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Mechanisms of dendritic cell-mediated natural killer cell activation

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Chapter I.

Introduction

Non-standard abbreviations

For readability issues, standard abbreviations, as listed by the European Journal of Immunology and reported in Appendix A, are not defined in the main text. Non-standard abbreviations used in this chapter are defined in the main text the first time they are used and are listed here. The same form is used in the plural.

AIM2, absent in melanoma 2
ASC, apoptosis-associated speck-like protein containing a CARD
ATP, adenosine-5'-triphosphate
BDCA-2, blood dendritic cell antigen 2
CARD, caspase activation and recruitment domain
CLR, C-type lectin receptors
CPPD, calcium pyrophosphate dihydrate crystals
DC-SIGN, DC-specific ICAM-3-grabbing non-integrin
FCAS, familial cold autoinflammatory syndrome
FLT3L, FMS-related tyrosine kinase 3 ligand
IKK, IκB kinase
iNOS, inducible nitric oxide synthase
IRAK, IL-1R associated kinase
IRF, interferon regulatory factor

KIR, killer cell Ig-like receptor

LC, Langherans cell

LGP2, laboratory of genetics and physiology 2

LRR, leucine-rich repeat

MAVS, mitochondrial anti-viral signaling

MDA5, melanoma differentiation associated gene 5

MDP, muramyl dipeptide

MMR, MΦ mannose receptor

Mo, monocytes

MSU, monosodium urate

MWS, Muckle-Well syndrome

NCR, natural cytotoxicity receptor

NKP, NK cell precursor

NLR, nucleotide-binding domain and leucine-rich repeat containing

NLRC4, NLR family, CARD domain containing 4

NLRP3, NLR family, pyrin domain containing 3

NOD, nucleotide oligomerization domain

PAK, p21-activated kinases

PGN, peptidoglycan

RHIM, RIP homotypic interaction motif

RIG-I, retinoic acid inducible gene I

RIP, receptor interacting protein

RLH, RIG-I-like helicases

Syk, spleen Tyr kinase

TAK1, TGF-β-activated protein kinase 1

TANK, TRAF family member-associated NF-kappa-B activator

Tip, TNF and iNOS producing

TIR, Toll/IL-1R

TIRAP, TIR domain-containing adaptor protein

TRADD, TNFR1-associated via death domain

TRAF6, TNFR-associated factor 6

TRAM, TRIF-related adaptor molecule

TRIF, TIR domain-containing adaptor inducing IFN-β

Dendritic cells

Adaptive immunity has long been the primary focus of immunological studies because of its extreme plasticity and seemingly higher complexity than innate immunity. Only 30 years ago, the mechanisms underlying the regulation of adaptive immunity were thought to be independent from innate immunity. Nowadays, it is becoming increasingly clear how deep the connection between the two branches of the immune system is. At the interface between innate and adaptive immunity, DC play a key role.

Overview

DC were identified in 1973 by Ralph Steinman and Zavil Cohn who proposed the name “dendritic cells” due to their distinct morphology, characterized by stellate shape and continous expansion and retraction of their processes (Steinman and Cohn 1973). Formally belonging to innate immunity because their receptors are germline-encoded, DC are essential regulators of T cell activation. Although other APC, such as B cells and MΦ, are able to prime T cells, those cells evolved to fulfill different tasks and are primarily focused either to antibody production or clearance of pathogens and cellular debris, respectively. By contrast, DC specifically evolved to initiate adaptive immunity and their antigen presentation capabilities

are far superior to those of B cells and macrophages (Banchereau and Steinman 1998).

According to the paradigm (Figure 1), immature DC capture antigens in peripheral tissues. Then, under the influence of inflammatory cytokines or triggered by microbial encounter, DC undergo a complex reprogramming of gene expression called “maturation”. During maturation, DC migrate to secondary lymphoid organs. Once there, the eventually fully-mature DC now express high levels of soluble and membrane-associated molecules that prime/activate T cells whose specificity matches the processed antigen displayed by DC.

Beside the well-documented antigen presentation and T cell activation, investigators have reported a role for DC in the maintenance of tolerance (Steinman and Nussenzweig 2002) and in NK cell activation (Fernandez, Lozier *et al.* 1999; Lucas, Schachterle *et al.* 2007; Newman and Riley 2007).

Development and subsets

Most of our knowledge on DC comes from the study of precursors (either monocytes, Mo, or BM HSC) differentiated *in vitro* in the presence of GM-CSF plus or minus IL-4. However, *in vivo* DC show different features of their *in vitro* counterparts and are less homogeneous. Moreover, mice deficient in GM-CSF seem to have a normal DC compartment (Vremec, Lieschke *et al.* 1997). This consideration is of particular

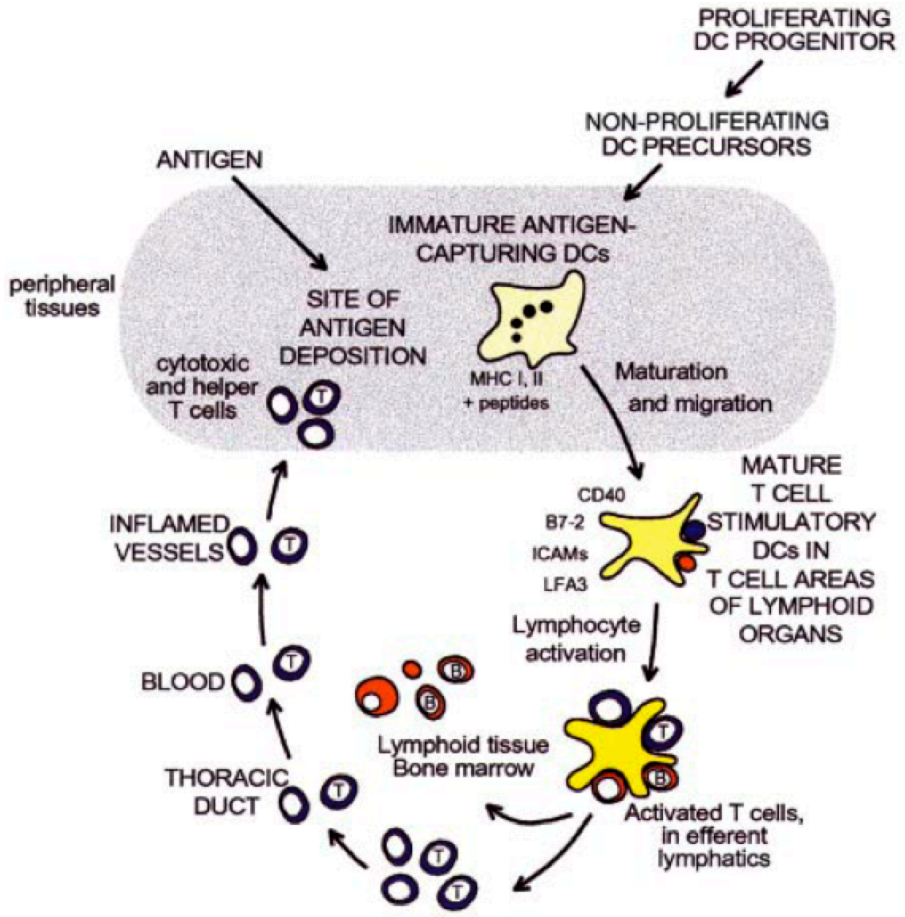

Figure 1: (from Bancherau and Steinman 1998). DC life cycle.

importance for human studies. Indeed, although Mo represent the major source of DC progenitors for the *in vitro* DC differentiation in humans, Mo-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. Indeed, the major cytokine directing DC development *in vivo* at the steady-state is FMS-related tyrosine kinase ligand

(FLT3L) (Maraskovsky, Brasel *et al.* 1996; McKenna, Stocking *et al.* 2000).

Recently, a cell type specialized in IFN-I production has been identified and called plasmacytoid DC (Liu 2005). Developmentally, it is only loosely related to classical DC, called conventional DC, and its physiology is quite different. Therefore, from now onwards, we will restrict our discussion to conventional DC.

Shortman and colleagues (Shortman and Naik 2007) divide DC according to a variety of criteria:

- Migratory versus lymphoid-tissue resident DC. According to the paradigm, DC behave as depicted in Figure 1, sampling the environment and migrating to secondary lymphoid organs upon infection/inflammation. By the time they have reached secondary lymphoid organs, they have already achieved a mature phenotype and shut down the phagocytic machinery. Langherans cells (LC) of the epidermis are the most common example of such DC. By contrast, lymphoid-tissue resident DC do not display a migratory behavior. They are immature and active in antigen uptake.
- Differential expression of markers. Migratory DC from different tissues can be distinguished by the expression of specific molecules. For example, epidermal LC have higher levels of langerin than dermal DC. Similarly, lymphoid-tissue resident DC can be divided, in mice, in

two major subsets: $CD8^-$ ($CD205^-$ $33D1^+$ $Sirp\alpha^+$) and $CD8^+$ ($CD205^+$ $33D1^-$ $Sirp\alpha^-$). The two subsets differ not only for the surface phenotype, but also at functional level. Indeed, $CD8^+$ DC are specialized in cross-presentation (uptake of extracellular antigens, processing and loading on MHC I molecules) and IL-12 secretion. $CD8^-$ DC can be further divided according to the presence or absence of CD4.

- Steady state versus inflammatory DC. Some DC population cannot be found at the steady state and emerge upon infection/inflammation. For instance, the so-called Tip DC (from TNF and inducible nitric oxide synthase, iNOS, produced by those cells) appear as a consequence of *Listeria monocytogenes* infection in mice. In humans, Mo-derived DC represent the most close counterpart of murine Tip DC. Not surprisingly, inflammatory DC may differentiate from inflammatory Mo.

All DC develop from HSC, similarly to other blood cells (Shortman and Naik 2007). Initially, it seemed obvious that DC would come from myeloid precursors, given that their closest relationship at phenotypic and functional levels is with MΦ. Moreover, HSC grown under the influence of GM-CSF give rise to DC, MΦ and granulocytes (Inaba, Inaba *et al.* 1993). Surprisingly, it was found that even lymphoid precursors can differentiate into DC (Ardavin, Wu *et al.* 1993). This was first

interpreted as a differential origin of the two major DC subsets at the steady state, specifically myeloid ancestry for $CD8^-$ DC and lymphoid lineage for $CD8^+$ DC. However, later on it was reported that both lymphoid and myeloid precursors can produce all known DC subsets, showing an unsuspected plasticity in DC development (Manz, Traver *et al.* 2001). Therefore, the commitment to the DC lineage should act downstream of these two precursors.

Recently, an intermediate DC precursor was identified in the BM and found to express the inflammatory Mo marker CX_3CR1, but not CD11c. However, upon transfer to irradiated or non-irradiated recipients, this precursor was 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 (at low levels) is acquired by immature DC and is upregulated during DC maturation. Indeed, the mature DC can be classified as the latest stage of DC development. Therefore, DC development is under control of both signals coming from the tissue environment, as for any other cell in the body, and microbial/inflammatory stimuli, a property specific of the immune system (Figure 2).

In the spleen, BrdU studies suggest that the turnover of DC is fast (3-5 days) (Kamath, Pooley *et al.* 2000; Diao, Winter *et al.* 2006) and relies on both the homeostatic proliferation of fully-developed DC and the differentiation of splenic DC precursors (Diao, Winter *et al.* 2006; Naik, Metcalf *et al.* 2006). It was

believed that, since splenic DC precursors are not able to self-renew, constant replenishment from blood-borne precursors must take place, but this notion has been recently challenged by experiments with parabiotic mice (Kabashima, Banks *et al.* 2005).

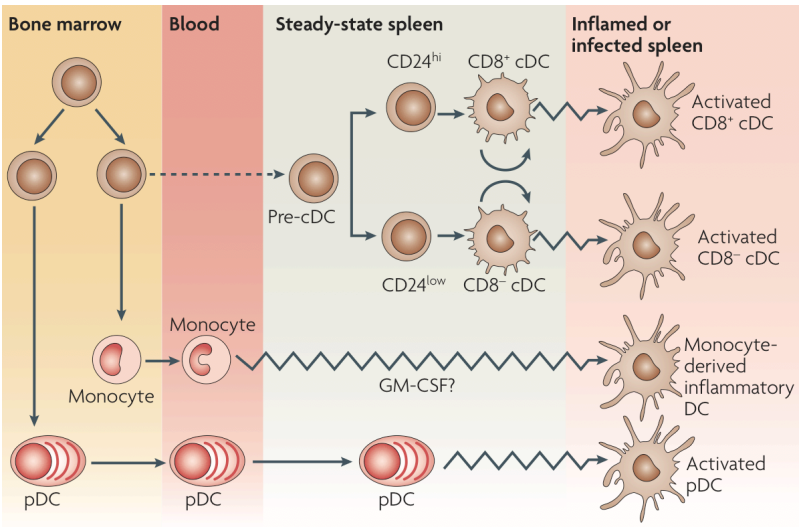

Figure 2: (from Shortman and Naik 2007). Developmental pathways to splenic DC.

Receptors

DC sense pathogens through PRR, receptors recognizing conserved structures exclusively espressed by certain classes of microbes, called PAMP. PRR include most receptors for Ag uptake, such as FcR and complement receptors. However, the most studied class of PRR is represented by TLR.

TLR

Hoffmann and colleagues initiated the TLR field in 1996 by observing that Toll-mutant flies were highly susceptible to fungal infection (Lemaitre, Nicolas *et al.* 1996; 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 positionally cloned the murine receptor for LPS, a homologue of *Drosophila melanogaster* Toll as well, thus named TLR4 (Poltorak, He *et al.* 1998). Since those pioneering 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 variety of both immune and non-immune cells, such as DC, MΦ, T cells, B cells, NK cells, epithelial cells, fibroblasts, adipocytes and others.

TLR are functionally competent as dimers, usually homo-dimers, with the exception of hetero-dimers between TLR2 and either TLR1 or TLR6 (Triantafilou, Gamper *et al.* 2006). The nucleic acid-sensing TLR3, TLR7, TLR8 and TLR9 are expressed in the ER compartment, whereas all other TLR are found on the plasma membrane. It has been proposed that the ER localization of TLR recognizing DNA and RNA is mandatory to avoid sensing of self nucleic acids (Barton, Kagan *et al.* 2006). By contrast, plasma membrane TLR sense a variety of microbial ligands, such as LPS, flagellin, peptidoglycan and

lipopeptides (Figure 3), which have no counterparts in higher organisms.

All TLR share key features on both structural and signaling

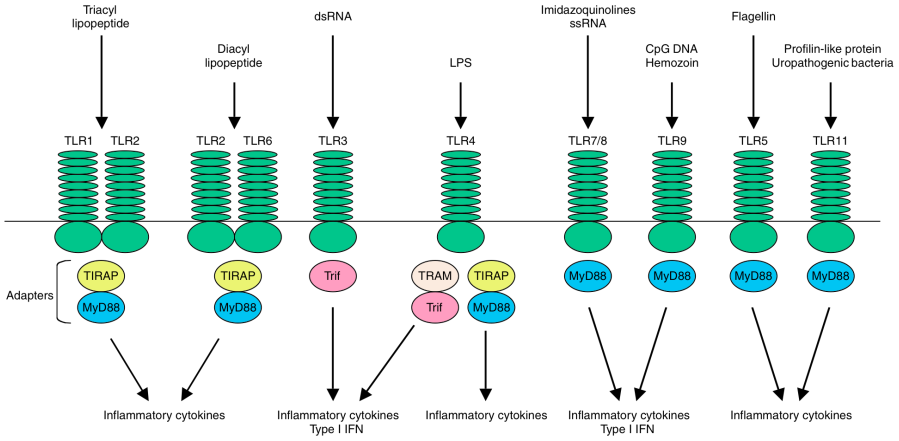

Figure 3: (from Kawai and Akira 2006). TLR ligands and adaptors.

levels. Each TLR is composed of three domains: a ligand-binding leucine-rich repeat (LRR) domain, a transmembrane domain and a cytosolic Toll/IL-1R (TIR) domain committed to signal transduction. The signaling cascade of all TLR is initiated by TIR domain-dependent heterophilic interactions with TIR domain-containing cytosolic adaptors and invariably culminates in the activation of NF-κB and AP-1.

So far, four TIR domain-containing adaptors have been described as positively mediating TLR signaling: MyD88, TIR domain-containing adaptor protein (TIRAP)/Mal, TIR domain-containing adaptor inducing IFN-β (TRIF) (also known as

TICAM1) and TRIF-related adaptor molecule (TRAM) (also known as TICAM2).

MyD88, together with TIRAP, has a chief role in TLR signaling (Kawai and Akira 2007) and is utilized by nearly all TLR, with the notable exception of TLR3. Upon receptor engagement by an appropriate ligand, MyD88 associates with the TLR and recruits members of the IRAK (IL-1R associated kinase) family: IRAK1, IRAK2, IRAK4 and IRAK-M. Only IRAK1 and IRAK4 are active Ser/Thr protein kinases, whereas IRAK2 and IRAK-M are not functional, suggesting that they may negatively regulate TLR signaling. As a consequence of recruitment, IRAK4 and IRAK1 are phosphorylated, dissociate from MyD88 and bind to TNFR-associated factor 6 (TRAF6). TRAF6 is an E3 ubiquitin ligase that, in complex with Ubc13 and Uev1A, promotes the synthesis of Lys 63-linked polyubiquitin chains. By ubiquitylation, TRAF6 activates TGF-β-activated protein kinase 1 (TAK1), a MAPKKK, that in turn forms a complex with TAB1, TAB2 and TAB3. TAK1 phosphorylates an unknown target, leading to the activation of IκB kinase (IKK), ultimately responsible for IκB degradation and NF-κB translocation to the nucleus. TAK1 also phosphorylates MKK3 and MKK6, two MAPKK, which subsequently activate the MAPK JNK and p38. 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, which can then associate as c-Jun homodimers or 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-κB and AP-1 direct the synthesis of the mRNA of several pro-inflammatory cytokines (Figure 4).

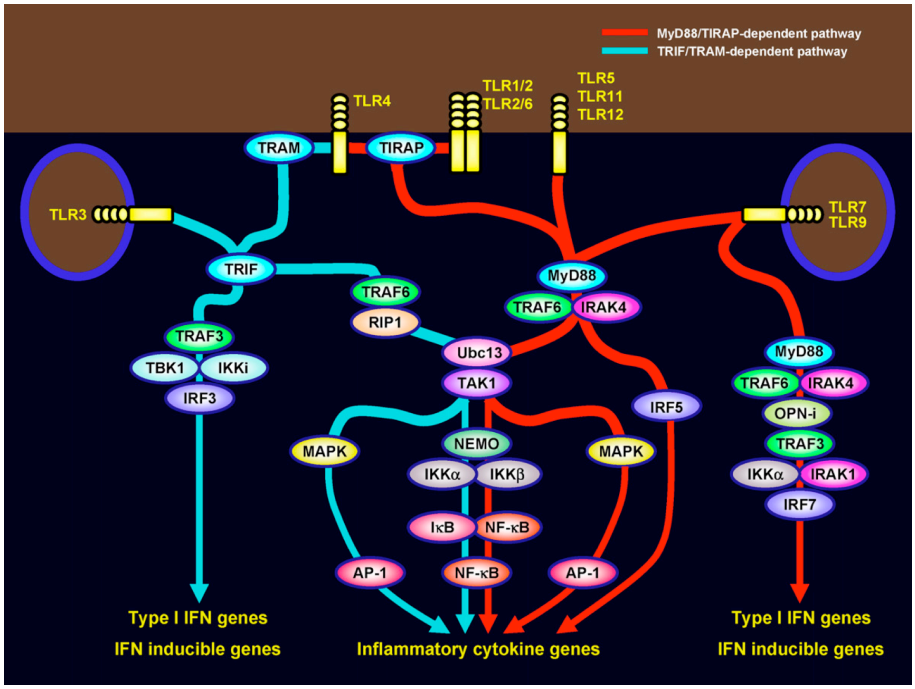

Figure 4: (from Kawai and Akira 2007). MyD88-dependent and -independent TLR signaling pathways.

TRIF mediates an MyD88-independent pathway triggered by TLR3 and TLR4 (Kawai and Akira 2007). In the case of TLR4, TRIF also needs the cooperation of another TIR domain-containing adaptor, TRAM. Notably, the TIRAP-MyD88 pathway is activated earlier than the TRAM-TRIF pathway. TLR4 engages with TIRAP and MyD88 on the cell surface. Once internalized, TLR4 traffics to the endosomes, where it

dissociates from TIRAP and MyD88, engaging and activating TRAM and TRIF (Kagan and Medzhitov 2006). When TRIF is activated by TLR4, it induces inflammatory responses via the recruitment of TRAF6, similarly to MyD88. However, TRIF exploits also a unique mechanism leading to NF-κB 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 members 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 (Figure 4).

On top of activating NF-κB and AP-1, similarly to MyD88, a distinctive feature of TRIF is its ability to induce the production of IFN-I and IFN-inducible genes, such as the chemokine IP-10 (Kawai and Akira 2006). TRIF mediates IFN-I production by recruiting TRAF3 and two non-canonical IKK, TBK1 and IKKi. These two IKK phosphorylate interferon regulatory factor (IRF) 3, that, in turn, forms a dimer and translocates to the nucleus to induce the expression of target genes, including IFN-α/β (Figure 4).

NLRP3 and inflammasomes

An early indicator of the role of nucleotide-binding domain and leucine-rich repeat containing (NLR) family, pyrin domain

containing 3 (NLRP3) in inflammation was its emerging association with immune disorders. Familial Cold Autoinflammatory Syndrome (FCAS) and Muckle-Well Syndrome (MWS) are two autosomal-dominant "autoinflammatory" conditions, which are characterized by intermittent episodes of rash, arthralgia, fever and conjunctivitis. In 2001 it was reported that both these diseases were linked to mutations in the *CIAS1* gene (Hoffman, Mueller *et al.* 2001), which encodes the NLRP3 protein (also called cryopyrin, NALP3 or PYPAF1) (Ting, Lovering *et al.* 2008). FCAS and MWS belong to the class of NLRP3/cryopyrin-associated periodic syndromes (CAPS), so what is the role of NLRP3 in these pathologies?

Martinon and colleagues began to answer this question by showing that NLRP3 is required for the maturation of the potent pro-inflammatory cytokine IL-1β (Martinon, Burns *et al.* 2002). This finding is clearly relevant in the clinical setting, as treating patients suffering from CAPS with the IL-1β inhibitors Anakinra or Rilonacept results in a striking resolution of their symptoms (Brydges, Mueller *et al.* 2009; Martinon, Mayor *et al.* 2009). Studies revealed that NLRP3 acts as part of a complex cascade involved in the activation and secretion of IL-1β. NLRP3 is incorporated into a large cytoplasmic caspase-1 activating complex called the inflammasome, which also contains the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD, caspase activation and recruitment domain), Cardinal and, of course, procaspase-1. Once the

activated inflammasome is assembled, the procaspase-1 zymogen self-cleaves to generate mature caspase-1, now able to activate precursors of many of the IL-1 family of cytokines through proteolytic processing (Martinon, Mayor *et al.* 2009). ProIL-1β and proIL-18 are converted to their biologically active forms by caspase-1; however, the IL-1α precursor does not require processing and is not a substrate of caspase-1 (Keller, Ruegg *et al.* 2008). Interestingly, NLRP3 also influences this cytokine; indeed, deficiency in NLRP3 impairs the release of IL-1α in response to adenosine-5'-triphosphate (ATP) stimulation (Keller, Ruegg *et al.* 2008). Therefore, active caspase-1 mediates both proteolytic processing and secretion of IL-1 family cytokines (Keller, Ruegg *et al.* 2008).

NLRP3 is the prototypic receptor able to organize an inflammasome, but it is by no means the only one. Other NLR have been reported to assemble alternative inflammasomes in response to different triggers (Martinon, Mayor *et al.* 2009). For instance, the NLRP1 inflammasome is responsive to the anthrax lethal toxin, whereas the NLR family CARD domain-containing 4 (NLRC4, also known as IPAF) inflammasome is able to sense bacterial virulence factors (mainly flagellin) injected through a type III or type IV secretion system. However, NLRP3 is unique in that it is activated by a variety of chemically unrelated stimuli. These include crystals, insoluble protein aggregates and other particulates (asbestos, silica, the Alzheimer's disease-related amyloid-β, the gout-causing agent monosodium urate, MSU, and the pseudogout-associated

calcium pyrophosphate dihydrate crystals, CPPD); ionophores, channel-activating or pore-forming toxins of both bacterial (nigericin and hemolysins from *L. monocytogenes*, *S. aureus*, *A. hydrophila*) and eukaryotic (maitotoxin) origin; and soluble purines (ATP) (Martinon, Mayor *et al.* 2009).

In trying to understand how NLRP3 functions, parallels were initially drawn with TLR. NLR family members, including NLRP3, contain a leucine-rich repeat domain, which is the portion of the TLR responsible for ligand binding (Martinon, Mayor *et al.* 2009). However, it now seems that, unlike TLR, NLRP3 does not directly bind the molecules that trigger its activation, but is activated by their common downstream effects, which explains why NLRP3 is able to sense such a huge variety of stimuli (Martinon, Mayor *et al.* 2009).

Attention has now turned to the identification of these common intracellular signals that might directly activate NLRP3. For example, phagocytosis of large particulates that activate the NLRP3 inflammasome is frequently unsuccessful, due to the sheer size of the particle. This is termed "frustrated" phagocytosis and induces ROS generation by NADPH oxidase (Dostert, Petrilli *et al.* 2008), but can lead to lysosomal rupture if phagocytosis progresses further (Hornung, Bauernfeind *et al.* 2008). 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 inflammasone, 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 triggered by the intermediary effect of massive K^+ efflux (Mariathasan, Weiss *et al.* 2006; Petrilli, Papin *et al.* 2007). Whilst 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.

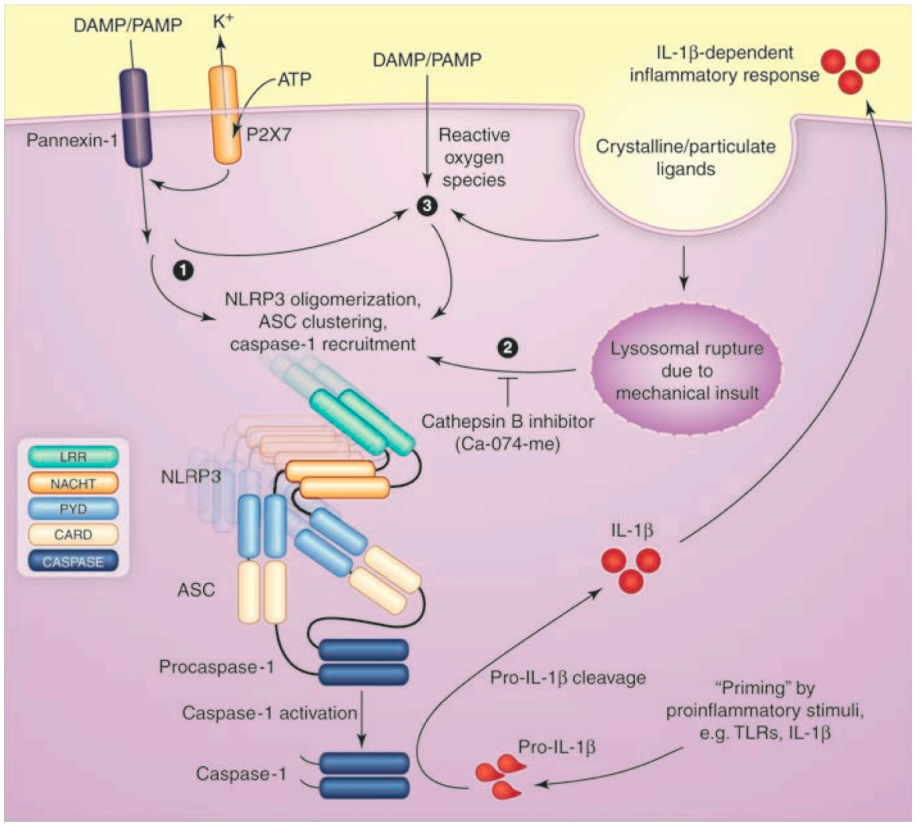

Figure 5: (from Schroder, Zhou *et al.* 2010). Current models for the activation of the NLRP3 inflammasome.

The NLRP3 inflammasome, however, does not act in isolation. It is strikingly unresponsive to its known triggers unless licensed by NF-κB 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-1β followed by a second signal controlling its release (Hogquist, Nett *et al.* 1991).

However, the interplay of TLR and the NLRP3 inflammasome is more complex. To illustrate, NLRP3-mediated cleavage of the constitutive IL-18 precursor (Semino, Angelini *et al.* 2005) still requires activation of both NF-κB and the inflammasome (Eisenbarth, Colegio *et al.* 2008). Therefore, in addition to TLR role in transcriptional regulation of cytokine precursors, TLR triggering must also be required for direct activation of inflammasomes. The issue was addressed by Bauernfeind and colleagues (Bauernfeind, Horvath *et al.* 2009), who identified the expression of *NLPR3* itself as the limiting factor in the NLRP3 inflammasome activation. Indeed, by increasing NLRP3 expression above threshold, NF-κB “primes” the inflammasome, namely renders it responsive to activating stimuli (Bauernfeind, Horvath *et al.* 2009).

Non-inflammasome NLR, RLH and CLR

Despite attention has mostly focused on the ability of certain members to form inflammasome complexes (Schroder, Zhou *et al.* 2010), NLR have other key functions. They are cytosolic receptors, homologues of the plant R (resistance) genes, which confer resistance to infection caused by fungal, viral, parasitic, and insect pathogens by inducing cell death of infected cells. In the NLR family, NOD1 and NOD2 were two of the first identified receptors. Both receptors sense a variety of bacterial peptidoglycan (PGN) moieties, even though the mechanisms by which PGN is delivered into the cytosol are unclear. Among others, the best known ligands are meso-diaminopimelic acid for NOD1 and muramyl dipeptide (MDP) for NOD2 (Ting, Duncan *et al.* 2010). NOD receptor signaling ultimately leads to NF-κB and MAPK activation. Upon ligand binding, NOD1 and NOD2 self-oligomerize and recruit the protein kinase RIP2. RIP2 converge at the level of TAK1 activation with the TLR pathway. As already depicted, TAK1 activation results in the nuclear translocation of NF-κB and AP-1. However, in the case of NOD receptors, the signaling through the MAPK-AP-1 axis requires CARD9 (Hsu, Zhang *et al.* 2007).

The RIG-I-like helicase (RLH) family, comprising the three members RIG-I (retinoic acid inducible gene I), MDA5 (melanoma differentiation associated gene 5) and LGP2 (laboratory of genetics and physiology 2), is specialized in the recognition of viral RNA in the cytoplasm. LGP2 seems to act

as a negative regulator of RIG-I, which is indispensable for IFN responses to many single-stranded RNA viruses, such as the influenza A virus. MDA5 is able to sense a variety of other RNA viruses and its detection capabilities poorly overlap with those of RIG-I, highlighting the extreme specificity of each member of the RLH family. This is a mandatory feature, since RLH are located in the cytosol, where host RNA is abundant. Still, RLH are activated only by viral RNA. Therefore, a molecular pattern intrinsic in viral RNA may be key in RLH discriminatory capabilities. To this respect, RIG-I has been particularly well studied. Figure 6 summarizes the putative RIG-I ligands identified so far (Rehwinkel and Reis e Sousa 2010).

MDA5 has not been as extensively studied as RIG-I and little is known about the structural requirements of its natural ligands. It is worth noting that the TLR3 ligand polyI:C activates MDA5, but when it is shortened to less than 1 kb, it becomes a RIG-I ligand (Kawai and Akira 2009).

To engage downstream pathways upon viral recognition, RLH form homotypic CARD-CARD interactions with the mitochondrial anti-viral signaling (MAVS) adaptor protein, resulting in the recruitment and activation of signaling molecules. The adaptors TRAF3, TRAF family member-associated NF-kappa-B activator (TANK) and TNFR1-associated via death domain (TRADD) and the protein kinases TBK1 and IKKi are responsible for activation of the transcription factors IRF3 and IRF7 and subsequent transcription of IFN-I

(Hornung and Latz 2010). In addition, RLH activate NF-κB for the production of pro-inflammatory cytokines via CARD9 and Bcl-10 (Poeck, Bscheider *et al.* 2010).

Excitingly, RIG-I has also been implicated in the indirect recognition of dsDNA. Several reports pointed to the existence

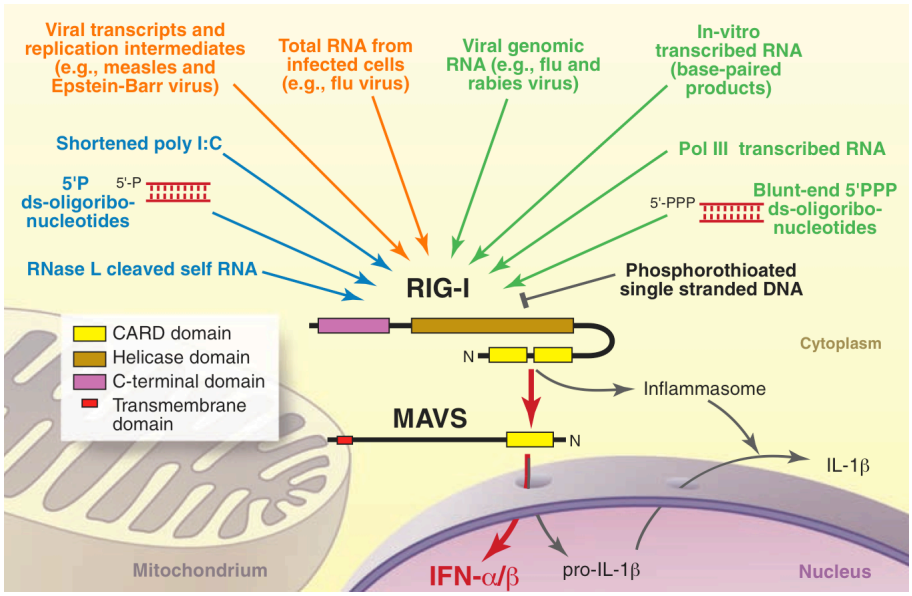

Figure 6: (from Rehwinkel and Reis e Sousa 2010). Putative RIG-I ligands.

of a cytosolic receptor promoting IFN-I production upon recognition of dsDNA or its synthetic equivalent polydA:dT, but the identity of such receptor has long remained elusive (Ishii, Coban *et al.* 2006; Stetson and Medzhitov 2006; Charrel-Dennis, Latz *et al.* 2008). Although polydA:dT cannot be sensed directly by RLH, it is transcribed by cytosolic RNA polymerase III into an uncapped RNA that triggers RIG-I (Ablasser,

Bauernfeind *et al.* 2009; Chiu, Macmillan *et al.* 2009), leading to IFN-I secretion. However, it seems that RIG-I has no role in the secretion of mature IL-1β in response to polydA:dT. Instead, the non-NLR absent in melanoma 2 (AIM2) inflammasome has been implicated in this process (Burckstummer, Baumann *et al.* 2009; Fernandes-Alnemri, Yu *et al.* 2009; Hornung, Ablasser *et al.* 2009; Roberts, Idris *et al.* 2009). Confusingly, RIG-I and MDA5 can form active inflammasomes in response to RNA viruses (Poeck, Bscheider *et al.* 2010). Since poly dA:dT is sensed by RIG-I through an RNA intermediate, it is not clear why RIG-I has not be implicated also in poly dA:dT-driven IL-1β release.

Another important class of PRR is represented by C-type lectin receptors (CLR). This family is specialized in the recognition of a wide range of carbohydrate structures and comprises both soluble and membrane-bound receptors. CLR can elicit inflammatory responses, similarly to TLR, NLR and RLH. Besides, a distinctive feature 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^{2+}-dependent manner (Geijtenbeek and Gringhuis 2009).

The MMR is an endocytic and phagocytic receptor that binds carbohydrate moieties on several pathogens, in particular viruses, fungi and mycobacteria. Moreover, 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, MMR binds endogenous molecules, such as neutrophil-derived myeloperoxidase, tissue plasminogen activator and lysosomal hydrolases (Allavena, Chieppa *et al.* 2004).

DC-SIGN is a type II transmembrane protein able to recognize both microbial and endogenous ligands, such as the HIV-1 gp120 envelope glycoprotein and ICAM-2 and ICAM-3 on resting T cells, respectively (Geijtenbeek, Torensma *et al.* 2000). Although DC-SIGN binds to the HIV-1 gp120, it does not function as a receptor for viral entry. Instead, DC carry DC-SIGN-bound HIV-1 through the lymphatics in a "Trojan horse" fashion, and it is eventually delivered to T cells (Geijtenbeek, Kwon *et al.* 2000). In this way, the infectivity period of HIV-1 is increased by several days. DC-SIGN-expressing DC are mainly located close to mucosal barriers. Indeed, lamina propria DC and dermal DC bear DC-SIGN. By contrast, LC do not express DC-SIGN but rather another CLR, Langerin, the hallmark protein of this cell type.

Dectin-1 (also known as CLEC7A) recognizes β-1,3-glucan and, as such, is able to detect a huge variety of fungal pathogens (including *Candida albicans* and *Aspergillus*

fumigatus) and mycobacteria. The commonly used stimulus zymosan, a preparation of yeast cell wall, is detected by both TLR2 and Dectin-1 (Brown, Taylor *et al.* 2002; Brown, Herre *et al.* 2003; Yoshitomi, Sakaguchi *et al.* 2005).

Recently, the signaling events downstream of CLR have attracted interest. From a biochemical perspective, CLR can be distinguished in receptors coupling to ITAM-containing adaptors, such as FcRγ and DAP12, and receptors inducing signaling pathways through the activation of protein kinases or phosphatases that either directly or indirectly interact with their cytoplasmic domains. Just to cite a few examples, to the first group belong Dectin-2 and CLEC4E, 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 examples are Dectin-1 and DC-SIGN, respectively.

The crosstalk between TLR and DC-SIGN requires the prior activation of NF-κB by TLR (or other NF-κB-triggering PRR). DC-SIGN engagement activates the Ser/Thr kinase RAF1 through a complex cascade involving the protein families Ras, Rho, p21-activated kinases (PAK) and Src. RAF1, in turn, induces the phosphorylation of the NF-κB subunit p65 at Ser276. Such phosphorylation seems to increase the transcriptional rate of NF-κB for selected genes, such as *Il8* and

Il10, but does not promote NF-κB nuclear translocation *per se*, providing a biochemical finding that explains why TLR cooperation is essential (Geijtenbeek and Gringhuis 2009).

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

DC linking innate and adaptive immunity

DC maturation is a terminal differentiation process through which the patrolling and poorly immunogenic immature DC switch to mature highly immunogenic DC. This process is characterized by a deep cytoskeletal reorganization with dendrite emission (Granucci, Petralia *et al.* 2003), migration to secondary lymphoid organs under the influence of CCL19 and CCL21 and enhanced Ag-presenting capacity. Indeed, mature DC upregulate MHC and costimulatory molecules, and produce chemokines and cytokines required for T cell activation and polarization.

DC maturation is triggered by microbial encounter or endogenous signals, such as inflammatory cytokines or molecules released by necrotic cells. DC maturation is a multi-step process. Initially, microbial or inflammatory stimuli induce the expression of intermediate levels of costimulatory molecules and promote migration, but cytokine secretion is poor. These features result in the so-called "licensed", or semi-mature

phenotype. Full DC maturation is accomplished during their CCR7-dependent migration toward the lymph nodes. Consistent with acquisition of costimulatory activity during maturation is the upregulation of CD86, CD80 and CD40 molecules (Banchereau and Steinman 1998).

The classical model of naive T cell activation postulates the requirement for three signals from the cognate APC: Ag-presentation in the context of MHC, costimulatory signals and soluble molecules that instruct T cells on the type of response required against the specific pathogen (Kalinski, Hilkens et al. 1999).

Differently from MΦ, Ag uptake in DC is not aimed at scavenging, but is specialized in processing and presentation on MHC molecules to T cells (Savina, Jancic et al. 2006). Immature DC can internalize particulate or soluble Ag via conventional phagocytosis, pinocytosis, receptor-mediated macropinocytosis and membrane ruffling (Banchereau and Steinman 1998).

The classical view of antigen presentation predicts MHC II loading for exogenous and MHC I loading for endogenous antigens. The peptides presented by MHC class I molecules are derived from proteins degraded mainly in the cytosol by the proteasome, whereas MHC class II molecules present peptides derived from proteins degraded in endosomal compartments by cathepsins and other hydrolytic enzymes. The ability of Ag

uptake decreases during the process of DC maturation, due to receptor internalization (Banchereau, Briere et al. 2000).

Costimulatory signals are required for full activation of naïve T cells. Consequently, antigen recognition in the absence of costimulation, such as in the case of immature DC, induces T cell anergy (Redmond W.L. & Sherman L.A., 2005). The best known costimulatory molecules on DC are the B7 family members CD80 and CD86, interacting with CD28 on T cells. Another crucial costimulatory molecule expressed by activated DC is CD40, which binds CD40L on T cells (Banchereau and Steinman 1998).

Different factors, including the type of stimulus and the particular PRR engaged, as well as the DC subset under investigation, induce a different pattern of cytokine release by activated DC (Kalinski, Hilkens et al. 1999). Using a global gene expression analysis, our group has studied the transcriptome reprogramming over time during DC maturation (Granucci, Vizzardelli et al. 2001). This study has shown that bacteria induce IL-2 production by DC, a cytokine essential for subsequent T cell proliferation. Only microbial molecules are effective stimuli of IL-2 secretion, whereas inflammatory cytokines lack this ability (Granucci, Feau et al. 2003).

Natural killer cells

NK cells are effector lymphocytes belonging to the innate immune system. Indeed, they are equipped with germ-line-encoded receptors not undergoing rearrangements as the TCR and the BCR do. As such, their responses do not show immunological memory, although this notion has been recently challenged by at least three independent studies (O'Leary, Goodarzi *et al.* 2006; Cooper, Elliott *et al.* 2009; Sun, Beilke *et al.* 2009). As they are currently defined, NK cells cannot be found in species lower than fish.

History and functions

Natural killing activity was discovered 40 years ago, shortly before the cells responsible for such phenomenon were identified. In particular settings, leukocytes from immunized animals showed no difference in cytotoxic responses in comparison to immunized animals. The same was true for healthy individuals compared to cancer patients (Takasugi, Mickey *et al.* 1973). The following step was realizing that such activity is dependent on a distinct subset of large granular lymphocytes showing spontaneous cytotoxic responses, thus named "natural killer" cells (Kiessling, Klein *et al.* 1975; Kiessling, Klein *et al.* 1975). Ten years later, the existence of NK cells, phenotipically and functionally distinct from T and B

cells, was definitely accepted. Since the beginning, NK cells have been widely studied in the context of anti-tumor immunity (Cerwenka and Lanier 2001), probably because of the circumstances of their discovery. A milestone in the field was the correlation of NK cell activity to *in vivo* resistance to tumor growth, particularly when NK cells are cultured with high doses of IL-2, giving rise to lymphokine-activated killers (LAK) cells (Grimm, Mazumder *et al.* 1982).

However, NK cells have a variety of different functions and their role is not restricted to cancer immune-surveillance. Indeed, they have been implicated in the immunity against pathogens (Lodoen and Lanier 2006; Vivier, Tomasello *et al.* 2008). Although examples of the role of NK cells in fighting bacteria and protozoa have been published, investigators have mainly focused in studying their contribution to anti-viral immunity (Biron, Nguyen *et al.* 1999), where cytotoxic responses, both "spontaneous" (by innate immunity) or "instructed" (by adaptive immunity), play a major role.

NK cells are equipped with cytolytic granules, *i.e.* lysosomes containing for the most part perforins and granzymes. Upon target recognition, these granules traffic to the contact zone with the susceptible target cell and fuse to the cell membrane, releasing their content which finally induces target cell lysis. In addition to this major mechanism, NK cells are able to induce cell death also via FasL, TNF and TRAIL.

NK cell functions are not restricted to cytotoxic responses. NK cells are one of the earliest source of IFN-γ (Martin-Fontecha, Thomsen *et al.* 2004), a chief cytokine in the defense against viruses. IFN-γ has also anti-bacterial effects. Indeed, it promotes the skewing of $CD4^+$ T cell polarization towards the Th1 lineage and activates macrophages (Boehm, Klamp *et al.* 1997). NK cells can also 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 (Loza and Perussia 2001; Orange and Ballas 2006; Cooper, Colonna *et al.* 2009).

NK cells are widely distributed throughout the body, an observation highlighting their fundamental role of innate sentinels. They comprise 2 to 18% of human circulating lymphocytes. In mice, they represent 2% of lymphocytes in the spleen, a percentage that can increase up to 10% in the lung (Vivier, Tomasello *et al.* 2008). Notably, NK cells are also found in lymph nodes of humans and mice in both steady-state and inflammatory conditions (Ferlazzo, Pack *et al.* 2004; Martin-Fontecha, Thomsen *et al.* 2004), where they exert immune-regulatory functions (Vivier, Tomasello *et al.* 2008).

NK cells bear a variety of activating and inhibitory receptors that can be triggered simultaneously upon interaction with target cells. The balance between positive and negative signals transduced by those receptors ultimately controls the outcome of NK cell responses. For this reason, understanding how NK

cell receptors work is of outstanding importance in view of possible therapeutic applications aimed to exploit the NK cell potential.

Receptors

The primary function of the immune system is to discriminate self and non-self entities, and NK cells make no exception. Differently from B and T cells, however, NK cell recognition relies on a huge repertoire of surface receptors rather than a single receptor. NK cell receptors are encoded in the germ line and do not undergo somatic cell genetic rearrangement. Several NK cell receptors are also expressed by other cells belonging to the so-called "transitional" immunity, notably γδ T cells and NKT cells (Steele, Oppenheim *et al.* 2000; Ogasawara and Lanier 2005).

Based on the signal delivered, either positive or negative, NK cell receptors are classified as activating or inhibitory, respectively.

Inhibitory receptors

Initially, NK cells were thought to recognize their target in a MHC-independent fashion. Indeed, they showed the straightforward ability to lyse target cells either totally lacking MHC-I or expressing allogeneic MHC-I. However, as noted by Karre and colleagues in a seminal paper (Karre, Ljunggren *et al.* 1986), NK cells are inhibited by MHC-I molecules, rather than simply ignoring them. The biological rationale behind this

behavior resides in the frequent downregulation of MHC-I by transformed or infected cells. Whereas activated $CD8^+$ T cells would be unable to kill target cells not expressing MHC-I, NK cells are specialized in spotting such cells. Therefore, MHC-I downregulation could be seen as an active mechanism of immune escape, in fact avoiding T cell recognition. However, NK cells act as a backup controller, rescuing a potential bug in immune-surveillance. The activation of NK cells by MHC-$I^{-/-}$ or MHC-I^{low} targets has been called "the missing-self hypothesis", meaning that the ubiquitously expressed MHC-I molecules are a marker of self and their absence is deciphered by the immune system as an hostile event (Bix, Liao *et al.* 1991; Lanier 2005).

Strikingly, mice and humans have different receptors mediating MHC-I recognition. Whereas murine NK cells exploit the C-type lectin-like Ly49 family of receptors (Karlhofer, Ribaudo *et al.* 1992; Yokoyama 2008), human counterparts rely on killer cell Ig-like receptors (KIR) (Colonna and Samaridis 1995; Wagtmann, Biassoni *et al.* 1995). This is one of the brightest examples of convergent evolution (Barten, Torkar *et al.* 2001), so extraordinary that, at the time of molecular cloning of Ly49 and KIR receptors, it was referred to as "the enigma of the natural killer cell" (Gumperz and Parham 1995). In addition to Ly49 and KIR receptors, both mouse and human NK cells have a third class of inhibitory receptors, namely the CD94/NKG2 family (Figure 7).

Signaling of inhibitory receptors

ITIM sequences (consensus: Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val) were first described for FcγRIIb (Muta, Kurosaki *et al.* 1994)

Inhibitory NK receptors for MHC

Species	Receptor	Ligands
Mouse	Ly49	H-2K, H-2D
Mouse	CD94/NKG2A	Qa-1^b
Human	KIR2DL	HLA-C
Human	KIR3DL	HLA-Bw4, HLA-A
Human	CD94/NKG2A (CD159a)	HLA-E
Human	CD85j, CD85d	HLA class I

Figure 7: (from Cerwenka and Lanier 2001). Inhibitory NK cell receptors recognizing MHC-I.

and are found in the cytoplasmic tail of all NK cell inhibitory receptors. Upon ligand recognition, the central Tyr residue of ITIM is phosphorylated. As a consequence, the phosphatases SHP-1, SHP-2, and SHIP are recruited at the cytoplasmic tail of the activated inhibitory receptor by means of their SH2 domain interacting with phospho-Tyr. SHP-1 and SHP-2 reduce the activation of a variety of signaling proteins, such as ZAP-70, Syk, PLCγ, LAT, FcεRIγ and Vav-1. By contrast, SHIP converts phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-3,4-bisphosphate, thereby dampening the Ca^{2+}-dependent signaling (Bolland and Ravetch 1999).

Ly49 inhibitory receptors

Ly49A, discovered by Yokoyama and colleagues almost 20 years ago (Karlhofer, Ribaudo *et al.* 1992), is the founding member of the Ly49 family, structurally C-type lectin-like receptors. Most Ly49 receptors are coupled to ITIM domains and, consequently, they deliver an inhibitory signal. However, notable exceptions are Ly49D and Ly49H, activating receptors that associate with the ITAM-bearing adaptor molecule DAP12. Ly49 receptors have no counterparts in human, with the exception of a poorly transcribed pseudogene in the chromosomal region 12p12.3-p13.2 (Westgaard, Berg *et al.* 1998). Remarkably, each NK cell transcribes only a subset of *Ly49* genes, that can be also expressed in a monoallelic fashion (Lanier 2005). It is likely that *Ly49* genes evolved by gene duplication and gene conversion (Mehta, Smith *et al.* 2001).

Ly49A is an homodimeric receptor binding to H-2D^{d}. The presence of a peptide loaded into the groove of the MHC molecule is required for Ly49A binding, although the peptide sequence does not seem to be important (Natarajan, Dimasi *et al.* 2002). Despite being a C-type lectin-like receptor, carbohydrate recognition is not required for ligand binding, which is merely dependent on protein-protein interactions (Lanier 2005).

Killer Cell Immunoglobulin-Like Receptors

The human KIR family comprises up to 15 genes on chromosome 19, but the precise number is different in different individuals. Computational analysis has revealed 37 haplotypes in the human population, each with a distinct set of *KIR* genes. Two haplotypes, named A and B, are widespread and represent most individuals (Uhrberg, Valiante *et al.* 1997). Only three genes are shared by all haplotypes: *KIR3DL2*, *KIR3DL3* and *KIR2DL4* (Lanier 2005). *KIR* genes are highly polymorphic and some alleles encode for functionally incompetent receptors (Hsu, Liu *et al.* 2002).

Human KIR are the functional equivalent of murine Ly49 receptors. However, they are structurally unrelated, being Ly49 receptors of the C-type lectin-like family, whereas KIR proteins belong to the Ig superclass (Colonna and Samaridis 1995; Wagtmann, Biassoni *et al.* 1995). Indeed, KIR can be categorized according to the number of Ig domains in their extracellular region: KIR2D have two Ig-like domains, whereas KIR3D possess three Ig-like domains. A further classification involves their cytoplasmic domain, which can be long (L), with one or two ITIM sequences, or short (S), without ITIM consensus. Instead, KIR bearing a short cytoplasmic domain recruit the adaptor molecule DAP12. As a consequence, KIR2DL and KIR3DL are inhibitory receptors, whilst KIR2DS and KIR3DS are activating receptors (Lanier 2005).

Similarly to Ly49 receptors, KIR recognize polymorphic determinants on MHC-I molecules. For a detailed list of KIR specific ligands, see Figure 8.

CD94/NKG2 Receptors

A single CD94 receptor and multiple NKG2 receptors are present in both humans and mice (Houchins, Yabe *et al.* 1991; Vance, Kraft *et al.* 1998), closely linked at their genomic locus. NKG2 family, similarly to Ly49 and KIR, comprises both activating and inhibitory receptors, NKG2C being the most extensively studied in the first group and NKG2A in the latter. The functional receptor is an heterodimer between CD94 and an NKG2 chain (Brooks, Posch *et al.* 1997). Indeed, CD94 lacks a cytoplasmic domain and relies on the partner NKG2 for downstream signaling. Similarly to the Ly49 family, CD94/NKG2 are structurally related to the C-type lectin-like transmembrane proteins. However, differently from both Ly49 and *KIR* genes, they show little allelic variation. This could be expected in that, rather than recognizing highly polymorphic conventional MHC-I molecules, their ligands are human HLA-E and mouse $Qa1^b$ (Braud, Allan *et al.* 1998; Vance, Kraft *et al.* 1998), non-conventional MHC-Ib molecules showing very limited polymorphism (Figures 7 and 8). HLA-E and $Qa1^b$ present peptides derived from the leader sequence of conventional MHC-I proteins (Aldrich, DeCloux *et al.* 1994).

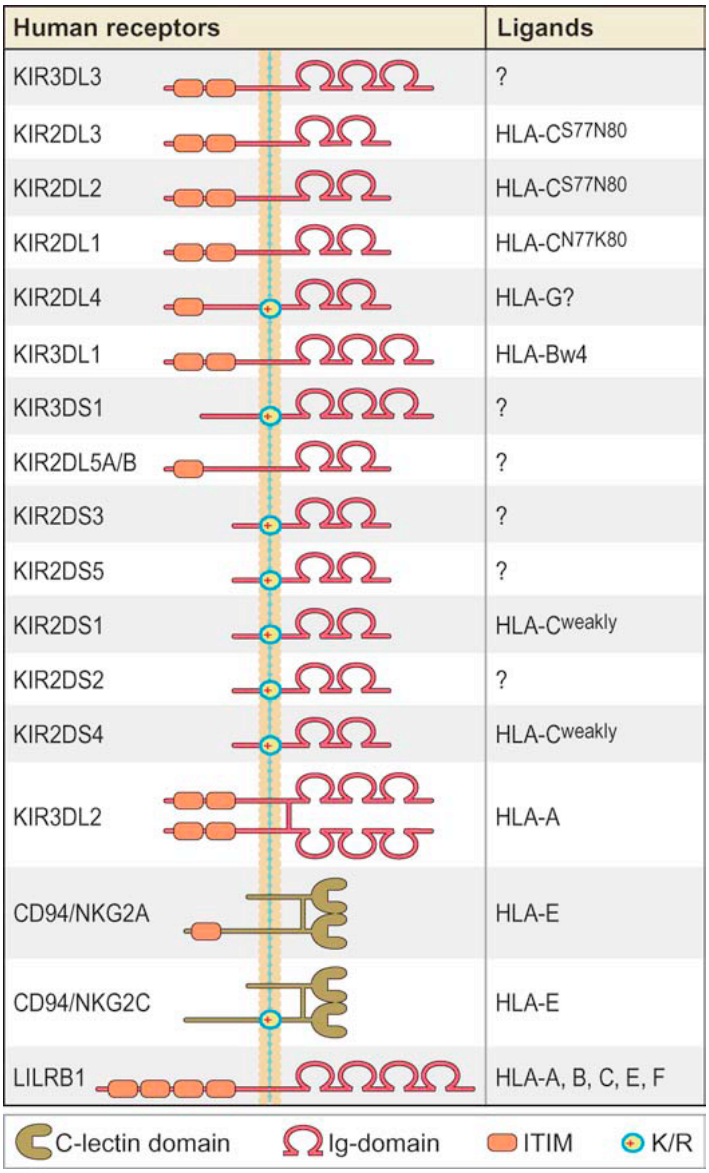

Figure 8: (from Lanier 2005). Human inhibitory NK cell receptors.

Since HLA-E and Qa1^b are not able to present peptides derived from their own digestion by the proteasome, their expression at the cell surface is impaired in the absence of conventional

MHC-I (and other non-conventional MHC-I molecules, such as HLA-G). Therefore, by monitoring the expression of HLA-E/Qa1^{b} through CD94/NKG2, NK cells can sense generalized MHC-I downregulation, resulting in missing self recognition. In this way, NK cells are able to monitor a large number of extensively polymorphic molecules with a limited set of moderately polymorphic receptors.

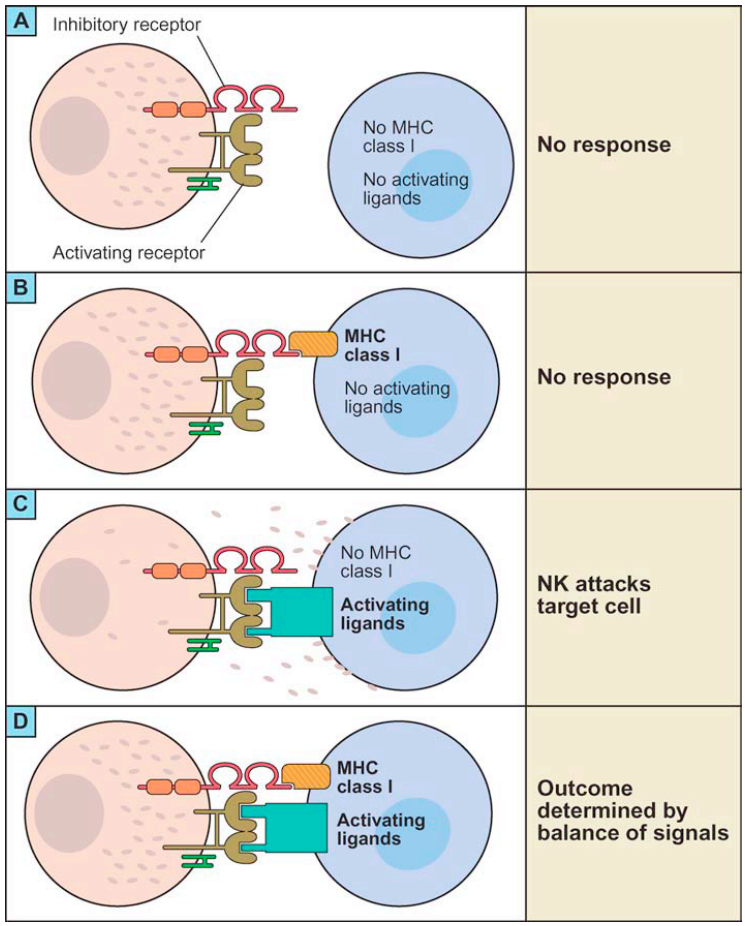

Figure 9: (from Lanier 2005). The revised missing self hypothesis.

Activating receptors

A major revision in the missing self hypothesis was the realization that NK cell activation is not only triggered by the lack of expression of ligands for inhibitory receptors, but also by the positive recognition of ligands for activating receptors (Figure 9). The balance between activating and inhibitory signals ultimately controls NK cell responses (Lanier 2005).

As already mentioned in the previous section, every family comprising inhibitory receptors recognizing MHC-I also encodes activating receptors. Therefore, most activating receptors belonging to the Ly49, KIR and NKG2 families bind MHC or MHC-like molecules, although the biological rationale behind such recognition is unclear. In addition to this patrolling strategy, NK cells can also sense conserved pathogen-encoded ligands and host-encoded non-MHC molecules (Figure 10).

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

Figure 10: (from Cerwenka and Lanier 2001). Activating NK cell receptors and their ligands.

Signaling of activating receptors

Several NK cell activating receptors are not capable of signaling in the absence of ITAM-containing adaptor molecules (Tassi, Klesney-Tait *et al.* 2006). Particularly, NK cells express three molecules providing ITAM sequences: FcεRIγ, DAP12 (alternatively named KARAP) and CD3ζ. Upon ligand binding and Tyr phosphorylation by Src family kinases in the ITAM region (consensus: Tyr-x-x-Leu/Ile-x(6-8)-Tyr-x-x-Leu/Ile), the newly formed phospho-Tyr becomes a docking site for the SH2 domain of the Tyr kinases Syk and Zap70. In this way, these kinases are effectively recruited to the adaptor and can start propagating the intracellular signal. Particularly, targets of Syk and Zap70 are LAT, NTAL, 3BP2 and SLP-76. Eventually, the signaling cascade triggers the activation of effector molecules such as PI3K, Vav, PLCγ (Figure 11), directly controlling, among others, actin rearrangements and granule polarization (Lanier 2005; Tassi, Klesney-Tait *et al.* 2006).

An alternative, non-ITAM based, strategy of signaling for activating receptors harnesses the adaptor molecule DAP10. DAP10 does not contain ITAM motifs but another Tyr-based sequence, Tyr-Ile-Asn-Met. Upon phosphorylation, this motif becomes a docking site for (Figure 12):

- the p85 subunit of PI3K, similarly to what happens for CD28, equipped with a similar sequence (Pages, Ragueneau *et al.* 1994).

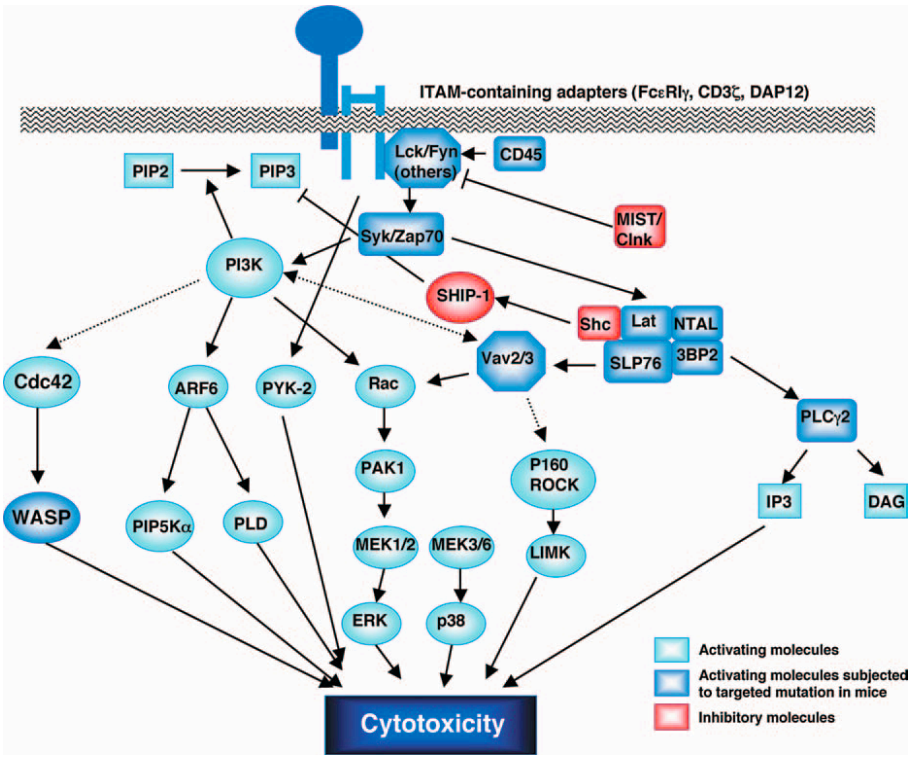

Figure 11: (from Tassi, Klesney-Tait *et al.* 2006). The signaling pathways of ITAM-containing adaptors.

- Grb-2, eventually 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 full activation mediated by receptors using DAP-10, NKG2D being the most important (Lanier 2005; Tassi, Klesney-Tait *et al.* 2006).

Recognition of self MHC molecules

Most activating receptors showing specificity for MHC and MHC-like molecules have evolved from/together the structurally related inhibitory receptors intended to sense missing self.

For instance, 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). Nonetheless, 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 (Lanier 2005). 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.

Indeed, 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 KIR with a short cytoplasmic tail are activating receptors binding DAP12. Similarly to the Ly49 family, activating KIR bind MHC-I with a much weaker affinity than the inhibitory counterparts (Winter, Gumperz *et al.* 1998). However, there is evidence supporting the involvement of KIR in autoimmune disorders. Particularly, 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 (Yen, Moore *et al.* 2001).

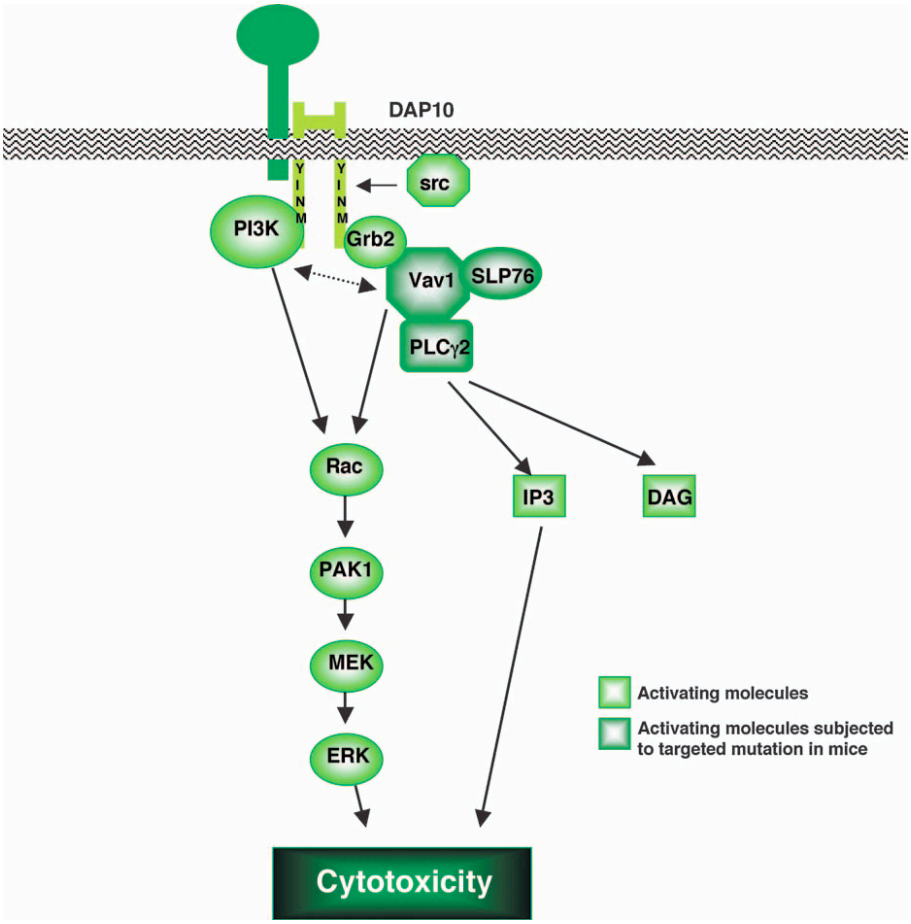

Figure 12: (from Tassi, Klesney-Tait *et al.* 2006). The signaling pathways of the DAP-10 adaptor.

As with Ly49 and KIR receptors, also the CD94/NKG2 family comprises both activating and inhibitory receptors; again, the

former show lower affinity for self ligands than the latter. For instance, both the inhibitory NKG2A and the activating NKG2C human receptors recognize HLA-E. However, differently from Ly49 and KIR, where a clear rationale for self-recognition by activating receptors has not been found yet, the benefit of self-recognition by activating receptors of the CD94/NKG2 family has been demonstrated in at least one scenario. Indeed, 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). Since *HSP60* is upregulated in stressed cells - and infection or transformation are stressful events - NK cells may consequently attack cells displaying abnormal behavior.

Recognition of host-encoded MHC-like molecules by NKG2D

The best characterized NK cell receptor recognizing cell surface glycoproteins structurally related to MHC class I is NKG2D. The name of this receptor is confusing. Indeed, 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). Strikingly, all CD8+ T cells in humans, irrespectively of their activation state, express NKG2D, whereas in mice only activated $CD8^+$ T cells 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, whereas the shorter can bind both DAP10 and DAP12. By contrast, humans express only the long isoform. In both species, however, NKG2D shows virtually no polymorphism.

Stimulation of NKG2D leads to cytotoxic responses and, sometimes, to the release of pro-inflammatory cytokines. The ligands for NKG2D are MICA, MICB, ULBP1, ULBP2, ULBP3, and ULBP4 in human, and the corresponding molecules (Rae-1, H60 and MULT1) in mouse (Radaev and Sun 2003). All these ligands are structurally 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, especially for CMV (Cerwenka and Lanier 2001). The highly polymorphic *MIC* and *ULBP* genes are upregulated by stressed cells, frequently transformed or virus-infected cells. *Rae-1* is induced in murine macrophages upon TLR signaling, but it is also expressed by a variety of tumor cell lines (Lanier 2005). Active immune escape mechanisms are put in place by tumors and viruses to counteract their recognition by NKG2D. Tumors were found to secrete soluble forms of NKG2D ligands, which function as decoys. HCMV encodes the UL16 protein that is able to bind and retain in the cytoplasm certain NKG2D ligands upregulated upon infection. Similarly, the MCMV m152 protein keeps Rae-1 proteins intracellularly (Cerwenka and Lanier 2001).

Recognition of host-encoded non-MHC molecules

This class of NK cell receptors includes a heterogeneous set of receptors, each with its own distinctive features.

- **NKR-P1 receptors**. NKR-P1 receptors belong to the family of C-type lectin-like transmembrane proteins. NK1.1 is a polymorphic antigen defining mouse NK cells in B6 mice (Glimcher, Shen *et al.* 1977) - but absent in other common inbred strains, such as BALB/c - and is a member of the NKR-P1 gene family. A reagent commonly used to study NK cells, the PK136 mAb, is supposed to bind NK1.1. In B6, it actually reacts with the product of the *Nkr-p1c* gene, but, in other strains, it can bind other NKR-P1 receptors (Carlyle, Martin *et al.* 1999). The ligand of NKR-P1C is not known yet. The NKR-P1 family comprises also inhibitory receptors, such as NKR-P1D, recognizing Clr-b, a glycoprotein expressed by all hematopoietic cells.
- **2B4**. The 2B4 receptor, also called CD244, and its ligand CD48 are members of the CD2 family (Ig-related). 2B4 is present both in mice and humans; however, while in mice this receptor seems to exert an inhibitory function, in humans 2B4 behaves as an activating receptor (Lanier 2005). 2B4 signaling is unique in NK cells. The signaling sequence of the cytoplasmic tail of 2B4 is Thr-x-Tyr-x-x-Leu/Ile, repeated four times. Upon Tyr phosphorylation, it seems that 2B4 recruits the phosphatases SHP-1 and

SHP2 and the adaptor SAP, ultimately leading to the activation of Vav1 and LAT (Tassi, Klesney-Tait *et al.* 2006).

- **DNAM-1 receptor**. CD226, or DNAM-1 receptor, is a member of the Ig superfamily. The ligands of DNAM-1, CD112 (PVR) and CD155 (nectin-2), are upregulated in tumor cells and promote NK cell cytotoxicity and cytokine production. PVR and nectin-2 are also present at the cell junctions 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).

Natural cytotoxicity receptors

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

NKp46 (encoded by *NCR1*) is expressed in both mice and humans and its expression is restricted to NK cells. At present, it is the best marker for NK cells, more reliable than the

classical CD49b or NK1.1, whose expression is not faithfully limited to NK cells (Walzer, Blery *et al.* 2007). Moreover, in contrast to most genes encoding NK cell receptors, whose transcription is stochastic and results in only a subset of NK cells expressing a given receptor, NKp46 is expressed by all NK cells (Moretta, Biassoni *et al.* 2000).

NKp44 (encoded by *NCR2*) associates with DAP12 and lacks an orthologue in mouse. Its expression on NK cells increases as a result of *in vitro* culture with IL-2 (Moretta, Biassoni *et al.* 2000). NKp30 and NKp46 associate with CD3ζ or FcεRIγ, but NKp30 (encoded by *NCR3*) is a pseudogene in mice (Walzer, Blery *et al.* 2007).

The ligands of NCR are mostly unknown. Cytotoxicity of NK cells against tumor cells is partially mediated by NCR, pointing to host-encoded ligands (Moretta, Biassoni *et al.* 2000). However, NKp46 has also been shown to recognize the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of Sendai virus (Mandelboim, Lieberman *et al.* 2001).

Recognition of MCMV by Ly49H

Ly49H is expressed by a subset of murine NK cells and associates to DAP12. Differently from other activating and inhibitory Ly49 receptors, Ly49H has no affinity for any H-2 molecule. However, it binds the MCMV-encoded m157 viral glycoprotein, expressed on the surface of infected cells, which is structurally related to MHC. The *Ly49H* gene is responsible

for the differential resistance to MCMV of different common inbred strains. To exemplify, B6 mice are resistant to MCMV and do have a *Ly49H* gene, whereas the susceptible BALB/C mice lack such gene. However, Ly49H-transgenic BALB/C mice are resistant to MCMV (Lanier 2005).

Strikingly, Ly49H$^+$ NK cells have been recently shown to possess memory-like features (Sun, Beilke *et al.* 2009; Ugolini and Vivier 2009), as depicted in Figure 13. Upon infection with MCMV, Ly49H$^+$ cells undergo proliferation, followed by contraction once the infection has been eliminated. Few virus-experienced NK cells survive this phase. However, these cells are more protective against MCMV infections than naïve NK cells when transferred to immunodeficient mice. They are also more efficient *in vitro* upon triggering of Ly49H or NK1.1 (Sun, Beilke *et al.* 2009).

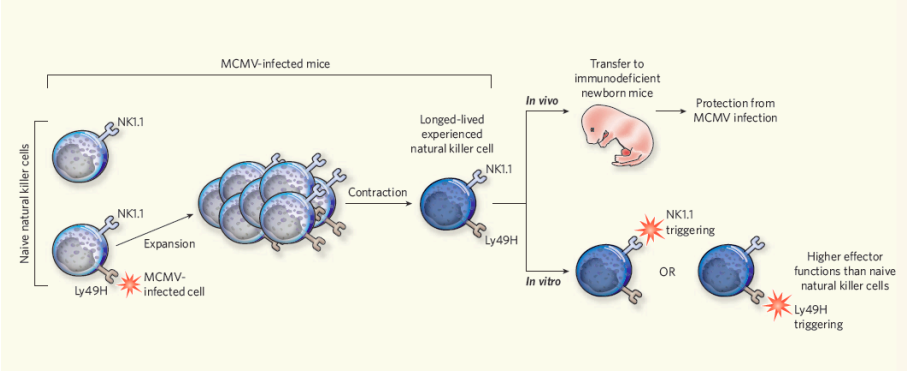

Figure 13: (from Ugolini and Vivier 2009). Adaptive immune features of Ly49H$^+$ NK cells.

TLR

TLR are the best-known PRR and their expression is not restricted to NK cells. On the contrary, they are mainly studied in myeloid cells, such as DC and MΦ. Nonetheless, both human and murine NK cells express TLR (Pisegna, Pirozzi et al. 2004; Schmidt, Leung et al. 2004; Sivori, Falco et al. 2004). These findings suggest that NK cells might recognize PAMP in a direct fashion. Indeed, some reports have demonstrated a role for TLR ligands in NK cell activation. Particularly, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9 agonists stimulate NK cell functions (Becker, Salaiza *et al.* 2003; Chalifour, Jeannin *et al.* 2004; Pisegna, Pirozzi *et al.* 2004; Schmidt, Leung *et al.* 2004; Sivori, Falco *et al.* 2004; Hart, Athie-Morales *et al.* 2005; Tsujimoto, Uchida *et al.* 2005; Lauzon, Mian *et al.* 2006).

However, this notion has been sometimes received with skepticism, with major concerns about the purity of NK cell preparations used in the experiments showing a role for direct TLR recognition on NK cells. Indeed, NK cell activation *in vivo* triggered by the candidate adjuvant polyI:C (TLR3 ligand) was shown to be indirect and mediated by a variety of accessory cells equipped with TLR and other PRR (McCartney, Vermi *et al.* 2009).

Despite this confusion, it seems clear that TLR stimulation alone is not able to significantly influence NK cell biology. However, TLR stimulation synergizes with cytokine treatment (usually IL-12, IL-18 and/or IFN-I) in terms of CD69

upregulation, IFN-γ release and cytotoxic effects (Sivori, Falco *et al.* 2004; Sivori, Carlomagno *et al.* 2006). It is worth noting that, even though NK cells express both intracellular and plasma membrane TLR, only the former have been thoroughly studied (Schmidt, Leung *et al.* 2004; Sivori, Falco *et al.* 2004; Hart, Athie-Morales *et al.* 2005; Sivori, Carlomagno *et al.* 2006; Lauzon, Mian *et al.* 2007; Sivori, Falco *et al.* 2007). In conclusion, the role of TLR stimulation in NK cell biology is still lacking exhaustive investigation and the reports published to date are conflicting (Lauzon, Mian *et al.* 2007).

CD16 and ADCC

CD16 (FcγRIIIA) was the first activating NK cell receptor to be identified. It is the low affinity receptor for IgG responsible for ADCC. In order to start the signaling pathway, it couples to the adaptors FcεRIγ and the CD3ζ chain in human, but only to FcεRIγ in mouse (Tassi, Klesney-Tait *et al.* 2006).

CD16 stimulation has been exploited in clinical settings. For instance, a therapy for EGFR-expressing cancers is based on a monoclonal antibody targeting the EGFR receptor. In addition to suppressing the EGFR signaling by interfering with ligand binding, such antibody has been shown to promote NK-cell mediated ADCC against tumor cells (Ashraf, Umana *et al.* 2009).

Development, licensing and subsets

NK cells comprise the third major lymphocyte population and can be distinguished from other lymphocytes by the absence of TCR and BCR. Indeed, a unique characteristic of NK cells is that, unlike other lymphocytes, their development does not require events involving antigen receptor gene rearrangement. Thus, they are present in SCID, RAG-deficient and athymic mice.

The NK cell precursor (NKP), defined as the cell able to give rise to mature NK cells but not any other blood cell type, differentiates from HSC and early lymphoid precursors in the BM (Huntington, Vosshenrich *et al.* 2007), although small populations of NKP have been identified in peri-natal liver and adult spleen and lymph nodes.

Afterward, the NKP differentiates further into immature NK cells, the stage immediately preceding mature NK cells. Immature NK cells express only a limited range of receptors and are not or only partially functional (Vosshenrich, Samson-Villeger *et al.* 2005). Indeed, they neither display cytotoxic capability against common target cell lines nor produce IFN-γ (Rosmaraki, Douagi *et al.* 2001). In mouse, they express NK1.1 but not CD49b, another typical marker of NK cells. For a detailed description of the phenotypic and functional characteristics of differentiating NK cells, refer to Figure 14.

Since NK cells are lymphocytes belonging to innate immunity and bearing invariant receptors, it may be argued that they

should be naturally tolerant to self. However, inhibitory receptors, which usually recognize distinct polymorphic variants of MHC-I, are not uniformly expressed on NK cells and their encoding genes are inherited and segregate independently from MHC-I genes. Consequently, some NK cells may not express any inhibitory receptor specific for self MHC-I, generating a potentially auto-reactive situation.

The groups led by Yokoyama and Raulet have extensively studied this topic and have shown that developing NK cells are not functionally competent when inhibitory receptors have not been engaged over the maturation process (Raulet and Vance 2006; Yokoyama and Kim 2006). This process has been referred to as “licensing”, meaning that compatibility between MHC-I and Ly49/KIR receptors licenses NK cells to kill target cells and produce cytokines. NK cell licensing resembles the positive selection of T cells in the thymus, where they encounter the appropriate self ligand. However, in the case of NK cells there is no evidence for negative selection or clonal deletion.

Even if the scientific community agrees on the general features of licensing, there is no consensus about the molecular mechanism underlying this process. Specifically, at least two models have been proposed (Figure 15). The disarming model speculates that NK cells lacking inhibitory receptors for self MHC-I are “disarmed”, *i.e.* hyporesponsive, a condition similar to T cell anergy.

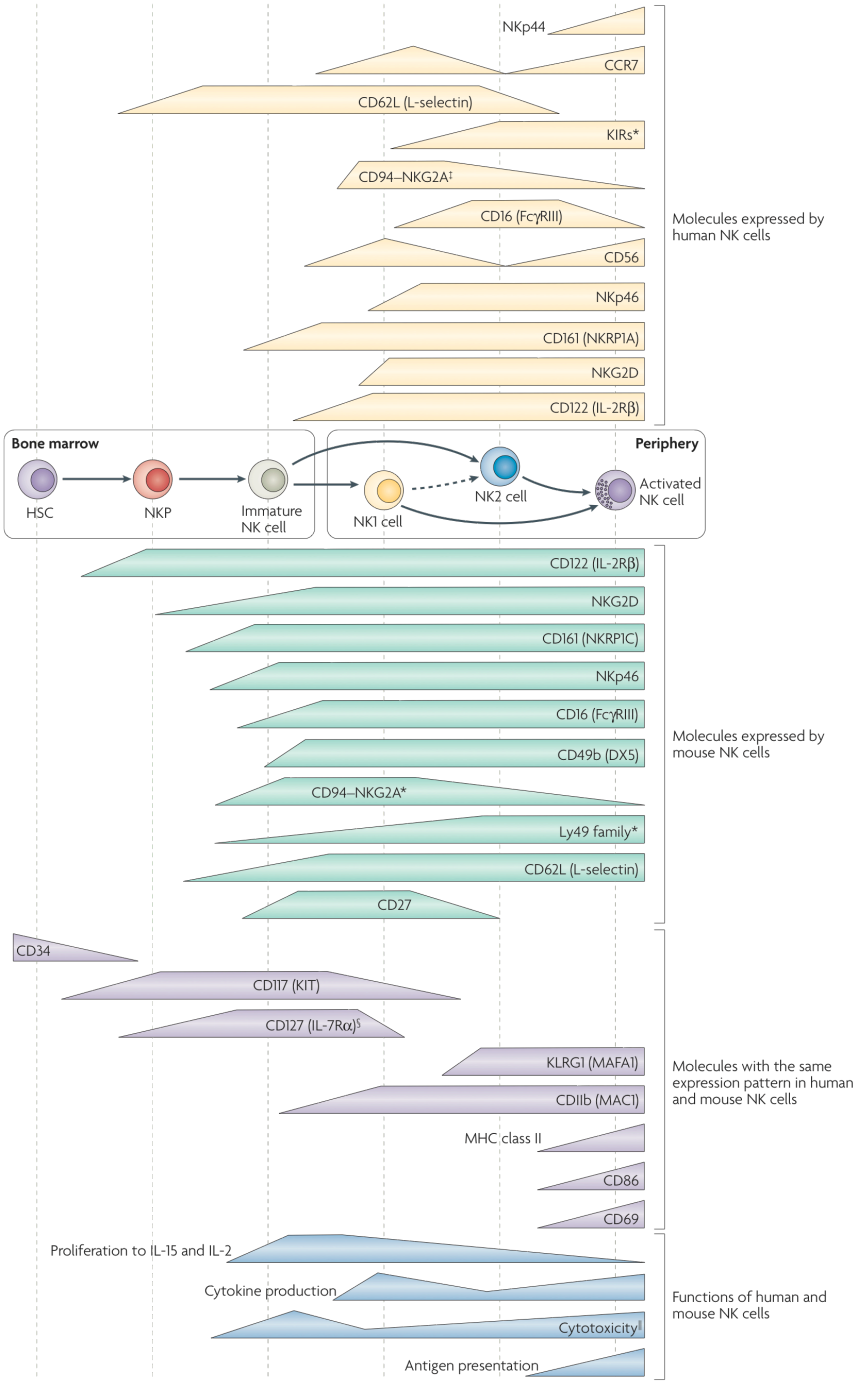
NKp44
CCR7
CD62L (L-selectin)
KIRs*
CD94–NKG2A‡
CD16 (FcγRIII)
CD56
NKp46
CD161 (NKRP1A)
NKG2D
CD122 (IL-2Rβ)
Molecules expressed by human NK cells
Bone marrow
Periphery
HSC
NKP
Immature NK cell
NK1 cell
NK2 cell
Activated NK cell
CD122 (IL-2Rβ)
NKG2D
CD161 (NKRP1C)
NKp46
CD16 (FcγRIII)
CD49b (DX5)
CD94–NKG2A*
Ly49 family*
CD62L (L-selectin)
CD27
Molecules expressed by mouse NK cells
CD34
CD117 (KIT)
CD127 (IL-7Rα)§
KLRG1 (MAFA1)
CD11b (MAC1)
MHC class II
CD86
CD69
Molecules with the same expression pattern in human and mouse NK cells
Proliferation to IL-15 and IL-2
Cytokine production
Cytotoxicity‖
Antigen presentation
Functions of human and mouse NK cells

Figure 14: (from Huntington, Vosshenrich *et al.* 2007). Phenotypic and functional characteristics of differentiating NK cells.

This model takes into account the presence of ligands for activating receptors on normal cells and emphasizes that missing self recognition is a balance between inhibition and activation. If unopposed by inhibitory signals, developing NK cells would be exposed to chronic stimulation, leading invariably to a sort of anergy. According to the disarming hypothesis, immature NK cells are genetically programmed to become functionally competent unless the environmental (over)stimulation renders them anergic (Raulet and Vance 2006).

By contrast, the arming model proposes that the default fate of developing NK cells is genetically programmed to hyporesponsiveness, unless rescued by appropriate signals. Specifically, this rescuing (or "arming") task would be fulfilled by the engagement of inhibitory receptors by self MHC-I molecules. Therefore, the inhibitory receptors would deliver a positive signal to NK cells in the immature stage of their development, whereas in the mature phase of NK cell life they would switch to delivering negative signals (Yokoyama and Kim 2006; Zanoni, Granucci *et al.* 2007; Yokoyama 2008).

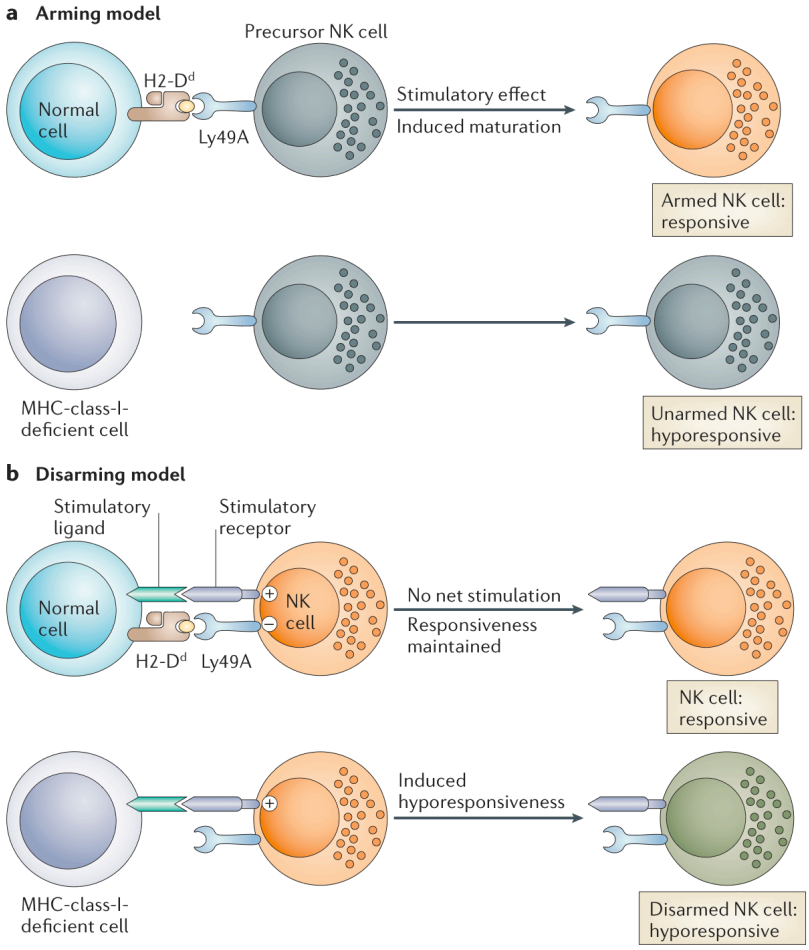

Figure 15: (from Raulet and Vance 2006). Comparison of arming and disarming models for NK cell tolerance to self.

Unlicensed NK cells are not completely devoid of functions. In inflammatory conditions, both licensed and unlicensed NK cells become activated. Examples of activation of unlicensed NK cells are viral (Dokun, Kim *et al.* 2001) and bacterial (Fernandez, Treiner *et al.* 2005) infections, cytokine treatment (Kim, Poursine-Laurent *et al.* 2005) and phorbol

ester/ionomycin stimulation (Fernandez, Treiner *et al.* 2005; Kim, Poursine-Laurent *et al.* 2005). These findings may provide a reason why potentially auto-reactive NK cells are maintained as anergic cells instead of being clonally deleted.

Mature NK cells are an extremely heterogeneous population because of the stochastic expression of inhibitory receptors. Still, it is possible to group NK cells in subsets on the basis of shared phenotypic and functional features.

In humans, two subsets can be easily distinguished (Cooper, Fehniger *et al.* 2001): $CD56^{dim}$ $CD16^{+}$ cells comprise the majority of circulating NK cells, whereas $CD56^{bright}$ (most being $CD16^{-}$) cells represent a minor fraction. Strikingly, this divergent phenotype is paralleled by a clear-cut distinction in functional features. $CD56^{dim}$ NK cells are highly cytotoxic but poor producers of IFN-γ. In addition, they express CD16 at high density, thereby showing potent ADCC responses. By contrast, $CD56^{bright}$ NK cells are specialized in releasing IFN-γ but show negligible cytotoxicity.

As depicted in Figure 16, these two subsets show a number of other differences. For instance, the high affinity heterotrimeric receptor for IL-2 is expressed only in the $CD56^{bright}$ subset. $CD56^{dim}$ cells express only the low affinity heterodimeric reptor for IL-2, lacking the α subunit. As a consequence, in response to low doses of IL-2, only the $CD56^{bright}$ cells proliferate extensively (Caligiuri, Murray *et al.* 1993).

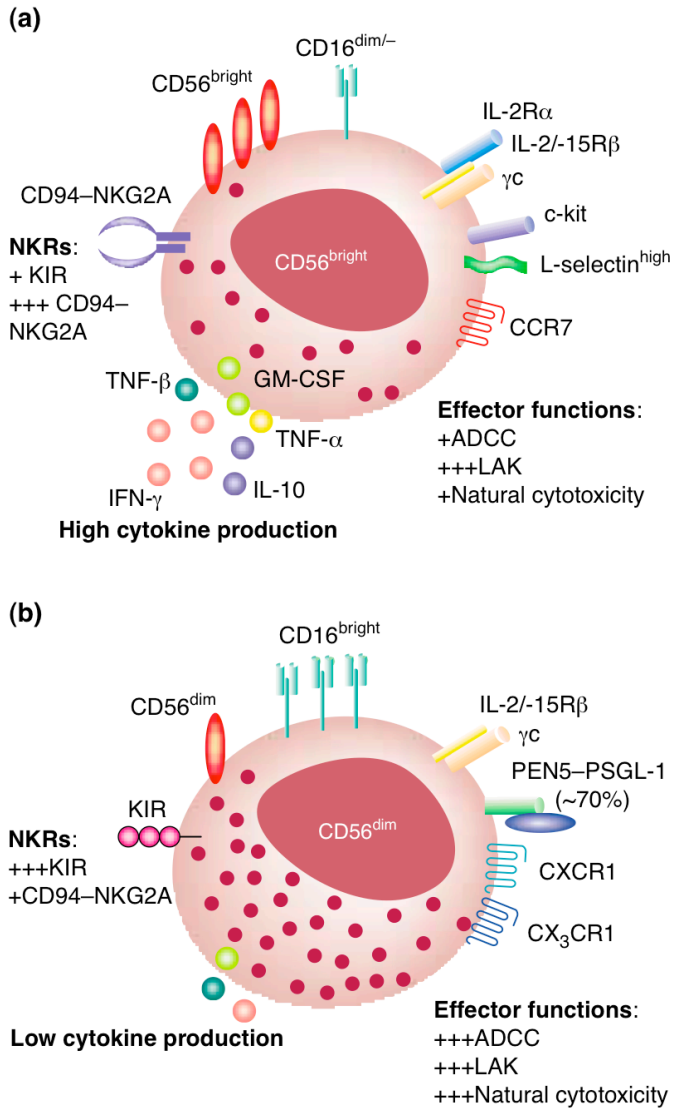

Figure 16: (from Cooper, Fehniger *et al.* 2001). Phenotypic and functional characteristics of human NK cell subsets.

Another major difference between these two subsets concerns the response to chemokines. CD56^{bright} NK cells express CCR7 and CXCR3. As a consequence, they are recruited to

secondary lymphoid organs and are responsive to IP-10. $CD56^{dim}$ NK cells express CXCR1 and CX_3CR1, allowing them to sense IL-8 and fractalkine, respectively (Campbell, Qin *et al.* 2001).

It has been speculated that the $CD56^{dim}$ subset may differentiate from the $CD56^{bright}$ subset. However, such hypothesis has never been formally proved (Cooper, Fehniger *et al.* 2001).

In mice, mature NK cells do not express CD56, hindering a precise comparison with human subsets. Nevertheless, both human and mouse NK cells express CD161 (NK1.1) and NKp46. However, CD161 is also expressed by some NKT and γδ T cells, whereas NKp46 expression is faithfully limited to pure $CD3^-$ NK cells. In addition, NKp46 is expressed by all NK cells, irrespective of their subsets or activation state, and is therefore the best marker for NK cells available nowadays (Moretta, Biassoni *et al.* 2000; Cooper, Fehniger *et al.* 2001; Huntington, Vosshenrich *et al.* 2007; Walzer, Blery *et al.* 2007).

Recently, Hayakawa and colleagues have suggested that CD27 expression may be useful to dissect murine NK cell subsets (Hayakawa and Smyth 2006). $CD27^+$ cells have many features common to $CD56^{bright}$ human NK cells, such as predominance in the lymph nodes and potent IFN-γ production. $CD27^-$ NK cells, similarly to the human $CD56^{dim}$ subset, are excluded from LN, but are the predominant population in spleen, lungs and blood. $CD27^+$ cells can be further distinguished in $CD11b^{hi}$ or

$CD11b^{lo}$, the former displaying higher cytotoxic responses than the latter.

DC-mediated NK cell activation

As already discussed, NK cells are equipped with receptors able to directly sense the presence of pathogens, most notably NKp46 (recognizing the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of Sendai virus), Ly49H (recognizing the MHC-like m157 protein of MCMV) and TLR (Mandelboim, Lieberman *et al.* 2001; Lanier 2005; Lauzon, Mian *et al.* 2006; Lauzon, Mian *et al.* 2007). However, activation of NK cells by most pathogens seems to be indirect and results from signals provided by accessory cells (Newman and Riley 2007). Even though there are clues supporting a primary role for DC (Lucas, Schachterle et al. 2007), other APC, such as MΦ, are able to act as accessory cells in NK cell activation (Tu, Bozorgzadeh *et al.* 2008). This is a key change of paradigm because NK cells have classically been regarded as innate lymphocytes autonomously responding to threats. Even the term “activation” rarely applied to NK cells. Indeed, differently from T cells, NK cells were not thought to exist in resting and activated states, and their actual name reflects this belief: NK cells were just “natural” responders.

This dogma was first challenged in 1999 by a pioneering work by Fernandez and colleagues (Fernandez, Lozier *et al.* 1999). They showed that *in vivo* DC expansion results in better anti-tumor immunity. However, such effect is dependent on the

presence of NK cells. Indeed, DC were able to activate NK cells in terms of production of IFN-γ and enhancement of cytotoxic responses. Moreover, the authors found that DC activated NK cells using both soluble and contact-dependent signals (Figure 17).

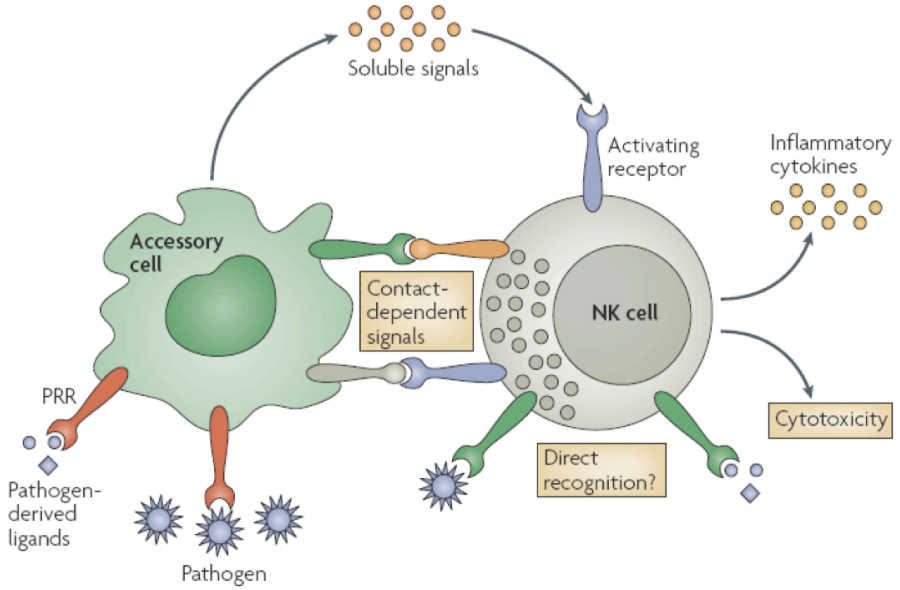

Figure 17: (from Newman and Riley 2007). Accessory cell-mediated NK cell activation.

Less than ten years later, the concept of NK cell activation by accessory cells was acknowledged in the scientific terminology. Lucas and colleagues speak of NK cell "priming" by DC and "restimulation" by target cells, words earlier reserved to T cells (Lucas, Schachterle *et al.* 2007). "Activated" or "primed" NK cells show a threshold of activation considerably lower than the "resting" counterparts upon "restimulation". Therefore, the

realization that the threshold of activation of NK cells is not constant but governed by accessory cells delineates a two-step mechanism (activation-response) controlling NK cell effector functions, in contrast to the one-step (response) mechanism favored until the end of last century.

Whereas the response stage can still be considered dependent on the balance between activating and inhibitory receptors recognizing molecules on target cells (provided that the same balance may be able to activate NK cells in certain circumstances, whilst totally failing in others, depending on the activation level of NK cells), obviously the activation stage must rely on different mechanisms. By analogy with T cell priming, membrane-bound costimulatory molecules and soluble cytokines expressed by DC have been investigated (Newman and Riley 2007). A strong finding supporting the concept that accessory cell-derived cytokines are key regulators of NK cell activation is that after incubation of blood mononuclear cells with TLR agonists, NK cells produce high amounts of IFN-γ. However, in the absence of accessory cells, IFN-γ is no longer produced.

Further evidence for the importance of accessory cells comes from studies using neutralizing antibodies or KO mice showing the importance of accessory cell-derived cytokines in NK cell responses to pathogens (Carson, Giri *et al.* 1994; Orange and Biron 1996; Hunter, Gabriel *et al.* 1997; Nguyen, Salazar-Mather *et al.* 2002; Nomura, Kawamura *et al.* 2002).

IL-12p70 has long been known as a potent inducer of IFN-γ, particularly in synergy with IL-18 (Chaix, Tessmer *et al.* 2008). Accordingly, neutralization of IL-12p70 *in vitro* and *in vivo* with blocking Ab impairs IFN-γ release by NK cells in response to a variety of pathogens and TLR agonists from viruses, bacteria and protozoans (Heinzel, Rerko *et al.* 1994; Scharton-Kersten, Afonso *et al.* 1995; Orange and Biron 1996; Nomura, Kawamura *et al.* 2002).

IFN-α/β enhance NK cell cytotoxicity (Gerosa, Gobbi *et al.* 2005) by upregulating FasL and perforin. A very well studied model showing the role of IFN-I in NK cell cytotoxic responses is the MCMV infection. Upon neutralization of IFN-I with blocking Ab, NK cell cytotoxic responses are dampened (Nguyen, Salazar-Mather *et al.* 2002). However, IFN-I are also known to promote NK cell-derived IFN-γ release in infections by viruses and protozoans (Malmgaard and Paludan 2003; Newman, Korbel *et al.* 2006).

IL-2 and IL-15 share the β and γ subunits of their heterotrimeric receptor. IL-15 has been repeatedly implicated in NK cell development and activation in a variety of settings (Carson, Giri *et al.* 1994; Ranson, Vosshenrich *et al.* 2003; Burkett, Koka *et al.* 2004; Ferlazzo, Pack *et al.* 2004; Ma, Koka *et al.* 2006; Lucas, Schachterle *et al.* 2007; Mortier, Woo *et al.* 2008; Huntington, Legrand *et al.* 2009). Similarly, high doses of IL-2 have been used for decades to generate LAK cells, *i.e.* NK cells showing powerful cytotoxic responses (Whiteside 2001). DC-

derived IL-2 is also crucial for IFN-γ release by NK cells in the context of anti-bacterial and anti-tumor immunity (Granucci, Zanoni *et al.* 2004; Zanoni, Foti *et al.* 2005).

The delivery of some cytokines released by accessory cells may require close contact with NK cells to be effective. This is surely the case for IL-15, which is trans-presented by IL-15Rα, thereby acting as a contact-dependent signal rather than a soluble signal (Burkett, Koka *et al.* 2004). Also DC-derived IL-12 must be delivered at the immune synapse with NK cells to effectively promote IFN-γ secretion, although this cytokine is a pure soluble molecule and is not trans-presented by membrane-bound proteins (Borg, Jalil *et al.* 2004). This requirement seems to be dictated by the need for polarizing and concentrating preformed IL-12-containing vesicles at the contact site.

It is worth noting that accessory cells may also release inhibitory cytokines dampening NK cell effector functions, most notably TGF-β (Li, Wan *et al.* 2006). For example, neutralization of TGF-β increases NK cell responses to *P. falciparum* (Newman, Korbel *et al.* 2006).

There is no full agreement about the signals involved in DC-mediated NK cell activation. First, DC respond to diverse PAMP by releasing different cytokines. Second, the experimental conditions may bias the results obtained. For instance, HSC differentiated in the presence of GM-CSF plus IL-4 rather than GM-CSF alone give rise to BM-DC profoundly different in their

functionality. Indeed, IL-4 can inhibit IL-2 production by PAMP-exposed DC (Guiducci, Valzasina *et al.* 2005; Schartz, Chaput *et al.* 2005). In human, DC cultured with IL-4 acquire the ability to activate NK cells independently of the presence of full microbial stimulation (Ferlazzo, Morandi *et al.* 2003). These findings may explain why investigators reported divergent conclusions about the role of IL-2 and IL-12 in DC-mediated NK cell activation (Zanoni, Granucci *et al.* 2007). The production of IL-2 rather than IL-12 seems a dichotomic choice, with the presence or absence of IL-4 controlling the "decision" of which of the two cytokines should be produced (Figure 18).

The location where DC-NK cell interactions occur *in vivo* is uncertain. The first contact may happen in peripheral tissues, where both resident and recruited DC would be able to activate NK cells (Moretta 2002). Then, DC exposed to pathogens become activated and migrate from the periphery to the DLN in a CCR7-dependent fashion (Weninger and von Andrian 2003), where they can probably stimulate resident and newly recruited NK cells. NK cell migration to LN is partially dependent on CXCR3 and DC are able to secrete the ligands for CXCR3 upon maturation (Martin-Fontecha, Thomsen *et al.* 2004). Therefore, DC first recruit and then activate NK cells in the DLN. Interestingly, since NK cells are an early source of IFN-γ, they have a positive role in $CD4^+$ T cell skewing towards a Th1 phenotype (Martin-Fontecha, Thomsen *et al.* 2004; Zanoni, Foti *et al.* 2005).

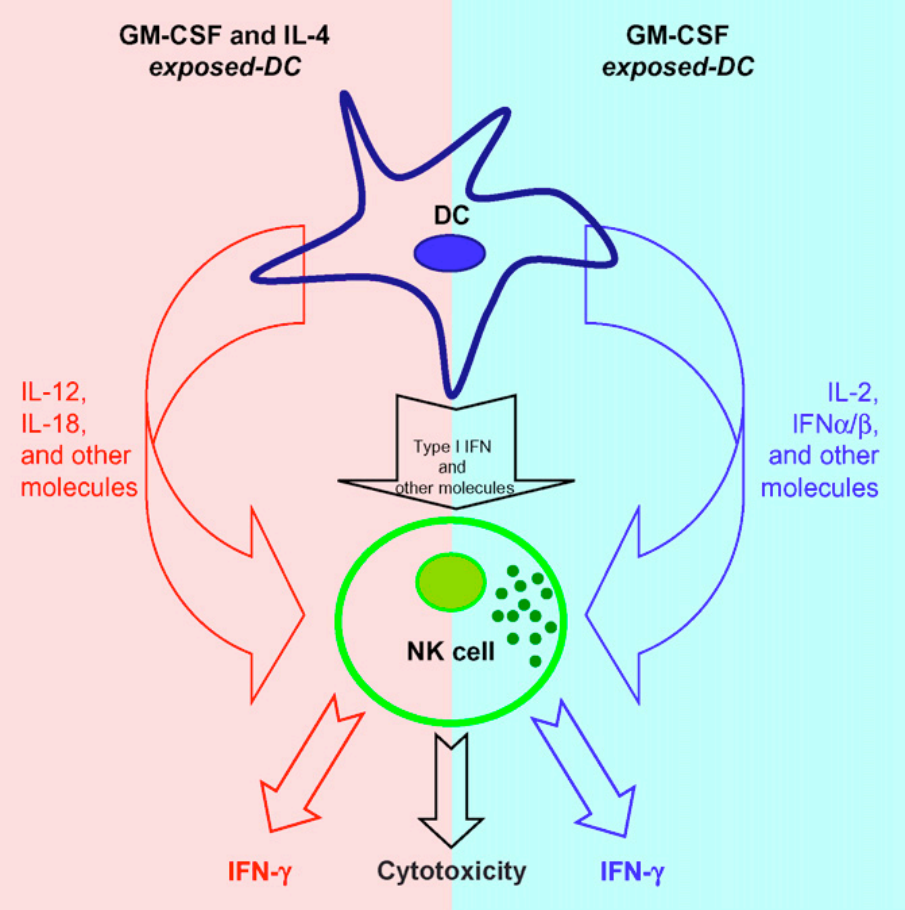

Figure 18: (from Zanoni, Granucci *et al.* 2007). Mediators of NK cell activation produced by activated DC differentiated in the presence of GM-CSF alone or GM-CSF and IL-4.

The DC-NK cell interactions are not unidirectional. Rather, they are a proper cross-talk (Della Chiesa, Sivori *et al.* 2005) with NK cells deeply influencing DC responses backwards. Immature DC are susceptible to NK cell-mediated lysis, but mature DC are protected. In humans, Ferlazzo and colleagues identified NKp30 as a crucial mediator of DC killing by NK cells

(Ferlazzo, Tsang *et al.* 2002). This observation might be interpreted as an active immuno-editing mechanism performed by NK cells, aimed to enhancing immune responses by preserving microbial-experienced DC while removing useless (or, worse, potentially tolerogenic) immature DC. For the same reason, NK cells may alternatively promote the maturation of DC by releasing proper stimulating factors. For instance, Rubartelli's group found that DC/NK cell interactions result in the release of the pro-inflammatory cytokine HMGB1 by NK cells, leading to DC maturation (Semino, Angelini *et al.* 2005). Other signals involved in NK cell-mediated DC activation are TNF-α (Gerosa, Baldani-Guerra *et al.* 2002; Vitale, Della Chiesa *et al.* 2005) and IFN-γ (Vitale, Della Chiesa *et al.* 2005).

In conclusion, DC-NK cell interactions are more complex than previously appreciated and have a profound impact in the regulation of immune responses. A better understanding of the mechanisms underlying this process would be invaluable for therapeutic applications involving the manipulation of the innate immune system.

Scope of the thesis

Chapter 2

Despite the role of NK cells in anti-tumor and anti-viral immunity has been deeply investigated, the mechanisms of NK cell activation in the context of anti-bacterial immunity remain obscure (Cerwenka and Lanier 2001). Recent studies suggest that NK cell activation during infections by a variety of pathogens is indirect and results from signals provided by accessory cells, most notably DC (Newman and Riley 2007). We aimed at investigating how DC activate NK cells in the presence of LPS, a major constituent of Gram-negative bacteria cell wall, recognized by TLR4 (Akira 2006). We decided to focus primarily on the secretion of NK cell-derived IFN-γ, because the anti-bacterial effects of NK cells are mainly mediated by that cytokine (Boehm, Klamp *et al.* 1997). We planned our study to span both *in vitro* and *in vivo* approaches. While the first were intended to clarify the molecular mechanisms of DC-mediated NK cell activation, the latter were meant to prove the relevance of our findings in an intact patho-physiological context.

Chapter 3

In the course of the study reported in Chapter 2, we needed to deepen our understanding of the mechanisms leading to the secretion of IL-1 family members, particularly IL-18. The release of IL-1 and IL-18 requires a unique double-step regulation, based on the activation of both NF-κB and inflammasomes (Martinon, Mayor *et al.* 2009). In order to study inflammasome activation, I joined Singapore Immunology Network for a one-year internship. There, I have also been involved in a project focused on understanding how the adaptive immune responses are shaped by stimuli triggering NLRP3, the best-known receptor able to assemble an inflammasome. In addition to some preliminary results, this side project led to a perspective review published in Eur. J. Immunol. and reported here.

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Chapter II.

Mechanisms of dendritic cell-mediated natural killer cell activation

Non-standard abbreviations

For readability issues, standard abbreviations, as listed by the European Journal of Immunology and reported in Appendix A, are not defined in the main text. Non-standard abbreviations used in this chapter are defined in the main text the first time they are used and are listed here. The same form is used in the plural.

BFA, brefeldin A
BM-DC, BM-derived DC
DOG, DTR-OVA-GFP
DOG-GFP dTg, CD11c.DOG-Ubiquitin.GFP double-transgenic
DT, diphtheria toxin
FLT3L, FMS-related tyrosine kinase 3 ligand
gbw, gram of body weight
IL-1RA, IL-1R antagonist
IL-18BP, IL-18 binding protein
MSU, monosodium urate
NLRP3, NLR family, pyrin domain containing 3

Materials and methods

Mice

All mice used in this study were on a B6 background. WT animals were supplied by Harlan Italy. *Il1r1*^{tm1Imx} (*Il1r1*$^{-/-}$), *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*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were supplied by S. Weiss (Helmholtz Centre for Infection, Germany) and J. Tschopp (University of Lausanne, Switzerland), respectively. CD11c.DOG (DTR-OVA-GFP) animals (Hochweller, Striegler *et al.* 2008) were a kind gift from G.J. Hämmerling (German Cancer Research Center DKFZ, Germany). Ubiquitin.GFP mice (Ikawa, Yamada *et al.* 1998) were obtained from M. Battaglia (San Raffaele Telethon Institute for Gene Therapy HSR-TIGET, Italy). All animals were housed under specific pathogen-free conditions and were used at 7-12 weeks of age in compliance with the Institutional Animal Care & Use Committee (University of Milano-Bicocca, Milan, Italy and Biological Resource Centre, A*STAR, Singapore) guidelines.

Cells

All cells were cultured in IMDM-10 complete medium (IMDM, 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 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 either GM-CSF- or FLT3L (FMS-related tyrosine kinase 3 ligand)-transduced B16 cell-conditioned medium as published (Inaba, Inaba *et al.* 1992; Granucci, Vizzardelli *et al.* 2001; Naik, Proietto *et al.* 2005).

Sirpα-positive and -negative DC were MACS-separated from bulk cultures of FLT3L-differentiated BM-DC in two steps: first, depletion of $CD45R^+$ plasmacytoid DC with the B220 Ab (BD Biosciences) and LD columns (Miltenyi Biotec); then, either positive or negative selection of $Sirp\alpha^+$ DC with the P84 Ab (BD Biosciences) and LS columns (Miltenyi Biotec). Purity was routinely more than 97% for both populations.

NK cells were purified from RBC-lysed splenocytes by MACS positive selection using CD49b (DX5) microbeads (Miltenyi Biotec) following manufacturer's instructions. Purity threshold to proceed with experiments was set to 90%. Alternatively, NK cells were first enriched by MACS negative selection for Ly-6G (1A8), CD19 (6D5) and CD3ε (145-2C11), using biotinylated Ab (BD Biosciences), streptavidin microbeads and LS columns (Miltenyi Biotec), then stained and FACS-sorted for high expression of CD49b (HMα2, BD Biosciences) with a FACSAria II cell sorter (BD Biosciences). Purity was always more than

99%. MACS-selected and FACS-sorted NK cells yielded similar results in the reported experiments.

YAC-1 cells were purchased from ATCC.

DC-NK cell co-culture

BM-DC (8E4/well) and NK cells (8E4/well for IFN-γ release assay, variable number for cytotoxicity assay according to the effector:target - E:T - ratio) 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 (1 μg/ml), shown contamination-free (Zanoni, Ostuni *et al.* 2009), monosodium urate (MSU) crystals (250 μg/ml) and Z-YVAD-FMK (10 μM) from Alexis Biochemicals; neutralizing anti-IL-1β (B122), anti-IL-1R1 (35F5), anti-IL-18Rβ (TC30-28E3), anti-IL-12(p40/p70) (C17.8) and all isotype controls from BD Biosciences; neutralizing anti-IL-18 (D048-3) from MBL International; neutralizing anti-CD70 (FR70) from eBioscience; recombinant IL-1RA (1 μg/ml), recombinant IL-18BPd/Fc chimera (1 μg/ml), recombinant human IgG1 Fc (1 μg/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 (IL-1RA and IL-18BPd/Fc chimera) were added 20 min prior to stimulation with LPS. Recombinant cytokines (rIL-18, rIFN-β and rIL-2) were added at the time of

stimulation. Following 6 h of co-culture, plates were briefly centrifuged and supernatants were collected for IFN-γ ELISA. Alternatively, 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 were from Biolegend (B122 and Poly5113 for IL-1β, XMG1.2 and R4-6A2 for IFN-γ) and MBL International (D047-3 and D048-6 for IL-18). Alternatively, kits from R&D Systems (IL-1β and IFN-γ Duoset ELISA development systems) and BD Biosciences (IFN-γ OptEIA ELISA set) were used. IFN-β was measured using pre-coated plates from PBL InterferonSource.

Cytotoxicity assay

Cytotoxicity was quantitated by a time-resolved fluorometric assay (Blomberg, Hautala *et al.* 1996) using the DELFIA EuTDA Cytotoxicity Reagents (Perkin-Elmer). 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 (2E4 target cells added to 8E4 DC and variable numbers

of effector NK cells according to the E:T ratio) for 3 additional 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, directly 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.

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 irradiated 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. 25% of donor BM cells came from mice deficient for the cytokine under examination, whereas the remaining 75% 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 (gbw) in PBS for 7 days. Mice were used for experiments only when the ablation of the target populations was more than 95% compared to animals that did not received DT.

To activate NK cells, mice were i.v. injected with 50 µg of LPS. After 5 h, mice were euthanized 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 Cytofix/Cytoperm reagents (BD Biosciences) according to the manufacturer's instructions and using the following Ab, all from BD Biosciences: anti-CD49b (DX5 or HMα2), anti-CD3ε (145-2C11) and anti-IFN-γ (XMG1.2) or its isotype control. Samples were acquired with a FACSCalibur flow cytometer (BD Biosciences).

In vivo NK cell migration to lymph nodes

NK cells and T cells were purified from RBC-lysed splenocytes using MACS technology (Miltenyi Biotec). NK cells were positively selected with anti-CD49b (DX5)-conjugated microbeads, whereas T cells were positively selected with anti-CD4- and anti-CD8-conjugated microbeads. Purified cells were loaded with 5 µM of CellTracker Probes (Invitrogen) for 30 min at 37°C following the manufacturer's suggestions. After washing, cells were allowed to sit for 15 additional min at 37°C to complete dye processing and secretion. Finally, 6E6 T cells and 3E6 NK cells were i.v. injected in recipient mice. 24 h later, the recipient mice were s.c. injected in the footpad with either 1E6 untreated or 1-h LPS-treated BM-DC. The following day, mice were euthanized and single cell suspensions of draining popliteal lymph nodes were prepared. Total cells were counted

and the fraction of migrated injected cells evaluated by FACS analysis on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

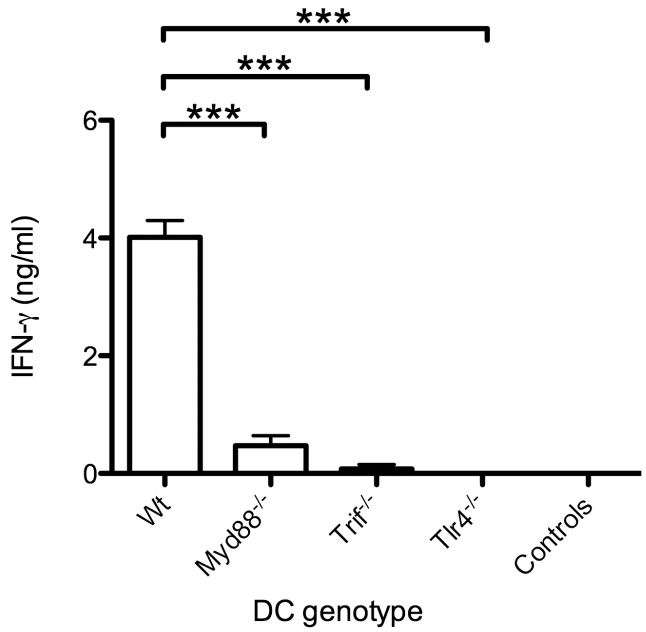
Figures report either the average of at least three independent experiments ± SEM or a representative experiment. All statistical analyses for significant differences were performed with Prism 5.0 (Graphpad) using the non-parametric one-tailed Mann-Whitney test, except when data were expressed as percentages relative to the full response. In that case, the one-tailed one-sample *t* test was adopted. *p*-values less than 0.05 were considered significant. Graphical representation of *p*-values is: * when $p<0.05$; ** when $p<0.01$; *** when $p<0.001$; ns, not significant.

Results

NK cells require MyD88, but not TLR4, to release IFN-γ when activated by LPS-stimulated DC

Despite NK cells have long been believed to operate independently from other cells of the immune system, there is recent evidence suggesting that accessory cells, such as DC and macrophages, are able to "prime" NK cells by lowering their threshold of activation upon target recognition. Moreover, NK cells may release IFN-γ when activated by mature DC (Fernandez, Lozier *et al.* 1999; Newman and Riley 2007). Upon LPS stimulation, DC acquired the ability to elicit IFN-γ production from NK cells. TLR4 and both the adaptors MyD88 and TRIF were required for this process (Figure 1A). By contrast, NK cells could undergo full activation even in the absence of TLR4 and TRIF, but not MyD88 (Figure 1B). Thus, although intracellular TLR expression by NK cells might have a role in promoting their direct activation (Sivori, Falco *et al.* 2004), the plasma membrane-bound TLR4 seems dispensable.

A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IFN-γ (ng/ml)
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Myd88-/-
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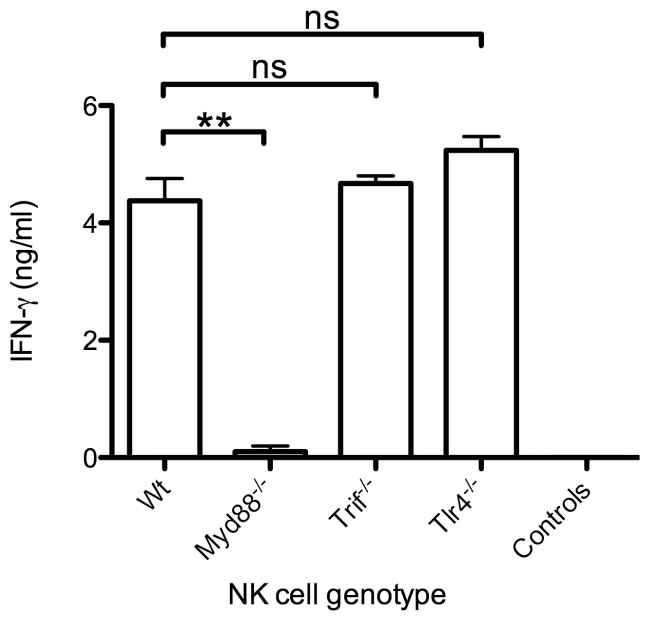
Controls
DC genotype

B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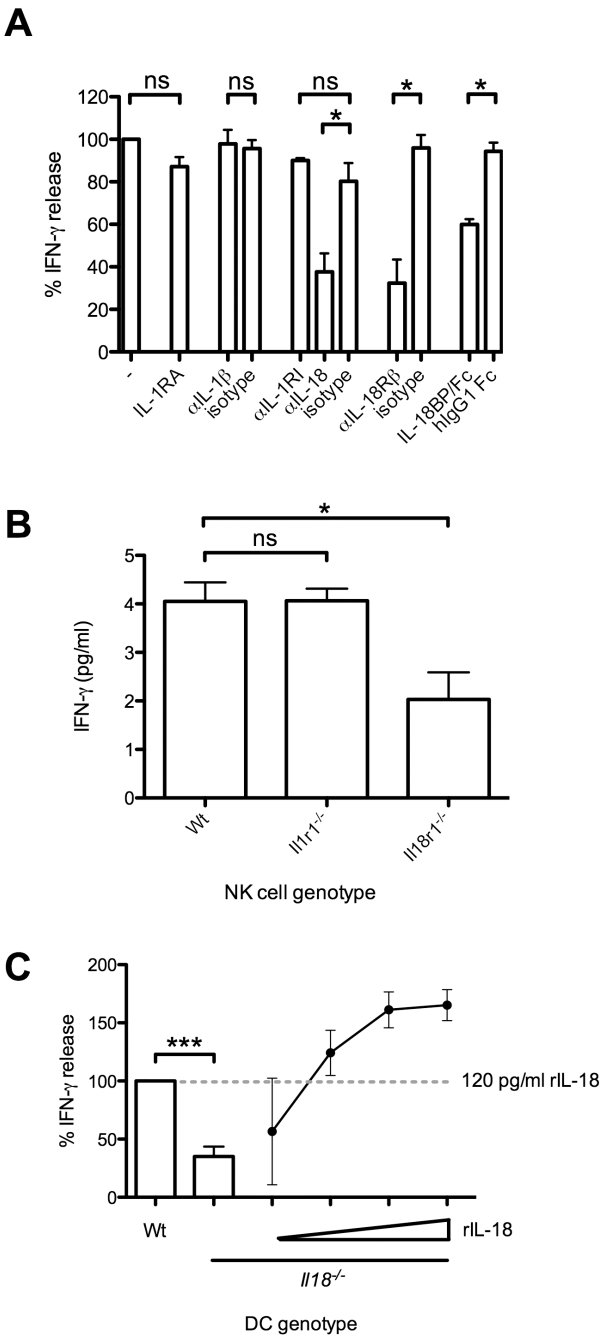
IFN-γ (ng/ml)
Wt
Myd88-/-
Trif-/-
Tlr4-/-
Controls
NK cell genotype

Figure 1. Differential requirement of TLR4 and the adaptors MyD88 and TRIF on DC and NK cells for IFN-γ release by NK cells activated by LPS-stimulated DC. Co-cultures of GM-CSF-derived BM-DC and NK cells were stimulated with LPS. 6 h later, cell-free supernatants were harvested and IFN-γ levels measured by ELISA. Controls: DC alone; NK cells alone; DC + NK cells; DC + LPS; NK cells + LPS. **(A)** LPS-stimulated DC of the indicated genotype were co-cultured with WT NK cells. **(B)** LPS-stimulated WT DC were co-cultured with NK cells of the indicated genotype. **(A-B)** $n \geq 4$. 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.

DC-derived IL-18 directly stimulates IFN-γ release by NK cells

The requirement for MyD88 but not TLR4 on NK cells (Figure 1B) for IFN-γ release in LPS-stimulated DC-NK cell co-cultures suggested the involvement of IL-1 family receptors, which signal through MyD88 (Dinarello 2009). Among others, IL-1α, IL-1β and IL-18 are members of the IL-1 family. IL-1α and IL-1β share the type I IL-1R, while IL-18 is recognized by IL-18R (Dinarello 2009). To investigate which member of the IL-1 family was required to induce NK cell activation, IL-1α, IL-1β and IL-18 were independently blocked by using neutralizing Ab or antagonists.

Specifically, IL-1α was indirectly inhibited by using either an Ab against IL-1R or the recombinant IL-1R antagonist (IL-1RA), another member of the IL-1 family (Dinarello 2009). IL-1β was blocked by the same reagents and also by a specific neutralizing Ab. NK cell-derived IFN-γ levels were unaffected by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C
200
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100
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% IFN-γ release
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120 pg/ml rIL-18
Wt
rIL-18
Il18-/-
DC genotype

Figure 2. IL-18, but not IL-1α/β, is required for DC-mediated NK cell activation. Co-cultures of GM-CSF-derived BM-DC and NK cells were stimulated with LPS. 6 h later, cell-free supernatants were harvested and IFN-γ levels measured by ELISA. **(A)** Cells were co-cultured in the presence of the indicated neutralizing Ab and recombinant proteins (or relevant isotype controls). **(B)** NK cells of the indicated genotype were co-cultured with LPS-stimulated WT DC. **(C)** LPS-stimulated DC of the indicated genotype were co-cultured with WT NK cells. In some conditions, increasing doses of rIL-18 were included in the 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)** $n \geq 3$. 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.

all the above treatments, ruling out a role for IL-1α and IL-1β in DC-mediated NK cell activation (Figure 2A). By contrast, blocking IL-18 with either recombinant IL-18 binding protein (IL-18BP) or neutralizing Ab against the cytokine itself or its receptor effectively dampened IFN-γ release by NK cells (Figure 2A).

These results were confirmed by using NK cells purified from mice deficient for either IL-18R or IL-1R. Indeed, *Il18r1*$^{-/-}$ but not *Il1r1*$^{-/-}$ NK cells showed a marked reduction in IFN-γ release (Figure 2B). Similarly, IL-18-deficient BM-DC could not fully activate NK cells, and the addition of recombinant IL-18 at the dose of 120 pg/ml completely restored IFN-γ production (Figure 2C). In summary, LPS-matured DC contribute to the induction of IFN-γ release by NK cells through the IL-18R-MyD88 axis.

DC-derived IFN-β is crucial for full NK cell activation in terms of IFN-γ release

The requirement for TRIF on DC to elicit IFN-γ release by NK cells (Figure 1A) might imply a role for IFN-β, the main cytokine controlled by the TRIF pathway (Yamamoto, Sato *et al.* 2002). We first confirmed that BM-DC release high amounts of IFN-β upon LPS stimulation (Figure 3A). To investigate the role of DC-derived IFN-β in NK cell activation, we took advantage of IFN-β-deficient DC, because Ab neutralizing IFN-β activity are not efficient (data not shown). We observed that IFN-β-deficient DC were much less capable to stimulate IFN-γ production by NK cells than the WT counterpart (Figure 3B). This phenotype was reversed by the addition of rIFN-β (Figure 3B), demonstrating that the impairment of NK cell activation was truly due to the absence of IFN-β and not to a possibly altered gene expression caused by either the insertion of the disrupting construct or IFN-β deficiency.

IL-12, IL-15, TNF-α and CD27/CD70 have no role in DC-mediated NK cell activation

IL-12p70 has been classically linked to NK cell activation and IFN-γ release (Ferlazzo, Tsang *et al.* 2002; Newman and Riley 2007). To investigate whether this was the case in our system, we blocked IL-12 in DC-NK cell co-cultures.

As shown in Figure 4A, the neutralization of IL-12 did not dampen IFN-γ levels. Moreover, we were not able to detect any IL-12p70 secretion by LPS-stimulated BM-DC (data not shown).

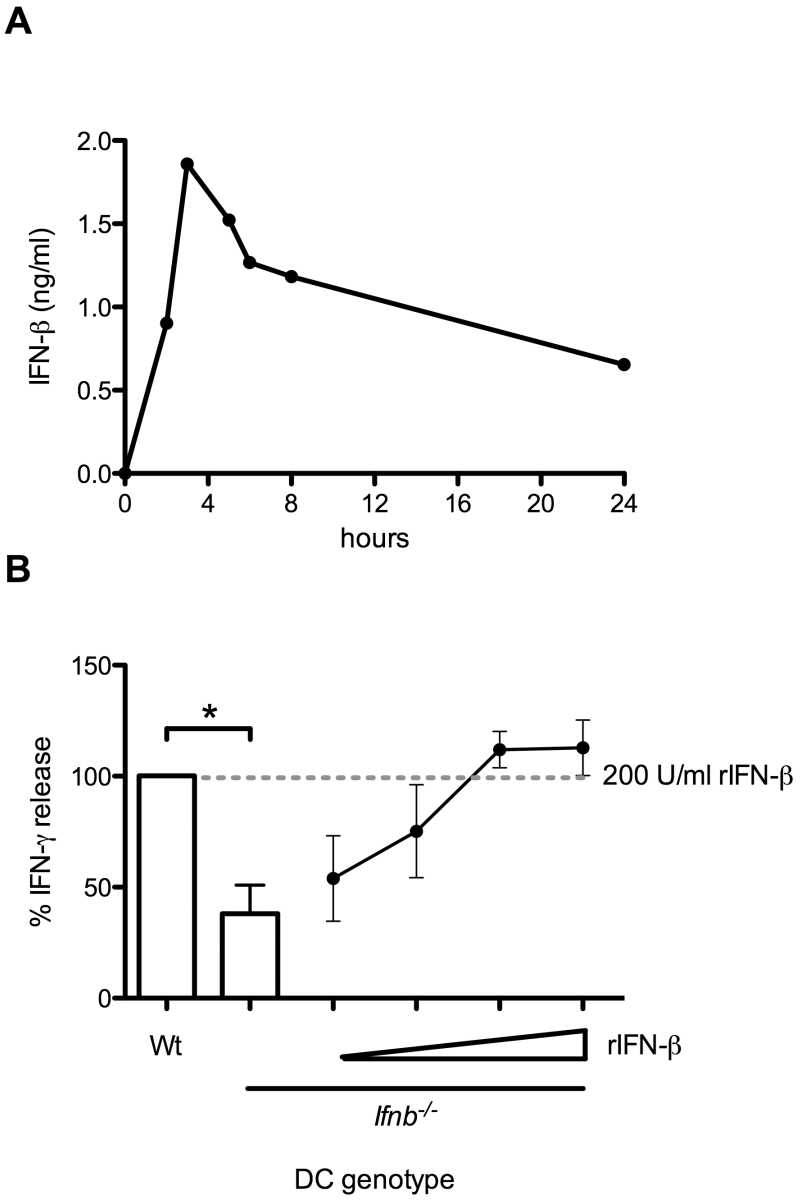
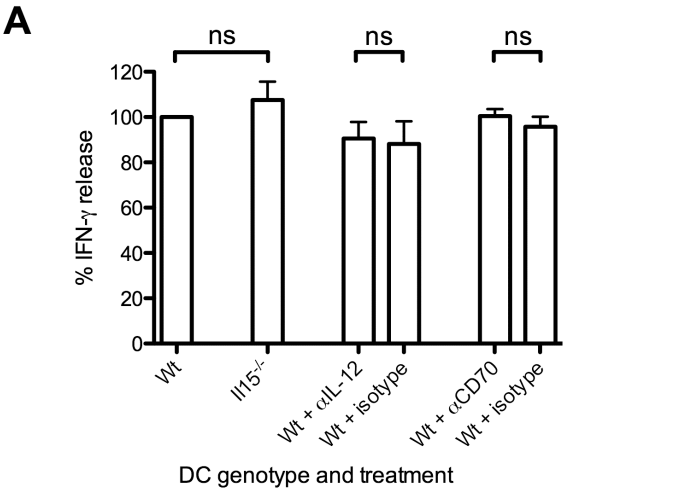


Figure 3. IFN-β is required for DC-mediated NK cell activation. **(A)** Kinetic measurement of IFN-β release by LPS-stimulated GM-CSF-derived BM-DC. **(B)** LPS-stimulated GM-CSF-derived BM-DC of the indicated genotype were co-cultured with WT NK cells. 6 h later, cell-free supernatants were harvested and IFN-γ levels measured by ELISA. In some conditions, increasing doses of rIFN-β were included in the co-culture. **(A)** A representative experiment out of three is shown. **(B)** Results are shown as percentage of IFN-γ release compared to NK cells activated by LPS-stimulated WT DC. $n \geq 3$. 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.

Recently, several investigators have reported a role for the trans-presented IL-15 in DC-mediated NK cell activation (Koka, Burkett *et al.* 2004; Lucas, Schachterle *et al.* 2007; Newman and Riley 2007; Mortier, Woo *et al.* 2008). Nevertheless, we failed to confirm those data, as IL-15 KO DC showed no defects in NK cell stimulation in respect to IFN-γ secretion (Figure 4A).

Similarly, we ruled out a role for CD70 (Figure 4A and data not shown) and TNF-α (data not shown), previously suggested to contribute to IFN-γ release by NK cells (Takeda, Oshima *et al.* 2000; Newman and Riley 2007).

In summary, we could not establish further requirements for DC-mediated NK cell activation in the context of LPS stimulation in addition to IL-2, previously published by our group (Granucci, Zanoni *et al.* 2004; Zanoni, Foti *et al.* 2005), and the here-reported IL-18 and IFN-β.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Il15-/-
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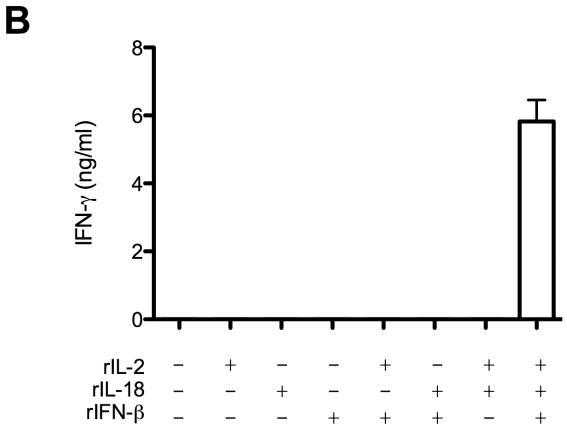
Wt + αCD70
Wt + isotype
DC genotype and treatment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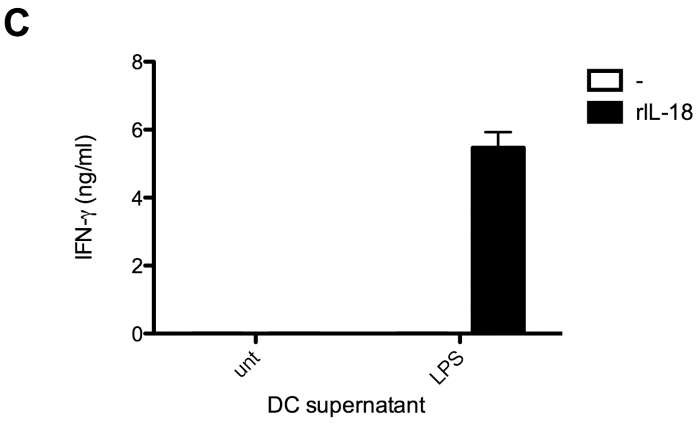
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IFN-γ (ng/ml)
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rIL-18
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LPS
DC supernatant

Figure 4. IL-2, IFN-β and the contact-dependent IL-18 are the DC-derived signals necessary and sufficient to induce full NK cell activation. **(A)** 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 relevant isotype controls) for 6 h. **(B)** NK cells were cultured for 6 h in the presence or absence of rIL-2 (1 ng/ml), rIL-18 (120 pg/ml) and rIFN-β (200 U/ml). **(C)** NK cells were cultured for 6 h in the presence of medium previously conditioned for 6 h by either untreated or LPS-stimulated DC in the presence or absence of rIL-18 (120 pg/ml). **(A-C)** At the end of the culture, IFN-γ levels were measured by ELISA. **(A)** Results are shown as percentage of IFN-γ release compared to NK cells activated by uninhibited LPS-stimulated WT DC. **(A-C)** $n \geq 3$. Statistical significance was determined by means of the one-tailed Mann-Whitney test, except for the *Il15*$^{-/-}$ condition **(A)** 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-2, IL-18 and IFN-β are necessary and sufficient to induce IFN-γ secretion by NK cells

Up to that point, we still did not know whether IL-2, IL-18 and IFN-β were not only necessary, but also sufficient to trigger NK cell activation. To address this question, we used recombinant cytokines at doses that revert the KO phenotype to WT levels (Figure 2C and 3B and data not shown). This approach is more reliable than using doses of recombinant cytokines similar to levels of natural secretion by WT DC as measured by ELISA, in that: a. the activity of recombinant versus natural cytokines is different; b. since natural secretion is a dynamic process, the measured levels of cytokines would change over time, while the addition of recombinant cytokines is performed at a single time point; and c. naturally secreted cytokines are active at the local

concentration in the limited space of the immune synapse, whereas the measured concentration refers to the cytokine upon dilution in the culture medium.

We found that the simultaneous stimulation of NK cells by IL-2, IL-18 and IFN-β elicits IFN-γ release, whereas the lack of either one of the three cytokines results in a totally ineffective stimulation (Figure 4B). Therefore, IL-2, IL-18 and IFN-β are necessary and sufficient to mediate DC-driven NK cell activation in terms of IFN-γ release.

IL-18 is the contact-dependent signal required in DC-NK cell interactions

The finding that three soluble cytokines are responsible for DC-mediated NK-cell secretion of IFN-γ was somehow unexpected, since it has been repeatedly shown that a productive DC-NK cell interaction demands at least one contact-dependent signal (Fernandez, Lozier *et al.* 1999; Newman and Riley 2007).

Although such requirement might be interpreted as dependence on a plasma membrane protein on DC, an alternative explanation exists. Certain soluble factors could be released in such low amounts to be effective only in the limited space surrounding the secretion source, where the local concentration is high enough. As they rapidly disperse, their concentration decreases to levels below a threshold of significant activity. In this way, they would behave as contact-dependent signals despite their soluble nature.

Rubartelli's group has shown that IL-18 is secreted at the immune synapse between DC and NK cells (Gardella, Andrei *et al.* 1999). Thus, it could well exhibit the properties of a contact-dependent, but soluble, factor. Indeed, it is common experience that IL-18 is hardly detectable. Even when DC are stimulated with LPS plus MSU, a kind of gold-standard stimulus to induce a huge release of caspase-1-dependent cytokines, such as IL-1β and IL-18, only few tens of pg/ml of IL-18 can be measured (Figure 7A).

To test whether IL-18 is the contact-dependent signal required in DC-mediated NK cell activation, we speculated that, while rIL-18 at the dose determined in Figure 2C mimics the physiological activity of the cytokine (putatively sensed by NK cells at the immune synapse with DC), naturally secreted IL-18, once dispersed in the culture medium, might be ineffective because too diluted. Therefore, we expected supernatants collected from LPS-stimulated DC to be insufficient to elicit IFN-γ release by NK cells, unless rIL-18 was added. Indeed, this was proven to be the case (Figure 4C), pointing out that IL-18 might require contact between DC and NK cells to fully exert its activity, while IL-2 and IFN-β are effective even upon dispersion in the culture medium.

IL-2 and IFN-β always cooperate to induce release of NK cell-derived IFN-γ

During the course of viral infections, systemic release of high amounts of type I IFN is common (Biron, Nguyen *et al.* 1999).

However, it was reported that IFN-α/β counteract NK cell secretion of IFN-γ induced by IL-12 (Nguyen, Cousens *et al.* 2000). Since IL-12 has no role in our system, while IL-2 has, we asked whether IFN-β could display an analogous inhibition of NK cell activation at high doses. However, we have always observed synergism between IFN-β and IL-2 in a dose-response fashion (Figure 5).

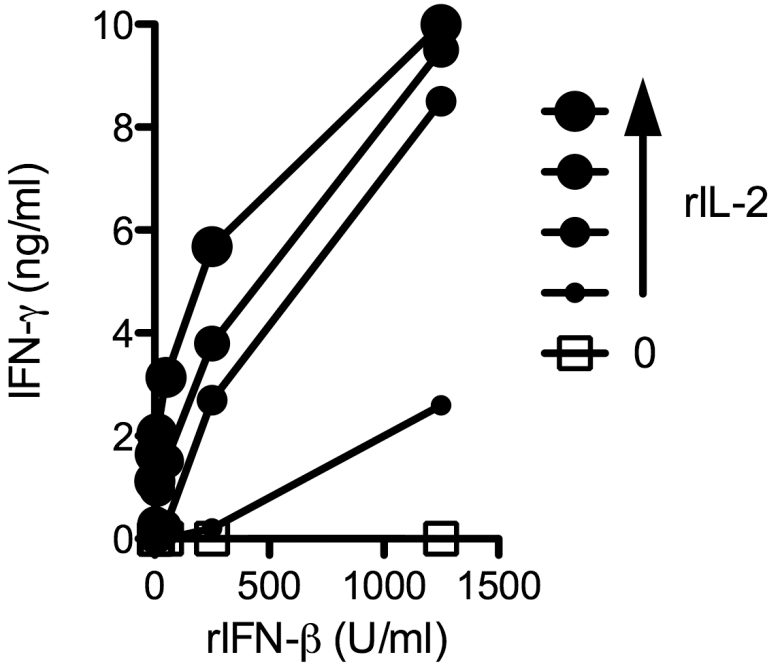


Figure 5. IL-2 and IFN-β show synergism in inducing IFN-γ release by NK cells. NK cells were cultured for 6 h with increasing doses of rIL-2 and rIFN-β in the presence of a fixed concentration of rIL-18 (120 pg/ml). A representative experiment out of three is shown.

Cytotoxic activity of NK cells requires IFN-β but not IL-18

Up to now we have checked the requirement of IL-18 and IFN-β for DC-mediated NK cell activation only in terms of IFN-γ release. However, NK cells have another major function, that is cytotoxicity against missing- or altered-self targets (Lanier 2005). As shown by experiments with KO cells, IL-18 displayed no role in the DC-induced priming of NK cell cytotoxic activity (Figure 6A). By contrast, IFN-β was essential, whereas IL-15 was again dispensable (Figure 6B).

IL-18 secretion by LPS-primed DC requires the presence of NK cells

Most IL-1 family cytokines show a double-step requirement for their secretion (Dinarello 2009). First, the precursor is induced by NF-κB activation. However, this is not the case for IL-18, whose precursor is constitutive (Dinarello 1999). NF-κB activation is nonetheless essential also for IL-18 in that it primes the molecular components required for the second step, generally known as inflammasomes (Bauernfeind, Horvath *et al.* 2009). Inflammasomes comprise and activate caspase-1, that in turn proteolitically processes the inactive precursor of IL-1β and IL-18 to the biologically active and mature form, which is immediately secreted (Martinon, Mayor *et al.* 2009). IL-18 makes no exception to this double-step rule. However, this raises the question about how LPS-activated DC may release mature IL-18, as LPS is known to activate NF-κB through TLR4

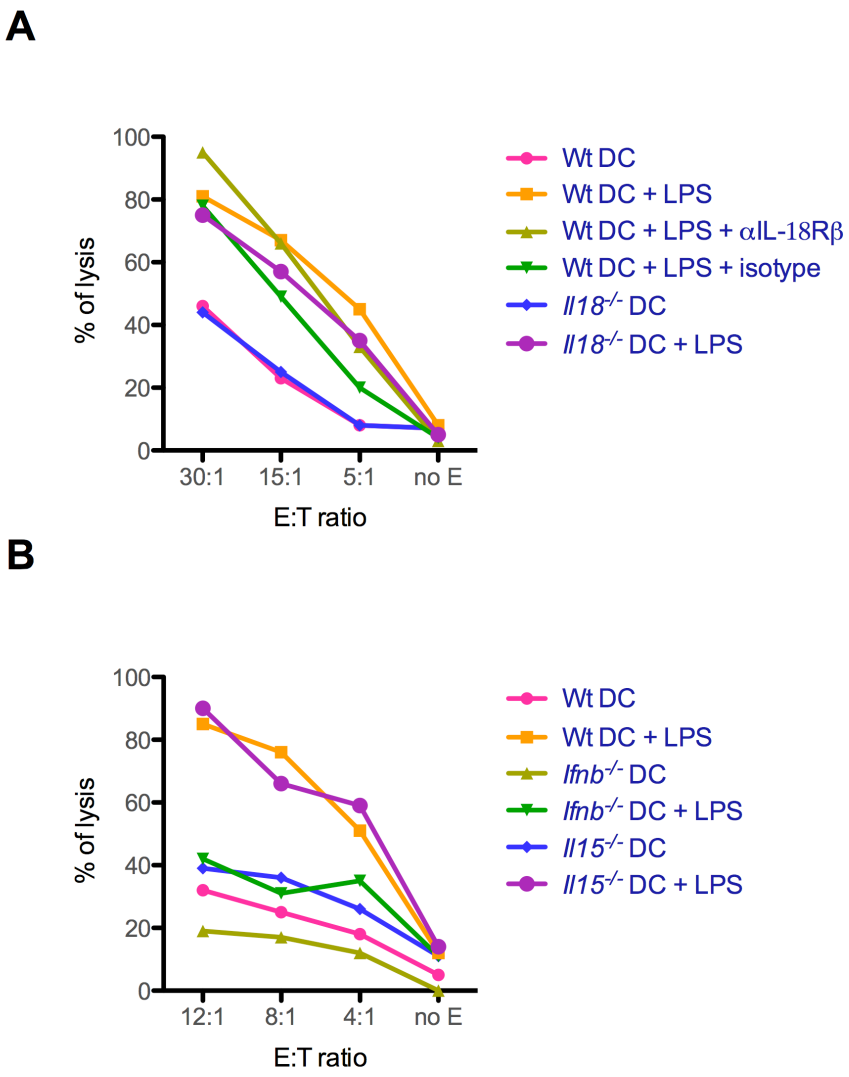


Figure 6. Cytotoxic activity by NK cells requires IFN-β but not IL-18. GM-CSF-derived BM-DC (8E4/well) of the indicated genotype, either untreated or stimulated by LPS, were co-cultured with variable numbers of effector WT NK cells for 6 h, in the presence or absence of the neutralizing anti-IL-18R Ab or its isotype control. Aftwerwards, TDA-loaded YAC-1 target cells (2E4/well) were added to wells 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 lysed cells, was measured and scaled according to maximum and spontaneous release. Two representative experiments out of six are shown.

but is not capable to stimulate any inflammasome (Dinarello 2009; Martinon, Mayor *et al.* 2009).

Nonetheless, we found a precise and specific requirement for DC-derived IL-18 in NK cell activation. First, we confirmed by PCR that IL-18 precursor is indeed constitutively expressed in BM-DC (data not shown). Then, we verified that inflammasomes are not activated upon LPS stimulation, as evident by the lack of secreted IL-1β and IL-18 (Figure 7A). We ruled out the involvement of an alternative pathway not based on the inflammasome-caspase-1 axis, as co-cultures of LPS-stimulated DC and NK cells treated with the caspase-1 inhibitor Z-YVAD-FMK were totally devoid of IFN-γ at the end of incubation (Figure 7B). However, the NLRP3 (NLR family, pyrin domain containing 3)-inflammasome was not involved, as demonstrated by the absence of difference in NK cell activation by NLRP3-deficient and WT DC (Figure 7B).

Another possibility was that during DC-NK cell crosstalk, NK cells themselves instruct DC to activate inflammasomes. Indeed, we could measure IL-1β in the supernatants only when LPS-stimulated DC were co-cultured in the presence of NK cells, although in limited amounts (Figure 7C). We were not able to detect any IL-18 (data not shown).

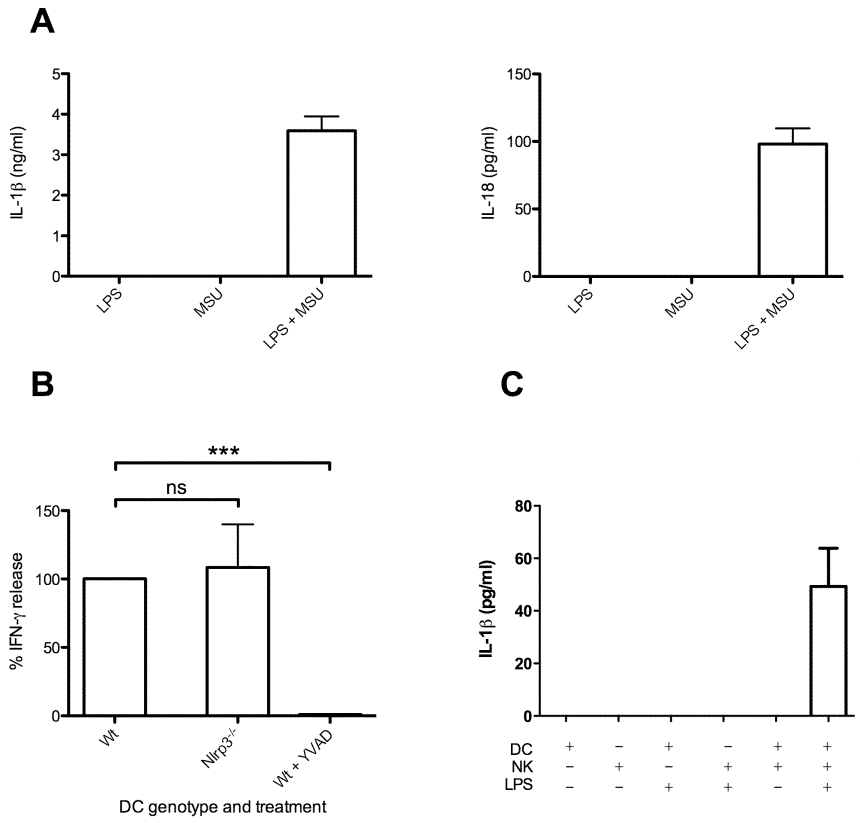


Figure 7. DC-derived IL-18 is likely released in response to an inflammasome-activating signal provided by NK cells. **(A)** GM-CSF-derived BM-DC were stimulated as indicated for 6 h. IL-1β and IL-18 were measured in cell-free supernatants at the end of the culture. **(B)** Co-cultures of GM-CSF-derived BM-DC of the indicated genotype and WT NK cells were stimulated with LPS, in the presence or absence of the caspase-1 inhibitor Z-YVAD-FMK (YVAD). 6 h later, cell-free supernatants were harvested and IFN-γ levels measured by ELISA. **(C)** GM-CSF-derived BM-DC and/or NK cells were cultured for 6 hours in the presence or absence of LPS. IL-1β levels were measured in cell-free supernatants at the end of the culture. **(A-C)** $n \geq 3$. Error bars depict SEM. **(B)** Results are shown as percentage of IFN-γ release relative to NK cells activated by uninhibited LPS-stimulated WT DC. 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.

Nevertheless, this circumstance might be expected as the levels of IL-18 were already very low even upon the strong stimulation with LPS plus MSU crystals. By contrast, in this condition the secretion of mature IL-1β was huge (Figure 7A). We are not aware of any reliable means to measure IL-18 at concentrations below 10 pg/ml.

Taken together, our data suggest that, to obtain IL-18 production, DC inflammasomes are first primed by LPS (Bauernfeind, Horvath *et al.* 2009) and then activated by an unknown mediator that requires the presence of NK cells. Upon this double stimulation, DC likely release minimal (but sufficient) amounts of IL-18 at the immune synapse (Gardella, Andrei *et al.* 1999), necessary for full stimulation of NK cells (Figure 2 and 4).

Depletion of DC *in vivo* dampens NK cell activation

In vitro, NK cells can be activated by accessory cells other than DC (Newman and Riley 2007). In order to test the relevance of DC in NK cell activation *in vivo*, we took advantage of mice bearing the human DTR under control of the CD11c promoter, the CD11c.DOG animals (Hochweller, Striegler *et al.* 2008). Differently from previously generated CD11c.DTR animals (Jung, Unutmaz *et al.* 2002), these mice allow multiple injections of DT, which ensure long-term DC ablation, because of more faithful transgene expression, truly limited to CD11c-positive cells. High expression of CD11c is required to achieve cell depletion, therefore DT is toxic only to DC. $CD11c^{low}$ cells

(including a fraction of NK cells) are unaffected in terms of both viability and functionality (data not shown).

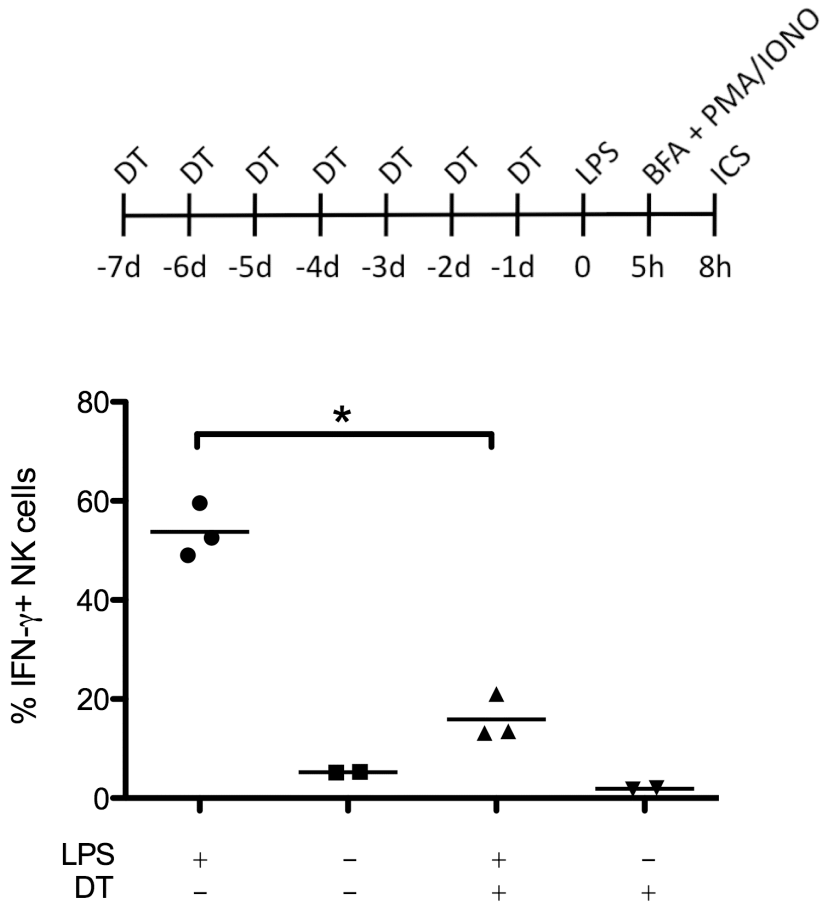


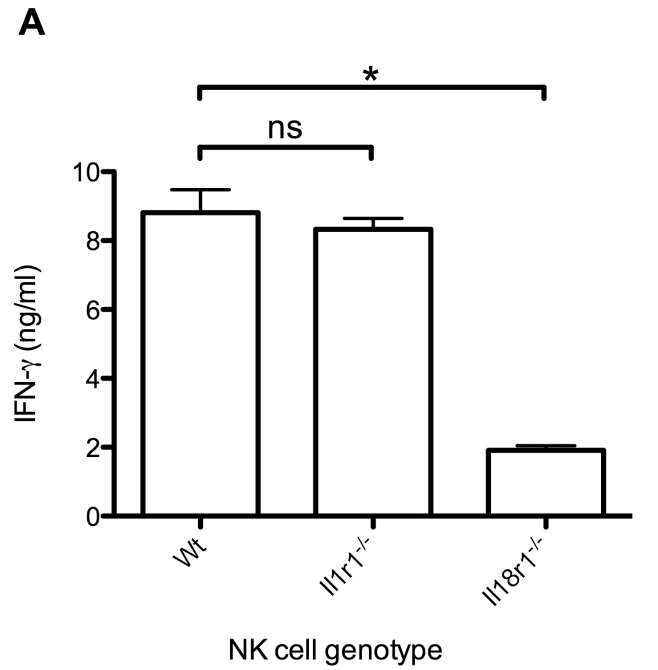
Figure 8. DC are the main accessory cells capable of NK cell activation *in vivo*. CD11c.DOG mice were daily i.p. injected with DT for 1 week. The following day, they were i.v. injected with LPS. 5 h later, splenocytes were restimulated *ex vivo* with PMA/ionomycin for 3 h in the presence of BFA. Intracellular staining for IFN-γ was then performed. NK cells were defined as $CD49b^{+}$ $CD3^{-}$. $n \geq 2$. 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.

In the absence of DC, the fraction of NK cells releasing IFN-γ upon LPS injection was reduced (Figure 8), highlighting a major role for DC, whose function in NK cell activation cannot be complemented by other accessory cells.

The Sirpα^{+} DC subset controls NK cell activation

Splenic CD11c^{high} DC are divided in two major subsets: Sirpα^{+} (CD8^{-} CD205^{-} 33D1^{+}) and Sirpα^{-} (CD8^{+} CD205^{+} 33D1^{-}), with different functional properties (Shortman and Naik 2007). Since DT administration to CD11c.DOG mice depletes CD11c^{high} cells, we asked whether NK cell activation capability belonged to both or only one of the two subsets. However, working with primary DC is tricky in terms of purity and number of recovered cells and their enrichment leads to significant perturbation of the maturation state. Therefore, as a compromise between physiological relevance and feasibility of manipulation, we took advantage of FLT3L-exposed cultures of BM cells, which give rise to DC subsets that recapitulate the *in vivo* counterparts features (Naik, Proietto *et al.* 2005).

Bulk FLT3L-differentiated BM-DC, preliminarily deprived of plasmacytoid DC, were indeed able to activate NK cells in a IL-18-dependent manner (Figure 9A), similarly to GM-CSF-differentiated BM-DC. When Sirpα^{+} and Sirpα^{-} subsets were separated, we observed that only Sirpα^{+} DC were able to activate NK cells (Figure 9B). Therefore, the *in vivo* counterpart CD8^{-} DC likely are the population of DC responsible for NK cell activation.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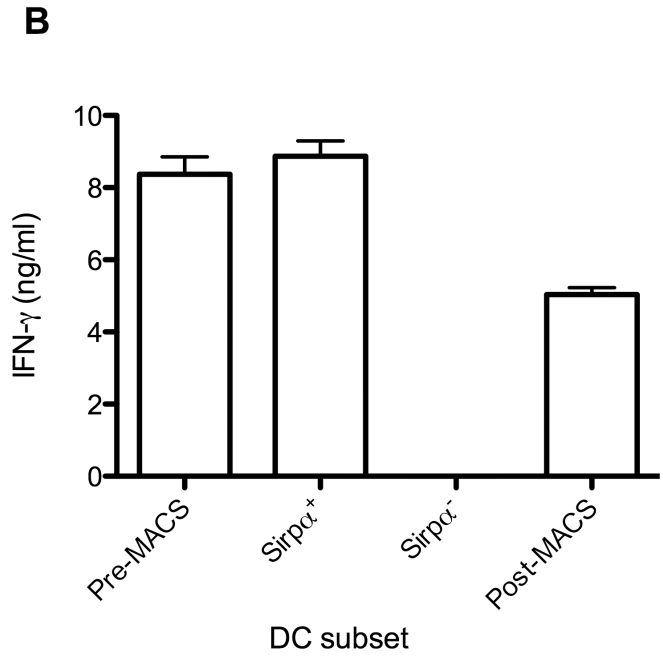
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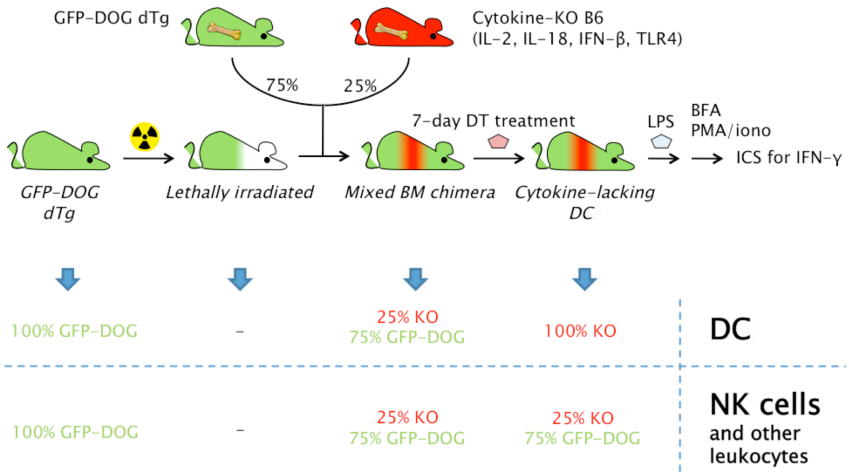
DC subset

Figure 9. Sirpα+ DC are the subset bearing NK cell-stimulatory capability. **(A)** Co-cultures of FLT3L-derived WT BM-DC depleted of $B220^+$ cells and NK cells of the indicated genotype were stimulated with LPS for 6 h. **(B)** FLT3L-derived WT BM-DC were first depleted of $B220^+$ cells (Pre-MACS), then separated on the basis of Sirpα expression. As control, the two populations resulting from purification were mixed together according to the pre-separation ratios (Post-MACS). LPS-stimulated DC were co-cultured with WT NK cells for 6 h. **(A-B)** At the end of the culture, IFN-γ levels were measured by ELISA. $n \geq 3$. 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.

DC-derived IL-2, IL-18 and IFN-β are required for full IFN-γ release by NK cells *in vivo*

Once established the role of DC in NK cell activation *in vivo* (Figure 8), we examined more specifically the molecular details of such interaction. Particularly, we tested the role *in vivo* of the three DC-derived cytokines necessary and sufficient to achieve NK cell activation *in vitro*: IL-2, IL-18 and IFN-β (Figure 4). To this aim, we generated mixed BM chimeras in which 75% of donor cells came from DOG-GFP dTg mice and 25% came from mice deficient for the cytokine under investigation. Upon injection of DT, only DOG-GFP dTg DC are ablated. Therefore, precursors from the DT-insensitive but cytokine-deficient fraction replenished the DC compartment over time. When DT was injected for long enough to allow full replenishment of the DC niche (one week in our studies), almost all DC were eventually KO for the cytokine under investigation, but normal in numbers (data not shown). By contrast, NK cells (and all other radiation-sensitive non-$CD11c^{high}$ cells) were represented

according to the initial ratio of reconstitution, namely 75% DOG-GFP dTg and 25% KO for a given cytokine, even after DT treatment (data not shown).



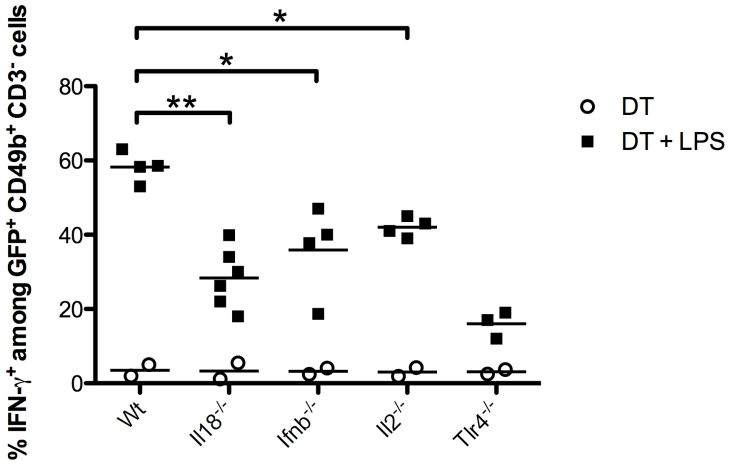


Figure 10. DC-derived IL-18, IFN-β and IL-2 are required for full NK cell activation *in vivo*. DOG-GFP dTg mice were lethally irradiated and reconstituted with BM donor cells coming for 75% from DOG-GFP dTg mice and for 25% from mice KO for the indicated gene. After full recovery (2 months), mice were daily i.p. injected with DT for 1 week. The following day, they were i.v. injected with LPS. 5 h later, splenocytes were restimulated *ex vivo* with PMA/ionomycin for 3 h in the presence of BFA. Intracellular staining for IFN-γ was then performed. NK cells were defined as $CD49b^+$ $CD3^-$. Only GFP^+ NK cells, not bearing any gene knock out, were analyzed. $n \geq 2$. 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.

Therefore, most radiation-sensitive non-$CD11c^{high}$ cells, including NK cells, did not bear any deficiency regardless of DT injection and were readily distinguishable by GFP expression. In this way, NK cells could develop in a more physiological environment compared to mice entirely KO for a particular gene, potentially subject to dysregulation of molecular and cellular balances. Even after 1 week of continuous depletion of DOG-GFP dTg DC, when nearly all DC were KO for a given cytokine, such deficiency was confined over a short time to a specific cellular population, thereby limiting side effects.

Beside DOG-GFP dTg donor cells, the KO partner cells came from mice lacking either IL-18, IFN-β, IL-2 or, as negative control, TLR4. LPS was injected in mixed bone marrow chimeras after 1 week of daily DT treatment and NK cell activation was assessed. Strikingly, the IFN-γ^+ fraction of NK cells was reduced when DC lacked either one of the three cytokines (Figure 10). Our findings confirm the requirement for

DC-derived IL-18, IFN-β and IL-2 in NK cell activation *in vivo* upon LPS stimulation.

Mature DC not only activate, but also recruit NK cells to secondary lymphoid organs

Successful NK cell activation by DC requires close encounter and contact, most likely in draining LN. We asked whether DC were able to fulfill this task by their own, thereby incorporating in a single cell type both NK cell recruitment and activation capabilities.

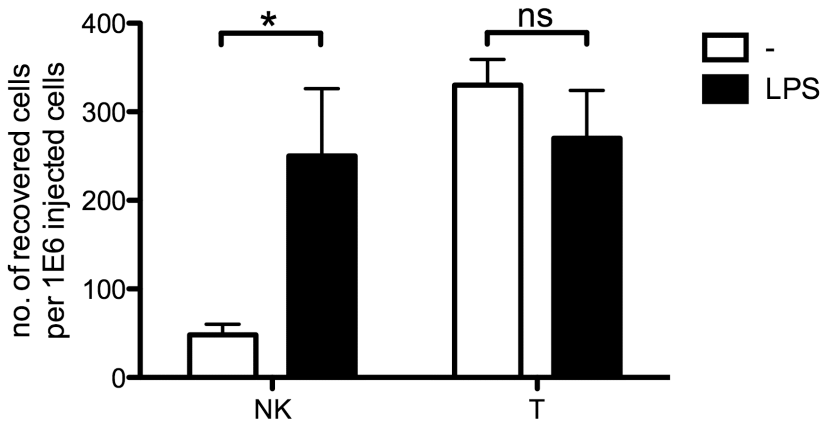


Figure 11. Mature BM-DC recruit NK cells to the draining lymph nodes. Purified NK and T cells were labeled with CellTracker Probes and i.v. injected in recipient mice. The following day, the mice were s.c. injected into the footpad with untreated (-) or LPS-stimulated (LPS) BM-DC. 24 h later, migration to the draining popliteal lymph node was assessed. $n \geq 4$. 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.

We i.v. injected lymphocytes, T or NK cells, labeled with CellTracker Probes, specifically designed for long-term tracing. The following day, BM-DC, either activated with LPS or left untreated, were s.c. injected in the footpad. Finally, 24 h later, we counted the numbers of injected lymphocytes that reached the draining lymph nodes. As shown in Figure 11, whereas the number of recruited T cells did not change in inflammatory (LPS) versus non-inflammatory (untreated) conditions in the absence of antigen, NK cells were actively attracted by mature BM-DC.

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Chapter III.

The controversial relationship between NLRP3, alum, danger signals and the next-generation adjuvants

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The NLRP3 inflammasome meets vaccines

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Abstract

Aluminum-containing adjuvants ("alum") have been the only licensed adjuvants for human vaccines for nearly a century and are still the most widely used. In spite of that, the mechanism of action of alum is enigmatic. A major advance came in the last years with the discovery of inflammasomes, supramolecular complexes regulating the activity of caspase-1 and, as a consequence, the release of pro-inflammatory IL-1 family members. In particular, the stress-sensing NLRP3-inflammasome has been implicated in alum adjuvanticity, although it is disputed to what degree NLRP3 mediates the immuno-stimulatory properties of alum. In the meantime, a number of other experimental adjuvants have been shown to activate the NLRP3 inflammasome as well. The combined exploitation of the self/non-self discriminating TLR pathways and the danger-triggered inflammasomes could be the basis of a long-needed rationale in the design of adjuvant systems, truly fitting the requirements to drive an effective and lasting immunity.

Aluminium-containing adjuvants in vaccines

The need for adjuvants arises from the poor immunogenicity of purified protein Ag in comparison to live microorganisms. Since their discovery in 1926 [1], billions of doses of alum have been administered to humans, without really knowing how or why they work. This lack of understanding led to adjuvants earning the title of "the immunologist's dirty little secret" [2], but more importantly it is holding back the development of new and improved adjuvants while the need for novel vaccines grows. Alum is still the only adjuvant authorized for human use in U.S., and just three proprietary alternatives have recently been licensed in Europe. Two kinds of alum adjuvants may be included in vaccines: either crystalline aluminium oxyhydroxide or amorphous aluminium hydroxyphosphate [3, 4].

Alum typically induces strong Th2 isotype humoral responses (dominated by IgG1 and IgE), but poor cellular responses [5]. However, the mechanisms underlying alum immuno-stimulation remain elusive. TLR had been thought likely candidates for mediators of alum adjuvanticity, but this has proven to be not the case [6, 7], which might have been somehow expected due to alum's non-microbial nature. In fact it is the physical structure of alum, whether crystalline or amorphous, which is crucial [8, 9]. Alum is insoluble in water, and so does not disperse easily following injection, leading to local retention of both the adjuvant and the Ag adsorbed to it [5]. This insoluble mass exacerbates the injury of injection and increases inflammation at the site,

using mechanisms that are just beginning to be understood [10]. This level of inflammation is key as it results in the recruitment of APC, specialized in Ag uptake and presentation. The APC at the site will then phagocytose particles of Ag-containing alum, and ultimately proceed to initiate the immune response to that Ag [10]. There are multiple steps in this process where the physical structure of the adjuvant is critical. First, it promotes inflammation by inducing cell damage [10]. The particulate nature of alum then allows it to be internalised far more efficiently by the recruited phagocytic cells, compared to soluble molecules [11]. Moreover, the ability of the adjuvant to physically bind Ag allows the immunogen to be retained in the tissue, which is important because unbound, soluble Ag redistribute quickly to secondary lymphoid organs through the lymph and elicit only transient, abortive T cell proliferation [10]. Last, the insoluble mass of aluminium compounds is regarded as a slowly dispersing depot ensuring Ag persistence, possibly ensuring maintenance of a memory pool of T cells [3].

It has been known for some time that PRR enable cells to sense and respond to the threats posed by pathogenic microorganisms, but how can they integrate the signals received from “sterile” non-pathogenic adjuvants such as alum? Recently, a key role for the stress-sensing receptor NLRP3 (nucleotide-binding domain and leucine-rich repeat containing (NLR) family pyrin domain containing 3) has been proposed [12-16].

NLRP3: linking stress and inflammation

An early indicator of the role of NLRP3 in inflammation was its emerging association with immune disorders. Familial Cold Autoinflammatory Syndrome (FCAS) and Muckle-Well Syndrome (MWS) are two autosomal-dominant "autoinflammatory" conditions, which are characterized by intermittent episodes of rash, arthralgia, fever and conjunctivitis. In 2001 it was reported that both these diseases were linked to mutations in the *CIAS1* gene [17], which encodes the NLRP3 protein (also called cryopyrin, NALP3 or PYPAF1) [18]. FCAS and MWS belong to the class of NLRP3/cryopyrin-associated periodic syndromes (CAPS), so what is the role of NLRP3 in these pathologies?

Martinon and colleagues began to answer this question by showing that NLRP3 is required for the maturation of the potent pro-inflammatory cytokine IL-1β [19]. This finding is clearly relevant in the clinical setting, as treating patients suffering from CAPS with the IL-1β inhibitors Anakinra or Rilonacept results in a striking resolution of their symptoms [20, 21]. Studies revealed that NLRP3 acts as part of a complex cascade involved in the activation and secretion of IL-1β. NLRP3 is incorporated into a large cytoplasmic caspase-1 activating complex called the inflammasome, which also contains the adaptor molecule ASC (apoptosis-associated speck-like protein containing a CARD, caspase activation and recruitment

domain), Cardinal and, of course, procaspase-1. Once the activated inflammasome is assembled, the procaspase-1 zymogen is able to autocatalytically activate, gaining the ability to proteolytically activate precursors of many of the IL-1 family of cytokines [21]. ProIL-1β, proIL-18 and proIL-33 are all converted to their biologically active forms by caspase-1; however, the IL-1α precursor does not require processing and is not a substrate of caspase-1 [22]. Interestingly, NLRP3 also influences this cytokine; indeed, deficiency in NLRP3 impairs the release of IL-1α in response to adenosine-5'-triphosphate (ATP) stimulation [22]. Therefore, active caspase-1 mediates both proteolytic processing and secretion of IL-1 family cytokines [22].

NLRP3 is the prototypic receptor able to organize an inflammasome, but it is by no means the only one. Other NLR have been reported to assemble alternative inflammasomes in response to different triggers (reviewed in [21]). However, NLRP3 is unique in that it is activated by a variety of chemically unrelated stimuli. These include crystals, insoluble protein aggregates and other particulates (asbestos, silica, the Alzheimer's disease-related amyloid-β, the gout-causing agent monosodium urate, MSU, and the pseudogout-associated calcium pyrophosphate dihydrate crystals, CPPD); ionophores, channel-activating or pore-forming toxins of both bacterial (nigericin and hemolysins from *L. monocytogenes*, *S. aureus*, *A. hydrophila*) and eukaryotic (maitotoxin) origin; and soluble purines (ATP) [21].

In trying to understand how NLRP3 functions, parallels were initially drawn with TLR. Some of the NLR family members, including NLRP3, contain a leucine-rich repeat domain, which is the portion of the TLR responsible for ligand binding [21]. However, it now seems that, unlike TLR, NLRP3 does not directly bind the molecules that trigger its activation, but is activated by their common downstream effects, which explains why NLRP3 is able to sense such a huge variety of stimuli [21]. Attention has now turned to the identification of these common intracellular signals that might directly activate NLRP3. For example, phagocytosis of large particulates that activate the NLRP3 inflammasome is frequently unsuccessful, due to the sheer size of the particle. This is termed 'frustrated' phagocytosis and induces ROS generation by NADPH oxidase [23], but can lead to lysosomal rupture if phagocytosis progresses further [15]. 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 inflammasone, via a mechanism not requiring any direct molecular interaction between NLRP3 and the particulates [15, 23]. In the case of both ATP and toxins, it seems that NLRP3 activation is triggered by the intermediary effect of massive K^+ efflux [24, 25]. Whilst 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.

The NLRP3 inflammasome, however, does not act in isolation. It is strikingly unresponsive to its known triggers unless licensed by NF-κB activating receptors, such as TLR, NOD or certain cytokine receptors [26]. 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-1β followed by a second signal controlling its release [27]. However, the interplay of TLR and the NLRP3 inflammasome is more complex. To illustrate, NLRP3-mediated cleavage of the constitutive IL-18 precursor [28] still requires activation of both NF-κB and the inflammasome [12]. Therefore, in addition to TLR role in transcriptional regulation of cytokine precursors, TLR triggering must also be required for direct activation of inflammasomes. The issue was addressed by Bauernfeind *et al.* [26], who identified expression of NLPR3 itself as the limiting factor in the NLRP3 inflammasome activation. Indeed, by increasing NLRP3 expression above threshold, NF-κB "primes" the inflammasome, namely renders it responsive to activating stimuli [26].

Role of NLRP3 in the properties of alum adjuvants

Given the central role of APC, particularly DC, in linking innate to adaptive immunity, researchers have spent considerable effort investigating how alum influences APC biology. Surprisingly, alum is unable to directly activate DC *in vitro* in

terms of upregulation of costimulatory molecules [10, 29, 30], although DC are activated indirectly by alum *in vivo* [10]. Despite a lack of DC maturation, alum does enhance Ag uptake and presentation [5, 11, 30, 31]. A major breakthrough came in 2007, when Re's group demonstrated that alum induces IL-1β and IL-18 release in a caspase-1 dependent way in LPS-primed human and murine DC *in vitro* [8], a reminder of the double-step requirement for inflammasome activation triggered by other particulates. The following year, it was found that uric acid is induced by alum *in vivo* and is responsible for alum immuno-stimulatory properties, suggesting a possible, indirect involvement of the NLRP3 inflammasome [10]. Shortly afterward, five concomitant reports showed that alum-mediated caspase-1 activation *in vitro* was NLRP3-dependent [12-16], and also required ASC, the protein bridging NLRP3 (and other NLR) and caspase-1 [12, 13, 15, 16]. Furthermore, the involvement of the NLRC4 (NLR family, CARD containing 4) inflammasome was excluded [13, 14, 16]. The mechanism *in vitro* seems to differ from what happens *in vivo*, as there is no intermediary role for two other NLRP3-activating danger signals, MSU and ATP [12, 15, 16]. Direct activation of NLRP3 required alum to be phagocytosed [12, 16], whereas ROS generation accounted only for a limited amount of NLRP3 activation in this setting [16].

Despite agreement on the involvement of NLRP3 *in vitro*, how this translates to *in vivo* responses is controversial [12-14, 16]. Considering alum's well known ability to elicit powerful humoral

responses, investigators have focused on Ag-specific antibody profiles and how they could be regulated by NLRP3. As expected, NLRP3 played no role in the induction of the Th1-associated isotype IgG2a [12-14, 16]. Other isotypes are less clearly influenced by NLRP3: for the Th2-related IgG1 and/or IgE, NLRP3 was shown to be either essential [12, 13], partially dispensable [16] or not required at all [14]. A number of reasons could explain such discrepancies. Most investigators took great care to avoid endotoxin contamination, with solutions ranging from clinical grade human serum albumin [14], through endotoxin-free ovalbumin preparations [16], to the human DT/TT (diphtheria toxin/tetanus toxoid) vaccine [13]. Thus, a role for undesired contaminants is unlikely. Another issue is the physico-chemical form of alum, especially bearing in mind knowledge of the critical role of the physical form of aluminium compounds in APC interactions. Some experiments [12, 16] were performed with Imject® Alum, a proprietary formulation available for research purposes, which is a 1:1 mixture of aluminium hydroxide and magnesium hydroxide that differs considerably from the alum adjuvants licensed for human use. Indeed, the aluminium phase of Imject® Alum was identified as amorphous aluminium hydroxycarbonate, discouraging the use of Imject® Alum to study the mechanism of action of aluminium-containing adjuvants [4]. Such differences might be critical, because while several particulates activate the NLRP3 inflammasome in a similar way [21], their physico-chemical properties are key. For example, whereas MSU crystals, the

etiological agent of gout, activates NLRP3, crystals of its analogue, allopurinol, do not, and allopurinol can even be used to *treat* gout [32]. Moreover, alum itself showed considerable lot-to-lot variation in the early stages of industrial production due to poor control of physico-chemical parameters, including particle size distribution, electrical charge and hydrated colloid nature of the precipitate [9]. Thus, not surprisingly two authors reported a failure in IL-1β secretion in response to aluminium/alum *powder* resuspended in physiological buffer [8, 32]. Therefore, although it has been shown that Imject® Alum is able to activate caspase-1 *in vitro* as much as licensed aluminium adjuvants do [8], the *in vivo* effects of a formulation with different physico-chemical parameters are unpredictable and could partially account for the differences observed in the above-mentioned studies [12-14, 16]. Finally, different experimental settings in terms of route of primary/booster immunisations, kinetic and Ag choice hinder a precise comparison [12-14, 16].

Cellular adaptive immunity induced by alum-mediated NLRP3 activation has not been investigated in such detail as the humoral response. Alum injection results in poor CTL activation and low delayed-type hypersensitivity reactions [9]. In the case of $CD4^+$ T cell polarisation, alum induces a well known bias toward a Th2 phenotype [9]. It is difficult to understand the specific role of NLRP3 activation in this setting, as $CD4^+$ T cell polarisation has been inferred indirectly by measuring the abundance of Th1 or Th2-related Ig isotypes [12, 13, 16].

Researchers have so far been unable to effectively address the relationship between NLRP3 and the Th17 phenotype, in part due to the absence of a clear Th17-driven humoral fingerprint. Two recent reports have now shown that Th17 and, to a lesser extent, Th1 skewing was enhanced when NLRP3 was constitutively active [20, 33], most likely resulting from an increased level of the Th17-polarizing cytokine IL-1β. Interestingly, in spite of that, alum immunisation results in Th2-type immunity, suggesting that other NLRP3-independent factors may skew the balance from Th17 to Th2 differentiation.

In looking for additional factors that might influence alum adjuvanticity, attention once again could turn to the TLR pathways. However, it should be remembered that previous studies with MyD88 [6, 12] and MyD88/TRIF [7] deficient mice clearly showed that these adaptors played no role in the effects of alum. This successfully ruled out the involvement of TLR, but because MyD88 is crucial also for IL-1 family receptor signalling [34], these results also cast doubt on the biological relevance of IL-1 family cytokines in the adjuvant effects of alum.

An additional layer of complexity is provided by Kool *et al.*, who showed an intermediary role of uric acid in alum immuno-stimulation [10]. The authors injected mice with an alum-Ag complex, and firstly observed the recruitment of a variety of innate immune cells to the site of injection. Among these cells were inflammatory monocytes that took up the Ag and migrated to the draining LN (DLN), while differentiating into DC. This

permitted Ag-specific $CD4^+$ T cell proliferation, in accordance with previous literature [9]. The importance of both tissue-resident and monocyte-derived DC was evident following their depletion, which significantly reduced $CD4^+$ T cell proliferation and Ag-specific IgG1 titers [10]. The other critical mediator of these effects was uric acid. After immunisation uric acid concentration increased, and if this was prevented (by treating mice with uricase) then the subsequent $CD4^+$ Ag-specific T cell proliferation was profoundly impaired [10]. This was likely related to the concomitant reduction of Ag-laden inflammatory monocytes in the DLN following uricase treatment [10].

So now we can begin to integrate all these data into a more complete understanding of the adjuvanticity of alum and the role of NLRP3. Although speculative, the process likely starts with alum induced necrosis. Dying cells release soluble uric acid, quickly generating supersaturated conditions locally that may lead to precipitation of MSU crystals [3, 29], which is the physical form of uric acid capable of stimulating the NLRP3 inflammasome [32]. Influx of infammatory monocytes and neutrophils follows, which, at least in the case of direct MSU injection, depends on the IL-1β/MyD88 axis [10, 35]. However, as noted above, the MyD88 axis was dispensable for alum adjuvanticity [6, 7, 12], but inflammatory monocyte-derived DC were required for $CD4^+$ T cell proliferation in the DLN [10], so how can it be so? This in fact leads to the question not of the relevance of MyD88, but of the relevance of T cell responses to the humoral reaction to alum. Since B cell isotype switching is

apparently able to occur in settings where monocyte-derived DC are not able to accumulate in the DLN, and T cell stimulation is limited, perhaps alum-induced IgG1 and IgE are produced independently of $CD4^+$ T cell help. For comparison with other studies [12-14, 16], it would be interesting to investigate whether IgG1 and IgE titers do in fact drop when alum-induced uric acid is depleted [10]. Translating these findings into humans is not straightforward, because humans lack a functional uricase enzyme, leading to steady-state plasma concentrations of uric acid much higher than other mammals [36].

The findings reported by Kool *et al.* [10] further challenge our knowledge of alum adjuvanticy. Both MSU and alum activate NRLP3 in a similar way *in vitro* [16, 32], yet alum requires uric acid *in vivo* to achieve inflammatory monocyte-dependent Ag uptake and $CD4^+$ T cell proliferation [10]. This reinforces the concept that NLRP3 can not be the only player in alum immuno-stimulation. This is, of course, expected, as MSU has long been known to mediate multiple biological effects, such as MAPK activation leading to IL-8 release [37]. Since alum injection generates uric acid (putatively MSU crystals), both the NLRP3-dependent and -independent properties of MSU should be taken into account. MSU might also be the intermediary responsible for the indirect activation of DC *in vivo* following alum injection [10, 29, 30, 38]. Finally, MSU was identified in a screening for endogenous danger signals in which the readout

was CTL induction [29]. In contrast, alum mounts poor CTL responses [9].

In summary, NLRP3 plays a critical role in alum adjuvanticity, but its effect on the final shape of the immune response is influenced by multiple other factors. This is exemplified by the relationship between alum, MSU and NLRP3, with the added input of MSU-stimulated NLRP3-independent mechanisms. Furthermore, it seems that, in the absence of other factors, NLRP3 activation may favour $CD4^+$ T cell skewing toward a Th17 phenotype [20, 33].

Exploiting NLRP3 in next-generation vaccines

Soon after reporting the involvement of NLRP3 in alum immuno-stimulatory properties, researchers wondered whether other particulate adjuvant candidates might harness the same pathway. Sharp *et al.* [39] found that poly(lactic-*co*-glycolic acid) (PLGA) and polystyrene microparticles do indeed activate caspase-1 through NLRP3 *in vitro* as efficiently as alum. Further investigation found that this required phagocytosis, K^+ efflux, lysosome acidification, and cathepsin B activity, but did not involve MSU, recapitulating the requirements of typical NLRP3-activating particulates [39]. *In vivo*, PGLA augmented Ag-specific IgG1 and IgG2b titers in a NLRP3-independent manner [***]. Other experimental adjuvants have also been shown to mediate IL-1β release in a NLRP3-dependent way, including QuilA, a saponin extracted from the bark of the

Quillaria saponaria tree, and Chitosan, a biodegradable polysaccharide derived from chitin [13].

Beside inflammasome-targeting molecules, a totally different portfolio of experimental adjuvants focuses on TLR ligands [40, 41]. Natural and artificial TLR ligands usually direct strong adaptive cellular immunity and Th1-biased $CD4^+$ T cell polarisation [6], which render them ideal candidate adjuvants in vaccines targeting diseases caused by intracellular pathogens. LPS (TLR4 ligand) elicits strong immune responses but is highly toxic and supports the concept that effectiveness of protection, strength of response and toxicity are tightly linked [42]. Indeed, a low-toxicity LPS derivative, 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL), shows significant loss of immuno-stimulatory activity [40]. Bearing in mind the two-step mechanism of NLRP3 activation, a number of novel adjuvants target both the TLR and inflammasome pathways. For example, Demento and colleagues [43] bound LPS onto the surface of PLGA nanoparticles. This resulted in a significant reduction of the toxicity normally associated with LPS administration, and markedly improved the adjuvant properties of the PGLA nanoparticles. Overall, this strategy notably elevated? both Ag-specific IgG titer and Th1 polarisation to a level comparable with that of the powerful adjuvant CFA [43]. Some such adjuvants have even been tested in humans, including the combination of MPL with QS21, a high-purity preparation of QuilA. In a randomised, double-blind study this novel adjuvant

outperformed CpG oligonucleotides in all the parameters tested [44].

Despite the efficacy of the combination adjuvants, the double step requirement for IL-1 release doesn't seem to apply *in vivo*. Alum or PLGA particle injection results in IL-1β release without any apparent need for inclusion of NF-κB activators in the vaccine formulation [39]. It's conceivable that endogenous mediators, such as danger signals released by cells injured upon particulate injection, activate NF-κB, thereby providing the "priming" signal. Even though TLR-mediated priming is not required for inflammasome function *in vivo*, coupling TLR and inflammasome targeting molecules in adjuvant formulations could still be beneficial, particularly for the Th1-skewing capability provided by TLR stimulation [6].

Vaccines often require booster doses to confer effective protection. Following the study by Guarda *et al.* [45], the role of NLRP3-triggering adjuvants should be specifically addressed in the context of primary and booster immunisations. Indeed, the authors reported that $CD4^+$ memory T cells can inhibit the activation of NLRP3 (and NLRP1) inflammasome in the cognate APC during Ag-specific interactions. The mechanism is based on the expression of TNF family ligands by T cells [45]. It would be possible to speculate that $CD4^+$ memory T cells generated upon primary immunisation could dampen the NLRP3-dependent effects of alum in booster immunisations. Nonetheless, booster injections are known to be effective. The

balance between T-cell mediated suppression and alum-directed re-activation of NLRP3 could still favour enhancement of immune responses. Moreover, all the NLRP3-independent effects should be preserved. Still, a precise understanding of that mechanism would be invaluable for discerning the different requirements for maximal efficacy of primary and booster immunisations.

Polly Matzinger proposed an integration of the classical self/non-self model with the danger model [46]: the immune system should attack non-self entities only when they are dangerous. In this respect, the IL-1 family cytokines, as critical regulators of inflammation, perfectly illustrate the point. TLR recognition (first signal) provides the discrimination between self and non-self, but not between dangerous and non-dangerous, as in the case of commensal bacteria. Alarm signals released by distressed or injured cells and sensed by inflammasomes could be interpreted as the second signal. Novel adjuvant systems should focus on providing both signals to elicit immunity to the “dangerous non-self” Ag, while carefully targeting appropriate receptors in order to polarise the immune system in the most beneficial way.

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Chapter IV.

Summary, conclusion and future perspectives

Non-standard abbreviations

For readability issues, standard abbreviations, as listed by the European Journal of Immunology and reported in Appendix A, are not defined in the main text. Non-standard abbreviations used in this chapter are defined in the main text the first time they are used and are listed here. The same form is used in the plural.

ASC, apoptosis-associated speck-like protein containing a CARD
CARD, caspase activation and recruitment domain
DT, diphtheria toxin
FLT3L, FMS-related tyrosine kinase 3 ligand
NLR, nucleotide-binding domain and leucine-rich repeat containing
NLRP3, NLR family, pyrin domain containing 3

Discussion

The cross-talk between DC and NK cells is relevant for immune responses to infectious agents and tumors (Fernandez, Lozier *et al.* 1999; Gerosa, Baldani-Guerra *et al.* 2002; Ferlazzo, Morandi *et al.* 2003). However, the molecular basis of such interaction was largely unknown. Here we defined the nature of the signals involved in DC-mediated NK cell activation, focusing on LPS stimulation, thereby mimicking the context of bacterial infections.

We first ruled out the possibility that NK cells were able to directly sense LPS and become activated in an autonomous way, without the need for accessory cells. Indeed, several studies reported a role for direct recognition of TLR agonists by NK cells (Becker, Salaiza *et al.* 2003; Chalifour, Jeannin *et al.* 2004; Sivori, Falco *et al.* 2004; Hart, Athie-Morales *et al.* 2005; Tsujimoto, Uchida *et al.* 2005; Lauzon, Mian *et al.* 2006; Sivori, Carlomagno *et al.* 2006; Sivori, Falco *et al.* 2007). This is not the case for TLR4, the receptor for LPS, because TLR4-deficient NK cells displayed no defect in IFN-γ release (Figure II.1B). By contrast, TLR4 was required on DC *in vitro* and *in vivo*, highlighting the accessory cell-dependent nature of NK cell activation in this context (Figure II.1A).

We demonstrated that DC produce IL-2, IL-18 and IFN-β in response to LPS (Figure II.2-3), and this is the minimal set of cytokines necessary and sufficient to activate NK cells in terms of IFN-γ release (Figure II.4).

However, DC-derived IL-18 is released only in the presence of NK cells (Figure II.7). Indeed, LPS stimulation alone is not able to trigger the secretion of IL-1 family members (Martinon, Mayor *et al.* 2009). In this respect, NK cells may provide the inflammasome-activating signal required for full processing and secretion of IL-18. We did not investigate the nature of such signal in the present study, but it would be of outstanding importance for future work. However, we were able to show that the best-known inflammasome, the nucleotide-binding domain and leucine-rich repeat containing (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome, was not involved in DC-mediated NK cell activation (Figure II.7). This suggests that other inflammasomes may be implicated. Indeed, when caspase-1 was inhibited in DC-NK cell co-cultures, NK cells were no longer able to secrete IFN-γ (Figure II.7). Also, preliminary data (not shown) demonstrate that DC deficient for ASC (associated speck-like protein containing a CARD, caspase activation and recruitment domain), an adaptor molecule shared by some inflammasomes, lose NK cell-stimulatory capability. Taken together, these findings strongly support the existence of a previously unappreciated mechanism of inflammasome activation that is dependent on the presence of lymphocytes. In line with that, investigators have recently

demonstrated other lymphocyte-mediated modulation of inflammasome activation. Tschopp and colleagues reported that effector and memory $CD4^+$ T cells can dampen the NLRP3 inflammasome activation (Guarda, Dostert *et al.* 2009). This ability relied on the expression of TNF superfamily receptors by T cells. Our results are also in agreement with a study published by the Rubartelli's group (Semino, Angelini *et al.* 2005), showing that IL-18 is released by human DC at the synaptic cleft only upon contact with NK cells.

Interestingly, it has been repeatedly shown that productive DC-NK cell interactions require both soluble and contact-dependent signals. However, we demonstrated that the soluble cytokines IL-2, IL-18 and IFN-β at physiological concentrations are able to fully activate NK cells (Figure II.4). This seeming discrepancy might be explained by at least two hypotheses, not mutually exclusive, and both centered on the peculiar biology of IL-18:

- LPS stimulation is not sufficient to induce IL-18 release by DC, because NK cell contact is required to activate inflammasomes. At first, the contact-dependency of DC-mediated NK cell activation, by analogy with T cell priming, was interpreted as the need for costimulatory membrane-bound proteins on DC (*e.g.* CD40 and CD80), putatively able to directly stimulate also NK cells (Newman and Riley 2007). By contrast, our data suggest a double-step mechanism. Initially, NK cells stimulate DC upon cell-cell contact. In turn, DC release the soluble IL-

18, that, together with the similarly soluble IL-2 and IFN-β, triggers IFN-γ release by NK cells. Therefore, the NK cell-mediated DC stimulation step, rather than the subsequent DC-mediated NK cell activation, would be properly contact-dependent.

- If the amount of cytokine being released is very low, it is possible that its concentration would be high enough to be biologically effective only in the limited space surrounding the secretion source. However, upon dilution the concentration drops to levels yielding a negligible activity. In this way, such soluble signals would behave more similarly to contact-dependent signals. Indeed, it has been shown that both IL-12 and IL-18 are released in low amounts even upon strong stimulation, and their secretion is confined at the immune synapse (Borg, Jalil *et al.* 2004; Semino, Angelini *et al.* 2005). Therefore, it is plausible that IL-18 confined secretion may account for the contact-dependency of DC-mediated NK cell activation.

It is worth noting that both hypotheses predict that supernatants from LPS-stimulated DC would not be able to activate NK cells. By contrast, the addition of rIL-18 at doses mimicking the activity at the immune synapse would be effective (and would bypass the contact-dependent step of induction of IL-18 release), when combined with IL-2 and IFN-β. Remarkably,

these predictions are in agreement with our observations (Figure II.4).

IL-18 exerts its biological functions through the MyD88-signaling IL-18R. Accordingly, both MyD88- and IL-18R-deficient NK cells show impaired IFN-γ production in co-cultures with LPS-stimulated DC (Figure II.1B-2). However, only MyD88 KO NK cells display a complete abrogation of IFN-γ release (Figure II.1B), whereas IL-18R NK cells retained a minimal secretion of such cytokine (Figure II.2B). Consistently, neutralization of IL-18 by different other means (with blocking antibodies or genetic ablation in DC) results in an almost identical impairment, yet not absolute, abrogation (Figure II.2A). We excluded any involvement of the MyD88-signaling IL-1α/β (Figure II.2A), but the IL-1 family comprises a variety of other members, most with unknown functions and, putatively, similar signaling through MyD88 (Dinarello 2009). IL-33 was not investigated. However, since IL-18, together with IL-2 and IFN-β, is sufficient to induce the secretion of NK cell-derived IFN-γ, the possible involvement of other IL-1 family members would be manifest (and significant) only in the absence of IL-18. Alternatively, since MyD88 is key for two important signaling pathways in the immune system (TLR and IL-1 family), it can be speculated that its abrogation might affect the normal balance of intracellular transduction networks even in the absence of infection/inflammation, possibly impairing a regular development of NK cells, that might be generically hyporesponsive.

In our system, we did not find a role for either IL-12p70 or IL-15 (Figure II.4A), linked to accessory cell-dependent NK cell activation in a variety of experimental settings (Carson, Giri *et al.* 1994; Ferlazzo, Tsang *et al.* 2002; Borg, Jalil *et al.* 2004; Ferlazzo, Pack *et al.* 2004; Lucas, Schachterle *et al.* 2007; Newman and Riley 2007; Huntington, Legrand *et al.* 2009). Mouse and human DC efficiently secrete bioactive IL-12 only when exposed to IL-4 (Hochrein, O'Keeffe *et al.* 2000). By contrast, microbial-stimulated IL-2 secretion is inhibited when DC are cultured in the presence of IL-4 (Guiducci, Valzasina *et al.* 2005; Schartz, Chaput *et al.* 2005). In addition, IL-4 is a semi-maturation stimulus for DC. Indeed, DC exposed to IL-4 acquire the ability to activate NK cells independently of the presence of microbial stimuli (Ferlazzo, Morandi *et al.* 2003). There is no biological rationale for differentiating mouse DC from HSC with GM-CSF plus IL-4, whereas, in human, IL-4 addition is only a technical trick to avoid the differentiation of monocyte precursors toward macrophages (Sallusto and Lanzavecchia 1994). For these reasons, DC differentiated with GM-CSF alone produce IL-2 but not IL-12 in response to LPS. Even in the absence of IL-12 production, such DC are still able to activate NK cells in an IL-2-dependent fashion (Granucci, Zanoni *et al.* 2004).

Focusing on the lack of a role for IL-15 in our system, it is worth noting that this cytokine shares the β and γ subunits of its heterotrimeric receptor with IL-2R. Even if the α subunit is different in IL-2R and IL-15R, IL-15Rα is not meant to transduce

intracellular signals. Rather, it is used to trans-present IL-15 to cells bearing the IL-15Rβγ, such as NK cells (Mortier, Woo *et al.* 2008). As such, it could be speculated that the signaling pathways of IL-2 and IL-15 may substantially (if not totally) overlap, with IL-2 signaling being a superset of IL-15 signaling, because of the additional IL-2Rα chain capable of signal transduction. Indeed, the main outcome of both IL-2- and IL-15-mediated stimulation of NK cells is STAT5 phosphorylation (Strbo, de Armas *et al.* 2008). In this respect, the distinct activity of these two cytokines might be achieved by the differential expression of their receptor α chains on target cells, rather than exploiting distinct signaling pathways. Following this line of thought, IL-2 might always complement IL-15 activity for NK cell activation, whereas the opposite would not be necessarily true. Accordingly, some pieces of evidence supporting a role for IL-15 have been generated using DC exposed to IL-4 (Koka, Burkett *et al.* 2004), which, as discussed above, cannot produce IL-2. In the future, it might be interesting to study how the minimal array of cytokines required for NK cell activation translates in terms of minimal set of STAT phosphorylation, taking into account the dichotomy between IL-2 and IL-12p70 and the possibly redundant role of IL-15.

An important finding of the present work is the unique role of DC for NK cell stimulation *in vivo* (Figure II.8). Our results are in line with experiments reported in the literature and similarly performed using the diphtheria toxin (DT)/DTR system to deplete DC (Lucas, Schachterle *et al.* 2007). However, we (data

not shown) and others (G.J. Hämmerling, personal communication) have observed a massive recruitment of monocytes in the spleen of DC-depleted mice, probably in order to differentiate into DC to replenish the empty DC niche. Even if this phenomenon does not seem to hinder the assessment of the role of DC in NK cell activation *in vivo*, we designed a more physiological experimental setting, based on mixed bone marrow chimeras (Figure II.10, top panel). Irradiated CD11c.DOG-Ubiquitin.GFP double-transgenic mice were reconstituted with BM donor cells mostly coming from the same strain, but with also a minor fraction isolated from mice lacking a given gene of interest. Upon prolonged DT injection, those mice develop a DC niche normal in size and characterized by a homogenous genotype carrying the selected deficiency. Since DC lacking TLR4 are totally unable to respond to LPS, mixed bone marrow chimeras designed to bear *Tlr4*$^{-/-}$ DC show unresponsiveness to LPS only in the DC compartment, while preserving the physiological number of DC. Using this model, we confirmed that CD11c^{hi} DC account for 60-70% of accessory cell-dependent NK cell-activation in the context of LPS stimulation. In addition, this condition served as negative control for other mixed bone marrow chimeras bearing a selective deficiency of IL-2, IL-18 or IFN-β restricted to the DC compartment. All those mice had reduced fractions of IFN-γ^{+} NK cells upon LPS challenge, corroborating our *in vitro* data (Figure II.10, bottom panel).

Since distinct DC subsets have different functions *in vivo* (Shortman and Naik 2007), we asked which subset is responsible for NK cell activation. To overcome technical limitations in splenic DC purification, we took advantage of the system reported by Naik and colleagues (Naik, Proietto *et al.* 2005). These authors showed that HSC cultured in the presence of FMS-related tyrosine kinase 3 ligand (FLT3L) give rise to DC functionally equivalent to their *in vivo* counterparts. Therefore, we generated BM-DC with FLT3L, and we were able to demonstrate that NK cell-stimulatory capability is confined to the Sirpα^{+} (CD8^{-}) subset (Figure II.9). We did not further investigate why Sirpα^{-} are not able to stimulate NK cells, but it would be interesting to assess whether IL-2, IL-18 and IFN-β are produced by such subset.

Given that the purpose of this project was to study NK cell activation in response to LPS, a product of Gram-negative bacteria, we focused more on IFN-γ release rather than cytotoxic responses. Indeed, IFN-γ is the main factor mediating Th1 polarization and phagocytic activation, *i.e.* the most suitable weapons of the immune system to fight bacterial infections (Boehm, Klamp *et al.* 1997; Ferlazzo, Morandi *et al.* 2003). However, we also investigated the molecular requirements of productive DC-NK cell interactions in terms of enhancement of cytotoxic responses (Figure II.6). DC-derived IL-2 at physiological concentrations was previously shown by our group to be dispensable for eliciting cytotoxic responses (Granucci, Zanoni *et al.* 2004). This should not be confused

with cultures of NK cells for several days in the presence of high doses of rIL-2, which results in potent cytotoxic LAK cells (Grimm, Mazumder *et al.* 1982; Whiteside 2001). In the present study we report that IL-18 is dispensable for DC-mediated induction of cytotoxic activity by NK cells, similarly to IL-2. However, in agreement with the literature (Nguyen, Salazar-Mather *et al.* 2002), we found that DC-derived IFN-β strongly boosts the cytotoxic responses of NK cells.

Our results shed new light on the mechanisms underlying NK cell activation *in vivo*. In addition to their well-studied role in anti-tumor and anti-viral immunity, NK cells are also important in controlling bacterial spread, even if this is much less appreciated. The most notable example is perhaps the immunity to *Listeria monocytogenes*, which has been shown to rely more on early production of IFN-γ by NK cells than on T cell responses (Dunn and North 1991).

A precise understanding of the basic biology governing NK cell responses might prove useful for designing targeted therapies. For instance, some drugs commercially available to treat $EGFR^+$ cancers are humanized antibodies against EGFR. It has been shown that the neutralization of the signaling pathway of EGFR by specific antibodies plays only a minor role in tumor regression. By contrast, antibodies with an Fc portion engineered to enhance NK cell-mediated ADCC are more effective (Ashraf, Umana *et al.* 2009). Such clinical achievements are based on basic understanding of NK cell

biology, with particular regard to the CD16 receptor properties, in this specific case.

Similarly, our data provide a framework to build possible future antibiotic therapies exploiting the immune system rather than inhibitors of bacterial growth, potentially subject to the phenomenon of resistance. Also, by selectively identifying which cytokines govern distinct immune responses, it would be possible to predict the effect of newly designed immunosuppressive regimens for transplanted individuals on specific responses, such as the immunity to infections. The potential is endless and translational research, which is founded on basic research, postulates a paradigm of drug discovery by rational design, rather than by random screening.

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Appendix A. Standard abbreviations

The abbreviations listed here are the standard abbreviations used without definition according to the European Journal of Immunology. The same form is used in the plural.

Å angstrom
aa amino acid
Ab antibody
Ag antigen
AIDS acquired immunodeficiency syndrome
ANOVA analysis of variance
AP-1 activator protein 1
APC antigen-presenting cell
AZT 3'-azido-3-deoxythymidine
BALF bronchoalveolar lavage fluid
BALT bronchus-associated lymphoid tissue
BCG Bacillus Calmette-Guérin
BCR B cell receptor
β2m β2 microglobulin
bFGF basic fibroblast growth factor
β-gal β-galactosidase
BM bone marrow

bp base pair

Bq Becquerel

BrdU 5-bromo-2'-deoxyuridine

BSA bovine serum albumin

BSE bovine spongiform encephalopathy

C region constant region of Ig

C/EBP CCAAT/enhancer-binding protein

CC CC chemokine

cDNA complementary deoxyribonucleic acid (cDNA)

CDR complementarity determining region

CFA complete Freund's adjuvant

CFSE carboxyfluorescein diacetate succinimidyl ester

CFU colony-forming unit

CHAPS 3-[(3-cholamidopropyl)dimethylammonio]
propanesulfonate

ChIP Chromatin immunoprecipitation

CHO Chinese hamster ovary

Ci curie

CIITA class II transactivator

CLIP class II-associated invariant-chain peptide

cM centimorgan

CMV cytomegalovirus

cNMP cyclic NMP (cAMP, cCMP, cGMP, cIMP, cUMP)

CNS central nervous system

CoA coenzyme A

Con A concanavalin A

COX cyclooxygenase

CpG cytosine guanine dinucleotide

cpm counts per minute

CREB cAMP response element binding protein

cRNA complementary RNA

CSF colony-stimulating factor

CTL cytotoxic T lymphocyte

CTLA cytolytic T lymphocyte-associate Ag

CXC CXC chemokine

3D three-dimensional

D region diversity region of Ig or TCR

Da dalton(s)

DAPI 4’,6-diamidino-2-phenylindole dihydrochloride

DC dendritic cell

Δ (delta) change

DMEM Dulbecco’s modified Eagle’s medium

DMSO dimethylsulfoxide

DNA deoxyribonucleic acid

DNase deoxyribonuclease

DNP dinitrophenyl

dNTP 2’-deoxynucleoside 5’-triphosphate (dATP, dCTP, dGTP, dTTP)

ds double-stranded

DTT dithiothreitol

E:T ratio effector to target ratio

EAE experimental autoimmune encephalomyelitis

EBV Epstein-Barr virus

EC50 50% effective concentration

ECL enhanced chemiluminescence
ECM extracellular matrix
ED50 50% effective dose
EDTA ethylenediamine tetraacetic acid
EGF epidermal growth factor
EGTA ethylene glycol-bis(b-aminoethyl ester)-N,N,N',N'-tetraacetic acid
ELISA enzyme-linked immunosorbent assay
ELISPOT enzyme-linked immunospot
EM electron microscopy
EMSA electrophoretic mobility shift assay
ER endoplasmic reticulum
ERK extracellular signal–regulated kinase
ES embryonic stem (cell)
Fab Ag-binding fragment
FACS fluorescence-activated cell sorter
Fc crystallizable fragment (of immunoglobulin)
FC(B)S fetal calf (bovine) serum
FGF fibroblast growth factor
FISH fluorescent *in situ* hybridization
FITC fluorescein isothiocyanate
FLICE Fas-associated death domain-like IL-1β-converting enzyme
FLIP FLICE inhibitory protein
FMLP formyl-methionyl-leucylphenylalanine
FOXP3/Foxp3 forkhead box p3 (human/mouse)
Fura 2-AM fura 2-acetoxymethyl ester

g gram

g unit of gravity

GABA γ-aminobutyric acid

GALT gut-associated lymphoid tissue

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GC germinal center

G-CSF granulocyte CSF

GFP green fluorescent protein

GM-CFU granulocyte-macrophage colony forming unit

GM-CSF granulocyte-macrophage CSF

gp glycoprotein

GPI glycosylphosphatidylinositol

GST glutathione S-transferase

GVH Graft-vs.host (reaction)

h hour

H chain heavy chain of Ig

H&E hematoxylin and eosin

HA hemagglutinin

HBSS Hanks' balanced salt solution

HCV hepatitis C virus

HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HGF hepatocyte growth factor

HIV human immunodeficiency virus

HLA human histocompatibility leukocyte Ag

HMG 3-hydroxy-3-methyl-glutaryl

HPLC high performance liquid chromatography

HRP horseradish peroxidase

HSC hematopoietic stem cell

HSP heat shock protein

HSV herpes simplex virus

HTLV human T lymphocyte virus

HUVEC human umbilical vein endothelial cells

i.m. intramuscular(ly)

i.n. intranasal(ly)

i.p. intraperitoneal(ly)

i.v. intravenous(ly)

IC50 inhibitory concentration of 50%

ICAM intercellular adhesion molecule

ICOS inducible costimulator

Id idiotype

ID50 50% infective or inhibiting dose

IDDM insulin-dependent diabetes mellitus

IDO indoleamine 2,3-dioxygenase

IFN interferon

Ig immunoglobulin

IGF insulin-like growth factor

IκB inhibitory NF-κB

IL interleukin

IMDM Iscove's modified Dulbecco's medium

IMEM Iscove's minimal essential medium

iNOS inducible nitric oxide synthase

IP immunoprecipitation

ITAM immunoreceptor tyrosine-based activation motif

ITIM immunoreceptor tyrosine-based inhibitory motif

IU international unit

J region joining region of Ig or TCR

JAK Janus kinase

JNK c-Jun NH_2-terminal kinase

k kilo

K_a association constant

K_D affinity constant

KO knockout

L ligand

L chain light chain of Ig

LAK lymphokine-activated killer

LD50 50% lethal dose

LN lymph node

LPS lipopolysaccharide

LTR long terminal repeat

LUC luciferase

m meter

M molar

mAb monoclonal Ab

MACS magnetic-activated cell sorting

MALT mucosa-associated lymphoid tissue

MAPK mitogen-activated protein kinase

MCP-1 monocyte chemoattractant protein-1

M-CSF macrophage CSF

2-ME 2-mercaptoethanol

MEK mitogen-activated protein kinase kinase

MEM minimum essential medium

MFI mean fluorescence intensity

mg milligram

µg microgram

MHC major histocompatibility complex

MIF macrophage migration inhibitory factor

min minute

MIP macrophage-inflammatory protein

mL milliliter

µL microliter

MLC mixed lymphocyte culture

MLN mesenteric lymph node

MLR mixed leukocyte reaction

MMP matrix metalloproteinase

MOI multiplicity of infection

MOPS 4-morpholinepropanesulfonic acid

MΦ macrophage

M_r relative molecular mass

mRNA messenger RNA

MS multiple sclerosis

MyD88 myeloid differentiating factor 88

n number in study or group

NAD nicotinamide adenine dinucleotide

NADH reduced NAD

NADP NAD phosphate

NADPH reduced NAD phosphate

NBT nitroblue tetrazolium

NDP nucleoside 5'-diphosphate (ADP, CTP, GDP, IDP, UDP)

NF nuclear factor

NFAT nuclear factor of activated T cells

NF-κB nuclear factor κB

NK cell natural killer cell

NKT cell natural killer T cell

NMP nucleoside 5'-monophosphate (AMP, CMP, GMP, IMP, UMP)

NO nitric oxide

NOD nonobese diabetic

NP-40 Nonidet P-40

nt nucleotide

NTP nucleoside 5'-triphosphate (ATP, CTP, GTP, ITP, UTP)

NTPase nucleoside 5'-triphosphatase (ATPase, CTPase, GTPase, ITPase, UTPase)

OD optical density

OVA ovalbumin

p probability

PAGE polyacrylamide gel electrophoresis

PAMP pathogen-associated molecular pattern

PBL peripheral blood lymphocyte

PBMC peripheral blood mononuclear cell

PBS phosphate-buffered saline

PCR polymerase chain reaction

PDGF platelet derived growth factor

PE phycoerythrin

PECAM-1 platelet endothelial cell adhesion molecule-1

PerCP peridinin chlorophyll protein

PFU plaque-forming unit
PHA phytohemagglutinin
PI propidium iodide
PI3K phosphatidylinositol 3-kinase
PIPES piperazine-N,N'-bis(2-ethane sulfonic acid)
PKC protein kinase C
PMA phorbol myristate acetate
PMN polymorphonuclear leukocyte
PMSF phenylmethylsulfonyl fluoride
polyI:C polyinosinic-polycytidylic acid
PRR pattern-recognition receptor
r recombinant
R receptor
RACE rapid amplification of cDNA end
RAG recombination-activating gene
RANTES regulated upon activation normal T-cell expressed and secreted
RBC red blood cell
RFLP restriction fragment length polymorphism
RNA ribonucleic acid
RNase ribonuclease
rpm revolutions per minute
rRNA ribosomal RNA
ROS reactive oxygen species
RSV respiratory syncytial virus
RT reverse transcription
RT-PCR reverse transcriptase polymerase chain reaction

s seconds
S Svedberg unit of sedimentation coefficient
s.c. subcutaneous(ly)
SCF stem cell factor
SCID severe combined immunodeficiency
SD standard deviation
SDS sodium dodecyl sulfate
SE standard error
SEM standard error of the mean
SHIP Src homology 2 domain-containing inositol 5'
phosphatase
SHP Src homology 2 domain-containing tyrosine phosphatase
siRNA small interfering RNA
SIV simian immunodeficiency virus
SOCS suppressor of cytokine signalling
SRBC sheep red blood cell
ss single-stranded
STAT signal transducer and activator of transcription
SV-40 simian virus 40
$t_{1/2}$ half-life
TAP transporter associated with Ag processing
Tat terminal deoxynucleotidyltransferase
TBS Tris-buffered saline
TBST TBS with Tween 20
TCR T cell receptor for antigen
TdT terminal deoxynucleotidyltransferase
Tg transgene/transgenic

TGF transforming growth factor

Th T helper (cell)

TLR Toll-like receptor

TNF tumor necrosis factor

TNP trinitrophenyl

TRAIL TNF-related apoptosis-inducing ligand

Treg regulatory T cell

Tris tris(hydroxymethyl)aminomethane

tRNA transfer RNA

TUNEL Tdt-mediated dUTP nick end labeling

U unit

UV ultraviolet

V region variable region of Ig

V(D)J variable (diversity) joining

v/v volume to volume ratio (%)

VCAM vascular cell adhesion molecule

wk week

WT wild-type

XID X-linked immunodeficiency

Zap70 ζ-associated protein 70

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