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MECHANISMS OF DENDRITIC CELL-MEDIATED NATURAL KILLER CELL ACTIVATION

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Introduction

1. DENDRITIC CELLS

Immunology has long been focused on antigens and lymphocytes, but the mere presence of these two parties does not always lead to immunity. A third party, the dendritic cell (DC) system of antigen presenting cells (APCs), is the initiator and modulator of the immune response. First visualized as Langerhans cells (LCs) in the skin in 1868, the characterization of DCs began only 25 years ago. It was known that "accessory" cells were necessary to generate a primary antibody response in culture, but it was only once DCs were identified and purified from contaminating lymphocytes and macrophages that their distinct function as APCs became apparent. Infected cells and tumours frequently lack the co-stimulatory molecules that drive clonal expansion of the T cell, the production of cytokines, and development into killer cells. DCs provide a means of solving these challenges. Located in most tissues, formally belonging to innate immunity because their receptors are germline-encoded, DC are essencial regulators of the T cell activation. Althrought other APC, such as B cells and MΦ, are able to prime T cells, those cells evolved to fulfill different tasks and are primarly focused either to antibody production or clearence of pathogens and cellular dibris, respectively. By contrast, DC specifically evolved to initiate adaptive immunity and their antigen presentation capabilities are for superior to those of B cells and macrophages (Banchereau and Steinman 1998). DCs capture and process antigens (Fig.1), and display large

amounts of MHC–peptide complexes at their surface. They upregulate their co-stimulatory molecules and migrate to lymphoid organs, the spleen and the lymph nodes, where they liaise with and activate antigen-specific T cells. All of these DC activities can be induced by infectious agents and inflammatory products, so that DCs are mobile sentinels that bring antigens to T cells and express costimulators for the induction of immunity. No other blood cell exhibits the shape and motility that give rise to the term "dendritic" cell. *In situ*, as in the skin, airways and lymphoid organs, DCs are stellate. The processes are long (10mm) and thin, either spiny or sheet-like. When alive DCs extend large, delicate processes or veils in many directions from the cell body. The shape and motility of DCs fit their functions, which are to capture antigens and select antigen-specific T cells (Banchereau and Steinman 1998). Beside the well-described role in antigen presentation and T cell activation, investigators have reported a role for DC in maintenance of tollerance (Steinman and Nussenzweig 2002) and in NK cells activation (Fernandez, Lozier et al. 1999; Lucas, Schachterle et al. 2007; Newman and Riley 2007).

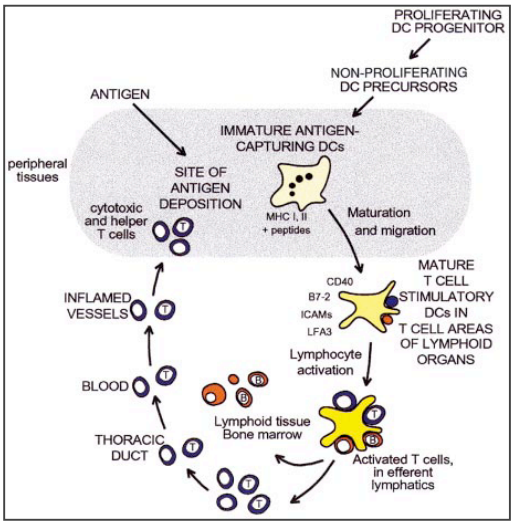

Fig.1 (from Banchereau and Steinman 1998).

1.1 DEVELOPMENT

Much of the evidence for this comes from studies on the development of DC in culture, under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF) in the presence or absence of TNF-α, IL-4, and c-kitL. To determine the dependence of the DC normally found within lymphoid organs on GM-CSF, Vremec and collegues have studied mice where GM-CSF or its receptor is absent, or where GM-CSF levels are exceptionally high; although GM-CSF can enhance DC numbers, the development of most of the DC of mouse lymphoid organs can proceed in the absence of this cytokine (Vremec, Lieschke et al. 1997).

In the presence of GM-CSF, DC can be generated either directly from a myeloid-committed precursor (that also gives rise to monocytes and granulocytes), or from a myelomonocytic

intermediate that gives rise to monocytes. Alternatively, PBMC cultured with GM-CSF and IL-4 can transiently differentiate into DC-like cells. DC can also arise from the most immature T cell precursors or bone marrow progenitors, which can also generate NK and B cells but not cells of the myeloid lineage. Interestingly, GM-CSF does not appear to be necessary for DC generation from lymphoid-committed precursors.

In addition, although DC can be generated *in vitro* using GM-CSF, the increased level of GM-CSF in GM-CSF transgenic mice does not increase the number of DC in lymphoid tissue suggesting that other growth factors are important for DC generation *in vivo* (Maraskovsky, Brasel et al 1996).

Indeed, althrough MΦ rapresent the major source of DC progenitors for the *in vitro* DC differentiation in humans, MΦ-derived DC do not represent steady-state but inflammatory DC and findings coming from GM-CSF-driven cultures might not be applied to steady-state DC. The major cytochine involved in DC development in steady state is FMS-related tyrosine kinase ligand (FLT3L) (Maraskovsky, Brasel et al. 1996; McKenna, Stocking et al. 2000).

Flt3L has been shown to stimulate the proliferation of hematopoietic stem and progenitor cells. Furthermore, *in vivo* administration of Flt3L has been shown to dramatically increase the numbers of hematopoietic progenitors in the bone marrow, peripheral blood and spleen, resulting in enhanced myelopoiesis and B lymphopoiesis. *In vivo* administration of Flt3L has a

profound effect upon the generation of functionally mature DC in multiple organs in mice. Both lymphoid- and myeloid-derived DC populations have been identified in the spleens of these mice. Furthermore, the *in vivo* administration of either GM-CSF, or GM-CSF and IL-4, or c-kitL, did not significantly increase the numbers of spleen DC, suggesting a distinct effect of Flt3L on *in vivo* DC generation (Maraskovsky, Brasel et al 1996).

DC cells can be divided following different criteria:

- **Migratory versus lymphoid-tissue-resident cDCs:** An important division of cDCs is into migratory DCs and lymphoid-tissue-resident DCs. The migratory DCs have the classical DC life history, serving as antigen-sampling sentinels in peripheral tissues, then migrating through the lymph to lymph nodes in response to danger signals; such migration to the lymph nodes also occurs in the steady state. The Langerhans cells (LCs) of the epidermis are the model migratory DCs. By contrast, the lymphoid-tissue-resident DCs have an immature phenotype and are active in antigen uptake and processing.
- **cDC subtypes:** The division of migratory cDCs is based on the peripheral tissue of origin. So, the epidermal LCs, even after arrival in the lymph node, are distinguished from the dermally derived DCs by high levels of expression of langerin. There is also a clear division between the lymphoid-tissue-resident cDCs, which is related to both surface phenotype and function, even within one lymphoid organ. In mice, lymphoid-tissue-resident cDCs can be separated into $CD8^+$ cDCs that express high levels

of CD8α on the cell surface, and $CD8^-$ cDCs that lack this marker. The $CD8^+$ and $CD8^-$ cDCs differ in immune functions, including cytokine production and the presentation of antigens on MHC class I molecules. The $CD8^-$ cDCs in turn are divisible into $CD4^+CD8^-$ ($CD4^+$) and $CD4^-CD8^-$ (double-negative) cDC subsets.

- **Inflammatory DCs:** Novel DC populations that are not found in the steady state appear as a consequence of infection or inflammation. These we term inflammatory DCs. One example is the DCs produced *in vivo* when DCs are stimulated by the influenza virus. Another example is the DCs that appear after the infection of mice with *Listeria monocytogenes*. These are termed Tip DCs as they produce tumour-necrosis factor (TNF) and inducible nitric-oxide synthase (iNOS).

  Recently, a cell type specialized in IFN-I production has been identified and called plasmacytoid DC. Developmentally, it is only loosely related to classical DC, called conventional DC, and its phisiology is quite different. Therefore, we will restrict our discussion to conventional DC. All DC develop from HSC, similarly to other blood cells (Fig.2)(Shortman and Naik 2007).

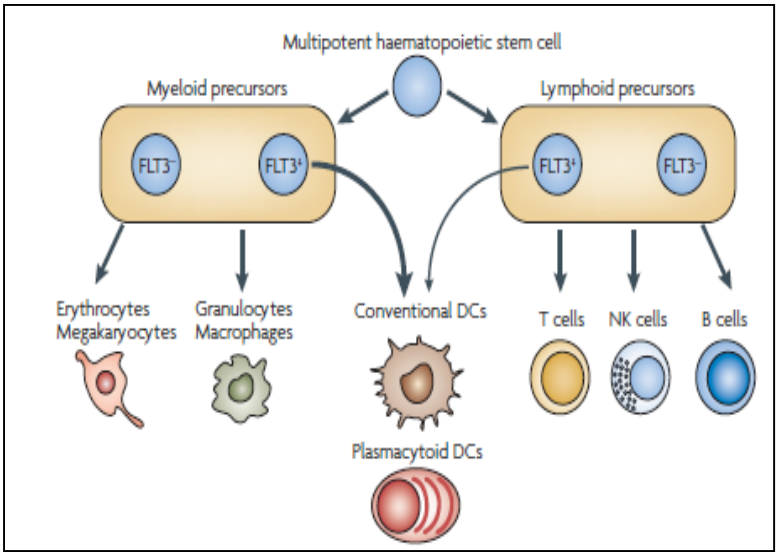

Fig.2 (from Shortman and Naik 2007): Dendritic-cell development from haematopoietic precursors.

Initially this origin was obvious, because DC cells are phenotipically and functionally very close to macrophages that have myeloid precursors. Moreover, HSC grown under the influence of GM-CSF give rise to DC, MΦ and granulocytes.

It was found that lymphoid precursors can differentiate into DC (Ardavin, Wu et al. 1993). It has been proposed that there are at least 2 classes of dendritic cells (DCs), $CD8a^+$ DCs derived from the lymphoid lineage and $CD8a^-$ DCs derived from the myeloid lineage. Here, the abilities of lymphoid- and myeloid-restricted progenitors to generate DCs are compared, and their overall contributions to the DC compartment are evaluated. It has previously been shown that primitive myeloid-committed progenitors (common myeloid progenitors CMPs) are efficient

precursors of both CD8α^+ and CD8α^- DCs *in vivo*. Here it is shown that the earliest lymphoid committed progenitors (common lymphoid progenitors CLPs) and CMPs and their progeny granulocyte-macrophage progenitors (GMPs) can give rise to functional DCs *in vitro* and *in vivo*. CLPs are more efficient in generating DCs than their T-lineage descendants, the early thymocyte progenitors and pro-T cells, and CMPs are more efficient DC precursors than the descendant GMPs, whereas pro-B cells and megakaryocyte-erythrocyte progenitors are incapable of generating DCs (Manz, Traver et al. 2001).

The intermediate DC precursor was identified in BM and found to express the inflammatory MF marker CX_3CR1 but not CD11c. However, upon transfer to irradiated or non-irradiated recipients, this precursor is able to give rise to both $CD8^+$ and $CD8^-$ DC (Fogg, Sibon et al. 2006).

More committed precursors express CD11c but not MHC-II. MHC-II expression is acquired by immature DC and is upregulated during DC maturation. Indeed, mature DC can be classified as the latest stage of DC development. Therefore, DC development is under control of both signals coming from tissue environment, as for any other cell in the body, and microbial/inflammatory stimuli, a property specific of the immune system (Diao, Winter et al 2006).

Bromodeoxyuridine labeling experiments indicated that DC subtypes have a rapid turnover (half-life, 1.5–2.9 days) in the spleen, with none being the precursor of another. Different DC

subtypes showed different kinetics of development from bone marrow precursors (Kamath, Pooley et al. 2006). DC turnover relies on homeostatic proliferation and differentiation of splenic DC precursors. It was belivied that, since splenic DC precursors are not able to self-renew, constant repleinshment from blood-borne precursors must take place, but this notion has been recently challenged by experiments with parabiotic mice (Kabashima, Banks et al 2005).

1.2 RECEPTORS

Control and elimination process of infectious pathogens involves recognition mode that, through transduction pathway, leading to the transcriptional regulation of genes of the immune response. The activation of innate immune cells (macrophages, monocytes, dendritic cells, neutrophils and epithelial cells) is mediated by germline encoded receptors, called PRRs (Pattern Recogntition Receptors), which recognize PAMPs (sugars, flagellin, peptidoglycan and lipopolysaccharides) inducing the immune response.

They may be associated with cellular membranes, such as:

- Toll-like receptors (TLRs)
- C-type receptors (clrs)

or present at cytosolic level such as:

- RIG-like helicase (LRLs)
- DNA sensors
- NOD-like receptors (NLRs)

In addition to recognizing the major classes of microbial pathogens (bacteria, viruses, yeasts, parasites) and their derivatives, they are sensors of endogenous components of inflammation, called DAMPs (Damage-Associated Molecular Patterns), such as nucleic acids, ATP and crystals uric acid resulting from damaged or dying cells.

1.2.1 TLR

Hoffman and colleagues initiated the TLR field in 1996 by observing that Toll-mutant flies were susceptible to fungal infection (Poltorak, He et al. 1998). Mammalian homologues of Toll were subsequently identified by computational alignments (Medzhitov, Preston-Hurlburt et al. 1997). In 1998, Beutler's group positionaly cloned the murine receptor for LPS, a homologue of *Drosophila melanogaster* Toll, named TLR-4 (Poltorak, He et al. 1998). Since those studies many other mammalian TLR have been identified: 10 in humans and 13 in mice (Kawai and Akira 2006). The crucial role of TLR in the immune response is highlighted by their expression on a variaty of immune and non-immune cells, such as DC, MΦ, T cells, B cells, NK cells, epitelial cells, fibroblasts, adipocytes and others.
Toll-like receptors are germ-lined encoded receptors of the innate immune system that are responsible for recognizing various invading pathogens through conserved motifs or "molecular signatures" found on the pathogen but not normally found in the host, termed pathogen-associated molecular

patterns. To date, 12 members of the TLR family have been identified in mammals, and they are responsible for recognizing pathogens as diverse as Gram-positive and Gram-negative bacteria, viruses, and fungi as well as protozoa. Upon binding their respective ligand, TLRs recruit signaling molecules to their intracellular signaling domain, leading to activation of nuclear factor B (NF-kB) and secretion of pro-inflammatory cytokines.

To date most of the TLRs have been shown to be able to recognize a particular molecular structure associated with a pathogen; TLR-4 recognizes lipopolysaccharide from Gram negative bacteria, TLR-3 senses double-stranded viral RNA, TLR-5 recognizes bacterial flagellin, TLR-7 and TLR-8 sense single-stranded viral RNA, whereas TLR-9 recognizes bacterial CpG DNA. TLR-2 though seems to be more promiscuous and to be able to recognize the most diverse set of pathogen-associated motifs including several components of Gram-positive bacteria such as peptidoglycan, lipoteichoic acid (LTA), lipoarabinomanan, lipoproteins, as well as different LPS from certain Gram-negative bacteria, yeast, spirochete, and fungi (Triantafilou, Gamper et al. 2006) (Fig.3).

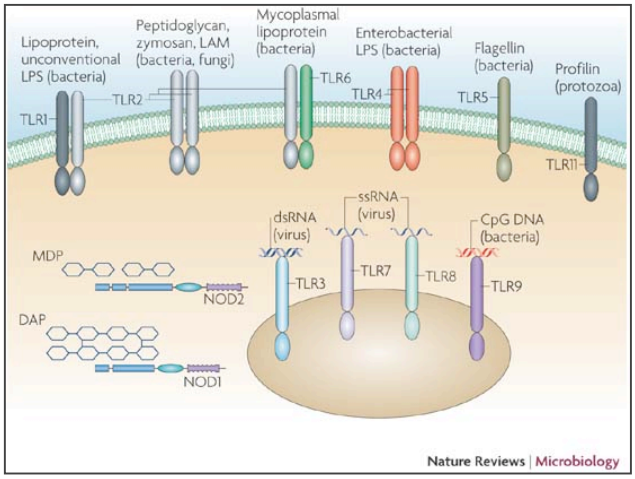

Fig. 3 (from Kaufmann 2007): TLR ligands.

All TLR share key features on both structural and signaling levels. TLRs are type I transmembrane glycoproteins comprising an extracellular, transmembrane, and intracellular signaling domain, respectively. The extracellular domains of TLRs are responsible for ligand recognition and contain 16–28 leucine-rich repeat (LRR) modules that provide the TLR ectodomains with a characteristic horseshoe-shaped folding. Ligand binding by TLR ectodomains readily triggers homo-/hetero-dimerization between TLRs, resulting in the recruitment of different adaptor proteins to intracellular TIR (Toll/interleukin (IL)-1 receptor) domains. The selective engagement of adaptors in turn defines the mode of signal transduction and, consequently, the biological outcome (Ostuni, Zanoni and Granucci 2010). The signaling cascade of TLR is iniziated by TIR domain-dependent heterophilic interaction with TIR

domain-containing cytosolic adaptors and invariably culminates in the activation of NF-kB and AP-1. Four TIR domain-containing adaptors have been described as positively mediating TLR signaling: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containig adaptor protein (TIRAP)/Mal, TIR domain-containig adaptor producing IFNb (TRIF) (also known as TICAM1) and TRIF-related adaptor molecule (TRAM) (also called TICAM2). Upon TLR activation, through its death domain, MyD88 interacts with the death domains of members of the IRAK (IL-1 receptor-associated kinase) family of protein kinases, including IRAK1, IRAK2, IRAK4 and IRAK-M. IRAK4 is initially activated, which in turn phosphorylates and activates IRAK1. It has been suggested that IRAK-M, which lacks intrinsic kinase activity, can negatively regulate TLR signaling by preventing the dissociation of IRAK4 and IRAK1 from MyD88. The function of IRAK2 remains unclear. After IRAK4 and IRAK1 have been sequentially phosphorylated, they dissociate from MyD88 and interact with TRAF6. TRAF6 is a RING domain E3 ubiquitin ligase, and together with E2, Ubc13 and Uev1A, it promotes Lys63-linked polyubiquitination of target proteins, including TRAF6 itself and NEMO. Lys63-linked ubiquitination is involved in protein interactions, activation of signaling pathways and subcellular localization. Ubiquitinated NEMO and TRAF6 subsequently recruit a protein kinase complex involving TAK1 (transforming growth factor-b-activated kinase-1) and

TABs (TAK1 binding proteins) (TAB1, TAB2 and TAB3), which then activates two distinct pathways involving the IKK complex and the mitogen-activated protein kinase (MAPK) (ERK, JNK, p38) pathway (Kawai and Akira 2006). Another MAPK, ERK, is activated in response to TLR ligands, but where the TLR signaling cascade branches out to activate ERK is obscure. JNK activation leads to the phosphorylation of c-Jun/c-Fos heterodimers comprising the transcription factor AP-1. The activation of ERK can induce the cellular levels of c-Fos, thus also upregulating AP-1 activity. Finally, the translocated transcription factors NF-kB and AP-1 direct the synthesis of the mRNA of several pro-inflammatory cytokines. (Fig. 4)

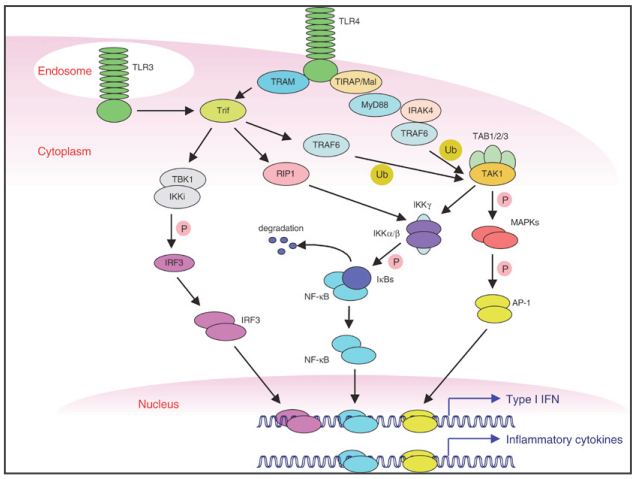

Fig.4 (from Kawai and Akira 2007): MyD88 dependent and independent TLR signaling pathway.

TRIF was identified as an essential adapter of the MyD88-independent pathway. TRIF, overexpression of which activates

IRF3 and NF-kB, is recruited to TLR3 and TLR4. TRIF-dependent signaling contributes to late phase activation of NF-kB and MAPK.

The N-terminal and the C-terminal regions of TRIF have distinct functions with regards to the recruitment of signaling molecules. The N-terminal region of TRIF activates both IFN-β and NF-kB promoters, whereas the C-terminal region activates NF-kB but not the IFN-β promoter. The N-terminal region recruits non-canonical IKKs, TBK1 (T2K, NAK) and IKKi (IKKe), which phosphorylate serine/threonine clusters present in the C-terminal region of IRF3. Phosphorylated IRF3 forms a dimer, which translocates from cytoplasm to the nucleus to induce expression of target genes including IFN-β.

In the case of TLR-4, TRIF also needs the cooperation of another TIR domain-containing adaptor, TRAM. The TIRAP-MyD88 pathway is activated earlier than the TRAM-TRIF pathway. TLR-4 engages with TIRAP and MyD88 on the cell surface. Once internalized, TLR-4 traffics to the endosomes, where it dissociates from TIRAP and MyD88, engaging and activating TRAM and TRIF (Kagan and Medzhintov 2006). When TRIF is activated by TLR-4, it induces inflammatory responses via the recruitment of TRAF6, similarly to MyD88. However, TRIF exploit also a unique mechanisms leading to NF-kB and MAPK activation (Kawai and Akira 2007). Indeed, the C-terminal region of TRIF contains a RIP homotypic interaction motif (RHIM), which mediates interaction with membranes of

the receptor interacting protein (RIP) family. In RIP1-deficient cells, TRIF-mediated production of inflammatory cytokines is abrogated, highlighting a chief role for this protein in the signaling of TRIF-dependent TLR. It is conceivable that RIP1 might cooperate with TRAF6 in facilitating TAK1 activation.

On top of activating NF-kB and AP-1, similarly to MyD88, a distintive feature of TRIF is its ability to induce the production of IFN-I and IFN-I-inducible genes, such as the chemokine IP-10 (Kawai and Akira 2006). TRIF mediates IFN-I production by recruitment TRAF3 and two non-canonical IKK, TBK1 and IKKi. These two IKK phosphorylate interferon regolatory factor (IRF) 3, that, in turn, forms a dimer and traslocate into the nucleus to induce the expression of target genes, including IFN-α/β.

1.2.2 NLRP3 AND INFLAMMASOMES

The innate immune system relies on its capacity to rapidly detect invading pathogenic microbes as foreign and to eliminate them. The discovery of Toll-like receptors (TLRs) provided a class of membrane receptors that sense extracellular microbes and trigger antipathogen signaling cascades. More recently, intracellular microbial sensors have been identified, including NOD-like receptors (NLRs). Some of the NLRs also sense nonmicrobial danger signals and form large cytoplasmic complexes called inflammasomes that link the sensing of microbial products and metabolic stress to the proteolytic activation of the proinflammatory cytokines IL-1β and IL-18.

The NLRP3 inflammasome has been associated with several autoinflammatory conditions including gout (Martinon, Mayor et al. 2009).

NALP3 (or NLRP3) is one of the most characterized NLRs, is composed of three domains:

- **LRR** (leucine-rich repeat) domain is situated at the C-terminus and is responsible for recognition and self-regulation of the ligand receptor.
- **NACHT** domain is involved in the process of oligomerization, similar to NB-ARC domain of Apaf1, a mediator of the apoptotic process.
- **PYD** (Pyrin Domain) is at the N-terminus and is required to call and activate the caspase-1, indirectly.

As a result of adequate stimulation, the NALP3 are assembled to give the inflammasome. Through PYD-PYD interaction, the N-terminal domains of the adaptor molecule, NLRP3 recruit ASC (PYD + CARD domain) and through CARD-CARD interaction with pro-caspase-1 triggers the autoproteolytic activity. The pro-caspase-1 is synthesized as a zymogen of 45 kDa, which becomes active after the cut on the C-terminal subunits (p10 and p20) and the formation of a eterotetramer (2p10 +2 p20).

The inflammasome NALP3 is activated by various mechanisms. Involvement of the P2X7 receptor ATP-dependent: a potassium channel that is assumed to activate the inflammasome using extracellular ATP under certain conditions, because the balance of the membrane potential is altered.

Implications of the channel pannexin-1: a model that can be bound or be considered independent from the activation of P2X7R ATP-dipendent, which leads the opening of this pore on the cell membrane and passage of microbial-derived particles such as MDP (Muramyl Dipeptide), by vesicles that allows them to flow into the cytosol.

Ligands particulates and crystalline material (asbestos, silica, MSU alum, microparticles of polystyrene): experiments in which the formation of ROS (Reactive Oxygen Species) is inhibited or attenuated, show that behind this event, there is phagocytosis of asbestos or silica, which results in the activation of NALP3. The other event sees the lysosomal wall rupture, caused by large crystals phagocytized, the latter shall and in the absence of exogenous material within lysosomes, an endogenous danger signal to the cell.

Some human diseases are associated with inflammasome activation, largely because of deregulation in the production of IL-1β:

- Criopirinopaties (HPFS). They are hereditary periodic fever syndromes, which occur with recurrent fever phenomena and strong inflammatory answers. Missense mutations linked to NACHT domain of NALP3 cause gain-of-function.
- Gout. Involves the alteration of the purine metabolism, with accumulation of uric acid crystals (MSU) and subsequent cellular response.
- Vitiligo. Progressive skin discoloration caused by the absence of

melanocytes, associated with mutations in the locus of NALP1.

- Auto-inflammatory diseases (such as FMF and PAPA, from alterations of the PYD of NALP3) (Martinon, Mayor et al. 2009; Keller, Ruegg et al. 2008).

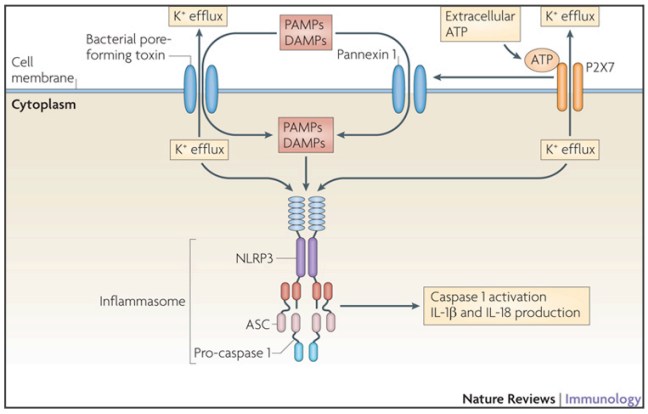

Fig.5 (from Tschopp and Schroder 2010): NLRP3 inflammasome activation.

NLRP3 is the prototypic receptor able to organize an inflammasome, but it is by no means the only one. The inflammasome assembler consists of several NLRs (MW about 700 kDa) and is responsible for activating the cysteine-aspartate protease caspase-1, responsible for the cleavage of precursor pro-inflammatory cytokines. There are three subfamilies of NLRs:

- NALPs. They are the central monomers (NALP1, NALP2, NALP3) activators of caspase-1 (the inflammasome).
- IPAF and NAIP. They have a different N-terminal domain (BIR and CARD domain), but both are involved in the inflammasome formation individually or in combination.

• NODs. They are not involved in the inflammasome formation (Martinon, Mayor et al. 2009).

In trying to understand how NLRP3 functions, parallels were initially drawn with TLR. NLRP3, as all members of the NLR family, contains a leucine-rich repeat domain, which is also the portion that binds TLR ligands, the difference between TLR and NLRP3 is that NLRP3 does not bind directly to the active molecule but due to the effects of the active molecules. This explains why the TLR only respond to specific stimuli while NLRP3 responds to a wider variety of stimuli (Martinon, Mayor 2009).

Attention has now turned to the identification of these common intracellular signals that might directly activate NLRP3. The activity of all currently known NALP3 activators is blocked by inhibiting efflux of K^+. Since little is known about activators of other NALPs such as NALP6 or NALP12, the assessment of the role of K^+ efflux in NALP inflammasomes in general is limited. The common trigger of NALP3 inflammasome activation is a decrease in physiological intracellular K^+ concentration (143mM). This notion is in line with the observation that some of the most potent activators of the NALP3 inflammasome are known K^+ channels. The microbial toxins nigericin, gramicidin, maitotoxin and a-toxin are K^+ channels and are all recognized to cause a marked depletion of intracellular K^+. ATP acts via the P2X7 receptor, which upon stimulation facilitates an immediate K^+ efflux. In addition to K^+, another intracellular compound

seems to be required for NALP3 activation, namely reactive oxygen species (ROS).

Elevated ROS levels have been previously observed in ATP-treated macrophages and cells from stressed mice, which in both cases resulted in caspase-1 activation. Although the pathway connecting ROS to inflammasome activation remains un-known, a downstream role of MAPK (p38 or ERK) was suggested (Petrilli, Papin et al. 2007). Molecules such as ROS or those released from rupturing lysosomes could well be one of the missing messengers linking the particulate stimuli with activation of the NLRP3 inflammasome, via a mechanism not requiring any direct molecular interaction between NLRP3 and the particulates (Dostert, Petrilli et al. 2008; Hornung, Bauernfeind et al. 2008).

In the case of both ATP and toxins, it seems that NLRP3 activation is friggered by the intermediary effect of massive K^+ efflux (Petrilli, Papin et al. 2007). While the release of intracellular ROS and K^+ efflux might appear unrelated, is noteworthy that all the conditions known to activate NLRP3 are related to perturbations of cellular homeostasis. Therefore, NLRP3 is an effective sensor of cellular stress. (Fig.6)

The NLRP3 inflammasome, however, does not act in isolation. It is unresponsive to its known triggers unless licensed by NF-kB activating receptors, such as TLR, nucleotide oligomerization domain (NOD) or certain cytokine receptors (Bauernfeind, Horvath et al. 2009). This requirement for a double step to

induce IL-1 family cytokine release has long been explained, at least in the case of the non-constitutive precursor of IL-1β, by a first stimulus (usually LPS) necessary for the transcription of proIL-1 β followed by a second signal controlling its release (Hogquist, Nett et al. 1991).

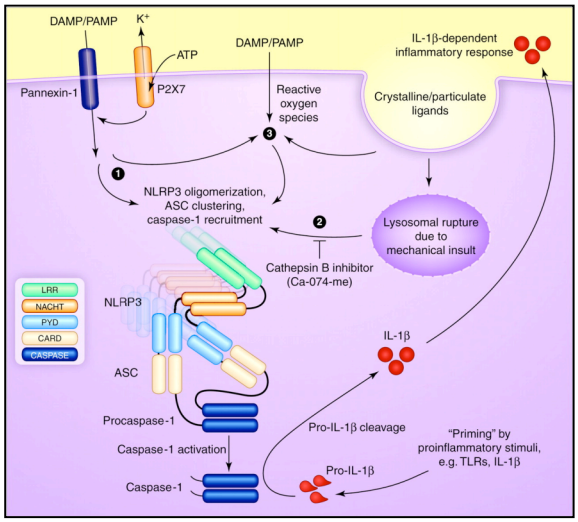

Fig.6 (from Schroder, Zhou et al. 2010): Current models for NLRP3 activation.

The correlation between TLRs and NLRP3 is more complex. The cleavage of pro-IL-18 (IL-18 precursor) NLRP3-mediated, requires activation of NF-kB and of the inflammasome. Moreover, in addition to the role of TLRs as transcriptional regulators of cytokine precursors, most likely the TLRs are required for direct activation of the inflammasome. This has affected Bauernfeind and colleagues who identified NLRP3 itself as the limiting factor in the activation of NLRP3 inflammasome. In fact, increasing the expression of NLRP3, NF-kB primes the inflammasome and makes it responsive to stimuli activating

(Beuernfeind, Horvath et al. 2009).

1.2.3 OTHER RECEPTORS

NLR (nucleotide-binding domain, leucine-rich repeat–containing) proteins have rapidly emerged as central regulators of immunity and inflammation with demonstrated relevance to human diseases.

Much attention has focused on the ability of several NLRs to activate the inflammasome complex and drive proteolytic processing of inflammatory cytokines; however, NLRs also regulate important inflammasome-independent functions in the immune system. NLRs can be categorized into functional subgroups that regulate

other crucial innate immune pathways, such as canonical and noncanonical nuclear factor kB (NF-kB), mitogen-activated protein kinase (MAPK), type I interferon (IFN), cytokines, chemokines, and reactive oxygen species (ROS) as well as ribonuclease L (RNase L) activation.

NOD1 and NOD2 were two of the first characterized members of the NLR family. NOD1 and NOD2 respond to the bacterial peptidoglycan–derived molecules meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively. NOD1 can also be activated by mesolanthionine, another peptidoglycan-associated diamino-amino acid. N-glycolylated MDP, which is made by mycobacteria and actinomycetes, is substantially more potent in its ability to elicit NOD2-dependent activation of NF-

kB than Nacylated MDP, generated by typical Gram-positive and Gram-negative bacteria (Ting, Duncan et al. 2010)

The signaling linked to the activation of the NOD receptor leads to the activation of NF-kB and MAPK. After ligand binding, NOD1 and NOD2 self-oligomerize and recruit the protein kinase RIP2, which converges at the level of TAK1 activation with the TLR pathway. The activation of TAK1 results in the nuclear translocation of NF-kB and AP-1. In the case of NOD receptors, the signal through MAPK-AP-1 also requires the involvement of CARD9 (Hsu, Zhang et al. 2007).

The RLR family has three members: retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP-2). These cytoplasmic proteins all share a central DExD/H-box RNA helicase domain. RIG-I and MDA5 also have two N-terminal caspase activation and recruitment domains (CARDs). CARDs allow for the interaction of activated RIG-I or MDA5 with the adaptor protein mitochondrial antiviral signaling which localizes to the outer mitochondrial membrane. RIG-I is indispensable for IFN responses to many single-stranded RNA viruses. These include negative-stranded viruses of the orthomyxovirus (such as influenza A virus) and paramyxovirus (such as measles, mumps, and Sendai virus) families and positive-stranded viruses like hepatitis C.

MDA5 is essential for protection from a different set of viruses, including picornaviruses (such as poliovirus and

encephalomyocarditis virus). Little is known about virus sensing by LGP2, which may instead primarily play a regulatory role. The virulence of some viruses, including some strains of influenza A virus, is due at least in part to a dysregulation of the innate immune response. MDA5 has not been studied in such detail as RAG1 and its natural ligands are not so known. However, it's known that the polyI:C, a TLR-3 ligand, activates MDA5 but when shortened to less than 1 kb becomes a RIG1 ligand (Kawai and Akira 2009). Therefore, understanding how RLRs become activated may allow the development of new

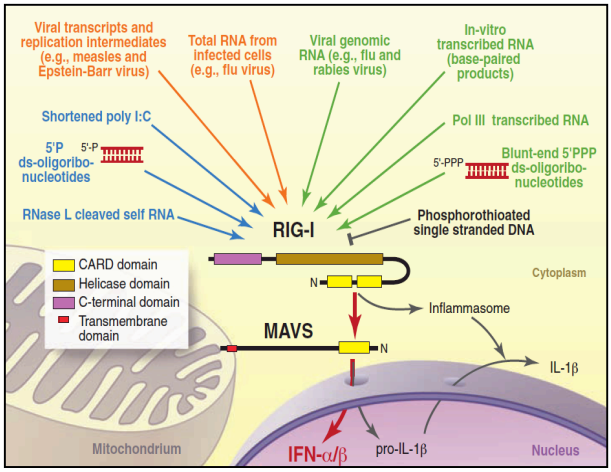

Fig.7 (from Rehwinkel and Reis e Sousa 2010): Putative RIG-I ligands.

strategies for the containment of viral spread and prevention of disease, as well as help to understand the basic principles underlying self/virus innate immune discrimination (Fig.7) (Rehwinkel and Reis e Sousa 2010).

To engage downstream pathways after recognizing a virus,

RLHs form homotypic CARD-CARD interactions with the adaptor protein MAVS, which results in the recruitment and activation of further signaling molecules to mitochondria-associated complexes. The adaptors TRAF3, TRAF family member-associated NF-kappa-B activator (TANK) and TNFR1-associated via deth domain (TRADD) and the kinases TBK1 and IKKi are responsible for activation of the transcription factors IRF3 and IRF7 and subsequent synthesis of type I interferon. RLHs additionally activate the proinflammatory NF-kB pathway for the production of cytokines such as interleukin 1β (IL-1β) and IL-6 via CARD9 and Bcl-10 (Poek, Bscheider et al. 2010). RIG-1 has also been implicated in the indirect recognition of dsDNA. Several reports pointed to the existence of a cytosolic promoting IFN-I production upon recognition of dsDNA or its syntetic equivalent polydA:dt, but the identity of such receptor has long remained elusive (Ishii, Coban et al. 2006; Stetson and Medzhintov 2006).

PolydA:dt cannot be sensed directly by RLH, it is transcribed by cytosolic RNA polimerasi III into an uncapped RNA that triggers RAG-I leading IFN-I secretion. It seems that RIG-I is not implicated instead in the secretion of mature IL-1 in response to polydA:dT; the non-NLR absent in melanoma 2 (AIM2) inflammasome has been implicated in this process (Hornung, Ablasser et al. 2009).

Confusingly, RIG-I/MDA5 can form active inflammasomes in response to RNA viruses; since polyda:dt is sensed by RAG-I

through an RNA intermediate; it is not clear why RIG-I has no implication also in polydA:dT-driven IL-1β release (Poeck, Bscheider et al. 2009).

Another important class of PRR are C-type lectin receptors (CLR). This family is specialized in the recognition of a wide range of carbohydrate structures and comprised both soluble and membrane-bound receptors. CLR can elicit inflammatory responses, as TLR, NLR and RLH. A distintive features of CLR is that many members of this family mediate scavenging functions, promoting receptor-mediated phagocytosis or endocytosis of microbes and dead cells. CLR include, among others, the MΦ mannose receptor (MMR), Dectin-1, Dectin-2, CLEC4E, CLEC9A, CD205, DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), blood dendritic cell antigen 2 (BDCA-2), and Langerin. They all possess at least one carbohydrate recognition domain and bind sugars in a Ca^{+}-dependent manner (Geijtenbeek and Gringhuis 2009).

The MMR are endocytic and phagocytic receptor that binds carbohydrate and several pathogens, in particolar viruses, fungi and mycobacteria. The high mannan content in the bacterial cell envelope of both Gram-positive and Gram-negative bacteria ensures efficient recognition by the MMR. In addition it binds endogenous molecole such as neutrophil-derived myeloperoxidase, tissue plasminogen activator and lisosoma hydrolases (Allavena, Chieppa et al. 2004).

DC-SIGN is a type II trans membrane protein able to recognize

microbial and endogenous ligands, such as the HIV-1 gp120 envelope glycoprotein and ICAM-2 and ICAM-3 on T cells (Geijtenbeek, Torensma et al. 2000). DC-SIGN cannot mediate HIV-1 entry but rather functions as a unique HIV-1 trans receptor facilitating HIV-1 infection of CD4/CCR5-positive T cells; DC cells instead carry DC-SIGN-bound HIV-1 through the lymphatics (Geijtenbeek, Kwon et al. 2000).

Dectin-1 (or CLEC7A) is a small type II transmembrane receptor containing one lectin-like carbohydrate recognition domain, which recognizes 1,3- and/or 1,6-linked glucans, intact yeast, an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail and a huge variety of fungal pathogens (including *Candida albicans* and *Aspergillus fumigatus*). The receptor is expressed at high levels on macrophages and neutrophils, and to a lesser extent on dendritic cells and a subpopulation of T cells. The commonly used stimulus zymosan, a preparation of yeast cell wall, is detected by both TLR-2 and Dectin-1 (Brown, Taylor et al. 2002).

Recently, the signaling events downstream of CLR have attracted interest. From a biochimical perspective, CLR can be distinguished in receptors coupling to ITAM-containing adaptors, such as FcRγ and DAP12, and receptors inducing signaling pathway through the activation of protein kinases or phosphatases that either directly or indirectly interact with their cytoplasmatic domains. For exemple, Dectin-2 and CLEC4E

belong to the first group, whereas Dectin-1 and DC-SIGN lie in the second group. According to a different criterion, CLR members can be grouped in receptors that are autonomously able to modulate the gene expression profiles and receptors that need the cooperation of other PRR, particularly TLR. Representative exemple are Dectin-1 and DC-SIGN. The crosstalk between TLR and DC-SIGN require the prior activation of NF-kB by TLR. DC-SIGN engagement activates the Ser/Thr kinase RAF1 through a complex cascade involving the protein family Ras, Rho, p21-activated kinase (PAK) and Src. RAF1, in turn, induces the phosphorylation of the NF-kB subunit p65 at Ser276. Such phophorylation seems to increase the transcriptional rate of NF-kB for delected genes, but does not promote NF-kB nuclear translocation, providing a biochemical finding that explains why TLR cooperation is essential (Geijtenbeek and Gringhuis 2009).

Dectin-1 is able to directly activate NF-kB via the spleen Tyr kinase (Syk)-CARD9-Bcl-10 cascade. The same pathway is exploited by Dectin-2, CLEC4E and CLEC9A, a described member of the CLR family in the recognition of necrotic cells (Sancho, Joffre et al. 2009).

2. NATURAL KILLER CELLS

NK cells are innate lymphocytes capable of recognizing and killing target cells and producing inflammatory cytokines, such as IFN-γ. In concert with other members of the innate response, NK cells are important for the initial control of many viral and bacterial pathogens. Unlike adaptive T and B lymphocytes, NK cells do not somatically rearrange their receptor genes, but rely upon a finite number of germ line-encoded inhibitory and activating NK receptors capable of recognizing MHC class I and class I-like molecules.

The existence of immunological memory in NK cells has recently been suggested in a model of chemical hapten-induced contact hypersensitivity; however, the precise mechanism and identity of the antigen-specific receptors responsible for mediating the recall responses were not defined (Cooper, Elliott et al. 2009; Sun, Beilke et al. 2009).

2.1 FUNCTIONS

Natural killing activity was discovered in 1973, before the discovery of the cells involved in this process. Lymphocytes of animals immunized or not immunized showed no difference in the cytotoxic response and the same was observed in healthy individuals compared with individuals with cancer. Soon after it was discovered that this activity depended on a specific cell subset consisting of granular lymphocytes capable of developing cytotoxic responses in a spontaneous. These cells

took the name of natural killer cells (Tagasugi, Mickey et al. 1973; Kiessling, Klein et al. 1975).

Years later, the existence of NK cells as cells phenotypically and functionally distinct from B cells and T cells was finally accepted. Studies on these cells were concentrated initially on their anti-tumor ability because of the circumstances of their discovery. The most important discovery in the field was the correlation between NK cell activity to resistance to tumor growth *in vivo*, especially when NK cells are grown in culture with high doses of IL-2, resulting in cell lymphokine-activated killers (LAK) (Cerwenka and Lanier 2001; Grimm, Mazumder et al. 1982).

NK cells, however, have different functions and their role is not only limited to the monitoring of tumor immunity, they are also involved in immunity against pathogens. Some examples of the role of NK cells in the battle against bacteria and protozoa have been published and attention has been devoted to the study of their contribution to anti-viral immunity where is very important the "spontaneous" (by innate immunity) or "induced" (by adaptive immunity) cytotoxic activity (Vivier, Tomasello et al. 2008; Biron, Nguyen et al. 1999).

NK cells have cytolytic granules, lysosomes containing mostly perforin and granzymes. After recognizing the target, these granules migrate into the area of contact with the possible target and fuse with the cell membrane, releasing their contents that induces lysis of the target cell. In addition, NK

cells are able to induce cell death via FasL, TNF and TRAIL. The functions of NK cells are not restricted to the cytotoxic activity, NK cells are in fact one of the main source of IFN-γ, which is the most important cytokine against viruses and bacteria. The IFN-γ also promotes the slope of $CD4^+$ T cells towards the Th1 lineage and activates macrophages. NK cells may secrete other cytokines such as IL-22, IL-3, GM-CSF, CSF-1, TNF-α, IL-5, IL-13, IL-10, and chemokines such as MIP-1, RANTES and IL-8 (Martin-Fontecha, Thomsen et al. 2004; Boehm, Klamp et al. 1997; Cooper, Colonna et al. 2009).

Consistent with their function as innate sentinels, NK cells are wide-spread throughout lymphoid and nonlymphoid tissues. In most tissues, NK cells represent a minor fraction of total lymphocytes (from 2% in mouse spleen to 10% in mouse lung and from 2% to 18% in human peripheral blood) (Vivier, Tomasello et al. 2009). NK cells were also found in the lymph nodes in both human and murine homeostatic conditions and in inflammatory conditions where they exert immune-regolatory functions (Ferlazzo, Pack et al.2004).

NK cells have a variety of activators and inhibitors receptor that can be activated simultaneously upon interaction with target cells. The balance between positive and negative signals transduced by these receptors controls the response of NK cells. For this reason, understanding the function of receptors in these cells is very important for potential therapeutic applications designed to use the potential of NK cells.

2.2 RECEPTORS

NK cells, like all cells of the immune system, have the primary function of discriminating self and non-self entities. Unlike T and B cells, NK cells for recognition rely on a vast repertoire of surface receptors rathen than a single receptor. The NK cell receptors are germ-line encoded and do not undergo somatic cell genetic rearrangement. Many of the NK cell receptors are also expressed on the surface of other cells such as $\gamma\delta$ T cells or NKT cells. According to the transmitted signal, the NK cell receptors can be divided into inhibitory receptors or activating receptors (Steele, Oppenheim et al. 2000; Ogasawara and Lanier 2005).

2.2.1 INHIBITORY RECEPTORS

Initially it was thought that NK cells recognize their targets in a MHC-independent manner, but they showed the ability to lyse target cells lacking MHC-I or expressing allogeneic MHC-I. Karre and colleagues demonstrated that NK cells are inhibited by MHC-I molecole (Karre, Ljunggren et al. 1986). The biological factor that has allowed us to identify this phenomenon is the frequent downregulation of MHC-I by infected or transformed cells. Whereas $CD8^+$ T cells are unable to kill target cells that do not express MHC-I, NK cells are specialized in the elimination of these kind of cells. The MHC-I down regulation could be seen as an active mechanism, to avoid recognition by

T cells. NK cell activation caused by target $MHC^{-/-}$ or MHC^{low} was defined "missing self" hypothesis. This means that the MHC molecules, expressed ubiquitously, are marker of self and their absence is deciphered by immune system as signals of danger (Lanier 2005).

In a curious way, both humans and mice have different receptors that will recognize MHC molecules; whereas the murine NK cells using the C-type lectin-like Ly49 family of receptors, humans rely on killer cell Ig-like receptors (KIR) (Karlhofer, Ribaudo et al. 1992; Colonna and Samaridis 1995; Wagtmann, Biassoni et al. 1995).

In addition to the Ly49 and KIR receptors, both mice and humans have a third class of inhibitory receptors called CD49/NKG2 family (Fig.8).

2.2.2 SIGNALING INHIBITORY RECEPTORS

In the cytoplasmic tail of inhibitory receptors of NK cells there are sequences called ITIM sequences, motifs composed of six aminoacids (tyrosine-isoleucine-X-XX-leucine, where X is a non-specific AA), the first described for FcγRIIb in 1994. Following the recognition of the ligand, ITIM motifs are phosphorylated (central tyrosine residue) and, as a consequence of phosphorylation, phosphatase SHP-1, SHP-2 and SHIP are recruited to the cytoplasmic tail of the activated inhibitory receptor. SHP-1 and SHP-2 reduces the activation of many signaling proteins such as ZAP-70, Syk and PLCγ. By contrast,

SHIP dampen the Ca^{2+}-dependent signaling (Bolland and Ravetch 1999).

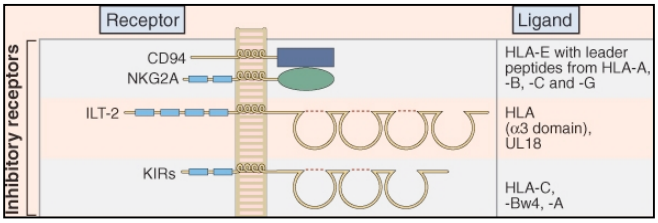

Fig.8 (from Abbas and Lichtmann 2005): Inhibitory NK cell receptors recognizing MHC-I.

2.2.3 Ly49 INHIBITORY RECEPTORS

Ly49A, discovered by Yokoyama about 20 years ago, is the founder of the Ly49 family, structurally C-Type lectin-like receptors. Many Ly49 are coupled to the ITIM domains and transduce inhibitory signals. Instead Ly49D and Ly49H, activating receptors associated with the adapter molecule DAP12. Ly49 receptors have not been described in humans (Westgaard, Berg et al. 1998).

Each NK cell transcribes only a subset of Ly49 genes that can only be expressed in a monoallelic fashion, it seems that the Ly49 genes evolved by gene duplication. Ly49A is a homodimer receptor bound to $H\text{-}2D^{d}$, the presence of a peptide loaded into the groove of the MHC molecule is required for Ly49A binding, even if the peptide sequence is not important (Lanier 2005; Mehta, Smith et al. 2001; Natarajan, Dimasi et al. 2002).

2.2.4 KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS

The human KIR family includes 15 genes on chromosome 19, but the exact number is different in different individuals, the computational analysis have in fact identified 37 different haplotypes in the human population, two of these are the most represented. KIR receptors are highly polymorphic, only 3 genes are shared by all haplotypes: KIR3DL2, KIR3DL3 and KIR2DL4 (Lanier 2005).

The human KIR are functional equivalents of murine Ly49 receptors, but are structurally different, in fact the Ly49 receptors belong to the family of the C-type lectin-like, while KIR proteins belong to the Ig superclass. KIR receptors are classified according to the number of Ig domains in the extracellular region: KIR2D has two Ig-like domains, KIR3D has three Ig-like domains. Another type of classification involves the cytoplasmic domain that can be long (L, with one or two ITIM sequences) or short (S, no consensus ITIM). Bearing a short cytoplasmic domain KIR recruit the adapter molecule DAP12, and hence KIR2DL and KIR3DL are inhibitory receptors, while KI2DS and KIR3DS are activating receptors (Lanier 2005). Like Ly49 receptors, KIR recognize polymorphic determinants on MHC-I molecules (Fig.9).

2.2.5 CD94/NKG2 RECEPTORS

In humans and mice a single CD94 receptor and many NKG2 receptors are present. The NKG2 family, such as Ly49 and KIR receptor, includes both activatory and inhibitory receptors, the most studied are NKG2C receptors (activatory receptor) and NKG2A (inhibitory receptor).

The functional receptor consists of a heterodimer composed of CD94 and an NKG2 chain (Brooks, Posch et al. 1997).

Like the Ly49 family, CD94/NKG2 proteins are structurally related transmembrane C-type lectin-, but unlike the *Ly49* and *KIR* genes, they have allelic small variations because they are able to recognize conventional highly polymorphic MHC-I molecules, their ligands are human HLA-E and mouse $Qa1^b$ (Braud, Allan et al.1998, Vance, Kraft et al. 1998).

HLA-E and $Qa1^b$ present peptides derived from the leader sequence of conventional MHC-I proteins. Since HLA-E and $Qa1^b$ are not able to present peptides derived from their own digestion by the proteasome, their expression on the cell surface impared in the absence of conventional MHC-I (and other non-conventional MHC-I molecules such as HLA-G).

Controlling HLA-E/$Qa1^b$ expression through CD94/NKG2, NK cells can sense a general downregulation of MHC-I, which concludes with the missing self recognition. In this way, NK cells are able to monitor a large number of polymorphic molecules with a moderately polymorphic receptors (Lanier 2005).

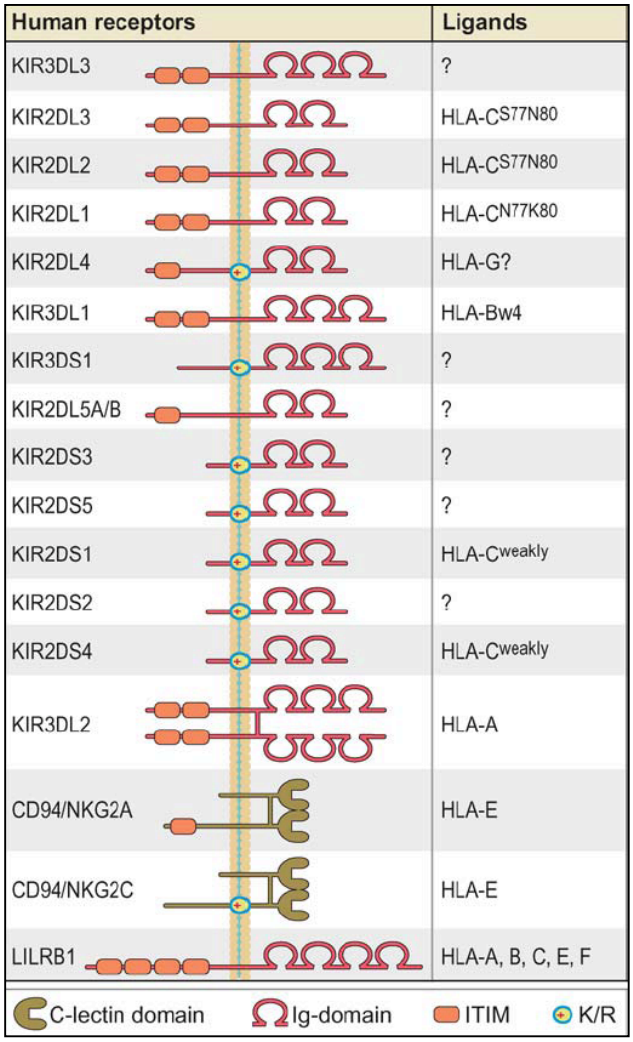

Fig.9 (from Lanier 2005): Human NK cell receptors for MHC class I.

2.2.6 ACTIVATING RECEPTORS

The major revision of the theory of the missing self is the realization of the fact that the activation of NK cells is not only due to the lack of ligand for inhibitory receptors, but also by the positive recognition of ligands for activating receptors (Fig. 10). The balance between activating and inhibitory signals controls

the response mediated by NK cells (Lanier 2005).

As mentionated previously, every family comprising inhibitory receptors recognizing MHC-I, also encodes activating receptors. Most activating receptors belonging to the Ly49, KIR and NKG2 families bind MHC-I or MHC-like molecole, although the biological mechanisms involved in this process is unclear. NK cells can also sense conserved pathogen-encoded ligands and host-encoded non-MHC molecules (Fig.11).

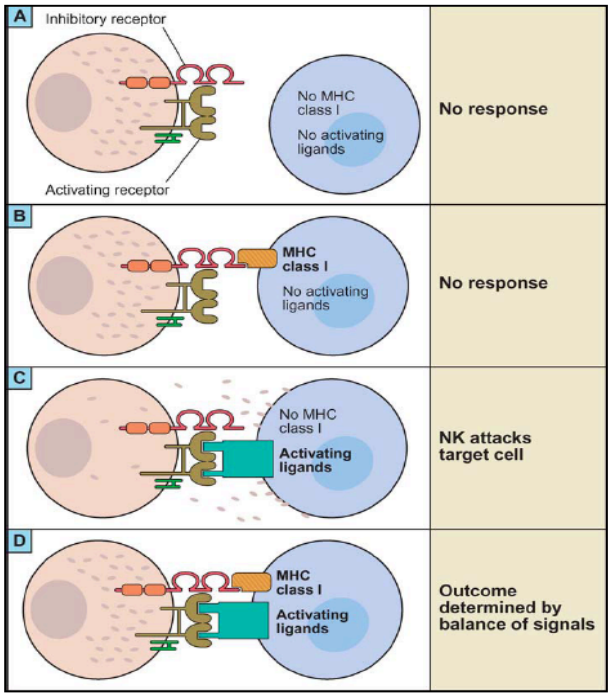

Fig.10 (from Lanier 2005): The missing self hypothesis.

Activating NK cell receptor complexes and their ligands				
Receptor	**Species**	**Signalling adaptor**	**Signalling Pathway**	**Ligand***
CD16	Mouse, human	FcεRIγ or CD3ζ	ZAP70/Syk	IgG
NKp30	Human	FcεRIγ or CD3ζ	ZAP70/Syk	?
NKp46	Mouse, human	FcεRIγ or CD3ζ	ZAP70/Syk	Influenza haemagglutinin, others?
NKR-P1C	Mouse	FcεRIγ or CD3ζ	ZAP70/Syk	?
KIR2DS	Human	DAP12	ZAP70/Syk	HLA-C, others?
CD94/NKG2C	Mouse, human	DAP12	ZAP70/Syk	HLA-E (Qa-1)
Ly49D	Mouse	DAP12	ZAP70/Syk	H-2D^d
Ly49H	Mouse	DAP12	ZAP70/Syk	MCMV-induced?
NKp44	Human	DAP12	ZAP70/Syk	Influenza haemagglutinin, others?
NKG2D	Mouse, human	DAP10	PI3K	MIC, ULBP (RAE-1, H60)
CD244	Mouse, human	SAP	?	CD48

Fig.11 (from Cerwenka and Lanier 2001): Activating NK cell receptors and ligands.

2.2.7 SIGNALING OF ACTIVATING RECEPTORS

Many NK cell activating receptors are not able to signaling in the absence of ITAM-containing adaptor molecule. In particular NK cells express three molecules providing ITAM sequences: FcεRLy, DAP12 (also named KARAP) and CD3ζ. After ligand binding and Tyrosine phosphorilation by Src family kinases in the ITAM region, the phospho-Tyr becames a docking site for the SH2 domain and Zap70. In this way these kinases are recruited to the adaptor and can start propagating the intracellular signal. Targets of Syk and Zap70 are LAT, NTAL, 3BP2 and slp-76, the signaling cascade triggers the activation of effector molecules such as PI3K, Vav and PLCγ controlling actin rearrangements and granule polarization (Lanier 2005; Tassi, Klesney-Tait et al. 2006).

An alternative, non-ITAM based, strategy of signaling for activating receptors passes by the adaptor molecule DAP10. DAP10 dos not contain ITAM motifs but another sequence. After phosphorylation this motif becames a docking site for:

- p85 subunit of IP3K, similarly to what happens for CD28,

equipe with a similar sequence (Pages, Ragueneau et al 1994).

- Grb-2, triggering effector molecules such as Vav, SLP-76 and PLCγ (Upshaw, Arneson et al. 2006).

Both branches of the DAP10 signaling pathway are required for a full activation mediate by receptors using DAP10, NKG2D being the most important (Lanier 2005, Tassi, Klesney-Tait et al. 2006).

2.2.8 RECOGNITION OF SELF MHC MOLECULES

Most activating receptors show specificity for MHC and MHC-like molecules, inhibitory receptors have developed related structures suitable to recognize the missing self. In B6 mice, the Ly49D receptor appears to mediate the rejection of H-2D^d bone marrow allografts (Nakamura, Linnemeyer et al. 1999), although a strong binding of Ly49D to H-2D^d has never been formally demonstrated (Hanke, Takizawa et al. 1999). Cross-linking of Ly49D with specific antibodies promotes cytokine release and cytotoxic responses by NK cells, confirming its nature of activating receptor. Indeed, Ly49D associates with DAP12. The reason why an activating receptor should recognize self molecules is elusive. Possibly, the high affinity ligands of activating Ly49 receptors may not be host proteins. In this frame, the interactions with H-2 might represent only a weak, biologically unimportant, cross-reactivity (Lanier 2005).

The similar DAP12-associated Ly49H receptor does not bind to any known H-2 molecule but binds with high affinity the MCMV

m157 glycoprotein (Brown, Dokun et al. 2001).

Human KIRs with a short cytoplasmatic tail are activating receptors binding DAP12. Similarly to the Ly49 family, activating KIR bind MHC-I with a much weaker affinity then the inhibitory couterparts. There is evidence supporting the involvement of KIR in autoimmune disorders. In particular, individuals with a KIR haplotype comprising the KIR2DS gene and HLA-C alleles that cannot bind their inhibitory KIR2DL receptors are more prone to certain types of arthritis (Winter, Gumpez et al. 1998; Yen, Moore et al. 2001).

As with Ly49 and KIR receptors, also the CD49/NKG2 family comprise both activating and inhibitory receptors; they show lower affinity for self ligands. Both the inhibitory NKG2A and the activating NKG2C human receptors recognize HLA-E. Michaelsson and colleagues noticed that HLA-E can present peptides from HSP60, but NKG2A loses the ability to detect HLA-E loaded with such peptides (Michaelsson, Teixeira de Matos et al. 2002).

2.2.9 RECOGNITION OF HOST-ENCODED MHC-LIKE MOLECULES BY NKG2D

NKG2D is the best characterized NK cell receptor recognizing cel surface glycoproteins structurally related to MHC-I. NKG2D has no relationship with the CD94/NKG2 family, does not form dimers with CD94, displays no homology with NKG2A, NKG2C, NKG2E and NKG2F (Lanier 2005).

All $CD8^+$ T cells in humans, irrespectively of their activation state, express NKG2D, whereas in mice only activated $CD8^+$ T cell express NKG2D. Another difference between humans and mice concerns the adaptor proteins mediating NKG2D signaling. In mouse, NKG2D is present in two different isoforms generated by alternative splicing. The longer isoform associates only with DAP10, the shorter one can bind both DAP10 and DAP12. By contrast, humans express only the long isoform; in both species NKG2D shows no polymorphism.

Stimulation of NKG2D leads to citotoxic responses and to the release of pro-inflammatory cytokines. The ligands for NKG2D are MICA, MICB, ULBP1, ULBP2, ULBP3 and ULBP4 in human, the corrisponding molecole (Rae-1, H60 and MULT1) in mouse (Radaev and Sun 2003). All the ligands are MHC-like molecules, but only MICA and MICB are encoded within the MHC locus.

NKG2D has a key role in anti-tumor and anti-viral responses, expecially for CMV (Cerwenka and Lanier 2001). *MIC* and *ULBP* genes are highly upregulated in stressed cells (transformed or virus-infected cells). *Rae-1* is induces in murine macrophages upon TLR signaling, but it is also espressed by a variety of tumor cell lines (Lanier 2005). Tumors were found to secrete soluble forms of NKG2D ligands, wich function as decoys. HCMV encodes the UL16 protein that is able to bind and retain in the cytoplasm certain NKG2D ligands upregulated upon infection. The MCMV m152 protein keeps Rae-1 proteins intracellularly (Cerwenka and Lanier 2001).

2.2.10 RECOGNITION OF HOST-ENCODED NON-MHC MOLECULES

This class of NK cell receptors includes a heterogeneous set of receptors.

- NKR-P1 receptors. NKR-P1 receptors belong to the family of C-type lectin-like trans membrane proteins. NK 1.1 is a polimorfic antigen defining NK cells in B6 mice, but absent in other inbreed strains such as BALB/c, and is a member of the NKR-P1 gene family (Glimcher, Shen et al. 1977). The NKR-P1 family comprises also inhibitory receptors, such as NKR-P1D, recognizing Clr-b, a glycoprotein espressed by all hematopoietic cells.
- 2B4. The 2B4 receptor, also called CD244, and its ligand CD48 are members of the CD2 family. It is present both in mice and humans; however, while in mice this receptor seems to exert an inhibitory function, in humans it behaves as an activating receptor (Lanier 2005). 2B4 signaling is unique in NK cells. Upon the posphorilation of the tyrosine in the sequence of the cytoplasmatic tail, it seems that 2B4 recruits the phosphatases SHP-1, SHP-2 and SAP (Tassi, Klesney-Tait et al. 2006).
- DNAM-1 receptor. DNAM-1 receptor (also called CD226) is a member of the Ig superfamily. The ligands of this receptor, CD112 (PVR) and CD155 (nectin-2), are upregulated in tumor cells and promote NK cell cytotoxicity and cytokine production. The ligands are also present at the cell junction on primary vascular endothelial cells and their binding by DNAM-1 is crucial

for trans-endothelial NK cell migration and diapedesis (Della Chiesa, Sivori et al. 2005). The interaction between DNAM-1 and its ligands has also been associated to the lysis of immature and mature DC by NK cells (Pende, Bottino et al. 2005).

2.2.11 NATURAL CYTOTOXICITY RECEPTORS

Natural cytotoxicity receptors (NCR) were cloned by the group of Alessandro and Lorenzo Moretta during the identification of receptors promoting NK cell cytotoxicity in a HLA-indipendent manner (Moretta, Biassoni et al. 2000) and were functionally named. NCR comprise NKp46, NKp30 and NKp44 (Sivori, Vitale et al. 1997; Pessino, Sivori et al. 1998; Pende, Parolini et al. 1999; Vitale, Bottino et al. 1998).

NKp46 (encoded by *NCR1*) is espressed both in mice and humans and its expression is restricted to NK cells. It is the best marker of NK cells, more reliable than the classical CD49b or NK1.1, whose expression is not limited to NK cells; moreover it is espressed by all NK cells (Walzer, Blery et al. 2007; Moretta, Biassoni et al. 2000).

NKp44 (encoder by *NCR2*) associates with DAP12 and lacks an orthologue in mouse. It's expression increases *in vivo* colture with IL-2 (Moretta, Biassoni et al. 2000).

NKp30 and NKp46 associated with CD3ζ or FcεRIγ, but NKp30 (encoder by *NCR3*) is a pseudogene in mice (Walzer, Blery et al. 2007).

Cytotoxicity of NK cells against tumor cell is partially mediated by NCR and NKp46 has also been shown to recognize the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of Sendai virus (Moretta, Biassoni et al. 2000; Mandelboim, Lieberman et al. 2001).

2.2.12 RECOGNITION OF MCMV BY LY49H

Ly49H is espressed by a subset of murine NK cells and is associated with DAP12. This receptor has no affinity for any H-2 molecule, but it binds the MCMV-encoded m157 viral glycoprotein, espressed on the surface of infected cells, wich is structurally related to MHC. The *Ly49H* gene is responsabile for the resistance to MCMV; B6 mice are resistant to MCMV and do have a Ly49H gene, while the susceptible BALB/c mice lack this gene. Accordingly, Ly49H-transgenic BALB/c mice are resistant to MCMV (Lanier 2005).

It's been shown that $Ly49H^+$ NK cells have the ability to develop memory (Ugolini e Vivier 2009) (Fig.12). After infection with MCMV, $Ly49^+$ cells undergo a proliferative phase, but if the infectious agent is removed there is a downturn. Few NK cells survive infection, but these cells are more protected against infection with MCMV compared to naive NK cells when transferred into immunodeficient mice. These cells are also more efficient *in vitro* upon triggering with Ly49H or NK1.1 (Sun, Beilke et al. 2009).

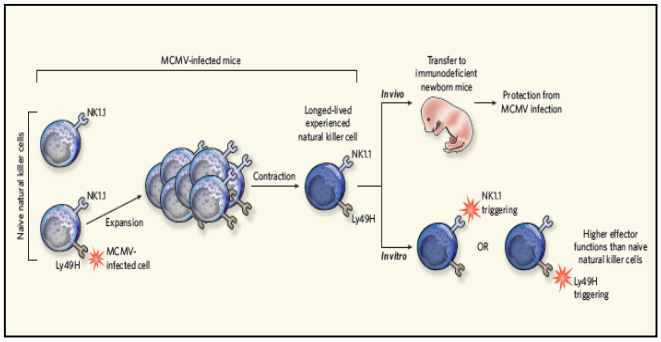

Fig.12 (from Ugolini and Vivier 2009): Natural killer cells and virus exposure.

2.2.13 TLR

TLRs are the most studied PRR and their expression is not restricted to NK cells. They are mainly studied in myeloid cells like DC and MΦ, both human and murine NK cells express TLR and this tells us then that NK cells can recognize PAMP directly. It has recently been shown that TLRs are involved in the activation of NK cells, in particular TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-8 and TLR-9 agonists stimulate NK cell functions (Pisegna, Pirozzi et al. 2004; Sivori, Falco et al. 2004; Becker, Salaiza et al. 2003; Lauzon, Mian et al. 2006). It is clear that the only TLR stimulation is not able to significantly influence the biology of NK cells. Stimulation of TLRs act in synergy with cytokine treatment (IL-12, IL-18 and/or IFN-I) in terms of up-regulation of CD69, the release of IFN-γ and cytotoxic effects. Furthermore, although NK cells express both intracellular and menbrane TLR, only the intracellular form was studied. In conclusion, stimulation of TLRs in NK cells is still incomplete

and data are contradictory (Sivori, Falco et al. 2004; Sivori, Carlomagno et al. 2006; Lauzon, Mian et al. 2007; Schmidt, Leung et al. 2004).

2.2.14 CD16 AND ADCC

The first activating NK cells receptor studied was CD16 (FcγRIIIA), this is the IgG receptor responsible for ADCC. In order to transduce the signal, it is coupled to the adapter molecule FcεRIy and the chain CD3ζ in humans, but only with FcεRIy in mice. CD16 has been used in clinical settings (Tassi, Klesney-Tait et al. 2006; Ashraf, Umana et al. 2009).

2.2.15 DEVELOPMENT, LICENSING AND SUBSETS

NK cells are the third population of lymphocytes with the main characteristic that their development do not require a rearrangement of antigen receptor gene. So they are present in SCID, RAG-deficient and athymic mice. The NK cell precursor (NKP) differentiates from hematopietic stem cells (HSCs) and early lymphoid precursors in the BM and is able to rise to mature NK cells but not other cell types. NKP small populations have been identified in the peri-natal liver and spleen and lymph nodes in adults (Huntington, Vosshenrich et al. 2007).

The step between NKP and mature NK cells include immature NK. These cells express only a limited range of receptors and are not functional or only partly. They do not show cytotoxic activity against common target cell lines and do not produce

IFN-γ (Rosmaraki, Douagi et al. 2001; Vosshenrich, Samson-Villeger et al. 2005) (Fig.13)

Since NK cells are lymphocytes of innate immunity and have invariants receptors, we can assume that they are tolerant to self; whereas the inhibitory receptors that normally recognize MHC-I polymorphisms are not uniformly expressed on NK cells and their coding genes segregate independently from MHC-I genes. As a result, some NK cells do not express specific receptors for self MHC-I, giving rise to potential self-reactive situations.

This aspect has been thoroughly studied and has been shown that NK cells are not functionally competent when the inhibitory receptors have not been involved in the process of maturation. This process has been called "licensing": the compatibility between MHC-I and Ly49/KIR receptors licenses NK cells to eliminate target cells and produce cytokines. This process is very similar to the process of positive selection of T cells in the thymus, the only difference is that in the case of NK cells there's not negative selection or clonal deletion. There are no certainties about the molecular mechanisms underlying this process even if two models have been proposed (Fig.14).

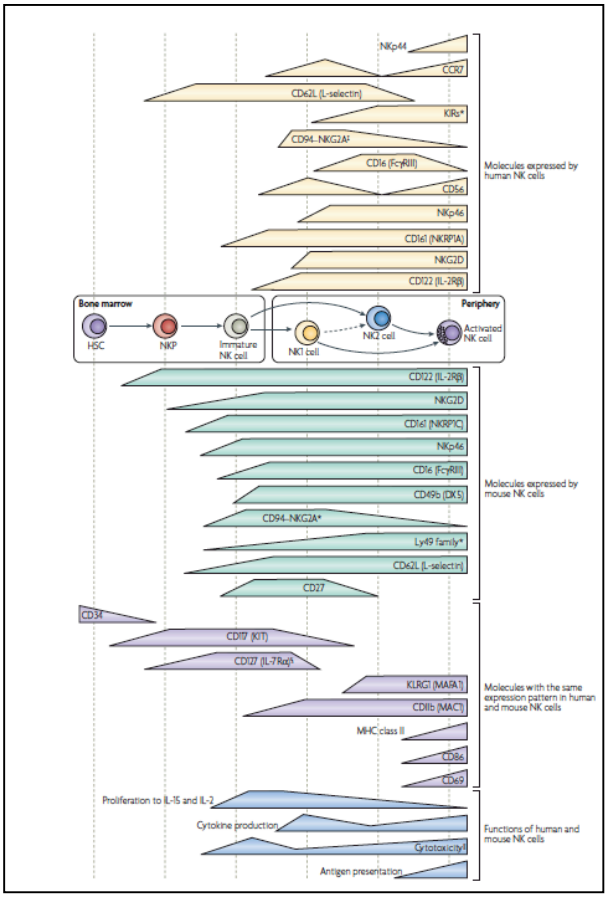

Fig.13 (from Huntington, Vosshenrich et al. 2007): Phenotypic and functional characteristics of human and mouse NK cells.

The disarming model states that NK cells lacking inhibitory receptors for self MHC-I are iper-responsive. This model consider the presence of ligands for activating receptors on normal cells and emphasize the fact that the recognition of missing self is in balance between inhibition and activation.

In the absence of inhibition, immature NK cells are genetically programmed to become functionally competent unless the

(over) stimulation of the environment makes them anergic (Raulet and Vance 2006).

The other model, the arming model, proposes a genetically programmed fate of NK cells during the development to the hyper-responsiveness unless subjected to appropriate stimuli. This particular rescuing (or "arming") task could be performed with the recruitment of inhibitory receptors by self MHC-I molecules. The inhibitory receptors may transduce a positive signal to NK cells in an immature stage of their development, while in the mature stage, NK cells switch to the delivering negative signals (Yokoyama 2008; Zanoni, Granucci et al. 2007).

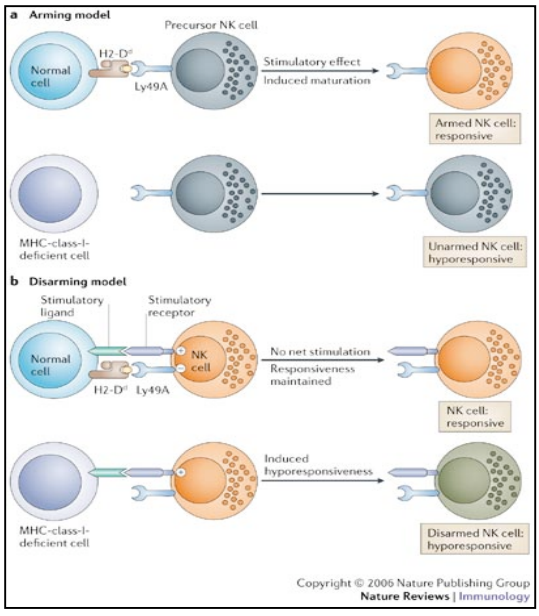

Fig.14 (from Raulet and Vance 2006): Comparison of arming and disarming as mechanisms of natural-killer-cell self-tolerance.

Unlicensed NK cells are not able to fully carry out their functions. In inflammatory conditions there are licensed and unlicensed activated NK cells. This could be the reason why self-reactive NK cells are maintained in an anergic state rather than be eliminated.

Mature NK cells are a highly heterogeneous population because the stochastic expression of inhibitory receptors, it is still possible to group the NK cells based on phenotype and functions. In humans we can easily distinguish two subsets: $CD56^{dim}$ $CD16^{+}$ cells are the majority of circulating NK cells while $CD56^{bright}$ (almost all $CD16^{-}$) that are a little fraction; obviously these populations have other different functions. $CD56^{dim}$ NK cells are cytotoxic but produce very few IFNγ, they express CD16 and show strong ADCC response. $CD56^{bright}$ NK cells are specialized in releasing IFN-γ but shows weak cytotoxic responses.

These two populations also show other differences, for example $CD56^{bright}$ express the heterotrimeric high-affinity IL-2 receptor and then in the presence of low doses of IL-2 only these cells can proliferate; $CD56^{dim}$ cells instead express only the heterotrimeric receptor for IL-2 with low affinity (Cooper, Fehniger et al. 2001; Caligiuri, Murray et al. 1993) (Fig.15).

The two subsets also differ in response to chemokines. $CD56^{bright}$ NK cells express CCR7 and CXCR3, they are then recruited into the secondary lymphoid organs and respond to IP-10, whereas $CD56^{dim}$ NK cells express CXCR1 and CX_3CR1

and therefore are able to sense IL-8 and fractalkine. It has also been suggested that the $CD56^{dim}$ cells can differentiate from $CD56^{bright}$ but this has not yet been tested (Cooper, Fehniger et al. 2001).

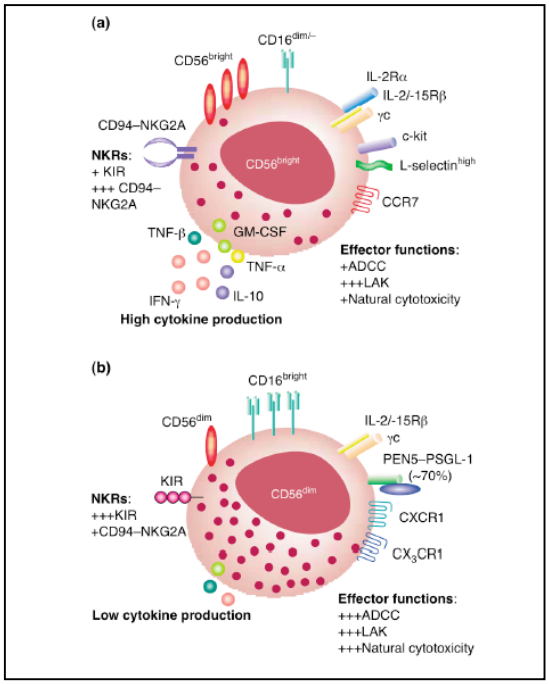

Fig.15 (from Cooper, Fehniger et al. 2001): Phenotypic and functional characteristics of human NK cell subsets.

Mature NK cells in mice do not express CD56, while in humans and in mice these cells express NK1.1 (CD161) and NKp46, the first is a marker also present on NKT cells and γδT cells while NKp46 is expressed by all NK cells irrespective of their subsets or activation state (Moretta, Biassoni et al. 2000; Cooper, Fehniger et al.2001; Huntington, Vosshenrich et al. 2007).

Hayakawa and colleagues recently suggested a possible role of CD27 in the distribution of murine NK cells in subsets. The $CD27^{+}$ cells are very similar to human $CD56^{bright}$ NK cells (the majority in the lymph nodes they produce large amounts of IFN-γ), while $CD27^{-}$ cells, such as human $CD56^{dim}$ NK cells, are not present in the lymph nodes but the cell population dominance in spleen, lungs and blood. $CD27^{+}$ NK cells can in turn be divided into $CD11b^{hi}$ (these cells show high cytotoxic activity) or $CD11b^{low}$ (Hayakawa, Smyth et al. 2006).

3. DC-MEDIATED NK CELLS ACTIVATION

NK cells have receptors that can recognize the presence of pathogens such as NKp46, Ly49H and TLR, despite that the activation of NK cells by the majority of pathogens is indirect and is due to signals released by accessory cells. Although the DC cells have a critical role, other APC such as MΦ appear to act as accessory cells in the activation of NK. Unlike T cells, it was thought that NK cells could exist in resting and activated states. In 1999, this concept has been reviewed by Fernandez and colleagues that have demonstrated how the expansion of DCs *in vivo* results in greater anti-tumor immunity, and this effect is dependent on NK cells. DC cells are able to activate NK cells with both soluble signals that contact-dependent signals (Lanier 2005; Newman and Riley 2007; Lucas, Schachterle et al. 2007; Tu, Bozorgzadeh et al 2008; Fernandez, Lozier et al. 1999) (Fig.16).

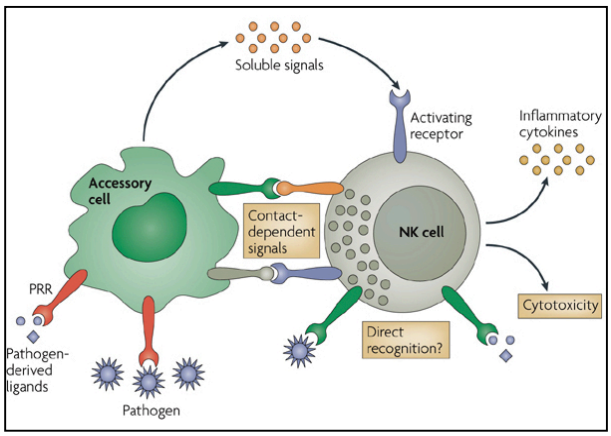

Fig.16 (from Newman and Riley 2007): Accessory cell-mediated NK cell activation.

10 years later, Lucas and colleagues proposed a study on NK cells "primed" by DC cells and "restimulated" by target cells. In this way we realized that the threshold of activation of NK cells is not constant but governed by accessory cells and it is a two-step mechanism (activation-response) that can control the ability of effector NK cells (Lucas, Schachterle et al. 2007).

While the response stage can be considered dependent on the balance between activating and inhibitory receptors able to recognize molecules on target cells (depending on the level of activation of NK cells that may be able to activate NK cells in certain circumstances or not), obviously the activation status depends on different mechanisms. Given the analogy with T cells, soluble cytokines and costimulatory molecules bound to the membrane released by DC were investigated (Newman and Riley 2007). An example that demonstrates the importance of cytokines released by DC in the activation of NK is as follows: the incubation of blood mononuclear cells with TLR agonists, leads NK cells to produce large amounts of IFN-γ, but if are removed accessory cells, IFN-γ is no longer produced. The most important evidence for the relevance of accessory cells derived from studies in which neutralizing antibodies are used and demonstrate the importance of cytokines released from accessory cells in the activation of NK in response to pathogens (Carson, Giri et al. 1994; Orange and Biron 1996; Nomura, Kawamura et al. 2002).

IL-12p70 was long considered a strong stimulus for the

production of IFN-γ especially in combination with IL-18 (Chaix, Tessmer et al. 2008). Using blocking antibodies to neutralize IL-12p70 *in vitro* and *in vivo* there is a decrease in the release of IFN-γ by NK cells in response to many pathogens and TLR agonists by viruses, bacteria and protozoa (Orange and Biron 1996; Nomura, Kawamura et al. 2002).

IFNα/β increases the NK cell cytotoxic up-regolating FasL and perforin (Gerosa, Gobbi et al. 2005). A model study shows how strong is the role of type I IFNs in the cytotoxic response of NK cells in infections with MCMV; if the production of type I INFs is neutralized with blocking antibodies, the cytotoxic response decreased (Nguyen, Salazar-Mather et al. 2002). The type I IFNs are also involved in the release of IFN-γ by NK cell-derived infections by viruses and protozoa (Newman, Korbel et al. 2006).

IL-2 and IL-15 share their β and γ subunits of their heterotrimeric receptor. IL-15 is strongly involved in the development and activation of NK cells (Carson, Giri et al. 1994; Ranson, Vosshenrich et al. 2003; Ferlazzo, Pack et al. 2004; Huntington, Legrand et al 2009). Similarly, high doses of IL-2 has been used for decades to generate LAK cells, NK cells that are able to initiate strong cytotoxic responses (Whiteside 2001). IL-2 also issued by the DC is involved in the release of IFN-γ by NK cells in anti-bacterial and anti-tumor immunity (Granucci, Zanoni et al. 2004; Zanoni, Foti et al. 2005). The release of some cytokines by accessory cells may require close

contact with NK cells to be effective. This is the case of IL-15 that is trans-presented IL-15Rα, acting as a contact dependent signal in addition to soluble signal. (Burkett, Koka et al. 2004). IL-15 secreted by DC must be channeled into the immune synapse together with the NK cells to promote an effective release of IFN-γ, and although this cytokine is a soluble molecule, the trans-presentation is made by trans-membrane proteins (Borg, Jalil et al. 2004). This statement seems dictated by the need to polarize and concentrate the vesicles containing preformed IL-15 in the site of contact. The accessory cells can also release cytokines capable of inhibiting the decrease of NK cell effector functions such as TGF-β. The neutralization of TGF-β increases the response of NK cells against *P.falciparum* (Li, Wan et al. 2006; Newman, Korbel et al. 2006).

There is no consensus on the signals issued by the DC to activate NK cells, in fact the DC release different cytokines in response to stimulation with different PAMPs and also we must also consider the experimental conditions used. For istance, HSC differentiate in the presence of GM-CSF plus IL-4 rather than GM-CSF alone gives very different functions to the BM-DC, indeed IL-4 can inhibit the production of IL-2 by DC exposed to PAMP (Guiducci, Valzasina et al. 2005; Schartz, Chaput et al. 2005).

In humans, DC cultured with IL-4 are able to activate NK cells regardless of the presence of bacterial stimulation (Ferlazzo, Morandi et al. 2003). This explains why there are different

conclusions about the role of IL-2 and IL-12 in the activation of NK cells mediated by DC. The production of IL-2 as well as the production of IL-12 appears to be a dichotomous choice, the presence or absence of IL-4 controls which of two cytokines should be produced (Zanoni, Granucci et al. 2007) (Fig. 17) . The site of DC-NK cell interaction is uncertain. The first contact occurs at the level of peripheral tissues, where resident and recruited DC are able to activate NK cells (Moretta 2002). DC exposed to the pathogen are activated and migrate from the periphery to draining lymph nodes (DLN) in a CCR7-dependent manner (Weninger and von Andrian 2003), and here they are able to stimulate resident and newly recruited NK cells. The migration of NK cells to lymph nodes depends in part on CXCR3 and DC are able to secrete CXCR3 ligands upon maturation (Martin-Fontecha, Thomsen et al. 2004). So DC first recruit then activate NK cells in the draining lymph node. Although NK cells are a source of IFN-γ, they also have a positive role in $CD4^+$ T cell skewing towards a Th1 phenotype (Martin-Fontecha, Thomsen et al. 2004; Zanoni, Foti et al. 2005).

The NK-DC interaction is not unidirectional. NK cells indeed profoundly affect the responses of DC (Della Chiesa, Sivori et al. 2005).

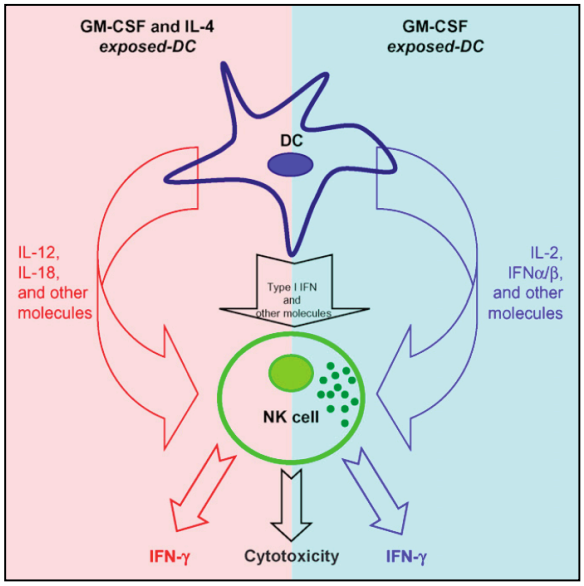

Fig.17 (from Zanoni, Granucci et al. 2007): Mediators of NK cell activation produced by activated DC differentiated in presence of GM-CSF alone or plus IL-4.

The immature DC are susceptible to lysis mediated by NK cells while the mature DC are protected. A mediator has been identified in humans, NKp30, which is crucial in the killing of DC cells by NK cells (Ferlazzo, Tsang et al. 2002). This observation could be interpreted as a mechanism of immuno-editing performed by NK cells, in order to increase the immune responses by preserving microbial-experienced DC while removing the useless or potentially tolerogenic immature DC. For the same reason, NK cells can alternately promote the maturation of DC by releasing appropriate stimulating factors.
Rubatelli and colleagues found that the interaction DC/NK results in the release of a pro-inflammatory cytokine, HMGB1, by NK cells, allowing the maturation of DC (Semino, Angelini et

al. 2005). Other signals involved in the activation of DC by NK cells are TNF-α and IFN-γ (Gerosa, Baldani-Guerra et al. 2002; Vitale, Della Chiesa et al. 2005).

In conclusion, the interaction DC/NK is very complex and has a strong importance in regulating the immune response. These mechanisms could be very important for any therapeutic applications involving the manipulation of the innate immune system.

4. SCOPE OF THE THESIS

The role of NK cells in anti-viral and anti-tumor immunity has been extensively studied, nevertheless the mechanisms involved in the activation of NK cells in an anti-bacterial remain to be fully understood. Recent studies have shown that activation of NK cells by various pathogens is indirect and is driven by accessory cells, particularly by DCs. (Cerwenka and Lanier 2001, Newman and Riley 2007).

We have studied how DC can activate NK cells in the presence of LPS, the main component of the Gram-negative cell wall, which is recognized by TLR-4 (Akira 2006). We decided to focus our attention primerely on the production of IFN-γ by NK cells because their anti-bacterial effects are mainly mediated by this cytokine (Boehm, Klamp et al. 1997). Here we show the molecular mechanisms underlying the activation of NK cells mediated by DC (*in vitro* and *in vivo*) upon LPS exposure.

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Chapter II.

Mechanisms of dendritic cell-mediated natural killer cell activation

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MATERIALS AND METHODS

Mice

All mice used in this study were on a B6 background. WT animals were supplied by Harlan Italy. $Il2^{tm1Hor}$ ($Il2^{-/-}$), $Il18r1^{tm1Aki}$ ($Il18r1^{-/-}$), $Il18^{tm1Aki}$ ($Il18^{-/-}$) and $Ticam1^{Lps2}$ (Trif $^{-/-}$) mice were purchased from the Jackson Laboratory. $Il15^{tm1Imx}$ ($Il15^{-/-}$) animals were from Taconic. $Myd88^{-/-}$ and $Tlr4^{-/-}$ mice were provided by S. Akira (IFReC, Japan). $Ifnb^{-/-}$ were supplied by S. Weiss (Helmholtz Centre for Infection, Germany); CD11c.DOG (DTR-OVA-GFP) animals (Hochweller, Striegler et al. 2008) were a gift from G. J. Hämmerling (German Cancer Research Center DKFZ, Germany). Ubiquitin.GFP mice (Ikawa, Yamada et al. 1998) were obtained from M. Battaglia (S. Raffaele Telethon Institute for Gene Therapy HSR-TIGET, Italy). NK-DTR mice (Walzer, T., Chiossone, L. and Vivier, E. 2007) were gently given by E. Vivier (Centre d'Immunologie de Marseille-Luminy (CIML) CNRS-INSERM-Université de la Méditerranée, France); Perforin knock out (PKOB) mice were obtained from B. Ludewig (University of Zurich, Switzerland); Beta-2 Microglobulin knock out (B2M) mice comes from CDTA D'Orleans (Département de Cryopréservation, Distribution, Typage et Archivage animal, University of Orléans, France) and mice deficient in Nalp3 ($ASC^{-/-}$) were supplied by Dixit Genentech (South San Francisco, CA). All the mice were breeded under specific pathogen-free conditions and were used

at 8-12 weeks of age in compliance with the Institutional Animal Care & Use Committee (University of Milano-Bicocca, Milan, Italy) guidelines.

Cells

All cells were cultured in IMDM-10 complete medium (IMDM, 10% heat-inactivated FBS, 2mM L-glutamine, 100 U/ml penicilin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol).

BM-derived DC (BM-DC) were generated by culturing BM cells in IMDM-10 containing 10% of GM-CSF-transduced B16 cell-conditionated medium as published (Inaba, Inaba et al. 1992; Granucci, Vizzardelli et al. 2001).

D1 cells (dendritic cells derived from the spleen) were cultured in IMDM-10 containing 30% of mGM-CSF-transduced NIH/3T3 cell-conditionated medium as published (Mortellaro, A., Urbano, M., Citterio, S. et al. 2009).

NK cells were purified from RBC-lysed splenocytes by MACS negative selection for CD49b (DX5), using biotinylated Ab (BD Biosciences), streptavidin microbeads and LS columns (Miltenyi Biotec), purity threshold to proceed with experiments was set to 87-90%. YAC-1 cells were purchased from ATCC.

DC-NK cell co-culture

BM-DC (1E5/well) and NK (5E4/well) cells were co-cultured in a flat-bottom 96-well plate in the presence or absence of the following reagents: ultrapure TLR-grade Re-form LPS from *E.*

Coli serotype R515 (1μg/ml), shown contamination-free (Zanoni, Ostuni et al. 2009), neutralizing anti-IL-1β, anti-IL-1R1, anti-IL-18Rβ, and all isotype controls from BD Bioscences; anti-IL-15 and anti-IL-15Rα from R&D Systems; neutralizing anti-IL-18 from MBL Interantional; recombinant IL-1RA (1μg/ml), recombinant IL-18BPd/Fc chimera (1μg/ml), recombinant human IgG1 Fc (1μg/ml) and recombinant IL-18 from R&D Systems; recombinant IFN-β from PBL InterferonSource; recombinant IL-2 from Peprotech. All neutralizing Ab and the relevant isotype controls were used at 10 μg/ml and were purchased in the no azide/low endotoxin format. Chemical inhibitors, Ab and recombinant proteins aimed to block cytokines were added 20 min prior to stimulation with LPS. Recombinant cytokines were added at the time of stimulation. Following 6 h of co-colture, plates were briefly centrifuged and supernatants were collected for an IFNγ ELISA analysis or TDA-loaded YAC-1 target cells were added for 3 additional h to perform cytotoxicity assays.

ELISA

Cell-free supernatants were collected at the indicated time points and analyzed following standard sandwich ELISA protocols. Ab pairs from BD Bioscence for IL-2, kits from e-Bioscences for TNF-α, IL-1β and IFN-γ were used. IFN-β was

measured using pre-coated plates from PBL InterferonSource.

Cytotoxicity assay

Cytotoxicity was quantitated by a time-resolved fluorometric assay using the DELFIA EuTDA Cytotoxicity Reagents (Perkin-Elmer) (Blomberg, Hautala et al. 1996). 5E6 YAC-1 target cells in 1 ml of IMDM-10 were labeled with 5 μl of BATDA reagent, the acetoxymethyl ester of the fluorescence enhancing ligand TDA, at 37°C for 30 min. Within cells, the ester bonds are hydrolized to form the hydrophilic TDA which no longer passes the membrane. Target cells were then washed 4 times in PBS and added to a 6 h DC-NK cell co-culture in 96-well plates for additional 3 h. Maximal release was produced by incubating target cells with DELFIA lysis solution, whereas spontaneous release was measured on target cells alone. 50 μl of cell-free supernatants, containing the TDA released by killed cells, were mixed to 150 μl of DELFIA europium solution for 10 min while shaking. Time-resolved fluorescence of the Eu:TDA chelate, correlating with the number of lysed cells, was measured with a Victor3 plate reader (Perkin-Elmer). Medium background was subtracted from all conditions. Results show the percentage of target cell lysis relative to maximum and spontaneous release controls.

Cytotoxicity *in vivo*

RBC-lysed splenocytes, 50% from wt mice and 50% from β2M

ko mice were labeled with 0.075 μM and 0.75 μM CFSE rispectively and 7E6 cells were injected i.v. into wt, IL-2 ko, IL-18 ko and IFN-β ko mice with or without LPS. After an O.N. incubation splenocytes of recipients mice were collected and then analized by a facs analysis in order to misure the percentage of wt and β2M ko cells. We have calculated the ratio between wt and β2M ko cells for both treated and non treated mice and then we have misured the % of specific kill = $100 - (100 \times ratio_{treated})/ratio_{untreated}$.

In vivo NK cell activation and IFN-γ secretion

In order to generate mixed BM chimeras, CD11c. DOG - Ubiquitin. GFP double-transgenic (DOG-GFP dTg) mice were irradated with 950 rads total body irradiation, reconstituted with i.v injected 5E6 BM cells, and allowed to recover for at least 2 months before use in experiments. 20% of donor BM cells came from mice deficient for the cytokine under examination, whereas the remaining 80% came from DOG-GFP dTg mice.

To deplete DC, mixed BM chimeras or CD11c.DOG mice received a daily i.p. injection of 16 ng of diphtheria toxin (DT, Sigma-Aldrich) per gram of body weight in PBS for 7 days. To activate NK cells, mice were injected with 50 μg of LPS. After 5h, mice were sacrificed and RBC-lysed splenocytes restimulated with PMA (50 ng/ml) and ionomycin (100 ng/ml) for 3 h in the presence of brefeldin A (BFA, 10 μg/ml) all from

Sigma-Aldrich. Intracellular staining was performed using Citofix/Cytoperm reagents (BD Biosciences) and using the following Ab, all from BD Biosciences: anti-CD49b (DX5 or Hmα2), anti-CD3ε and anti-IFN-γ or its isotype control. Samples were acquired with a FACSalibur flow cytometer (BD Biosciences).

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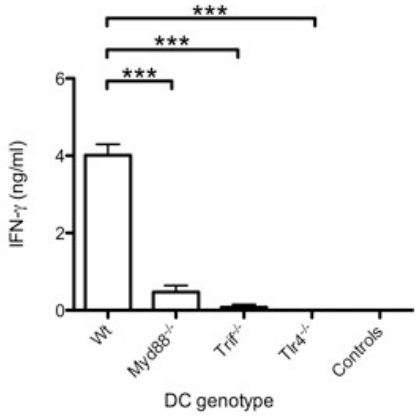
RESULTS

MyD88, but not TLR-4, is important for the release of IFN-γ by NK cells activated by LPS-stimulated DC

Initially it was thought that NK cells were acting independently of other cells of the immune system, but recently it became clear that accessory cells, such as macrophages and DC, are able to "prime" NK cells increasing their ability to recognize targets. In particular, NK cells are able to release large amounts of IFN-γ when activated by mature DC (Fernandez, Lozier et al. 1999; Newman and Riley 2007).

Following stimulation with LPS, the DC become capable of stimulating the production of IFN-γ by NK cells. This process requires the intervention of both TLR-4 and the adapters MyD88 and TRIF, but in a curious way, there has been a full activation of NK cells even in the absence of TLR-4 and TRIF, but not in the absence of MyD88 (Fig.1). Although the intracellular expression of TLR by NK cells appears to play an important role in promoting the direct activation of these cells, TLR-4 bound to the plasma membrane appears dispensable (Sivori, Falco et al. 2004).

A



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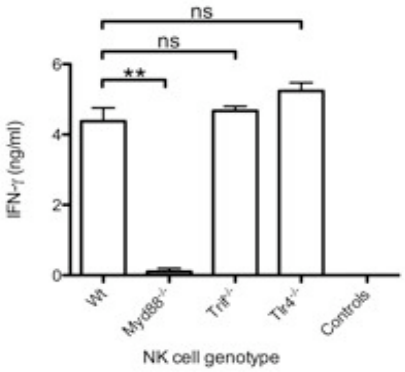


Fig.1 - Different use of TLR-4 and adapters MyD88 and TRIF on DC and NK cells for the production of IFN-γ by NK cells activated by LPS-stimulated DC. Co-cultures of GM-CSF-derived BM-DC and NK cells were stimulated with LPS. After an O.N. incubation, was measured by ELISA analysis, the level of IFN-γ produced in the supernatants. The controls used were as follows: DC alone, NK cells alone, DC + NK cells, DC + LPS, NK cells + LPS. (**A**) DC cells of indicated genotypes, stimulated with LPS, were co-cultured with wt NK

cells. (**B**) wt DC cells stimulated with LPS were co-cultured with NK cells of indicated genotypes. (A-B) n ≥ 4. Error bars depict SEM. Statistical significance was determined by means of the one-tailed Mann-Whitney test: * when p<0.05; ** when p<0.01; *** when p< 0.001; ns, not significant.

IL-18 released by DC directly stimulates the production of IFN-γ by NK cells

The involvement of MyD88 but not TLR-4 or TRIF for the production of IFN-γ by NK cells stimulated by LPS-activated DC (Fig.1B) suggests an involvement of IL-1 family receptors that transduce the signal through MyD88 (Dinarello 2009). To be able to figure out which IL-1 family member was involved in the activation of NK cells, IL-1α, IL-1β and IL-18 were blocked independently, using neutralizing antibodies or antagonists. In particular, IL-1α is inhibited in an indirect manner using either an Ab against IL-1R or recombinant IL-1R antagonist (IL-1RA), another member IL-1 family (Dinarello 2009). IL-1β was blocked with the same reagents and also with specific neutralizing antibodies. The production of IFN-γ by NK cells was not affected by treatments performed and it was shown that both IL-1α and IL-1β are not involved in the activation of NK cells by DC. Conversely by blocking IL-18 with recombinant IL-18 binding protein (IL-18BP) or neutralizing Ab against the cytokine or its receptor actually decreases the secretion of IFN-γ by NK cells (Fig.2A).

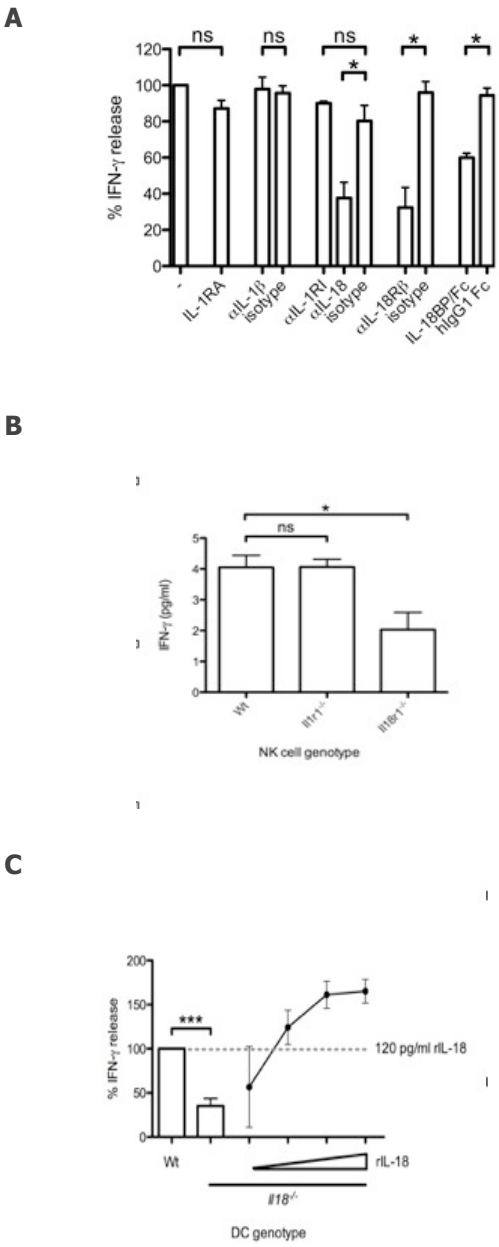


Fig.2 – IL-18, but not IL-1α/β, is required for NK activation. Co-cultures of GM-CSF-derived BM-DC and NK cells were stimulated with LPS. After an O.N. incubation, was measured by ELISA analysis, the level of IFN-γ produced in the supernatants. (**A**) The cells were co-cultured in the

presence of the indicated neutralizing antibodies and recombinant proteins (or isotype controls). (**B**) NK cells of the indicated genotypes were co-cultured with wt DC stimulated with LPS. (**C**) DC stimulated with LPS of the indicated genotypes were co-cultured with wt NK cells. In some cases, increasing doses of rIL-18 have been added to co-culture. (**A,B**) Results are shown as percentage of IFN-γ release relative to NK cells activated by uninhibited LPS-stimulated wt DC. (**A,C**) n ≥ 3. Error bars depict SEM. Statistical significance was determined by means of the one-tailed Mann-Whitney test, except for the IL-1RA (**A**) and the $Il18^{-/-}$ (**C**) conditions that were tested against 100% for which the one-tailed one-sample t test was adopted: * when $p<0.05$; ** when $p<0.01$; *** when $p<0.001$; ns, not significant.

These results were confirmed using purified NK cells deficient for both IL-18R and IL-1R. Instead $Il18r1^{-/-}$ but not $Ilr1^{-/-}$ NK cells showed a marked reduction in the release of IFN-γ (Fig.2B). Similarly, IL-18-deficient BM-DC were not able to fully activate NK cells and the addition of 120 pg/ml of recombinant IL-18 restored the production of IFN-γ (Fig.2C). In conclusion, LPS-matured DC contributed to the production of IFN-γ by NK cells through IL-18R-MyD88 pathway.

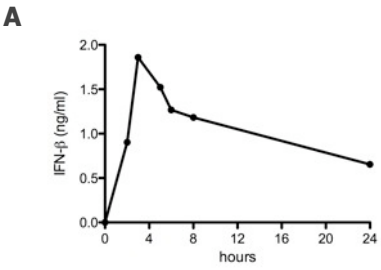
IFN-β released from DC is essential for a full NK cells activation in terms of production of IFN-γ

The involvement of TRIF pathway on DC to allow the production of IFN-γ by NK cells might imply the involvement of IFN-β which is the most important cytokine controlled by this

pathway (Yamamoto, Sato et al. 2002) (Fig.1A).

First, we confirmed that after stimulation with LPS the BM-DC are capable of releasing large amounts of IFN-β (Fig.3A). In order to understand the role of IFN-β in the activation of NK cells, we used IFN-β deficient DC because the antibody capable of neutralizing the activity of IFN-β are not effective enough. We observed that IFN-β deficient DC are much less able to stimulate the production of IFN-γ by NK cells compared to the wt control (Fig.3B).

By adding rIFN-β to the co-culture we observed a recovery in the production of IFN-γ, demonstrating that the decrease in the activation of NK cells is really due to the absence of IFN-β and not to a possible alteration of gene expression caused by the insertion of the disrupting construct or IFN-β deficiency.



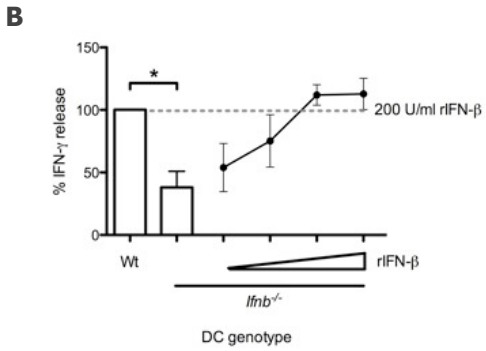
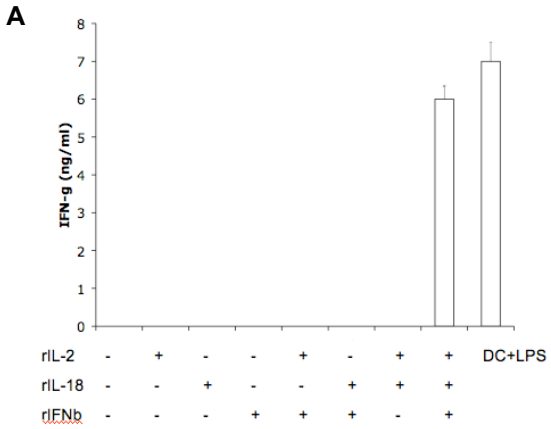


Fig.3 – IFN-β is essencial for th NK cell activation by DC. (**A**) Kinetic measurement of IFN-β release by GM-CSF BM-DC stimulated with LPS. (**B**) GM-CSF BM-DC stimulated with LPS of the indicated genotypes were co-cultured with WT NK cells. After an O.N. incubation, was measured by ELISA analysis, the level of IFN-γ produced in the supernatants. In some cases, increasing doses of rIFN-β have been added to co-culture. (**A**) A representative experiment out of three is shown. (**B**) Results are shown as percentage of IFN-g release compared to NK cells activated by LPS-stimulated WT DC. n ≥ 3. Error bars depict SEM. Statistical significance was determined by means of the one-tailed one-sample t test; * when $p<0.05$; ** when $p<0.01$; *** when $p<0.001$; ns, not significant.

IL-2, IL-18 and IFN-β are necessary and sufficient to induced IFN-γ secretion by NK cells

We investigated whether IL-2, IL-18 and IFN-β were necessary and sufficient for full activation of NK cells, we used recombinant cytokines at doses able to reverse the phenotype

of ko to wt levels (Fig.2C and Fig.3B). This method is more reliable than using doses of recombinant cytokine equal to those secreted by wt DC in natural conditions because the activity of recombinant cytokines is different from that of natural cytokines, the natural secretion is a dynamic process and because the cytokines are released naturally active only at the level of immune synapse in a limited space and a local concentration while the measured concentration in the experiment carried out refers to a cytokine diluted in culture medium. We observed that the simultaneous stimulation of NK cells with IL-2, IL-18 and IFN-β promotes the release of IFN-γ, while removing only one of three cytokine, the stimulation is ineffective (Fig.4A). We can therefore conclude that IL-2, IL-18 and IFN-β are necessary and sufficient for the DC-mediated NK cells activation that leads to IFN-γ release.



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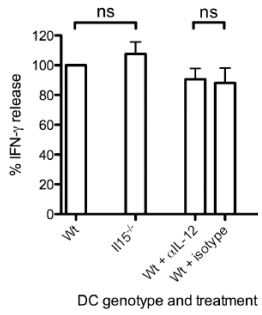


Fig.4 – IL-2, IL-18 and IFN-β are necessary and sufficient to mediate DC-driven NK cell activation. (**A**) NK cells were cultured in the presence or absence of rIL-2 (1 ng/ml), rIL-18 (120 pg/ml) and rIFN-b (200 U/ml). After an O.N. incubation, was measured by ELISA analysis, the level of IFN-γ produced in the supernatants. (**B**) Co-cultures of GM-CSF-derived BM-DC of the indicated genotype and wt NK cells were stimulated with LPS in the presence of the indicated neutralizing Ab (or isotype controls) O.N. Results are shown as percentage of IFN-γ levels relase compared to NK cells activated by uninhibited LPS-stimulated wt DC. n ≥ 3. Statistical significance was determined by means of the one-tailed Mann-Whitney test, except for the $Il15^{-/-}$ condition that was tested against 100% for which the one-tailed one-sample *t* test was adopted: * when $p<0.05$; ** when $p<0.01$; *** when $p<0.001$; ns, not significant.

IL-12, IL-15 have no role in the activation of NK cells

IL-12p70 is usually associated with activation of NK cells and the release of IFN-γ (Ferlazzo, Tsang et al. 2002; Newman and Riley 2007). To test the role of IL-12 in our experimental

setting, we blocked IL-12 in DC-NK co-cultures. Neutralization of IL-12 does not decrease the levels of secreted IFN-γ (Fig.4B) and we have not been able to measure the secretion of IL-12p70 by BM-DC stimulated with LPS. Recently it was reported a role for trans-presented IL-15 in the DC-mediated NK cell activation (Koka, Burkett et al. 2004; Lucas, Schacht et al. 2007; Newman and Riley 2007; Mortier, Woo et al. 2007). In contrast to published results, we were not able to define a role for IL-15. Indeed IL-15-deficient DC were equally able as wt DC in activating NK cells (Fig.4B) (Takeda, Oshima et al. 2000; Newman and Riley 2007). In conclusion, DC-derived, IL-18 and IFN-β (as we report here) are necessary and sufficient in the activation of NK cells in presence of LPS (Granucci, Zanoni et al. 2004; Zanoni, Foti et al. 2005).

DC-NK cells interaction requires contact dependent signal given by IL-18

The importance of three soluble cytokines in activating DC-NK cells was quite surprising since it is reported that NK cells require contact-dependent signals for the production of IFN-γ (Fernandez, Lozier et al. 1999; Newman and Riley 2007). It can be hypothezised that some soluble factors could be released in small amounts in a confined space around the site of secretion to reach a local concentration sufficient to activate interacting cells.

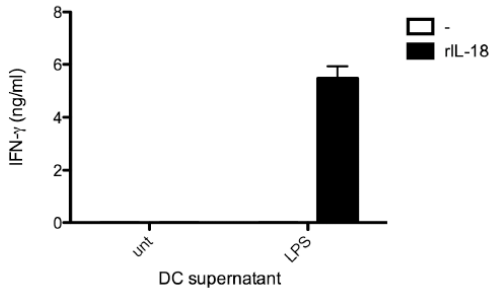


Fig.5 – IL-2, IFN-β and the contact-dependent IL-18 are necessary and sufficient to mediate DC-driven NK cell activation. NK cells were cultered for 6h in the presence of medium previously conditioned for 6h by either untreated or LPS-stimulated DC in the presence or absence of rIL-18 (120 pg/ml). At the end of the culture, IFN-γ levels were measured by ELISA. n ≥ 3. Error bars depict SEM. Statistical significance was determined by means of the one-tailed Mann-Whitney test.

Depletion of DC *in vivo* dampens NK cell activation

To study the activation of NK cells by DC *in vivo*, we used mice that express the diphtheria toxin (DT) receptor under the control of the CD11c promoter (Hochweller, Striegler et al. 2008). Unlike mice CD11c.DTR (Jung, Unutmaz et al. 2002) created previously, these mice after repeated injections of DT show a durable $CD11c^{+}$ high and low cell ablation. In the absence of DC, the production of IFN-γ by NK cells was reduced, this is the best signal of the importance of DC in the activation of NK cells.

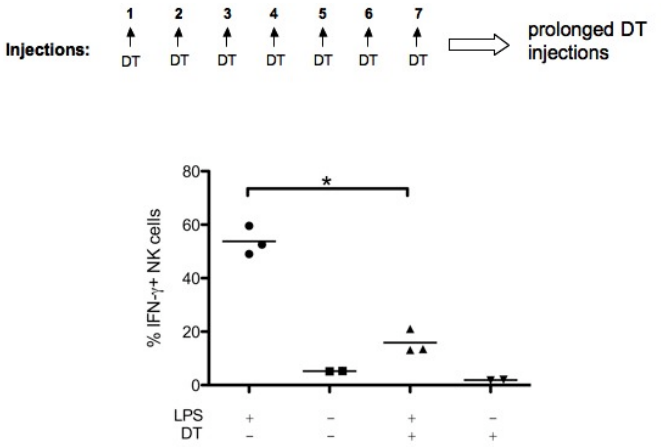


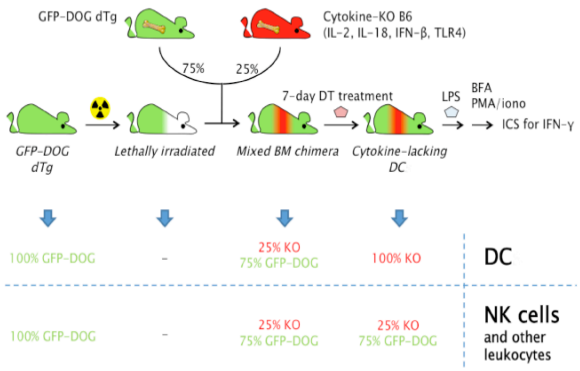
Fig.6 – DC cells are the accessory cells involved in NK cells activation *in vivo.* CD11c.DOG mice were injected with DT daily for a week, the next day were injected with LPS i.v. After 5 hours, the splenocytes were restimulated *ex vivo* with PMA/ionomycin for 3 hours in the presence of BFA. It was later made an intracellular staining for IFN-γ, NK cells were defined $CD49b^{+}$ $CD3^{-}$. n ≥ 2. Error bars depict SEM. Statistical significance was determined by means of the one-tailed Mann-Whitney test: * when $p<0.05$; ** when $p<0.01$; *** when $p<0.001$; ns, not significant.

IL-2, IL-18 and IFN-β are required for a full NK cell activation *in vivo*

Once we have established the importance of DCs in the activation of NK cells, we studied more thoroughly the mechanisms underlying their interaction. We have focused our attention, in a *in vivo* context, in particular on the three cytokines secreted by DC that have been shown to be important for activation of NK cells *in vitro*: IL-2, IL-18 and IFN-

β. To this end mixed BM chimeras were created in which 80% of donor cells came from DOG-GFP mice and 20% came from cells from mice deficient for the three cytokines examined. Following the injection of DT, only the DOG-GFP DC cells are eliminated and thus the precursors insensitive to DT, but deficient for the three cytokines are able to repopulate the cleared compartment. When the DT is injected for the right period (7 days) in order to eliminate all DOG-GFP DC cells, most of the DCs should be ko for the considered cytokines but normal in numbers.

NK cells and all $CD11c^{high}$ cells will be present in the same proportion after treatment with DT (80% DOG-GFP, 20% KO).



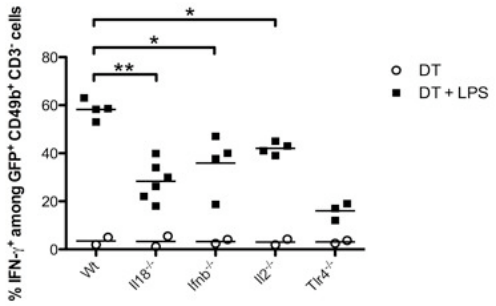


Fig.7 – IL-2, IL-18 and IFN-β are required for a full NK cells activation *in vivo*. DOG-GFP mice were lethally irradiated and then reconstituted with BM cells from donor mice 80% DOG-GFP and 20% mice ko for the cytokines under investigation. After two months of recovering, the mice were injected daily i.p. with DT for a week and the eighth day they were treated i.v. with LPS. After 5 hours the splenocytes were restimulated *ex vivo* with PMA/ionomycin for 3 hours in the presence of BFA and was performed an intracellular staining for IFN-γ. NK cells were defined as $CD49b^+$ $CD3^-$. Only GFP^+ NK cells, not bearing any gene knock out, were analyzed. n ≥ 2. Error bars depict SEM. Statistical significance was determined by means of the one-tailed Mann-Whitney test: * when $p<0.05$; ** when $p<0.01$; *** when $p<0.001$; ns, not significant.

The most radio-sensitive cells non-$CD11c^{high}$ (including NK cells) do not show any deficiency following injection of DT and can be easily distinguished by the expression of GFP. In this way, NK cells can develop in a more physiological environment compared with mice completely ko for a particular gene that normally undergo molecular and cellular disregulation. After a

week of deletions, although almost all DC cells are ko for the cytokine of interest, the deficiency is still restricted to a cellular compartment (DC) and then the effects of deficiency are limited. In addition to DOG-GFP donor cells, the knockout cells derived from mice lacking IL-18, IL-2, IFN-β or TLR-4 as a control. LPS was injected into chimeras after a week of treatment with DT and then was evaluated NK cell activation. The population of NK cell IFN-γ^+ was greatly reduced when DC lack one of the three cytokines (Fig. 7). As we have seen *in vitro*, we observed the involvement of IL-18, IL-2 and IFN-β in the activation of NK cells *in vivo* too following stimulation with LPS.

Cytotoxic activity of NK cells requie IFN-β

Up to now we have observed the importance of IFN-β, IL-18 and IL-2 in the NK activation mediated by DC only in terms of IFN-γ production. However NK cells have another important function that is cytotoxicity against missing- or altered-self targets (Lanier 2005). In our experiments, we had observed that IL-18 and IL-2 ko cells displayed no role in the NK cell cytotoxic activity DC-mediated (Fig.8A); by contrast IFN-β is essencial for the NK cells cytotoxic activity (Fig.8B).

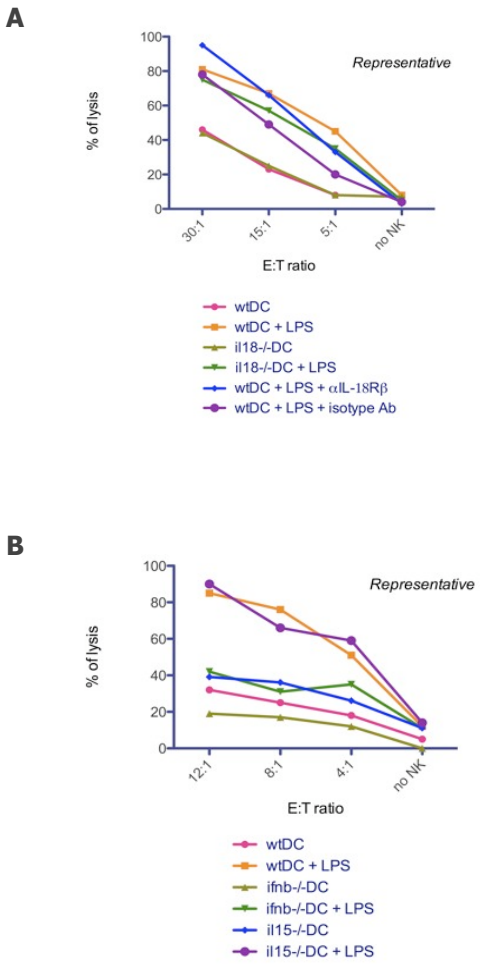


Fig.8 – Cytotoxic activity by NK cells require IFN-β. GM-CSF derived BM-DC (8E4/well) of the indicated genotipe, stimulated or not with LPS, were co-coltured with wt NK cells for 6 h, in the presence or absence of the neutralizing anti-IL-18R Ab or its isotype control. TDA-loaded YAC-1 target cells (2E4/well) were added to the co-colture for 3 additional hours. Target cells killed by effector cells released TDA in the culture medium. TDA-containing supernatants were introduced in europium solution at the end of the culture. Eu and TDA form a stable and highly fluorescent chelate. The fluorescent signal, directly correlating with the amount of lysated cells, was

measured and scaled according to maximum and spontaneous release. Two representative experiments out af six are shown.

Cytotoxic activity of NK cells requires IFN-β *in vivo*

NK cells are able to activate their cytotoxic activity in responce to cells that don't express MHC or that express low MHC levels (Lanier 2005). To confirm the importance of DC-released IFN-β in this process *in vivo* and in a more physiological context, we have injected 50% of wt splenocytes and 50% of β2microglobulin ko splenocytes labeled with two different concentration of CFSE into WT mice and mice deficient for IL-2, IL-18 and IFN-β. After 24 h, we have valuated the percentage of lysated cells with FACScaibur, mesuring the ratio between wt and β2M ko cells.

We have observed that the killing percentage is lower when we have used INF-β ko recipient mice, demonstrating that INF-β, but not IL-2 or IL-18, is important in NK cytotoxic activity.

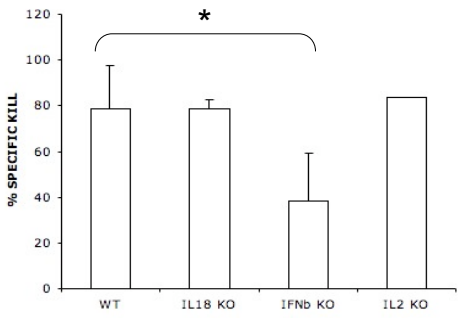


Fig.9 – Cytotoxic activity by NK cells require IFN-β *in vivo*. 7E6 RBC-lysed splenocytes, 50% from wt donor and 50% from β2M ko donor, were labeled with 0.075 μM and 0.75 μM CFSE rispectively and then injected, with or without LPS, i.v. into wt and IL-2, IL-18, IFN-β ko recipient mice. After an O.N incubation, splenocytes from the recipient mice were collected and analyzed with a facs analysis. We have mesured the percentage of ko or wt cells in the different mice and we have calculated the ratio between this two population. we were able to obtain the percentage of specific killing using this formula: % specific killing = $100 - (100 \times ratio_{treated})/ratio_{untreated}$

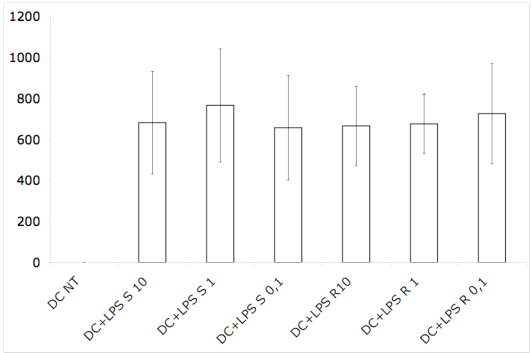
Possible NK cells involvement in the inflammasome activation

Since IL-18 has proved to be important in the activation of NK cells and because IL-18, as IL-1β, is secreted after activation of the inflammasome and cleavage of the precursor by caspase-1 (Keller, Rüegg et al. 2008), we hypothesized a possible involvement of NK cells in this process. It is also known that the activation of the inflammasome in macrophages requires a double stimulation with TLR ligands, such as ATP or muramil dipeptide (MDP) (Netea, Van de Veerdonk et al. 2008). We wanted to test whether the same stimuli were required also for the inflammasome activation in DCs or whether LPS per se was a sufficient stimulus. We tested two different types of LPS: rough LPS (R), used in all previous experiments, and smooth LPS (S) that differs from the previous for the presence of an O-polysaccharide structure (Jiang, Georgel et al. 2005; Gangloff,

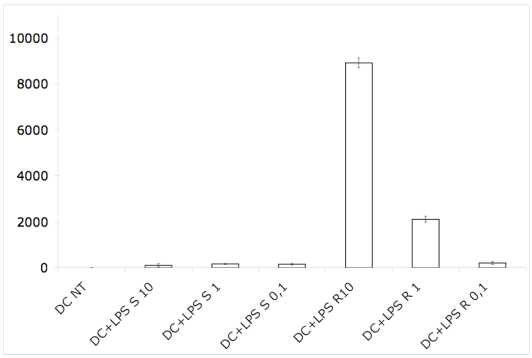
Zahringer et al. 2002).

The two types of LPS were used at concentrations of 10 μg/ml, 1 μg/ml and 0.1 μg/ml and as proof of inflammasome activation we measured the production of pro-IL-1β (intracellular form of cytokine) and IL-1β (secreted form), because IL-18 is very difficoult to detect. The production of pro-IL-1β is neither LPS nor dose-dependent, while DC produce IL-1β only using LPS R and in a dose dependent manner (Fig.10 A-B). We investigated whether the same phenomenon occurs in the presence of NK cells measuring the production of IFN-γ, and the result is the same (Fig.10 C). Assuming that IL-18 behaves in the same manner as IL-1β, knowing the role of IL-18 in NK-DC interaction, we can say that inflammasome also plays a role in this process. It would be interesting to see which inflammasome is involved.

A



B



C

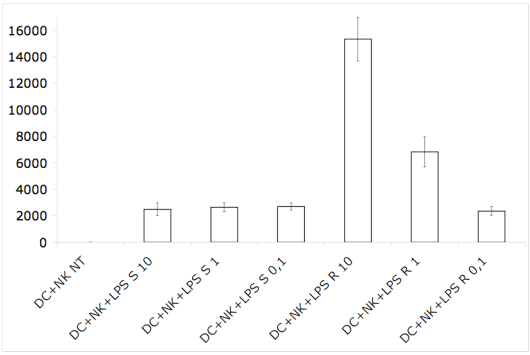


Fig.10 – Possible involvement of NK cells in the inflammasome activation. (**A-B**) 1E5 wt GM-CSF-derived BM-DC were stimulated with LPS R and LPS S at three different concentration (10, 1, 0,1 μg/ml) and after an O.N. incubation the production of pro-IL-1β and IL-1β were measured by ELISA. (**C**) 1E5 wt GM-CSF BM-DC stimulated with LPS S and LPS R (10, 1, 0,1 μg/ml) and 5E4 wt NK cells were co-coltured O.N. The production of IFN-γ were measured by ELISA.

This process is ASC-dependent, but Nalp3, P2X indipendent

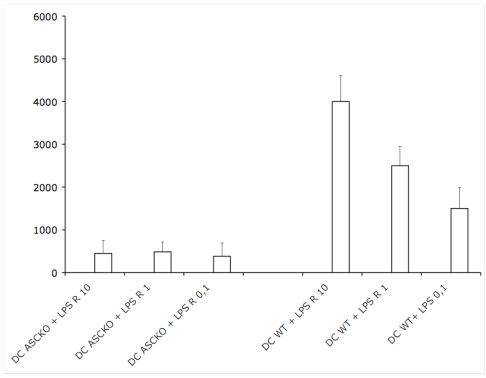
There are three subfamilies of NLRs:

- NALPs: they are the central monomers (eg NALP1, NALP2 and NALP3) of the platform that can activate caspase-1.
- IPAF and NAIP: they have a different N-terminal domain but both are involved in the formation of the inflammasome individually or in combination.
- NODS: they have different functions during the assembly of the inflammasome.

All types of inflammasomes lead to the same effect: the secretion of IL-1β and IL-18. This occurs through a cascade of signals using different molecules including the adapter molecule ASC (associated speck-like protein containing CARD, caspase activation and recruitment domain) whose involvement has been demonstrated using ASC-deficient DC stimulated with LPS S and LPS R at three different concentration. (Fig.11A).

We subsequently evaluated the role of ATP-gated P2X receptors using $P2X^{-/-}$ DCs. We observed a lack of involvement of these receptors in the inflammasome activation. The fact that P2X receptors are not involved proves once again that in the case of DC a single stimulus is sufficient for the inflammasome activation. (Fig.11 B).

A



B

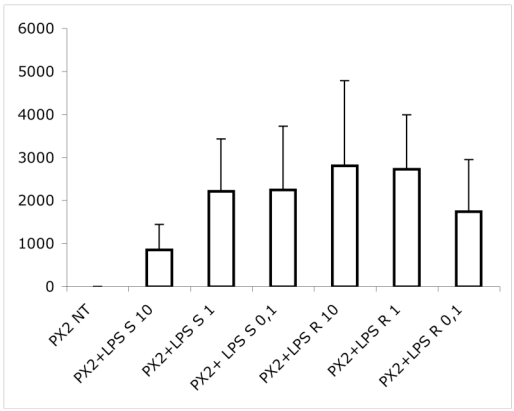


Fig.11 – Role of ASC and P2X ATP-dependent receptors in the inflammasome activation. 1E5 ASC or P2X ko GM-CSF-derived BM-DC were stimulated with LPS R and LPS S at three different concentration (10, 1, 0,1 μg/ml) and after an O.N. incubation the production of IL-1β were measured by ELISA.

This process seems so tied to one of the inflammasomes, but not NALP3, using ASC in the signal cascade that leads to IL-18 and IL-1β secretion.

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Chapter III.

Summary, conclusion and future perspectives

DISCUSSION

The interaction between DC and NK cells is very important for immune responses to tumors and infectious agents (Fernandez, Lozier et al. 1999; Gerosa, Baldani-Guerra et al. 2002; Ferlazzo, Morandi et al. 2003). The molecular basis of this interaction is unclear. In this study we defined the nature of the signals involved in this interaction in conditions mimiking bacterial infection.

First of all we have escluded the possibility that LPS can act directly on NK cells, without the need of accessory cells. Many studies report a direct recognition of TLR agonists by NK cells (Becker, Salaiza et al. 2003; Sivori, Falco et al. 2004; Lauzon, Mian et al. 2006; Sivori, Carlomagno et al. 2006; Sivori, Falco et al. 2007). This is not the case of TLR-4, as shown by TLR4 deficient NK cells. In contrast TLR-4 is important for DC and this was demonstrated both *in vitro* and *in vivo*. We have shown that DC produce IL-2, IL-18 and IFN-β after stimulation with LPS, and these three cytokines are necessary and sufficient to elicit IFN-γ production by NK cells.

It has been shown that NK-DC interactions require both soluble and contact-dependent signals. We have shown, however, that soluble cytokines IL-2, IL-18 and INF-β are able to fully activate NK cells at physiological concentration. This discrepancy with the literature can be explained by two hypotheses focused primarily on the role of IL-18:

The stimulation with LPS is not sufficient to induce the production of IL-18 by DC, for the inflammasome activation NK cells contact is required. At first, the dependence on contact with the DC for the activation of NK cells, as for the priming of T cells, was interpreted as a necessity of costimulatory molecules bound to the DCs membrane (such as CD40 and CD80) that are capable directly to stimulate NK cells (Newman and Riley 2007). However, our data show a dual mechanism. Initially, NK cells stimulate DC through cell-cell contact, DC release soluble IL-18 with IL-2 and IFN-β, allowing the release of IFN-γ by NK cells. Therefore, the NK cell–mediated DC stimulation and the subsequent DC-mediated NK cell activation, would be contact dependent.

If the amount of cytokines released is very low, it is possible that the concentration may be sufficient to be biologically effective only in a small area surrounding the site of secretion. In this way, the soluble signals resemble more similarly to conctat-dependent signals. It has been shown that IL-12 and IL-18 are released in small amounts even after stimulation, and their secretion is confined to the immune synapse (Borg, Jalil et al. 2004; Semino, Angelini et al. 2005). It's possible that the secretion of IL-18 may be important for the contact dependency of DC-mediated NK cells activation.

Both hypotheses suggest that the supernatants of DC stimulated with LPS are not able to activate NK cells. In contrast, the addition of rIL-18 at doses that mimic the activity

to immune synapses may be effective and may bypass the step contact dependent induction of release of IL-18 when combined with IL-2 and IFN-β. This hypothesis seems to be in agreement with our observations.

IL-18 performs its functions through IL-18R using the pathway mediated by MyD88. In fact, MyD88- and IL-18R- deficient NK cells show less production of IFN-γ when co-cultured with DC stimulated with LPS. However, only MyD88 deficient NK cells showed a complete abrogation of the secretion of this cytokine. Similarly, the neutralization of IL-18 with blocking antibodies and genetic ablation gave the same result, namely a decrease, but not absolute, in the cytokine production. In the NK cell activation process we exclude any involvement of IL-1α/β, although the IL-1 family includes a number of other members whose functions are not yet fully known but they likely use MyD88 pathway (Dinarello 2009).

Sice IL-18 together with IL-2 and IFN-β is sufficient to induce a full activation of NK cells for the production of IFN-γ, most likely the importance of IL-1 family in this process is visible and significant only in the absence of IL-18. Since MyD88 is important for two signal transduction pathways (TLR and IL-1family) it can be also thought that its elimination will affect the balance of intracellular transduction network in the absence of infection/ inflammation, perhaps preventing the development of regular NK cells, that might be generically hyporesponsive.

In our experiments we did not find any involvement of IL-

12p70 or IL-15, cytokines that are usually associated with activation of NK cells by accessory cells in many experimental settings (Carson, Giri et al. 1994; Ferlazzo, Tsang et al. 2002; Borg, Jalil et al. 2004; Ferlazzo, Pack et al. 2004; Newman and Riley 2007, Huntington, Legrand et al. 2009). Mouse and human DC secrete IL-12 only in the presence of IL-4 (Hochrein, O'Keeffe et al. 2000). In contrast, the production of IL-2 following microbial stimulation fails in the presence of IL-4 (Guiducci, Valzasina et al. 2005) that is, in addition, a semi-maturation stimulus for DC. DC exposed to IL-4 acquire the capacity to activate NK cells regardless of the presence of microbial stimuli (Ferlazzo. Morandi et al. 2003). In human, the addition of IL-4 is only a method to allow differentiation of monocytic precursors into macrophages (Sallusto and Lanzavecchia 1994). For this reason, DC differentiated with only GM-CSF produce IL-2 but not IL-12 in response to LPS, whereas in the absence of IL-12, the DC are still able to activate NK cells in IL-2 dependent manner (Granucci, Zanoni et al. 2004). In our system we could not identify a role for IL-15. This cytokine shares β and γ subunits of its heterotrimeric receptor with IL-2R. Although the subunits is different in IL-2R and IL-15R, IL-15Rα is unable to transduce the intracellular signal, but is used to trans-present IL-15 to cells that have IL-15R$\beta\gamma$, like NK cells (Mortier, Woo et al. 2008). It can therefore be assumed that the signal transduction pathway used for the production of IL-2 and IL-15 are partly overlapping; the best

result of the stimulation of NK cells mediated by IL-2 and IL-15 is the phosphorylation of STAT5 (Strbo, de Armas et al. 2008). According to this, the different activities of these two cytokines can be distinguished by different expression of the α chain of their receptor on target cells using two different pathways for signal transduction. There is sufficient evidence regarding the role of IL-15 in experiments in which DC exposed to IL-4 did not produce IL-2 (Koka, Burkett et al. 2004). We therefore hypothesize that IL-2 is able to complement the activity of IL-15 in the activation of NK cells, while the reverse is not necessarily true. In future experiments we will better assess the role of IL-15 in the NK-DC interaction trying to focus our attention on the type of stimulus provided by the secretion of this cytokine and the role of DC and NK cells in cytokine secretion. Another important finding of this study is the unique role of DCs in the activation of NK cells *in vivo*. To conclusively establish the role of DCs in the activation of NK cells *in vivo*, we developed an experimental model based on mixed bone marrow chimeras. DC11c.DOG-GFP double transgenic mice were irradiated and then reconstituted with BM cells from donor mice of the same strain and with a small percentage from mice deficient for the gene of interest. Following prolonged DT injections, these mice develop normal-sized niches which are characterized by heterogeneous cells deficient for the gene set. Since DC without TLR-4 may not respond to stimulation with LPS, the chimeras reconstituted with $TLR4^{-/-}$ DC do not respond

to stimulation with LPS only in the DC compartment, while keeping the physiological number of DCs. In this way we confirmed that $CD11c^{hi}$ DC are involved for 60-70% in cell-dependent NK cells activation after stimulation with LPS. These mice served as negative control for the other chimeras reconstituted with DC deficient for IL-2, IL-18 and IFN-β. These mice have a number of NK cell IFN-γ^+ much lower after stimulation with LPS, and this confirms the data we have obtained *in vitro*.

We paid more attention to the production of IFN-γ rather than cytotoxic activity of NK cells because the scope of this work is to study the activation of NK cells in response to the LPS that is present in the gram-negative bacteria wall. IFN-γ is the most important factor in mediating the phagocytic activation and Th1 polarization, it is the most powerful weapon against bacterial infections (Bohem, Klamp et al. 1997; Ferlazzo, Morandi et al. 2003). We then paid attention on NK-DC interaction with regard to the possible increase in the cytotoxic response. IL-2 released from DC to physiological concentrations was previously demonstrated by our laboratory to be dispensable for the development of a cytotoxic response (Granucci, Zanoni et al. 2004). This should not be confused with the cultures of NK cells grown for days in the presence of rIL-2 at high doses, which transforms into LAK cells with a potent cytotoxic activity (Grimm, Mazumder et al. 1982; Whiteside 2001). Here we show that IL-18 and IL-2 are not necessary to activate the cytotoxic

activity of NK cells, but IFN-β, exactly as reported in the literature (Nguyen, Salazar-Mather et al. 2002), potently induces cytotoxic response of NK cells both *in vitro* and *in vivo*.
A better knowledge of the mechanism of NK cells activation could be infinitely useful for developing targeted therapies. For example, the drugs available to treat $EGFR^+$ tumors are humanized antibodies against EGFR although it has been scientifically proven that the neutralization of the EGFR pathway with specific antibodies play a minor role in the fight against cancer progression. Our findings reveal novel aspects of the molecular mechanisms that contribute to DC–NK interactions both *in vitro* and *in vivo* and define the involvement of IL-2, IL-18 and IFN-β. Understanding the regulation of innate immune responses and defining the critical mediators places us closer to effectively manipulate these responses to improve therapeutic outcomes.

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