytes, including both those of the innate and adaptive immune system. This includes

CD4 T cells, most notably Th17 cells, and γτ T cells, NK cells, LTi cells and LTi-like cells. Regulation of IL-22 expression by these different subsets has some common qualities, such as similar activation receptors and transcription factors, but there also unique mechanisms (Zenewicz L and Flavell R 2008).

DC cells as source of IL-22

Studies have identified IL-22 expression in $CD11c^+$ cells presumed to be dendritic cells even if the stimuli which can drive IL-22 production from CD11c+ cells have not been fully elucidated yet. Zheng Y et al. reported that IL-22 is crucial in innate immunity against the infection with *C.rodentium*. They reported that WT and Rag2 KO mice produce a comparable production of IL-22 in response to infection. They suggested lamina propria CD11c+ DCs stimulated with IL-23 may be the source of IL-22 However, they could not exclude that other immune cells, like NK cells, could produce this cytokine (Zheng et al. 2008). Becker C et al. reported that lamina propria CD11c+ cells were able to produce IL-22 in response to LPS and CpG and IL-23 in a model of acute colitis in mice (Becker C et al. 2009). Romani et al. reported that CD11c+ DC cells of peyer's patches were able to produce IL-22 in response to IL-23 stimulation in a mouse model of *Candida albicans* infection (De Luca et al. 2010).

NK cells as source of IL-22

IL-22 transcripts were first detected in human peripheral blood NK cells stimulated with IL-12 and IL-2 (Wolk K, Kunz S et al. 2002) and subsequently in mouse NK1.1$^+$ cells (Zenewicz LA et al. 2008). In one study, analysis of human tonsils and Peyer's Patches (PPs) revealed an NK cell subset expressing the receptor NKp44, a marker that is absent on circulating NK cells (Cella M et al. 2009). NKp44$^+$ cells are predominantly found in the mucosal areas covering the lymphoid aggregates. Transcriptional and functional profiles reveal that mucosal NKp44$^+$ cells are not proficient at typical NK cell functions such as release of perforin and IFN-γ. Instead, NKp44$^+$ cells isolated from the mucosae produce IL-22. Although IL-22 is frequently produced together with IL-17 by CD4$^+$ T helper 17 (Th17) cells (Liang et al. 2006; Wilson et al. 2007), NKp44$^+$ cells do not produce IL-17 (Cella M et al. 2009). Corresponding analysis of NK cells in the murine gut yielded very similar findings (Cella et al. 2009; Sanos et al. 2009). Analysis of NK cells in the lamina propria and intraepithelial surfaces of the mouse small intestine with the use of multiple markers such as NKp46, NK1.1, CD127, and c-Kit (not NKp44, which is only expressed in humans) revealed substantial heterogeneity. A subpopulation of NKp46$^+$ cells express CD127 (IL-7Rα) and c-Kit, lack Ly49 receptors, and are poor producer of perforin and IFN-γ. Interestingly, the classical NK cell marker NK1.1 is expressed only on a small subset of

these cells. Functional characterization of NKp46^{+}CD127^{+}c-Kit^{+}NK1.1^{-} and NKp46^{+}CD127^{+}c-Kit^{+}NK1^{+} cells confirmed expression of the IL-22 transcript (Sanos et al. 2009) and protein (Cella et al. 2009), comparable to human NKp44^{+} cells. Moreover, NKp46^{+}CD127^{+}c-Kit^{+}NK1.1^{-} and NKp46^{+}CD127^{+}c-Kit^{+}NK1.1^{+} cells express the GM-CSF transcript (Satoh-Takayama et al. 2008). A second subpopulation of NKp46^{+} cells lacks CD127 and bears features typical of classical NK cells, such as coexpression of NK1.1, various Ly49 receptors, perforin, and IFN-γ. Collectively, these studies demonstrate the conservation of a unique NK cell subset that specializes in IL-22 secretion in both humans and mice (**fig.6**).

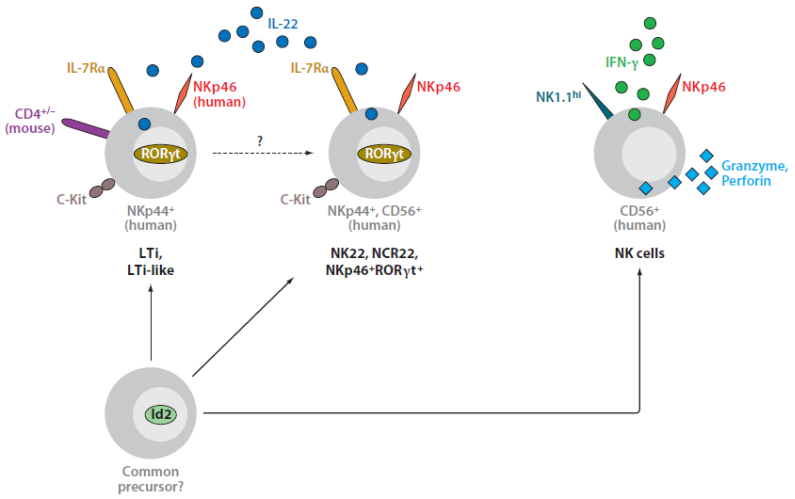


Fig.6: IL-22 producing NK subtypes.

Innate lymphoid cells as source of IL-22

ILCs are an emerging family of innate lymphocytes that share the requirement for a Id2–dependent precursor cell for development and do not require the recombination of an antigen-specific receptor (Spits H and Di Santo J 2011). There are several classes of ILCs that have the ability to secrete T_H1 cell–associated cytokines or T_H2 cell–associated cytokines and also populations that express IL-22. Those IL-22-producing ILCs share several common phenotypic and transcriptional similarities, largely lacking expression of most lineage markers (TCRαβ, TCRγδ, CD3, CD8, CD5, CD19, B220, Gr-1, CD11b, F4/80, Ter119 and NK1.1) while expressing several characteristic ILC markers (CD90, CD127, CD132). IL-22-producing ILCs are responsive to common γ-chain cytokines, such as IL-7, which can promote the expansion of various ILC populations (Satoh-Takayama N et al. 2008). In addition to requiring the transcriptional inhibitor Id2, the IL-22-producing ILCs also require the transcription factor RORγt for their development and express IL-23R (Spits H and Di Santo J 2011), whose ligation is sufficient to induce IL-22 production. Among the IL-22 producing ILCs, specific cell population can be defined on the basis of surface marker expression, coexpression of other cytokines, localization, transcriptional regulation and biological functions (**fig.7**).

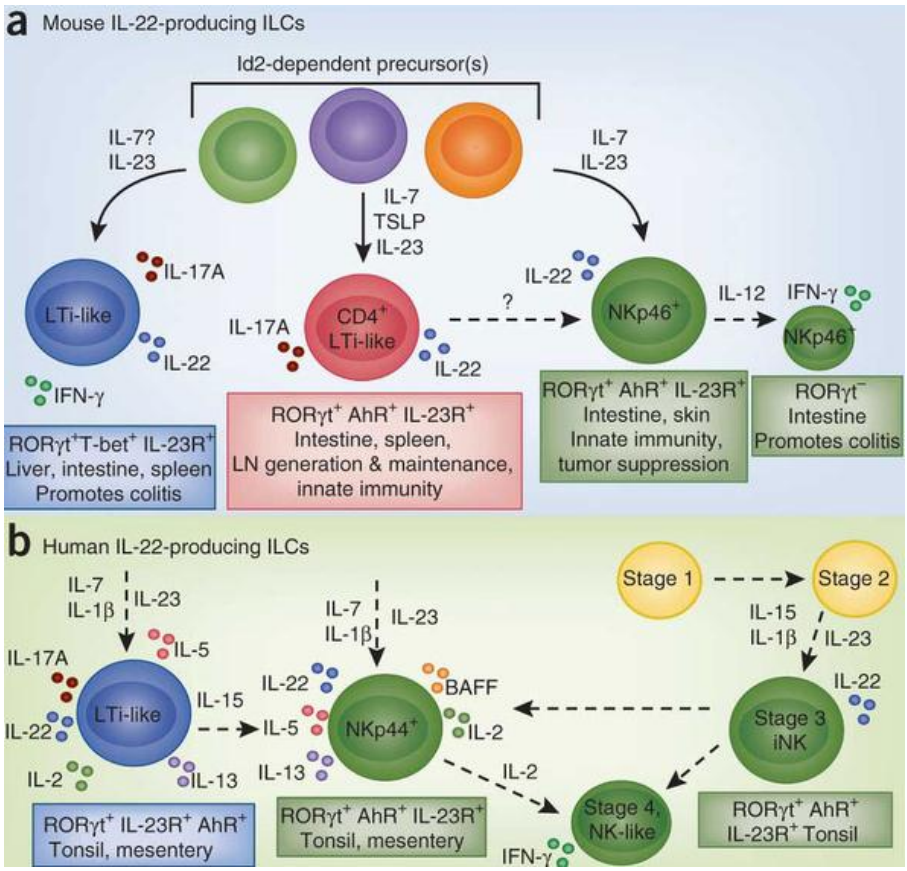


Fig.7: regulation, function and lineage relationship of IL-22-producing ILCs. ILCs have been identified as a critical source of IL-22 both in mouse (a) and in human (b)

IL-22 expression and regulation in Tcell population

IL-22 was originally found to be expressed in mouse and human T cells but not B lymphocytes (Dumoutier L, Louahed and Renauld 2000; Wolk K, Kunz S et al.2002). Since then, IL-22 expression has been reported in a broad range of T cell types. In humans, the major

producers of IL-22 seem to be cells of the T_H1 and T_H22 helper T cell subsets and IL-22-producing cytotoxic T cells ('T_C22 cells'), with expression also observed in T_H17 cells, IL-17-producing cytotoxic T cells ('T_C17 cells') and γδ T cells (Sonnemberg et al. 2011). In mice, high expression of IL-22 is observed in T_H17 and T_C17 cells, as well as in the innate-like γδ T cells and NKT cells (**fig.8**). IL-22 is produced by $CD3^+$ ($CD4^-CD8^-$) oligoclonal γδ cells that are detected in the skin epidermis, gut and lung epithelia and at low frequencies in human blood (Bonneville M, O'Brian RL 2010). IL-22 expression by γδ T cells can be driven by IL-1b, IL-23 and agonists of AhR, and IL-22 is coexpressed with IL-17A, IL-17F, IFNg or TNF (Sutton CE et al. 2009; Martin B, Hirota K 2009). Human T_H1 cells have substantial IL-22 production (Volpe E et al. 2008) and were the initial subset demonstrated to produce this cytokine (Volpe et al. 2009).

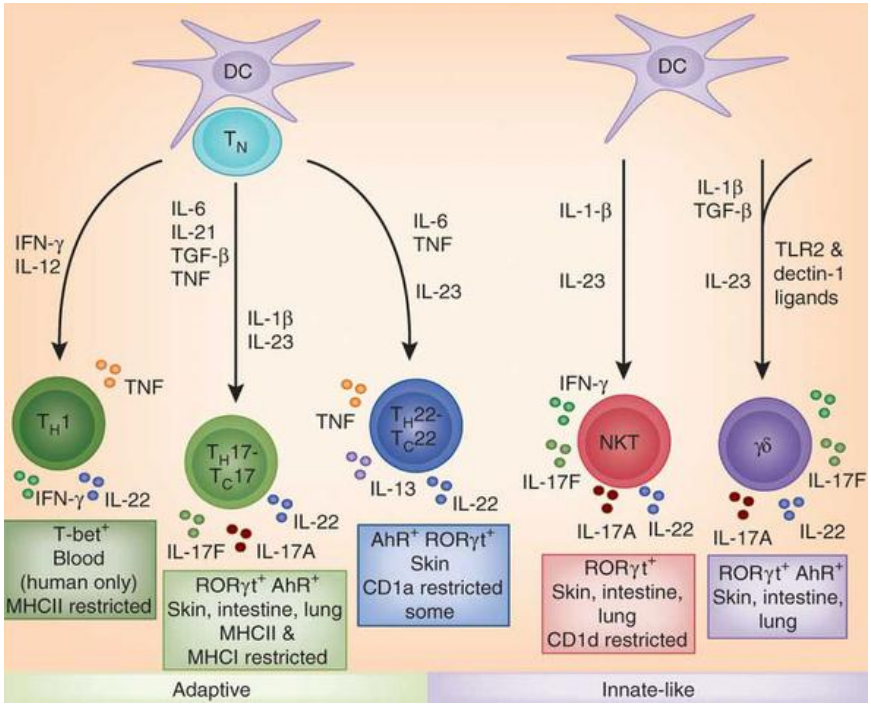


Fig.8: Differentiation, regulation and function of IL-22 producing T cell populations.

IL-22 is produced by $CD3^+$ ($CD4^-CD8^-$) oligoclonal γδ cells that are detected in the skin epidermis, gut and lung epithelia and at low frequencies in human blood (Bonneville M, O'Brian RL 2010). IL-22 expression by γδ T cells can be driven by IL-1b, IL-23 and agonists of AhR, and IL-22 is coexpressed with IL-17A, IL-17F, IFNg or TNF (Sutton CE et al. 2009; Martin B, Hirota K 2009). Human T_H1 cells have substantial IL-22 production (Volpe E et al. 2008) and were the initial subset demonstrated to produce this cytokine (Volpe et al.2009). Although human T_H17 cells also make IL-22, human *IL22* expression

correlates better with expression of the T_H1 subset markers IFN-γ and T-bet than with expression of the T_H17 subset markers IL-17A and AhR. However, an associative relevance for IL-22 expression in human T_H1 cells has yet to be defined in relation to either an immune or inflammatory response. In contrast to human T_H1 cells, mouse T_H1 cells seem to express IL-22 relatively poorly (Liang SC et al. 2006). Major producers of IL-22 in mice are T_H17 cells. Differentiation of naive T cells to the T_H17 lineage requires interaction between the cognate TCR and major histocompatibility complex class II and stimulation with the cytokines IL-6, IL-1β and TGF-β for differentiation, with IL-23 being critical for maintaining expansion of the differentiated subset and optimal IL-22 expression (Liang SC et al. 2006). Similar to its production in mice, the production of IL-22 by human T_H17 cells depends on TGF-β, IL-1β, IL-23 and AhR (Veldhoen M et al. 2008). Although TGF-β is required for T_H17 differentiation in mice and humans, limiting concentrations are required for optimal IL-22 expression, as higher concentrations suppress IL-22 production but not IL-17A production (Ciric B, El-behi et al. 2009). Mouse and human $CD8^+$ T cells can also express IL-22 when differentiated into T_C17 cells by mechanisms similar to those for T_H17 differentiation (Ciric B, El-behi et al. 2009), and these cells are detected in substantial numbers in the blood and/or tissue of humans and mice after infection, cancer and autoimmunity. In addition to

differentiating into T_H1, T_H17 and T_C17 cells, $CD4^+$ T cells and $CD8^+$ T cells can differentiate into the T_H22 and T_C22 lineages, respectively, which express IL-22 but not IFN-γ or IL-17A. Human $CD4^+$ T_H22 and $CD8^+$ T_C22 subsets have been detected in inflamed and diseased skin and clones established with this stable expression profile (Res PC et al. 2010; Nograles KE et al. 2009). The existence of T_H22 cells in mice remains unclear.

1.4.2 IL-22 receptor and signal transduction

IL-22 signals through a distinct class II receptor (IL-22R) composed of the subunits IL-22 R1 (IL-22RA1) and IL-10R2 (IL-22R) which are independently shared with IL-20 and IL-24 and with IL-10 and IL-26 respectively (Xie MH et al. 2000). IL-22 binds first to IL-22R1, and then the IL-22-IL-22R1 complex binds IL-10R2 to propagate downstream signals. IL-22 induces the activation of the JAK-STAT pathway inducing phospholrilation of Jak1 and Tyk2 kinases and the STAT1, STAT3 and STAT5 transcription factors. In addition to the JAK-STAT pathway IL-22 can also activate ERK, JNK and p38 MAP kinase in a rat hepatoma cell line and in human intestinal epithelial cell line HT29 (Lejeune D et al. 2002) **(fig.9)**.

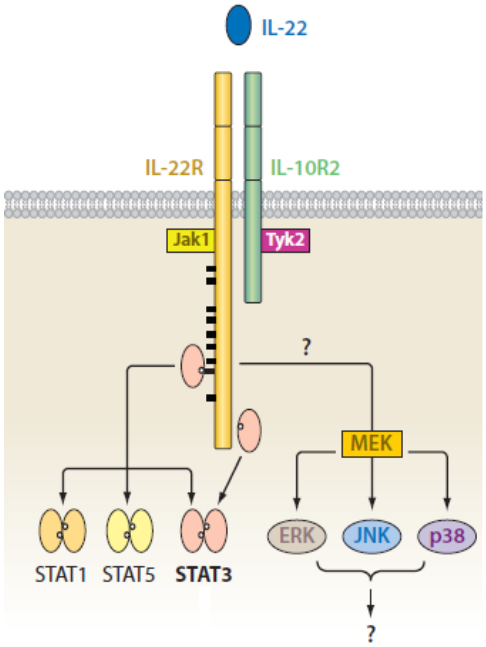


Fig.9: receptor and downstream signalling of IL-22. IL-22 mainly signal through STAT3. IL-22R can associate with STAT3 in a tyrosine-independent manner.

Several research groups have searched for IL-22 targets and have studied the expression of the IL-22 complex components. IL-10R2 is ubiquitously expressed, in fact it is shared between different cytokines (Wolk K, Kunz S et al. 2004), while the expression of IL-22R chains establish whether a cell is an IL-22 target or not. IL-22 doesn't act on immune cells in fact no expression of IL-22R was detected in bone marrow, blood mononuclear cells, thymus or spleen and in others immune cells such as NK cells, B cells, Tcells, macrophages and mature or immature DC (Wolk K, Kunz S et al. 2004). IL-22R was

found to be expressed in organs such as skin, and those from the digestive (pancreas, small intestine, liver and colon) and the respiratory (lung and trachea) system with the highest expression found in the skin and pancreas (Ouyang Y et al. 2010) (**fig.10**).

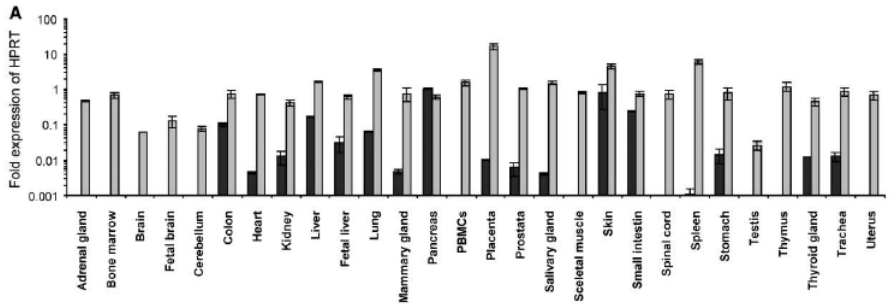


Fig.10: IL-22R and IL10R expression in tissues.

IL-22 binds not only IL-22 receptor but also a soluble receptor called IL-22 binding protein (IL-22RA2).

1.4.3 IL-22 binding protein (IL-22BP)

IL-22 BP represents a CFR2 member that lacks the intracellular and transmembrane domain. it is encoded by an independent gene that lacks sequences for membrane anchorage. The pathways that regulate the expression of this receptor and its function *in vivo* are yet to be characterized. Expression of IL-22BP mRNA has been demonstrated in some human tissues like placenta and breast, in lymphatic organs (thymus, spleen and lymph nodes), in gastrointestinal system (stomach and colon), in the lungs and in the

skin (Wolk K et al. 2004). Inflammatory states have been connected to a downregulation of IL-22 BP expression. In fact, treatment of mice with bacterial LPS, while inducing IL-22 expression in various organs, reduced IL-22 BP expression in the lymph nodes (Wolk K et al. 2004). Additionally, high IL-22 expression in a mouse colitis model was associated with decreased IL-22 BP expression in the inflamed intestine (Wolk K, Witte E et al. 2007). IL-22 appears to be the only IL-10 family member that, once secreted, can be tightly regulated in its activity by an inhibitory soluble receptor.

1.4.4 IL-22 promoter

IL-22 promoter has been studied in CD4+ T cells as they are the main IL-22 producer cells. STAT3 and Notch transcription factor regulates IL-22 production from both Th1 and Th17 cells. STAT3 is essential for the differentiation of Th17 cells and for the expression of the Th17 key transcription factor RoR$\gamma\tau$ and RoRα in response to TGF-β, IL-6 and IL-21 (**fig.11**). STAT3-deficient T cells are impaired in the expression of IL-22 (Ouyang W et al. 2011).

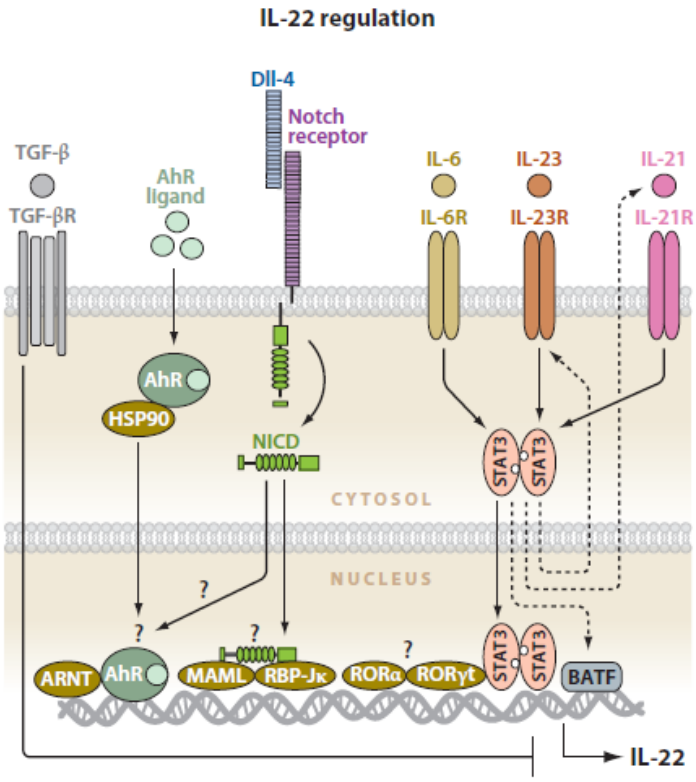


Fig.11: IL-22 promoter in Th17 cells

The retinoic acid-related orphan nuclear receptor (RORγt)

The retinoic acid-related orphan nuclear receptor RORγt is necessary and sufficient to drive Th17 development. Interestingly, most, but not all, cell types that produce IL-22 express RORγt as well. The exact involvement of RORγt and RORα in the transcriptional regulation of IL-22 is not well understood. RORα-deficient T cells show normal IL-

22 expression under Th17 condition in vitro, and forced expression is not sufficient to induce IL-22. Likewise, overexpression of RORγt alone is not sufficient for IL-22 expression in murine (Ivanov II et al. 2006) or human T cells (Wilson NJ et al. 2007). RORγt-deficient cells, however, show strongly reduced IL-22 expression in vitro in the presence of TGF-β/IL-6 or TGF-β/IL-6/IL-23 (Yang XO et al. 2008) or TGF-β/IL-21/IL-23 (Nurieva L et al. 2007). However, Duhen et al. (Duhen et al 2009) report that human Th22 cells produce IL-22 while expressing low to undetectable levels of RORγt. When considered together, these findings suggest that RORγt acts in combination with other pathways to regulate IL-22 expression. Interestingly, only RORα/RORγt-double-deficient T cells totally lack IL-22 expression (Yang XO et al. 2008).

The cytosolic Aryl hydrocarbon receptor (AhR)

The cytosolic aryl hydrocarbon receptor has been identified as a crucial factor in the development of Th17 cells. AhR was suggest as link between the adaptive immune system and environmental factors because it recognizes numerous small xenobiotic and natural molecules, such as 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) and the tryptophan photometabolite 6-formylindolo(3,2-b) carbazolo (FITC). Th17 cell express particularly high levels of AhR in mouse and human (Veldohem et al. 2008; Quintana FJ et al. 2008). AhR

deficiency impairs but does not prevent Th17 development. Interestingly, AhR is essential for IL-22 expression in Th17 cells (Veldohem et al. 2008), γδ T cells (Martin B et al. 2009), and human Th22 cells (Duhen T et al. 2009). $CD4^+$ T cells from AhR-deficient mice can develop Th17 cell responses, but when confronted with AhR ligand they fail to produce IL-22. Th17 cells from AhR-deficient mice cannot produce IL-22 despite the presence of IL-23 under inflammatory conditions such as immunization with CFA or injection with heat-killed mycobacteria (Veldohem et al. 2008). The exact mode of action of AhR remains elusive. AhR is apparently associated with other Th17-related factors to activate *Il22* transcription, given that, in AhR-deficient T cells, forced expression of AhR under neutral, Th1, or Th2 conditions does not induce IL-22 even in the presence of its ligand FICZ (Veldohem et al. 2009).

1.4.5 The functions of IL-22

IL-22 seems to be a novel type of immune mediator, which although produced by immune cells does not affect these cells but rather regulates the function of certain tissue cells (Wolk K et al. 2004).

Effect of IL-22 on epidermal cells

Keratinocytes are the major target cells of IL-22 in the skin (Wolk K et al. 2004). In 2004, it was described an increased expression of the antimicrobial proteins β-defensin (BD)-2, BD3 and S100A7 (psoriasin) (Wolk K et al. 2004). Later on, IL-22 influence was analyzed in more detail using microarray (Wolk k et al. 2006; Boniface et al. 2005). The broad range of investigated gene expressions revealed regulation of only a limited number of genes by IL-22. These genes can mainly be attributed to three important keratinocyte functions: their antimicrobial defense, their migratory capacity, and their terminal differentiation. In fact, IL-22 affects the keratinocytes in a manner that leads to enhanced antimicrobial defense and mobility and an inhibition of terminal differentiation (Wolk k et al. 2006; Boniface et al. 2007). Aside from the induction of β-defensins and S100A7 as described above, other antimicrobial proteins of the S100 protein family such as S100A8 and S100A9 were found by these studies to be up-regulated (Liu H et al. 2007). BD2 is especially effective in inhibiting gram-negative bacteria, whereas the spectrum of BD3 clearly includes gram-positive bacteria. S100A7 was demonstrated as a major player in the resistance against *E. coli*. The enhanced migration capacity of keratinocytes is facilitated by IL-22 through enhanced expression of the extracellular matrix-degrading enzymes matrix metalloproteinase (MMP)1 and MMP3 (Wolk k et al. 2006;

Boniface et al. 2005). In fact, IL-22-treated keratinocytes showed enhanced migration on collagen matrix. Moreover, IL-22 decreased the keratinocyte expression of annexin A9, which plays a role in the regulation of actin-cytoskeleton dynamics.

Effect of IL-22 on hepatocytes

Hepatocytes represent another important IL-22 target cell population. In 2000, the Renauld group demonstrated an IL-22-induced mRNA expression of several acute phase proteins such as serum amyloid A (SAA), α1-antichymotrypsin and haptoglobin in HepG2 human hepatoma cells and, concordantly, an increase of SAA mRNA expression in the liver of IL-22-treated mice (Dumoutier L et al. 2000). Furthermore, it was shown that IL-22 also directly induced production of LPS binding protein (LBP) in primary human hepatocytes and HepG2 cells *in vitro* and found increased blood levels of LBP in mice after IL-22 application, which was accompanied by enhanced hepatic but not renal or pulmonary LBP expression (Wolk K et al. 2007). Therefore, IL-22 promotes the hepatic production of proteins with systemically inflammation-limiting and protective roles which, in fact, favor the degradation of proteases known to be produced in large amounts during inflammation (α1-antichymotrypsin), enhance the opsonization and phagocytosis of gram-negative bacteria (SAA), protect against hemoglobin-induced

oxidative tissue damage (haptoglobin), and neutralize bacterial components and therefore limit their immunostimulatory capacity (high LBP levels). Additionally, IL-22 protects against liver injury. In 2004, the Gao group investigated a T-cell-mediated murine hepatitis model induced by injection of ConA, which is characterized by T-cell IL-22 production, and showed that the use of neutralizing anti-IL-22 antibodies worsened liver injury, while pretreatment with IL-22 prevented it (Radaeva S et al. 2004). In a subsequent study by the group of R. A. Flavell, the protective role of IL-22 in ConA-mediated liver injury was emphasized by use of IL-22-deficient mice, which were highly susceptible in this hepatitis model, in contrast to IL-17-deficient mice, which were equally as susceptible as wild type mice (Zenewicz LA et al. 2007). The exact principle behind the protective action of IL-22 still remains to be resolved. It seems to be clear that IL-22 protection is not associated with lower immune activation (Zenewicz LA et al. 2007).

Effect of IL-22 on respiratory and gastrointestinal cells

A study by the Kolls group investigated effects of IL-22 on respiratory epithelial cells by use of microarray analysis. The authors found an upregulation of G-CSF, the antimicrobial proteins BD2, S100A7 and S100A12, the neutrophilic granulocyte-attracting chemokines CXCL1 and CXCL5, and thyroid oxidase 2 in human bronchial epithelial cells,

as well as an up-regulation of the antimicrobial protein lipocalin (LCN)-2, CXCL5, polymeric immunoglobulin receptor, and the mucous protein Muc 1 in mouse tracheal epithelial cells by IL-22 (Ajuila et al. 2008). One of the first described effects of IL-22 on cells from the gastrointestinal tract was that of pancreatic acinar cells. Importantly, the pancreas is the organ with the highest IL-22R1 expression to be analyzed so far, especially in the pancreatic islets. In murine pancreatic acinar cells, IL-22 induced the expression of osteopontin and pancreatitis-associated protein (PAP) 1/RegIIIα, an effect that was also seen in the mouse pancreas *in vivo* (Aggarwal et al. 2001).

1.4.6 IL-22 roles in host defense

Given the fact that IL-22 increase the expression of a range of antimicrobially acting proteins in various epithelia suggests a role for this cytokine in the innate immune defense, especially against extracellular bacteria. Moreover, further mechanisms used by IL-22 to promote the defense against intracellular bacteria, fungi, viruses and parasites are currently in exploration.

IL-22 role in bacterial infection

IL-22 is critical for limiting bacterial replication and dissemination, probably in part by inducing the expression of antimicrobial peptides from epithelial cells at these barrier surfaces. Mice deficient in IL-22

or IL-23, the latter a cytokine produced by activated dendritic cells that is critical for IL-22 expression *in vivo*, show rapid mortality in response to each pathogen, which suggests that a potent early innate IL-22-dependent response is essential for protective immunity to infection (Zheng Y et al. 2008; Aujla et al.2008). Demonstrating the functional importance of IL-22 in promoting barrier immunity, two studies have determined that IL-22 is essential for host protective immunity to the extracellular Gram-negative pathogens *Klebsiella pneumoniae* in the lung (Zheng Y et al. 2008) and *Citrobacter rodentium* in the intestine (Aujla et al. 2008). *Citrobacter rodentium*, a gram-negative bacterium, attaches to murine colon epithelial cells and induces acute infectious colitis, mimicking attaching and effacing bacterial infections in the human. Upon *C. rodentium* infection, IL-23 and IL-22 are quickly upregulated in the colon. IL-23 induces IL-22 production from innate immune cell types including LTi cells, $NKp46^{+}$ NK-like cells, and DCs (Wolk K et al. 2009; Cella M et al. 2009). IL-22-deficient mice display much more severe epithelial damage as well as systemic bacterial dissemination. Many IL-$22^{-/-}$ mice succumb within the second week after infection. IL-22 is also required for host defense against *Klebsiella pneumoniae* infection in the lung (Zheng Y et al. 2008). Blocking IL-22 results in early death of the infected animals. IL-22 synergizes with IL-17 in this model to induce lung repair, proinflammatory chemokines and cytokines, and Lipocalin-2,

which is required for lung epithelial cells to kill *K. pneumoniae*. *Salmonella enterica* is another gram-negative bacterium that can cause systemic infection or gastroenteritis in humans. Th1 cells and IFN-γ are essential for the clearance of *Salmonella* infections. However, during intestinal infection with *Salmonella*, IL-23 induces the production of IL-22 and IL-17. Increased IL-22 and Th17 responses also have been associated with *Mycobacterium tuberculosis, Leishmania donovani*, and *Shigella flexneri* infections. The influence of IL-22 on the elicitation of host protective immunity is dependent on the pathogen, as IL-22 seems to have no substantial role in host defense after infection with *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Listeria monocytogenes* or *Schistosoma mansoni* (Zenewicz LA et al. 2007; Wilson MS et al. 2010). IL-22 may not be required for immunity to these specific infectious agents because, unlike *C. rodentium* or *K. pneuomiae*, these pathogens do not become intimately associated with the barrier surface or selectively induce functional or pathological changes in epithelial cells.

IL-22 role in others infectious models

Contrary to its protective function during bacterial infections, IL-22 can cause damage in the intestine during parasite *Toxoplasma gondii* infection (Munoz A et al. 2009). IL-22 is dispensable for acute or chronic systemic infections (Wilson M et al. 2010). When the ileum is

infected perorally, *T. gondii* causes massive necrosis, leading to death in C57BL/6 mice. IL-22$^{-/-}$ mice are resistant to the immunopathology induced by *T. gondii*. Blocking IL-22 slows the death rate and alleviates the ileum damage and inflammation after the infection (Munoz A et al. 2009). The downstream mediators of IL-22 in this model are unclear. Although this is still in the early stages of investigation, IL-22 does not seem to have a substantial direct role in immunity to viral pathogens. For example, IL-22 deficiency does not influence the outcome of influenza infection (Guo H and Topham DJ 2010). However, IL-22 may have an important role in providing protective innate immunity when the adaptive immune system is impaired, such as after infection with human immunodeficiency virus.

IL-22 role in fungal defence

As for its role in viral defense, knowledge of the contribution of IL-22 to immunity against fungal infections is also limited. From the broad range of antimicrobial proteins produced in response to IL-22, some, including BD2, BD3, S100A8 and S100A9 are also known to be active against *C. albicans* (Feng Z et al. 2005). Conti et al. investigated the role of IL-22 in fungal infections using a mouse model of oropharyngeal candidiasis induced by sublingual infection with *C. albicans* (Conti et al. 2009). In this model the pathogen was

completely cleared in wild-type mice, which was associated with a high infiltration of neutrophilic granulocytes into the tongue. IL-22-deficient mice were only mildly susceptible, although they lost weight after infection. In fact, they showed low fungal burden and robust neutrophilic infiltrates, consistent with the minor role of IL-22 in chemokine production. The Romani group described a more essential role for IL-22 in the defense against *C. albicans* using an early intra-gastric and systemic infection model (De Luca A. et al. 2005). The authors found that IL-22-deficient mice were less able to control the growth of yeasts or hyphae administered through the different infection routes and showed hyphae penetration through the stomach mucosa after intra-gastric administration. Importantly, in the absence of IFN-γ or IL-17RA, IL-22 was strongly elevated in the mice's stomachs and impressively mediated antifungal protection (De Luca A. et al. 2005). In line with this study, humans with a low natural bioactivity of IL-22, IL-17A and IL-17F resulting from the systemic presence of neutralizing auto-antibodies against IL-22, IL-17A and IL-17F were shown to ineffectively clear *Candida* infections, as demonstrated in patients suffering from chronic mucocutaneous candidiasis in the context of autoimmune polyendocrine syndrome type I (Conti HR et al. 2009; Kisand K et al. 2010).

1.4.7 The role of IL-22 in autoimmunity

IL-22 is highly expressed in several different chronic inflammatory conditions, including psoriasis, IBD and rheumatoid arthritis (Wolk K et al. 2006; Ikeuchi H et al. 2005). The up-regulation of IL-22 is positive correlated with the disease activity or exacerbation. It cannot be inferred if IL-22 is a cause of the inflammation and/or a result of it. As such, small animal disease models have been employed to help elucidate the role of IL-22 in inflammation. Using either gene-deficient mice or administration of neutralizing antibodies, investigators have studied the role of IL-22 in various inflammatory conditions. These models have identified both inflammatory and protective roles for IL-22. The best studied function for IL-22 is within the skin; IL-22 is inflammatory during skin inflammation. Transgenic mice engineered to over-express IL-22 have an aberrant skin phenotype that resembles psoriasis (Wolk K et al. 2009). The IL-22 transgenic pups are born with shiny and stiff skin and die several days post-birth. Histological analysis of the skin reveals epidermal thickening and that the dermal layer contains infiltrating macrophages. Using IL-22-deficient mice, Zheng *et al.* showed that in the absence of IL-22, IL-23-mediated dermal inflammation was reduced (Zheng Y et al. 2007). Another group has also shown that IL-22 is inflammatory in a T-cell-mediated model of psoriasis (Ma H et al. 2008). These data are consistent with *in vitro*

studies in which IL-22 was found to mediate keratinocyte proliferation and epithelial hyperplasia (Wolk K et al. 2009; Ma H et al. 2008). Furthermore, using three-dimensional epidermis culture systems, Wolk *et al.* showed that IL-22 is important for epidermal remodeling (Wolk K et al. 2009). Thus, though induction of proliferation, IL-22 induces keratinocyte migration, leading to the hyperplasia of keratinocyte layers, and results in a thickening of the epidermis. In addition to psoriasis, an inflammatory role for IL-22 has been found in rheumatoid arthritis (Geboes et al. 2009). Using an experimental model in which mice are immunized against collagen generating an autoimmune response in the joints, mice deficient in IL-22 had decreased incidence of arthritis and pannus formation. IL-22 may promote osteoclastogenesis leading to bone erosion in rheumatoid arthritis. IL-22 also has a protective role in inflammation. The dual nature of this cytokine, protective versus inflammatory, likely depends on the inflammatory context, which includes, but is not limited to, the duration and amount of IL-22 present, the overall cytokine milieu and the tissues involved. IL-22 is also protective during IBD. Genome-wide linkage analysis of IBD patients has identified mutations in the genes encoding IL-22 and the IL-10Rβ subunit of the IL-22 receptor complex (Glocker E O et al. 2009). The IL-22 receptor complex is highly expressed within the GI tract and in the inflamed colon, IL-22 is expressed by CD4 T cells, likely T_h17 cells,

and innate lymphocytes such NK cells and LTi-like cells (Zenewicz L A et al. 2008). Using different experimental models of IBD—DSS-induced colitis which is thought to be mainly driven by innate immune response cells and CD4+ $CD45RB^{high}$ T-cell-mediated colitis in which naive T cells devoid of regulatory T cells are transferred into T-cell-deficient mice where they proliferate unimpeded leading to colitis—IL-22 has been shown to be protective in both cases. Furthermore, Sugimoto *et al.* showed that IL-22 can be therapeutic to IBD; gene therapy transfer of the *Il22* gene into the colons of already inflamed mice resulted in amelioration of the inflammation (Sugimoto K et al. 2008). Several molecules appear to be important for the mechanism of how IL-22 provides protection within the GI tract. IL-22 induces expression of several anti-microbial molecules in the GI tract, including Reg3γ, lipocalin-2 and β-defensins (Wolk K et al. 2004; Zheng Y et al. 2008). These proteins may be important in the control of pathogenic micro-organisms within the gut. IL-22 also induces expression of mucins, a large heavily glycosylated family of proteins that forms a protective layer in the GI tract allowing for separation of commensal and pathogenic flora from the epithelium and hereby minimizing the immune response (Sugimoto et al. 2008). Furthermore, IL-22 has direct effects on the colonic epithelium allowing for its proliferation and thereby contributing to its integrity.

1.4.8 IL-22 in tumor development

There are few studies that focus on IL-22 effects on tumor cells. Weber at al. investigated the IL-22 influence on the proliferation, apoptosis, and cell cycle regulation in murine breast cancer cells (EMT6) (Weber GF et al. 2006). The cytokine induced a cell cycle arrest of these cells in the G2-M phase and reduced the proliferation rate by approximately 50%. Interestingly, IL-22 did not induce apoptosis of EMT6 cells. Ziesché et al. demonstrated that IL-22 induced inducible nitric oxide synthase in human DLD-1 colon carcinoma cells and in Caco-2 colon carcinoma cells (Ziesche E et al. 2007). About a contribution of IL-22 to tumor cell survival was reported also by Zhang et al. The authors described that IL-22 was highly expressed in primary tumor tissue of nonsmall cell lung carcinoma and the overexpression of IL-22 protected lung cancer cell lines from chemotherapeutic drug-induced death (Zhang W et al. 2008).

1.4.9 IL-22 as a therapeutic target

As discuss human IL-22 shares 25% identity with human IL-10 and 79% homology with mouse IL-22 (Dumoutier L et al 2000). Augmented expression of IL-22 has been documented in several diseases states and furthermore, spontaneous mutations arising in

the human population that affect the IL-22-IL-22R pathway correlate with defects in barrier immunity. These date suggest that targeting the IL-22-IL-22R pathway may yield new therapeutic potential for the treatment of certain human diseases. IL-22 expression is detected in many inflammatory and infectious human diseases. For example, higher concentrations of IL-22 derived from T_H17 cells are observed in the peripheral blood and tissues of patients with psoriasis or arthritis, and on the basis of preclinical model studies, it is predicted that IL-22 promotes pathological inflammation in these disease settings (Wolk K et al. 2006; Boniface K et al. 2007). In contrast to its concentration in psoriasis, higher concentrations of IL-22 from T_H22 and T_C22 cells are observed in the inflamed skin of patients with atopic dermatitis (Nograles et al. 2009). IL-22 concentrations are also higher in the peripheral blood and intestine of patients with inflammatory bowel disease. Although IL-22 is tissue protective in mouse models (Sugimoto et al. 2008; Zenewicz et al. 2008), its role in human intestinal inflammation has been debated, as IL-22 expression correlates with proinflammatory gene expression (Pene J et al. 2008; Liu H et al. 2007; Kleinschek MA et al. 2009). Infection with *Leishmania donovani*, which causes a lethal visceral disease, is associated with T_H17 cell expression of IL-22 and is also positively correlated with disease protection. Further, patients with cystic fibrosis who have exacerbated infection with *Pseudomonas*

aeruginosa have more T cell secretion of IL-22 in lung-draining lymph nodes (Aujla SJ et al. 2008). This induction of IL-22 expression in infectious settings is consistent with its identified role in mouse models for the promotion of immunity at barrier surfaces. Thus far, most reports have examined T cell expression of IL-22 in human disease; however, given the characterization and critical importance of IL-22 expression by innate cells, it will also be important to examine innate sources of IL-22, such as ILCs, in future studies. Some spontaneously arising mutations in the human population may shed light on the *in vivo* functions of human IL-22. For example, patients suffering from hyper–immunoglobulin E syndrome (Job's syndrome) have a hypomorphic mutation in the gene encoding STAT3, a critical transcription factor involved in signaling upstream and downstream of IL-22 expression (Zenewicz LA et al. 2008). These patients have impaired T_H17 differentiation and lower IL-22 production. Consistent with the role of IL-22 in promoting immunity to fungal and bacterial pathogens at barrier surfaces in mouse models, patients with this syndrome also suffer severe and recurrent secondary infections in the skin, lung and intestine (Ma CS et al. 2008; Minegishi et al. 2007). Furthermore, patients with autoimmune polyendocrine syndrome 1 have high serum titers of autoantibodies that neutralize IL-17A, IL-17F and IL-22 and also have less production of IL-22 protein. This impairment in IL-22 production correlates with the development of

chronic mucocutaneous candidiasis in these patients (Kisand K et al. 2010; Puel A et al. 2010). The role of IL-22 in autoimmune diseases affecting organs not representing physiologically outer body barriers is currently not finally clarified and may probably depend on the specific organ. The Becher group investigated the role of IL-22 in experimental autoimmune encephalomyelitis (EAE), the mouse model of the human disease multiple sclerosis that affects 0.1% of the people. Using IL-22-deficient mice, they showed that IL-22 is not required for the development of EAE (Kreymborg K et al. 2007). The Aizawa group described that IL-22-Ig gene therapy was effective in controlling experimental autoimmune myocarditis in rats (Chang H et al. 2006). These authors found evidences that the IL-22 target cells in that model were non inflammatory cells such as fibroblasts, smooth muscle cells, and endothelial cells. In rheumatoid arthritis (RA), a chronic inflammatory disease that affects up to 1% of people, the IL-22 expression in synovial tissues and the number of IL-22-positive CD4+ cells in the blood were increased (Shen H et al. 2009). *In vitro*, IL-22 at high concentrations significantly increased the production of CCL2 and the proliferation of synovial fibroblasts derived from RA patients (Ikeuchi H et al. 2005). Interestingly, Geboes et al. reported about a pathogenetic role of IL-22 in collagen-induced arthritis, an animal model of RA (Geboes L et al. 2009). Surprisingly, the less severe course of arthritis observed in the absence of IL-22 was

associated with increased production of CII-specific and total IgG antibodies. Furthermore, the authors found that IL-22 promoted osteoclastogenesis *in vitro*.

SCOPE OF THE THESIS

The aim of this thesis was to characterize the stimuli and the intracellular pathways which are responsible for IL-22 production in bone marrow dendritic cells (BMDC). Through a functional genomics approach we analyzed the genes modulated in BMDC after the infections with different gram+ bacteria. Surprisingly, we detected a strong production of the novel cytokine IL-22. Although it is known that CD4+ cells (Th17 subtype), NK cells (NK22 and NKp46+ subtypes), lymphoid tissue-inducer cells (LTi) are able to produce IL-22 through the activation of different transcription factors, little it is known about BMDC. Therefore, in the first part of this study we characterized the stimuli responsible for IL-22 production in *in-vitro* stimulated BMDC. Since bacteria are recognized by different PRR present on BMDC surface, we investigated the contribution of individual TLRs and c-type lectin receptors involved in IL-22 production upon stimulation. In the second part, we focused on IL-22 gene regulation through the characterization of the intracellular signalling cascades including the MAP kinases and transcription factors gene families.

REFERENCES

Aggarwal S, Xie MH, Maruoka M, Foster J, Gurney AL **Acinar cells of the pancreas are a target of IL-22.** J. *Interferon cytokine Res* 21, 1047-53 (2001)

Akira S and Takeda K **Toll-like receptor signalling.** *Nat. Rev. Immunol.* **4**, 499–511 (2004)

Akira S, Uematsu S and Takeuchi O **Pathogen recognition and innate immunity.** *Cell* **124**, 783–801 (2006)

Alexopoulou L, Holt AC, Medzhitov R and Flavell RA **Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3**. *Nature* **413**, 732–738 (2001)

Aujla SJ, Chann YR, Zheng M, Fei M *et al*. **IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia.** *Nat. Med*. 14, 275–281 (2008)

Becker C et al. **STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing** *JEM*, 1465-1471 (2009)

Boniface K et al. **A role for T cell-derived interleukin 22 in psoriatic skin inflammation.** *Clin. Exp. Immunol*. 150, 407–415 (2007)

Boniface K, Bernard FX, Garcia M, Gurney AL, Morel F **IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes.** ***J.Immunol*** 174, 3695-702 (2005)

Bonneville M, O'Brien R. and Born WK **γδ T cell effector functions: a blend of innate prog ramming and acquired plasticity**. *Nat. Rev. Immunol.* 10, 467–478 (2010)

Cao W, Zhang L, Rosen DB, Bover L, Watanabe G, Bao M, Lanier LL, Liu YJ **BDCA2/Fc εRIγ complex signals through a novel BCR-like pathway in human plasmacytoid dendritic cells**. *PLoS Biol.* **5**, e248 (2007)

Cella M, Fuchs A, Vermi W, Facchetti F, Otero K, Lennerz JK, Doherty JM, Mills JC, Colonna M **A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity** *Nature*, 722–725 (2009)

Chang H, Hanawa H, Liu H, Yoshida T, Hayashi M, Watanabe R, Abe S, Toba K, Yoshida K, Elnaggar R, Minagawa S, Okura Y, Kato K, Kodama M, Maruyama H, Miyazaki J, Aizawa Y **Hydrodynamic-based delivery of an interleukin-22-Ig fusion gene ameliorates experimental autoimmune myocarditis in rats.** *J Immunol* 177, 3635–3643 (2006)

Chen ZJ **Ubiquitin signalling in the NF-kB pathway.** *Nat. Cell. Biol.* 7, 758-765 (2005)

Ciric B, El-behi M, Cabrera R, Zhang G-X and Rostami A **IL-23 drives pathogenic IL-17-producing CD8$^+$ T cells.** *J. Immunol*. 182, 5296–5305 (2009)

Conti H, F. Shen, N. Nayyar, E. Stocum, J.N. Sun, M.J. Lindemann *et al.* **Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis** *J Exp Med*, 206 2299–311 (2009)

Cros J, Cagnad N, Woollard K, Patey N, Zang SY *et al*. **Human CD14^{dim} monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors**. *Immunity* **33**, 375–386 (2010)

De Luca A, Zelanti T, D'angelo C, Zaganella S *et al*. And Romani L **IL-22 defines a novel immune pathway of antifungal resistance.** *Mucosal Immunol*. 3, 361–373 (2010)

Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. **Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells.** *Nat. Immunol.* 10, 857–63 (2009)

Dumoutier L, Louahed J and Renauld JC **Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9**. *J. Immunol*. 164, 1814–1819 (2000)

Dumoutier L, Van Roost E, Colau D and Renauld JC **Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor.** *Proc. Natl. Acad. Sci. USA 97,* 10144–10149 (2000)

Feng Z, Jiang B, Chandra B, M. Ghannoum, S. Nelson, A. Weinberg**Human beta-defensins: differential activity against candidal species and regulation by *Candida albicans*** J Dent Res, 84 445–450 (2005)

Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM and Maniatis T **IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway.** *Nat. Immunol.* **4**, 491–496 (2003)

Geboes L, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, Renauld JC, Matthys P **Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice.** *Arthritis Rheum* 60 390–395 (2009)

Geissmann F. Siewere MH, Manz MG, Jung S *et al*. **Development of monocytes, macrophages, and dendritic cells.** *Science* **327**, 656–661 (2010)

Glocker EO, Kotlarz D, Boztug K, et al. **Inflammatory bowel disease and mutations affecting the interleukin-10 receptor.** *N. Engl. J. Med.,* 361, 2033 (2009)

Gordon S & Taylor PR **Monocyte and macrophage heterogeneity**. *Nature Rev. Immunol.* **5**, 953–964 (2005)

Goriely S, Neurath MF and Goldman M **How microorganisms tip the balance between interleukin-12 family members.** *Nature Rev. Immunol.* **8**, 81–86 (2008)

Gringhuis SI et al. **Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and SYK.** *Nature Immunol.* **10**, 203–213 (2009)

Gringhuis SI, den Dunnel J, Litjens M, von Het Hof B, van Kooyk Y, Geijtenbeek TB **C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-κB.** *Immunity* **26**, 605–616 (2007)

Gross O et al. **Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity.** *Nature* **442**, 651–656 (2006)

Gross O et al. **Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence.** *Nature* 459, 433-436 (2009)

Guo H and Topham DJ **IL-22 production by pulmonary natural killer cells and the potential role of IL-22 during primary influenza infection.** *J. Virol*. 84, 7750–7759 (2010)

Hashimoto C, Hudson KL, Anderson KV **The tool gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.** *Cell*, 52, 269-79 (1998)

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, **Underhill DM and Aderem A The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.** *Nature* **410**, 1099–1103 (2001)

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K and Akira S **Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway**. *Nat. Immunol.* **3**, 196–200 (2002)

Henri S et al. **The dendritic cell populations of mouse lymph nodes.** *J. Immunol.* 167, 741–748 (2001)

Hochrein H et al. **Differential production of IL-12, IFN-α and IFN-γ by mouse dendritic cell subsets.** *J. Immunol*. 166, 5448-5455 (2001)

Huang Q, Yang J, Lin Y, Walker C, Cheng J, Liu ZG and Su B **Differential regulation of interleukin 1 receptor and Toll-like receptor signaling by MEKK3.** *Nat. Immunol.* **5**: 98–103 (2004)

Iannacone M, Moseman EA, Tonti E, Bosurgi L, Junt T *et al*. **Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus.** *Nature* **465**, 1079–1083 (2010)

Ikeuchi H, Kuroiwa T, Hiramatsu N, et al. **Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine.** *Arthritis Rheum.,* 52, 1037 (2008)

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, et al. **The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17$^+$ T helper cells.** *Cell* 126, 1121–33 (2006)

Iwasaki A and Kelsall BL **Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3α, MIP-3β, and secondary lymphoid organ chemokine.** *J. Exp. Med.* 191, 1381–1394 (2000)

Janeway CD jr **How the immune response system works to protect the host from infection: a personal view**. *Nat. Proc. Sci USA* 98 7461-7467 (2001)

Karin M and Greten FR **NF-kappaB: linking inflammation and immunity to cancer development and progression.** *Nat. Rev. Immunol.* **5**, 749–759 (2005)

Kawai T & Akira S **Toll-like receptors and their crosstalk with other innate receptors in infection and immunity.** *Immunity* **34**, 637–650 (2011)

Kawai T, Adachi O, Ogawa T, Takeda K and Akira S **Unresponsiveness of MyD88-deficient mice to endotoxin**. *Immunity* **11**, 115–122 (1999)

Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K and Akira S **Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes.** *J. Immunol.* **167**, 5887–5894 (2001)

Kisand K et al. **Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines.** *J. Exp. Med*. 207, 299–308 (2010)

Kleinschek MA et al. **Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation.** *J. Exp. Med.* 206, 525–534 (2009)

Kreymborg K, Etzensperger R, Dumoutier L, Haak S, Rebollo A, Buch T, Heppner FL, Renauld JC, Becher B **IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis.** *J Immunol* 179, 8098–8104 (2007)

Kronin V et al. **Differential effect of $CD8^+$ and $CD8^-$ dendritic cells in the stimulation of secondary $CD4^+$ T cells.** *Int. Immunol.* 13, 465–473 (2001)

Lejeune D, Dumoutier L, Costantinescu S, Kruijer W, Stinson J and Reanould JC **Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line.** *J. Biol. Chem*. 277, 33676–33682 (2002)

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. **The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potemt antifungal response in Drosophila adults.** *Cell*, 86, 93-83 (1996)

Liang S.C., Tan X, Luxenberg D, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser L **Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides** *J. Exp. Med*., 203, 2271–2279 (2006)

Liang SC, Nickerson-Nutter C, Pittman DD, Carrier Y et al.and Fousier LA **IL-22 induces an acute-phase response.** *J. Immunol*. 185, 5531–5538 (2010).

Liu H et al. **The expression of interleukin-22 and S100A7, A8, A9 mRNA in patients with psoriasis vulgaris.** *J. Huazhong Univ. Sci. Technolog. Med. Sci.* 27, 605–607 (2007)

Liu YJ **IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors.** *Annu. Rev. Immunol.* **23**, 275–306 (2005)

Ma CS et al. **Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3.** *J. Exp. Med.* 205, 1551–1557 (2008)

Ma HL, Liang S, Li J, et al. **IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation.** *J. Clin. Invest*., 118, 597 (2009)

Maldonado-López R et al. **CD8α^+ and CD8α^- subclasses of dendritic cells direct the development of distinct T helper cells *in vivo*.** *J. Exp. Med.* 189, 587–592 (1999)

Martin B, Hirota K, Cua DJ, Stockinger B and Veldhoen M **Interleukin-17-producing γδ T cells selectively expand in response to pathogen products and environmental signals.** *Immunity* 31, 321–330 (2009).

McGaha TL, Chen Y, Ravishankar B, van Rooijen N & Karlsson MC **Marginal zone macrophages suppress innate and adaptive immunity to apoptotic cells in the spleen.** *Blood* **117**, 5403–5412 (2011)

Medzhitov R, Preston-hurlburt P, Janeway Jr CA **A human homologue of the drosofila Toll protein signals activation of adaptive immunity.** *Nature* 388, 394-397 (1997)

Minegishi Y et al. **Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome**. *Nature* 448, 1058–1062 (2007)

Moore KW, de Waal MR, Coffman RL, O'Garra A **Interleukin-10 and the interleukin-10 receptor.** *Annu. Rev. Immunol*. 19, 683-765 (2001)

Moser M and Murphy KM **Dendritic cell regulation of T_H1–T_H2 development.** *Nature Immunol.* 1, 199–205 (2000)

Mosser DM and Edwards JP **Exploring the full spectrum of macrophage activation.** *Nature Rev. Immunol.* **8**, 958–969 (2008)

Munoz, M. *et al*. **Interleukin (IL)-23 mediates Toxoplasma gondii-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17.** *J. Exp. Med*. 206, 3047–3059 (2009)

Murray PJ & Wynn TA **Obstacles and opportunities for understanding macrophage polarization.** *J. Leukoc. Biol.* **89**, 557–563 (2011)

Nograles KE et al. **IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells**. J. *Allergy Clin. Immunol*. 123, 1244–1252 (2009)

Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulus AD et al. **Essential autocrine regulation by IL-21 in the generation of inflammatory T cells.** *Nature* 448, 480-483 (2007)

Oshiumi H, Matsumoto M, Funami K, Akazawa T and Seya T **TICAM-1 an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction.** *Nat. Immunol.* **4**, 161–167 (2003)

Osorio F & Reis ESC **Myeloid C-type lectin receptors in pathogen recognition and host defense.** *Immunity* **34**, 651–664 (2011)

Ouyang W et al. **Regulation and function of the IL-10 family cytokines in inflammation and disease** *Ann. Rev. Immunol*. 29, 71-109 (2011)

Pène J et al. **Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes.** *J. Immunol*. 180, 7423–7430 (2008)

Pestka S *et al*. **Interleukin-10 and related cytokines and receptors**. *Annu. Rev. Immunol.* 22, 929–979 (2004)

Poltrak A, He X, Smirnova I, LinMY, Huffel CV, Du X et al. **Defective LPS signaling in C3H/HeJ and C57/10ScCr mice: mutation in Tlr4 gene.** *Science* 282, 2085-2088 (1998)

Puel A et al. **Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I.** *J. Exp. Med.* 207, 291–297 (2010)

Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, et al. **Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor.** *Nature* 453, 65–71 (2008)

Radaeva S, Sun R, Pan HN, Hong F, Gao B. ***Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation.*** *Hepatology* , 39, 1332 (2004)

Rawlings DJ, Sommer K and Moreno-Garcia ME **The CARMA1 signalosome links the signalling machinery of adaptive and innate immunity in lymphocytes.** *Nature Rev. Immunol.* **6**, 799–812 (2006)

Reis e Sousa C et al. ***In vivo*** **microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas.** *J. Exp. Med.* 186, 1819–1829 (1997)

Res PC et al. **Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis.** *PLoS ONE* 5, e14108 (2010)

Rock FL, Hardiman G, Timans JC et al. A **family of human receptors structurally related to Drosophila Toll.** *Proc. Natl Acad. Sci USA* 95, 588-593 (1998)

Rogers NC, Slack EC, Edwards AD, Note MA, Schulz O et al. and Reis e Sousa C **SYK-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins**. *Immunity* **22**, 507–517 (2005)

Rothfuchs AG, Bafica A, Feng CG, Egen JG, William DL, Brown GD **Dectin-1 interaction with Mycobacterium tuberculosis leads to enhanced IL-12p40 production by splenic dendritic cells**. *J. Immunol.* **179**, 3463–3471 (2007)

Rutschman R *et al*. **Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production.** J. *Immunol.* **166**, 2173–2177 (2001)

Saijo S et al. **Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans.** *Nature Immunol.* **8**, 39–46 (2007)

Sanos SL, Bui VL, Mortha A, Oberle K, Heners C, Johner C, Diefenbach A **RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells** *Nat. Immunol*., 10, 83–91 (2009)

Sato K et al. **Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor γ chain to induce innate immune responses.** *J. Biol. Chem.* **281**, 38854–38866 (2006)

Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O and Akira S **Essential function for the kinase TAK1 in innate and adaptive immune responses.** *Nat. Immunol.* **6**, 1087–1095 (2005)

Satoh-Takayama N, Vosshenrich CA, Lesjoir V, Pattier S et al. **Microbial flora drives interleukin 22 production in intestinal NKp46$^+$ cells that provide innate mucosal immune defense.** *Immunity* 29, 958–970 (2008)

Schulz SM, Kohler G, Schutze N et al. **Protctive immunity to sistemic infection with attenuated Salmonella enterica serovar enteridiis in the absence of IL-12 is associated with IL-23-dependent IL-22, but not IL-17**. *J. Immunol*. 181, 7891-901 (2008)

Schulze-Tanzil G, Zreiqat H, Sabat R, Kohl B, Halder A, Muller RD, John T **Interleukin-10 and articular cartilage: experimental therapeuticel approaches in cartilage disorders.** *Curr. Gene Ther* 9, 306-315 (2009)

Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R and Hiscott J **Triggering the interferon antiviral response through an IKK-related pathway.** *Science* **300**, 1148–1151 (2003)

Shen H, Goodall JC, Hill Gaston JS **Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis.** *Arthritis Rheum* 60, 1647–1656 (2009)

Shortman K and Yong-jun Lin **Mouse and human dendritic cells subtypes Nat. Review** 2, 151-161 (2002)

Spits H and Di Santo JP **The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling.** *Nat. Immunol.* 12, 21–27 (2011)

Sonnemberg GF, Fousier L and Artis D. **Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surface by IL-22** *Nature Immunol*. 5, 383-390 (2011)

Sugimoto K, Ogawa K, Mizoguchi E, Shimomura Y *et al*. **IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis.** *J. Clin. Invest.* 118, 534–544 (2008).

Sugimoto K, Ohata M, Miyoshi J, Ishizaki H, Tsuboi N, Masuda A, Yoshikai Y, Takamoto M, Sugane K, Matsuo S, Shimada Y and Matsuguchi T **A serine/threonine kinase Cot/Tpl2 modulates bacterial DNA-induced IL-12 production and Th cell differentiation.** *J. Clin. Invest.* **114**, 857–866 (2004)

Sutton CE et al. **Interleukin-1 and IL-23 induce innate IL-17 production from γδ T cells, amplifying Th17 responses and autoimmunity.** *Immunity* 31, 331–341 (2009)

Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano SI, Honda K, Ohba Y, Mak TW and Taniguchi T **Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors.** *Nature* **434**: 243–249 (2005)

Takeda K and Akira S **Toll-like receptors in innate immunity**. *Int. Immunol.* **17**, 1–14 (2005)

Theofilopoulus AN et al. **Tipe I interferon (alpha/beta) in immunity and autoimmunity.** *Int. Immunol.* 17, 1-14 (2005)

Tsujimura H, Tamura T, Kong HJ, Nishiyama A, Ishii KJ, Klinman DM and Ozato K **Toll-like receptor 9 signaling activates NF-kappaB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells.** *J. Immunol.* **172**, 6820–6827 (2004)

Underhill DM, Rossnagle E, Lowell CA and Simmons RM **Dectin-1 activates SYK tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production**. *Blood* **106**, 2543–2550 (2005)

van Kooyk Y and Rabinovich GA **Protein-glycan interactions in the control of innate and adaptive immune responses.** *Nature Immunol.* **9**, 593–601 (2008)

Veldhoen M et al. **The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins.** *Nature* 453, 106–109 (2008)

Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, et al. **The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins**. *Nature* 453,106–9 (2009)

Volpe E et al. **A critical function for transforming growth factor-b, IL-23 and proinflammatory cytokines in driving and modulating human Th-17 responses**. *Nat. Immunol.* 9, 650-657 (2008)

Volpe E et al. **Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production.** *Blood* 114, 3610–3614 (2009)

Vremec D, Pooley J, Hochrein H, Wu L and Shortman K **CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen.** *J. Immunol.* 164, 2978–2986 (2000)

Weber GF, Gaertner FC, Erl W, Janssen KP, Blechert B, Holzmann B, Weighardt H, Essler M **IL-22-mediated tumor growth reduction correlates with inhibition of ERK1/2 and AKT phosphorylation and induction of cell cycle arrest in the G2-M phase.** *J Immunol* 177, 8266–8272 (2006)

Wilson MS, Feng CG, Barber DL, Yarovinsky F *et al*. **Redundant and pathogenic roles for IL-22 in mycobacterial, protozoan, and helminth infections**. *J. Immunol*. 184, 4378–4390 (2010)

Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F *et al.* **Development, cytokine profile and function of human interleukin 17-producing helper T cells** *Nat. Immunol*., 8, 950–957 (2007)

Wolk K, Haugen HS, Xu W, et al. **IL-22 and IL-20 are key mediators of the epidermal alterations in psoriasis while IL-17 and IFN-gamma are not.** *J. Mol. Med*., 87, 523 (2009)

Wolk K, Kunz S, Asadullah K and Sabat R **Cutting edge: immune cells as sources and targets of the IL-10 family members?** *J. Immunol*. 168, 5397–5402 (2002)

Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K and Sabat R **IL-22 increases the innate immunity of tissues**. *Immunity* 21, 241–254 (2004).

Wolk K, Witte E, Hoffmann U et al. **IL-22 induces lipopolisaccharide-binfding protein in hepatocytes: a potential systemic role for IL-22 in Crohn's disease.** *J. Immunol.* 178, 5973-5981 (2007)

Wolk K, Witte E and Witte K and Sabat R **Biology of IL-22** Semin Immunopathol. (2009)

Wolk K, Witte E, Hoffmann U, Doecke WD et al. **IL-22 induces lipopolysaccaride-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease**. *J. Immunol*. 178, 5973-81 (2007)

Wolk K, Witte E, Kunz S, Asadullah K and Sabat R **IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis.** *Eur. J. Immunol*. 36, 1309–1323 (2006)

Xie MH, Aggare S, Ho WH, Foster J, Zhang Z *et al*. **Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2–4 and IL-22R.** *J. Biol. Chem*. 275, 31335–31339 (2000)

Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K and Akira S **Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.** *Science* **301**, 640–643 (2003)

Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, et al. **STAT3 regulates cytokine-mediated generation of inflammatory helper T cells.** *J. Biol. Chem.* 282, 9358–63 (2007)

Zelensky AN and Gready JE The C-type lectin-like domain superfamily. *FEBS J.* **272**, 6179–6217 (2005)

Zenewicz LA, Yancolopulus GD, Valenzuela DM, Murphy AJ, Karow M and Flavell RA **Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation.** *Immunity* 27, 647–659 (2007)

Zenewicz LA, Yancolopulus GD, Valenzuela DM, Murphy AJ, Karow M and Flavell RA Innate **and adaptive interleukin-22 protects mice from inflammatory bowel disease.** *Immunity* 29, 947–957 (2008)

Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA and Ghosh S **A toll-like receptor that prevents infection by uropathogenic bacteria.** *Science* **303**, 1522–1526 (2004)

Zhang W, Chen Y, Wei H, Zheng C, Sun R, Zhang J, Tian Z **Antiapoptotic activity of autocrine interleukin-22 and therapeutic effects of interleukin-22-small interfering RNA on human lung cancer xenografts.** *Clin Cancer Res* 14, 6432–6439 (2008)

Zheng Y, Danilenko DM, Valdez P, et al. **Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis.** *Nature,* 445, 648 (2007)

Zheng Y, Valder PA, Danilenko DM, Hu Y et al. and Ouyang W **Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens**. *Nat. Med*. 14, 282–289 (2008)

Ziesche E, Bachmann M, Kleinert H, Pfeilschifter J, Muhl H **The interleukin-22/STAT3 pathway potentiates expression of inducible nitric-oxide synthase in human colon carcinoma cells.** *J Biol Chem* 282, 16006–16015 (2007)

Chapter 2: MATERIALS AND METHODS

2.1 Mice

Seven week-old C57/BL6 mice were purchased from Harlan Laboratories. MyD88 KO mice were provided by S. Akira (IFReC, Japan). Mice were then backcrossed and kept under specific pathogen-free condition.

2.2 Cell cultures

2.2.1 D1 cells

D1 cells are grown factor-dependent DC culture system in which mouse spleen-derived DC are maintained in a homogenous immature state (Winzler C et al. 1997). D1 were maintained in vitro in Iscove's Dulbecco medium (IMDM, Euroclone) supplemented with 10% heat-unactivated fetal bovine serum (Gibco, Australia), 100U/ml penicillin (Euroclone), 100ug/ml streptomycin (Euroclone), 2mM L-glutammine (Euroclone) and 50uM β-mercaptoethanol (Sigma) plus 30% R1 medium (supernatant derived from NIH3T3 fibroblast transfected with GM-CSF). Cells were incubated at 37^0C and 5% CO_2. When D1

cells were about 80% of confluence in plate, they were collected and splitted (2,5x10^6 cells/plate with 10ml fresh medium).

2.2.2 Bone marrow dendritic cells (BMDC)

Bone marrow was collected from both wild type and MyD88 KO C57/BL6 seven week-old mice. After mice sacrifice, femurs and tibia were collected and bone marrow washed out with sterile PBS w/o Ca2+ and Mg2+. 6x10^6 cells were seeded in bacterial Petri Dish (Steriln) with 10m of IMDM (Euroclone) supplemented with 10% heat-inactivated FBS (EC, Euroclone), 2mM L-glutamine (Euroclone), 100U/ml penicillin (Euroclone), 100ug/ml streptomycin (Euroclone) 50uM β-mercaptoethanol (Sigma) and 15% of GM-CSF transduced B16 tumor cells (20ng/ml GM-CSF). Cells were then incubated at 37°C and 7% CO_2. After 3 days 10ml of fresh medium were added and at day 6 10ml of medium were replaced with other ones fresh. After 7-10 day of culture, cells were analyzed by flow cytometer (FACScalibur, Becton Dikinson) for CD11c and B7.2 expression. BMDC that results between 75%-85% CD11c positive and less than 20% B7.2 positive (immature state) were used to perform assays.

2.2.3 Bone marrow macrophages (BMMϕ)

Bone marrow was flushed out from tibia and femurs of C57/BL6 mice as previous described. 7x10^6 cells were seeded in bacterial Petri Dish (Sterilin) with 10m of complete grow medium were cultured in

DMEM (Euroclone) supplemented with 10% heat-inactivated FBS (EC, Euroclone), 2mM L-glutamine, 100U/ml penicillin, 100ug/ml streptomycin 50uM β–mercaptoethanol and 30% L929 medium (L929 are tumor cells transduced with M-CSF). Cells were then incubated at 37°C and 7% CO_2. After 7 day of culture, supernatant was discarded and macrophages were collected from the bottom of plate with trypsin-EDTA (Sigma). Cells were then used to perform assays.

2.3 *Ex-vivo* cells

2.3.1 Splenocytes

Splenocytes were derived from the spleen of WT C57/BL6 mice. After mice sacrifice, spleen was isolated and manually disaggregated in 5ml of cold PBS w/o Ca^{2+} and Mg^{2+} using a sterile syringe. Cells were filtrated on 70μm strainer (Euroclone) and cultured in complete IMDM medium (as previous described). For experimental setting, splenocytes were seeded $2x10^6$cells/ml and incubated at 37°C and 7% CO_2.

2.3.2 Dendritic cells (DCs) purification

DCs were obtained from spleen of WT C57/BL6 mice. After mice sacrifice, spleen was isolated and injected with Collagenase VIII (Sigma) resuspended in collagenase buffer (Hepes-NaOH 10mM pH 7,4, NaCl 150mM, KCl 5mM, $MgCl_2$ 1mM and $CaCl_2$ 5mM; to volume

with dH_20). After 30 minutes incubation at 37°C, spleen was manually disaggregated and cells filtrated on 70μm strainer (Euroclone). After red bloods cells lysis, splenocytes were stained with anti-CD11c MACS microbeads (Myltenyi Biotech) diluted in purification buffer (0,5% BSA, EDTA 2mM in PBS w/o Ca^{2+} and Mg^{2+}). CD11c cells were positively selected on MS column according to the manufacture's recommendation (MS columns Myltenyi Biotech). Cells purity was analyzed by FACS (FacsCalibur, Becton Dikinson). Only cells between 90%-95% CD11c and over 95% MHCII positive were used to perform assays.

2.3.3 Macrophages cells purification

Spleen macrophages

Macrophages were obtained from spleen of WT C57/BL6 mice as previous described for DCs. After red bloods cells lysis, splenocytes were first stained with anti-CD11b biotinilated antibody for 20 minutes and then with MACS streptavidin beads (Myltenyi Biotech). Macrophages were positively selected on MS column (MS columns Myltenyi Biotech) according to the manufacture's recommendations. Cells purity was analyzed by FACS (FacsCalibur, Becton Dikinson). Only cells between 90%-95% CD11b positive were used to perform assays.

Peritoneum macrophages

Macrophages were obtained by injecting 3ml of cold PBS into peritoneum of C57/BL6 mice and recovering it after a lavage. Cells were then stained with anti-F4/80 antibody and sorted by MoFlo cells sorter (Beckton Dikinson). The obtained macrophages were 99% F4/80 positive.

2.4 Bacteria

2.4.1 Growing conditions

Lactobacillus paracasei (Lp) B21060 strain was supplied from Bracco SpA (Milan). This strain was grown in MRS broth (Fluka) at 37C under agitation. Bacteria were collected and used for experiments at OD_{600} 0f 0.8 that correspond to the exponential phase of growth.

Lactococcus lactis (Lc) MG1363 was provided by Jerry Wells (TNO, Netherlands). This strain was grown in M-17 broth supplemented with 0,5% glucose, at 30 C in a water bath. Bacteria were collected and used for experiments at OD_{600} of 1 that correspond to the exponential phase of growth.

Listeria innocua (Li) was grown in brain heart infusion (BHI, Fluka) at 37C under agitation. Bacteria were collected at OD_{600} of 0,6 that correspond to the exponential phase of growth.

Listeria monocytogenes (Lm) EGD pNF8 strain was provided by Olivier Dussurget from Pasteur Institut. This strain carried pNF8 plasmid, which contains the gene encoding for GFP protein and erythromycin resistance. *Lm* was grown in BHI supplemented with 5ug/ml erythromycin (to preserve plasmid into the strain) under agitation at 37C to OD_{600} of 0,6.

All bacteria used were stocked at -80C in 10% glycerol until use. Colonies forming units (CFU) were determined by plating serial ten-fold dilutions on agar plates and counting colonies after growth at 30°-37°C for 24h.

2.4.2 Infections with bacteria

Bacterial infections were performed *in vitro*. Frozen bacteria were thawed from glycerol stock and washed twice with cold PBS. After being suspended into their own medium, they were added to cells at the selected multiplicity of infection (MOI). 1h post infection bacteria were removed and fresh medium with 50ug/ml gentamycin (Sigma) was added. The real MOI of infection was checked by plating serial ten-fold dilutions on agar plates and counting colonies forming units (CFU).

2.4.3 Microarray experiments

D1 cells were seeded at 4,5x10^5 cells/ml in a bacterial culture plates (Sterilin) with 10ml of complete IMDM medium without penicillin and streptomycin. D1 cells were then infected with different gram+ bacteria: *L.paracasei* (MOI 1:1000), *L.lactis* (MOI 1:1000), *L.innocua* (MOI 1:1000), *L.monocytogenes* (MOI 1:20). 4h, 8h, 12h and 24h post infection supernatants were discarded and cells were collected in Trizol Reagent (Invitrogen). RNA was isolated and purified on a QIAGEN RNeasy column (QIAGEN mini kit). RNA quality was assessed by Agilent 2100 Bioanalyzer RNA 6000 Nano chip (Agilent Technologies, Palo Alto, CA). The fragmented biotinylated cDNA was hybridized on the MOE430A GeneChip (affymetrix, Santa Clara, CA) and scanned.

2.5 *In-vitro* cells stimulation

2.5.1 Dendritic cells stimulation

BMDC were seeded 1,5x10^6 cells/ml in 400ul of complete IMDM medium in a 24-weel plate (Grainer). The following stimuli were used: 5ug/ml rLPS (Alexis), 10ug/ml zymosan (Sigma), 5ug/ml CpG (Primm), 20ug/ml poly IC, 1ug/ml imiquimod, 100ng/ml Pam3Cys, 100ng/ml MALP2, 50ng/ml Flagellin, 100ng/ml profillin (TLRs kit from AXXORA), curdlan 10ug/ml (Vinci-biochem), 20ng/ml recombinant mouse IL-23 (eBIOSCIENCE).

CD11c+ cells were seeded 2,5x10^6 cells/ml in 100ul of complete IMDM medium in a 96-well plate (Grainer). CD11c DCs were stimulated with 20ng/ml of mouse recombinant IL-23, 10ug/ml LPS, 5ug/ml CpG, 20ug/ml zymosan.

After 20h supernatants were collected and tested by ELISA assay.

2.5.2 Macrophages stimulation

BMMϕ were seeded 1,5x10^6 cells/ml in a 24-multiwell plate in 400ul of complete DMEM medium. Spleen- and peritoneal-derived macrophages were seeded 2,5x10^6 cells/ml in complete IMDM medium in a 96-well plate. All macrophages types were stimulated with 5ug/ml rLPS, 10ug/ml zymosan, curdlan 10ug/ml and 20ng/ml of mouse recombinant IL-23. After 20h supernatants were collected and tested by ELISA assay.

2.6 Experiment with chemical inhibitors

BMDC were seeded 1,5x10^6 cells/ml in a 24-well plate in 400ul of complete IMDM medium. Cells were pretreated with chemicals inhibitors for 30 minutes and then stimulated with TLRs agonists (LPS 5ug/ml, CpG 5ug/ml and Zymosan 10ug/ml) or with Dectin-1 ligand curdlan 10ug/ml. After 20h supernatants were collected and tested by ELISA assay. The following inhibitors (Sigma) were used to selectively block MAP kinases: SP600125 (20uM) was used to block JNK, PD98059 (20uM) was used to block ERK and SB203580 was used

to block p38. The following inhibitors (Sigma) were used to selectively block transcription factors: Bay11-7082 (10uM) from Sigma was used to block NF-kB, cyclosporine A o FK506 (10uM) from Vinci-Biochem was used to block NFAT and CH-223191 (10uM) from Vinci-Biochem was used as AhR antagonist.

2.7 Citotoxicity assay

Cytotoxicity assays were performed using cell titer blue assay (Promega). BMDC were seeded $3x10^4$/well in a 96 multiwell plate and pretreated for 30 minutes with chemical inhibitors at the concentration previous described. Cells were then stimulated with TLRs agonists (LPS 5ug/ml, CpG 5ug/ml and Zymosan 10ug/ml) or with Dectin-1 ligand curdlan 10ug/ml. After 20h of incubation at 37°C, cells titer blue was added to each well. After 4 hours absorbance at 570nm and 600nm was read and cells vitality valuated according to manufacturer's recommendations.

2.8 IL-23 blocking experiments

Splenocytes were seeded $2x10^6$cells/ml in complete IMDM medium. Cells were treated with IgG1 control antibody (eBIOSCIENCE) or with blocking antibody against IL-23 (eBIOSCIENCE) and stimulated with recombinant mouse IL-23 (eBIOSCIENCE). BMDC were seeded $1,5x10^6$ cells/ml in a 24-well plate in 400ul of complete IMDM medium and stimulated with TLRs agonist in presence of α-IL23 or IgG1

antibodies. After 20h supernatants were collected and tested by ELISA assay.

2.9 Quantitative real time PCR (qRT-PCR)

To perform qRT-PCR total RNA was isolated with Trizol Reagent (Invitrogen) from all cell types and from tissues (spleen and liver), by disrupting and homogenizing them in Trizol Ragent using TissueLyser (QIAGEN). RNA was purified with RNeasy mini or micro kit (QIAGEN). DNAse digestion was carried out in the column during RNA extraction (RNase-free DNase Set, QIAGEN RNA quantity and quality were evaluated with NanoDrop spectrophotometer (ND-1000, Thermo Scientific). 900ng of RNA were reverse transcribed to cDNA with random primers (High Capacity cDNA Reverse Trenscription kit, Applied Biosystem). qRT-PCR was performed starting from 10ng cDNA for each sample using specific primer sets for the selected gene and 18S as housekeeping gene. q-PCR was run on 7500 machine (Applied Biosystem) with Power Sybr Green PCR Master Mix (Applied Biosystem). Primers were designed with Primer3 sofware (http://frodo.wi.mit.edu/) and checked with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) m-fold (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) IDT oligo analyser (http://eu.idtdna.com/analyser/Applications/OligoAnalyzer/).

Primers were validated for dimers formation and amplification efficiency. Only primers with amplification efficiency between 85% and 115% were accepted (Primm). Primers sequences are reported in **table1**. To test IL-23R mRNA we used Taqman probes (Applied Biosystem) and qRT-PCR was performed using Taqman PCR master mix (Applied Biosystem). The raw data (Ct, threshold cycle) were obtained with Applied Biosystem software. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ nethod ($DCt=Ct_{target}-Ct_{18S}$, $\Delta\Delta Ct=\Delta Ct_{stimulated}-\Delta\Delta Ct_{not\ treated}$) using 18S as the housekeeping gene.

2.10 Cytokines Detection

Cytokine amounts were measured by ELISA assays according to manufacturer's instructions using the following kits: IL-22 (Bender MedSystem), IL-23, TNFalpha, IL-2 (eBIOSCIENCE).

2.11 FACS analysis

FACS (FACScalibur, Becton Dickinson) analysis were performed to assess BMDC maturation and to verify purification process quality. We used 2.4G2 antibody to block non-specific antibody binding to FcγIII and FcγII. The following antibodies were used: anti-CD11c-APC (eBIOSCIENCE), anti-IA^b-PE-MHCII (BioLegend), biotinilated anti-CD11b (BD Bioscience), anti biotinilated-F4/80 (BD Bioscience),

streptavidin-PE (BD Bioscience). Analysis was performed by using CellQuestPro software (BD).

Table1: primers sequences reported 5'-3'

TLR1 F	AGTCAGCCTCAAGCATTTGG
TLR1 R	GCTCAACCCCAGGAACTGTA
TLR2 F	CTCCCAGATGCTTCGTTGTT
TLR2 R	CCTGCTTCCAGAGTCTCCAG
TLR3 F	AGACACACAGGCTGAGCAGT
TLR3 R	GAAATGTTCCCAGACCCAGT
TLR4 F	CAGCAAAGTCCCTGATGACA
TLR4 R	TCCAGCCACTGAAGTTCTGA
TLR5 F	TCAAACACCTGGATGCTCAC
TLR5 R	AGACACACCGTCTTCCTGCT
TLR6 F	AGCATGACCCCGTTCTCTAA
TLR6 R	CTCAGGGCTTTTGTTCTTGG
TLR7 F	TGCTGTGTGGTTTGTCTGGT
TLR7 R	ACCTTTGTGTGCTCCTGGAC
TLR8 F	TGGTTATGTTGGCTGCTCTG
TLR8 R	GGGATGTGGATGAAGTCCTG
TLR9 F	TCTGTACCCCGTTTCTCTGC
TLR9 R	GTTGGACAGGTGGACGAAGT
TLR11 F	TTGATAGGCAGAGGCTCCAT
TLR11 R	TTCCACTCCACTTTCCCTTG
Dectin-1 F	GGAATCCTGTGCTTTGTGGT
Dectin-1 R	TTCTGTGGGCTTGTGGTTCT
18S F	CGAAAGCATTTGCCAAGAAT
18S R	AGTCGGCATCGTTTATGGTC
AhR F	CCCAGCAGGAACTAAAGCAC
AhR R	AAGGCACACTGTCCACCTCT
Rorγt F	GAAGGCAAATACGGTGGTGT
Rorγt R	GGGCAATCTCATCCTCAGAA
IL-22 F	GCTCAACTTCACCCTGGAAG
IL-22 R	TCTGGATGTTCTGGTCGTCA

REFERENCES

Winzler C, Rovere P, Rescigno M, Granucci F, Penna G et al. **Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures.** *J. Exp. Med.* 185, 317-328 (1997)

Chapter 3: RESULTS

3.1 CHARACTERIZATION OF THE STIMULI INVOLVED IN IL-22 PRODUCTION BY DENDRITIC CELLS

3.1.1 The dendritic cell line D1 up-regulates IL-22 mRNA in response to bacterial infections

Dendritic cells (DCs) sense the host environment for the presence of microorganisms and respond to danger stimuli. After the encounter with a pathogen, DCs undergo to a genetic reprogramming leading to the acquisition of different functional phenotypes that switch on the appropriate immune response. A functional genomics approach has been used to understand the molecular mechanisms regulated in DCs after the interaction with different gram+ bacteria. Thus, D1 cells, a mouse spleen derived DCs line (see materials and methods), were infected with *L.paracasei, L.lactis, L.innocua* and *L.monocytogenes* in a time course experiment. All bacteria were used at a multiplicity of infection (MOI) of 1:1000 with the exception of *L.monocytogenes* that was used at a MOI of 1:20 because higher MOI caused cell death. The different MOI were chosen to induce an optimal DCs

activation that was evaluated by FACS analysis of CD86, CD80, MHCII and CD40 activation markers. D1 cells transcriptome was analyzed at 4h, 8h and 24h after infection and an unsupervised analysis to identify the common differentially expressed genes (DEG) was conducted. Analyzing all modulated DEGs, a strong regulation of IL-22 mRNA was observed (**fig.1**).

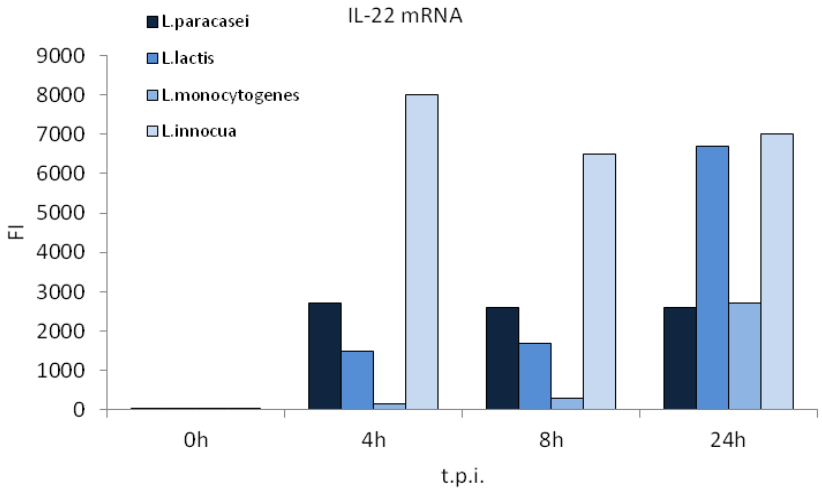
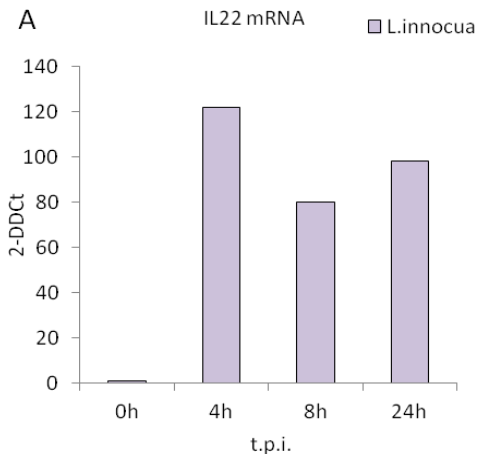


Fig.1: Microarray raw data of IL-22 gene expression in D1 cells infected with: *L.paracasei* (MOI 1:1000), *L.lactis* (MOI 1:1000), *L.monocytogenes* (MOI 1:20), *L.innocua* (MOI 1:1000). Samples were collected 4h, 8h and 24h post infection and hybridized onto the MOE430A GeneChip (Affymetrix). FI: fluorescence intensity; tpi: time post infection.

The interleukin gene was rapidly induced at high level *by L.innocua* 4h post infection (p.i.) and maintained at 8h and 24h. The same expression profile was maintained by *L.paracasei,* although the

fluorescent intensity was lower. On the contrary, D1 cells infected with *L.lactis* and *L.monocytogenes* showed an up-regulation of IL-22 mRNA 24h p.i. (**fig.1**). Microarray results were validated by quantitative real time PCR (qRT-PCR). IL-22 mRNA expression was tested in D1 cells infected with the gram+ bacteria. IL-22 mRNA was induced at highest level in response to *L.innocua* infection. In fact, IL-22 mRNA was fast up-regulated 4h post infection and maintained at high levels until 24h (**fig.2a**). On the contrary IL-22 mRNA was up-regulated 24h p.i. in response to *L.monocytogenes and L.lactis*. *L.paracasei* modulated IL-22 mRNA at lower levels 4h p.i. and maintained the same level until 24h (**fig2b**). The data confirmed the results previously shown by microarray analysis indicating that DCs are able to produce IL-22 in response to a gram+ bacteria challenge.



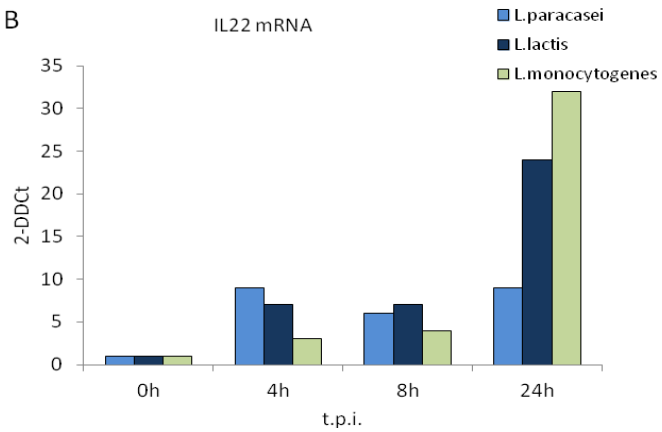
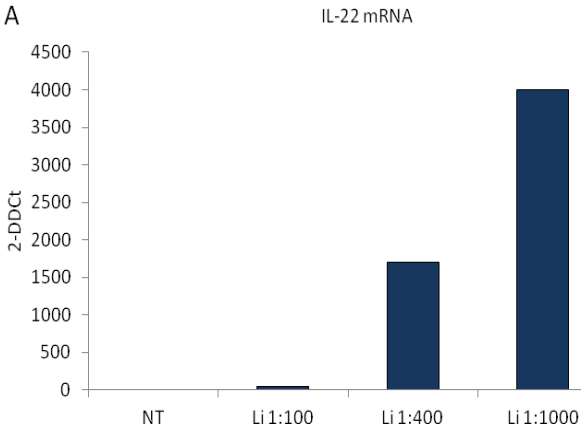


Fig.2: Microarray data validation by q-RT-PCR. D1 cells infected with (A) *L.innocua* (MOI 1:1000) and (B) *L.paracasei* (MOI 1:1000), *L.lactis* (MOI 1:1000), *L.monocytogenes* (MOI 1:20), of IL-22 gene expression in D1 cells infected with *L.innocua* (MOI 1:1000); mRNA data were normalized against 18S and then expressed as fold stimulation over control (0h, not treated cells). T.p.i.: time post infection.

3.1.2 BMDC up-regulate IL-22 mRNA in response to *L.innocua* infection

To further verify whether IL-22 was produced in other DCs models, we tested IL-22 mRNA expression in bone marrow-derived DC (BMDC) generated *in vitro* and stimulated with *L.innocua. L.innocua* was used because it was shown to induce a high amount of IL-22 in D1 cells. Bone marrow was derived from femurs and tibia of C57BL/6 mice and BMDC were differentiated *in vitro* by using medium supplemented with GM-CSF (see materials and methods). The

differentiation process was monitored by flow cytometry (FACS) after 8 day of culture. Cells between 75%-85% positive for CD11c and less than 20% B7.2 positive were used to perform the experiments. BMDC ($4,5x10^5$cells/ml) were infected with different multiplicity of infection (MOI) of *L.innocua* (1:100 1:400 1:1000). IL-22 production was measured both by qRT-PCR and by ELISA assay. A dose-response effect was observed at different MOI tested both in mRNA fold change increase and in protein production (**fig.3**).



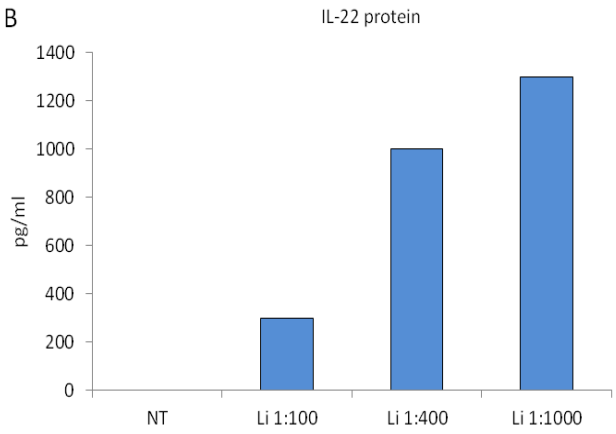


Fig.3: BMDC produced IL-22 stimulated with *L.innocua* (MOI 1:100, 1:440, 1:1000). Cells were seeded 4,5x10^5 cells/ml and stimulated for 24h. **A)** IL-22 analysis by qRT-PCR: data were normalized against 18S and then expressed as fold stimulation over control (NT, not treated cells). **B)** IL-22 ELISA assay: data are reported as pg/ml.

3.1.3 TLRs stimulation induce IL-22 production by BMDC

BMDC were able to produce IL-22 in response to gram+ bacterial infection. It is know that several pattern recognition receptors (PRR) present on DCs surface are responsible for both the recognition and the bacteria up-take. To investigate which receptors were involved in IL-22 production by BMDC, we analyzed the contribution of individual TLRs in its ability to induce the cytokine production. The basal TLRs mRNA expression on BMDC surface was characterized by qRT-PCR (**fig.4**). BMDC were collected 8 day post differentiation and analyzed by FACS. They were 85% CD11c and 11% B7.2 positive. Cells (10^6) were collected in trizol reagent and total RNA was isolated on Qiagen

micro column (see materials and methods). qRT-PCR was performed by using Sybr green and the primers pairs were selected and tested as reported in the materials and methods section. We characterized the expression of 10 different TLRs. The data in **fig.4** show that TLR1, TLR2, TLR4, TLR6, TLR7, TLR8 and TLR9 are expressed at moderate level (Ct between 25 and 23) whereas TLR3 (Ct=27,4), TLR5 (Ct=30) and TLR11 (Ct=32,5) are expressed at low level. In **table1** the Ct values of TLRs and 18S housekeeping are reported.

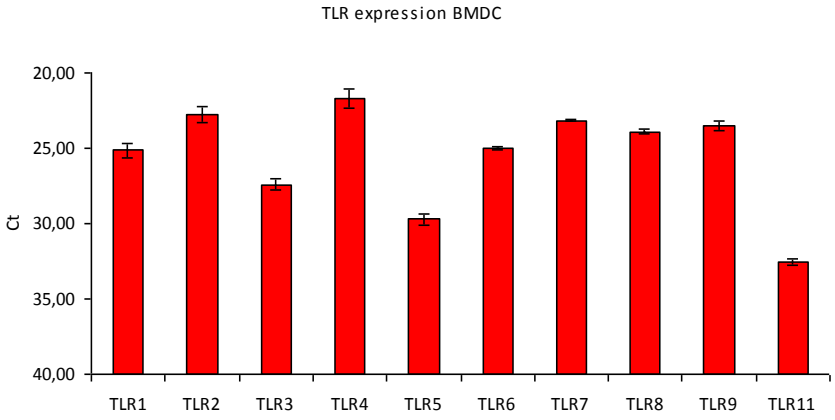


Fig.4: TLR expression on BMDC. At day 8 of colture, cells (85% CD11c and 11% B7.2 positive) were collected in trizol and qRT-PCR was performed. The expression of each TLR is reported as Ct value (±SD). Ct: cycle threshold.

Table1: Cycle threshold (Ct) value for each TLR.

	Ct
TLR1	25,13
TLR2	22,80
TLR3	27,44
TLR4	21,69
TLR5	29,73
TLR6	24,98
TLR7	23,14
TLR8	23,89
TLR9	23,51
TLR11	32,55
18S	7,30

To define which TLR was able to induce IL-22 production, we stimulated BMDC with different TLRs ligands. BMDC ($1,5x10^6$ cells/ml) were stimulated with the following TLR agonists: LPS 5ug/ml (TLR4-CD14), Zymosan 10ug/ml (TLR2-TLR6, Dectin-1, CD36), CpG 5ug/ml (TLR9), polyIC 1ug/ml (TLR3), Pam3Cys 100ng/ml (TLR1-TLR2), MALP2 100ng/ml (TLR2-TLR6), imiquimod 1ug/ml (TLR7), Profillin 100ng/ml (TLR11), flagellin 50ng/ml (TLR5). After 20h supernatants were collected and tested for IL-22 production by ELISA assay. IL-22 was produced in response to stimulation of all TLRs ligands with the exception of flagellin and profillin. Cells stimulated with zymosan (1400pg/ml), LPS (1000pg/ml) and CpG (1200pg/ml) induced the highest amount of IL-22. Malp2, Pam3Cys and polyIC

induced a production of IL-22 close to the detection limits of the assay (**fig.5**).

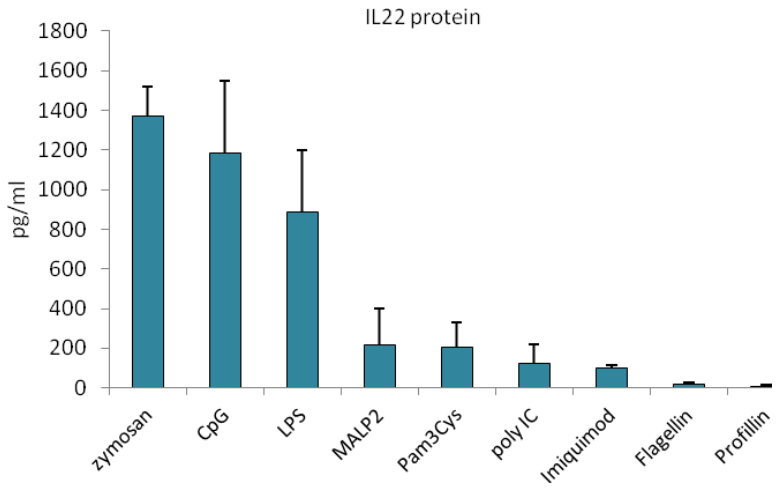


Fig.5: TLR activation induces IL-22 production in BMDC. BMDC ($1,5x10^6$ cells/ml) were stimulated with different TLR agonists. After 20h, supernatants were collected and tested for IL-22 production by ELISA (pg/ml). The data show the mean values of three independent experiments (± SD).

3.1.4 MyD88 is involved in IL-22 production by BMDC

Since IL-22 was induced in BMDC after TLRs stimulation, we decided to study the regulation of IL-22 in DCs. As previously shown TLR4, TLR9, TLR1, TLR2 and TLR6 are able to induce the highest IL-22 amount. These TLRs transduce the intracellular signal through the use of adaptor molecule MyD88 and therefore, we tested whether DCs-induced IL-22 production was MyD88 dependent. Both wild type

(WT) and MyD88 KO BMDC (1,5x10^6 cells/ml) were stimulated with the different TLRs agonists. After 20h supernatants were collected and tested for IL-22 production. The results in **fig.6** show that IL-22 production induced by CpG was completely abrogated in the absence of MyD88 as well as in response to Malp2 (TLR2-TLR6) and Pam3Cys (TLR1-TLR2) stimulation. A residual amount of IL-22 was detected after stimulation with LPS, probably due to the CD14 component of TLR4 complex.

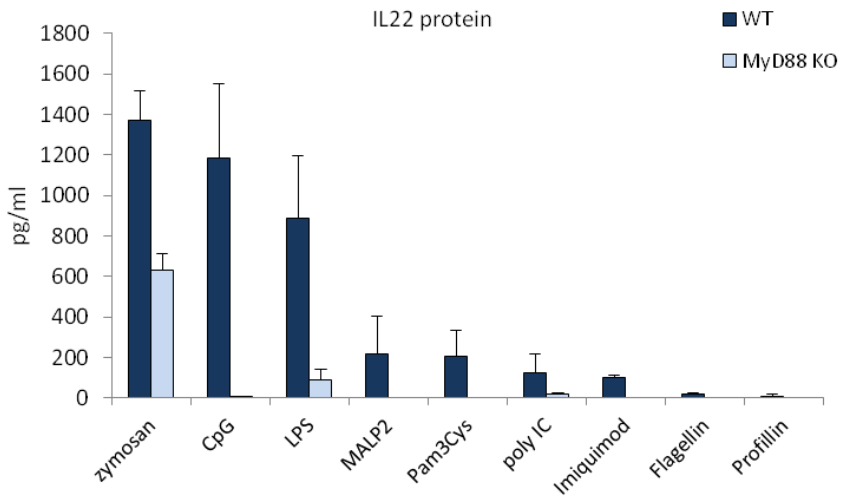


Fig.6: IL-22 production in BMDC is MyD88 dependent. BMDC from both WT and MyD88 KO mice were stimulated with TLRs ligands zymosan (10ug/ml), CpG (5ug/ml), LPS (5ug/ml), (Malp2 100ng/ml), Pam3Cys (100ng/ml), polyIC (1ug/ml), imiquimod (1ug/ml), flagellin (50ng/ml) and profillin (100ng/ml). After 20h supernatants were collected and tested by ELISA assay. The data show the mean values of four independent experiments (± SD).

MyD88KO BMDC were still able to produce a considerable amount of IL-22 (600pg/ml) in response to zymosan, suggesting that others pathogen recognition receptors (PRRs) may be involved in this response. Zymosan, is a complex molecules derived from the fungal wall digestion and it is usually used to mimic fungal infections. It is known to be recognized by cells through TLR2-TLR6, trough the c-type lectin receptor Dectin-1, which recognized β-glucans, and through the scavenger receptor CD36. In response to zymosan stimulation, BMDC could produce IL-22 not only trough TLR signaling pathway but also through a TLR-independent one. In fact, while TLR2 and TLR6 signal trough MyD88, Dectin-1 and CD36 use a different adaptor molecules to transduce the intracellular signaling. In conclusion, IL-22 production in BMDC is dependent both on a MyD88-dependent and independent pathway.

3.1.5 BMDC produce IL-22 in response to the c-type lectin receptor Dectin-1

Since MyD88KO BMDC stimulated with zymosan were still able to produce a high amount of IL-22, we investigated the stimulation of others PRR different from TLRs. Zymosan is known to contain β-glucans structures, which are recognized by Dectin-1 receptor. Dectin-1 is able to transduce intracellular signaling through the spleen kinase (SyK) pathway. qRT-PCR was performed to evaluate the

mRNA basal level of this receptor. Dectin-1 mRNA resulted expressed at intermediate level (Ct=23). Following receptor expression characterization, both WT and MyD88 KO BMDC (1,5x10^6 cells/ml) were stimulated with curdlan (10ug/ml) a pure β-glucan Dectin-1 agonist. After 20h supernatants were collected and analyzed for IL-22 production. The data shown in **fig.7** indicated that curdlan induced a strong IL-22 protein production both from WT and MyD88KO BMDC (1100pg/ml). Therefore, we concluded that in BMDC, IL-22 is produced also in response to stimulation of c-type lectins receptors confirming the existence of a MyD88-independent way. In particular, IL-22 can be produced by BMDC following the recognition of both bacterial and fungal cell wall components.

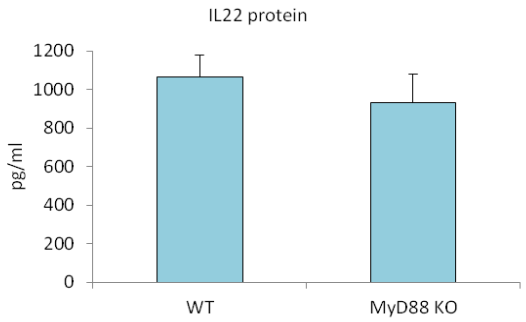


Fig.7: WT and MyD88 KO BMDC produce the same amount of IL-22. Cells (1,5x10^6 cells/ml) were stimulated for 20h with curdlan (10ug/ml). IL-22 production was measured by ELISA assay. The data represente the mean values of three independent experiments (± SD).

3.1.6 IL-22 production is not induced by IL-23 in BMDC

It has been reported that IL-22 gene regulation is dependent on IL-23 stimulation in total splenocytes, CD4+ T cells subtype Th17 (Zheng Y et al. 2007), NKp46+ cells from the gut (Cella M et al. 2009) and Lymphoid Tissue Inducer cells (LTi). In all these studies, it has been reported that, in response to TLRs stimulation, DCs are able to produce IL-23 which in turns induce IL-22 production by the target cells expressing the correspondent IL23R. For this reason, we investigated whether IL-22 production was dependent on IL-23 production observed after TLRs stimulation. BMDC were stimulated with either TLRs ligands or with mouse recombinant IL-23 (mrIL-23). Splenocytes were used as positive control. Both BMDC ($1,5x10^6$cells/ml) and total splenocytes ($2x10^6$cells/ml) from C57/BL6 mice were stimulated with 20ng/ml of mrIL-23. After 20h supernatants were collected and tested for IL-22 production. The data reported in **fig.8** show that BMDC were not able to produce IL-22 in response to IL-23 whereas total splenocytes produced the cytokine as expected (1200 pg/ml).

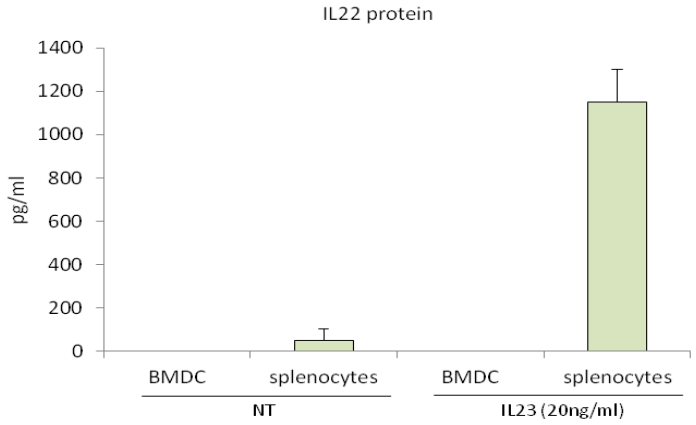


fig.8: IL-22 protein production in BMDC and total splenocytes after IL-23 stimulation. BMDC (1,5x10^{6}cells/ml) and splenocytes (2x10^{6}cells/ml) were stimulated with 20ng/ml of mrIL-23. After 20h supernatants were collected and tested for IL-22 by ELISA assay. The data show the mean values of three independent experiments (± SD).

To understand the unresponsiveness of BMDC to IL-23, several experiments to verify whether DCs express IL23R were performed. IL-23 expression was investigated by q-RT-PCR and by flow citometry (FACS), using both specific IL-23R primers and antibody, respectively. Unfortunately, we were not able to have reproducible data and therefore we could not obtain information about IL-23R expression in BMDC although IL23R mRNA seemed to be expressed at very low levels (BMDC IL-23r subunit: Ct 30; IL12rb1 subunit: Ct 29). Because of these results, blocking experiments with anti-IL-23 antibody (αIL-23) were performed. First, TLRs dependent IL-23 production was

measured. BMDC (1,5x10^6 cells/ml) were stimulated with LPS (5ug/ml), CpG (5ug/ml) and zymosan (10ug/ml) which were demonstrated to induce the strongest IL-22 production by BMDC. After 20h supernatants were collected and analyzed for IL-23 production. The data reported in **fig.9** show that BMDC produce high amount of IL-23 in response to LPS, CpG and zymosan.

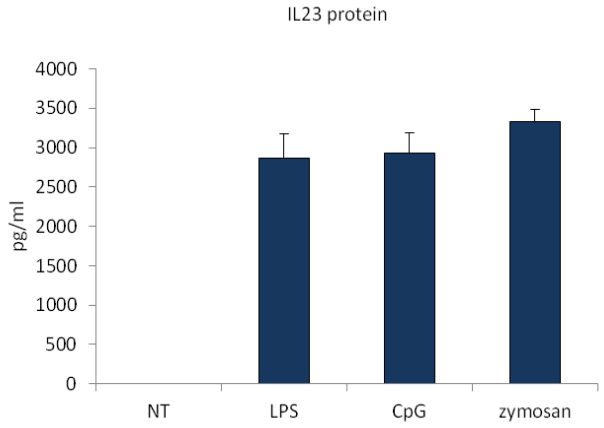


Fig.9: BMDC produce IL-23 after TLRs triggering. BMDC (1,5x10^6 cells/ml) were stimulated with the following TLR agonists: LPS (5ug/ml), CpG (5ug/ml) and Zymosan (10ug/ml). After 20h the supernatants were collected and tested for IL-23. The data show the mean values of three independent experiments (± SD).

Next, IL-22 production was measured in BMDC stimulated with the same TLRs ligands in presence of αIL-23 or the isotype control antibody IgG1 (IgG1ab). The functionality and the aspecific effects of

these antibodies were tested by stimulating total splenocytes with IL-23 in presence or absence of αIL-23 or IgG1ab. The optimal concentration for the assays was determined by a dose-response experiment on splenocytes. Splenocytes ($2x10^6$cells/ml) were stimulated with 2ug/ml of both αIL-23 and IgG1ab in presence or absence of mrIL-23 (5ng/ml). After 20h supernatants were collected and analyzed for IL-22 production. The data reported in **fig.10** indicate that the two antibodies alone did not induce any cytokine production. Splenocytes stimulated with 5ng/ml of mrIL-23 produced 1100pg/ml of IL-22 as well as splenocytes stimulated with IL-23 in presence of IgG1. On the contrary, 50% of reduction in IL-22 production (500pg/ml vs 1100pg/ml of stimulated cells) was measured in response to IL-23 in presence of αIL-23. The IgG1ab and αIL-23 could not be used at higher concentration because we observed an aspecific IL-22 production.

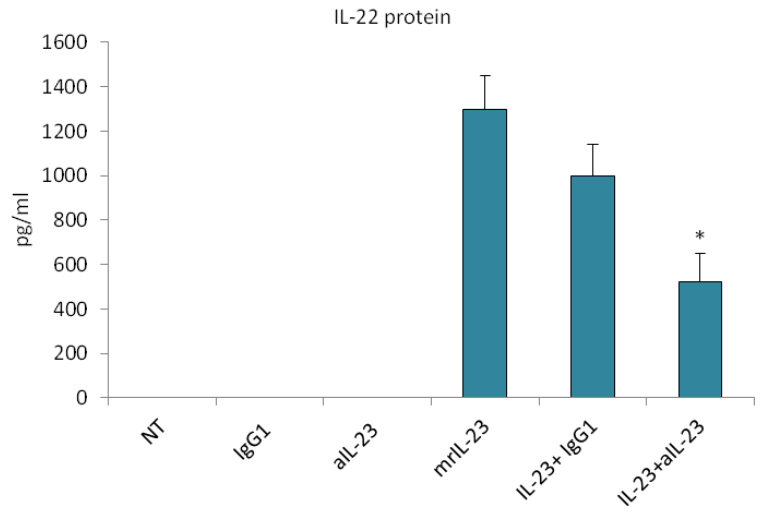


Fig.10: IL-22 production in total splenocytes stimulated with IL-23 (5ng/ml) in presence or absence of αIL-23 antibody. Splenocytes ($2x10^6$cells/ml) were stimulated for 20h with: IgG1 antibody (2ug/ml) plus or minus mrIL-23, αIL-23 (2ug/ml) plus or minus IL-23 or with mrIL-23 (5ng/ml) alone. The data show the mean values of three independent experiments (± SD). Statistical analysis: Student's T test *p<0,05

In a second set of experiments, we stimulated BMDC ($1,5x10^6$cells/ml) with LPS, CpG and zymosan in the presence of IgG1 or αIL-23 antibodies. After 20h the supernatants were collected and tested for IL-22 production. The data reported in **fig.11** show that IL-22 production by BMDC was directly dependent on TLRs stimulation and not indirectly mediated by IL-23 present in BMDC supernatants after TLRs triggering. In fact, BMDC stimulated with TLRs ligands in presence of αIL-23 produced the same amount of IL-22 as well as

BMDC stimulated with TLRs agonists alone. So, we could conclude that IL23 is not able to induce IL22 production in BMDC.

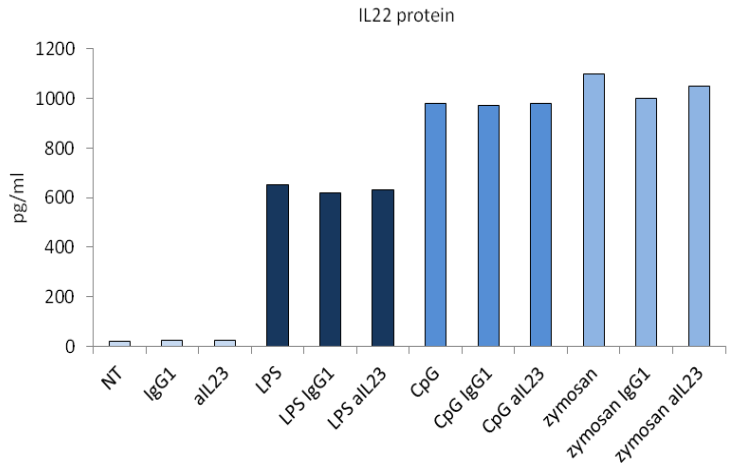


Fig.11: IL-22 production in BMDC is directly produced by TLR triggering and not indirectly mediated by IL-23. BMDC ($1,5x10^6$ cells/ml) were stimulated for 20h in presence of LPS (5ug/ml), CpG (5ug/ml) and zymosan (10ug/ml) plus or minus αIL23 (2ug/ml), IgG1(2ug/ml) and rmIL23 (5ng/ml). IL-22 production was tested by ELISA assay.

3.2 CHARACTERIZATION OF THE INTRACELLULAR PATHWAYS INVOLVED IN IL-22 TRANSCRIPTION

The intracellular signaling responsible for IL-22 transcription has not been fully elucidated yet. It is known that STAT3 induce IL-22 production downstream to IL23R after IL23 binding. The citosolic aryl hydrocarbon receptor (AhR) and the nuclear Ror$\gamma\tau$ receptor have been reported to be required for IL-22 transcription in Th17 cells (Ouyang W et al. 2010). It is known that TLRs stimulation in both DCs and macrophages lead to production of several cytokines and chemokines through a MyD88-dependent pathway, which in turns lead to AP-1 and NF-kB transcription factors phosphorylation. Nevertheless, no information is available about the intracellular signaling required for IL-22 production after TLRs activation. For these reasons, we decided to characterize the intracellular signaling responsible for IL-22 production by BMDC after PRR stimulation.

3.2.1 The transcription factor AP-1 is necessary for IL-22 production in BMDC

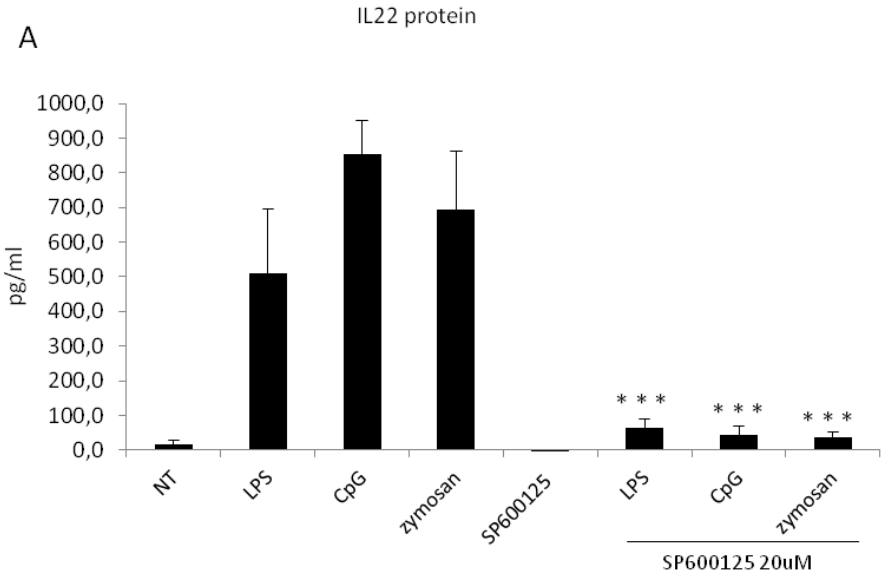
The transcription factor AP-1 is a heterodimer composed of two different subunits derived from one of the four AP-1 subfamily: JUN (JunB, JunD, c-Jun), FOS (FosB, Fra1, Fra2), ATF (B-ATF, ATF2, ATF3, ATF4) and MAF (c-MAF, MafA, MafB). JNK, ERK and p38 MAP kinases are responsible for the phosphorylation of AP-1 transcription factors

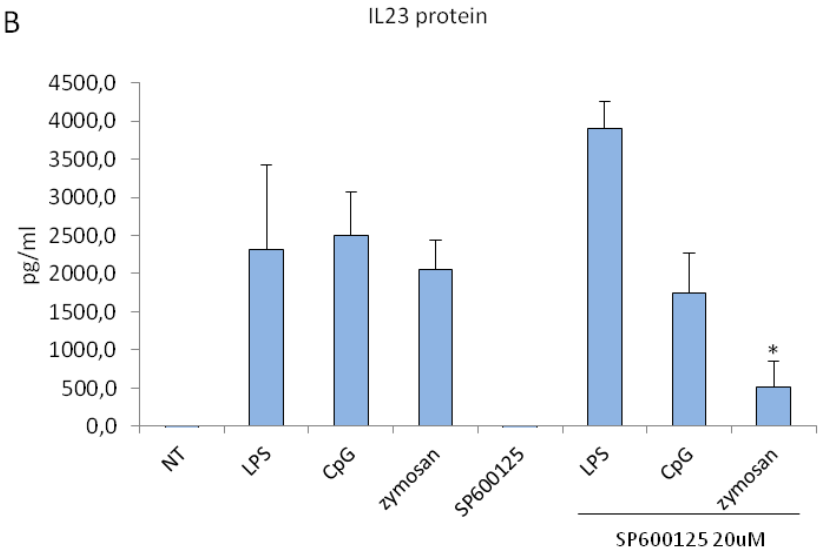
subunits. To study whether AP-1 was involved in IL-22 production, experiments with specific chemical inhibitors were performed to block the functionality of JNK, ERK and p38 MAP kinases. The experimental setting used was the same for each experiment. BMDC ($1,5x10^6$ cells/ml) were pretreated for 30 minutes with the specific inhibitors. Cells were then stimulated with TLR agonists LPS (5ug/ml), CpG (5ug/ml) and zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-22 by ELISA assay. The optimal inhibitor concentrations were determined by dose-response experiments. The toxic effects of compounds were tested by cytotoxicity assay with the cell titer blue assay (see materials and methods). Cell death within 10% was considered acceptable. TNFa and IL-23 production was tested in each assay as controls.

JNK and ERK MAP kinases are required for IL-22 gene transcription

JNK MAP kinase was blocked by using the specific inhibitor SP600125 at the concentration of 20uM. The data reported in **fig.12a** indicated a reduction higher than 93% of IL-22 production in BMDC stimulated with LPS, CpG and zymosan in presence of SP600125. As control of inhibitor specificity, we also tested the involvement of JNK in IL-23 and TNFa gene transcription. In **fig.12b**, the results show that JNK is not required for IL-23 production in BMDC stimulated with LPS and CpG. On the contrary, JNK seems to be necessary for IL-23 production in response to zymosan, suggesting that probably this kinase could be

involved in the intracellular signalling downstream the c-type lectin receptors. The data represented in **fig.12c**, show the amount of TNFa produced by BMDC stimulated with LPS (18ng/ml), CpG (14ng/ml) and zymosan (16ng/ml) in presence or absence of JNK inhibitor. No significative reduction of TNFa was measured blocking JNK in response to stimulation with LPS (17ng/ml) and zymosan (13ng/ml). On the contrary, a reduction of 60% was measured in TNFa production when CpG was used in the presence of SP600125. Taken together, these data suggested that JNK is necessary for IL-22 production in BMDC stimulated with LPS, CpG and zymosan. JNK is also involved in IL-23 transcription in BMDC stimulated with zymosan and is required for TNFa transcription in BMDC stimulated with CpG.


A
IL22 protein
pg/ml
1000,0
900,0
800,0
700,0
600,0
500,0
400,0
300,0
200,0
100,0
0,0
* * *
* * *
* * *
NT
LPS
CpG
zymosan
SP600125
LPS
CpG
zymosan
SP600125 20uM


B
IL23 protein
pg/ml
4500,0
4000,0
3500,0
3000,0
2500,0
2000,0
1500,0
1000,0
500,0
0,0
*
NT
LPS
CpG
zymosan
SP600125
LPS
CpG
zymosan
SP600125 20uM

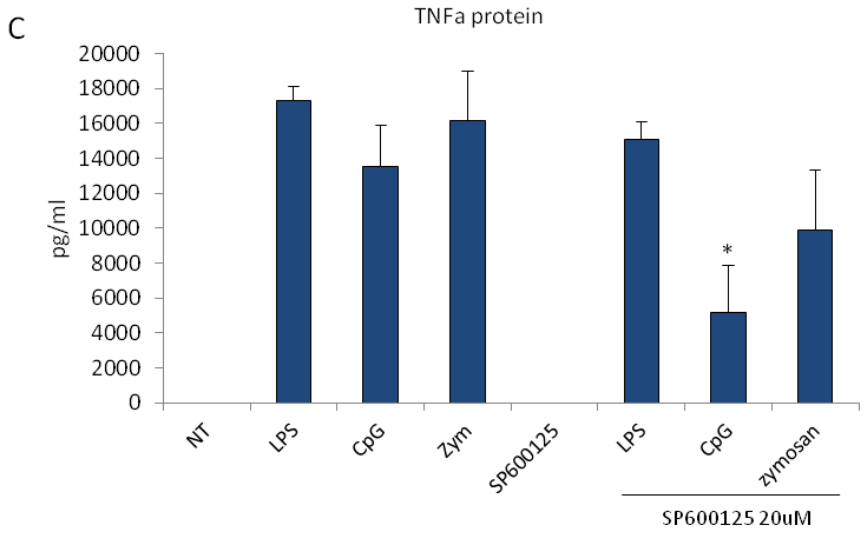
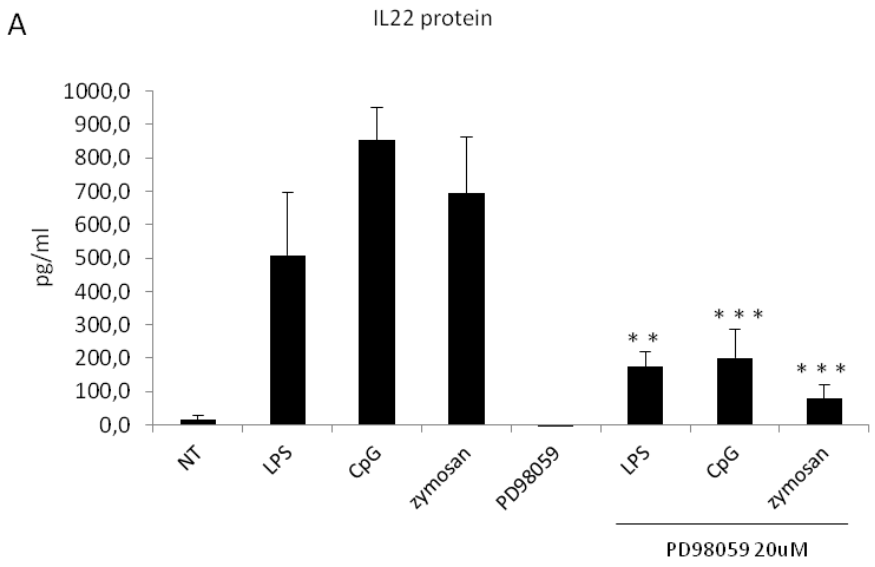


Fig.12: JNK MAP kinase involvement in transcription of IL-22, IL-23 and TNFa in BMDC. Cells (1,5x10^{6}cells/ml) were pretreated 30 minutes with SP600125 (20uM) and then stimulated with TLR agonist LPS (5ug/ml), CpG (5ug/ml) and Zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-22 **(A)**, IL-23 **(B)** and TNFa **(C)** production by ELISA. The data show the mean values of five independent experiments for IL-22 (± SD) and three experiments for both IL-23 and TNFa (± SD). Statistical significance is shown *** p<0,001; ** p<0,01; *p<0,05 (Student't T test)

ERK MAP kinase was blocked by using the specific inhibitor PD98059 at the concentration of 20uM. The data reported in **fig.13a** indicate a reduction of 75% in IL-22 production in BMDC stimulated with LPS and CpG in presence of PD98059. A stronger reduction, more than

90%, was measured in BMDC stimulated with zymosan and ERK inhibitor. These data indicated that ERK is required for IL-22 transcription. In **fig.13b**, the results show that ERK was not necessary for IL-23 transcription in BMDC stimulated with LPS, CpG and zymosan. In fact, we measured no reduction in IL-23 production by blocking ERK with PD98059. The same result was obtained for TNFa. Data represented in **fig.13c**, shown that ERK is not required for TNFa production in BMDC stimulated with LPS, CpG and zymosan in presence of PD98059.



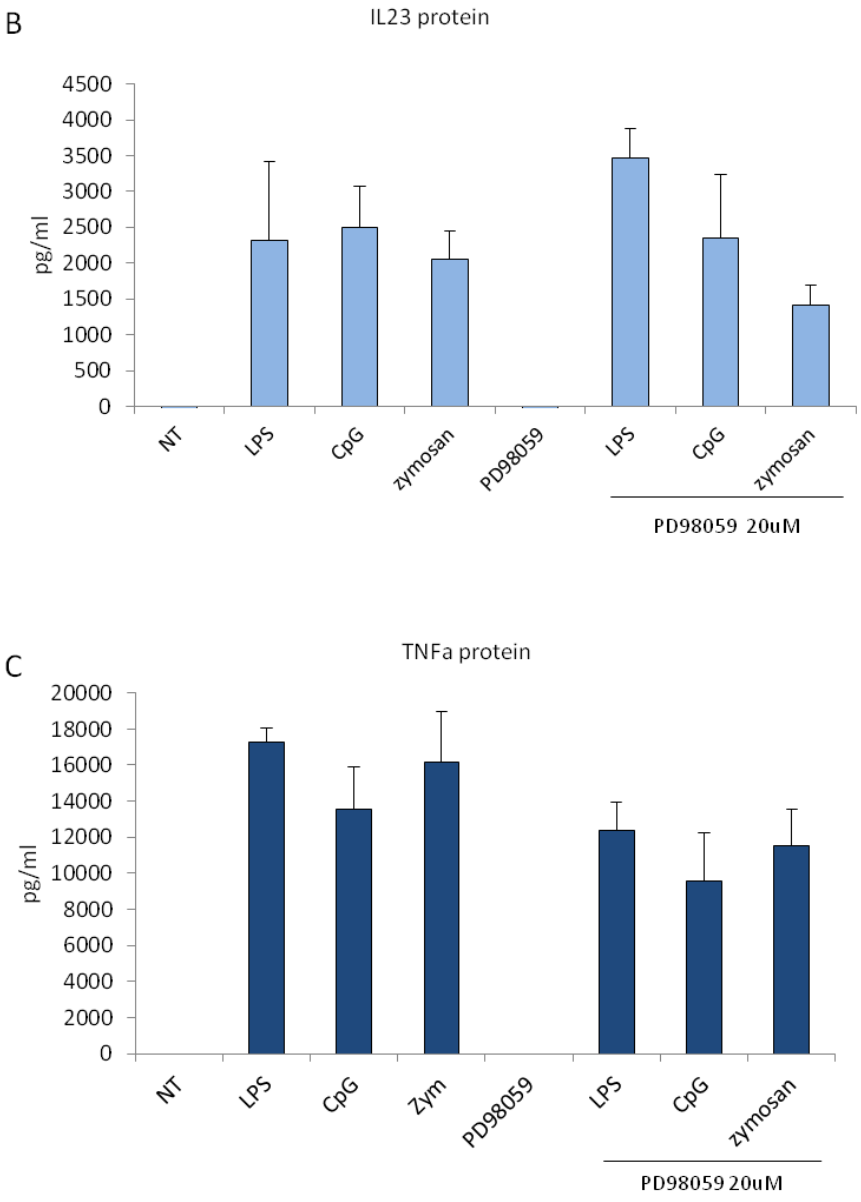


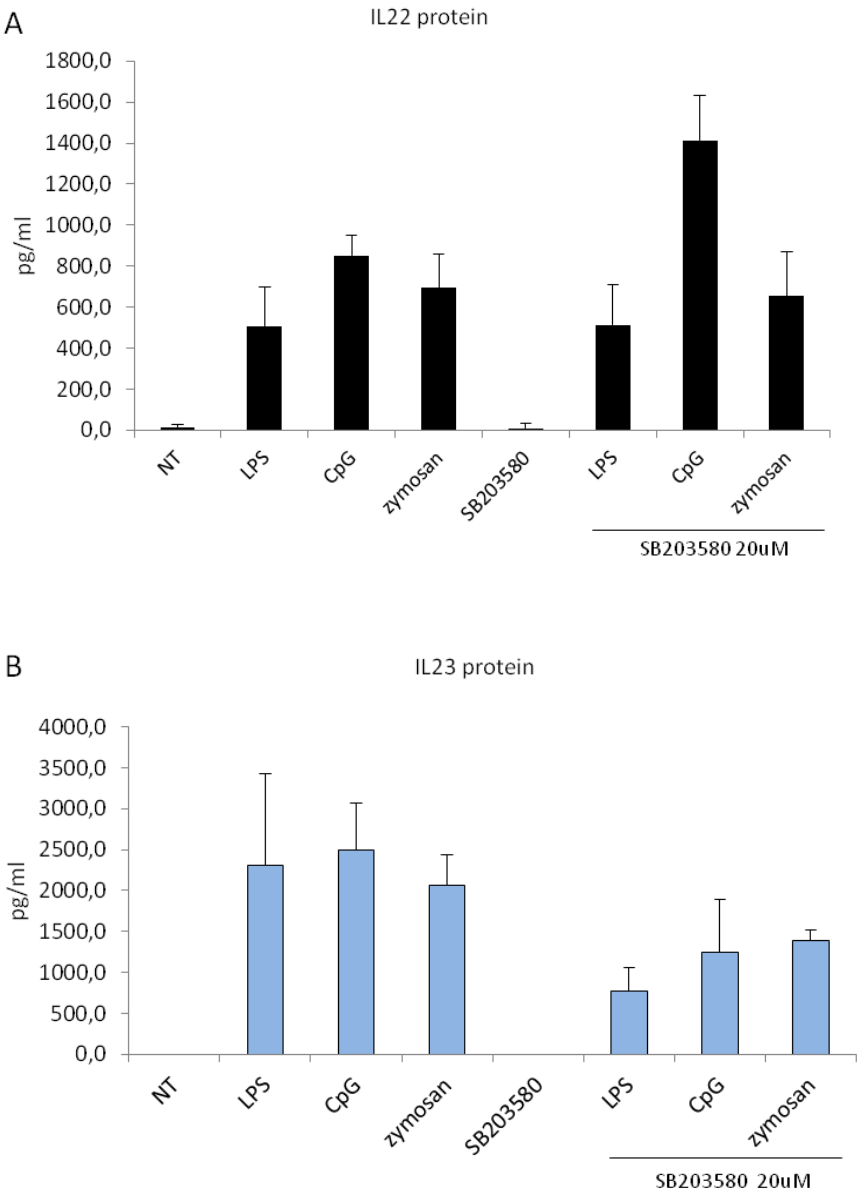
Fig.13: ERK MAP kinase involvement in IL-22, IL-23 and TNFa in BMDC. Cells ($1,5x10^6$cells/ml) were pretreated 30 minutes with PD98059 (20uM) and then

stimulated with TLR agonist LPS (5ug/ml), CpG (5ug/ml) and Zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-22 **(A)**, IL-23 **(B)** and TNFa **(C)** production by ELISA. The data show the mean values of five independent experiments for IL-22 (± SD), three independent experiments for both IL-23 and TNFa (± SD). Statistical significance is shown, *** p<0,001; ** p<0,01; *p<0,05 (Student't T test)

p38 MAP kinase is not required for IL-22 transcription

p38 MAP kinase was block by using the specific inhibitor SB203580 at the concentration of 20uM. The data reported in **fig.14a** indicated that p38 was not necessary for IL-22 transcription in response to LPS, CpG and zymosan. In fact, no IL-22 reduction was measured. The same results were obtained for both IL-23 and TNFa. In **fig.14b** and **fig.14c** respectively, the data show that p38 was not involved in both IL-23 and TNFa production, since no decrease of these two cytokines was measured. Taken together, these data suggested that p38 is not required for IL-22, IL-23 and TNFa transcription in BMDC after TLRs stimulation.

In summary, we can conclude that JNK and ERK kinases are necessary for IL-22 transcription in BMDC and have to be functional at the same time. These data also suggested that AP-1 is absolutely necessary for IL-22 transcription in BMDC.


A
IL22 protein
pg/ml
1800,0
1600,0
1400,0
1200,0
1000,0
800,0
600,0
400,0
200,0
0,0
NT
LPS
CpG
zymosan
SB203580
LPS
CpG
zymosan
SB203580 20uM
B
IL23 protein
pg/ml
4000,0
3500,0
3000,0
2500,0
2000,0
1500,0
1000,0
500,0
0,0
NT
LPS
CpG
zymosan
SB203580
LPS
CpG
zymosan
SB203580 20uM

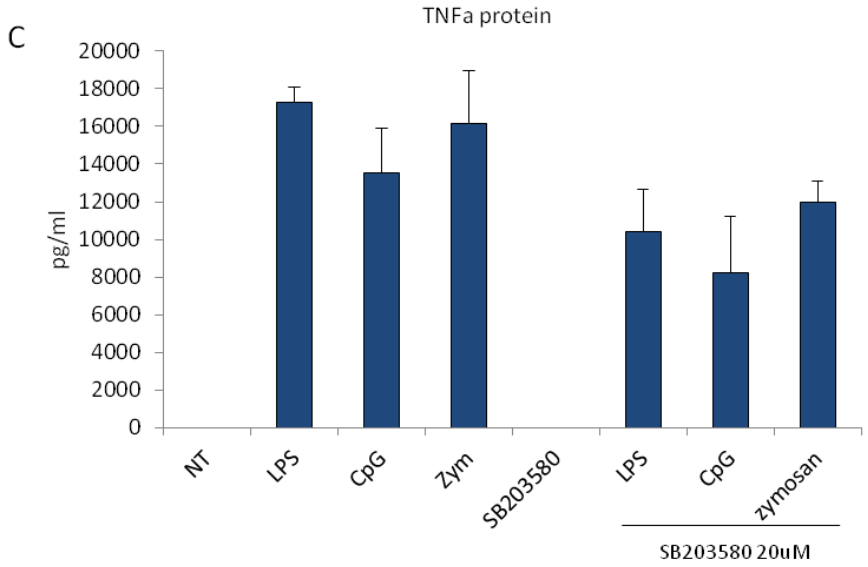
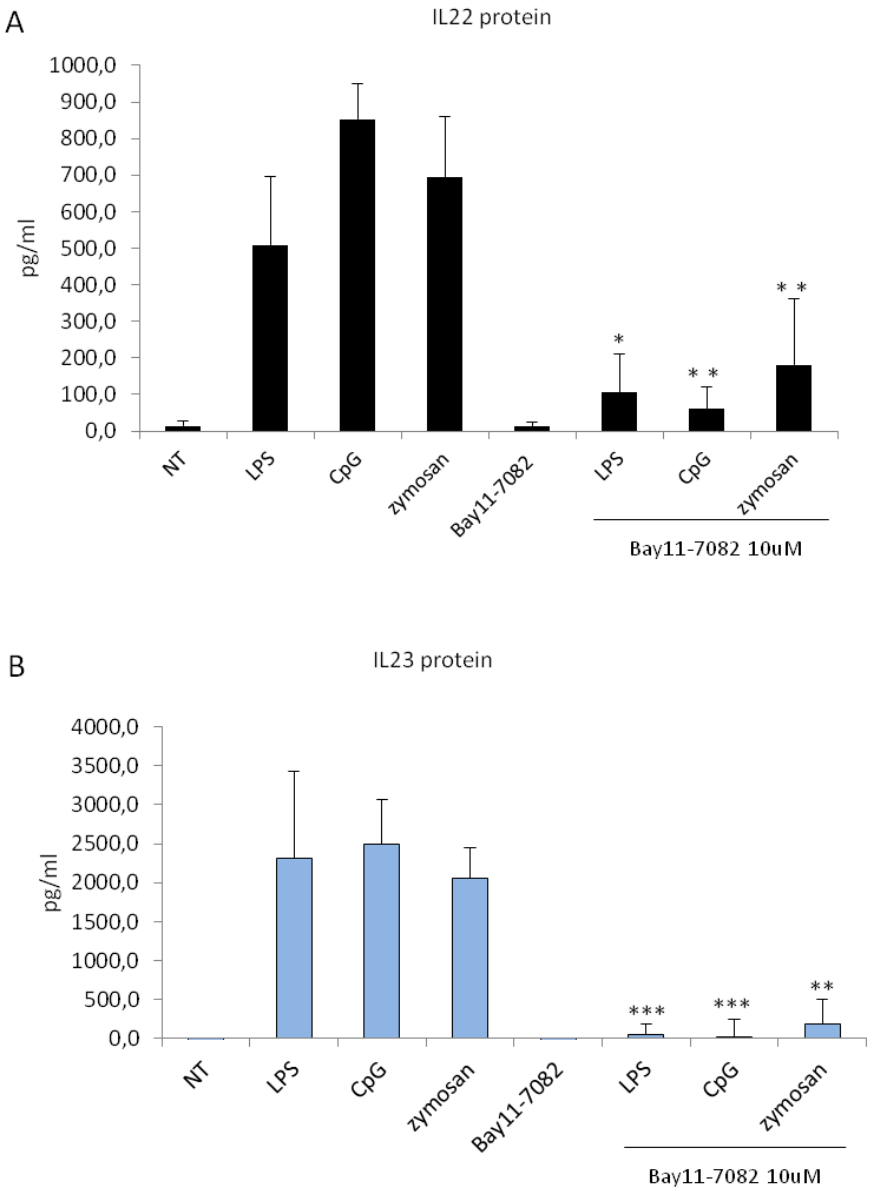


Fig.14: p38 MAP kinase involvement in IL-22, IL-23 and TNFa in BMDC. Cells ($1,5x10^6$cells/ml) were pretreated 30 minutes with SB203580 (20uM) and then stimulated with TLR agonist LPS (5ug/ml), CpG (5ug/ml) and Zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-22 **(A)**, IL-23 **(B)** and TNFa **(C)** production by ELISA. The data show the mean values of five independent experiments for IL-22(± SD) and three experiments for both IL-23 and TNFa(± SD). Statistical significance is shown, *p<0,05 (Student't T test).

3.2.2 NF-kB transcription factor is required for IL-22 transcription in BMDC

It is known that NF-kB is required for cytokines and chemokines gene regulation in response to TLRs stimulation. Therefore, the involvement of NF-kB in IL-22 production was measured by performing experiments with the specific inhibitor Bay11-7082.

BMDC (1,5x10^6 cells/ml) were pretreated for 30 minutes with Bay11-7082 at the concentration of 10uM. Cells were then stimulated with TLR agonists LPS (5ug/ml), CpG (5ug/ml) and zymosan (10ug/ml). The optimal inhibitor concentration was determined by dose-response experiments and the compound toxicity was tested by citotoxicity assays (see materials and methods). After 20h supernatants were collected and analyzed for IL-22, IL-23 and TNFa production. Data in **fig.15a** show a reduction of 70% of IL-22 production in BMDC stimulated with LPS and CpG in presence of Bay11-7082 and a reduction of 60% in BMDC stimulated with zymosan. The data **fig.15b** shows that NF-kB is required for IL-23 production. In fact, IL-23 reduction higher than 85% was measured in BMDC stimulated with LPS, CpG and zymosan in presence of Bay11-7082. The data in **fig.15c**, show that NF-kB seems to be involved in TNFa production in response to CpG stimulation. In fact, by blocking NF-kB, we did not detect any TNFa reduction in response to LPS and zymosan whereas a reduction of 60% was measured in response to CpG.


A
IL22 protein
pg/ml
1000,0
900,0
800,0
700,0
600,0
500,0
400,0
300,0
200,0
100,0
0,0
*
* *
* *
NT
LPS
CpG
zymosan
Bay11-7082
LPS
CpG
zymosan
Bay11-7082 10uM
B
IL23 protein
pg/ml
4000,0
3500,0
3000,0
2500,0
2000,0
1500,0
1000,0
500,0
0,0
***
***
**
NT
LPS
CpG
zymosan
Bay11-7082
LPS
CpG
zymosan
Bay11-7082 10uM

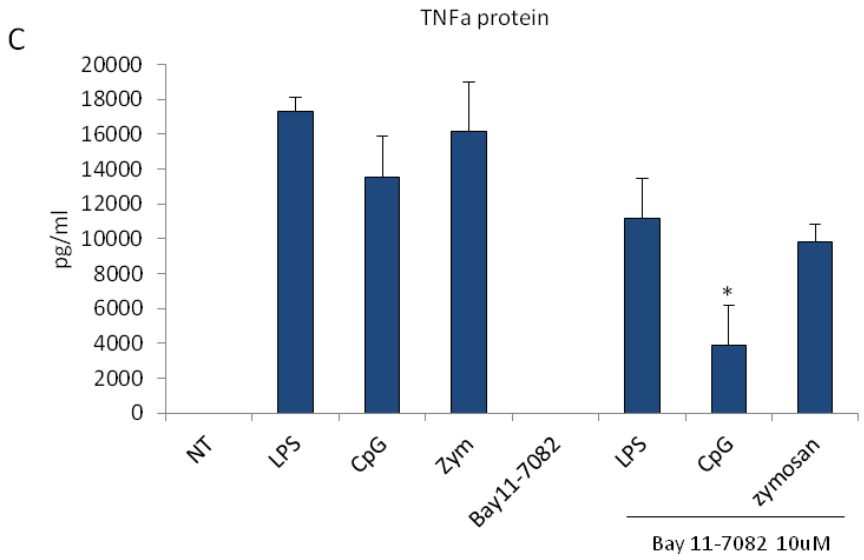


Fig.15: Involvement of NF-kB in IL-22, IL-23 and TNFa in BMDC. Cells (1,5x10^{6}cells/ml) were pretreated 30 minutes with Bay11-7082 (10uM) and then stimulated with TLR agonist LPS (5ug/ml), CpG (5ug/ml) and Zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-22 **(A)**, IL-23 **(B)** and TNFa **(C)** production by ELISA. The data show the mean values of seven independent experiments for IL-22 (± SD) and four independent experiments for both IL-23 and TNFa (± SD). Statistical significance is shown, *** p<0,001; ** p<0,01; *p<0,05 (Student't T test)

3.2.3 NFAT is not necessary for IL-22 transcription in BMDC

It is known that some cytokines, such as IL-2, require NFAT for gene regulation. Therefore, we evaluated the involvement of NFAT for IL-22 transcription in BMDC by blocking calcineurin with cyclosporine.

Calcineurin is a calcium-dependent serine-threonine phosphatase. It activates the T cells and can be blocked by drugs. Calcineurin activates by dephosphorylation the cytoplasmic nuclear factor of activated T cell (NFATc). The activated NFATc is then translocated into the nucleus, where it up-regulates the expression of IL-2, which, in turn, stimulates the growth and differentiation of T cells. BMDC ($1,5x10^6$ cells/ml) were pretreated for 30 minutes with cyclosporine A at the concentration of 10uM to block calcineurin. After 20h supernatants were collected and tested for IL-22 and IL-2 production. IL-2 was used as positive control of the assay. As shown in **fig.16**, there was no reduction in IL-22 production, suggesting NFAT is not involved in IL-22 transcription. On the contrary, the production of IL-2 was completed abrogated in BMDC stimulated with CpG and Zymosan in presence of cyclosporine (**fig.17**).

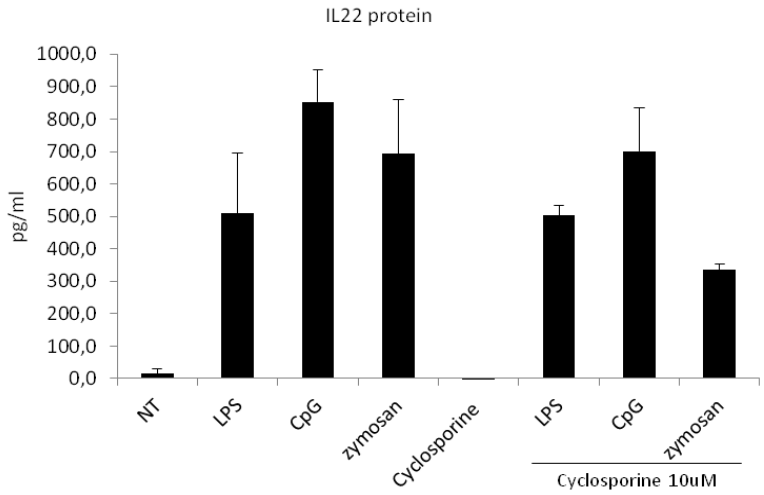


Fig.16: Involvement of NFAT in IL-22 in BMDC. Cells (1,5x10^{6}cells/ml) were pretreated 30 minutes with cyclosporine (10uM) and then stimulated with TLR agonist LPS (5ug/ml), CpG (5ug/ml) and Zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-22 production by ELISA. The data show the mean values of three independent experiments (± SD). Statistical significance is shown, *p<0,05 (Student't T test).

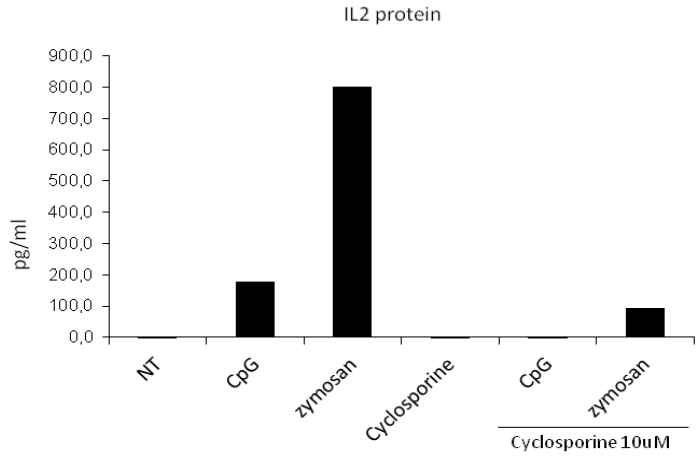


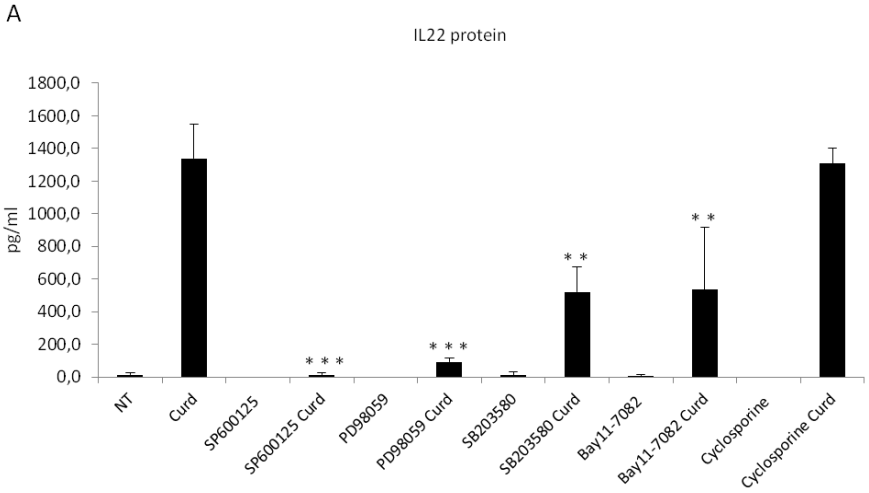
Fig.17: Involvement of NFAT in IL-2 in BMDC. Cells (1,5x10^{6}cells/ml) were pretreated 30 minutes with cyclosporine (10uM) and then stimulated with TLR agonists CpG (5ug/ml) and Zymosan (10ug/ml). After 20h supernatants were collected and tested for IL-2 production by ELISA. The data are representative of two independent experiments.

3.2.4 AP-1 and NF-kB but not NFAT transcription factors are required for IL-22 transcription downstream Dectin-1 receptor

We previously showed that BMDC was able to produce IL-22 in response to curdlan stimulation through a MyD88-independent pathway. Curdlan binds Dectin-1 receptor that transduces the intracellular signaling through SyK kinase that leads to the activation of NF-kB and AP-1 transcription factors. Therefore we performed

experiments to understand the involvement of MAP kinases, NF-kB and also of NFAT in IL-22 production by BMDC stimulated with curdlan. BMDC ($1,5x10^6$ cells/ml) were pretreated for 30 minutes with chemical inhibitors and then stimulated with curdlan (10ug/ml). After 20h supernatants were collected and tested for IL-22. As positive control we measured TNFa production. As shown in **fig18a**, by blocking JNK and ERK kinases with the respective specific inhibitors SP600125 (20uM) and PD98059 (20uM), a reduction of 97% and 92% was measured in BMDC respectively. Surprisingly a reduction of 50% was measured by blocking p38 kinase with SB302085 (20uM). The same percentage of reduction was obtained by blocking NF-kB with Bay11-7082 (10uM). No reduction in IL-22 production was measured blocking calcineurin through cyclosporine. Taken together these data suggested that, in BMDC stimulated with curdlan, JNK and ERK MAP kinase are necessary for IL-22 transcription; p38 MAP kinase and NF-kB play a role even if in their absence the cells are still able to produce a considerable level of IL-22. No reduction in TNFa production was measured as shown in **fig.18b.** From these results, we can conclude that the intracellular pathways responsible for IL-22 production in BMDC are specific in response to the PRRs that is activated.

A


IL22 protein
pg/ml
1800,0
1600,0
1400,0
1200,0
1000,0
800,0
600,0
400,0
200,0
0,0
* * *
* * *
* *
* *
NT
Curd
SP600125
SP600125 Curd
PD98059
PD98059 Curd
SB203580
SB203580 Curd
Bay11-7082
Bay11-7082 Curd
Cyclosporine
Cyclosporine Curd

B

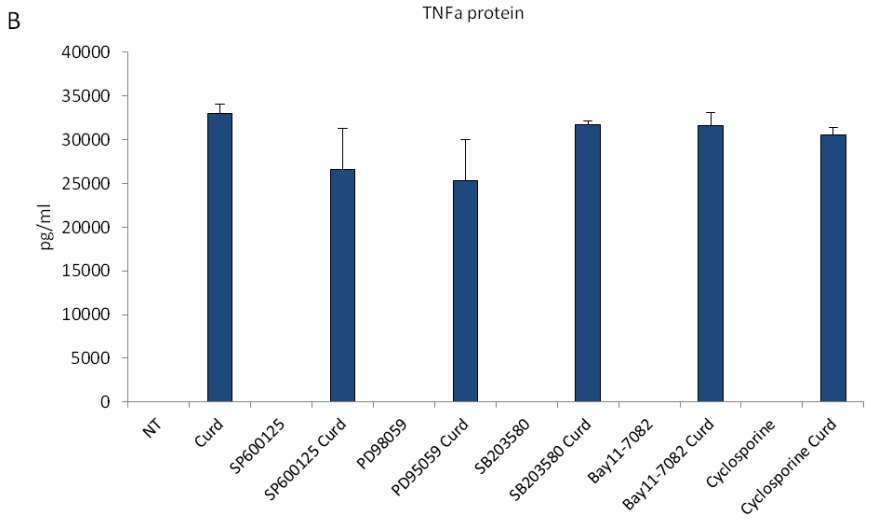

TNFa protein
pg/ml
40000
35000
30000
25000
20000
15000
10000
5000
0
NT
Curd
SP600125
SP600125 Curd
PD98059
PD95059 Curd
SB203580
SB203580 Curd
Bay11-7082
Bay11-7082 Curd
Cyclosporine
Cyclosporine Curd

Fig.18: AP-1 and NF-kB but not NFAT transcription factor are involved in IL-22 production downstream Dectin-1 receptor in BMDC. Cells (1,5x10^{6}cells/ml) were pretreated 30 minutes with MAP kinases inhibitors JNK SP600125(20uM), ERK PD98059(20uM), p38 SB203580(20uM), NF-kB inhibitor Bay11-0782(10uM) and NFAT inhibitor cyclosporine (10uM). Supernatants were collected and analyzed for IL-22 (A) and TNFa (B) production 20h post stimulation with curdlan (10ug/ml). The data show the mean values of four independent experiments (± SD). Statistical significance is shown, *** p<0,001; ** p<0,01; *p<0,05 (Student't T test). Curd: curdlan.

3.2.5 The nuclear factor AHR but not RORgt is required for IL-22 transcription in BMDC

Aryl hydrocarbon receptor (AhR) is a nuclear receptor that has been reported to be essential in CD4+cells (Th17 subtype) for the production of IL-22 in response to IL-23 (Stockinger B et al. 2009). The physiological ligands of this receptor are unknown, but it has been reported to bind several exogenous ligands such as natural plant flavonoids, polyphenolics and indoles, as well as synthetic polycyclic aromatic hydrocarbons and dioxin-like compounds. AhR is a cytosolic transcription factor that is normally inactive, bound to several co-chaperones. Upon ligand binding to chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the chaperones dissociate resulting in AhR translocating into the nucleus and dimerizing with ARNT (*AhR nuclear translocator*), leading to changes

in gene transcription (Stevens E et al. 2009). Therefore, we studied the role of AhR in producing IL-22 in BMDC stimulated with TLR agonists and curdlan in presence of AhR antagonist. The mRNA expression of AhR in BMDC was evaluated by qRT-PCR. AhR mRNA resulted expressed at intermediate level (Ct=23) in BMDC. Therefore, BMDC were pre-treated 30 minutes with AhR antagonist CH-223191, a chemical compound that binds with high affinity AhR resulting in sequestration in the cytosol. Cells were then stimulated with 5ug/ml LPS, 5ug/ml CpG and 10ug/ml curdlan. After 20h the supernatants were collected and tested for IL-22 production. A reduction of 70% was measured in IL-22 production suggesting that AhR play a role in IL-22 transcription in BMDC (**fig.19**).

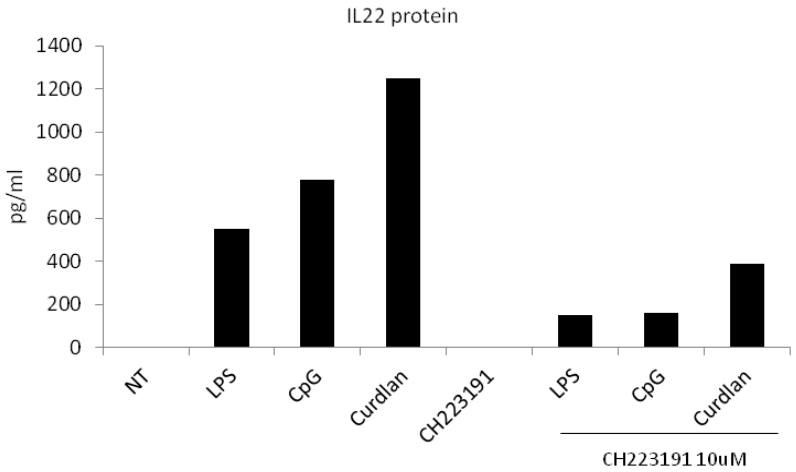


Fig.19: AhR is necessary for IL-22 production in BMDC. Cells (1,5x10^6cells/ml) were pretreated 30 minutes with AhR antagonist CH223191 (10uM). Supernatants were collected and analyzed for IL-22 production 20h post stimulation with LPS (5ug/ml), CpG (5ug/ml) and curdlan (10ug/ml). The data show a representative of two independent experiments.

The other receptor involved in IL-22 production by Th17 cells is Retinoic related orphan receptor (RoR$\gamma\tau$). We decided to test whether RoR$\gamma\tau$ could have a role in IL-22 production by BMDC. RoR$\gamma\tau$ mRNA expression was evaluated by qRT-PCR. RoR$\gamma\tau$ mRNA expression in the spleen and in the liver was used as positive controls. These two organs are rich in Th17 cells that have been described to express the transcription factor. Spleen and liver were obtained from C57/BL6 mice and were homogenized by using tissue lyser instrument (see materials and methods). As shown in **fig.20**, the RoR$\gamma\tau$ mRNA was not expressed in BMDC (Ct=38) whereas it was

expressed at intermediate levels in the spleen (Ct=26) and in the liver (Ct=24) cells. These data suggested that RoRγτ is not required for IL-22 production in BMDC as in Th17 cells. In **table2** Ct values of RoRγτ and 18S housekeeping genes are reported.

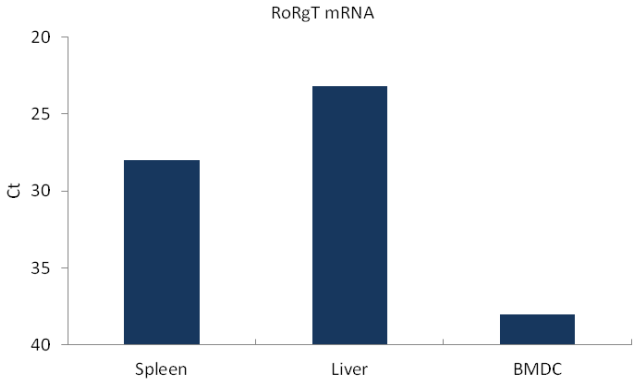


Fig.20: RoRγτ is not expressed in BMDC. Cells (1,5x10^{6}cells) were collected in trizol as well as sample from spleen and liver. After reverse transcription q-RTPCR was performed. Ct values of RoRγτ mRNA expression are reported. Ct: cycle threshold.

Table2: Cycle threshold (Ct) value of RoRγτ and 18S mRNA

	Ct RoRγτ	**18S**
Spleen	28	7,5
Liver	23,2	7,2
BMDC	38	7,4

3.3 IL-22 PRODUCTION BY MACROPHAGES

We previously showed that IL-22 is produced by BMDC in response to TLRs stimulation through a MyD88-dependent pathway. We concluded that IL22 can be produced in BMDC in IL-23 independent manner. Phagocytes other than DCs express high level of PRR. Therefore, we decided to study whether cell types expressing PRR were able to produce IL22 in response to PRR ligands. Within the phagocytic populations, macrophages are known to express several PRRs, including TLRs, c-type lectin, scavenger and Fc receptors (Murray PJ and Wynn T 2011). Therefore, we decided to test whether macrophages stimulated with TLRs agonists and curdlan were able to produce IL-22.

3.3.1 Bone marrow macrophages are not able to produce IL-22 in response to TLRs stimulation.

Bone marrow derived macrophages (BMMϕ) were obtained from bone marrow of C57/BL6 mice and *in-vitro* differentiated for 8 days in medium supplemented with M-CSF (see materials and methods). TLRs and Dectin-1 mRNA expression in macrophages was analyzed by qRT-PCR. As shown in **fig.21** TLR1, TLR2, TLR4, TLR6, TLR7, TLR8 and TLR9 were expressed at intermediate level (Ct>25) as well as Dectin-1 receptor (Ct=26). TLR5 (Ct=29) was expressed at low level whereas

TLR11 that was not expressed (Ct=38). In **table3** Ct values are reported.

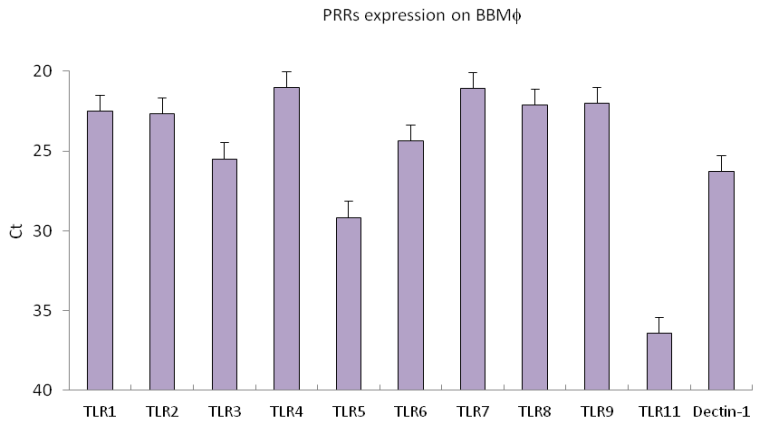


Fig.21: TLR expression on BMMϕ. At day 8 of colture, cells were collected in trizol and qRT-PCR was performed. The expression of each TLR is reported as Ct value (± SD).

Table3: Cycle threshold (Ct) value for each TLR and Dectin-1

	BMMϕ
TLR1	22,50
TLR2	22,68
TLR3	25,49
TLR4	21,01
TLR5	29,16
TLR6	24,35
TLR7	21,10
TLR8	22,11
TLR9	22,03
TLR11	36,42
Dectin-1	26,27
18S	7,3

BMMϕ were then seeded at different concentrations (ranging from $1,5x10^6$ to $4x10^6$cells/ml) and stimulated with TLRs agonists LPS (5ug/ml), CpG (5ug/ml) and zymosan (10ug/ml), with curdlan (10ug/ml) and with mrIL-23 (20ng/ml). After 24h and 48h supernatants were collected and analyzed for IL-22. To test macrophages activation we measured TNFa production. BMMϕ were not able to produce IL-22 in response to TLRs stimulation and curdlan. BMMϕ were properly activated since we measured TNFa production in response to LPS, CpG and curdlan (**fig.22**).

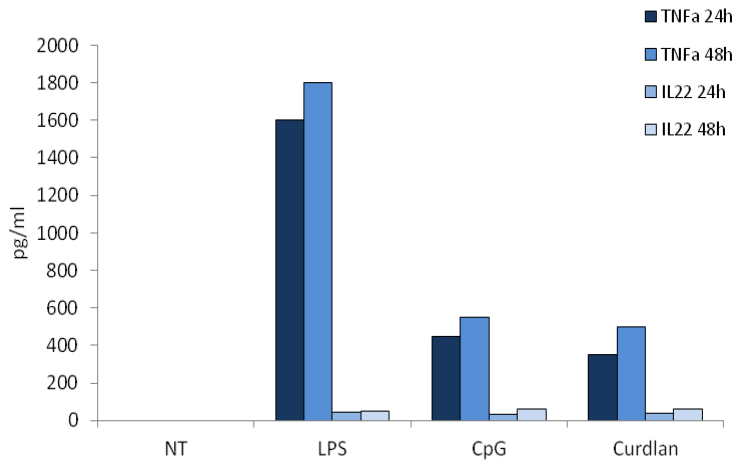


Fig.22: IL-22 and TNFa production in BBMϕ. Cells (1,5x10^{6}cells/ml) were stimulated with LPS (5ug/ml), CpG (5ug/ml) and curdlan (10ug/ml). After 24h and 48h supernatants were collected and analyzed for IL-22 and TNFa production. Data show a representative of two independent experiments.

The results indicated that BMMϕ are not able to produce IL-22. Therefore, we decided to investigate IL-22 production in *ex-vivo* derived macrophages.

3.3.2 Macrophages from peritoneum and spleen are not able to produce IL-22 in response to TLRs and Dectin-1 stimulation.

IL-22 production by *ex-vivo* macrophages in response to TLR stimulation was studied using spleen- and peritoneum-derived macrophages of C57/BL6 mice.

Splenic macrophages

Macrophages were purified from spleen of C57/BL6 mice (see materials and methods). They were positively selected for CD11b+ through MACS beads (94% positive for CD11b after FACS analysys). Splenic macrophages (2,5x10^6cells/ml) were then stimulated in vitro with LPS (5ug/ml), zymosan (10ug/ml), curdlan (10ug/ml) and also with mrIL23 (20ng/ml). We stimulated also total spenocytes and negative fraction (including Tcells, Bcells, NKcells and CD11c+ cells) as positive controls. After 20h supernatants were collected and tested for IL-22 production. No IL-22 production was detected in all three purified fractions in response to TLRs and Dectin-1 stimulation. Splenocytes and negative fraction stimulated with mrIL-23 produced 1000pg/ml and 600pg/ml of IL-22 respectively whereas no IL-22 was produced by CD11b+ (**fig.23a**). The cells were properly activated since high amount of TNFa was measured in response to TLR ligands stimulation (**fig.23b**).

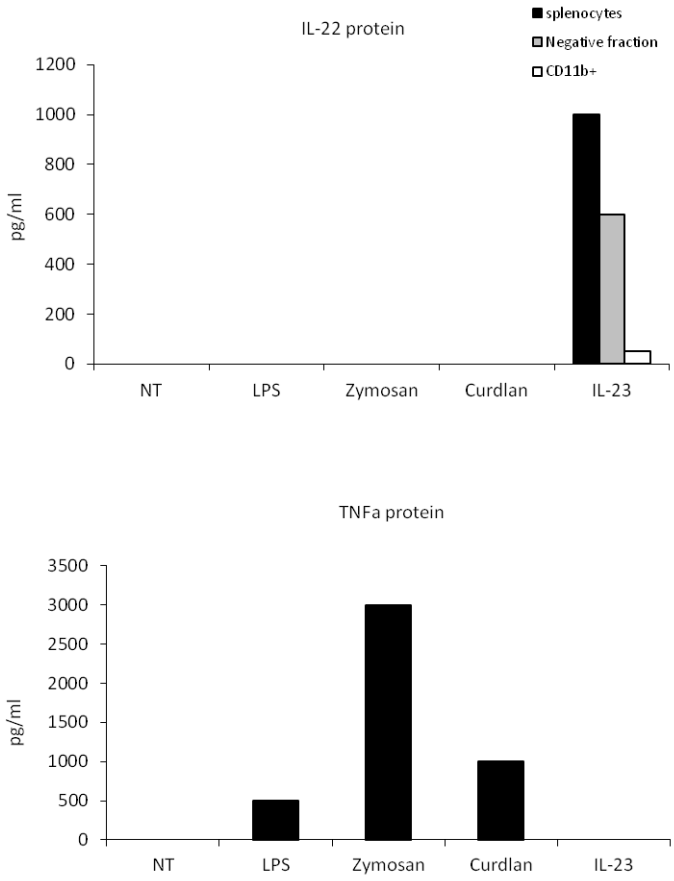
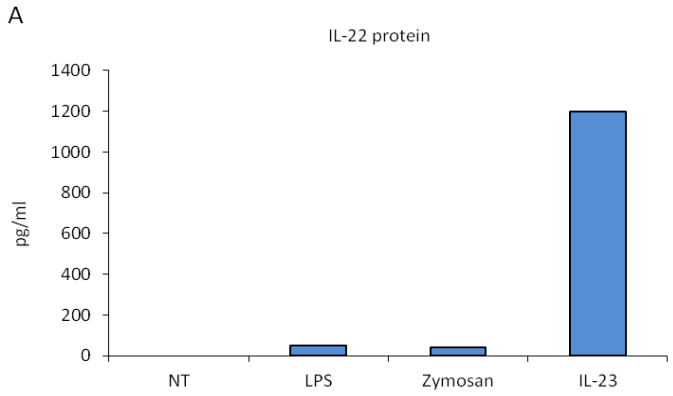


Fig.23: Spleen-derived macrophages are not able to produce IL-22 stimulation after TLR and Dectin-1 stimulation. Total splenocytes ($2x10^6$cells/ml), negative fraction ($2,5x10^6$cells/ml), CD11b+ cells ($2,5x10^6$ cells/ml) were stimulated with TLRs agonists (LPS 5ug/ml and Zym 10ug/ml), Curdlan 10ug/ml and mrIL-23 20ng/ml. After 20h supernatants were collected and tested for IL-22 production **(A-**top panel**)** and supernatants from CD11b+ cells were also tested for TNFa production **(B-**bottom panel**)**. Data show a representative of two independent experiments.

Peritoneal macrophages

Peritoneal macrophages were derived from peritoneum of C57/BL6 mice (see materials and methods). They were positively sorted for F4/80 by MoFlo cytometer with a purity of 99%. Macrophages (2,5x10^{6}cells/ml) were stimulated *in-vitro* with LPS (5ug/ml), zymosan (10ug/ml) and mrIL-23 (20ng/ml). After 20h supernatants were collected and tested for IL-22 and TNFa production. As shown in **fig.24a** no IL-22 production was measured in response to LPS and zymosan. On the contrary, 1200pg/ml of IL-22 were measured in response to IL-23 stimulation. F4/80+ macrophages produced high amount of TNFa in response to LPS, zymosan and mrIL-23 stimulation (**fig.24b).**



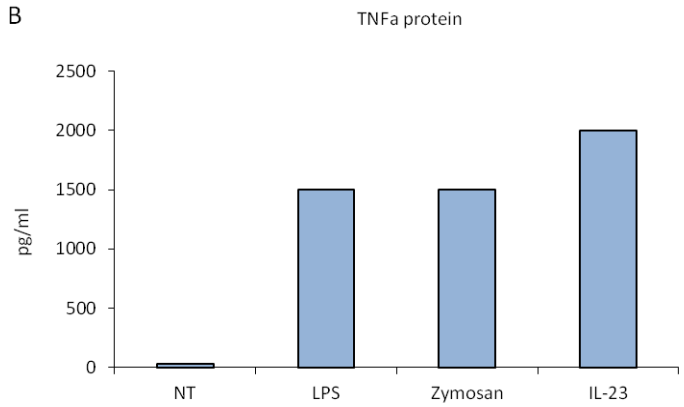


Fig.24: Peritoneum-derived macrophages (purity> 98%) are not able to produce IL-22 stimulation after TLR and Dectin-1 stimulation. F4/80+ cells ($2,5x10^6$ cells/ml) were stimulated with TLRs agonists (LPS 5ug/ml and Zym 10ug/ml) and IL-23 (20ng/ml). After 20h supernatants were collected and tested for IL-22 production **(A)** and supernatants from F4/80+ cells were also tested for TNFa production **(B)**.

From these experiments, we can conclude that different populations of *ex-vivo* macrophages are impaired in the production of IL-22 in response to PRR ligands. In addition, peritoneal macrophages seem to be able to produce IL-22 in response to IL-23. We are presently characterizing the F4/80 positive cells in the peritoneal population that is able to respond to IL-23 since it has been demonstrated that DCs sub-population also expresses F4/80.

3.4 IL-22 PRODUCTION BY *EX-VIVO* CD11c+ CELLS

Finally, we tested IL-22 production by *ex-vivo* CD11c+ cells. It previously was reported that intestinal CD11c+ (Zheng Y et al. 2008) and Peyer's patches DCs (De Luca et al. 2010) can produce IL-22 in response to IL-23. Lamina propria CD11c+ (Becker C et al. JEM 2009) was described to produce IL-22 in response to LPS and CpG stimulation. The stimuli which can elicit IL-22 production from *ex-vivo* CD11c+ DCs have not been completely clarified yet. Therefore, we tested whether CD11c+ stimulated with TLRs agonists and curdlan were able to produce IL-22. TLRs and Dectin-1 expression on CD11c+ DC was first tested. CD11c+ DCs were purified from spleen of C57/BL6 mice. Total splenocytes were positive selected for CD11c (see materials and methods). Cells ($2,5x10^6$) were collected in trizol reagent, total RNA was isolated and qRT-PCR was performed (see materials and methods). TLR1, TLR2, TLR6 and TLR9 were well expressed in CD11c+ DCs whereas TLR4 (Ct= 28) as well Dectin-1 (Ct= 27,4) were expressed al lower level (**fig.25)**. **Table 4** summarized the Ct values.

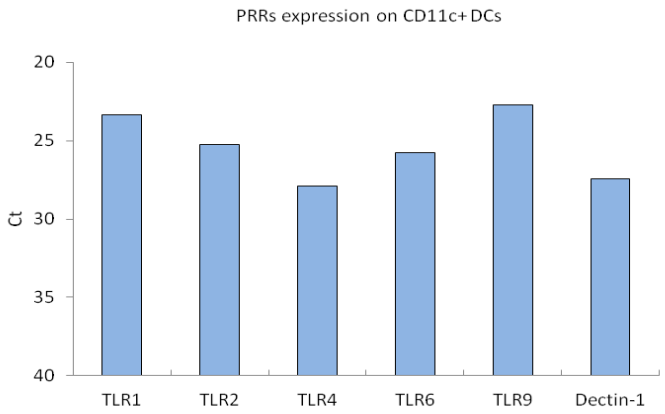


fig.25: TLR expression on CD11c+ cells. After MACS beads purification, cells (95% CD11c+) were collected in trizol qPCR was performed to obtained basal level of expression. The expression of each TLR is reported as Ct values.

Table4: Cycle threshold (Ct) value for each TLR and Dectin-1.

	CD11c+
TLR1	23,37
TLR2	25,26
TLR4	27,91
TLR6	25,78
TLR9	22,73
Dectin-1	27,41
18S	7,8

To verify IL-22 production, cells ($2{,}5x10^6$cells/ml) were then stimulated with LPS (10ug/ml), CpG (10ug/ml), zymosan (10ug/ml) and curdlan (10ug/ml). As positive control, CD11c+ cells were

stimulated with 20ng/ml of mrIL-23. After 20h supernatants were collected and tested for IL-22 production. CD11c+ cells produce great amount of IL-22 (1000pg/ml) in response to IL-23 and lower amounts in response to TLR agonists LPS (200pg/ml), CpG (300pg/ml) and zymosan (350pg/ml). No IL-22 production was detected in response to curdlan (**fig.26**).

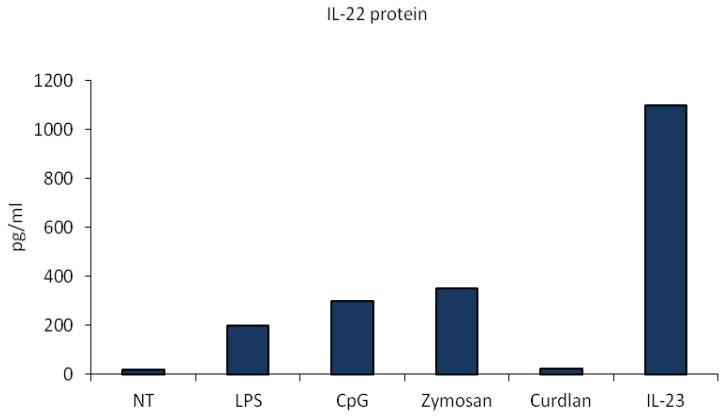


Fig.26: CD11c+ DC cells produce low levels of IL-22 in response to TLR stimulation. CD11c+ DCs (94% purity for CD11c and 98% purity of MHCII) were stimulated with TLRs agonists LPS (5ug/ml), CpG (5ug/ml) and zymosan (10ug/ml), with curdlan 10ug/ml and IL-23 (20ng/ml). After 20h supernatants were collected and tested for IL-22 production by ELISA. Data show a representative of two independent experiments.

From these results we concluded that *ex-vivo* CD11+ DCs are able to produce IL22 in response to TLR ligands although at lower levels compared with BMDC. Opposite to BMDC, CD11c+ cells are also able

to produced IL-22 in response to IL-23. Presently we are investigating which cell population within the CD11c+ cells is responsible for IL-22 production.

REFERENCES

Becker C, Pickert G, Neufert C, Ouyang W, Neurath MF **STAT3 links IL-22 signalling in intestinal epithelial cells to mucosal wound healing** *JEM* 1465-1471 (2009)

Cella M, Fuchs A, Vermi W, Facchetti F, Otero K and Colonna M **A human natural killer subset provides an innate source of IL-22 for mucosal immunity** *Nature*, 722-725 (2009)

De Luca A, Zelanti T, D'angelo C, Zaganella S *et al*. And Romani L **IL-22 defines a novel immune pathway of antifungal resistance.** *Mucosal Immunol*. 3, 361–373 (2010)

Murray PJ & Wynn TA **Obstacles and opportunities for understanding macrophage polarization.** *J. Leukoc. Biol.* **89**, 557–563 (2011)

Ouyang W et al. **Regulation and function of the IL-10 family cytokines in inflammation and disease** *Ann. Rev. Immunol*. 29, 71-109 (2011)

Stevens EA, Mezrich JD, Bradfield CA **The aryl hydrocarbon receptor: a perspective on potential roles in the immune system** *Immunology* 127, 299-311 (2009)

Stockinger B, Esser C, Ranung A The aryl hydrocarbon receptor in immunity Trends in immunology 9, 447-454 (2009)

Zheng Y, Valder PA, Danilenko DM, Hu Y et al. and Ouyang W **Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens**. *Nat. Med*. 14, 282–289 (2008)

Chapter 4: DISCUSSION

We studied the genes modulated in DCs after the interaction with gram+ bacteria by a functional genomic approach. Among the 1500 genes modulated, the mRNA up-regulation of the novel cytokine IL-22 was detected. Although it is known that CD4+ cells (Th17 subtype), NK cells (NK22 and NKp46+ subtypes), lymphoid tissue-inducer cells (LTi) (Sonnernberg GF et al. 2011) are able to produce IL-22 under several conditions and through the activation of different transcription factors, only few and controversial data are available on IL-22 production by DCs. In the first part of this study we characterized the stimuli able to induce IL-22 by BMDC. It was reported that peyer's patches (De Luca et al. 2010), intestinal (Zheng Y et al. 2008) and lamina propria (Becker C et al. 2009) CD11c+ DCs are able to secrete IL-22 in response to IL-23. In our study, we first provide the evidence that BMDC are able to produce IL-22 in response TLRs stimulation through the activation of a MyD88-dependent pathway. LPS, CpG and zymosan have been shown to induce the highest IL-22 production by BMDC through the activation of TLR4, TLR9 and TLR2-TLR6 respectively. We demonstrated that IL-22 production by TLR9 is completely MyD88-dependent since in MyD88 KO BMDC was completely abrogated. In response to LPS, we

measured a residual amount of IL-22 production, probably due to CD14 component of TLR4 complex. In response to zymosan we still measured a considerable amount of IL-22. This data suggested that BMDC are able to produce IL-22 through a MyD88-independent pathway. Zymosan is a complex stimulus derived from fungal wall digestions that is recognized by DCs through TLR2-TLR6, the c-type lectin Dectin-1 and the scavenger receptor CD36. We reported that Dectin-1 could induce IL-22 production through Syk kinase-dependent pathway after binding the specific ligand curdlan. This data showed that in BMDC, IL-22 is produced also by c-type lectin receptor stimulation. Recently, Gessner MA et al. have identified a role for IL-22 in early innate immune responsiveness to *A.fumigatus* lung infection (Gessner et al. 2012). Induction of IL-22 was significantly dependent on *A.fumigatus* recognition by β-glucan receptor Dectin-1. These results highlight that IL-22 could be produced by cells through different pathways. In contrast with data reported for CD11c+ DCs, we measured no IL-22 production in BMDC stimulated with IL-23. It was not reported that BMDC express IL-23 receptor. Therefore, the expression of IL-23R has been tested both by qRT-PCR and citofluorimetry but not definite data were obtained. Our study provided the evidence that IL-22 production in BMDC is directly dependent on TLRs and c-type lectin stimulation. Therefore, it was interesting to deeply investigate the intracellular signaling

which lead to IL-22 production in BMDC downstream the adaptor molecule MyD88 and Syk kinase, respectively. The transcription factors involved in IL-22 production have been studied only in Th17 cells. Ouyang W et al. previously reported that STAT3, aryl hydrocarbon receptor (AhR), RoRγT and Batf3 (member of AP-1 family) are required for IL-22 transcription in response to IL-23 stimulation (Ouyang W et al. 2011). Our study first demonstrated that in BMDC, JNK and ERK MAP kinases are required to be functional at the same time to induce IL-22 after stimulation with bacterial and fungi components. On the contrary, p38 MAP kinase is not required for IL-22 production through TLRs stimulation whereas we demonstrated that it is necessary in Syk kinase pathway. This data suggested that IL-22 transcription is finely regulated depending on the receptor stimulated. A recent study reported that in DCs curdlan induce an up-regulation of p38 activity stronger than LPS do (Huang G et al. 2012), suggesting that this kinase is required for AP-1 phosphorilation after fungal infections. Anyway, according to data reported for Th17 cells by Ouyang's group, we demonstrated that AP-1 is necessary for IL-22 transcription in BMDC although in response to PRR stimulation. We also confirmed that AhR is required for IL-22 transcription in BMDC stimulated with TLRs ligand and curdlan whereas in Th17 it is required after IL-23 stimulation (Ouyang et al 2011). On the contrary, RoR$\gamma\tau$ is not express in BMDC and it is not

required for IL-22 transcription as in Th17 cells. No information has been reported about the involvement of NF-kB in IL-22 regulations. According to data reported for several cytokines, we also confirmed that NF-kB is required for IL-22 production in BMDC stimulated with PRRs ligands. We provided the evidence that BMDC are able to produce IL-22 after TLRs and c-type lectin stimulation and that AP-1 and NFkB are required for gene transcription. It remains to examine whether these pathways are also present in *ex-vivo* cells or in *in-vivo* models. In contrast with the results obtained stimulating BMDC *in-vitro*, ex-vivo spleen CD11c+ DCs stimulated with LPS, CpG and zymosan release low amount of IL-22. These data could be explained by the fact that TLRs mRNA expression is different between BMDC and CD11c+ DCs. While TLR4 expression in DCs is 4-fold lower than in BMDC, TLR9 is expressed at the same level. This data could be explained by the presence of CD11c+ plasmacytoid DCs in CD11c+ DCs fraction that express high level of TLR9. Our results seem to confirm the data obtained by Becker's group that suggested CD11c+ DCs from lamina propria of mice produced low amount of IL-22 in response to CpG stimulation although it remain to be demonstrated that this response is not mediated by IL-23 since CD11c+ produce IL-22 upon stimulation with IL-23 (Becker C et al. 2009). Moreover, previous studies conducted in our lab, showed that CD11c+ DCs derived from spleen of mice injected i.v. with LPS produced IL-22

(data not shown). Therefore, all these data suggested that IL-22 produced by splenic DCs could have a role during systemic bacterial infection by directly stimulation of PRR on DCs surface. CD11c+ DCs from different tissues were reported to produce high level of IL-22 in response to IL-23 (De Luca et al 2010 and Zheng Y et al. 2008). According to this data, we showed that spleen derived CD11c+ DCs produce high level of IL-22 in response to IL-23. These results highlight the major role for IL-23 in IL-22 regulation in the tissues (Sonnenberg G, Fouser L and Artis D 2011). It is know that in the spleen there are different CD11c+ DCs subtypes. Therefore, we suggest that IL-23 is the major stimulus responsible for IL-22 production by CD11c+ DCs even if there could be a sub-population able to respond to TLRs stimulation. Presently, we are characterizing the CD11c+ DCs subpopulation that is able to respond to either IL-23 or TLRs. To confirm that IL-22 production is directly dependent on TLRs and not mediated by IL-23 produced by DCs after TLR triggering, experiments using IL-23 receptor KO mice will be performed.

Our results on IL-22 production by macrophages showed that BMMϕ are not able to produce IL-22 in response to TLRs ligands and curdlan stimulation. Nevertheless, we demonstrated that the expression of PRRs on BMMϕ is similar to BMDC. This data could be explained by the fact that macrophages could lack of some transcription factors necessary for IL-22 transcription downstream both TLRs and c-type

lectin receptors. We could also speculate that IL-22 is produced by BMDC and not by BMMϕ because of their distinct function. In fact, while macrophages are specialized in ingesting and processing foreign materials and recruit additional macrophages through inflammatory chemokines, DCs are able to distinguish between different pathogens and to elicit the appropriate immune response through secretion of several cytokines. We propose that DC not only influence T lymphocytes but could also crosstalk with non immune cells through IL-22. Our *in-vitro* studies were also confirmed in *ex-vivo* macrophages derived from spleen and peritoneum of C57/BL6 mice. We showed that these macrophages were not able to produce IL-22 in response to TLRs ligands and curdlan. On the contrary, peritoneal macrophages are able to produce IL-22 in response to IL-23. Presently we are characterizing the F4/80 cell population to determine which fraction is responsible for IL-22 production. In fact, it has been reported that certain DC subtype are able to express F4/80 antigen.

CONCLUSIONS and FUTURE PERSPECTIVES

In this study we characterized the stimuli and the intracellular signaling required for IL-22 production in DCs. IL-22, a novel cytokine that belongs to the IL-10 family, has been described to have both inflammatory and anti-inflammatory role. IL-22 does not seem to

influence directly immune cells since IL-22R1 chain of IL-22 heterodimeric receptor complex is present only in a range of non immune tissues (skin, liver, respiratory system and gastrointestinal tract). Therefore, IL-22 production by DCs highlighted a new role for antigen presenting cells in innate immunity. Through IL-22 DCs can directly cross talk with different non immune cells and instruct them to produce innate immunity mediators to protect the host against bacteria and fungi infections and also against liver injury. In autoimmune disease such as IBD, psoriasis and arthritis rheumatoid IL-22 has been showed to have a pro-inflammatory role and its up-regulation often correlates with disease exacerbation (Wolk K et al. 2006; Geboes et al. 2009; Ikeuchi et al. 2009). Recent data reported the involvement of Il-22 also in tumor development (Weber GF et al. 2006; Ziesche et al. 2007). In all these pathologies the fine balance of this cytokine is required and the understanding its expression and regulation is important for the development of IL-22 as a potential drug target.

REFERENCES

Becker C et al. **STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing** *JEM*, 1465-1471 (2009)

De Luca A, Zelanti T, D'angelo C, Zaganella S *et al*. And Romani L **IL-22 defines a novel immune pathway of antifungal resistance.** *Mucosal Immunol*. 3, 361–373 (2010)

Gessner MA, Werner JL, Lilly LM, Nelson MP, Metz AE, Dunaway CW, Chan YR, Ouyang W, Brown GD, Weaver CT, Steele C. **Dectin-1-dependent interleukin-22 contributes to early innate lung defense against Aspergillus fumigatus. *Infect Immun.* 80, 410-417 (2012)**

Geboes L, Dumoutier L, Kelchtermans H, Schurgers E, Mitera T, Renauld JC, Matthys P **Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice.** *Arthritis Rheum* 60 390–395 (2009)

Huang G, Wang Y, Vogel P and Chi H **Signaling via the kinase p38a programs DCs to drive Th17 differentiation and autoimmune disease inflammation.** *Nat. Imm.* 13, 152-159 (2012)

Ikeuchi H, Kuroiwa T, Hiramatsu N, et al. **Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine.** *Arthritis Rheum.,* 52, 1037 (2008)

Ouyang W et al. **Regulation and function of the IL-10 family cytokines in inflammation and disease** *Ann. Rev. Immunol*. 29, 71-109 (2011)

Sonnemberg GF, Fousier L and Artis D. **Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surface by IL-22** *Nature Immunol*. 5, 383-390 (2011)

Weber GF, Gaertner FC, Erl W, Janssen KP, Blechert B, Holzmann B, Weighardt H, Essler M **IL-22-mediated tumor growth reduction correlates with inhibition of ERK1/2 and AKT phosphorylation and induction of cell cycle arrest in the G2-M phase.** *J Immunol* 177, 8266–8272 (2006)

Ziesche E, Bachmann M, Kleinert H, Pfeilschifter J, Muhl H **The interleukin-22/STAT3 pathway potentiates expression of inducible nitric-oxide synthase in human colon carcinoma cells.** *J Biol Chem* 282, 16006–16015 (2007)

Wolk K, Witte E, Kunz S, Asadullah K and Sabat R **IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis.** *Eur. J. Immunol*. 36, 1309–1323 (2006)

Zheng Y, Valder PA, Danilenko DM, Hu Y et al. and Ouyang W **Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens**. *Nat. Med*. 14, 282–289 (2008)

Non vorrei solo ringraziare, ma dedicare un pensiero alle persone che mi sono da sempre vicine e a quelle che ho incrociato in questi anni. Un grosso grazie va alla mia famiglia, a “The Ivanhoe”, il mio babbo, per tutte le schiscette che mi ha preparato e per il solito messaggio “che tr prendi?” Per avermi fatto da taxista avanti e indietro dalla stazione. Grazie a “madre” che a suo modo mi ha sempre spronato a fare del mio meglio e ad ingoiare con furbizia i rospi senza prendermela troppo (per quanto possibile). Grazie alla Leo, perché lei è la sorella fuma fashion che mi presta i vestiti, mi fa ridere imitando Toretto alle rotonde e mi fa ascoltare la musica ruzza alle 7.30 del mattino. Grazie al nonno Carletto perchè a 90 anni è proprio argento vivo e la domenica a pranzo mi chiede sempre cosa faccio. Partendo dalla ricerca arriviamo, non so come, ad un excursus sulla storia italiana. Grazie a chi quotidianamente ha vissuto il laboratorio con me. Grazie alla Papagna mia, Angela, perché è quella che senza dubbio mi ha capito di più di tutti, che mi sgrida quando serve e gioisce con me dei successi. Grazie per il post lab, per le cene, le risate, per avermi resa più mangiona e “alcolista”. Grazie alla Towers, Anna, per avere condiviso con me 4 anni fatti di lavoro, di cambiamenti, di gioie e di ingiustizie, che dire…ci siamo fatte le ossa! Grazie alla CB, Caterina, perché i congressi non sarebbero stati la stessa cosa, per le risate che ci facciamo e perché reggi le mie battute. Il mio battesimo del volo l'ho fatto con te! Grazie alle ultime arrivate, alla Pemi, Samantha, perché è il controllo positivo che fa un sacco di domande e alla Ripa, Chiara, perché come stravolgi i nomi tu non lo fa nessuno.

Grazie anche alla Briga, Daniela, perché quando piove ed entra in studio mette l'ombrello dell'inter dentro il cestino dei rifiuti (brava!) e alla Vergans, Elena, perché è la fashion stylist. Grazie Marco per i topi e soprattutto sto ancora aspettando il saccottino! Grazie alla Franka per avermi lasciato la sua scrivania e fatto scoprire che si può vivere anche con un po' di disordine. Grazie ad Ivan per i consigli e a Tatiana per le chiacchierate. Grazie a Maria per avermi dato la possibilità di svolgere la tesi nel suo laboratorio. Grazie a Joe per le sterile cages e per il lavoro che fa, sempre con il sorriso. Grazie a tutti i miei amici, perché insieme siamo un bel gruppo e ci divertiamo, perchè mi ascoltate incuriositi quando vi racconto dei topolini. Il grazie più grande però va a Michele, che è sempre al mio fianco. Non importa cosa sono, dove sono, cosa faccio, lui c'è sempre. Mi vuole bene per come sono ed è fiero di me, questo è ciò che conta davvero.