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PhD

Program in Translational and Molecular Medicine

DIMET

GENE EXPRESSION PROFILING IN HOST-PATHOGEN INTERACTIONS AND IDENTIFICATION OF THE MOLECULAR MECHANISMS INVOLVED IN DENDRITIC CELLS ACTIVATION

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CHAPTER 1 - GENERAL INTRODUCTION

1.1. HOST-PATHOGEN INTERACTIONS AND FUNCTIONAL GENOMICS

The human body is constantly exposed to microbes that usually only colonize the host harmlessly, but that may cause infectious diseases, sometimes leading to fatal outcomes [1]. The human body has developed a variety of host defence mechanisms that comprise: physical, mechanical or biochemical barriers and two inducible immune defence systems. The innate and the adaptive immune systems are sequentially activated during activation and work cooperatively to eradicate the microbial agent.

The response of host cells to pathogenic microorganisms are among the most-well studied examples of cellular response to external stimuli. The advent of microarray technology has greatly expanded our ability to monitor changes in the abundance of transcripts. Over the past 10 years, more than 200 papers have been published that document changes in gene expression that occur on infection of 25 different host cell types with 26 different species of bacteria, 30 different types of viruses, as well as yeasts, protozoa and helminths [2, 3]. These data have shown that host cells undergo marked reprogramming of their transcriptome during infection, which indicates that this might be a central component of host defence. Recent studies suggested that there is an initial

common host response largely characterised by features of the innate immune response; however, specific host gene expression patterns that reflect differences between bacteria of different species can also be discerned [4]. A detail understanding of the common responses is likely to give insight into the basic molecular mechanisms governing these interactions, whereas gene that are regulated in a cell-specific or pathogen-specific matter will provide information about specific gene expression programs that regulate specific cells upon pathogen encounter [5].

Functional genomics involving genome-wide expression analysis is rapidly finding applications in clinical medicine [6]. This approach is changing the way we define health and disease, allowing an unbiased view of the global changes in gene expression that are occurring. The most common application of functional genomics has been direct towards class prediction and has yielded some impressive results in cancer research and tumor diagnosis. Because inflammation is a very complex process it can be a new field of functional genomics application as a mean to unravel the basic biological processes. This approach has been recently described by the group of Ginsburg, who showed that peripheral blood gene expression can be used to characterize host response to infection [79]. Using three human viral challenge cohorts for Rhinovirus (HRV), respiratory syncytial virus (RSV) and influenza A, they developed a robust blood mRNA expression signature that

classifies symptomatic human respiratory viral infection. Moreover this 'acute respiratory viral' bio-signature of disease could distinguish bacterial and viral respiratory infections. This study highlights the important concept that capturing the host response to pathogen exposure may serve as the basis for both diagnostic testing as well as a window into the fundamental biology of infection.

1.1.1 Microarray data analysis: classification algorithms

In past years a wide range of methods for microarray data analysis has evolved, ranging from simple fold change (FC) approaches to complex algorithms. In particular many classification methods are extensively used in microarray research.

Classification algorithms are used to discover new categories within a data set (class discovery; unsupervised classification) or assigned cases to a given category predefined (class prediction; supervised classification) [84].

Hierarchical cluster analysis that graphically presents results in a dendrogram is probably the most common unsupervised classification algorithm used. Another representation that can be used for class discovery is the Principal component analysis (PCA). This exploratory multivariate statistical technique reduces the dimensionality of the data set and identifies for each sample the most representative variables (principal component).

Supervised classification algorithms are typically developed on a 'training' data set and validated on an independent 'testing' set. One major limitation of supervised method is overfitting the predictor. This occurs when an excessively complex model with too many parameters is developed from a small sample of 'training' data. The model will fit the original data but may predict poorly for independent data [85]. Class prediction applications include identification of diagnostic markers of various states, outcomes, or response to treatment. Having a small set of probes is often desirable when considering diagnostic tools, therefore researchers try to identify the smallest possible set of genes that can still achieve good predictive performance. The list of genes selected by a classifier must be large enough to be robust against noise and small enough to be readily applied in a clinical setting.

Random Forest is a supervised classification algorithm developed by Leo Breiman that uses an ensemble of decision trees [76]. It has several characteristic that make it ideal for microarray data sets: it can handle thousands of variables, it can be used when there are many more variables than observations and it has good predictive performance even where most variables are noise.

1.2. DENDRITIC CELLS (DCS)

Many cell types participate to the innate immune response such as macrophages polymorphonuclear leucocytes and dendritic cells.

In 1973, Ralph Steinman and Zanvil Cohn reported the identification of a novel cell type in the peripheral lymphoid organs of mice [8]. As a result of their distinct morphology, the authors proposed to term these cells "dendritic" cells. DCs represent a special type of leukocytes able to alert the immune system for the presence of infections and responsible for the activation and control of both innate and adaptive immune responses [9,10]. After the encounter with a pathogen, DCs efficiently process antigens for their presentation in association with the major histocompatibility complex (MHC) molecules. However, before DCs can prime the adaptive immune response, they must complete a full maturation process that is initiated by direct exposure to microbial ligands. Interaction with pathogens results in a DCs activation state that leads to their migration to the T cell-area of lymph nodes where the antigen-specific cells of the adaptive immune response can be primed.

Early studies on DCs indicated they were of bone marrow origin and developing precursors migrated from the bone marrow to the blood. DCs have been found in heart, liver, thyroid, pancreas, bladder, kidney, ureter, gut, lungs and skin. Fully developed DCs

have also been observed in the circulatory networks of the body, including blood and afferent lymphatics. The last ones represent DCs emigrating from peripheral organs into lymphoid tissues. Within organs and lymphoid tissues, DCs can be subdivided into a number of sub-populations.

1.2.1 DC subtypes

There are different DC subtypes, each with a particular location and specialized function in the immune system. Different DC subtypes have been described according to the surface expression of particular markers and tissue distribution [11-13]. In mouse, immature DCs are characterized by the expression of CD11c (the integrin alpha X-chain), low levels of the costimulatory molecules CD80 and CD86, and low levels of MHC class II; these molecules can be up-regulated at the cell surface upon activation [14].

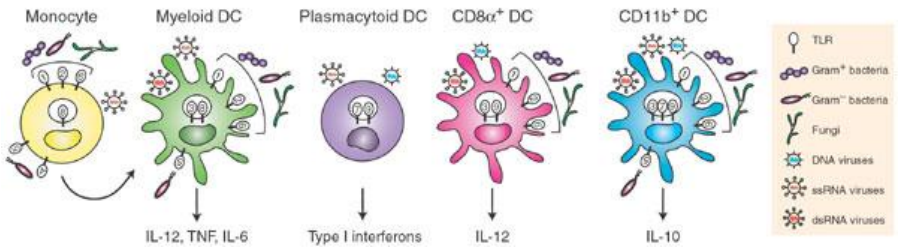

Fig.1 DC sub-populations, the microorganisms that they recognize and the main cytokines that they produce.

Interestingly DCs can also express the T cell markers CD4 and CD8. Another molecule that has been used to identify mouse DC

subtypes is the CD11b; using CD4, CD8 and CD11b markers, five distinct DC classes have been identified. Three DC classes have been observed in the spleen, $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$. The $CD8^-$ DCs reside mostly in the marginal zone while the $CD8^+$ are mainly located in the T cell area [13]. Murine DCs expressing CD8α were initially defined as a lymphoid subtype, while DCs lacking CD8α were defined as myeloid DCs. However $CD8\alpha^+$ and $CD8\alpha^-$ have now been derived from both common myeloid and common lymphoid progenitors and it has been demonstrated that $CD8\alpha^-$ can develop into $CD8\alpha^+$ DCs *in vivo* [15]. Another DC population has been identified in all lymph nodes. These lymph node DCs are $CD4^-CD8^-CD11b^+$ and express also moderate levels of the scavenger receptor CD205 [16]. Finally, in skin-draining lymph nodes, an additional DC subtype has been found, it expresses high levels of Langerin, a molecule typically produced by Langerhans cells (LC), an immature population of DCs located in the skin [14]. Recently a sixth DC sub-population, called interferon (IFN)-producing plasmacytoid DCs (pDCs), has been identified in mouse blood and lymph nodes [17].

In humans, DC subtypes are less characterized. Human DC do not express CD8 and in spleen and tonsils DCs differentially positive for CD11b, CD11c and CD4 have been identified [13]. Most of the information on human DC subtypes, and their possible origin derive from *in vitro* studies. Blood monocytes are the most

commonly used precursors to generate DCs in culture. Following incubation with inflammatory products, these DCs can reach the mature phenotype showing high levels of MHC class II and costimulatory molecules [18]. The second human DC subtype are the IFN-producing plasmacytoid DCs [19]. They are phenotypically characterized as $CD11c^{-}CD45RA^{+}CD123^{+}$.

1.2.2 How DCs sense microbes

The receptor repertoire of DCs includes a broad family of pattern recognition receptors (PRR) that recognize microbial components that, whether pathogenic or not, serve as ligands to alert the immune response. One of the main PRR family, called Toll like receptors (TLR), senses a distinct repertoire of conserved molecules expressed by fungi, viruses, bacteria and protozoa. The TLR field was initiated by J. Hoffmann and colleagues in 1996, thanks to the observation that Toll-mutant flies were highly susceptible to fungal infection [20,21]. Mammalian homologues were identified initially on the basis of their homology to the *Drosophila* Toll protein, the developmental protein required for antifungal immune responses in adult fly [22]. In 1998 one mammalian homologue named TLR4 was positionally identified by B. Beutler and colleagues as the LPS receptor, encoded by the *Lps* locus, known to be required in mice to respond to Gram-negative bacteria [21]. Since then, many other mammalian TLR have been identified: 10 in humans (TLR1-10) and 12 in mice (TLR1-9,

TLR10-13). TLR1, TLR2, TLR4, TLR5 and TLR6 are located at the cell surface; in contrast, TLR3, TLR7, TLR8 and TLR9, all of that are involved in the recognition of microbial nucleic-acid-like structures, are not present on the cell surface [24-26] but rather in the endoplasmic reticulum (ER). TLR molecules are characterized by an extracellular domain with a leucin-rich repeats (LRR) and a cytoplasmic domain (TIR domain) similar to that of IL-1R family [27,28]. Functional analysis of each mammalian TLR member has revealed that they recognize different microbial ligands, such as LPS, lipoproteins, peptydoglycan, bacterial CpG DNA, single and double-stranded RNA, bacterial flagellin, bacterial profilin. All these ligands are mostly conserved among pathogens but are not found in mammal cells.

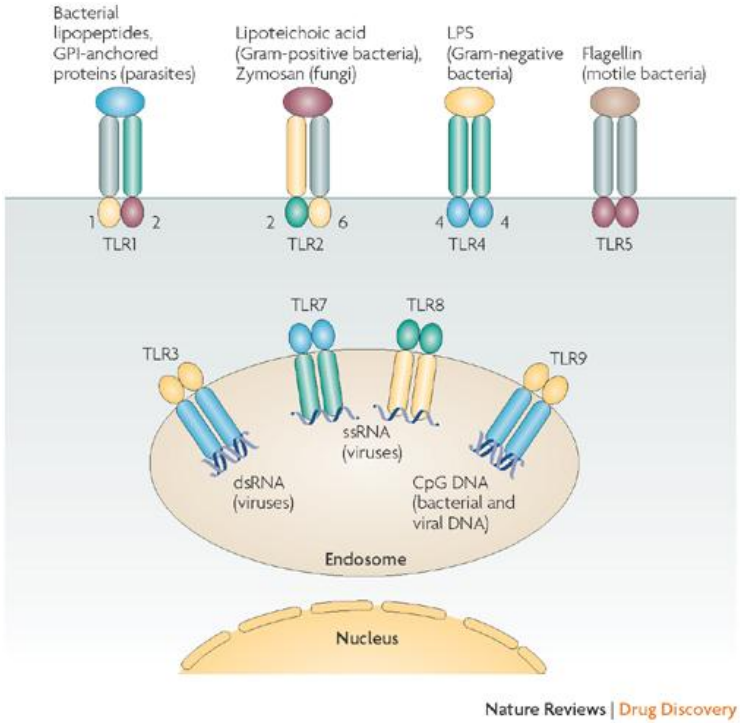

Fig.2 TLRs and their ligands.

The association of TLRs with MyD88, which is utilized by all TLRs except TLR3, recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK 1. Activated IRAK-4 phosphorylates IRAK-1, which subsequently associates with tumour necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 activates transforming growth factor beta activated kinase (TAK1) and TAK-binding proteins 1 (TAB1) and 2 (TAB2). This complex in turn activates the transcription factor (NFkB) and activator protein 1 (AP-1) through the canonical IkB kinase (IKK) complex and the mitogen activated protein kinase pathway, respectively. The kinase activity

of the IKK complex is modulated by its IKKg subunit, the transcription factor NFkB modulator (NEMO). The activation of IKK complex by TAK1 leads to the phosphorilation of IkB, resulting in the translocation of NFkB into the nucleus, which induces the expression of inflammatory cytokines including TNF-α, IL-6 and IL-1β. Activation of the Myd88-dependent pathway results also in the activation of MAPKs such as p38, ERK and JNK, which leads to the activation of AP-1. In addition to this MyD88 dependent pathway some TLR family members have unique signalling pathway involved in type I IFN production [29]. TLR3 and TLR4, which recognize dsRNA and LPS derived from gram negative bacterial infection respectively, can induce interferons through the adaptor molecule TRIF [30,31]. In cells derived from TRIF knock out mice, TLR3 and TLR4-dependent IFNα and subsequent IFN response are defective. Unlike TLR3, which can directly recruit TRIF, TLR4 uses the adaptor TRAM to transduce signal from TRIF [30,33]. TRIF interacts with both receptor-interacting protein 1 (RIP1) and TRAF6 and cooperatively with these two proteins activates NFkB to induce expression of proinflammatory cytokines. Furthermore, TRIF activates also TRAF family members-associated NFkB activator (TANK) binding kinase 1 (TBK1) via TRAF3. In turn, TBK1 phosporylates directly two transcription factors, IRF-3 and IRF-7

allowing them to translocate into the nucleus and induce IFN-α and IFN inducible genes.

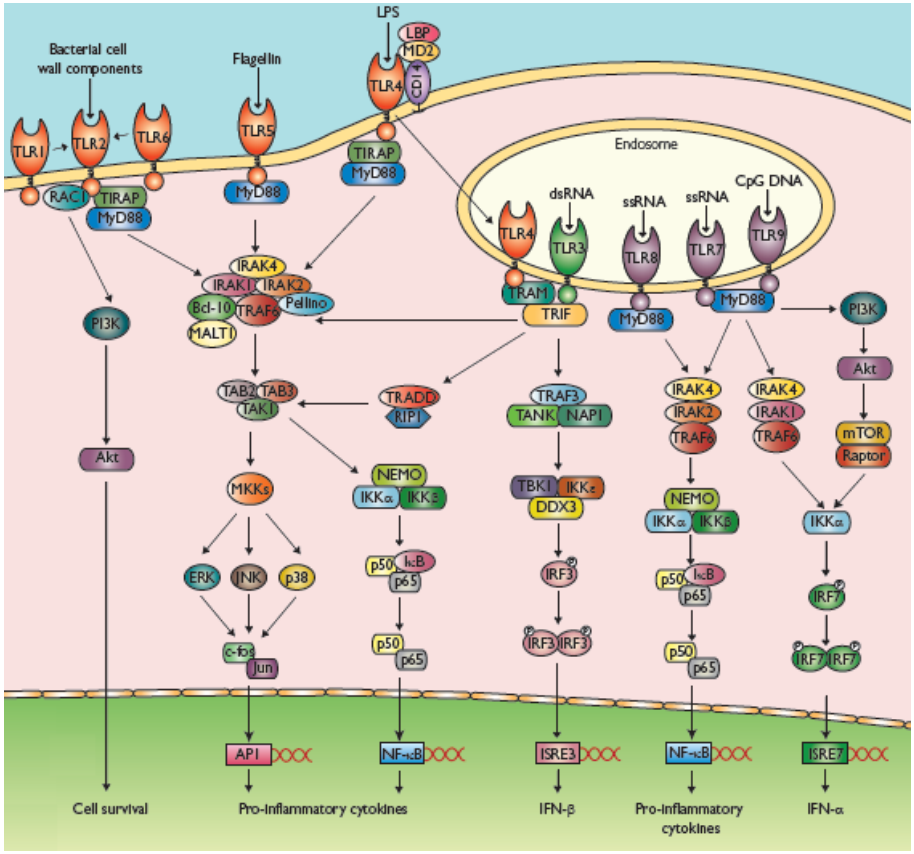

Fig.3 TLR signalling. Ligand binding to their cognate TLR induces two signalling pathways, the Myd88-dependent and the Myd88-independent pathways.

Even if TLR are the most studied receptors family, DCs express many other receptors that are fundamental for their responses. Many pathogens reach the cell cytoplasm so that hosts have had to evolve mechanisms to detect intracellular pathogens. Not all TLRs are able to activate IRF3 and a TLR able to target the IRF3 pathway in response to gram-positive bacterial infection has not been identified so far. The activation of the TBK1/IRF3 pathway from within the cell through cytoplasmic sensor is therefore very important. For virus two proteins have been identified, called retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated protein-5 (MDA-5) [30,31]. Both proteins contain a DEXD/H BOX RNA HELICASE that binds viral dsRNA and a structure that resemble a caspase recruitment domain (CARD) for signal transduction. Binding of dsRNA stimulates CARD to signal to the IFNα promoter through IRF3 [35]. RIG-1 and MDA-5 use the mitochondrial membrane bound protein IFNβ promoter stimulator 1 (IPS1) as a specific adaptor [36]. Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 are other intracellular receptors that contain a nucleotide binding oligomerization domain and bind in the cytosol the core structure of bacterial peptydoglycans. The NOD protein family has been shown to act as intracellular receptor of bacterial lipids [37] and recently the role of NOD1 in producing chemokines and recruiting immune cells has been demonstrated [38].

Another receptors family, important for DC immune responses, is the C-type lectin family. This family of receptors recognizes a wide range of carbohydrate structures. These receptors include the MMR, Dectin-1, DEC-205, DC-SIGN, BDCA-2, and Langerin. They all possess at least one carbohydrate recognition domain and bind sugars in a variety of secondary and tertiary structures [39]. Most of these molecules are involved in receptor-mediated phagocytosis or endocytosis of microbes.

1.2.3 DCs interactions with other immune cells

Upon activation, DCs can secrete a diversified panel of chemokines that attract different cell types at different times of the immune response. They also express a unique set of costimulatory molecules which permit the activation of naïve T cells. Through the cytokines they secrete as well as the surface molecules they express, DCs can affect the functions of T cells, B cells, natural killer (NK) cells and natural killer T (NKT) cells. Cytokines may represent products of DC and/or induce the differentiation of immature DCs into mature DCs [40].

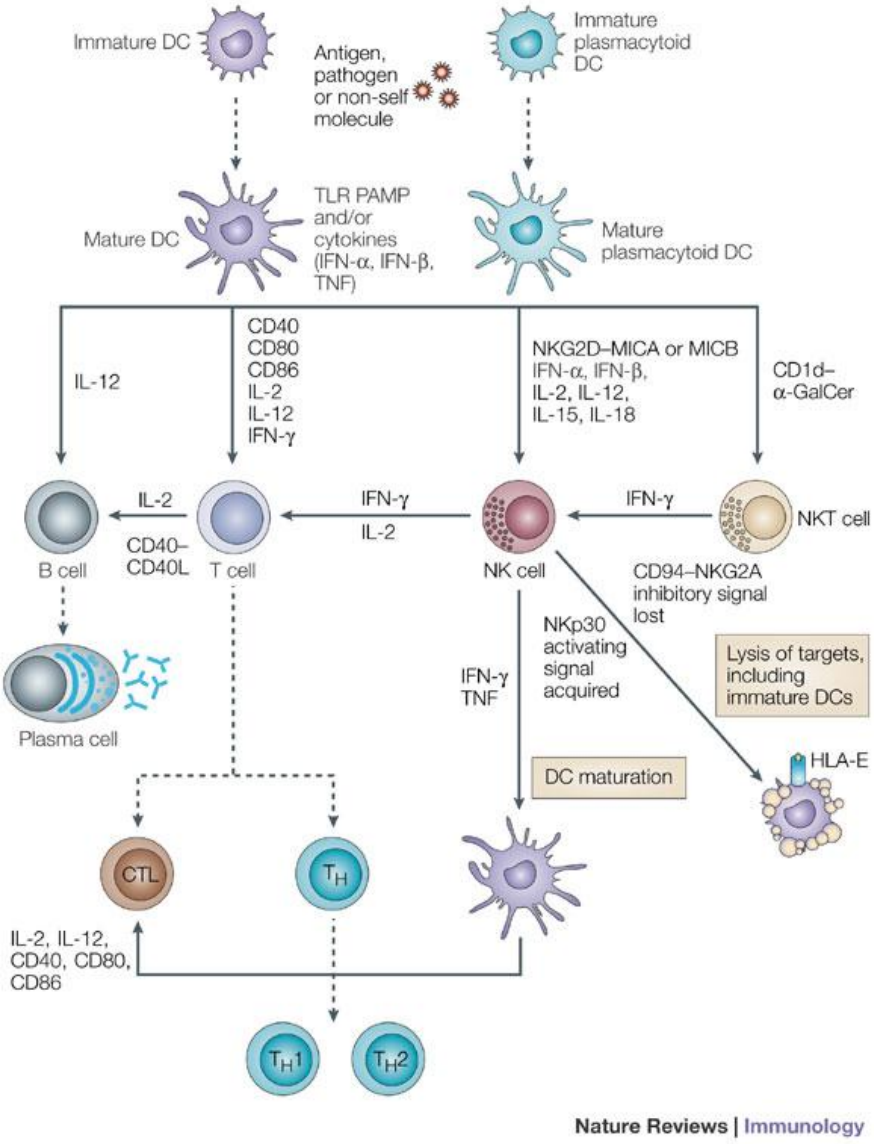

Fig.4 DCs interactions with T cells, B cells, NK cells and NKT cells through the surface molecule they express and the cytokines they express.

DCs process antigens and present them in the context of peptide–MHC complexes to T cells. In the presence of appropriate signals

from co-stimulatory molecules (CD40, CD80 and CD86) and cytokines (interferon-g (IFN-g), interleukin-2 (IL-2) and IL-12), DCs induce the activation of naïve T cells and the generation of $CD8^+$cytotoxic T lymphocytes (CTLs) or polarization of $CD4^+$ T helper (Th) into different cell lineages: Th1, Th2, Th17 or T regulatory (T_{reg}). The effector cell types are characterized by their synthesis of specific cytokines and their immuno-regulatory functions, as indicated on the figure 5. The differentiation along different lineages involves different cytokines and the activation of distinct signalling cascades and transcription factors that result in the induction of additional cyto/chemokines and cyto/chemokine receptors, which may be part of positive and negative feedback loops.

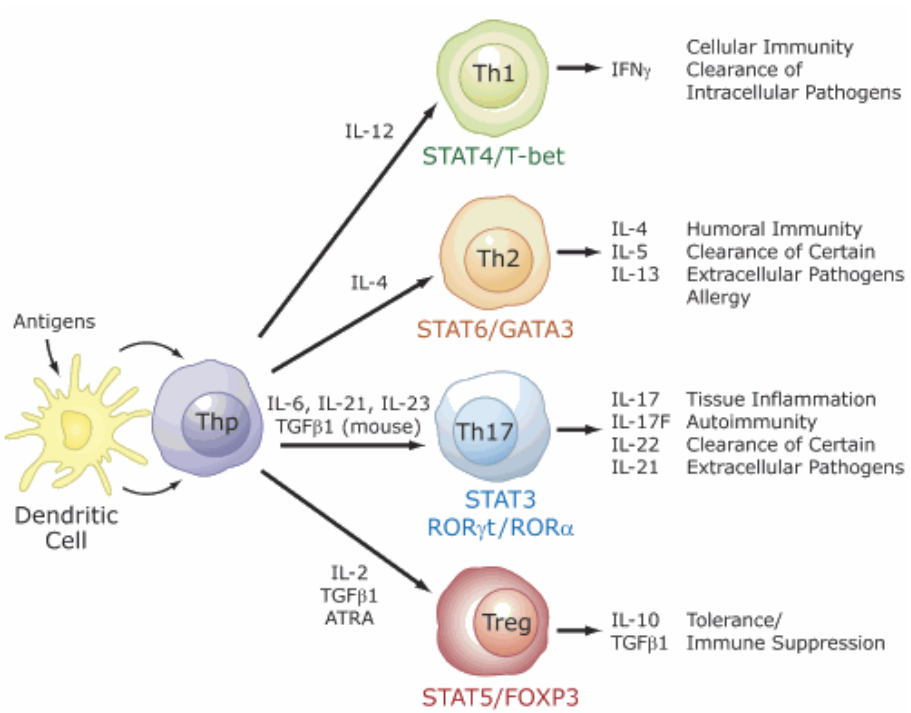

Fig.5 T helper cell differentiation. Prompted by different types of cytokines produced by DCs and other sources, undifferentiated T helper cells can develop into the Th1, Th2, Th17 or Treg lineage.

DCs can also affect the functions of B cells, generally by complex interactions that involve signals originating from T cells (CD40–CD40 ligand (CD40L) interactions and IL-2 secretion) that have interacted with activated DCs. Interplay between DCs and NKT cells also occurs. Functional NKT-cell ligands, such as α-galactosylceramide (α-GalCer), are presented to NKT cells by CD1d molecules at the cell surface of DCs. Selective targeting of α-GalCer to DCs induces a stronger and more prolonged NKT-cell response. Reciprocally, NKT cells can promote the maturation of

DCs. DCs affect the functions of NK cells and can induce their activation through pathways that require both cell–cell contact (NKG2D (NK group 2, member D)–MICA (MHC-class-I-polypeptide-related sequence A) and/or MICB) and cytokines (IFN-α, IFN-β, IL-2, IL-12, IL-15 and IL-18). DC-derived signals elicit NK-cell-mediated cytolysis, as well as NK-cell-mediated cytokine production. Reciprocally, NK cells can affect DC functions by being involved in DC maturation and DC elimination.

1.2.4 DCs and pathogens

Several studies have shown that DCs can internalize latex beads and zymosan [41-44], but also apoptotic bodies [45] as well as microbes such as *Saccaromyces cerevisiae*, *Corynebacterium parvum*, *Staphylococcus aureus* [42], *Leishmania spp* [47] and *Borrelia burgdorferi* [44]. The ability of DCs to phagocytose particulates or bacteria is optimal when DCs are immature, whereas this capacity is reduced, but not abolished, in mature DCs [46]. Upon attachment, DCs engulf the microorganism by actively surrounding it with pseudopodia. This process is facilitated by Fc-type and complement-type receptor-mediated endocytosis. The movement of the pseudopodia in activated DCs involves actin-binding proteins, and it can be blocked by the drug cytochalasin D, which stops the polymerization of actin and inhibits phagocytosis. The rearrangement of the cytoskeleton, associated with DCs motility, involves the depolymerization of the actin [49]. Once a bacterium

has been fully internalized into the phagosome, fusion of the phagosome with other intracellular vacuoles or granules takes place. Processing of bacterial molecules for antigen presentation occurs in lysosomes following their fusion with phagosomes. This process may take several hours, as antigen presentation of bacterial antigens is not observed earlier than 6 hours following infection [50]. Cytokine and chemokines genes as well as IFN-inducible genes are differentially expressed in the first few hours, whereas at later time points, apoptotic and anti-apoptotic genes as well as genes involved in the T cell activation function are induced.

Stimuli that induce DCs activation and maturation increase the efficiency of antigen processing for both class I and class II pathways and the half-life of peptide+MHC complexes at the cell surface that otherwise are rapidly internalized and recycled [50-52].

TNFα, IL1β and IL-6 are readily detected in DCs infected with either Gram-positive or Gram-negative bacteria. Biologically active p70 IL-12 is produced by myeloid mouse DCs only in a very small amount after bacterial encounter, as compared to human monocyte derived DCs [53]. Recently, using a global gene expression analysis applied to the study of mouse DCs maturation, it has been demonstrated that bacteria or bacterial cell products induce DCs to produce IL-2 transiently at early time points after stimulation. The first observation was made for DCs stimulated with Gram-negative bacteria [54]. Afterward, it was shown that many different

microbial stimuli are able to induce IL-2 production by DCs while none of the inflammatory cytokine tested was able to elicit IL-2 production by DCs [55], confirming the hypothesis that DCs are extremely plastic and can modulate their responses depending on the nature of the stimulus. DC-derived IL-2 has been associated with regulatory functions in adaptive immune responses in mice, but an intriguing possibility is that this IL-2 produced at early time points after DCs activation might also have a role in activating cells of the innate immune system [54]. Consistent with acquisition of co-stimulatory activity during maturation is the up-regulation of CD86 and CD40 molecules. The up-regulation of the co-stimulatory molecules and the coordinated translocation of MHC molecules at the cell surface are essential molecular events for the subsequent antigen presentation and activation of both $CD4^+$ and $CD8^+$ T cells.

A microarray study, where human monocyte-derived DCs were exposed to a diverse set of organisms and components (a gram-*E.coli* and its cells wall component lipopolysaccharide, a fungus *C.albicans* and yeast cell wall-derived mannan, an RNA virus influenza A and double strand RNA), showed a large-scale change in gene expression demonstrating that DCs are able to undergo a marked transformation in their cellular phenotype [5]. They identified a core DC response to any pathogen but also specific genes regulated by each pathogen. These differential responses

demonstrate that human monocyte-derived DCs are flexible in their responses and may even exhibit a diversity of responses similar to that of the different DC subtypes.

1.3. INTERLEUKIN 22 (IL-22)

In 2000, Renauld's group described a secreted a-helical protein that they had discovered during a search for differentially expressed genes in interleukin (IL)-9-stimulated murine BW5147 T-lymphoma cells [56]. Due to its primary structure similarity to the anti-inflammatory and immunosuppressive cytokine IL-10, this protein was named IL-TIF for 'IL-10 related T cell-derived inducible factor'. Shortly thereafter, the human counterpart was identified in two studies and the cytokine was quickly renamed IL-22 [57-58].

IL-22 is a member of the IL-10 cytokine family that comprises IL-10, IL-19, IL-20, IL-24, IL-26, IL-28a, IL-28b and IL-29. The last three members show amino acid (aa) sequences similarity to the type I interferons. All IL-10 family members exert their biological effects via heterodimeric receptor complexes composed of a type I receptor chain (R1) and a type 2 receptor chain (R2). These receptor chains belong to the cytokine receptor family class 2 (CRF2). As a convention the R1 chains are defined as the receptor chains with the longer intracellular moiety able to bind signal transducers and activators of transcription (STAT) molecules.

Some of the IL-10 family members share receptor chains or even whole receptor complexes.

	IL-10R1	IL-20R1	IL-22R1	IL-28R1
IL-10R2	IL-10	IL-26	IL-22	IL-28 IL-29
IL-20R2	?	IL-19 IL-20 IL-24	IL-20 IL-24	?

Fig.6 Combination of receptor chains in receptor complexes enabling the effects of the IL-10 family members.

Despite the structural relation and the use of similar or partly identical receptors the novel IL-10 family members do not seem to be functionally related to the IL-10. IL-22 in particular represents a novel type of immune mediator [59].

1.3.1 The IL-22 encoding gene

The human *IL22* gene is located on the longer arm of chromosome 12, on 12q15, approximately 52 kbp and 99 kbp upstream from the *IL26* and *IFNG* locus respectively, and it has the same transcriptional orientation as these two genes (minus strand). IL22 gene comprises five exons.

The mouse IL22 gene was mapped to chromosome 10D2, also near the IFNG locus. It comprises six exons, in effect the counterpart to the human exon 1 is split into two exons. The IL22

gene (*Iltifa*) was found to be duplicated *(Iltifb*) in some mouse strains such as C57BL/6, 129 and FVB. The second copy of the gene is very similar to the first one except for a deletion including 603 bp from the putative promoter and the first non-coding exon and some variations within both the introns and the hexons. The biological significance of the *IL22* gene duplication is unclear. So far no evidence for the expression of *Iltifb* could be obtained [60].

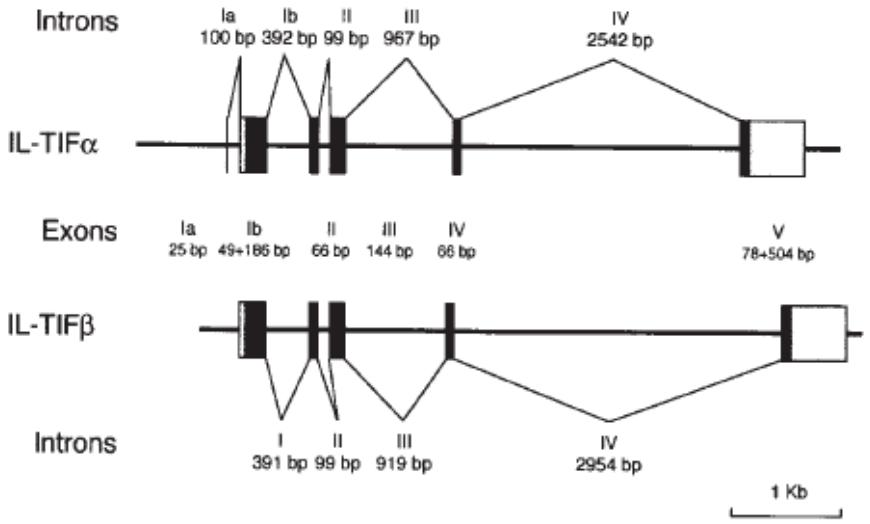

Fig.7 Exon-intron organization of mouse *Iltifa* and *Iltifb*.

1.3.2 The structure of secreted IL-22

The human *IL22* and mouse *IL22* genes each encode proteins of 179 aa in length, which are almost identical to the length of the IL-10 immature protein (178aa). After splitting the signal peptide, IL-22 is secreted as a protein of 146 aa. Amino acid sequence

alignment revealed an identity with IL-10 of 22% in the mouse and 25% in the human. Despite this relatively weak aa sequence similarities there is a strong resemblance with respect to the secondary structure (a-helical structure).

1.3.3 The cellular sources of IL-22

The first data indicated that IL-22 was expressed only in activated human T cells and, at lower levels, in activated human NK cells [61]. Among the T cells subset, IL-22 was preferentially expressed by the $CD4^+$ fraction. No expression of IL-22 was found in macrophages, immature or mature DCs that were generated *in vitro* from human blood monocytes. Three years ago Fouser's group showed that IL-22 was expressed at higher amount by Th17 cells than by Th1 or Th2 [66]. They also found that IL-23 enhances the expansion of IL-22 producing cells during Th17 differentiation.

In 2007 Wu and Ouyang showed that IL-22 produced by Th17 mediates the acanthosis induced by IL-23 [67]. Flavell's group discovered that IL-22 expressing Th17 provided protection during acute liver inflammation [68].

In 2008 Kolls' group confirmed that IL-22 expression *in vivo* is regulated by IL-23 and showed that IL-22 produced by Th17 is involved in mucosal host defence against *Klebsiella pneumoniae* [69]. At the some time Sauvage and Ouyang demonstrated that during *Citrobacter rodentium* infection IL-22 was induced in the colon of wild-type mice. Moreover they showed that IL-22 pathway is still

active in Rag2 knockout mice; therefore T and B cells are not the sources of IL-22 in their model. They suggested that DCs may be one of the sources of IL-22 but they could not exclude other innate immune cells, especially NK cells [70].

In 2009 NKT cells (T cells subset characterized by both T cell and NK cell markers that recognize glycolipid antigens), derived from murine splenocytes, were found to produce IL-22 upon stimulation with a-galactosyl ceramide [71]. In a very recent report, a distinct NK cell population ($NKp46^{+}$) was described as an innate source of IL-22 in human and mouse lymphoid tissue [72-73]. In parallel, human fetal lymphoid tissue inducer (LTi) cells and mouse adult LTi-like cells in secondary lymphoid tissues were found to release IL-22 that mediates host defence against extracellular pathogens [74]. After LPS intraperitoneal injection in mice IL-22 expression was found in various organs, including the gut, liver, stomach, kidney, lungs, heart, thymus and spleen. The exact cellular sources of IL-22 in these mice were not clear [62]. Very recently Becker's group using an experimental mouse model of colitis demonstrated that lamina propria cells isolated from colon produce IL-22 upon stimulation with LPS, CpG and lipoteichoic acid [75]. Because performing immunostaining of colon cross sections they observed a striking colocalization of CD11c and IL-22, they suggest that DCs may be an important source of IL-22 in colitis.

1.3.4 The IL-22 receptor

The IL-22 receptor complex is composed of IL-22R1 and IL-10R2. A comparison of human and mouse IL-22R1 nt and aa sequences revealed that their identity is 78% at the nt level and 72% at the aa level. IL-10R2 has been shown to be ubiquitously expressed; therefore the expression of the IL-22R1 chain should determine whether a cell is an IL-22 target or not. Sabat's group demonstrated that neither resting nor stimulated human immune cells (macrophages, DCs, B, T, NK cells) express IL-22R1 [61]. The IL-10R2 was highly expressed and regulated in these cells. In contrast to immune cells, expression of IL-22R1 was detected in a range of human tissues including skin, small intestine, colon, liver, lung, kidney and pancreas. In line with the results obtained with blood immune cell subpopulations, no IL-22R1 was detected in bone marrow, PBMCs, thymus and spleen. Sabat's group also demonstrated that IL-22 does not influence the release of different cytokines by the tested immune cells.

In line with the data obtained in human system, IL-22R1 mRNA has been reported to be expressed in mice in liver, kidney, and lung tissue, but not in the brain, spleen or heart [62].

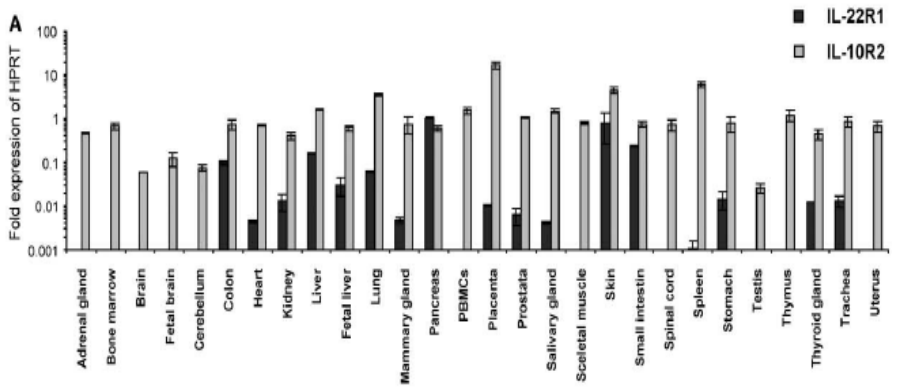

Fig.8 Expression of functional IL-22 receptor complex in different human tissues. Gene expression was anlyzed by qPCR relative to HPRT expression.

1.3.5 The IL-22 binding protein

In addition to the cell surface associated IL-22 receptor complex, there is a secreted, single-chain IL-22 binding receptor named IL-22 binding protein (IL-22BP). This protein corresponds to the isolated extracellular moieties of integral membrane CRF2 receptors. IL-22BP is the only known soluble receptor within the CRF2 family encoded by an independent gene. The gene encoding human IL-22BP is located on chromosome 6q24.1 approximately 152 kbp upstream from the locus encoding IL-20R1, and approximately 24 kbp downstream from the *IFNGR1* locus, and has the same transcriptional orientation of these two genes (minus strand).

The mouse counterpart of human IL-22BP is encoded by a gene located on chromosome 10A3, also near the *Ifngr* and the *Il20r1* loci. In human there are three different splice variants, only variant 2 is soluble receptor for IL-22, the role of variant 1 and 3 remains still open. In mice there is only one splice variant corresponding to human variant 2 [59].

Expression of IL-22BP mRNA (variant 2) has been demonstrated in some human tissues: the female reproductive system (placenta and breast), the lymphatic organs (thymus, spleen, lymph nodes), the gastrointestinal system (stomach and colon), the lungs and the skin. In mice the highest IL-22BP constitutive expression has been found in lymph nodes and a weaker expression in the lungs, spleen and thymus. No expression could be detected in kidney, liver and heart [64].

The affinity of IL-22 to IL-22BP appears to be at least four times higher than that of IL-22 to IL-22R1. Although IL-22BP inhibits IL-22 effects *in vitro*, its role under physiological conditions *in vivo* may be different. Soluble receptor may positively regulate cytokine action, they may prolong cytokine half-life and might be necessary for their systemic effects facilitating cytokine arrival in remote parts of the body. Sabat's group showed, in colon of patient affected by Crohn's disease (CD), a strongly increase in IL-22 expression and at the same time a decrease in IL-22BP expression. This comports an alteration in the IL-22BP/IL-22 ratio and so IL-22 produced in

the colon should have local as well as systemic effect. These data suggested a mainly inhibitory role of IL-22BP *in vivo* too [65].

1.3.6 The IL-22 induced signal transduction

IL-22 and its receptor complex induce activation of JAK1 (Janus Kinase-1) and TYK2 (non-receptor protein Tyrosine Kinase-2) but not JAK2. The IL-10R2 chain recruits TYK2 and then activates MAPK (Mitogen Activated Protein Kinase) p38, ERK and JNK. The IL-22R1 recruits JAK1 that further causes phosphorylation of STAT1 (Signal Transducers and Activators of Transcription Factor-1), STAT3, and STAT5 on tyrosine residues, which then migrate to the nucleus, where they activate the transcription of target genes [59] .

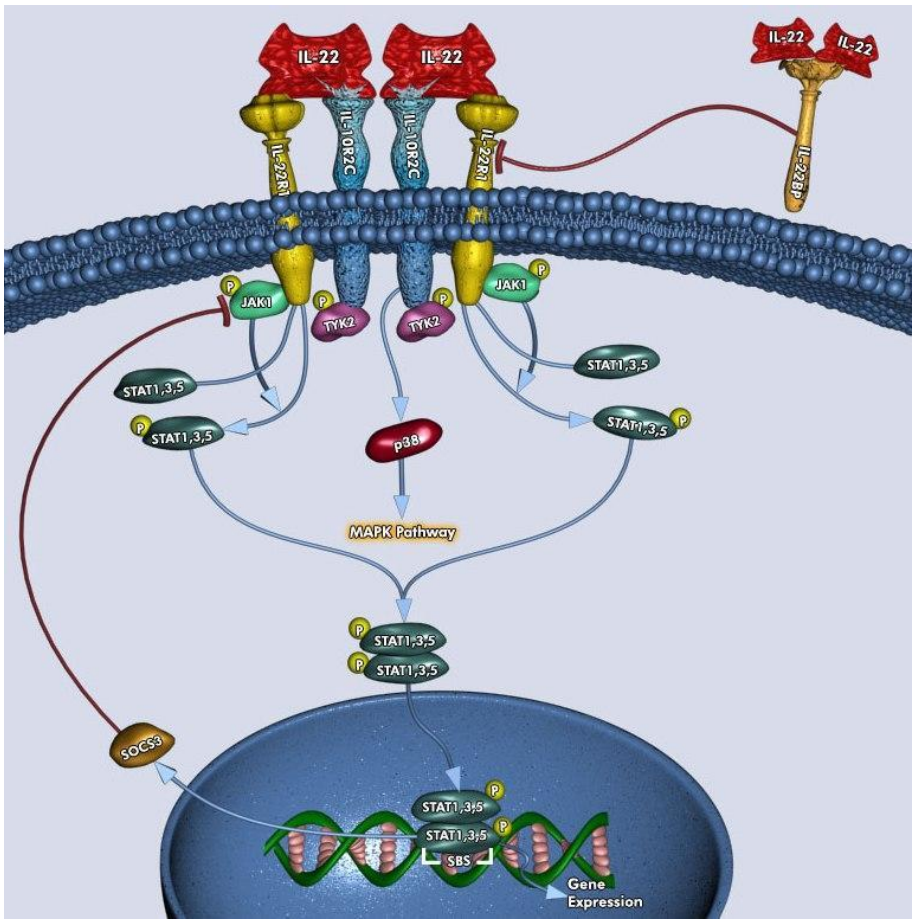

Fig.9 IL-22 signalling pathway.

1.3.7 Biological effects of IL-22

IL-22 does not serve as the means of communications between leukocytes given that it does not seem to directly influence immune cells. The expression of IL-22R1 indicates that the most important target cells of IL-22 are cells of the digestive tract, skin, lungs, pancreas, liver and kidney (see section **1.3.4**). The currently

published data indicate for this cytokine both anti-inflammatory and pro-inflammatory roles.

IL-22 induces up regulation of different antimicrobial peptides such as b-defensin 2 (hBD2), hBD3, S100A7 (psoriasin), S100A8 (calgranulin A) and S100A9 (calgranulin B) in human keratinocytes [61,66]. IL-22-induced hBD2 expression was also detected in primary hepatocytes. Gurney's group showed induction of PAP1 mRNA (member of the Reg family protein) in pancreas of mice after IL-22 injection. PAP1 plays an important role in the protection against tissue injury. In hepatocytes IL-22 stimulation up regulates the expression of the acute phase reactants serum amyloid A, a1-antichimotripsin and haptoglobin and induces Lipopolysaccharide-Binding protein (LBP). Acute phase proteins function to restore the homeostasis after a challenge and have a protective role during liver injury. At constitutive concentrations LBP is essential for the host to sense bacteria and to stimulate innate immunity cells to initiate an appropriate inflammatory response. High concentrations of LBP neutralize bacterial components and therefore limit their immune stimulatory activity [65]. High LBP levels induced by T cell-derived IL-22 in the later phase of the immune reaction might promote the clearance of pathogens. Injection of recombinant IL-22 prior to treatment with different inflammatory molecules reduced the amount of hepatocyte damage in the liver. In addition, injection of IL-22

neutralizing antibodies, immediately after the inflammatory molecules, exacerbated hepatic injury [77]. Moreover IL-22 deficient mice have more extensive liver damage in response to Concanavalin A-mediated hepatitis than wild-type mice. It seems that the increase liver damage observed may be due to increased susceptibility of hepatocytes to the destructive immune response [68].

Since LPS injection induces IL-22 gene expression in mouse tissues, it is very likely that IL-22 plays a role in the immune response to bacterial infection. Recently three different groups showed a protective role for IL-22 during gram$^-$ infections (*C.rodentium*, *K.pneumoniae*, *S.enterica*). Sauvage and Ouyang showed that IL-22 is indispensable for host survival during the early phase of infection with *C.rodentium* [70]. In fact infection of IL-22 knockout mice with this gram$^-$ bacterium results in increased intestinal epithelial damage, systemic bacterial burden and mortality. IL-22 is required for the direct induction of the Reg family of antimicrobial proteins in colonic epithelial cells. IL-22 was found to be important also in maintaining host defence against *K.pneumonia*. [69]. IL-22 increased lung epithelial cell proliferation and increased transepithelial resistance to injury. Specifically IL-22 up regulates several host defence genes, including *Lcn2* (lipocalin-2), *Cxcl1*, *Cxcl5* and *Cxcl9* in primary mouse tracheal epithelial cells; Lipocalin-2 is a protein known to have a function in innate

immune response through its ability to sequester iron from bacterial organisms such as *E.coli*. Finally Alber's group demonstrated that IL-22 is required for protection against hepatocyte necrosis during salmonellosis [86]. Recently IL-22 produced by particular NK cell subsets and Lymphoid tissue inducer-like cells (LTi-like cells) was proposed as critical mediator in mucosal host defence [83].

Increased levels of IL-22 have been demonstrated in patients with different autoimmune diseases (rheumatoid arthritis, psoriasis, Crohn's disease) and the levels were correlated with disease severity indicating the IL-22-importance in the pathogenesis of these diseases [67,80,82]. IL-22 is able to induce a skin phenotype resembling human lesional psoriatic skin. In fact Ouyang's group demonstrated that IL-22 produced by Th17 mediates IL-23-induced dermal inflammation and acanthosis [67]. Nojima's group proposed a pro-inflammatory role for IL-22 in rheumatoid arthritis (RA). They suggested that IL-22 produced by synovial fibroblasts and macrophages, may promote inflammatory responses in RA synovial fibroblast proliferation and production of chemokines [82]. IL-22 is strongly expressed in the inflamed intestine and in the blood of Crohn's disease patients. IL-22 signalling in intestinal epithelial cell resulted in increased expression of pro-inflammatory cytokines and in increased cell migration [80]. In addition high expression levels of IL-22 were detected in primary tumor tissue,

malignant pleural effusion and serum of patients with non-small cell lung carcinoma (NSCLC). Treatment with IL-22R1 blocking antibodies or IL-22 RNA interference (RNAi) *in vitro* resulted in the apoptosis of lung cancer cells. Furthermore, administration of IL-22-RNAi plasmid inhibited the growth of human lung cancer cell in BALB/c nude mice [78].

1.4. SCOPE OF THE THESIS

The scope of this thesis was to investigate the molecular mechanisms involved in dendritic cells (DCs) activation in response to bacteria and TLR ligands. Host-pathogen interactions are inevitably complex. Therefore, we applied a functional genomics approach to study the genes that are induced in DCs during activation. Microarray experiments generate a huge amount of data that can be analysed in several ways in order to obtain different kinds of information.

In the first part of this thesis we considered microarray data of dendritic cells stimulated with different microorganisms and molecules in order to identify inflammatory biomarkers that can describe, at molecular level, the dendritic cells status in terms of inflammation. The outcome is reported in chapter 2 as submitted to PlosONE.

In the second part we deeply analyzed dendritic cells-bacteria interactions in order to find out specific molecular mechanisms

induced in DCs upon infections. During microarray data analysis we showed a strong modulation of a new cytokine: IL-22. Therefore, we focused our attention on studying IL-22 production and its regulation in DCs *in vitro* and *in vivo*. The obtained results are described and discussed in chapter 3.

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CHAPTER 2 - GENE EXPRESSION PROFILES IDENTIFY INFLAMMATORY SIGNATURES IN DENDRITIC CELLS

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2.1. ABSTRACT

Dendritic cells (DCs) constitute a heterogeneous group of antigen-presenting leukocytes important in activation of both innate and adaptive immunity. We studied the gene expression patterns of DCs incubated with reagents inducing their activation or inhibition. Total RNA was isolated from DCs and gene expression profiling was performed with oligonucleotide microarrays. Using a supervised learning algorithm based on Random Forest, we generated a molecular signature of inflammation from a training set of 77 samples. We then validated this molecular signature in a testing set of 38 samples. Supervised analysis identified a set of 44 genes that distinguished very accurately between inflammatory and non inflammatory samples. The diagnostic performance of the signature genes was assessed against an independent set of samples, by qRT-PCR. Our findings suggest that the gene expression signature of DCs can provide a molecular classification for use in the selection of anti-inflammatory or adjuvant molecules with specific effects on DC activity.

2.2. INTRODUCTION

Dendritic cells (DCs) are bone marrow-derived cells present in all lymphoid and non lymphoid organs. They play a role in immune regulation, inducing tolerance and preventing autoimmunity, inducing anti-tumor immunity and protecting against infectious agents. DCs constitute a heterogeneous group of cells with different origins (both myeloid and lymphoid), anatomic locations, cell surface phenotypes and functions, but all these cells are efficient antigen-presenting cells in the induction of naive, memory, effector and regulatory T-cell responses [1,2,3].

DCs have several pattern recognition receptors (such as Toll-like receptors). During infection or inflammation, these receptors interact with microbe-associated molecules (such as LPS, bacterial DNA and double-stranded RNA), resulting in DC activation [4,5]. Endogenous TLR ligands are also released in conditions of inflammation, such as cell injury, and induce similar activation programs [6,7]. These programs affect various DC functions, such as migration to draining lymph nodes for antigen presentation, costimulation and the production of a specific cytokine profile determining the type of T-cell response to be developed. This process is known as maturation and it enables DCs to initiate and direct the acquired immune system (B and T cells) and, ultimately, to mount an antigen (Ag)-specific response [8].

Global transcriptomic analysis has recently been shown to be a

powerful approach yielding new insight into the biology of specific cell subsets or tissues, by providing information about their specific gene expression programs [9,10,11,12]. Moreover, the analysis of genome-wide expression profiles is now a widely used technique for the identification of diagnostic markers of various disease states, outcomes, or responses to treatment [13,14,15,16,17]. Markers are selected by scoring each individual gene on the basis of the extent to which its expression pattern discriminates between different classes of disease or between cases and controls. The disease status of new patients is predicted with classifiers tuned to the expression levels of the marker genes. One potential problem with expression-based classification is that cellular heterogeneity within tissues and genetic heterogeneity between samples may decrease the discriminatory power of individual genes in complex diseases [18,19]. As DCs are involved in various diseases involving the immune system, from inflammatory diseases to cancer, the identification of molecular markers in DCs specific to inflammation is of potential clinical and pharmaceutical value. In this study, we identified a genetic signature of inflammation in mouse DCs. We chose to study mice, because they are widely used in models of many immunological diseases. These findings may lead to the identification of a prospective signature of inflammation and should increase our understanding of the biological processes underlying chronic inflammatory diseases.

2.3. RESULTS

2.3.1 Sample selection and processing

In total, 115 samples were analyzed to develop a prognostic molecular assay of DC activation. Seventy-seven arrays were used for the training set and 38 were used for the testing set. We analyzed different samples of the DC line D1 [20] treated with inflammatory stimuli including bacteria (*Listeria monocytogenes*), helminths (*Schistosoma* eggs), protozoa (*Leishmania* promastigotes) and TLR ligands (LPS, poly I:C and zymosan) and samples of D1 cells treated with dexamethasone, *Schistosoma* SLA and *Leishmania* amastigotes, all of which are known to down-regulate the inflammatory response [21,22]. The microarray data used were either generated in this study or derived from previous experiments [21,23,24]. **Table 1** describes the sample dataset used in this study. We amplified total RNA and hybridized it to an Affymetrix mouse MG-U74Av2 GeneChip oligonucleotide microarray containing 12,488 probe sets. The resulting microarray signal intensities for all 12,488 probe sets were normalized and the background was subtracted.

Table1. Characteristic of the Microarray Data Set used

Cell type	Stimulus	N° of Arrays	Class ass
D1	None	14	Non Inflamm
D1	DEX	6	Non Inflamm
D1	Leishmania Ama	8	Non Inflamm
D1	Shistosoma SLA	8	Non Inflamm
D1	CpG	10	Inflamm
D1	Leishmania Pro	8	Inflamm
D1	Listeria EGD	20	Inflamm
D1	LPS	8	Inflamm
D1	PAM3CYS	10	Inflamm
D1	PolyI:C	10	Inflamm
D1	Shistosoma Eggs	8	Inflamm
D1	Zymosan	15	Inflamm

Total Arrays in class 1 (Non inflammatory)	36
Total Arrays in class 2 (Inflammatory)	79

2.3.2 Multivariate analysis reveals the existence of an inflammatory state for dendritic cells

Principal component analysis (PCA) makes it possible to visualize correlations in datasets by compressing information into a small number of dimensions. PCA was carried out on the data for DCs treated with stimuli inducing activation via various receptors, including the Toll-like receptors. Projection of the samples onto a plane corresponding to the first two dimensions derived from PCA resulted in a clear separation along the first dimension (**Fig.1**). Control and experimental samples treated with anti-inflammatory stimuli were projected toward positive values of the first dimension and samples with signs of activation were projected toward negative values. The PCA data suggest that DCs in different

functional states could be separated on the bases of differences in inflammatory stimulation.

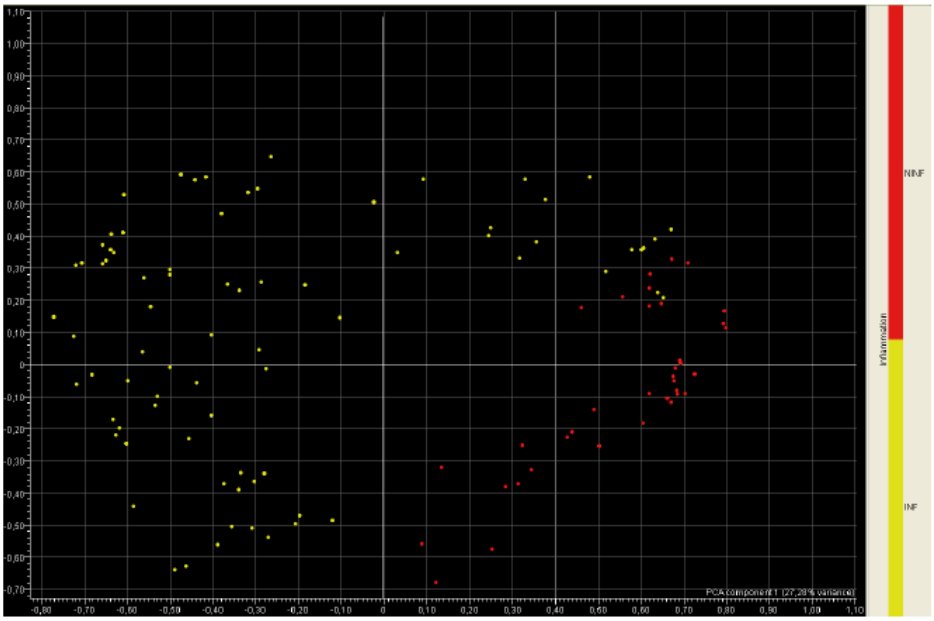
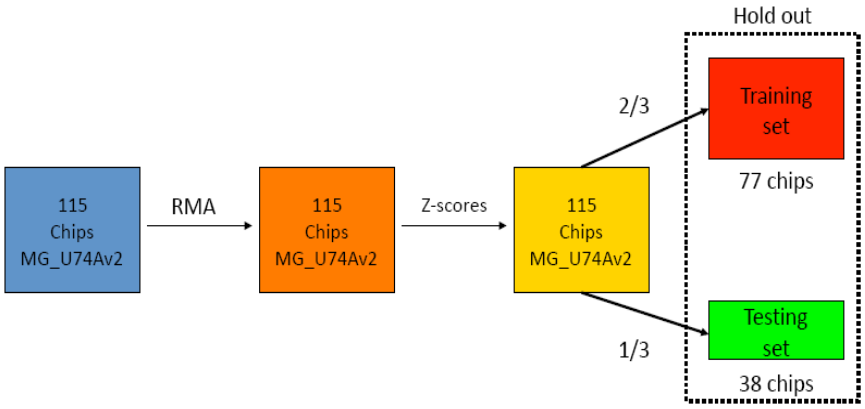


Fig.1 PCA score plot.
Seventy-nine "inflammatory" observations (red) and 36 "non inflammatory" observations (yellow) used to generate and test the random forest model. Genome-wide gene expression data were collected with DNA-microarray technology and the normalized hybridization signals were analyzed by PCA. A score plot with the first and second principal component axes is shown. Inflamed samples are mostly projected toward negative values of the first PC axis, whereas samples from controls and non inflamed samples are projected toward positive values.

2.3.3 Data analysis strategy for the selection of classifier genes

We carried out a step-wise analysis to determine whether it was possible to select a gene expression signature of inflammation: (a) an expression index was calculated with RMA [25]; (b) sample classification: genes capable of discriminating between the two groups were identified by comparing groups of samples in the inflammatory group with those in the non inflammatory group (training set); (c) independent validation of classifier genes: the genes selected were used to classify an independent group of samples (testing set); (d) validation of the genetic signature by quantitative RT-PCR (qRT-PCR) on independent DCs samples prepared with different stimuli. The procedure used for the selection and preparation of microarrays is shown in **supplementary Fig.1**.



Supplementary Fig.1 Training and test sets, as used for the development of a classifier for the predictive analysis of microarrays. All samples were chosen based on the stimulus used for DC activation. The classifier, the random forest, was developed on the basis of two thirds of the samples (77 samples) and was then validated on the remaining one third (38 samples).

2.3.4 Transcriptional signatures discriminate between inflammatory and steady-state cellular phenotypes

Raw intensity values from microarray hybridization were normalized with the robust multiarray average in the R-package for statistical computing (available at www.R-project.org). A random forest classification model was built from a training set (50 observations in conditions of inflammation, 27 observations in non inflammatory conditions) obtained from the genome-wide gene expression analysis of DCs incubated with different stimuli. All the samples were assigned to training or testing sets: two thirds of the samples (n=77) were assigned to the training sets, the remaining

third being assigned to testing sets (n=38; supplementary Figure 1). The results obtained for the untreated samples and those treated with non inflammatory stimuli were very similar and these two groups of samples were therefore considered to belong to the same class (data not shown). This approach resulted in the identification of 54 genes distinguishing accurately between the two classes of samples, as demonstrated by analysis with the testing set (data not shown; **Table 2**). The molecular signature was illustrated with a heat map based on Euclidean distance (**Fig.2**). We identified 18 genes down-regulated by inflammation and 36 up-regulated by inflammation. Unsupervised clustering analysis confirmed the robustness of the set of genes identified, with very clear distinction between samples treated with and without inflammatory stimuli (**Fig.2**). Gene Ontology was used to classify the modulated genes in terms of function. The genes selected encoded proteins involved in the immune response (47%), cell death (31%), cell differentiation (44%) and metabolic process (42%), as shown in **Fig.3**.

The down-regulated genes were found to encode proteins involved in the biological pathways of cell division (CCNB2, Kif20a, CDC20), lipid metabolic process (DALG, Elovl6, Sgpp1), response to stimuli (Hdac5, H2-DM, IL1rl1, IL18r1, LTA4H), and metabolic processes (Txndc16, Tep1, Eif4ebp2, MAN2B1, Znrf2). Two of these genes, Tm7sf3 and TSPAN8, could not be functionally

classified. The up-regulated genes encoded proteins involved in the immune response (CD40, CD86, CCL22, IL1b, Sqstm1, TAP1, TNFRSF1B, IL-6, IL12b, IFIT1, CCR7, IRF8, Isg15, Nfkbia, Nfkbiz, Stat5a), cell death (Daxx, Gadd45B, Serpinb9, TRAF1, TNFRSF1B), cytokine metabolic processes (IL1b, Nfkb1, IL12b, IL-6, Stat5a) and cell differentiation (CD40, Clic4, CDKN1A, IRF8, SKIL).

Table2. Fifty-four Genes able to predict Inflammatory Signatures in DC

Affymetrix ID	Gene Title	Gene Symbol
100423_F_AT	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 5A	STAT5A
100540_AT	LEUKOTRIENE A4 HYDROLASE	LTA4H
100584_AT	ANNEXIN A4	ANXA4
100588_AT	PROTEASOME (PROSOME, MACROPAIN) 28 SUBUNIT, BETA	PSME2
100779_AT	INTERLEUKIN 12B	IL12B
100981_AT	INTERFERON-INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 1	IFIT1
101072_AT	EUKARYOTIC TRANSLATION INITIATION FACTOR 4E BINDING PROTEIN 2	EIF4EBP2
101144_AT	INTERLEUKIN 18 RECEPTOR 1	IL18R1
101995_AT	SEQUESTOSOME 1	SQSTM1
102218_AT	INTERLEUKIN 6	IL6
102310_AT	CHEMOKINE (C-C MOTIF) LIGAND 22	CCL22
102779_AT	GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE 45 BETA	GADD45B
102831_S_AT	CD86 ANTIGEN	CD86
103035_AT	TRANSPORTER 1, ATP-BINDING CASSETTE, SUB-FAMILY B (MDR/TAP)	TAP1
103040_AT	CD83 ANTIGEN	CD83
103254_AT	TRAF TYPE ZINC FINGER DOMAIN CONTAINING 1	TRAFD1
103402_AT	TRANSMEMBRANE 7 SUPERFAMILY MEMBER 3	TM7SF3
103486_AT	INTERLEUKIN 1 BETA	IL1B
103494_AT	TETRASPANIN 8	TSPAN8
103665_AT	ELOVL FAMILY MEMBER 6, ELONGATION OF LONG CHAIN FATTY ACIDS (YEAST)	ELOVL6
104149_AT	NUCLEAR FACTOR OF KAPPA LIGHT CHAIN GENE ENHANCER IN B-CELLS INHIBITOR, ALPHA	NFKBIA
104376_AT	HISTONE DEACETYLASE 5	HDAC5
104418_AT	ZINC AND RING FINGER 2	ZNRF2
104443_AT	CHEMOKINE (C-C MOTIF) RECEPTOR 7	CCR7
160501_AT	KINESIN FAMILY MEMBER 20A	KIF20A
160608_AT	RAB20, MEMBER RAS ONCOGENE FAMILY	RAB20
161005_AT	RIKEN CDNA 5730420B22 GENE	TXNDC16
92962_AT	CD40 ANTIGEN	CD40
98092_AT	HISTOCOMPATIBILITY 2, CLASS II, LOCUS DMA	H2-DMA
98367_AT	TELOMERASE ASSOCIATED PROTEIN 1	TEP1
98448_AT	RIKEN CDNA E330036I19 GENE	DAGLB
94186_AT	TNF RECEPTOR-ASSOCIATED FACTOR 1	TRAF1
94256_AT	CHLORIDE INTRACELLULAR CHANNEL 4 (MITOCHONDRIAL)	CLIC4
94294_AT	CYCLIN B2	CCNB2
94501_AT	SPHINGOSINE-1-PHOSPHATE PHOSPHATASE 1	SGPP1
94752_S_AT	SKI-LIKE	SKIL
94814_AT	GUANINE NUCLEOTIDE BINDING PROTEIN, ALPHA INHIBITING 3	GNAI3
94881_AT	CYCLIN-DEPENDENT KINASE INHIBITOR 1A (P21)	CDKN1A
94928_AT	TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY, MEMBER 1B	TNFRSF1B
95024_AT	UBIQUITIN SPECIFIC PEPTIDASE 18	USP18
96120_AT	DNAJ (HSP40) HOMOLOG, SUBFAMILY B, MEMBER 6	DNAJB6
96125_AT	FAS DEATH DOMAIN-ASSOCIATED PROTEIN	DAXX
96319_AT	RIKEN CDNA 2310042N09 GENE	CDC20
96515_AT	INTERLEUKIN 4 INDUCED 1	IL4I1
96752_AT	INTERCELLULAR ADHESION MOLECULE	ICAM1
96935_AT	PDZK1 INTERACTING PROTEIN 1	PDZK1IP1
98002_AT	INTERFERON REGULATORY FACTOR 8	IRF8
98405_AT	SERINE (OR CYSTEINE) PEPTIDASE INHIBITOR, CLADE B, MEMBER 9	SERPINB9
98427_S_AT	NUCLEAR FACTOR OF KAPPA LIGHT CHAIN GENE ENHANCER IN B-CELLS 1, P105	NFKB1
98500_AT	INTERLEUKIN 1 RECEPTOR-LIKE 1	IL1RL1
98822_AT	INTERFERON, ALPHA-INDUCIBLE PROTEIN	ISG15
98988_AT	NUCLEAR FACTOR OF KAPPA LIGHT POLYPEPTIDE GENE ENHANCER IN B-CELLS INHIBITOR, ZETA	NFKBIZ
99562_AT	MANNOSIDASE 2, ALPHA B1	MAN2B1
99982_AT	NUCLEAR FACTOR OF KAPPA LIGHT CHAIN GENE ENHANCER IN B-CELLS INHIBITOR, BETA	NFKBIB

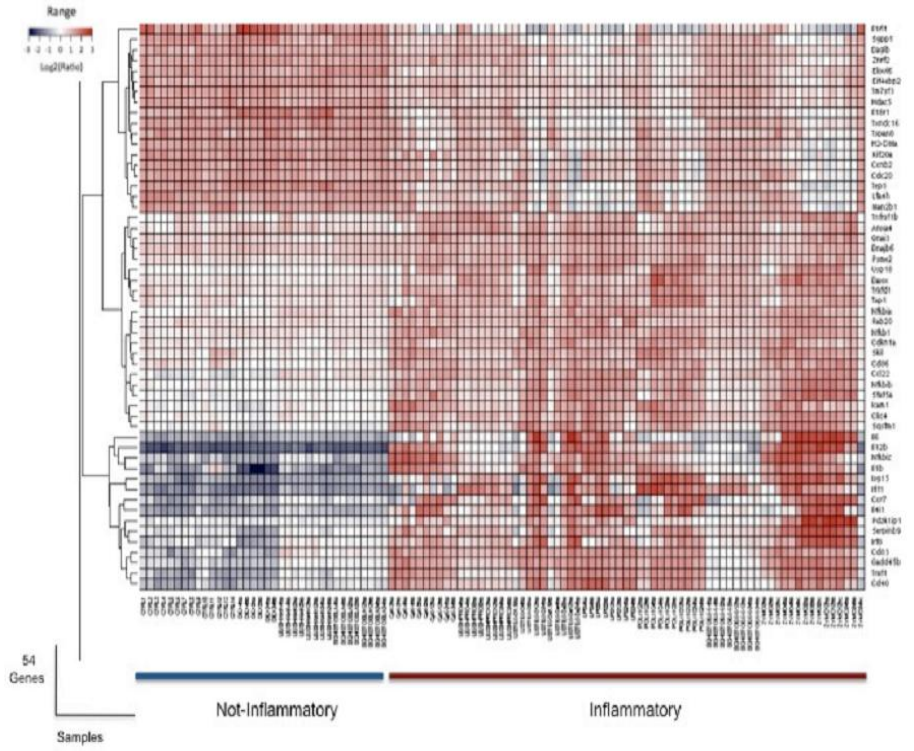


Fig.2 Heat map of the dendritic cell-specific signature.
Genes with different levels of expression in DCs treated with inflammatory stimuli and in those treated with anti-inflammatory molecules. Each column represents a sample and each row represents a gene. Levels of gene expression are indicated on a colour scale, with red corresponding to the highest level of expression and blue corresponding to the lowest level. The Log_2 ratio is expressed with respect to the mean expression level of each gene.

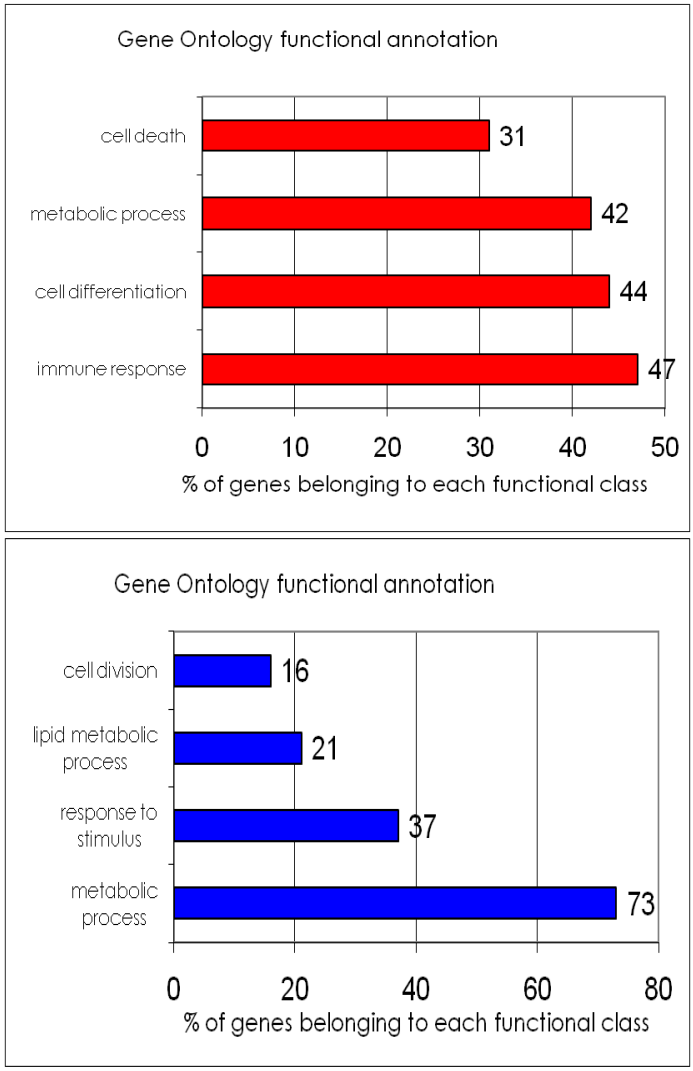


Fig.3 Gene Ontology annotation.
Chart illustrating the pathways in which the proteins encoded by the genes within the genetic signature are involved. DC-specific genes were functionally annotated with DAVID (http://niaid.abcc.ncifcrf.gov/). The genes overexpressed in DCs during activation (red) included genes involved in immune responses (47 %), cell differentiation (44%, metabolic process (42%) and cell death (31%). The genes down-regulated in DCs (blue) included genes involved metabolic process (73 %) and the response to stimuli (37%).

2.3.5 Real-time reverse transcriptase (RT)-PCR validation of microarray observations

Despite the accuracy of the classifier for random forest-based class assignment in a test set of DC samples, the mean expression index of transcripts was low. We therefore performed qRT-PCR to confirm the relative expression levels recorded for the DC samples with Affymetrix technology. We used the 18s rRNA gene as a housekeeping gene for the normalization of target gene expression. We prepared independent DC line D1 samples by treating the cells for 24 h with known signals, such as 10 μg/ml LPS, 20 μg/ml Poly I:C and 500 ng/ml zymosan, and with 10^{-8} M dexamethasone, 10^{-8} M vitamin D and 50 ng/ml IL10, which are known to have anti-inflammatory activity [26,27,28,29,30]. All the RNA samples in the study were converted to cDNA using the same reverse-transcription cocktail and procedure. The pattern of gene expression observed on qRT-PCR confirmed the microarray data. We assessed the predictive value of genes by calculating the median levels of expression for that gene in the known inflammatory and anti-inflammatory samples and then determining the mean expression level for the gene. These values were used to determine whether, for a given stimulus, the level of expression of the gene could be used to assign the sample to the correct class. We therefore subjected all the genes to the same test, under different stimulation conditions. A score of 1 was assigned if the expression

level exceeded the mean expression value for the gene for a sample to be inflammatory or was less than the mean value for a sample to be anti-inflammatory. A score of 0 was awarded in all other cases (**supplementary Fig.2**). Poly I:C stimulation was correctly classified by 94 % (51/54) of the genes, whereas LPS and zymosan stimulations were correctly classified by 89 % (48/54) and 85 % (46/54) of the genes, respectively. As predicted, dexamethasone was the best anti-inflammatory reagent, correctly classified by 100% (54/54) of the genes. IL10 and vitamin D were identified as anti-inflammatory stimuli by 96 % (52/54) and 89 % (48/54) of the genes, respectively (**Fig.4**).

Vitamin D up-regulated IL1b, Rab20, NFKBIA, NFKBIZ and Skil. These genes are up-regulated by TLRs ligands (**Fig.4**). The effect of vitamin D on IL-1b gene expression has already been demonstrated in primary mouse keratinocytes and other cell types, whereas the induction of proteins involved in intracellular trafficking has not previously been shown [31,32,33,34]. Rab20 was recently identified as a potential regulator of connexin 43 trafficking [35].

We then assessed the power of the selected genetic signatures to classify samples treated with different reagents probing different types of receptors. We treated DCs with live bacteria, such as *Listeria monocytogenes* and *Lactobacillus paracasei*, 10 μM nimesulide and 1000 U/ml IFNα to modify their functional state. As

expected, live bacteria induced the strongest inflammatory signature response in DCs (**Fig.5A**): *Listeria monocytogenes* and *Lactobacillus paracasei* were correctly classified as inflammatory by 98 % (53/54) and 94 % (51/54) of the genes, respectively. *Listeria monocytogenes* is known to induce the production of type I IFNs. We therefore expected this bacterium to induce the interferon-responsive genes IFIT1 and Isg15. Levels of transcription for IL1b, Pdzk1ip1, Isg15, IFIT1, USP18 were strongly reduced by *Lactobacillus paracasei*. We therefore conclude that *Lactobacillus paracasei* is less able to induce a type I IFN response than other genes of the inflammatory signature, suggesting that commensal bacteria may specifically modulate inflammatory genes in DCs (Fig. 5A). Finally, we measured the effect of IFNα and nimesulide treatment on DCs. We compared the results obtained with nimesulide and IFNα with the signatures obtained with dexamethasone, IL10 and vitamin D (**Fig.5B**). Nimesulide and IFNα were classified as anti-inflammatory by 98% (53/54) and 80% (43/54) of the genes, respectively. ISG15, IFIT1, USP18 are known to be regulated by IFNα whereas CLIC4, TRAFD1 and IL4Ii have not been shown to be affected by IFNα. This confirms the role of IFNα in the regulation of DC activity [36,37].

We were able to classify samples treated with IFNα, IL10, vitamin D as non inflammatory with this system, whereas live bacteria (*Listeria* and *Lactobacillus*) were classified as inflammatory on the

basis of the strong inflammatory signatures induced. LPS and Poly I:C were found to be stronger inducers of DC activation than zymosan (46/54), at least in our experimental conditions. Zymosan has also been shown to induce a regulatory phenotype in DCs [38].

NI	INF	Gene Name	DEX	VitD	IL-10	LPS	poly I:C	ZymA
		IL-6	0,61	1,02	0,91	195,66	10,83	105,66
		IL12B	0,23	0,87	0,62	139,32	28,66	306,94
		PDZK1lp1	0,98	0,38	0,18	32,59	1,02	11,91
		IL-4I1	0,43	0,74	0,54	20,73	36,38	19,89
		CCL22	0,18	0,33	0,42	16,14	17,23	7,76
		CD83	0,50	0,32	0,53	16,06	23,28	7,31
		GADD45B	0,80	0,73	0,84	14,90	20,20	7,76
		IL1B	0,16	11,02	1,58	14,57	6,54	9,72
		CD40	0,53	0,54	0,66	13,10	15,04	6,22
		NFKBIZ	0,72	1,75	1,19	12,25	1,94	2,02
		RAB20	0,48	4,19	1,22	11,52	9,96	5,03
		CCR7	0,44	0,47	0,56	11,38	25,14	6,67
		TRAF1	0,57	0,94	1,09	10,15	14,88	6,85
		STAT5A	0,88	0,71	0,75	7,38	6,37	3,53
		ISG15	0,31	0,96	0,74	5,07	17,46	7,30
		NFKBIA	0,77	1,95	1,01	4,48	3,83	3,85
		IRF8	0,25	0,83	0,67	3,92	11,21	4,22
		ICAM1	0,95	1,02	0,86	3,83	4,74	3,21
		NFKBIB	0,55	0,55	0,52	3,38	3,30	2,15
		TNFRSF1B	0,58	0,60	0,70	2,51	3,05	1,60
		SQTM1	0,94	1,07	0,87	2,16	3,53	2,43
		IFIT1	0,47	1,46	0,86	2,08	17,57	5,91
		SKIL	0,76	1,61	0,81	1,97	1,38	1,29
		GNAI3	0,88	1,10	1,28	1,93	1,63	1,23
		USP18	0,56	0,60	0,73	1,93	3,50	1,80
		NFKB1	0,63	0,59	0,71	1,83	1,98	1,42
		CD86	1,12	1,16	1,52	1,69	1,75	1,64
		CLIC4	0,79	1,00	0,99	1,55	2,25	1,33
		TAP1	0,57	1,27	0,92	1,42	3,37	2,22
		PSME2B-PS	0,46	0,59	0,65	1,41	2,31	1,33
		ANXA4	0,51	0,65	0,59	1,28	1,47	1,40
		DNAJB6	0,81	1,34	1,51	1,25	2,06	1,40
		TRAFD1	0,64	1,19	1,14	1,16	2,69	1,84
		SERPINB9	0,31	0,56	0,52	1,16	2,52	1,76
		CDKN1a	0,37	0,63	0,65	0,83	2,28	1,50
		DAXX	0,54	0,91	0,79	0,75	1,89	1,25
		EIF4EBP2	0,75	0,71	0,73	0,63	0,53	0,53
		ELOVL6	0,86	0,64	1,03	0,61	0,29	0,56
		TM7SF3	0,87	1,09	1,19	0,51	0,34	0,72
		TSPAN8	2,38	0,93	1,59	0,50	0,27	0,52
		CCNB2	1,01	0,98	0,97	0,45	0,20	0,40
		ZNRF2	0,82	0,77	1,10	0,45	0,26	0,71
		LTA4H	0,59	0,66	0,92	0,43	0,16	0,44
		IL18R1	1,87	1,07	0,88	0,41	0,38	0,46
		TEP1	0,68	0,87	1,34	0,40	0,34	0,61
		HDAC5	0,95	0,82	1,00	0,39	0,48	0,47
		KIF20A	1,22	0,76	0,72	0,36	0,15	0,40
		H2-DMa	1,05	1,15	0,88	0,34	0,36	0,62
		CDC20	1,03	0,69	0,73	0,31	0,17	0,22
		DAGLB	0,66	0,46	0,68	0,31	0,28	0,44
		TXNDC16	1,12	1,03	1,07	0,28	0,19	0,40
		SGPP1	1,34	0,91	0,84	0,26	0,22	0,51
		MAN2B1	0,93	0,95	0,83	0,22	0,19	0,40
		IL1RL1	1,28	0,76	0,70	0,07	0,02	0,13

	$2^{-\Delta\Delta Ct}$
I	100-1000
H	20-100
G	10-20
F	3-10
E	1,5-3
D	1,0-1,5
C	0,6-1,0
B	0,3-0,6
A	0-0,3

Fig.4 Sample validation by quantitative real-time PCR.
qRT-PCR confirmation of classifier transcript levels in inflamed samples derived from DCs treated with TLR ligands (LPS, PolyI:C and zymosan) and in non inflamed samples treated with anti-inflammatory stimuli (dexamethasone, vitamin D and IL-10) for 24 h. Reactions were performed in two wells, normalized with respect to 18s rRNA levels. The results in the table are expressed relative to the corresponding level of expression of each transcript in the untreated sample. Data are presented as mean fold changes in classifier gene transcript levels in three independent experiments per group. The columns in the left reflect the pattern of expression as determined by microarray analysis. NI: Non inflamed samples; INF; Inflamed samples.

A

NI	INF	Gene Name	DEX	VitD	IL-10	Lp	Lm
		IL12B	0,23	0,87	0,62	7955,01	22383,56
		IL-6	0,61	1,02	0,91	258,95	3544,28
		PDZK1lp1	0,98	0,38	0,18	12,91	119,38
		IL-4I1	0,43	0,74	0,54	45,62	92,01
		IFIT1	0,47	1,46	0,86	0,70	67,36
		RAB20	0,48	4,19	1,22	42,84	55,62
		CCR7	0,44	0,47	0,56	28,40	51,72
		GADD45B	0,80	0,73	0,84	31,41	46,55
		IRF8	0,25	0,83	0,67	38,32	41,17
		CD83	0,50	0,32	0,53	42,22	40,54
		ISG15	0,31	0,96	0,74	1,17	29,92
		CCL22	0,18	0,33	0,42	41,93	28,86
		CD40	0,53	0,54	0,66	29,60	28,58
		IL1B	0,16	11,02	1,58	3,04	27,89
		TRAF1	0,57	0,94	1,09	17,46	20,85
		NFKBIZ	0,72	1,75	1,19	6,15	18,51
		STAT5A	0,88	0,71	0,75	15,25	13,30
		GNAI3	0,88	1,10	1,28	8,79	8,77
		SERPINB9	0,31	0,56	0,52	11,58	8,23
		CLIC4	0,79	1,00	0,99	5,03	6,78
		NFKBIB	0,55	0,55	0,52	10,47	6,27
		NFKBIA	0,77	1,95	1,01	7,63	6,27
		DAXX	0,54	0,91	0,79	2,52	6,26
		DNAJB6	0,81	1,34	1,51	7,05	6,23
		ICAM1	0,95	1,02	0,86	4,91	6,16
		TNFRSF1B	0,58	0,60	0,70	4,23	6,05
		SQTM1	0,94	1,07	0,87	4,34	5,88
		USP18	0,56	0,60	0,73	1,42	5,65
		TRAFD1	0,64	1,19	1,14	2,19	5,50
		SKIL	0,76	1,61	0,81	4,15	4,77
		TAP1	0,57	1,27	0,92	2,38	4,66
		NFKB1	0,63	0,59	0,71	2,79	3,94
		PSME2B-PS	0,46	0,59	0,65	2,98	3,56
		CD86	1,12	1,16	1,52	1,79	3,39
		ANXA4	0,51	0,65	0,59	3,15	2,09
		CDKN1a	0,37	0,63	0,65	1,60	0,50
		EIF4EBP2	0,75	0,71	0,73	0,51	0,48
		ZNRF2	0,82	0,77	1,10	0,31	0,34
		DAGLB	0,66	0,46	0,68	0,36	0,32
		LTA4H	0,59	0,66	0,92	0,35	0,26
		ELOVL6	0,86	0,64	1,03	0,68	0,25
		TM7SF3	0,87	1,09	1,19	0,56	0,25
		TEP1	0,68	0,87	1,34	0,24	0,23
		SGPP1	1,34	0,91	0,84	0,22	0,20
		HDAC5	0,95	0,82	1,00	0,24	0,20
		H2-DMa	1,05	1,15	0,88	0,24	0,18
		TXNDC16	1,12	1,03	1,07	0,16	0,18
		CDC20	1,03	0,69	0,73	0,06	0,16
		CCNB2	1,01	0,98	0,97	0,03	0,13
		KIF20A	1,22	0,76	0,72	0,04	0,12
		IL18R1	1,87	1,07	0,88	0,15	0,12
		MAN2B1	0,93	0,95	0,83	0,10	0,11
		TSPAN8	2,38	0,93	1,59	0,09	0,11
		IL1RL1	1,28	0,76	0,70	0,01	0,02

B

NI	INF	Gene Name	DEX	VitD	IL-10	Nim	IFNa
		IL-6	0,61	1,02	0,91	0,40	0,61
		IL12B	0,23	0,87	0,62	0,33	0,52
		PDZK1Ip1	0,98	0,38	0,18	0,81	0,63
		IL-4I1	0,43	0,74	0,54	0,67	2,27
		CCL22	0,18	0,33	0,42	0,60	0,60
		CD83	0,50	0,32	0,53	0,71	1,56
		GADD45B	0,80	0,73	0,84	0,70	1,52
		IL1B	0,16	11,02	1,58	0,83	0,64
		CD40	0,53	0,54	0,66	0,68	1,60
		NFKBIZ	0,72	1,75	1,19	1,09	0,92
		RAB20	0,48	4,19	1,22	0,86	1,37
		CCR7	0,44	0,47	0,56	0,54	1,11
		TRAF1	0,57	0,94	1,09	0,62	1,35
		STAT5A	0,88	0,71	0,75	0,83	1,04
		ISG15	0,31	0,96	0,74	0,88	11,03
		NFKBIA	0,77	1,95	1,01	1,26	1,02
		IRF8	0,25	0,83	0,67	0,87	0,59
		ICAM1	0,95	1,02	0,86	0,80	1,09
		NFKBIB	0,55	0,55	0,52	0,92	0,89
		TNFRSF1B	0,58	0,60	0,70	0,80	1,02
		SQTM1	0,94	1,07	0,87	1,01	1,39
		IFIT1	0,47	1,46	0,86	1,06	12,02
		SKIL	0,76	1,61	0,81	1,01	1,36
		GNAI3	0,88	1,10	1,28	0,89	0,99
		USP18	0,56	0,60	0,73	0,92	3,06
		NFKB1	0,63	0,59	0,71	0,72	0,77
		CD86	1,12	1,16	1,52	1,08	1,26
		CLIC4	0,79	1,00	0,99	0,48	1,83
		TAP1	0,57	1,27	0,92	0,84	1,67
		PSME2B-PS	0,46	0,59	0,65	0,95	0,97
		ANXA4	0,51	0,65	0,59	0,86	0,78
		DNAJB6	0,81	1,34	1,51	0,90	1,11
		TRAFD1	0,64	1,19	1,14	0,85	2,14
		SERPINB9	0,31	0,56	0,52	0,97	0,74
		CDKN1a	0,37	0,63	0,65	0,86	0,48
		DAXX	0,54	0,91	0,79	0,89	1,56
		EIF4EBP2	0,75	0,71	0,73	0,99	0,68
		ELOVL6	0,86	0,64	1,03	ND	0,90
		TM7SF3	0,87	1,09	1,19	0,87	0,63
		TSPAN8	2,38	0,93	1,59	0,86	1,42
		CCNB2	1,01	0,98	0,97	0,79	0,68
		ZNRF2	0,82	0,77	1,10	0,79	0,99
		LTA4H	0,59	0,66	0,92	ND	0,37
		IL18R1	1,87	1,07	0,88	0,83	1,08
		TEP1	0,68	0,87	1,34	0,89	0,85
		HDAC5	0,95	0,82	1,00	0,95	1,01
		KIF20A	1,22	0,76	0,72	0,84	0,85
		H2-DMa	1,05	1,15	0,88	0,92	0,88
		CDC20	1,03	0,69	0,73	0,79	0,77
		DAGLB	0,66	0,46	0,68	ND	0,73
		TXNDC16	1,12	1,03	1,07	0,86	0,99
		SGPP1	1,34	0,91	0,84	0,99	0,90
		MAN2B1	0,93	0,95	0,83	0,86	0,87
		IL1RL1	1,28	0,76	0,70	0,86	0,66

	$2^{-\Delta\Delta Ct}$
I	100-1000
H	20-100
G	10-20
F	3-10
E	1,5-3
D	1,0-1,5
C	0,6-1,0
B	0,3-0,6
A	0-0,3

Fig.5 q-Real Ttime PCR analysis of gene expression on independent samples for class prediction.

A) Expression levels of 54 genes in DCs treated with the bacteria *Listeria monocytogenes* (Lm) and *Lactobacillus paracasei* (Lp) for 24 h.

B) DC samples treated with nimesulide and IFNα for 24 h. Comparison with cells treated with dexamethasone (DEX), IL-10 and vitamin D (VitD). Data are presented as mean fold changes in classifier gene transcript levels in three independent experiments per group. NI: Non inflamed samples; INF; Inflamed samples.

A

Gene Name	M noINF	M INF	A (no INF,INF)	DEX	VitD	IL-10	LPS	polyI:C	ZymA
IL-6	0,91	105,66	53,28	1	1	1	1	0	1
IL12B	0,62	139,32	69,97	1	1	1	1	0	1
PDZK1Ip1	0,38	11,91	6,14	1	1	1	1	0	1
IL-4I1	0,54	20,73	10,64	1	1	1	1	1	1
CCL22	0,33	16,14	8,23	1	1	1	1	1	0
CD83	0,50	16,06	8,28	1	1	1	1	1	0
GADD45B	0,80	14,90	7,85	1	1	1	1	1	0
IL1B	1,58	9,72	5,65	1	0	1	1	1	1
CD40	0,54	13,10	6,82	1	1	1	1	1	0
NFKBIZ	1,19	2,02	1,60	1	0	1	1	1	1
RAB20	1,22	9,96	5,59	1	1	1	1	1	0
CCR7	0,47	11,38	5,92	1	1	1	1	1	1
TRAF1	0,94	10,15	5,54	1	1	1	1	1	1
STAT5A	0,75	6,37	3,56	1	1	1	1	1	0
ISG15	0,74	7,30	4,02	1	1	1	1	1	1
NFKBIA	1,01	3,85	2,43	1	1	1	1	1	1
IRF8	0,67	4,22	2,45	1	1	1	1	1	1
ICAM1	0,95	3,83	2,39	1	1	1	1	1	1
NFKBIB	0,55	3,30	1,92	1	1	1	1	1	1
TNFRSF1B	0,60	2,51	1,55	1	1	1	1	1	1
SQTM1	0,94	2,43	1,69	1	1	1	1	1	1
IFIT1	0,86	5,91	3,39	1	1	1	0	1	1
SKIL	0,81	1,38	1,10	1	0	1	1	1	1
GNAI3	1,10	1,63	1,37	1	1	1	1	1	0
USP18	0,60	1,93	1,26	1	1	1	1	1	1
NFKB1	0,63	1,83	1,23	1	1	1	1	1	1
CD86	1,16	1,69	1,43	1	1	0	1	1	1
CLIC4	0,99	1,55	1,27	1	1	1	1	1	1
TAP1	0,92	2,22	1,57	1	1	1	0	1	1
PSME2B-PS	0,59	1,41	1,00	1	1	1	1	1	1
ANXA4	0,59	1,40	0,99	1	1	1	1	1	1
DNAJB6	1,34	1,40	1,37	1	1	0	0	1	1
TRAFD1	1,14	1,84	1,49	1	1	1	0	1	1
SERPINB9	0,52	1,76	1,14	1	1	1	1	1	1
CDKN1a	0,63	1,50	1,06	1	1	1	0	1	1
DAXX	0,79	1,25	1,02	1	1	1	0	1	1
EIF4EBP2	0,73	0,53	0,63	1	1	1	1	1	1
ELOVL6	0,86	0,56	0,71	1	0	1	1	1	1
TM7SF3	1,09	0,51	0,80	1	1	1	1	1	1
TSPAN8	1,59	0,50	1,05	1	0	1	1	1	1
CCNB2	0,98	0,40	0,69	1	1	1	1	1	1
ZNRF2	0,82	0,45	0,63	1	1	1	1	1	0
LTA4H	0,66	0,43	0,54	1	1	1	1	1	1
IL18R1	1,07	0,41	0,74	1	1	1	1	1	1
TEP1	0,87	0,40	0,63	1	1	1	1	1	1
HDAC5	0,95	0,47	0,71	1	1	1	1	1	1
KIF20A	0,76	0,36	0,56	1	1	1	1	1	1
H2-DMa	1,05	0,36	0,71	1	1	1	1	1	1
CDC20	0,73	0,22	0,47	1	1	1	1	1	1
DAGLB	0,66	0,31	0,48	1	0	1	1	1	1
TXNDC16	1,07	0,28	0,68	1	1	1	1	1	1
SGPP1	0,91	0,26	0,58	1	1	1	1	1	1
MAN2B1	0,93	0,22	0,57	1	1	1	1	1	1
IL1RL1	0,76	0,07	0,42	1	1	1	1	1	1
			Tot Genes	54	48	52	48	51	46
			% of Genes	100	89	96	89	94	85

B

Gene Name	M noINF	M INF	A (no INF,INF)	DEX	VitD	IL-10	Lp	Lm
IL12B	0,62	139,32	69,97	1	1	1	1	1
IL-6	0,91	105,66	53,28	1	1	1	1	1
PDZK1lp1	0,51	11,91	6,21	1	1	1	1	1
IL-4I1	0,54	20,73	10,64	1	1	1	1	1
IFIT1	0,86	5,91	3,39	1	1	1	0	1
RAB20	1,22	9,96	5,59	1	1	1	1	1
CCR7	0,47	11,38	5,92	1	1	1	1	1
GADD45B	0,80	14,90	7,85	1	1	1	1	1
IRF8	0,67	4,22	2,45	1	1	1	1	1
CD83	0,50	16,06	8,28	1	1	1	1	1
ISG15	0,74	7,30	4,02	1	1	1	0	1
CCL22	0,33	16,14	8,23	1	1	1	1	1
CD40	0,54	13,10	6,82	1	1	1	1	1
IL1B	1,58	9,72	5,65	1	0	1	0	1
TRAF1	0,94	10,15	5,54	1	1	1	1	1
NFKBIZ	1,19	2,02	1,60	1	0	1	1	1
STAT5A	0,75	6,37	3,56	1	1	1	1	1
GNAI3	1,10	1,63	1,37	1	1	1	1	1
SERPINB9	0,52	1,76	1,14	1	1	1	1	1
CLIC4	0,99	1,55	1,27	1	1	1	1	1
NFKBIB	0,55	3,30	1,92	1	1	1	1	1
NFKBIA	1,01	3,85	2,43	1	1	1	1	1
DAXX	0,79	1,25	1,02	1	1	1	1	1
DNAJB6	1,34	1,40	1,37	1	1	0	1	1
ICAM1	0,95	3,83	2,39	1	1	1	1	1
TNFRSF1B	0,60	2,51	1,55	1	1	1	1	1
SQTM1	0,94	2,43	1,69	1	1	1	1	1
USP18	0,60	1,93	1,26	1	1	1	1	1
TRAFD1	1,14	1,84	1,49	1	1	1	1	1
SKIL	0,81	1,38	1,10	1	0	1	1	1
TAP1	0,92	2,22	1,57	1	1	1	1	1
NFKB1	0,63	1,83	1,23	1	1	1	1	1
PSME2B-PS	0,59	1,41	1,00	1	1	1	1	1
CD86	1,16	1,69	1,43	1	1	0	1	1
ANXA4	0,59	1,40	0,99	1	1	1	1	1
CDKN1a	0,63	1,50	1,06	1	1	1	1	0
EIF4EBP2	0,73	0,53	0,63	1	1	1	1	1
ZNRF2	0,82	0,45	0,63	1	1	1	1	1
DAGLB	0,66	0,31	0,48	1	0	1	1	1
LTA4H	0,66	0,43	0,54	1	1	1	1	1
ELOVL6	0,86	0,56	0,71	1	0	1	1	1
TM7SF3	1,09	0,51	0,80	1	1	1	1	1
TEP1	0,87	0,40	0,63	1	1	1	1	1
SGPP1	0,91	0,26	0,58	1	1	1	1	1
HDAC5	0,95	0,47	0,71	1	1	1	1	1
H2-DMa	1,05	0,36	0,71	1	1	1	1	1
TXNDC16	1,07	0,28	0,68	1	1	1	1	1
CDC20	0,73	0,22	0,47	1	1	1	1	1
CCNB2	0,98	0,40	0,69	1	1	1	1	1
KIF20A	0,76	0,36	0,56	1	1	1	1	1
IL18R1	1,07	0,41	0,74	1	1	1	1	1
MAN2B1	0,93	0,22	0,57	1	1	1	1	1
TSPAN8	1,59	0,50	1,05	1	0	1	1	1
IL1RL1	0,76	0,07	0,42	1	1	1	1	1
			Tot Genes	54	48	52	51	53
			% of Genes	100	89	96	**94**	**98**

C

Gene Name	M noINF	M INF	A (no INF,INF)	DEX	VitD	IL-10	Nim	IFNα
IL-6	0,91	105,66	53,28	1	1	1	1	1
IL12B	0,62	139,32	69,97	1	1	1	1	1
PDZK1lp1	0,51	11,91	6,21	1	1	1	1	1
IL-4I1	0,54	20,73	10,64	1	1	1	1	1
CCL22	0,33	16,14	8,23	1	1	1	1	1
CD83	0,50	16,06	8,28	1	1	1	1	1
GADD45B	0,80	14,90	7,85	1	1	1	1	1
IL1B	1,58	9,72	5,65	1	0	1	1	1
CD40	0,54	13,10	6,82	1	1	1	1	1
NFKBIZ	1,19	2,02	1,60	1	0	1	1	1
RAB20	1,22	9,96	5,59	1	1	1	1	1
CCR7	0,47	11,38	5,92	1	1	1	1	1
TRAF1	0,94	10,15	5,54	1	1	1	1	1
STAT5A	0,75	6,37	3,56	1	1	1	1	1
ISG15	0,74	7,30	4,02	1	1	1	1	0
NFKBIA	1,01	3,85	2,43	1	1	1	1	1
IRF8	0,67	4,22	2,45	1	1	1	1	1
ICAM1	0,95	3,83	2,39	1	1	1	1	1
NFKBIB	0,55	3,30	1,92	1	1	1	1	1
TNFRSF1B	0,60	2,51	1,55	1	1	1	1	1
SQTM1	0,94	2,43	1,69	1	1	1	1	1
IFIT1	0,86	5,91	3,39	1	1	1	1	0
SKIL	0,81	1,38	1,10	1	0	1	1	0
GNAI3	1,10	1,63	1,37	1	1	1	1	1
USP18	0,60	1,93	1,26	1	1	1	1	0
NFKB1	0,63	1,83	1,23	1	1	1	1	1
CD86	1,16	1,69	1,43	1	1	0	1	1
CLIC4	0,99	1,55	1,27	1	1	1	1	0
TAP1	0,92	2,22	1,57	1	1	1	1	0
PSME2B-PS	0,59	1,41	1,00	1	1	1	1	1
ANXA4	0,59	1,40	0,99	1	1	1	1	1
DNAJB6	1,34	1,40	1,37	1	1	0	1	1
TRAFD1	1,14	1,84	1,49	1	1	1	1	0
SERPINB9	0,52	1,76	1,14	1	1	1	1	1
CDKN1a	0,63	1,50	1,06	1	1	1	1	1
DAXX	0,79	1,25	1,02	1	1	1	1	0
EIF4EBP2	0,73	0,53	0,63	1	1	1	1	1
ELOVL6	0,86	0,56	0,71	1	0	1	ND	1
TM7SF3	1,09	0,51	0,80	1	1	1	1	0
TSPAN8	1,59	0,50	1,05	1	0	1	0	1
CCNB2	0,98	0,40	0,69	1	1	1	1	0
ZNRF2	0,82	0,45	0,63	1	1	1	1	1
LTA4H	0,66	0,43	0,54	1	1	1	ND	0
IL18R1	1,07	0,41	0,74	1	1	1	1	1
TEP1	0,87	0,40	0,63	1	1	1	1	1
HDAC5	0,95	0,47	0,71	1	1	1	1	1
KIF20A	0,76	0,36	0,56	1	1	1	1	1
H2-DMa	1,05	0,36	0,71	1	1	1	1	1
CDC20	0,73	0,22	0,47	1	1	1	1	1
DAGLB	0,66	0,31	0,48	1	0	1	ND	1
TXNDC16	1,07	0,28	0,68	1	1	1	1	1
SGPP1	0,91	0,26	0,58	1	1	1	1	1
MAN2B1	0,93	0,22	0,57	1	1	1	1	1
IL1RL1	0,76	0,07	0,42	1	1	1	1	1
			Tot Genes	54	48	52	50	43
			% of Genes	100	89	96	**93**	**80**

Supplementary Fig.2 Selection of genes discriminating between DC phenotypes. **A)** We investigated the predictive value of genes by calculating the median level of expression for the gene in the inflamed and non inflamed samples (LPS, PolyIC, zymosan, dexamethasone, IL-10 and vitamin D and then calculating mean expression levels for that gene. **B-C)** These values were used to assess whether, for a particular stimulus (*Listeria monocytogenes*, *Lactobacillus paracasei*, nimesulide or IFN*a*), the expression level of the gene could be used to assign the sample to the correct class. A score of 1 was assigned if the expression level exceeded the mean value for inflammatory treatment or was below the mean level for anti-inflammatory treatment. A score of 0 was assigned in all other cases.

2.3.6 Transcription signatures are dendritic cell-specific

We assessed the specificity of the classifier genes by analyzing 44 of the 54 genes that gave consistent results for different samples by qRT-PCR, in the macrophage cell line MT2 [39]. The MT2 cells were stimulated with 10^{-8} M dexamethasone or 10 μg/ml LPS. The genes of the inflammation signature were not well expressed in MT2 cells if compared to the D1 cells (**Fig. 6**). Only 41% (18/44) of the genes showed the expected pattern of change in expression in response to LPS stimulation in MT2 cells. Indeed, some genes displayed opposite patterns of expression in the two cell lines tested. Il18r1 was induced in MT2 cells but down-regulated in DCs in response to LPS. We therefore conclude that the inflammatory signature identified in this study is specific to DCs.

			D1		MT2	
NI	INF	Gene Name	DEX	LPS	DEX	LPS
		IL-6	0,61	195,66	0,51	269,71
		IL12B	0,23	139,32	ND	ND
		PDZK1lp1	0,98	32,59	0,73	0,24
		IL-4I1	0,43	20,73	0,82	0,93
		CCL22	0,18	16,14	0,77	2,62
		CD83	0,50	16,06	0,88	0,78
		GADD45B	0,80	14,90	0,69	1,43
		IL1B	0,16	14,57	0,37	132,22
		CD40	0,53	13,10	0,78	4,50
		RAB20	0,48	11,52	0,86	2,50
		CCR7	0,44	11,38	1,79	2,41
		TRAF1	0,57	10,15	0,55	25,05
		STAT5A	0,88	7,38	1,02	0,49
		ISG15	0,31	5,07	0,60	4,00
		NFKBIA	0,77	4,48	1,03	3,65
		IRF8	0,25	3,92	0,69	0,64
		ICAM1	0,95	3,83	0,84	0,71
		NFKBIB	0,55	3,38	1,21	1,45
		TNFRSF1B	0,58	2,51	0,59	2,76
		SQTM1	0,94	2,16	0,76	2,03
		SKIL	0,76	1,97	0,63	0,87
		GNAI3	0,88	1,93	0,82	0,86
		NFKB1	0,63	1,83	1,22	0,52
		CLIC4	0,79	1,55	0,81	0,56
		TAP1	0,57	1,42	0,64	1,02
		PSME2B-PS	0,46	1,41	0,62	1,30
		ANXA	0,51	1,28	0,49	0,65
		DNAJB6	0,81	1,25	0,87	0,97
		TRAFD1	0,64	1,16	0,76	0,83
		SERPINB9	0,31	0,73	0,80	2,18
		EIF4EBP2	0,75	0,63	0,80	0,71
		TSPAN8	2,38	0,50	0,59	0,35
		CCNB2	1,01	0,45	0,73	0,68
		ZNRF2	0,82	0,45	0,75	0,61
		IL18R1	1,87	0,41	0,39	1,59
		TEP1	0,68	0,40	0,73	0,58
		HDAC5	0,95	0,39	1,01	0,41
		KIF20A	1,22	0,36	1,11	0,62
		H2DMa	1,05	0,34	0,66	0,68
		CDC20	1,03	0,31	0,80	0,71
		TXNDC16	1,12	0,28	0,92	0,55
		SGPP1	1,34	0,26	0,78	0,56
		MAN2B1	0,93	0,22	0,74	0,35
		IL1RL1	1,28	0,07	0,83	0,58

Fig.6 Specificity of the genetic signature. Real-time PCR confirmation of 44 inflammatory signature genes in the DC line D1 and the absence of this signature in MT2 cells. Both cell lines were treated with LPS (10 µg/ml) and 10^{-8} M dexamethasone (DEX) for 24 h. Data are presented as mean fold changes in classifier gene transcript levels in three independent experiments per group.

2.3.7 Classifier genes can be used to predict the inflammatory process in DCs *in vivo*

Preclinical animal models of inflammation and infections have become important tools for improving our understanding of the regulation of inflammatory reactions in general and for the development of novel treatment strategies to modulate excessive, deleterious inflammatory reactions. We measured the gene expression signature associated with inflammation in *ex vivo* splenic DCs derived from mice treated with the endotoxin LPS, and dexamethasone with the aim of converting our *in vitro* DC assay into a useful tool for preclinical mouse models of inflammatory diseases.

Splenic DCs detect antigens derived from the blood and are widely used as a model system for testing treatments that affect DC recruitment or for detecting DC activation during systemic infection. We analyzed the pattern of expression of the 44 genes in splenic DCs *in vivo*. We treated a group of mice with 50 μg of LPS/mouse or dexamethasone and, after 5 h, $CD11c^+$ cells were purified from spleen by magnetic bead separation. Cell purity was checked by FACS analysis and 90% of the cells were found to be $CD11c^+$ (data not shown) then assessed the prognostic value of the 44 genes by qRT-PCR: 91% (40/44) and 80% (35/44) of the genes correctly predicted the inflammatory or non inflammatory phenotype of splenic DCs, confirming that the inflammatory

signature selected in this study was also induced in DCs *in vivo* (**Fig.7**, **supplementary Fig.3**). We were unable to confirm the induction of IL12b, IRF8 and NFkb1 by LPS *in vivo*, possibly due to the choice of an inappropriate time point for the analysis.

			D1		splenic DC	
NI	INF	Gene Name	DEX	LPS	DEX	LPS
		IL-6	0,61	195,66	0,24	4,33
		IL12B	0,23	139,32	0,74	0,52
		PDZK1Ip1	0,98	32,59	0,11	81,81
		IL-4I1	0,43	20,73	0,36	5,50
		CCL22	0,18	16,14	0,60	3,93
		CD83	0,50	16,06	0,34	2,35
		GADD45B	0,80	14,90	0,50	12,83
		IL1B	0,16	14,57	0,27	8,10
		CD40	0,53	13,10	0,60	6,45
		RAB20	0,48	11,52	0,70	7,36
		CCR7	0,44	11,38	0,46	23,56
		TRAF1	0,57	10,15	0,56	3,43
		STAT5A	0,88	7,38	0,69	5,74
		ISG15	0,31	5,07	0,50	65,98
		NFKBIA	0,77	4,48	0,63	6,75
		IRF8	0,25	3,92	0,90	0,80
		ICAM1	0,95	3,83	0,64	4,43
		NFKBIB	0,55	3,38	0,79	3,91
		TNFRSF1B	0,58	2,51	0,54	3,37
		SQTM1	0,94	2,16	0,83	3,18
		SKIL	0,76	1,97	0,44	1,82
		GNAI3	0,88	1,93	0,91	1,85
		NFKB1	0,63	1,83	1,26	0,92
		CLIC4	0,79	1,55	0,48	14,78
		TAP1	0,57	1,42	0,66	2,47
		PSME2B-PS	0,46	1,41	0,50	2,94
		ANXA4	0,51	1,28	0,62	7,16
		DNAJB6	0,81	1,25	0,71	1,49
		TRAFD1	0,64	1,16	1,01	4,74
		SERPINB9	0,31	0,73	0,36	9,18
		EIF4EBP2	0,75	0,63	0,76	0,41
		TSPAN8	2,38	0,50	0,08	0,05
		CCNB2	1,01	0,45	1,06	0,34
		ZNRF2	0,82	0,45	0,80	0,24
		IL18R1	1,87	0,41	0,51	0,78
		TEP1	0,68	0,40	0,64	0,41
		HDAC5	0,95	0,39	0,78	0,18
		KIF20A	1,22	0,36	1,36	0,68
		H2-DMa	1,05	0,34	2,17	0,09
		CDC20	1,03	0,31	0,85	0,31
		TXNDC16	1,12	0,28	0,88	0,10
		SGPP1	1,34	0,26	0,82	0,25
		MAN2B1	0,93	0,22	0,70	0,10
		IL1RL1	1,28	0,07	0,54	1,26

Fig.7 *In vivo* validation of the DC-specific inflammatory signature.
C57BL/6 mice were treated with LPS and, after 5 h, $CD11c^+$ cells were isolated from spleen and tested for the inflammatory signature. Data are presented as mean fold changes in classifier gene transcript levels in three independent experiments.

A

Gene Name	A (no INF,INF)	MT2 DEX	MT2 LPS
IL-6	53,28	1	1
IL12B	69,97	0	1
PDZK1lp1	6,14	1	0
IL-4I1	10,64	1	0
CCL22	8,23	1	0
CD83	8,28	1	0
GADD45B	7,85	1	0
IL1B	5,65	1	1
CD40	6,82	1	0
RAB20	5,59	1	0
CCR7	5,92	1	0
TRAF1	5,54	1	1
STAT5A	3,56	1	0
ISG15	4,02	1	0
NFKBIA	2,43	1	1
IRF8	2,45	1	0
ICAM1	2,39	1	0
NFKBIB	1,92	1	0
TNFRSF1B	1,55	1	1
SQTM1	1,69	1	1
SKIL	1,10	1	0
GNAI3	1,37	1	0
NFKB1	1,23	1	0
CLIC4	1,27	1	0
TAP1	1,57	1	0
PSME2B-PS	1,00	1	1
ANXA	0,99	1	0
DNAJB6	1,37	1	0
TRAFD1	1,49	1	0
SERPINB9	1,14	1	1
EIF4EBP2	0,63	1	0
TSPAN8	1,05	0	1
CCNB2	0,69	1	1
ZNRF2	0,63	1	1
IL18R1	0,74	0	0
TEP1	0,63	1	1
HDAC5	0,71	1	1
KIF20A	0,56	1	0
H2DMa	0,71	0	1
CDC20	0,47	1	0
TXNDC16	0,68	1	1
SGPP1	0,58	1	1
MAN2B1	0,57	1	1
IL1RL1	0,42	1	0
	Tot Genes	40	18
	% of Genes	91	41

B

Gene Name	A (no INF,INF)	splenic DC DEX	splenic DC LPS
IL-6	53,28	1	0
IL12B	69,97	1	0
PDZK1lp1	6,14	1	1
IL-4I1	10,64	1	0
CCL22	8,23	1	0
CD83	8,28	1	0
GADD45B	7,85	1	1
IL1B	5,65	1	1
CD40	6,82	1	0
RAB20	5,59	1	1
CCR7	5,92	1	1
TRAF1	5,54	1	0
STAT5A	3,56	1	1
ISG15	4,02	1	1
NFKBIA	2,43	1	1
IRF8	2,45	1	0
ICAM1	2,39	1	1
NFKBIB	1,92	1	1
TNFRSF1B	1,55	1	1
SQTM1	1,69	1	1
SKIL	1,10	1	1
GNAI3	1,37	1	1
NFKB1	1,23	0	0
CLIC4	1,27	1	1
TAP1	1,57	1	1
PSME2B-PS	1,00	1	1
ANXA4	0,99	1	1
DNAJB6	1,37	1	1
TRAFD1	1,49	1	1
SERPINB9	1,14	1	1
EIF4EBP2	0,63	1	1
TSPAN8	1,05	0	1
CCNB2	0,69	1	1
ZNRF2	0,63	1	1
IL18R1	0,74	1	1
TEP1	0,63	1	1
HDAC5	0,71	1	1
KIF20A	0,56	0	1
H2-DMa	0,71	1	1
CDC20	0,47	1	1
TXNDC16	0,68	1	1
SGPP1	0,58	1	1
MAN2B1	0,57	1	1
IL1RL1	0,42	0	1
	Tot Genes	40	35
	% of Genes	91	80

Supplementary Fig. 3 Selection of genes discriminating between different DC phenotypes *in vitro* and *in vivo*.
Class predictor genes were identified on the basis of their mean levels of expression with known stimuli and their classification performance was determined in MT2 cells (**A**) and in *ex-vivo* CD11c$^+$ cells derived from the spleens of mice trated with LPS (**B**).

2.4. DISCUSSION

The development and marketing of microarray platforms has led to extensive investigation of global gene expression profiles in health and disease. The expression profiling of diverse healthy tissues provides a comprehensive view of the range of transcriptional regulation in physiological conditions [35,40,41,42]. Similarly, the identification of gene expression signatures indicative of disease subtypes improves our understanding of the molecular basis of disease [13,14,43]. Small sample size and the large number of measurements required for each sample currently limit the efficacy of gene expression profiling, leading to efforts to develop new analytical methods. Gene expression profiles have recently found applications in diagnosis, prognosis and the provision of predictive information, and in the classification of human cancers and inflammatory diseases [44,45].

In this study, we used the random forest algorithm [46] to identify specific transcriptional signatures of inflammation in DCs and to evaluate whether these molecular signatures could be used to determine the activation state of DCs *in vitro* and *in vivo*. We found that the selected predictive genes up-regulated during DCs activation fell into distinct functional classes, with major involvement in immune system processes (47%) cell differentiation (44%), metabolic process (42%) and cell death (31%). Gene Ontology analysis identified the genes involved in immune system

processes as Nfkbia, Nfkbib, Stat5a, IL1b, TAP1, IL12b, IL-6, Nfkb1, IRF8, CCL22, Sqstm1, Nfkb1, CD40, IFIT1, CCR7, Isg15, CD83 and TRAFD1. The genes involved in cell differentiation identified were Skil, Traf1, Clic4, Gadd45b, TNFRSF1B, SERPINB9, Sqstm1 and Cdkn1a. Gene Ontology analysis also identified genes involved in cell death, such as Traf1, Sqstm1, Nfkb1, Gadd45b, TNFRSF1B, SERPINB9 and DAXX. The down-regulated predictive genes were identified as involved in cell division (CCNB2, Kif20a, CDC20), lipid metabolism (DAGLB, Elovl6, LTA4H, Sgpp1), defense responses (Hdac5, IL1rl1, IL18r1, LTA4H) and metabolic processes (Txndc16, Hdac5, IL1rl1, DAGLB, LTA4H, Tep1, Sgpp1, H2-DMa, Eif4ebp2, MAN2B1, Elovl6, Znrf2, CDC20).

Our findings clearly demonstrate that the gene expression profiling of DCs reliably distinguishes between activating and non activating stimuli. We validated our findings on an independent set of samples treated with molecules inducing the activity of different receptors and DC activation. Dexamethasone, IL10 and vitamin D were used as typical anti-inflammatory stimuli. They were generally classified as non activating signals. By contrast, TLR ligands and whole bacteria, which are widely recognized as inflammatory signals, were classified as such by this system. The signature expression profile identified here consists of 44 highly predictive genes. These 44 genes provide a unique gene expression profile

indicative of activation in DCs and providing important biological insight into the host response mediated by DCs. Genes already identified as involved in inflammation and DC activation were selected, including those encoding the interleukins IL-6, IL-12b and IL-1b [47], the chemokine CCL22 [48], the membrane molecules CD40, CD83, CD86, ICAM1, and CCR7, and the transcription factors STAT5a and IRF8, although the precise role of IRF8 in DC activation is currently unknown.

Some of the genes selected have no known role in inflammation. For example, the Pdzk1ip1 gene encodes a protein associated with various tumors when abundant. This protein seems to play a role in Akt activation and its gene was strongly induced by *L. monocytogenes* and LPS, whereas no overexpression was associated with poly I:C treatment. Thus, the induction of this gene is dependent on surface TLR stimulation. Rab20 encodes a protein that regulates intracellular trafficking and may play an important role in inflammation. Rab20 has been shown to interact with connexin 45 [35]. IL4I1 was recently identified as an oxidase active against *l*-amino acids with potential effects on lymphocyte proliferation. It is strongly induced by DC activation.

The genes encoding proteins involved in the NF-kB pathway were more strongly induced by bacteria than TLR ligands. Inhibitor genes, such as NFKBIA and NFKBIB, were also overexpressed after stimulation with bacteria, consistent with the activation of

regulatory mechanisms that control the inflammation. The down-regulated genes include a large proportion of membrane proteins, such as IL18r1, IL1rl1, TSPAN8 and Tm7sf3, and genes encoding proteins with enzymatic activity, such as MAN2B1 (an m-mannosidase), LTA4H (leukotriene A4 hydrolase), Txndc16 (thioredoxin domain-containing 16), Sgpp1 (sphingosine-1-phosphate phosphatase 1), Hdac5 (histone deacetylase 5). The role of this down-regulation is currently unknown and requires further investigation.

We used the dendritic cell line D1 to optimize the sensitivity and precision of our gene expression profiling. We decided to determine an inflammation signature for DCs rather than for any other type of leukocyte, because DCs link innate and adaptive immunity [49]. The prediction of DC activation state is therefore of potential value for the testing of exogenous molecules with potential anti-inflammatory or adjuvant activity in DCs, to favor the repression or induction of T-cell responses. We validated the inflammatory signature *in vivo,* by testing the response in splenic DCs from mice treated with LPS and dexamethasone. Most of the genes (80%) studied successfully characterized the activation state of splenic DCs, and differentiated the profile of these cells from that of DCs derived from mice treated with dexamethasone.

In conclusion, we used a meta-analysis of microarray data to identify gene modules predictive of DC activation. Accuracy and

simplicity are essential characteristics of predictors for molecular assays. The predictive accuracy of predictors generally ranges from 65% to 100% (mean, 82%) [50]. It is therefore important to identify the best way to select a suitable classifier for data, to maximize accuracy. In this study we used random forest methods for the selection of genes and the classification of microarray data [46,51]. The potential of array-based multidimensional predictors to outperform traditional parameters is increasing for biomarker discovery. The number of array-based studies is likely to increase exponentially, particularly in the field of inflammatory diseases. Such studies have been widely used for cancer classification [52,53]. In this study, we identified and validated a prognostic gene expression signature in DCs associated with inflammation. The signaling events affected by many of the genes in this signature occur in pathways essential for the immune response and cell activation. These genes may therefore be suitable targets, alone or in combination, for trials of anti-inflammatory and adjuvant treatments. We have demonstrated that a genome-wide systems biology approach may have advantages over traditional methods for biomarker discovery. Moreover, the small number of genes in our signature makes it possible to use simple, conventional assays, such as quantitative reverse transcriptase-polymerase chain reaction [54]. The increasing availability of laboratory diagnosis by polymerase chain reaction has opened up new possibilities for

genomic testing based on the use of genetic signatures, in routine clinical conditions.

2.5. MATERIALS AND METHODS

2.5.1 Cell culture

D1 cells [20] were maintained *in vitro* in Iscove's modified Dulbecco's medium (IMDM, Euroclone) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, origin: Australia), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Euroclone) and 50 μM β-mercaptoethanol (Sigma) plus 30% R1 medium (supernatant from NIH3T3 fibroblasts transfected with GM-CSF).

MT2 cells are immortalized macrophages. They were derived from mouse thymus as previously described [39]. They were cultured in IMDM (Euroclone) supplemented with 5% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Euroclone) and 50 μM β-mercaptoethanol (Sigma).

2.5.2 Microarray dataset

We obtained published gene expression datasets for microarray experiments performed with D1 cells, dexamethasone (10^{-8} M;[21,22,23] and the live microorganisms *Schistosoma mansoni (*MOI

1:200 parasites/cell*)* and *Leishmania mexicana* (MOI 1:8) . All the other samples were prepared specifically for this study. D1 cells were infected with *Listeria monocytogenes* EGD (MOI 1:20; provided by P. Cossard, Pasteur Institute, France) or treated with various TLR agonists: rLPS (10 μg/ml, Alexis, serotype R515), CpG (10 μg/ml, Sigma), poly I:C (20 μg/ml, Amersham), Pam3Cys (1 μM, Sigma) and zymosan (500 ng/ml, Sigma).

2.5.3 Microarray assay

We harvested 10^7 D1 cells in the immature state or after 4 h, 8 h, 12 h or 24 h of stimulation. Total RNA was isolated with Trizol Reagent (Invitrogen, Life Technologies, Karlruhe, Germany) and purified on a Qiagen RNeasy column (Qiagen, Hilden, Germany) to remove small fragments. RNA quality was assessed on an Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Only samples with intact total RNA profiles (retention of both ribosomal bands and the broad central peak of mRNA) were used for the microarray and quantitative RT-PCR gene expression analyses. *In vitro* transcription (IVT) products were generated and oligonucleotide array hybridization and scanning were carried out according to the instructions supplied by Affymetrix (Santa Clara, CA). We used 10 to 16 μg of total RNA from each sample and T7-linked oligo-dT primers for first-strand cDNA synthesis. The fragmented biotinylated cDNA (15 μg) was

hybridized onto the MG-U74Av2 GeneChip (Affymetrix), using the recommended procedures for prehybridization, hybridization, washing and staining with streptavidin–phycoerythrin (SAPE).

2.5.4 Microarray data analysis and supervised class prediction

Array images were analyzed with the RMA algorithm [25]. Samples displaying a signal ratio >3.0 for the β-actin and GAPDH probe sets were considered to be poor-quality targets and were excluded from the dataset.

The final dataset contained the results for 115 arrays (79 DC samples subjected to pro-inflammatory stimuli and 36 samples with anti-inflammatory reagents). A single log scale normalized expression measure for each probe set was obtained from the low-level data files (CEL files), by the robust multiarray analysis (RMA) procedure [25]. The data were subjected to Z-score-based transformation. A diagnostic model was obtained by applying the random forest (RF) method to the training set [46]. The model was based on 1000 bootstrap samples of the training set, with 1000 classification trees generated with a view to classifying cases as "inflammatory" and "not-inflammatory" on the basis of microarray gene expression measurements. RF is a method of the "decision tree classifiers" family, but it works on a collection of trees (a 'forest') rather than a single tree. In a decision tree, each node

represents an attribute — in our case, the probeset — and the terminal nodes (the 'leaves') represent the attribute producing the best separation between the classes –("inflammatory" and "not inflammatory" in this analysis) of a dataset. RF feeds each tree with an independent subset of attributes from a training set and individual instances are classified by a voting procedure, with the majority of the decision trees in the collection indicating the appropriate classification. Finally, during the classification process, RF determines the relative importance of each attribute, through various methods, such as calculation of the Gini Index, which assesses the importance of the variable and carries out accurate variable selection.

2.5.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

D1 cells were infected with *Listeria monocytogenes* EGD (multiplicities of infection, MOI, 1:20) and *Lactobacillus paracasei* (MOI 1:1000) or were treated with various stimuli: sLPS (10 μg/ml, Alexis, serotype SO55:B5), poly I:C (20 μg/ml, Amersham), zymosan (500 ng/ml, Sigma), dexamethasone (10^{-8} M, Sigma), vitamin D (10^{-8} M, Sigma), IL-10 (50 ng/ml, Immunok), IFNα (1000 U/ml, PBL Biomedical Laboratories) and Nimesulide (10 μM, Cayman).

We harvested $5x10^6$ D1 cells either at 0 h or after 24 h of

stimulation. Total RNA was isolated with Trizol Reagent (Invitrogen) and purified on a Qiagen RNeasy column (Mini kit, Qiagen). DNase digestion was carried out in the column during RNA extraction (RNase-free DNase Set, Qiagen). RNA quantity and quality was evaluated spectrophotometrically (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific). We reverse transcribed 1 μg of total RNA with random primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was performed on 10 ng of total cDNA from independent samples, using primer sets specific for 54 selected genes and the 18s housekeeping gene. qRT-PCR was carried out on a 7500 machine (Applied Biosystems), with Power SYBR Green PCR Master Mix (Applied Biosystems). Assays were carried out in duplicate. Primers were designed with Primer3 software (http://frodo.wi.mit.edu/) and checked with other tools (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi; m-fold, http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi; IDT oligo analyzer,http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primers were validated, and only primers with an amplification efficiency of 85 to 115% were accepted (Primm srl, Italy). Primer sequences are reported in **supplementary Table 1**. The raw data (Ct, threshold cycle) were obtained with Applied Biosystems software. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = Ct_{target}-Ct_{18s}$, $\Delta\Delta Ct = \Delta Ct_{stimulated} - \Delta Ct_{not\ treated}$),

using 18s as the housekeeping gene.

Gene symbol	NCBI Reference Sequence	Primer Name	primer sequences 5'-3'
18s	NR_003278	m18s_F1	CGAAAGCATTTGCCAAGAAT
		m18s_R1	AGTCGGCATCGTTTATGGTC
Txndc16	NM_172597	5730420B22Rik_F	CCAGGAATGGAAACCTCCTC
		5730420B22Rik_R	CACCTCTGCTTCTTTCTGACACTCTA
ANXA4	NM_013471	ANXA4_F	GGGCTGTCTGATTGAGATCC
		ANXA4_R	TATTGCTGCTGGTATGTCTGG
CCL22	NM_009137	CCL22_F	TCTCGTCCTTCTTGCTGTGG
		CCL22_R	CACTAAACGTGATGGCAGAGG
CCNB2	NM_007630	CCNB2_F	CAACAAGCAGCCGAAACC
		CCNB2_R	GAGCAGAGCATCAGAGAAAGC
CCR7	NM_007719	CCR7_F	GGCTCTCCTTGTCATTTTCC
		CCR7_R	CCTTAAAGTTCCGCACATCC
CD40	NM_011611, NM_170701,	CD40_F	GGTCCATCTAGGGCAGTGTG
	NM_170702, NM_170703, NM_170704	CD40_R	GGTCCATCTAGGGCAGTGTG
CD83	NM_009856	CD83_F	GTGGCACTGAGAGTGTGGAG
		CD83_R	TTGGATCGTCAGGGAATAGG
CD86	NM_019388	CD86_F	ATCAAGGACATGGGCTCGTA
		CD86_R	GAAGTTGGCGATCACTGACA
CDC20	NM_023223	CDC20_F2	GCACTCACTGCTTCAACTGG
		CDC20_R2	TGGCTTCCTTTGCTTTGC
Cdkn1a	NM_007669	Cdkn1a_F	CTTGTCGCTGTCTTGCACTC
		Cdkn1a_R	AATCTGTCAGGCTGGTCTGC
Clic4	NM_013885	Clic4_F	TGATGGTGAAAGCATTGGAA
		Clic4_R	TGCAGGTTTCCTTTTCAGGT
DAXX	NM_007829	DAXX_F	GGCGAGAGACAGAAGAGACG
		DAXX_R	CTAGGACCCTCACCAGATTCC
DNAJB6	NM_011847,NM_001037941	DNAJB6_F	ACGACAAATATGGCAAAGAAGG
		DNAJB6_R	TGAAGACATCATCTGGGTTCC
Daglb	NM_144915	E330036I19RIK_F	GCTACTGTTCCAGCGACTCC
		E330036I19RIK_R	CTGAACCACCCTCCTCTTCC
Eif4ebp2	NM_010124	Eif4ebp2 _F	GACGCTGTTCTCCACAACG
		Eif4ebp2_R	GGTGACTCCAGGGATATTGG
Elovl6	NM_130450	Elovl6_F	GGTGACTCCAGGGATATTGG
		Elovl6_R	GTGGGAGTAGCACTGGTCGT
Gadd45b	NM_008655	Gadd45b_F	CTCCTGGTCACGAACTGTCA
		Gadd45b_R	CCCATTGGTTATTGCCTCTG
GNAI3	NM_010306	GNAI3_F2	TGAGGACGGCTATTCAGAGG
		GNAI3_R2	CCCCAAAATCAATCTTCAACC

Gene symbol	NCBI Reference Sequence	Primer Name	primer sequences 5'-3'
H2-DMa	NM_010386	H2-Dma_F	CTGCCTTCGGATCTCCTAGA
		H2-Dma_R	GTCACCTGAGCACGGTCTCT
Hdac5	NM_001077696	Hdac5_F	CGTGGACTGGGATATTCACC
		Hdac5_R	GAAAGAAGTTCCCGTTGTCG
Icam1	NM_010493	Icam1_F3	GCCTTGGTAGAGGTGACTGAG
		Icam1_R3	GACCGGAGCTGAAAAGTTGTA
IFIT1	NM_008331	IFIT1_F	CAGCACATCTTGCCAAAGC
		IFIT1_R	TAGCCAGAGGAAGGTGATGC
IL12b	NM_008352	IL12b_F	CCTGGACCTCAGACCAGAGA
		IL12b_R	AGAGTCTCGCCTCCTTTGTG
Il18r1	NM_008365	IL18r1_F	TTCAAAGGCAGTGCTTCACA
		IL18r1_R	GTACGTTCCCTCATCCTCCA
IL-1b	NM_008361	IL-1b_F	AGCTTCAAATCTCGCAGCAG
		IL-1b_R	TGTCCTCATCCTGGAAGGTC
Il1rl1	NM_001025602	Il1rl1_F	CTCCAAGAGCCAAGGTTCAG
		Il1rl1_R	CTCCAAGAGCCAAGGTTCAG
IL4i1	NM_010215	IL4i1_F2	TAGCAGCCAAGATGCTCAGT
		IL4i1_R2	CCCTATCCAGCCTGTCTTCT
IL6	NM_031168	IL-6_F	GGACTGATGCTGGTGACAAC
		IL-6_R	CCTCCGACTTGTGAAGTGGT
IRF8	NM_008320	IRF8_F	TGACACCAACCAGTTCATCC
		IRF8_R	GAAACTCCTCCCCGAAGC
Isg15	NM_015783	Isg15_F	CCAGGACGGTCTTACCCTTT
		Isg15_R	CTTTCGTTCCTCACCAGGAT
Kif20a	NM_009004	Kif20a_F	AGCAGATTCGACAGGAGGAA
		Kif20a_R	GGCACCTATCCGACTTTCAG
LTA4H	NM_008517	LTA4H_F	CTCATAGCTGGACAGGAAACC
		LTA4H_R	GAACTTTTCCCCAAACAACC
MAN2B1	NM_010764	MAN2B1_F	ATACCTGTGCTGGGATGTGC
		MAN2B1_R	GTTCGGTAAAACCCTTTCTGG
Nfkb1	NM_008689	mNfkb1_F	GCCTGCAAAGGTTATCGTTC
		mNfkb1_R	TCCTGCTGTTACGGTGCATA
Nfkbia	NM_010907	Nfkbia_F	AGCAGTCTTGACGCAGACCT
		Nfkbia_R	GCCAGGTAGCCGTGAGTAGA

Gene symbol	NCBI Reference Sequence	Primer Name	primer sequences 5'-3'
Nfkbib	NM_010908	Nfkbib_F	CCTGAGGACGAGGACGATAA
		Nfkbib_R	CCACTGTGAACCACGATGTC
Nfkbiz	NM_030612	Nfkbiz_F	GTGGAGGCAAAGGATCGTAA
		Nfkbiz_R	TCACGAAAGACAGGCAACTG
Pdzk1ip1	NM_026018	Pdzk1ip1_F	ATTGGAAACAAGGCAGATGG
		Pdzk1ip1_R	TCTCGTAGGCATTCTTGTGCT
Psme2b-PS	NM_011190, NM_001029855	Psme2_F	ACAGACAAGCAGGAGAAGAAGG
		Psme2_R	CTTAACCAAAGCGAGCAAGG
Rab20	NM_011227	Rab20_F	GATGTGCTTCGAGACCAGTG
		Rab20_R	GATCTGACTCCTCAGCCCTCT
SERPINB9	NM_009256	SerpinB9_F	TTAACAGCCTGGATGGAAGC
		SerpinB9_R	AAGTCAGCCTTGTCCTCTTGG
Sgpp1	NM_030750	Sgpp1_F	TGGGCATCTTCTCTTTCACC
		Sgpp1_R	CCAGGGTATAAGCAGCGTGT
Skil	NM_001039090	Skil_F	TGCACCTGTGACTCAACCTT
		Skil_R	TCTTGGAGTTCCTGCCTGTC
Sqstm1	NM_011018	Sqtm1_F	CTGTGGTGGGAACTCGCTAT
		Sqtm1_R	GGGAAAGATGAGCTTGCTGT
Stat5a	NM_011488	Stat5a_F	ACCTGGATGAGAGCATGGAT
		Stat5a_R	GAGCTTCTAGCGGAGGTGAA
TAP1	NM_013683	TAP1_F	ACTGAGGACACAGCCAACG
		TAP1_R	CAGGGTGACCAGAGTGAGG
Tep1	NM_009351	Tep1_F	AAGCACAGAAGTCCCAATGC
		Tep1_R	ACCTGGTCAGTGGTTTCAGG
Tm7sf3	NM_026281	Tm7sf3_F	ACATCCTGGCTTGTGGAGTC
		Tm7sf3_R	GAGGACGTTCAGGGTGATGT
TNFRSF1B	NM_011610	TNFRSF1B_F	CTCTAAGTGCCATCCCAAGG
		TNFRSF1B_R	TTGAACCCAACGATGTAAGG
Traf1	NM_009421	Traf1_F	ACCTGTCCCTCTTCATCGTG
		Traf1_R	ATGCTCTCGGTTGTTCTGGT
TRAFD1	NM_172275	Trafd1_F2	GGTAGAGCAGGGCTTCTGG
		Trafd1_R2	TCAATGAGCAGTTCCTCTGG
TSPAN8	NM_146010	TSPAN8_F2	GTTCAAGTGCTGTGGCTTGG
		TSPAN8_R2	GGAACTCCCCTGATAAGTTGC
USP18	NM_011909	USP18_F	GAACTCTTTGCCGTGATTGC
		USP18_R	AGCAGAACCACTTTCCATCC
Znfr2	AF513708	Znfr2_F2	CGCACTTGTTTGGAGGATT
		Znfr2_R2	CCCCGTATCTTTACTCAGCAC

Supplementary Table 1 Real Time primer sequences

2.5.6 *In vivo* experiment

Six-week-old C57BL/6 were injected with rLPS (50 μg/mouse, Alexis, serotype R515), dexamethasone (50 μg/mouse, Sigma), or with PBS as a control. The spleen was removed five hours later, and $CD11c^+$ cells (DCs) were purified by magnetic bead separation (Miltenyi Biotec). C57BL/6 mice were purchased from Charles River and were maintained in our animal facility at the University of Milano-Bicocca. All experiments were performed using protocols approved by University of Milano-Bicocca Animal Care and Use Committee. Mice were housed in containment facilities of the animal facility and maintained on a regular 12:12 hour light:dark cycle with food and water ad libitum.

2.6. ACKNOWLEDGMENTS

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CHAPTER 3 - IL-22 EXPRESSION AND REGULATION IN DENDRITIC CELLS AND IN TOTAL SPLENOCYTES

3.1. RESULTS

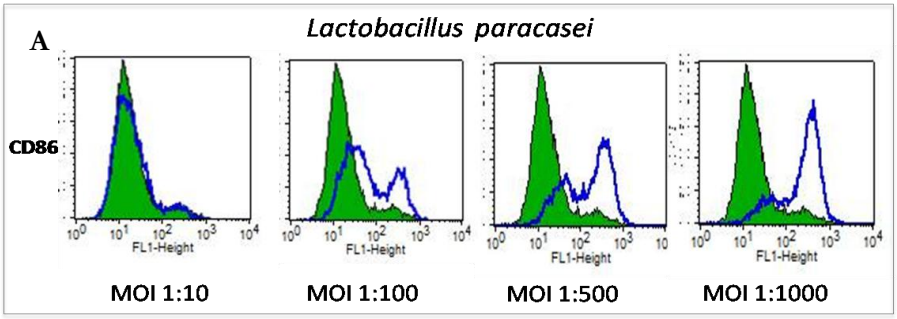
3.1.1 IL-22 expression

D1 cells up-regulate IL-22 mRNA upon infection with gram$^+$ bacteria

Dendritic cells (DCs) are extremely versatile antigen presenting cells (APCs) that sense the host environment and respond to danger. Direct contact with many pathogens leads to the maturation of DCs and to an active transcriptional response leading to the acquisition of diverse DCs functional phenotypes that orchestrate the appropriate immune response (see §1.2.3, 1.2.4). To study the molecular mechanisms induced in dendritic cells upon bacterial infection, we performed microarray experiments. D1 cells [1], a mouse spleen-derived dendritic cell line, were infected with different gram$^+$ bacteria (*Lactobacillus paracasei*, *Lactococcus lactis*, *Listeria monocytogenes, Listeria innocua*). All bacteria were used at a multiplicity of infection (MOI) of 1:1000 except for *L.monocytogens* that was used at a MOI of 1:20, because higher MOI was inducing a pronounced cellular death. The

different MOI have been chosen to induce an optimal DCs activation. For this reason, we tested the following maturation markers: CD80, CD86, MHCII. FACS analysis for CD86 is reported as example in **Fig.1**. D1 cell transcriptome was analyzed at 4 h, 8 h and 24 hours post infection (p.i.). Performing an unsupervised analysis, amongst the common differentially expressed genes (DEGs) we found the gene coding for interleukin-22. This cytokine was up-regulated by all tested bacteria although at different levels and with different kinetic (**Fig.2**). The gene was rapidly induced at high levels by *L.innocua* at 4h and maintained at 8 h and 24 h. The same expression profile was induced by *L.paracasei* although the fluorescence levels detected were lower. In contrast, D1 cells infected with *L.monocytogenes* and *L.lactis* showed up-regulation mainly at 24 h. Quantitative real-time PCR (qPCR) is a commonly used validation tool to confirm gene expression results obtained from microarray analysis, because provides a more precise quantification over a wider dynamic range of expression [12]. Therefore, we validated microarray data analysing IL-22 mRNA expression, in D1 cells infected with the different gram$^+$ bacteria, by qPCR. IL-22 was induced in all the samples under investigation but at highest levels in DCs infected with *L.innocua* (**Fig.3A**). *L.paracasei* and *L.innocua* up-regulated IL-22 mRNA, about 8-fold and 100-fold respectively over untreated cells, and maintained the same level of induction during the time point tested (**Fig.3B**).

L.lactis and *L.monocytogenes* affected IL-22 expression mainly 24 hours p.i. (about 20-fold, **Fig.3B**).



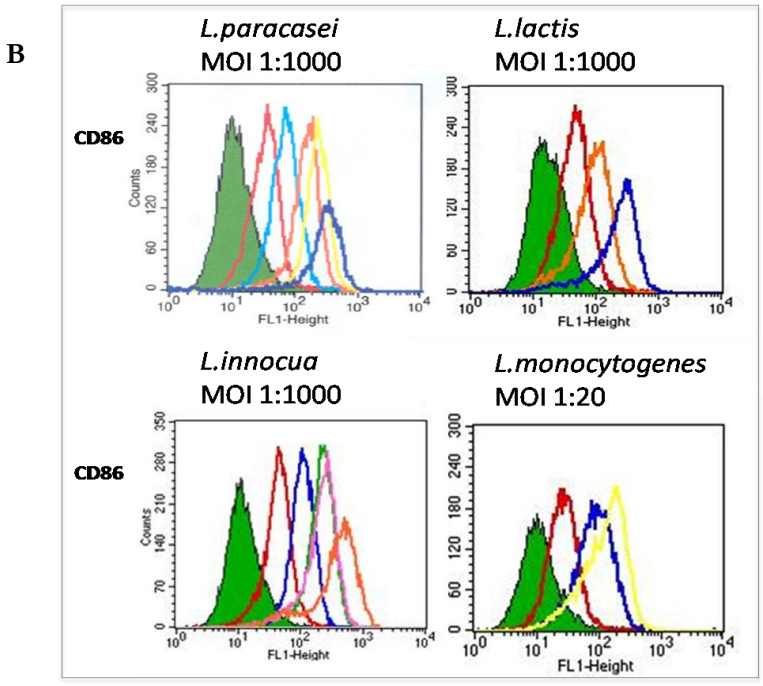


Fig.1 FACS analysis of D1 cells infected with gram$^+$ bacteria. **A)** CD86 expression on D1 at 24 h post infection with *L.paracasei* at different MOI (1:10, 1:100, 1:500, 1:1000) **B)** Expression of CD86 on D1 cells infected with different bacteria (*Lactobacillus paracasei* MOI 1:1000, *Lactococcus lactis* MOI 1:1000, *Listeria monocytogenes* MOI 1:20, *Listeria innocua* MOI 1:1000), at 4 h, 8 h and 24 h post infection.

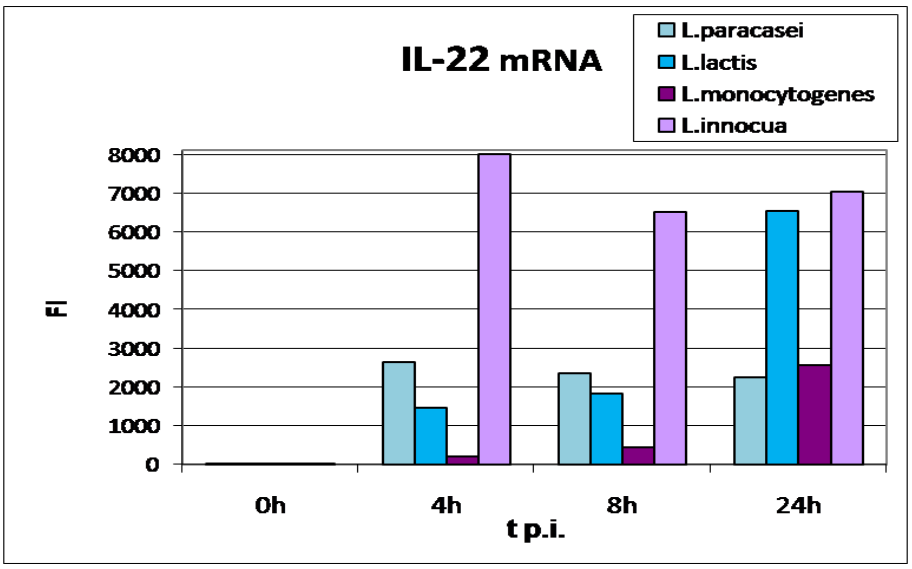


Fig.2 Microarray raw data of IL-22 gene expression in D1 cells infected with: *Lactobacillus paracasei* MOI 1:1000, *Lactococcus lactis* MOI 1:1000, *Listeria monocytogenes* MOI 1:20, *Listeria innocua* MOI 1:1000. Samples were collected at 4 h, 8 h, 24 h) post infection and hybridized onto the MOE430A GeneChip (Affymetrix). FI, fluorescence intensity.

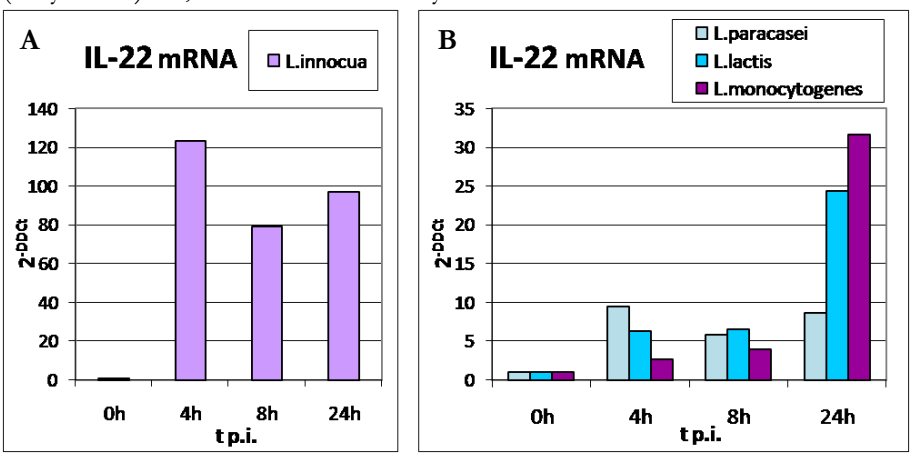


Fig.3 Microarray data validation by q-Real Time PCR. D1 cells infected with: **A)** *Listeria innocua* MOI 1:1000 **B)** *Lactobacillus paracasei* MOI 1:1000, *Lactococcus lactis* MOI 1:1000 and *Listeria monocytogenes* MOI 1:20;. mRNA data are normalized against 18s and then expressed as fold stimulation over control (0h, not treated cells).

D1 cells modulate IL-22 gene expression following TLR triggering

Since whole bacteria stimulate several receptor types on DCs, at the same time, we decided to investigate the individual TLR contribution to IL-22 induction. For this purpose, we analyzed IL-22 gene expression in D1 cells that have been treated with various TLR agonists: LPS (10 μg/ml), zymosan (5 μg/ml) and polyIC (20 μg/ml). At different time points post stimulation (2 h, 4 h, 8 h, 24 h) mRNA was collected and IL-22 mRNA level was analyzed by qPCR. As illustrated in **Fig.4**, all the ligands tested are able to up-regulate IL-22 gene expression although with different potency and kinetic. In particular, LPS and polyIC induced higher IL-22 mRNA at 2 h post treatment (35- fold induction over not stimulated cells). On the contrary, zymosan up-regulated IL-22 mainly at late time points (40-fold at 8 h and 24 h).

These results indicate that IL-22 is modulated by TLR ligands through the activation of TLR4/Cd14, TLR3 and TLR2/Dectin-1.

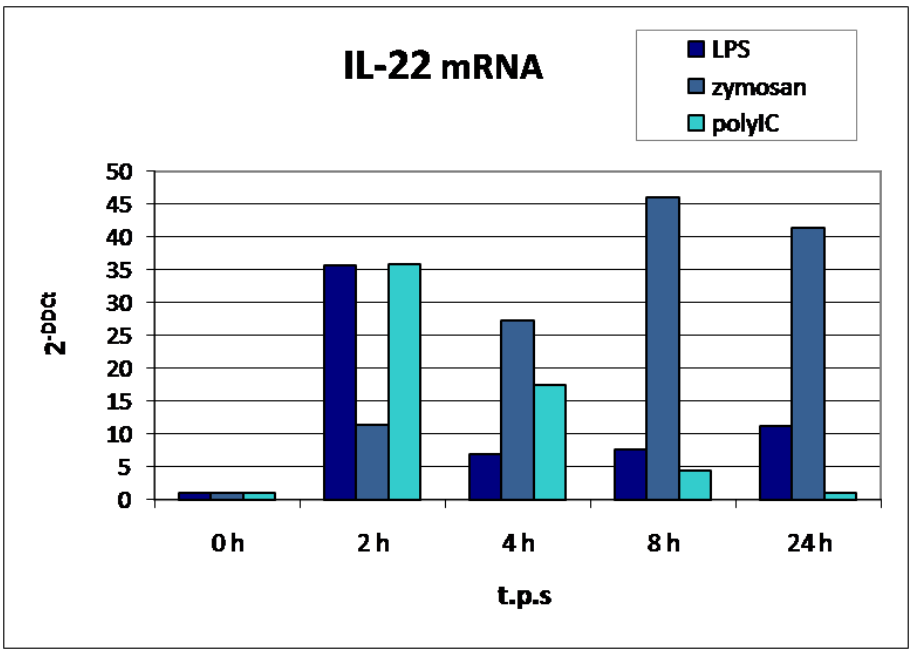


Fig.4 IL-22 gene expression in D1 cells treated with these TLR agonists: LPS (10 μg/ml), zymosan (5 μg/ml) and polyIC (20 μg/ml). At different time points post stimulation (t.p.s: 2 h, 4 h, 8 h, 24 h) mRNA was collected and IL-22 mRNA level analyzed by qPCR. mRNA data are normalized against 18s and then expressed as fold stimulation over control (0h, not treated cells).

D1 cells express IL-22 at mRNA and protein level

To test whether the modulation of IL-22 mRNA corresponded to the IL-22 protein production, we measured in the same samples IL-22 by q- Real Time PCR and by ELISA.

D1 cells ($5x10^5$ cells/ml) were treated with LPS (10 μg/ml) and at 2 h and 24 h post stimulation mRNA and supernatant were collected. As shown in **Fig.5**, LPS was able to up-regulate IL-22 production already at 2 h post treatment. The transcript was more induced at 4 h than at 24 h (20-fold stimulation over untreated

cells, **Fig.5A**). The IL-22 protein was detected as early as 2 h (100 pg/ml) but reached the maximal level of production (300 pg/ml) at 24 h probably because there was an accumulation in the supernatant during the 24 h analyzed (**Fig.5B**).

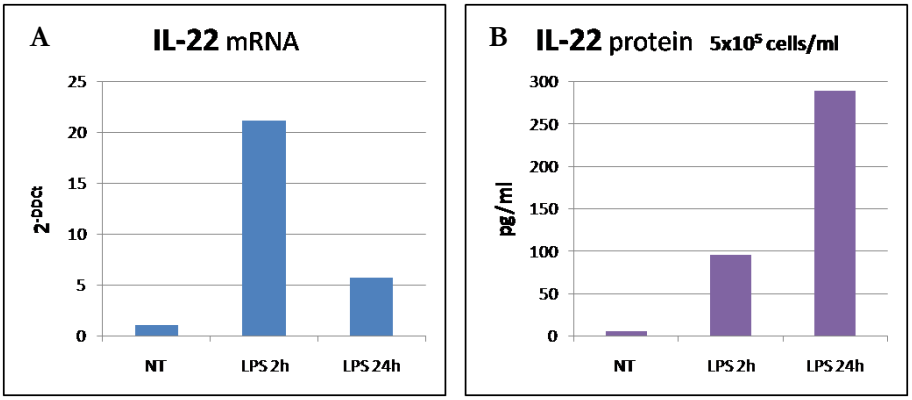


Fig.5 IL-22 production at transcriptional (**A**) and protein (**B**) level in D1 cells upon LPS stimulation at 2 h and 24 h post treatment.
D1 cells were cultured at cell concentration of $5x10^5$ cells/ml and stimulated with LPS 10 μg/ml. mRNA data are normalized against 18s and then expressed as fold stimulation over control (NT, not treated cells)

LPS up-regulates IL-22 mRNA in BMDC and in splenic DCs

To verify whether IL-22 was produced also in other cellular models that are generally used to study DC biology, we tested IL-22 mRNA expression in bone marrow-derived DC (BMDC) generated *in vitro* or in *ex-vivo* DCs (splenic DCs).

Bone marrow cells were derived from C57BL/6 mice and were differentiated *in vitro* using medium supplemented with GM-CSF

(see §3.3.1). The differentiation process was monitored by Flow cytometry (FACS) and cells were used in assays when they were 75-85% CD11c positive. BMDC ($1,5x10^6$ cells/ml) were stimulated with LPS (10 μg/ml) for 20 h and then IL-22 gene expression was quantified by q-Real Time PCR. **Fig.6A** shows the results: untreated DCs expressed IL-22 at very low level (Threshold cycle, Ct=36) but after induction with LPS, we observed an up-regulation of 60- fold (Ct= 30) that corresponded to about 250 pg/ml of IL-22 (**Fig.6B**). Moreover, we demonstrated that BMDC up-regulated IL-22 mRNA and produced IL-22 protein also 20 h post infection with *Listeria innocua* (different MOI: 1:100, 1:440, 1:1030). A dose-response effect was observed at the different MOI tested (**Fig.6**), confirming the correspondence between mRNA and protein level at the different MOI.

Since we have shown that some TLR agonists were able to induce IL-22 mRNA expression in D1 cells, we analyzed into details which TLR ligands are responsible for IL-22 production in BMDC. Therefore, BMDC ($1,5x10^6$ cells/ml) were treated with different molecules triggering TLR4/Cd14 (10 μg/ml LPS), TLR3 (20 μg/ml polyIC), TLR9 (5 μg/ml CpG), TLR1/TLR2 (100 ng/ml Pam3cys), TLR2/TLR6 (100 ng/ml MALP2), TLR7 (1 μg/ml Imiquimod), TLR11 (100 ng/ml Profilin); TLR5 (50 ng/ml Flagellin) and TLR2/Dectin-1 (5 μg/ml Zymosan). After 20 h we analyzed IL-22 expression (**Fig.7**). IL-22 production was detected

after stimulation with all ligands tested, with the exception of Profillin. Cells stimulated with LPS and CpG induced the highest levels of IL-22 (400 and 500 pg/ml respectively), followed by Pam3cys (200 pg/ml), MALP2 (200 pg/ml) and Zymosan (100 pg/ml). PolyIC and Flagellin induced the lowest levels of IL-22 (<50 pg/ml).

To assess whether also *ex-vivo* DCs were able to produce IL-22, splenic DCs were purified by $CD11c^+$ cell sorting from murine splenocytes after depletion of NK cells, T cells, B cells, granulocytes. The sorted cells were 96% $CD11c^+$ (splenic DCs). We stimulated *in vitro* splenic DCs with LPS (10 μg/ml) for 20 h and IL-22 mRNA was measured by Real Time PCR. We could confirm previous data obtained in BMDC: untreated splenic DCs express IL-22 at very low level (Ct=35) but after induction with LPS we observed an up-regulation of about 200 fold (Ct= 27).

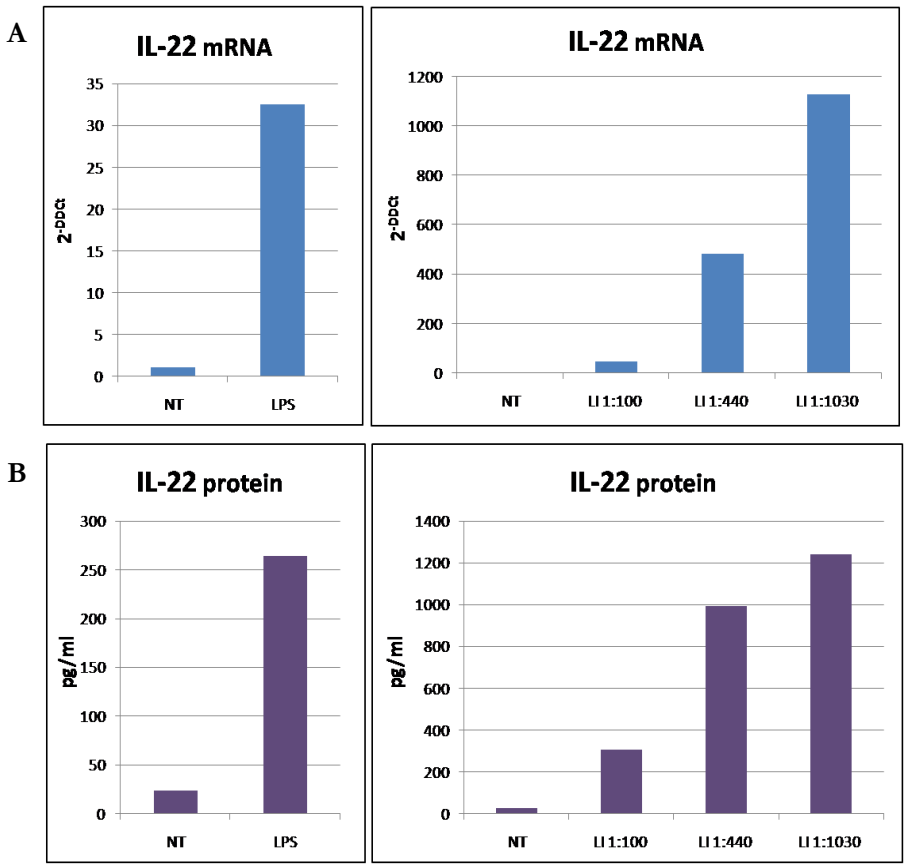


Fig.6 BMDC produce IL-22 upon stimulation with LPS or *Listeria innocua*. Simulations were performed at cell concentration of $1.5x10^6$ cells/ml for 20 h. We used LPS 10 μg/ml or *Listeria innocua* (LI) at different multiplicity of infection (MOI 1:100, MOI 1:440, MOI 1:1030).
A) mRNA data are normalized against 18s and then expressed as fold stimulation over control (NT, not treated cells). **B)** protein levels are expressed as pg/ml.

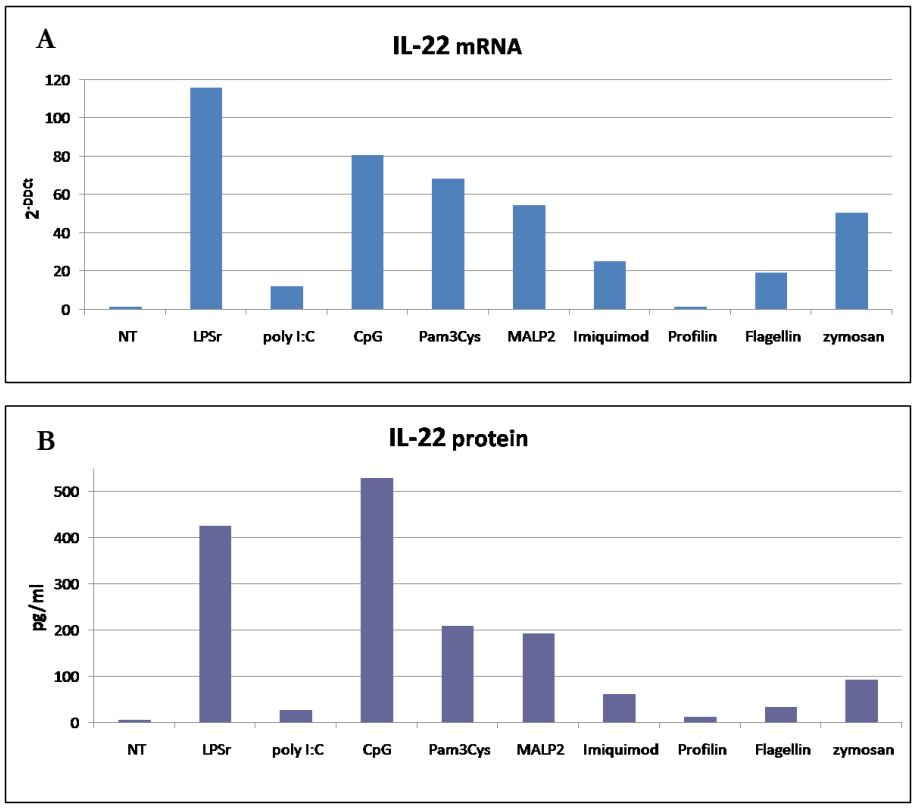


Fig.7 TLR triggering induces IL-22 expression in BMDC.
BMDC were cultured at a concentration of $1.5x10^6$ cell/ml and treated with the following TLR ligands: LPS (10 μg/ml), polyIC (20 μg/ml), Cpg (5 μg/ml), Pam3Cys (100 ng/ml), MALP2 (100 ng/ml), Imiquimod (1 μg/ml), Profilin (100 ng/ml), Flagellin (50 ng/ml), Zymosan (5 μg/ml). **A)** mRNA and **B)** supernatant were collected after 20 h.

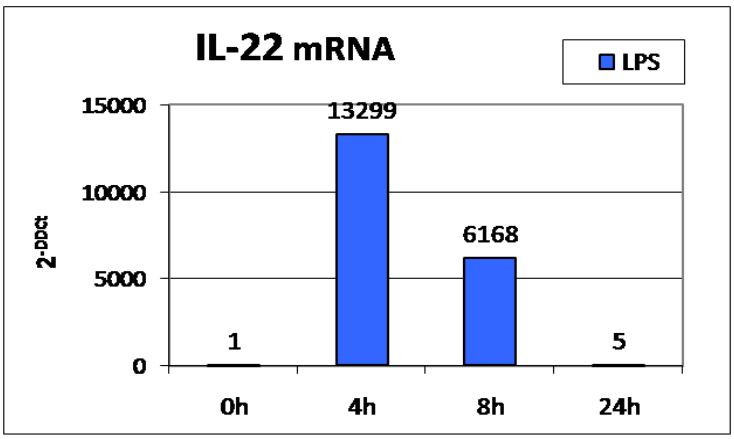
IL-22 is produced *in vivo* in mouse model of inflammation and systemic infection

To test if IL-22 was induced *in vivo*, we employed two different mouse models: a mouse model of systemic inflammation induced

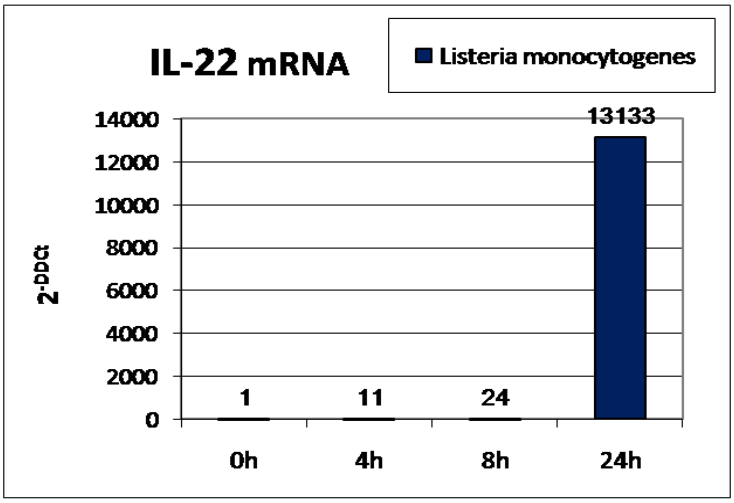
by LPS and a mouse model of systemic infection with gram$^+$ bacteria. C57BL/6 mice were intravenously (i.v.) injected with LPS (50 μg/mouse) and after 4 h, 8 h and 24 h the spleens were collected. **Fig.8A** shows that the IL-22 mRNA was up-regulated, about 13000-fold, as early as 4 h after injection. To verify whether also gram$^+$ bacteria could up-regulate IL-22 expression, mice were i.v. injected with *Listeria innocua* ($1.4x10^8$ CFU/mouse) or *Listeria monocytogenes* (10^6 CFU/mouse) and the spleens were isolated. As previously demonstrated *in vitro* using D1 cells, infection with *Listeria monocytogenes* induced *in vivo* a late IL-22 gene expression at the same level as seen with LPS (13000-fold at 24 h post infection, **Fig.8B**). On the contrary, systemic infection with *Listeria innocua* induced a lower IL-22 expression (20-fold) at 4 h post injection that decreased at 24 h (5-fold). IL-22 mRNA returned to basal level within 72 h post infection (**Fig.8C**). The data suggested that or *L.innocua* was not able to induce a strong up-regulation of IL-22 *in vivo* or the MOI used for the infection was not optimal to observe a strong IL-22 modulation.

Our results are in agreement with previous published data. It is well known that, in mice, intra peritoneal (i.p) injection of LPS (2 μg/mouse) induces IL-22 gene expression in several organs (gut, thymus, spleen, lung, liver, kidney, stomach, heart) within 2 h post injection, even if the cellular source of this cytokine is not known [2].

A


IL-22 mRNA
LPS
15000
10000
5000
0
2-DDct
13299
6168
1
5
0h
4h
8h
24h

B


IL-22 mRNA
Listeria monocytogenes
14000
12000
10000
8000
6000
4000
2000
0
2-DDct
13133
1
11
24
0h
4h
8h
24h

C

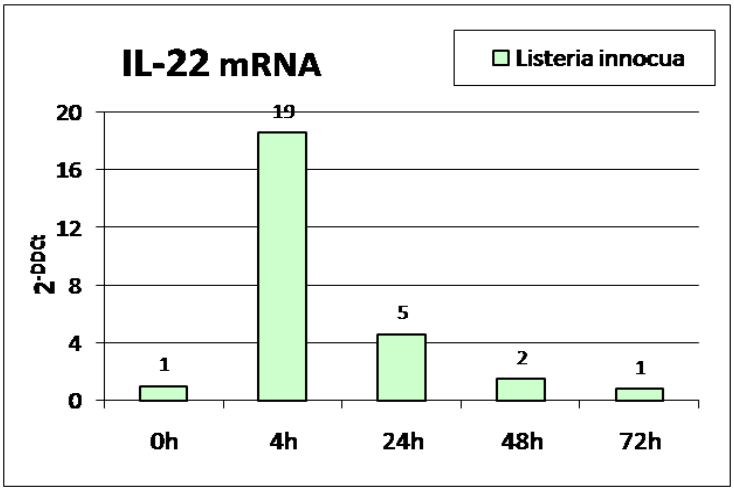

IL-22 mRNA
Listeria innocua
20
16
12
8
4
0
2-DDct
19
5
2
1
1
0h
4h
24h
48h
72h

Fig.8 IL-22 production *in vivo* in total spleen.
C57BL/6 mice were i.v. injected with: **A)** LPS (50 μg/mouse), **B)** *Listeria monocytoegenes* (10^6 CFU/mouse) or **C)** *Listeria innocua* ($1.4x10^8$ CFU/mouse). IL-22 mRNA expression was analysed in the spleen at 4 h, 8 h, 24 h post injection for LPS and *L.monocytogenes*, and at 4 h, 24 h, 48 h and 72 h post infection for *L. innocua*. mRNA data are normalized against 18s and then expressed as fold stimulation over control (NT, mice injected with PBS).

Since our *in vitro* data showed that DCs was able to produce IL-22, we tested whether DCs could be also the cellular source of IL-22 *in vivo* in the mouse model of systemic inflammation. C57BL/6 mice were i.v. injected with PBS or LPS (50 μg/mouse) and after 3 h total splenocytes were isolated. We purified $CD11c^+$ cells and $CD11c^-$ cells by magnetic beads separation (see §3.3.2). To assess the purity, the obtained splenic DCs were analyzed by FACS; they were 85% and 95% $CD11c^+$ and $MHCII^+$ respectively (data not shown). Since CD11c was reported to be weakly expressed on NK cells [14], we tested the % of $NK1.1^+$ cells in the positive fraction; $NK1.1^+$ were less than 6% in the $CD11c^+$ cell preparations.
$CD11c^+$ cells (splenic DCs) and $CD11c^-$ cells derived from untreated or LPS-stimulated mice, were then cultured *in vitro* ($3x10^6$ cells/ml) for 20 h in the presence or absence of LPS (10 μg/ml). The *in vivo* LPS stimulation induced 200 pg/ml of IL-22 protein in $CD11c^+$ (**Fig.9A**) and only 60 pg/ml in $CD11c^-$ cells (**Fig.9B**), whereas the *in vitro* LPS stimulation induced IL-22 protein production only in $CD11c^+$ cells (200 pg/ml, **Fig.9**). This discrepancy between *in vivo* and *in vitro* IL-22 induction, in $CD11c^-$

fraction, might reflect an indirect mechanism of gene induction by LPS in these cells.

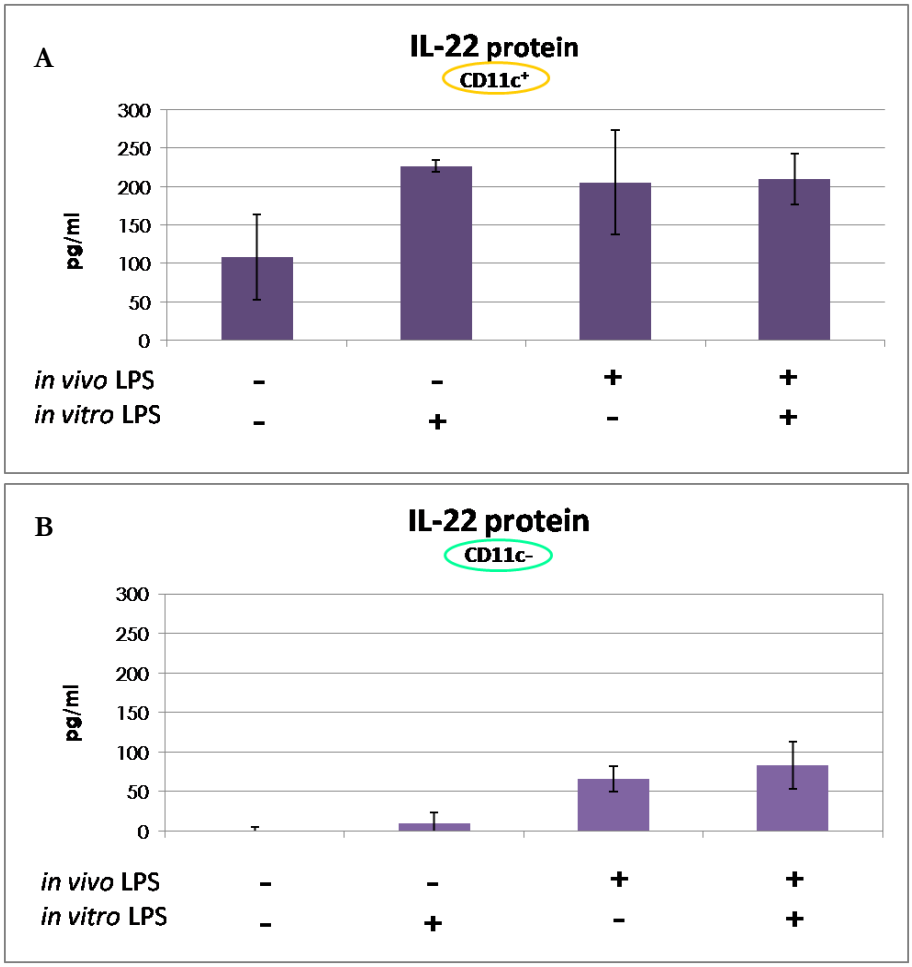


Fig.9 IL-22 protein level in **A)** splenic DCs ($CD11c^+$) and **B)** $CD11c^-$ cells isolated, by magnetic beads separation, from spleen of mice untreated or i.v. injected with LPS (50 μg/mouse). Both cell populations obtained were then cultured *in vitro* at cellular concentrations of $3x10^6$ cells/ml for 20 h in the presence or absence of LPS (10 μg/ml). Data show mean values of three mice ± SD.

3.1.2 Regulation of IL-22 production

Myd88 is involved in IL-22 production by BMDC

Since IL-22 was induced in DCs upon TLR triggering or infection with gram$^+$ bacteria *in vitro* and *in vivo,* we decided to investigate how IL-22 production is regulated in DCs. We have shown that the TLRs involved in IL-22 induction were mainly: TLR4, TLR9, TLR1, TLR2 and TLR6. All these TLRs signal through Myd88, therefore we tested whether DC-IL-22 production was Myd88 dependent. BMDC were derived from wt or Myd88$^{-/-}$ mice as previously described. BMDC (1,5x10^6 cells/ml) were treated *in vitro* with LPS (10 μg/ml), taxol (100 μM), CpG (5 μg/ml), zymosan (5 μg/ml) and polyIC (50 μg/ml). IL-22 expression was measured after 20 h of stimulation. The IL-22 production induced by CpG, through TLR9 triggering, was completely abrogated in the absence of Myd88 (**Fig.10**). In contrast, LPS, that signals through the complex TLR4/Cd14, was able to up-regulate IL-22 also in BMDC ko, though at lower level (40-fold instead of 120-fold increase for mRNA, **Fig.10A**, 60 pg/ml instead of 800 pg/ml, **Fig.10B**). These data suggested that Cd14 could be involved in IL-22 modulation. As shown in **Fig.10B**, taxol, a selective TLR4 agonist, was not able to induce IL-22 production in BMDC Myd88 ko. So we can state that the residual response to LPS found in BMDC ko is mediated by Cd14 pathway. **Fig.10C** shows that stimulation with zymosan up-regulated IL-22 production in absence of Myd88 too, although

we observed a reduction in IL-22 levels (60 pg/ml instead of 150 pg/ml). This data suggests that zymosan signalling leading to IL-22 production is only partially Myd88 dependent. As shown in **Fig.10**, stimulation of BMDC with polyIC that trigger TLR3 (Myd88-independent pathway), induced the same level of IL-22 in wt and ko mice. The IL-22 levels were lower than for other TLR agonists (IL-22 mRNA 10-fold induction, IL-22 protein 30 pg/ml, **Fig.10**) suggesting that TLR3 signalling do not lead a high IL-22 production.

Additionally, we infected wt DC and Myd88 ko DC with live bacteria *L.innocua* (MOI 1:100, 1:400, 1:1000) and, to our surprise, the IL-22 production following *L. innocua* infection turned out to be totally Myd88 dependent (**Fig.11**).

Therefore, we can conclude that IL-22 production in BMDC strongly depends on Myd88 even though a Myd88-independent pathway may exist. This pathway produces lower levels of IL-22 if compared to the Myd88 signalling.

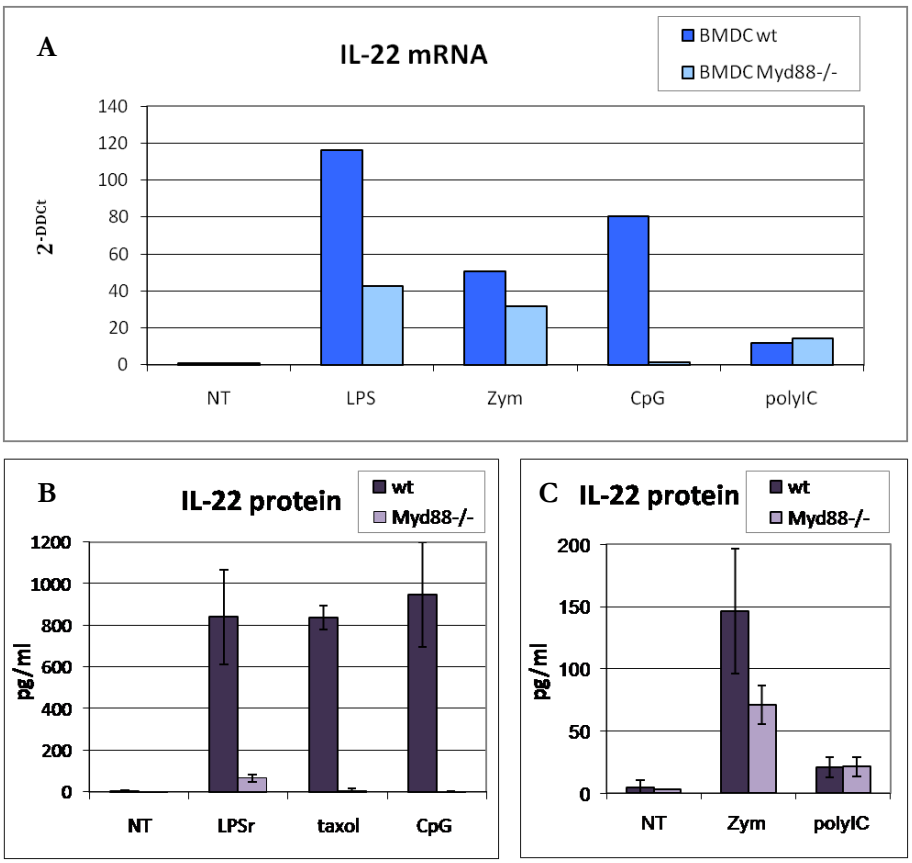


Fig.10 IL-22 production by BMDC isolated from wt or Myd88$^{-/-}$ C57BL/6 mice. BMDC were cultured at a concentration of 1.5x10^{6} cell/ml and treated for 20 h with several TLR ligands **A)** IL-22 mRNA expression in BMDC upon stimulation with: LPS (10 μg/ml), Zymosan (5 μg/ml), CpG (5 μg/ml) and polyIC (50 μg/ml). IL-22 protein levels in BMDC treated with: **B)** LPS (10 μg/ml), taxol (100 μM) , CpG (5 μg/ml) **C)** Zymosan (5 μg/ml) and polyIC (50 μg/ml). Protein data show mean values of three independent experiments ± SD. mRNA data show value of one of the three experiments as example.

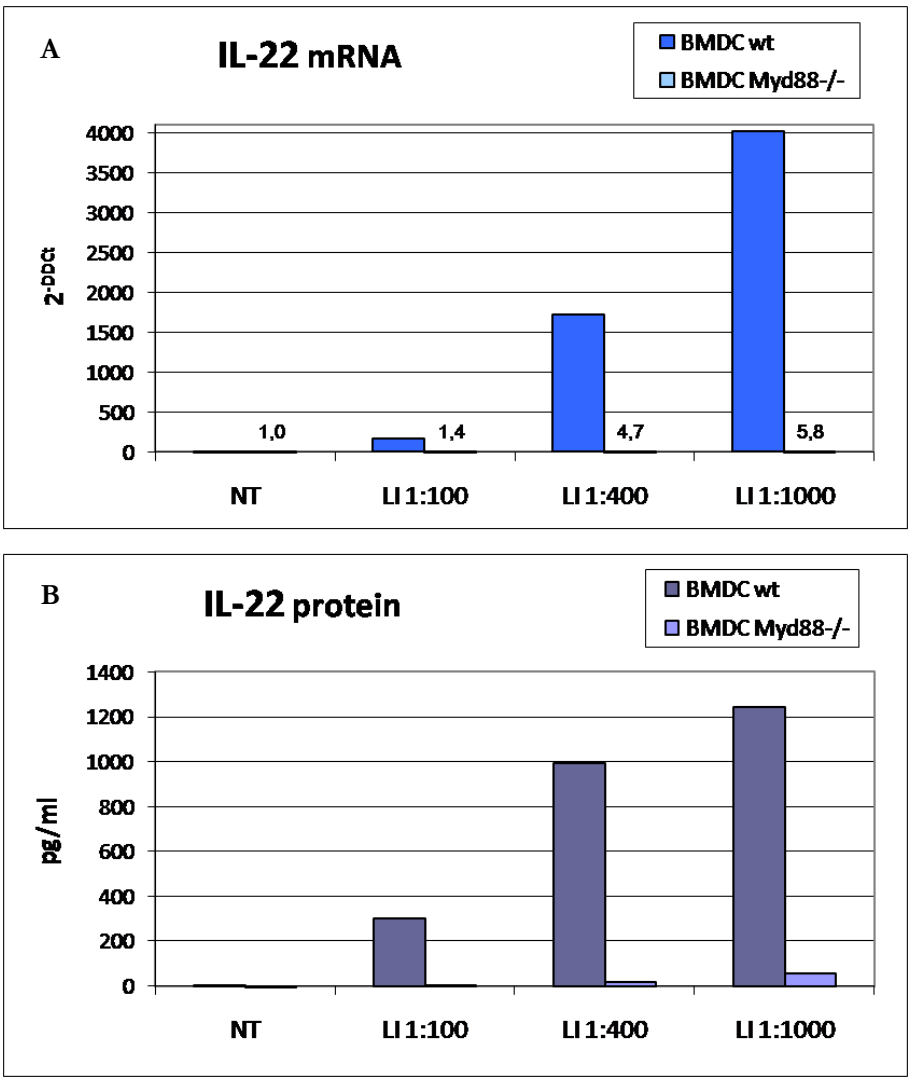


Fig.11 IL-22 production by BMDC isolated from wt or Myd88$^{-/-}$ C57BL/6 mice infected with *Listeria innocua* (MOI 1:100, 1:400, 1:1000). **A)** IL-22 mRNA and **B)** IL-22 protein were measured at 20 h post infection.

BMDC do not produce efficiently IL-22 upon IL-23 stimulation

It has been shown that IL-22 production by total splenocytes, Th17 cells, *ex-vivo* colon culture, peritoneal exudate cells and NK cells is IL-23 dependent [3,18,19]. To investigate whether LPS-induced IL-22 production was dependent on IL-23, as first, we measured IL-23 and IL-22 in the supernatant of BMDC ($1,5x10^6$ cells/ml) treated with LPS (10 μg/ml) or infected with *L.innocua* (MOI 1:500). As shown in **Fig.12A**, both the stimuli used were able to induce IL-23 production as early as 4 h post stimulation (500 pg/ml upon LPS, 180 pg/ml upon *L. innocua*). The IL-23 amounts did not correlate with IL-22 levels. In fact, as reported in **Fig.12B**, BMDC produced higher amounts of IL-22 upon infection with *L.innocua* than upon TLR4/Cd14 triggering. To verify if BMDC are indeed able to respond to IL-23, BMDC ($1,5x10^6$ cells/ml) were directly stimulated with recombinant IL-23 (20-40 ng/ml). In BMDC IL23 triggering does not induce IL-22 mRNA up-regulation, not even 20 h post stimulation (**Fig. 13A**), and leads to very small amounts of IL-22 protein (20 pg/ml, **Fig.13B**). To understand why BMDC do not respond to IL-23, we tested the expression of IL23R by qPCR (for this assay published primers were used [5]). We measured only low expression levels of IL23R in BMDC (BMDC IL-23r subunit: Ct 35,9; IL12rb1 subunit: Ct 31,1). Analysis of IL-23R expression by monoclonal antibody is

under investigation.

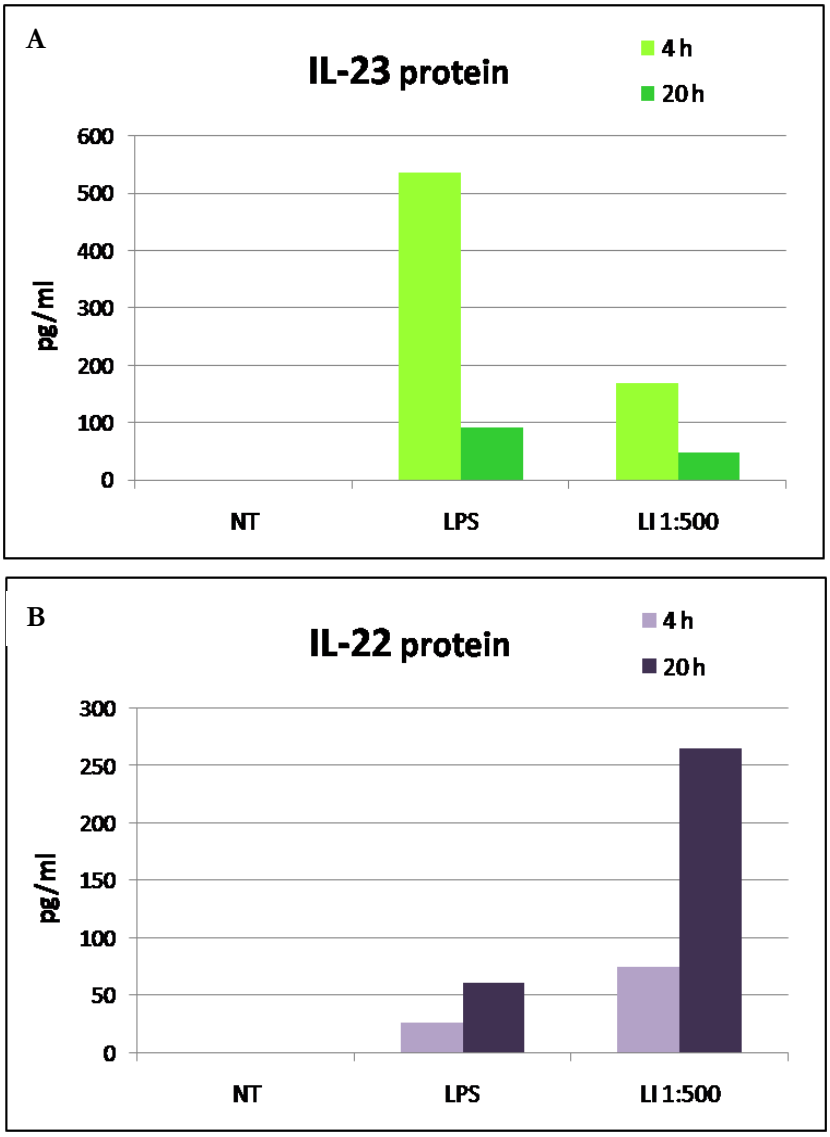


Fig.12 BMDC treated with LPS (10 μg/ml) or infected with *L.innocua* (MOI 1:500). At 4 h and 20 h post stimulation we collected supernatant and we tested by ELISA IL-23 (A) and IL-22 (B) levels.

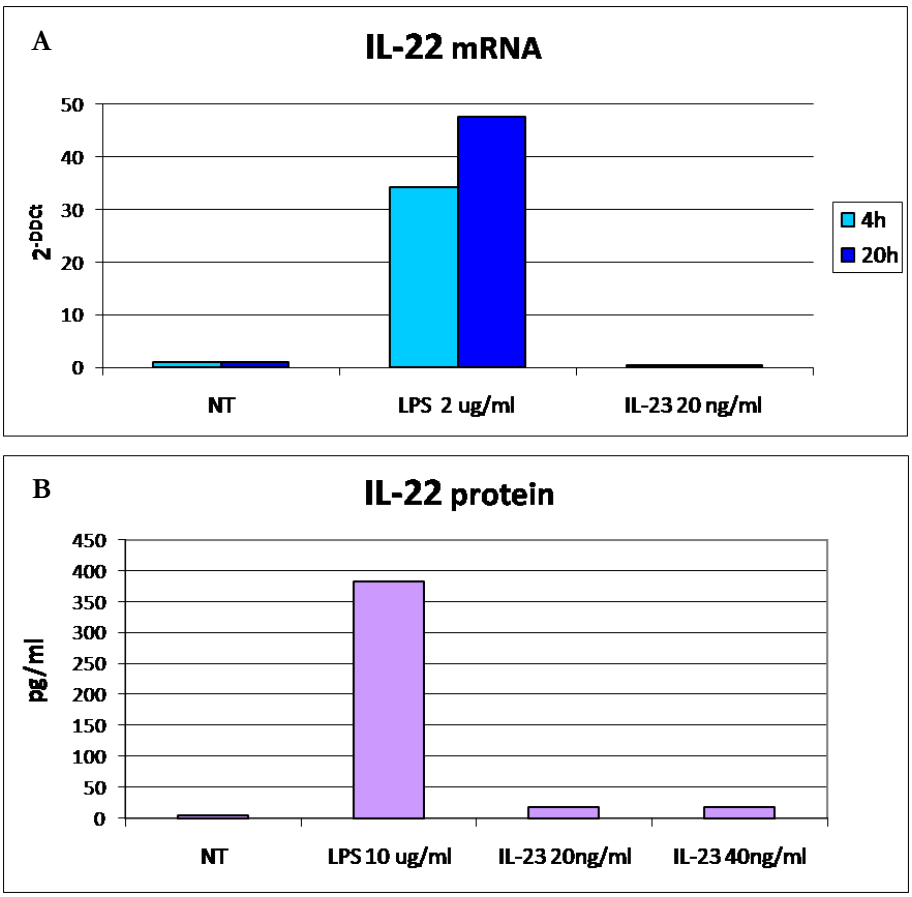


Fig.13 IL-22 production by BMDC after IL-23 or LPS stimulation as positive control. BMDC were cultured at $1.5x10^6$ cells/ml and stimulated with IL-23 (20-40 ng/ml) or LPS (2-10 μg/ml) for 4-20 h. IL-22 mRNA levels were measured both at 4 h and at 20 h**. A)** mRNA data are normalized against 18s and then expressed as fold stimulation over control (NT, not treated cells).
B) IL-22 protein levels were measured only 20 h post stimulation.

IL-22 upon LPS stimulation is induced with a very rapid kinetic

To get more information about the regulation of IL-22 production we performed a kinetic experiment. BMDC were stimulated with LPS and at different time points (30 min, 1 h, 2 h, 4 h, 20 h, 24 h) mRNA was collected (**Fig.14**). We observed that in BMDC the IL-22 mRNA expression was already up-regulated at 30 minutes post LPS stimulation (80-fold increase) and reached the maximum level within 2 h post stimulation. The up-regulation decreased at 20 h post treatment (20-fold increase) and returned to basal level at 24 hours.

To test whether new protein synthesis was required for IL-22 secretion, we used Cycloheximide (CHX) to block the protein synthesis. BMDC ($1,5x10^6$ cells/ml) were pretreated for 30 min with CHX and then stimulated with LPS (10 μg/ml). At 30 min and at 4 h post LPS-stimulation mRNA and supernatant were collected. As shown in **Fig.15**, the block of protein synthesis induced a strong reduction of IL-22 secretion in the medium. This suggested that IL-22 is not preformed in the cytoplasm but that *de novo* protein synthesis is required for its secretion. The pretreatment with cycloheximide did not impair LPS dependent IL-22 mRNA up-regulation (data not shown) suggesting that the transcription factors, necessary for IL-22 induction, were independent of *de novo* protein synthesis.

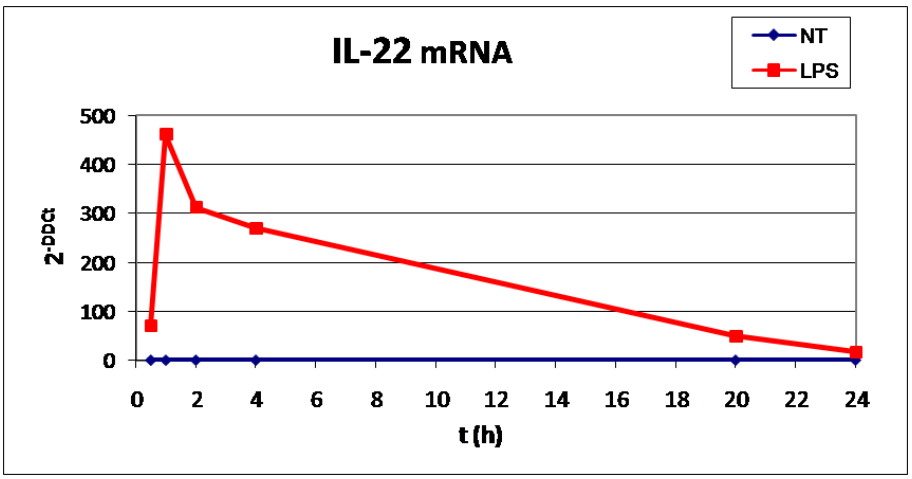


Fig.14 IL-22 induction kinetic. BMDC were stimulated with LPS (10 μg/ml) and mRNA was collected at 30 min, 1 h, 2 h, 4 h, 20 h, 24 h upon treatment.

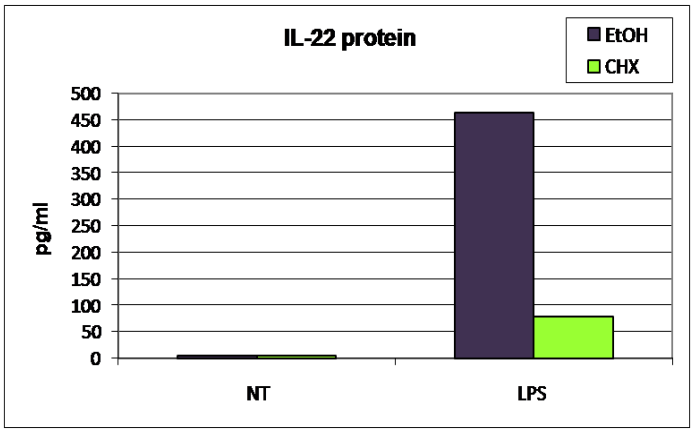


Fig.15 IL-22 induction by LPS after protein synthesis inhibition. BMDC were pretreated for 30 min with Cycloheximide (5 μg/ml) and then stimulated with LPS (10 μg/ml). At 4 h post stimulation we collected supernatant and we measured IL-22 by ELISA.

A soluble factor is involved in IL-22 induction by LPS in BMDC

We have shown that in BMDC IL-22 gene expression induced by LPS is Myd88-dependent and IL-23-independent. To better understand the mechanisms activated by LPS that lead to IL-22 production, we performed an experiment culturing BMDC in the presence or absence of DC-derived conditioned supernatant. BMDC, wt or Myd88$^{-/-}$, were untreated or stimulated with LPS (2 μg/ml) or LPS plus Polymixin (100 μg/ml) for 1 h. Then the medium has been washed out and BMDC cultured for additional 3 h in fresh medium supplemented with Polymixin in order to block hypothetical residual LPS. In **Fig.16** we reported IL-22 levels produced within 4 h by BMDC wt or Myd88 ko. We used these generated conditioned medium as stimulus for others BMDC, wt or Myd88$^{-/-}$. After 20 h we tested IL-22 levels by ELISA. The IL-22 values showed in **Fig.16**, were subtracted from the values found in the ELISA performed at 20 h. We observed that BMDC wt stimulated with conditioned medium derived from BMDC wt stimulated with LPS were able to secrete IL-22 even if at a lower level than when directly stimulated with LPS (200 pg/ml instead of 1000 pg/ml, **Fig.17**). Moreover, we showed no IL-22 induction when BMDC wt were treated with BMDC Myd88 ko conditioned supernatant. In addition, analysing BMDC Myd88 ko response, we found that they produced more IL-22 when stimulated with

BMDC wt-conditioned supernatant than when directly stimulated with LPS (200 pg/ml instead of 50 pg/ml, **Fig.18**). The data suggest that, in BMDC wt, LPS induces, through Myd88, a soluble factor that can re-signal (autocrine loop), in a Myd88-independent way, to up-regulate IL-22 (**Fig.19**).

To identify the hypothetical soluble factor we tested 23 different cytokines in supernatant of BMDC wt ($0,5x10^6$ cells/ml) stimulated for 4 h with LPS. The results are reported in **Fig.20**. We identified high amounts of TNFα, IL-6,.... We performed BMDC stimulation with TNFα, IL-12 and IL-2 but we did not detect IL-22 production (data not shown). We plan to make experiment using IL-9 because it is already known that this cytokine can induce IL-22 up-regulation [16].

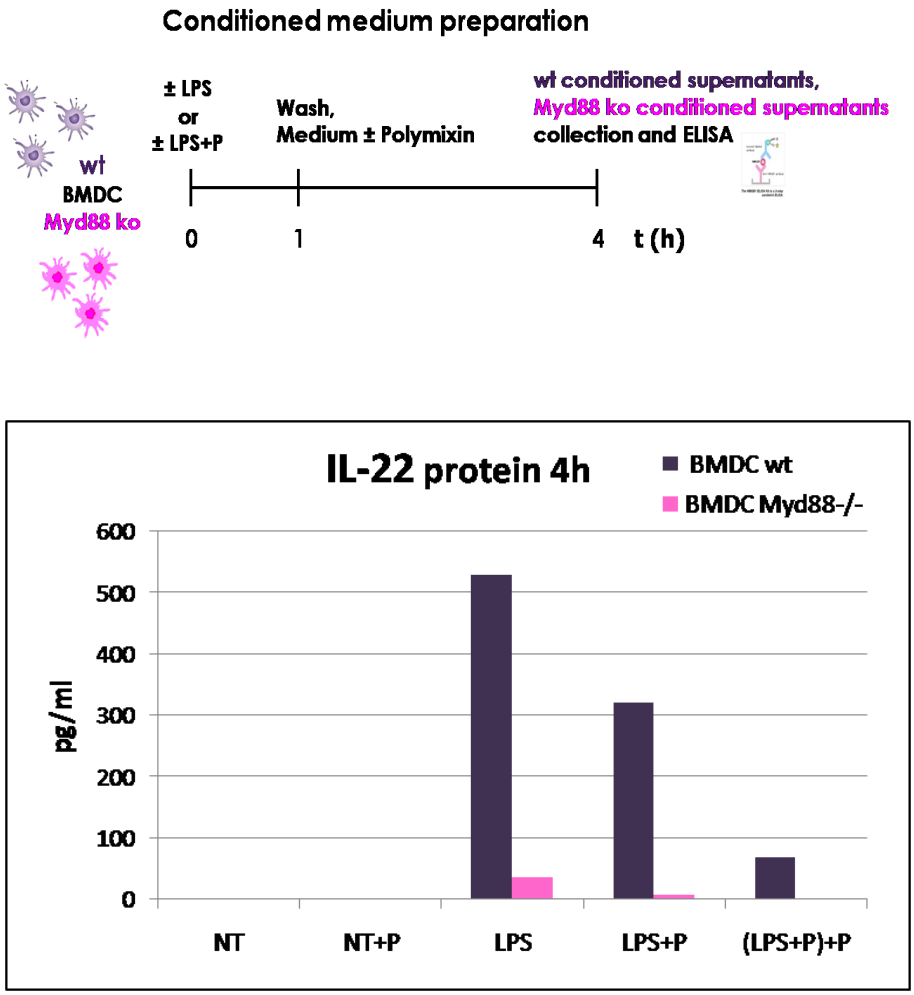


Fig. 16 IL-22 levels in BMDC wt or Myd88 ko conditioned supernatant used as stimulus for others BMDC were not treated (NT) or treated for 1 h with LPS (2 μg/ml). After several washes fresh medium plus/minus Polymixin (100 μg/ml) was added. 3 h later the supernatant were collected and tested by ELISA.

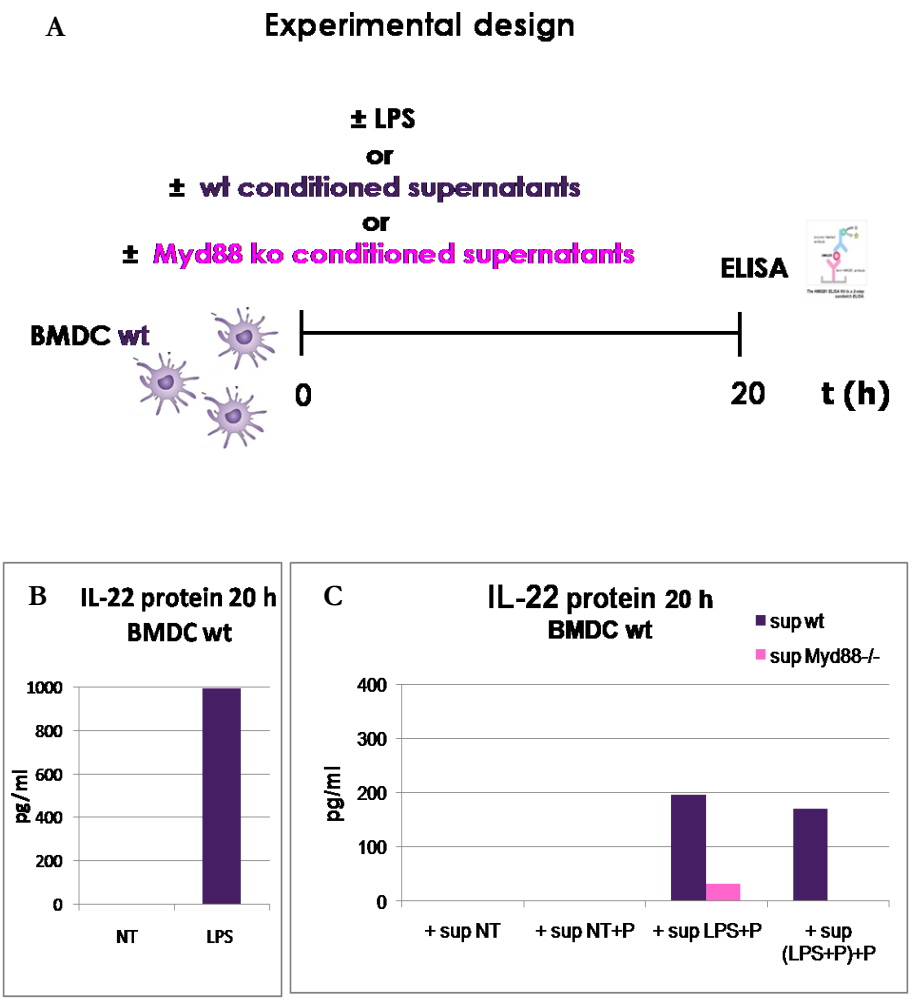


Fig.17 IL-22 production by BMDC wt under different stimuli. **A)** Experimental design. BMDC wt were treated **B)** directly with LPS (2µg/ml) or **C)** with 67% of conditioned medium derived from BMDC wt or Myd88$^{-/-}$ stimulated for 4 h with LPS (2µg/ml) ± Polymixin. For each sample we reported IL-22 level after subtraction of the IL-22 value provided by the conditioned medium used (see **Fig.16**)

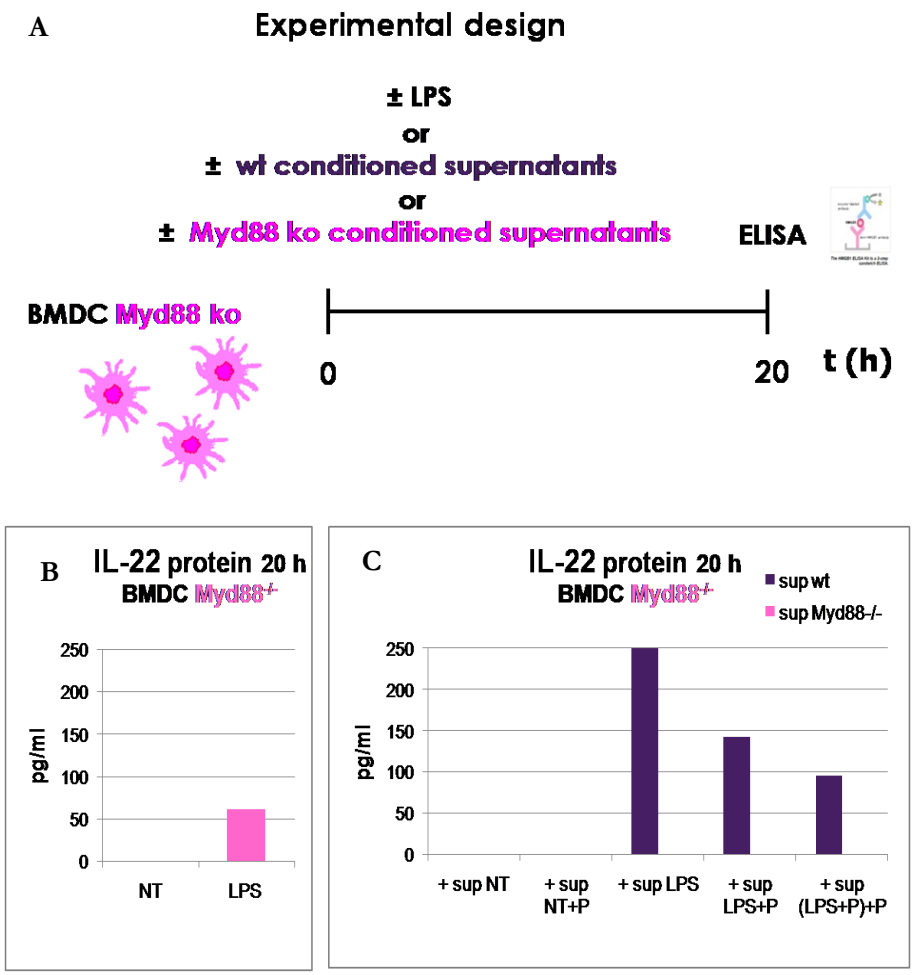


Fig.18 IL-22 production by BMDC Myd88$^{-/-}$ under different stimuli. **A)** Experimental design. BMDC Myd88$^{-/-}$ were treated **B)** directly with LPS (2µg/ml) or **C)** with 67% of conditioned medium derived from BMDC wt or Myd88$^{-/-}$ stimulated for 4 h with LPS (2µg/ml) ± Polymixin. For each sample we reported IL-22 level after subtraction of the IL-22 value provided by the conditioned medium used (see **Fig.16**)

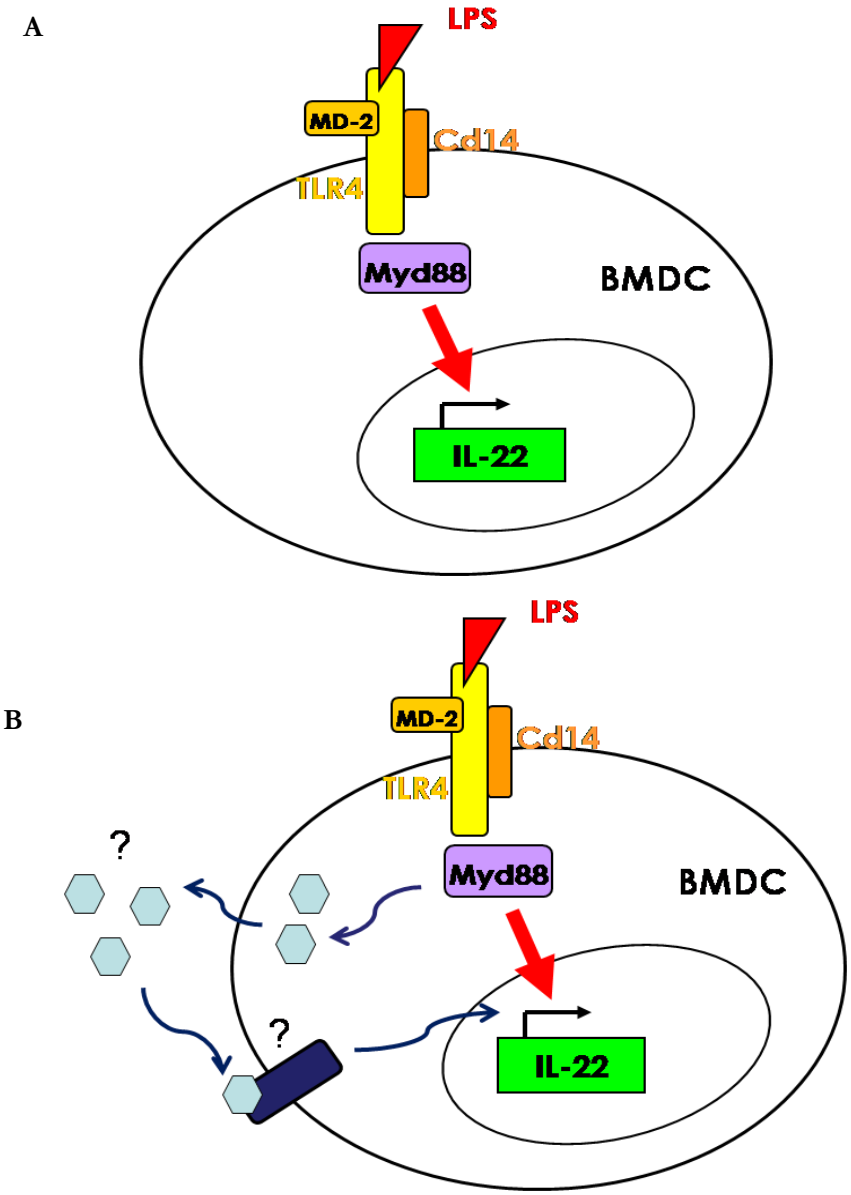


Fig.19 LPS signalling pathways involved in IL-22 production by BMDC. **A)** Main pathway: LPS directly, through Myd88, induces a strong IL-22 production. **B)** Secondary pathway: in a Myd88-dependent way, LPS induces a soluble factor that can re-signal in a Myd88-independent way to up-regulate IL-22.

	NT	LPS
	pg/ml	pg/ml
IL-3	0	5
IL-17	0	6
IL-4	0	6
IFNγ	0	8
IL-5	1	12
IL1α	1	28
IL-12p70	1	31
IL-10	1	59
GM-CSF	6	69
IL-2	1	134
IL-13	0	166
G-CSF	2	211
IL-9	0	211
IL1β	2	243
Eotaxin	0	792
Rantes	15	1057
MCP-1	307	2685
IL-12p40	31	3132
IL-6	3	3635
MIP-1α	9	4520
KC	8	4674
MIP-1β	104	5131
TNFα	0	19120

Fig.20 mouse 23-plex Cytokine/Chemokine panel tested in supernatant of BMDC ($0,5x10^6$ cells/ml) not treated (NT) or stimulated with 10 μg/ml LPS for 4 h.

IL-23 induce IL-22 production in the spleen

Given that IL-23 is involved in IL-22 induction in several cellular types, total splenocytes were isolated from C57BL/6 mice and were *in vitro* stimulated with IL-23 (20 ng/ml) for 20 h. In agreement with published data, we confirmed that wt splenocytes are able to secrete IL-22 after IL-23R engagement (1000 pg/ml at 20 h, **Fig.21**). To investigate the modulation of IL-22 gene expression induced by IL-23, we performed a kinetic experiment. After IL-23 stimulation, mRNA was collected at different time points (1 h, 2 h, 4 h, 24 h). As shown in **Fig.22**, we found a slow kinetic of IL-22 induction by IL-23. We measured IL-22 mRNA up-regulation as early as 1 h post stimulation but the IL-22 induction reached the maximum at 24 h.

Our previous data highlighted an important role for Myd88 in the signaling implicated in IL-22 up-regulation in BMDC. To verify if Myd88 was involved also in the pathway initiated by IL-23, total splenocytes ($2x10^6$ cells/ml), isolated from wt or $Myd88^{-/-}$ C57BL/6 mice, were stimulated *in vitro* with IL-23 (20 ng/ml) and after 20 h IL-22 was measured in the supernatant by ELISA. As illustrated in **Fig.23**, surprisingly IL-23 dependent IL-22 secretion by splenocytes was Myd88-dependent. These data indicate that IL-22 induction by IL-23 is indirect and it is mediated by a soluble factor that activates the Myd88 signalling pathway (**Fig.24**).

We next started to investigate the possible soluble factor responsible for IL-22 production in total splenocytes. Since IL-1b and IL-18 are known to signal through Myd88, we tested whether these cytokines were involved in IL-22 induction. Hence, total splenocytes ($2x10^6$ cells/ml) were treated for 20 h with IL-18 (20 ng/ml) or IL-1β (20 ng/ml). **Fig.25** shows we found that IL-18 and IL-1β stimulation induce IL-22 protein production, although at lower levels (about 100 pg/ml) if compared to IL-23 stimulation (about 1000 pg/ml). Therefore we can conclude that in the splenocytes IL-22 production is partially dependent on IL-1β and IL-18. Other groups demonstrated a role for IL-18, in combination with other cytokines (IL-12, IL-23, IL-15), in IL-22 production by NK cells [15]. We are currently performing additional experiments by using different cytokine combinations.

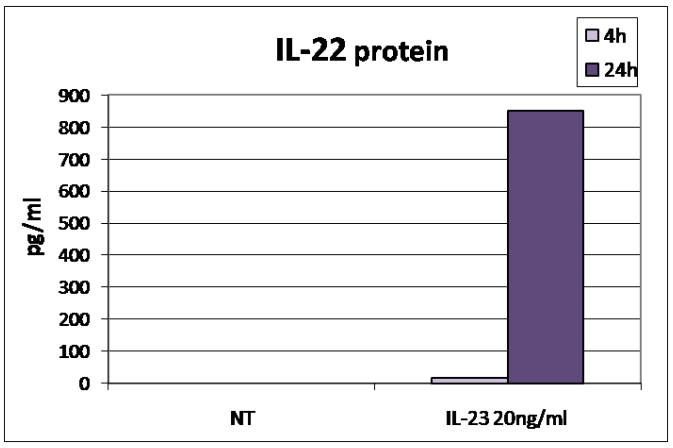


Fig.21 IL-22 secretion by splenocytes treated with IL-23 (20 ng/ml). ELISA was performed on supernatant of primary resplenocytes ($2x10^6$ cells/ml) *in vitro* stimulated for 4 h - 20 h.

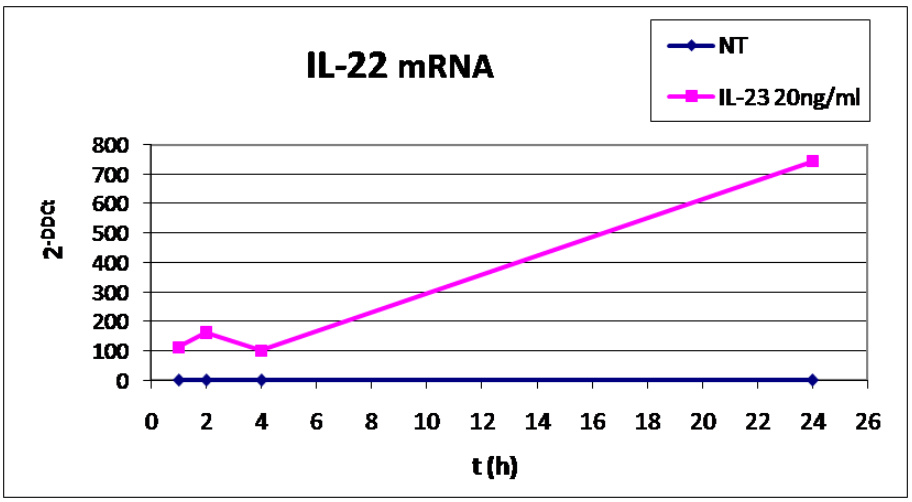


Fig.22 IL-22 induction kinetic. Total splenocytes ($2x10^6$ cells/ml) were stimulated with IL-23 (20 ng/ml) and mRNA was collected at different time points (1 h, 2 h, 4 h, 24h). mRNA data are normalized against 18s and then expressed as fold stimulation over control (NT, not treated cells).

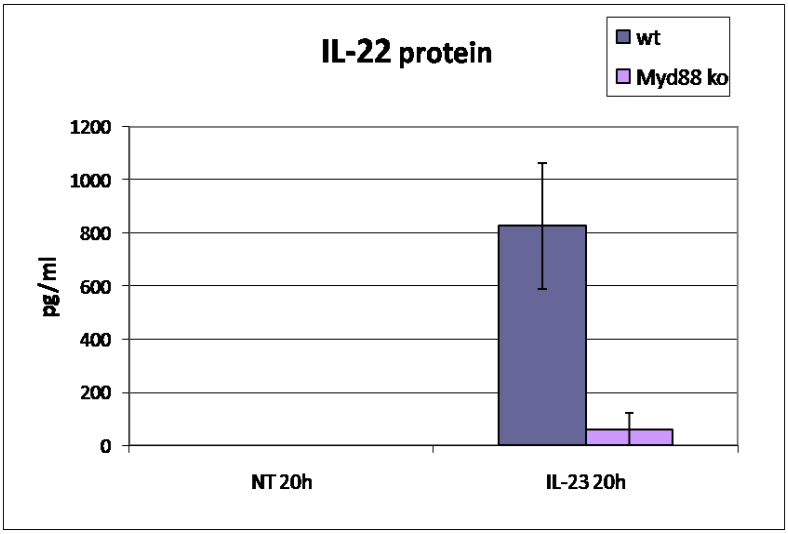


Fig.23 IL-22 secretion by splenocytes wt or Myd88$^{-/-}$ after IL-23R triggering. Total splenocytes were derived from C57BL/6 mice wt or Myd88$^{-/-}$, cultured at $2x10^6$ cells/ml and stimulated with IL-23 (20 ng/ml). After 20 h IL-22 was measured by ELISA in the supernatants. Data show mean values of five mice ± SD.

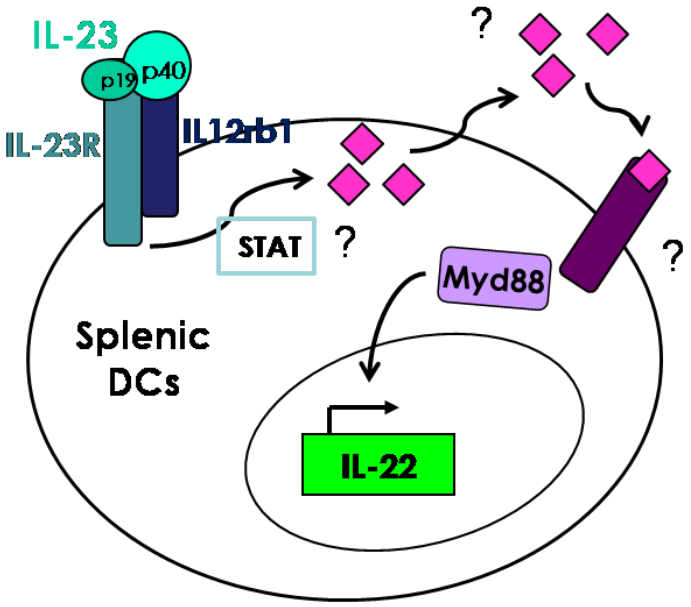


Fig.24 IL-23 signalling pathway involved in IL-22 production by splenic DCs. Indirect mechanism: IL-23, through its receptor, induces a soluble factor that then re-signals through Myd88.

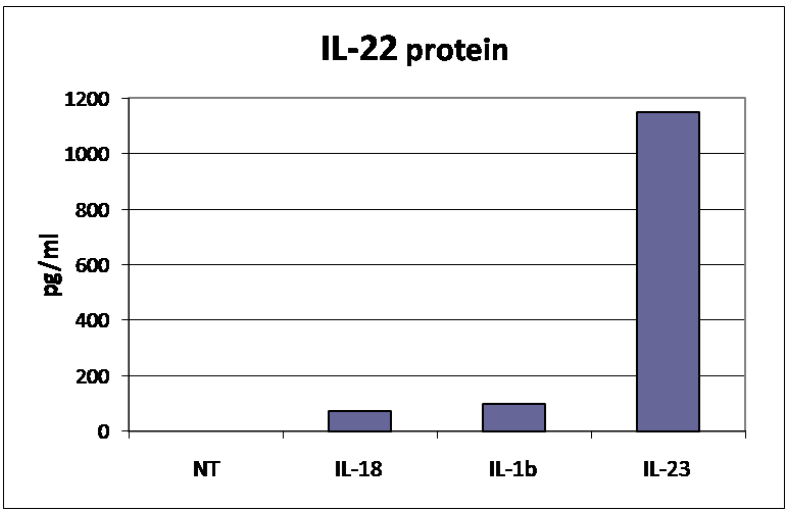


Fig.25 IL-22 secretion by splenocytes treated with different stimuli. ELISA was performed on supernatant of primary splenocytes ($2x10^6$ cells/ml) *in vitro* stimulated for 20 h with IL-18 (20 ng/ml), IL-1β (20 ng/ml) or IL-23 as positive control.

IL-22 induction by IL-23 in splenic DCs is Myd88 dependent

To determine whether IL-23 is implicated in IL-22 modulation in splenic DCs, we purified, from spleen of C57BL/6 mice wt or Myd88 ko, $CD11c^+$ (splenic DCs)/$CD11^-$ cells by magnetic beads separation as previously described. As illustrated in **Fig.26**, both cell populations, when purified from wt mice, were able to produce IL-22 following IL-23R triggering (about 1200 pg/ml) as opposed to $CD11c^+$/$CD11c^-$ cells derived from Myd88 ko mice where a strong reduction of IL-22 production was measured. Moreover, we observed that the decrease was more pronounced in the $CD11c^+$ than in the $CD11c^-$ cells (20 pg/ml and 120 pg/ml respectively). The data suggest that Myd88 has a more key role in $CD11c^+$ cells. The cellular source in the $CD11c^-$ population is under investigation since either T cells or NKcells or LTi-like cells (Lymphoid tissue inducer-like cells) may respond to IL-23 producing IL-22.

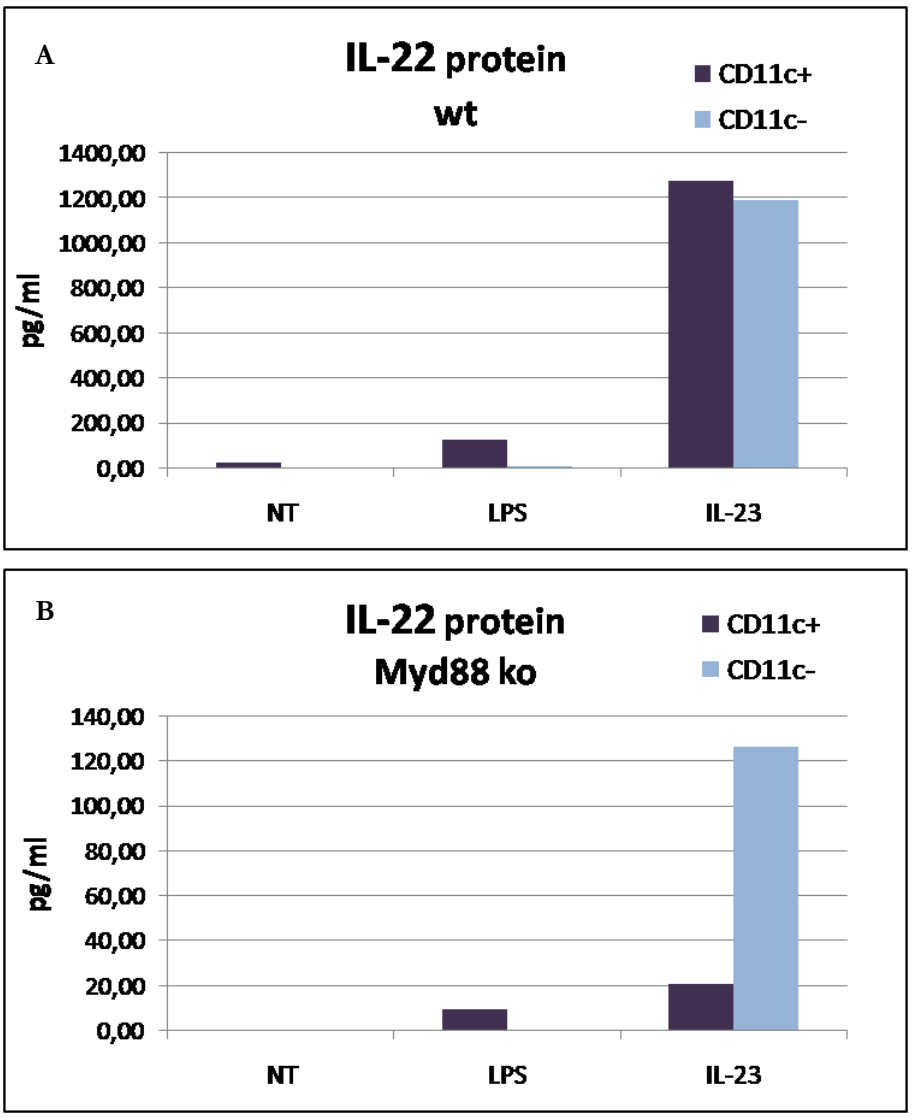


Fig.26 IL-22 production by CD11c$^+$ cells isolated from the spleen of wt (**A**) or Myd88 ko (**B**) mice. Splenic DCs were cultured at 3x10^6 cells/ml and stimulated with IL-23 (20 ng/ml) for 20 h. IL-22 protein levels were measured by ELISA. FACS analysis: CD11c$^+$ cells were >85% CD11c$^+$, >95% MHCII$^+$, <6% NK1.1$^+$.

3.1.3 IL-22 functional role

Mouse hepatocytes response to IL-22-stimualtion

Finally, we performed preliminary experiments in order to investigate the IL-22 functional role. We analysed, by quantitative Real Time PCR, the IL-22 receptor expression in different tissues or cells, to identify possible IL-22 targets. In agreement with published data [13], we found that the IL10R2 chain was expressed constitutively in different tissues (spleen, lymph nodes, liver, kidney, pancreas)/cells (D1 cells, BMDC, splenic DC, NK cells, T cells, splenocytes, hepatocytes, L929, NIH3T3) whereas IL22R1 chain was expressed only in non immune tissues (liver, kidney, pancreas)/cells (hepatocytes, L929) (**Fig.27**). Since IL22R is expressed in the liver (IL22R1 Ct= 29,8, IL10R2 Ct= 25,7), we decided to investigate the effects of IL-22 on primary mouse hepatocytes isolated from CD1 mice (see §3.3.2). A pilot microarray experiment was performed on hepatocytes ($0,5x10^6$ cells/ml) treated for 24 h with IL-22 (50 ng/ml). Microarray data were analyzed using AMDA software. DEG (differentially expressed genes) were selected with LIMMA (linear models for microarray data) method setting a threshold pvalue of 10^{-2}. 387 DEG were selected: 204 up-regulated by IL-22 and 183 down-regulated. Microarray data were expressed as Fold Change (FC) over control (Fluorescence intensity at 24h/Fluorescence intensity at 0h) and only DEG with FC over 1,5 were considered. IL-22 is

able to up-regulate 155 genes and to down-regulate 135 genes in these settings. The most interesting induced genes are presented in **Fig.28**. In agreement with previously published data [7], IL-22 up-regulated the expression of an acute phase protein (SAA-4, serum amyloid A4, 3-fold induction). Furthermore, IL-22 up-regulated the following genes: CXCL5 (chemokine C-X-C motif ligand 5; 4-fold), IL1r1 (interleukin receptor type 1; 4-fold), Hamp1/2 (hepcidin antimicrobial peptide; 3,5-fold), IL-33 (interleukin 33; 2,5-fold) and Cd14 (Cd14 antigen; 1,5-fold).

CXCL5, also known as ENA-78 or SCYB5, is a chemokine involved in inflammatory cells recruitment, especially neutrophils. Hamp1/Hamp2 are known as liver-expressed antimicrobial peptides. They are regulated by inflammatory cytokines (IL-6, IL-1β) and by LPS. They are mainly involved in innate immunity and in iron metabolism regulation. IL1r1 is a receptor implicated in several cytokine induced inflammatory response. IL-33 is a recent discovered cytokine. It was initially discovered as a nuclear factor (NF-HEV) abundantly expressed in high endothelial venules from lymphoid organs. IL-33 may act both as a cytokine and as a nuclear factor. It is reported to be able to induce T helper type 2 associated cytokines. IL-33 is induced by IL-1b and by IFNγ. Cd14 is an accessory molecule important for LPS responsiveness.

In conclusion, the microarray experiment suggests that IL-22 enhances the innate immunity activity of hepatocytes by up-

regulating the expression of inflammatory cytokines and antimicrobial peptides. This suggests that a cross-talk between hepatocytes and IL-22 secreting cells may exist in the liver.

A

	IL22R1	IL10R2
	Ct	Ct
spleen	36,00	24,65
lymph nodes	39,10	24,14
liver	29,88	25,77
kidney	29,29	22,10
pancreas	28,08	27,34

B

	IL22R1	IL10R2
	Ct	Ct
D1 cells	*40,00*	21,54
BMDC	38,24	21,45
Splenic DC	39,00	25,35
NK cells	*40,00*	23,85
T cells	*40,00*	26,06
splenocytes	38,98	23,87
primary hepatocytes	26,20	24,77
Hepa 1-6	29,50	24,95
MT2	37,37	23,08
L929	32,30	24,76
NIH3T3	*40,00*	25,27

Fig.27 IL-22 receptor mRNA expression in different murine tissue (**A**) and cells (**B**) without stimulation. qPCR raw data, threshold cycle (Ct), regarding both IL-22 receptor chains: IL22R1 and IL10R2.

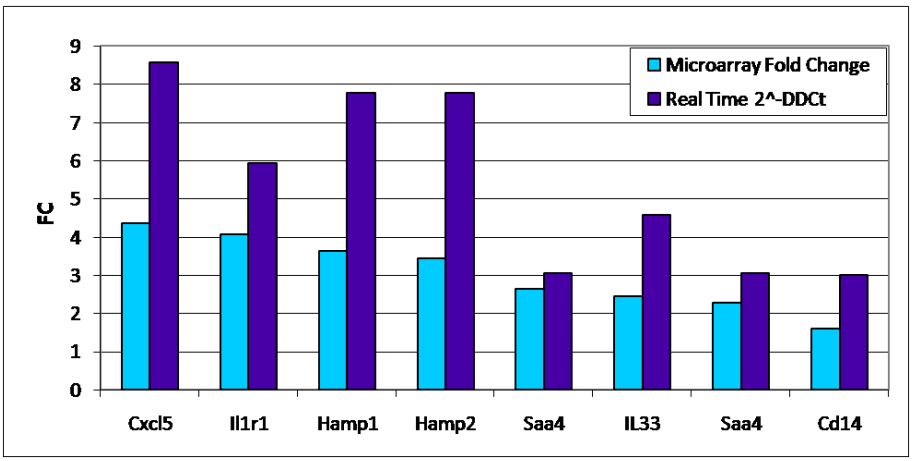


Fig.28 Mouse hepatocytes response to IL-22 stimulation.
Primary hepatocytes were derived from CD1 mice and treated *in vitro* with IL-22 (50 ng/ml). After 24 h mRNA was collected and processed for the hybridization on MOE430 A Array. The data we obtained were validated by Real Time PCR. Microarray data are expressed as Fold Change over control (Fluorescence intensity at 24h/Fluorescence intensity at 0h), Real Time data are expressed as 2^{-DDCt}.

3.2. DISCUSSION

IL-22 was discovered in 2000 by Renauld's group as a cytokine produced by murine BW5147 T-lymphoma cells stimulated with IL-9 [16]. Despite its structural relationship with IL-10, IL-22 represents a novel type of immune mediator that acts only on non immune cells. IL-22 was found to be a critical mediator of the early mucosal defence against gram$^-$ bacteria (*C.rodentium*, *K.pneumoniae*, *S.enterica*) [17-19] and increased levels of this cytokine have been demonstrated in patients with different autoimmune diseases (rheumatoid arthritis, psoriasis, Crohn's diseases) [20-22]. Prior studies have shown that IL-22 is produced mainly by T cells (Th_{17}) and NK cells [23]. Although some studies suggested that CD11c$^+$ DCs could be also a cellular source of IL-22, the data are presently controversial [18,24]. In addition, limited information is available on the stimuli responsible for IL-22 induction.
Our study provides the first evidence that DCs are able to produce IL-22 upon infection with gram$^+$ bacteria or upon TLR triggering. In this thesis, we demonstrate that Myd88 has a key role in the induction of IL-22 by several stimuli. Finally, we suggest a new role for IL-22 in the liver.

Our data show that DCs up-regulate IL-22 mRNA upon infection with different gram$^+$ bacteria (*L.innocua*, *L.paracasei*, *L.lactis*,

L.monocytogenes). *L.paracasei* and *L.innocua* up-regulate IL-22 mRNA during all the time point tested. On the contrary, *L.lactis* and *L.monocytogenes* affect IL-22 expression mainly 24 hours post infection. These data have been also demonstrated *in vivo*. It seems that a proper induction of IL-22 is very important because experiments performed using the mouse model of *L.monocytogenes* infection, have shown that IL-22 does not affect immunity against this pathogen. IL-22 deficient mice appear to have normal innate and adaptive immune responses against *Listeria monocytogenes* [25]. Recently others groups showed a protective role for IL-22 during gram$^-$ infections (*C.rodentium*, *K.pneumoniae*, *S.enterica*) [17-19]. It has been suggested that IL-22 is indispensable for the host survival during the early phase of infection and that an appropriate induction of this cytokine is beneficial to the host immune response.

In this thesis, we clearly demonstrate that mouse DCs (D1 cells, BMDC, splenic DC) induce IL-22 mRNA and secrete IL-22 protein upon LPS stimulation. In 2004, Sabat's group showed no IL-22 expression in immature or mature DCs generated *in vitro* from human blood monocytes [13]. Only few data are available on mouse DCs. Nevertheless, very recently Becker's group suggests that mouse CD11c$^+$ cells isolated from the lamina propria and stimulated with CpG are able to produce IL-22 [24].

In addition, our data show that an increase in IL-22 mRNA is correlated to an increase in IL-22 protein secretion. Performing the LPS-stimulation in the presence of cycloheximide, a protein synthesis inhibitor, we find that the up-regulation of IL-22 mRNA is not dependent on *de novo* protein synthesis. Moreover, we demonstrate that the secretion of IL-22 in the supernatant requires protein synthesis.

Furthermore, in this study we analyzed which TLR ligands are responsible for IL-22 production in BMDC. IL-22 production was detected after triggering of TLR4/Cd14, TLR9, TLR1/TLR2, TLR2/TLR6, TLR7, TLR11, TLR2/Dectin-1, TLR3 but not after TLR5 triggering. LPS and CpG seems to be the major inducers. Therefore we can conclude that TLR signalling is involved in IL-22 production. To understand which TLR contributes mainly to the induction of this cytokine, we performed experiments using ko mice. We compared BMDC derived from wt and Myd88 ko mice. Both BMDC were stimulated with LPS (TLR4/Cd14), taxol (TLR4), CpG (TLR9) and polyIC (TLR3). We show that IL-22 induction through TLR9 is completely abrogated in the absence of Myd88. In contrast, IL-22 induction by LPS is not completely abrogated in Myd88 ko cells suggesting a role for Cd14 signalling. Moreover, using taxol, a specific TLR4 agonist, we observed no IL-22 production in Myd88 ko BMDC. PolyIC, that signals in a

Myd88-independent way, induces the same amounts of IL-22 in BMDC wt and ko, even though the IL-22 produced by PolyIC is very low. These data indicate that, in BMDC, Myd88 is important for IL-22 induction but dispensable.

Published data indicate that IL-22 production by splenocytes, Th17 cells, ex-vivo colon culture, peritoneal exudate cells and NK cells is IL-23 dependent [3,18,19]. We have shown that LPS-stimulated BMDC produce IL-23, however we demonstrated that this cytokine is not important for IL-22 production, at least in BMDC. In contrast, splenic DCs ($CD11c^+$ cells) are able to secrete high level of IL-22 after IL-23 stimulation. A possible explanation may be that BMDC, *in vitro* differentiated using GM-CSF, do not express IL-23R efficiently. Instead, in splenic $CD11c^+$ cells we found higher IL-23R mRNA levels. To solve this issue, we are going to test different *in vitro* generated BMDC, for example using FLT3L (FMS-related tyrosine kinase 3 ligand) [29], to see whether we can obtain a cell population that expresses IL-23R. In addition, analysis of IL-23R expression by monoclonal antibody, in BMDC and in different splenic DCs population, will be investigated in order to define which cell populations are able to respond to IL-23 *in vitro* and *in vivo*.

In conclusion, the data obtained in BMDC suggest that it is possible to obtain IL-22 production by an IL-23 independent mechanism.

To get more information about IL-22 production regulation, we performed a kinetic experiment. We found that IL-22 induction by LPS in BMDC is very fast and it reaches the highest expression level within 2 h. This information suggests that there probably is a direct signalling pathway activated by LPS. To assess if also an indirect mechanism does exist, we carried out experiments using conditioned medium derived from BMDC, wt or Myd88 ko, stimulated with LPS. The results suggest that an indirect signalling is present after LPS-induction. In fact, conditioned medium derived from LPS-stimulated BMDC wt is able to induce IL-22, even if at low level. This effect is abrogated when conditioned medium from LPS-stimulated BMDC Myd88 ko are used. Furthermore we show that BMDC Myd88 ko produce more IL-22 when stimulated with BMDC wt conditioned supernatant than when directly stimulated with LPS.

Therefore, we conclude that, through a myd88-dependent pathway, LPS induces a soluble factor that can re-signal in a Myd88-independent way to up-regulate IL-22. However, the IL-22 amounts achieved through this indirect mechanism are lower than when the direct pathway is triggered. We suggest as hypothetical factor IL-9 because it is already known that it can induce IL-22 up-regulation [16] and because we detected this cytokine in supernatants of BMDC stimulated for 4 h with LPS. Moreover in order to identify the soluble factor we plan to measure different

cytokines in BMDC wt supernatants compared to BMDC Myd88 ko supernatants.

In this thesis, we verify that DCs produce IL-22 also *in vivo,* in a mouse model of systemic inflammation induced by LPS. We demonstrate that the *in vivo* LPS-stimulation is able to induce IL-22 protein production by splenic $CD11c^+$ cells and by $CD11c^-$ cells although at lower levels. On the contrary, the *in vitro* stimulation, of the same cell populations, by LPS affects IL-22 production only in $CD11c^+$ cells. This discrepancy between *in vivo* and *in vitro* IL-22 induction in $CD11c^-$ fraction, might reflect an indirect mechanism of gene induction by LPS in these cells. We hypothesized that, *in vivo,* LPS acts on $CD11c^+$ cells that produce a soluble factor, may be IL-23, which than re-signal on $CD11c^-$ cells making them able to produce IL-22. When the stimulation is performed *in vitro,* we have only $CD11c^-$ cells that don't respond directly to LPS; in fact $CD11c^-$ cells include mainly T cells, some NK cells, B cells and Lymphoid tissue inducer-like cells (LTi-like cells). Our hypothesis is supported by others published data indicating that IL-22 is produced by T cells and NK cells in response to IL-23 produced by APCs [3,27]. Moreover, DCs appear to be the early initiators of an innate IL-23-regulated cascade of IL-22 production in salmonellosis [19].

In agreement with published data, we confirmed that wt splenocytes are able to secrete IL-22 after IL-23R engagement. Furthermore, we demonstrated that both splenic DCs ($CD11c^+$) and $CD11c^-$ cells produce IL-22 upon stimulation with IL-23. Surprisingly, we found that also induction of IL-22 through IL-23 pathway is Myd88-dependent. Without Myd88 we have a 60-fold and a 10-fold reduction in the IL-22 production by $CD11c^+$ and by $CD11c^-$ cells respectively. Hence, Myd88 seems to be more important in $CD11c^+$ cells than in $CD11c^-$ cells.

These results indicate that IL-23 effect on IL-22 induction is indirect. We hypothesize that IL-23 induces a soluble factor that then re-signals in a Myd88-dependent way to produce IL-22. In total splenocytes IL-22 mRNA reach the higher level at 24 h upon IL-23 stimulation.

Since IL-18 and IL-1β engage the Myd88 adaptor to signal, we used these cytokines for splenocytes stimulation. We show IL-22 production even if at low level, suggesting that there may be a contribution from these cytokines. Other groups demonstrated a role for IL-18, in combination with other cytokines (IL-12, IL-23, IL-15), in IL-22 production by NK cells [15]. Therefore, experiments are going on to test the ability of different cytokine combinations to induce IL-22 production by splenocytes.

In summary, the data discussed above suggest that IL-22 may be produced through different pathways and that in different cell types there are different signalling leading to IL-22 induction. DCs are able to secrete several cytokines known to shape the adaptive immune response. The finding that DCs produce IL-22, a cytokine that affects only non immune cells, suggests that DCs are very important also in the cross-talk with non immune cells.

At present, little is known about the molecular mechanisms involved in IL-22 induction. Published data indicate that the glucocorticoid dexamethasone is able to suppress significantly IL-22 production mediated by *Staphylococcus epidermidis*; therefore, steroids may impair crucial aspects of tissue protection provided by IL-22: anti-apoptotic functions, mucosal host-defense [26]. Very recent data show that DAP10, an adaptor protein involved in NK cell activation, is required for IL-15 to induce IL-22 production by NK cells [27]. Finally, the group of B.Stokinger discovered a role for the aryl hydrocarbon receptor (AhR) in the IL-22 secretion by Th17 cells [29].

The role of IL-22 *in vivo* is controversial. This cytokine has been shown to have both anti- and pro-inflammatory roles. For example, IL-22 has a protective role during gram$^-$ infections and induces up-regulation of different antimicrobial peptides in

keratinocytes and in colonic epithelial cells [17-19, 13]. In the liver, Alber's group demonstrated that IL-22 is required for protection against hepatocyte necrosis during salmonellosis [4]. Finally, very recently published data demonstrated that IL-22 produced by NK cells inhibit the growth of *M.tubercolosis* in human macrophages. In their model, IL-22 seems to enhance phagolysosomal fusion [27]. So, in different infection models IL-22 seems to have a beneficial role because it induces a protective immune response. Nevertheless, in other contexts, a deregulation of this cytokine may be responsible for the pathological effect. Actually, increased levels of IL-22 have been demonstrated in patients with different autoimmune diseases (rheumatoid arthritis, psoriasis, Crohn's disease) and IL-22 levels were correlated with disease severity indicating the IL-22-importance in the pathogenesis of these diseases [20-22]. In fact Ouyang's group demonstrated that IL-22 produced by Th17 mediates IL-23-induced dermal inflammation and acanthosis [20]. Nojima's group proposed a proinflammatory role for IL-22 in rheumatoid arthritis [22]. High IL-22 is strongly expressed in the inflamed intestine and in the blood of Crohn's disease patients [21].

Finally, in this study we performed a preliminary experiment in order to unravel, at molecular level, the IL-22 role in primary hepatocytes. By microarray experiment, we found 387 DEG and the most relevant seems to be: Hamp1/2 that is an antimicrobial

peptide, also involved in iron metabolism, and CXCL5 important for neutrophil recruitment. We suggest that IL-22 in the liver, besides its antiapoptotic effect [31], can promote innate immune response through neutrophil recruitment and antimicrobial peptides secretion during systemic infection.

3.3. MATERIALS AND METHODS

3.3.1 Cell culture

D1 cells [8] were maintained *in vitro* in Iscove's modified Dulbecco's medium (IMDM, Euroclone) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, origin: Australia), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Euroclone) and 50 μM β-mercaptoethanol (Sigma) plus 30% R1 medium (supernatant from NIH3T3 fibroblasts transfected with GM-CSF). Cells were incubated at 37°C, 5%CO_2.

BMDC preparation Bone marrow cells from C57BL/6 mice and Myd88 ko mice were cultured in IMDM (Euroclone, Milan, Italy) supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin (all from Euroclone), 50 μM β-mercaptoethnol (Sigma), (IMDM complete medium) and 20% supernatant of GM-CSF transduced B16 tumor cells (20 ng/ml GM-CSF) [9]. Isolated cells were seeded at $6x10^6$ cells/ bacterial Petri Dish with 10 ml of grow medium (IMDM complete, 20% B16). Cells were incubated at 37°C, 7%CO_2. At day 3 10 ml of fresh medium were added, at day 6 10 ml of medium were changed. After 7-10 days of culture, cells were analyzed by Flow cytometry (FACS) for CD11c expression and used in assays when 75-85% of cells were CD11c positive.

3.3.2 *Ex-vivo* cells

Splenocytes Primary splenocytes were isolated breaking up the spleen of wt or Myd88$^{-/-}$ C57BL/6 mice. The obtained cells were cultured in complete IMDM in an incubator with 5%CO_2 at 37°C.

Dendritic cells purification DCs were purified from C57BL/6 mice. For purification DCs were positively selected from splenocytes by magnetic beads or by cell sorting.

In the first protocol, after red blood cells lysis cells were stained with anti-CD11c magnetic beads (Miltenyi Biotech). Cells were positively selected with MS columns, according to the manufacturer's recommendations. DCs obtained were 85% CD11c positive and 95% MHCII positive. In the second protocol, splenocytes, after red blood cells lysis, were depleted of T cells ($CD3^+$, TCR^+), B cells ($Cd19^+$), NK cells ($DX5^+$), granulocytes ($Gr1^+$). The remaining cells were then stained with anti-CD11c and sorted on FACScan platform. DCs obtained were 97% CD11c positive. $CD11c^+$ cells were cultured at $2{,}5x10^6$ cells/ml in complete IMDM.

Hepatocytes Primary hepatocytes, isolated from Cd1 mice, were purchased from Abcellute and reactivated following their protocol. Cells were cultured in William's medium supplemented with 2% heat-inactivated FBS, 2mM L-glutamine, 100U/ml penicillin, 100

μg/ml streptomycin (all from Euroclone). Heptaocytes were incubated at 37°C, 5% CO_2.

Where indicated cells were treated with : 20 ng/ml recombinant mouse IL-23 (eBioscience), 50 ng/ml recombinant mouse IL-22 (R&D), 5 μg/ml Cycloheximide (Sigma), 100 μg/ml Polymyxin B (InvivoGen), 2-5-10 μg/ml rLPS (Alexis), 5 μg/ml zymosan (Sigma), 5 μg/ml CpG (Primm), 100 μM taxol, 20 μg/ml polyIC, 1 μg/ml imiquimod, 100 ng/ml Pam3cys, 100 ng/ml MALP2, 50 ng/ml Flagellin, 100 ng/ml Profillin (all from AXXORA).

3.3.3 *In vivo* experiments

Seven-week-old C57BL/6 mice were injected with PBS (control), rLPS (50 μg/mouse, Alexis, serotype R515) or infected with different bacteria (*Listeria monocytogenes, Listeria innocua*). Spleen and liver were removed, at different time points, and placed in RNA later (Ambion) in order to preserve mRNA. In other experiments the spleen was isolated at 3 hours post injection and used for the isolation of $CD11c^+$ cells.

C57BL/6 wt mice were purchased from Charles River and were maintained in our animal facility at the University of Milano-Bicocca. C57BL/6 $Myd88^{-/-}$ were purchased from/were kindly provided by S. Akira. All experiments were performed using protocols approved by the University of Milano-Bicocca Animal

Care and Use Committee. Mice were housed under pathogen-free conditions and maintained on a regular 12:12 hour light:dark cycle with food and water ad libitum.

3.3.4 Bacteria

Growing conditions

Lactobacillus paracasei (Lp) B21060 strain was nicely supplied by Bracco SpA (Milano). This strain was grown, in MRS Broth (Fluka) at 37°C under agitation, to an OD_{600} of 0.8, corresponding to the exponential phase of growth.

Lactococcus lactis (Lc) MG1363 strain was kindly provided by Jerry Wells (TNO, Netherlands). This bacterium was grown in M-17 broth, supplemented with 0,5% glucose, at 30°C in a water bath to an OD_{600} of 1, corresponding to the log phase of growth.

Listeria innocua (Li) was grown in brain heart infusion (BHI, Fluka) at 37°C under agitation to an OD_{600} of 0.6 that corresponds to the exponential phase of growth.

Listeria monocytogenes (Lm) EGD pNF8 was kindly provided by Olivier Dussurget from Institut Pasteur. This strain carries the pNF8 plasmid, which contains the gene encoding the GFP protein. The replicative plasmid is maintained in the strains by growing them, under agitation, on brain heart infusion (BHI, Fluka) containing 5 μg/ml of erythromycin. *L.monocytogenes* was grown to a midlogarithmic phase (OD_{600}=0.6).

Bacteria were stored as 10% glycerol stocks at –80°C in small aliquots until use. The concentrations were quantified by plating serial dilutions on agar plates and counting colonies after growth at 30-37°C for 24-36 hours.

Infections with bacteria For *in vitro* infections, bacteria were thawed from glycerol stocks, washed in PBS, diluted into appropriate media and added onto cells at the selected multiplicity of infection (MOI). At 1 h post infection bacteria were removed and fresh medium with gentamycin (50 μg/ml) was added. The real number of bacteria was verified by plating serial dilutions of the diluted stimulus on appropriate agar plates. For intravenous infections with *Lm* or *Li,* bacteria were thawed from glycerol stocks, washed and resuspended in PBS, and 200 μl were injected in the lateral tail vein. The infection dose was controlled by plating serial dilutions on agar plates and counting colonies after growth at 37°C for 24-36 hours. Mice were injected with lethal doses of *Lm* ($1x10^6$ CFU) or with $1.4x10^8$ CFU of *Li.*

3.3.5 Microarray experiments

D1 cells were infected with different gram$^+$ bacteria: *L.paracasei* (MOI 1:1000), *L.lactis* (MOI 1:1000), *L.monocytogenes* (MOI 1:40), *L.innocua* (MOI 1:1000). We harvested 10^7 D1 cells in the immature state or after 4 h, 8 h or 24 h of stimulation. Total RNA was isolated with Trizol Reagent (Invitrogen, Life Technologies,

Karlruhe, Germany) and purified on a Qiagen RNeasy column (Qiagen, Hilden, Germany) to remove small fragments. RNA quality was assessed on an Agilent 2100 Bioanalyzer RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Only samples with intact total RNA profiles (retention of both ribosomal bands and the broad central peak of mRNA) were used for the microarray analyses. *In vitro* transcription (IVT) products were generated and oligonucleotide array hybridization and scanning were carried out according to the instructions supplied by Affymetrix (Santa Clara, CA). We used 10 μg of total RNA from each sample and T7-linked oligo-dT primers for first-strand cDNA synthesis. The fragmented biotinylated cDNA (10 μg) was hybridized onto the MOE430A GeneChip (Affymetrix), using the recommended procedures for prehybridization, hybridization, washing and staining with streptavidin–phycoerythrin (SAPE). These arrays contain 22600 probe sets that represent 14500 mouse genes.

3.3.6 Microarray data analysis

Array images were analyzed with the RMA algorithm. Samples displaying a signal ratio >3.0 for the β-actin and GAPDH probe sets were considered to be poor-quality targets and were excluded from the dataset. A single log scale normalized expression measure for each probe set was obtained from the low-level data files (CEL

files), by the robust multiarray analysis (RMA) procedure [10]. The data were subjected to Z-score-based transformation. For the selection of differentially expressed gene (DEG) we used the AMDA (Automated Microarray Data Analysis) software that is based on LIMMA (Linear Models for Microarray Data) [11].

3.3.7 Quantitative Real Time Polymerase Chain Reaction (q-Real Time PCR)

Total RNA was isolated with Trizol Reagent (Invitrogen) and purified on a Qiagen RNeasy column (Mini or micro kit, Qiagen). We followed manufacturer's recommendations for cells or for tissues. When processing tissues, disruption and homogenization step was performed using the TissueLyser (Qiagen). DNase digestion was carried out in the column during RNA extraction (RNase-free DNase Set, Qiagen). RNA quantity and quality was evaluated spectrophotometrically (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific). Generally we reverse transcribed 1 μg of total RNA with random primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative Real Time PCR (qRT-PCR) was performed on 10 ng of total cDNA from independent samples, using primer sets specific for the selected genes and the 18s housekeeping gene. qRT-PCR was carried out on a 7500 machine (Applied Biosystems), with Power SYBR Green PCR Master Mix (Applied Biosystems). Assays were carried out in duplicate. Primers were

designed with Primer3 software (http://frodo.wi.mit.edu/) and checked with other tools (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi; m-fold, http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi; IDT oligo analyzer,http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primers were validated, and only primers with an amplification efficiency of 85 to 115% were accepted (Primm srl, Italy). Primer sequences are reported in **Table 1**. The raw data (Ct, threshold cycle) were obtained with Applied Biosystems software. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = Ct_{target}-Ct_{18s}$, $\Delta\Delta Ct = \Delta Ct_{stimulated} - \Delta\Delta Ct_{not\ treated}$), using 18s as the housekeeping gene (we used HPRT1 for hepatocyte samples).

		Primer sequence 5'-3'
IL-22	F_3	GCTCAACTTCACCCTGGAAG
	R_3	TCTGGATGTTCTGGTCGTCA
IL-22R1	F_1	CTACGTGTGCCGAGTGAAGA
	R_1	GCCCAGATAACAGAGCAAGC
IL-10R2	F_1	TGGAGTGAACCCATCTGTGA
	R_1	CCAGGACGGAGACTATGAGG
18s	F_1	CGAAAGCATTTGCCAAGAAT
	R_1	AGTCGGCATCGTTTATGGTC
IL1r1	F_2	GGGCTCATTTGTCTCATGGT
	R_2	GCCGTGCATTTTATTTGGAG
Hamp1/2	F_1	TCTCCTGCTTCTCCTCCTTG
	R_1	ATGTCTGCCCTGCTTTCTTC

SAA-4	F_2	GTCAGTGGTGATGGCTGGTA
	R_2	AGGTTGTCCCGATAGGCTCT
IL-33	F_2	CTGCATGAGACTCCGTTCTG
	R_2	CCGTGGATAGGCAGAGAAGT
CD14	F_2	AACCTGGAAGCCAGAGAACA
	R_2	GTTTGAGCAGCCCAGATAGG
CXCL5	F_1	GCCCTACGGTGGAAGTCATA
	R_1	GCATTCCGCTTAGCTTTCTTT
HPRT1	F_1	CAGGCCAGACTTTGTTGGAT
	R_1	GGCTTTGTATTTGGCTTTTCC
IL23r	F_3	TGAAAGAGACCCTACATCCCTTGA
	R_3	CAGAAAATTGGAAGTTGGGATATGTT
IL12rb1	F_3	ATGGCTGCTGCGTTGAGAA
	R_3	AGCACTCATAGTCTGTCTTGGA

Table 1 Real Time primer sequences.

3.3.8 Cytokines detection

IL-22 protein levels were measured by ELISA (Bender MedSystems) according to manufacturer's instructions.

IL-23 protein levels were quantified by ELISA (eBioscience) according to recommended standard protocols.

BMDC supernatants were also analysed using Bioplex (Bio-Rad) for the detection of 23 different cytokines and chemokines (standard mouse 23-plex panel).

3.3.9 FACS analysis

To assess the maturation of BMDC and to verify the quality of purification processes, flow cytometry analysis was performed. In order to block non-specific binding of antibodies to the FcγIII and FcγII receptors we used the 2.4G2 antibody. We used the following antibodies: anti-CD11c-APC (BioLegend), anti-CD86-PE (BD Biosciences), anti-MHCII (BD Biosciences), anti-NK1.1-PE (BD Biosciences). The corresponding primary-labelled isotype control antibodies were used for staining controls. Cells were analysed on a FACScalibur (BD, Heidelberg, Germany) using the corresponding CellQuest software.

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CHAPTER 4 - CONCLUSIONS

4.1. SUMMARY

In this thesis we used a functional genomic approach to study host-pathogen interactions [1]. We analyzed the interaction from the host point of view and in particular from the dendritic cells point of view. Dendritic cells (DCs) constitute a heterogeneous group of antigen-presenting leukocytes important in activation of both innate and adaptive immunity [2].

In the first part of this thesis we explored the possibility to use dendritic cell transcriptomes to generate biomarkers of inflammation that may be useful to test DC activation *in vitro* and *in vivo*. We considered DC transcriptomes upon stimulation with different microorganisms and molecules able to induce activation (*Schistosoma mansoni eggs*, *Leishmania Mexicana promastigote*, *Listeria monocytogenes*, LPS, CpG, polyIC, Pam3cys, zymosan) or inhibition (*Schistosoma SLA*, *Leishmania Mexicana amastigote*, dexamethasone). We applied a supervised classification method, random forest algorithm, in order to identify an inflammatory signature that can describe, at molecular level, the dendritic cells status in terms of inflammation. The informative genes were selected using a training set (77 samples) and then validated on a testing set (38 samples).

The 54 predictive genes selected are able to distinguish very accurately between inflammatory and non inflammatory samples. Amongst these we found genes well known to be involved in the inflammatory process (Icam1, IL-6), as well as genes not tightly correlated with inflammation (Hdac5, Gadd45b). Surprisingly, some genes with unknown function (Txndc16, Isg15) were also selected. The diagnostic performance of the generated signature was assessed against an independent set of samples (D1 cells treated with IFNa, DEX, vitamin D, IL-10, *Lactobacillus paracasei*, *Listeria monocytogenes*, LPS, poly I:C, PAM3CYS, zymosan for 24h), by qPCR. Moreover, we validated the inflammatory signature *in vivo,* by testing the response in splenic DCs isolated from mice treated with LPS and dexamethasone. Most of the studied genes (80%), successfully characterized the activation state of splenic DCs, and differentiated the profile of these cells from that of DCs derived from untreated mice. The small number of genes in our signature allows to use simple, conventional assays, such as quantitative reverse transcriptase-polymerase chain reaction. The increasing availability of laboratory diagnosis by polymerase chain reaction has opened up new possibilities for genomic testing based on the use of genetic signatures, in routine clinical conditions.

A second major outcome from whole gene expression studies is the discovery of the molecular pathways induced in complex systems such as host-bacteria interactions [3]. Therefore, in the

second part of this thesis we analyzed dendritic cells-bacteria interactions and we focused on the identification of the specific molecular mechanisms induced in DCs upon infections. Among the 1500 genes modulated in DCs by bacteria, we have detected the up-regulation of the cytokine IL22. This interleukin has been shown to have both pro- and anti-inflammatory activity in different chronic inflammatory diseases, in mouse models of infection and in liver injury [4,5]. IL-22 was described to be produced mainly by $CD4^+$ T cells, in particular by Th_{17}, and by NK cells. Only few and controversial data are available on IL-22 production by DC. IL-22 does not seem to influence directly immune cells since the 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) [6]. Therefore, in this study we focused on how IL-22 is produced and regulated in DCs. We first demonstrated that IL-22 is produced by DCs in different *in vitro* systems (D1 cells, BMDC, splenic DCs) and *in vivo* by using mouse models of systemic inflammation and infection induced by LPS and $gram^+$ bacteria. We showed that IL-22 up-regulation by LPS reaches the maximum level within 2 h post stimulation. Instead IL-22 is induced by IL-23 in total splenocytes with a different kinetic suggesting the existence of different regulatory pathways in different cell types. Using cellular systems (BMDC and splenocytes) derived from wt or Myd88 ko mice, we established that Myd88 has

a key role in IL-22 up-regulation induced by LPS, as well as, by IL-23. We identified a Myd88-dependent and IL-23-independent production of IL-22 in BMDC upon LPS stimulation. Moreover, we found an IL-23-dependent and Myd88-dependent up-regulation of IL-22 after induction with IL-23 in total splenocytes and in splenic DCs. Finally, we performed a preliminary experiment in order to unravel IL-22 role in the liver since we could measure IL-22 receptor expression in this organ. Microarray experiment on primary hepatocytes, stimulated for 24 h with recombinant IL-22, suggests a role for IL-22 in the liver as a cytokine that promotes innate immune activation in terms of induction of antimicrobial peptides and secretion of chemokines that recruit phagocytes. The cellular source of IL-22 in the liver is currently under investigation.

4.2. CONCLUSIONS AND FUTURE PERSPECTIVES

The functional genomic approach used in this thesis generated two principal outcomes: we derived genetic biomarkers of DCs activation and we found a new cytokine (IL-22) produced by DCs upon stimulation with gram$^+$ bacteria or with TLR agonists.

We can perform an *in vitro* assay using the selected informative genes to test the inflammatory/non inflammatory property of new compounds. In addition, the approach described in this study can also be used to develop a diagnostic predictive test for the

classification of inflammatory diseases processing new data sets (microarray on healthy donor and patient).

The production of IL-22 by DCs highlighted a new role for this antigen presenting cells in innate immunity. By IL-22 production they can directly cross-talk with different non immune cells and instruct them to produce innate immunity mediators. IL-22 levels are strongly deregulated in some inflammatory-based pathologies. Therefore, a fine balance of this cytokine is required and the understanding of its regulation will allow to identify the pathway that could be targeted pharmacologically.

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