UNIVERSITY OF MILAN

Department of Biotechnologies and Translational Medicine

Ph.D. Course in Experimental Pathology and Neuropathology

XXVI° Cycle

CROSS-TALK BETWEEN HUMAN NK CELLS AND MACROPHAGES: INFLUENCE OF THE TUMOR MICRO-ENVIRONMENT

MED/04

Director of studies: Prof. Massimo Locati

Second supervisor: Dott. Domenico Mavilio

Ph.D. thesis of:

Irene Mattiola

Matr. R09291

ACADEMIC YEAR 2012/2013

To G.,

always with me

Contents

ABSTRACT

Natural killer (NK) cells are important effectors of innate immune responses providing cellular immunity against tumor-transformed and virally-infected cells. The existence of cross-talks between NK cells and myeloid cells, in particular dendritic cells, is well established, but information on the cross-talk between NK cells and macrophages is scanty. These interactions have been analyzed using an *in vitro* reconstituted tumoral micro-environment, as a simplified model to define soluble factors involved and/or cell contact dependency.

Autologous human NK cells and monocyte-derived macrophages were obtained from buffy coats of healthy donors after magnetic beads cell purification. Macrophages were polarized into M0, M1 and M2, using well described stimuli. First, the influence of human polarized macrophages on NK cell anti-tumoral activities was studied. The co-cultures between NK cells and macrophages were performed in direct contact or by treating NK cells with macrophage-conditioned media. Activating receptors expression and degranulation ability (CD107a assay) of NK cells were evaluated by flow cytometry. IFN-γ production by NK cells was quantified by RT-PCR and ELISA. Then, the effect of NK cell-derived IFN-γ on macrophage polarization was assessed. Gene expression of markers, cytokines and chemokines well described to characterized M1 or M2 polarization were evaluated by RT-PCR. In parallel, cytokine and chemokine secretion were detected by ELISA.

M1 polarization was required to enhance IFN-γ production and degranulation by resting NK cells. M1 ability to activate NK cells was further confirmed by the upregulation of CD69 activation marker. Importantly, either soluble mediators and direct contact interactions were involved in this process. However, the level of expression of NKp44 and NKG2D resulted increased only when NK cells were treated with M1-conditioned medium (M1-primed NK cells). Higher NKp44 and NKG2D expression correlated with enhanced NK cell degranulation towards altered cells. Although both NK cell subsets upregulated both receptors, M1-secreted IL-1β was responsible for NKp44 induction on $CD56^{dim}$ population, whereas IFN-β released by M1 favored increased expression of NKG2D by the $CD56^{bright}$ counterpart. Importantly, M1 secretion of IFN-β triggered NK cell expression of IL-15 and IL-15Rα, inducing a mechanism of IL-15 cis-presentation. IL-15 cis-presentation strongly enhanced IFN-γ secretion, that was further sustained by 2B4-CD48 interactions during direct co-cultures. On the contrary, NKG2D upregulation was responsible for increased degranulation by M1-primed NK cells. In parallel, IL-15 trans-presentation mediated by

M1, together with NKG2D and NKp30 engagement, were needed to trigger NK cell degranulation during direct contact interactions.

On the other hand, IFN-γ secreted by M1-primed NK cells was sufficient not only to downmodulate CD206 and ALOX15 expression by alternatively-activated macrophages, but also to induce pro-inflammatory cytokine (IL-1β and IL-15) and chemokine (CCL-5, CXCL-9 and CXCL-10) production. Importantly, also CD80 and IL-15Rα, which expression is strictly associated to M1 phenotype, were upregulated.

In conclusion, we demonstrate for the first time in a human model that IL-15/IL-15Rα complex plays a key role in the crosstalk between NK cells and M1 polarized macrophages. Both, cis and trans-presented IL-15 favors NK cell secretion of high amount of IFN-γ and enhances NK cell cytotoxic activity towards tumor cells. Furthermore, having determined a functional correlation between M1-derived IL-1β and NKp44 expression, we propose new effects of IL-1β on NK cell biology. Finally, we demonstrate that IFN-γ provided by activated NK cells is sufficient to partially revert the anti-inflammatory phenotype typical of alternatively-activated macrophages into a pro-inflammatory one. This confers to NK cells a potential involvement in TAMs re-education.

INTRODUCTION

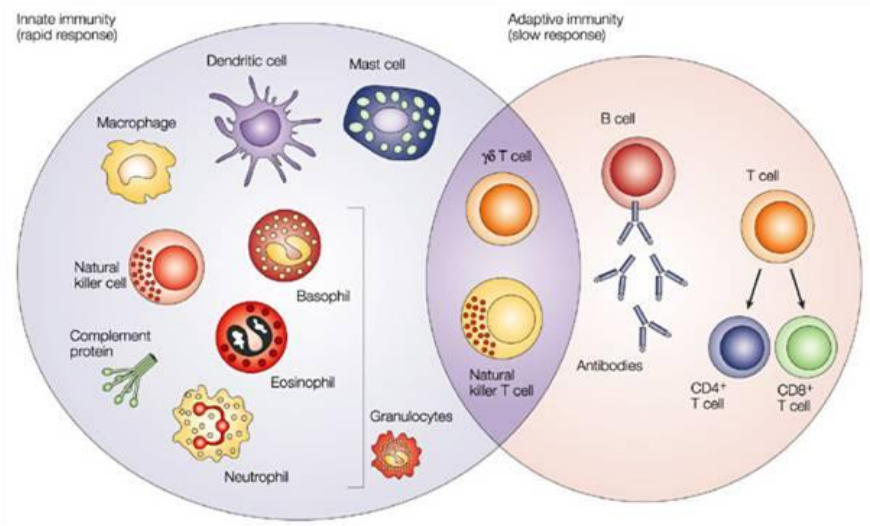
1. Immune system

Immune system can be defined as a system of biological structures aimed to protect the organism from diseases. It represents our defense against infections (bacteria, viruses, parasitic worms) and it has the role to eliminate whatever is recognized as "foreign". Indeed, the main feature of the immune system is the capability to distinguish "*self*" cells, marked by peculiar cell surface receptors, from *"non-self"* cells, such as altered or infected cells. In addition, the immune system participates to tissue remodeling, by removing dead or damaged cells. Having a functional immune system is fundamental, as disorders of immune system can result in autoimmune diseases, inflammatory diseases and cancer. In accordance with its complexity, the immune system is organized in different compartments. First, it is possible to discriminate between external defenses and internal defenses. External defenses are represented by anatomical barriers, body secretion, commensal flora and cilia. On the contrary, internal defenses are composed by immune cells, which can be further divided into two groups: innate immune cells and adaptive immune cells. If the functions of innate immunity are principally mediated by immune cells, adaptive immunity comprises both humoral and cell-mediated responses. Innate and adaptive immunity are characterized by many differences. First, if innate immune cells are activated rapidly after "injuries", within minutes or hours, adaptive immune cells require more time to be active and participate to immune responses after days. It is due to the fact that adaptive immune cells need to develop a variant receptor machinery able to recognize specific micro-organismic moieties and thus mounting a specific response to specific pathogens. In addition, adaptive immune cells develop an immunological memory, which allows faster responses upon further infections. On the contrary, innate immune cells are not able to recognize specific pathogens, since they express limited repertoire of invariant immune receptors. Furthermore, they can not develop an immunological memory. However, it represents the first line of defense of the immune system and it plays a very critical role in initiating adaptive immune responses.

1.1 Innate immunity

Innate immunity is composed by different cell types characterized by different features (Figure 1). Monocytes, macrophages, Dendritic Cells (DCs), mast cells, granulocytes, which comprise

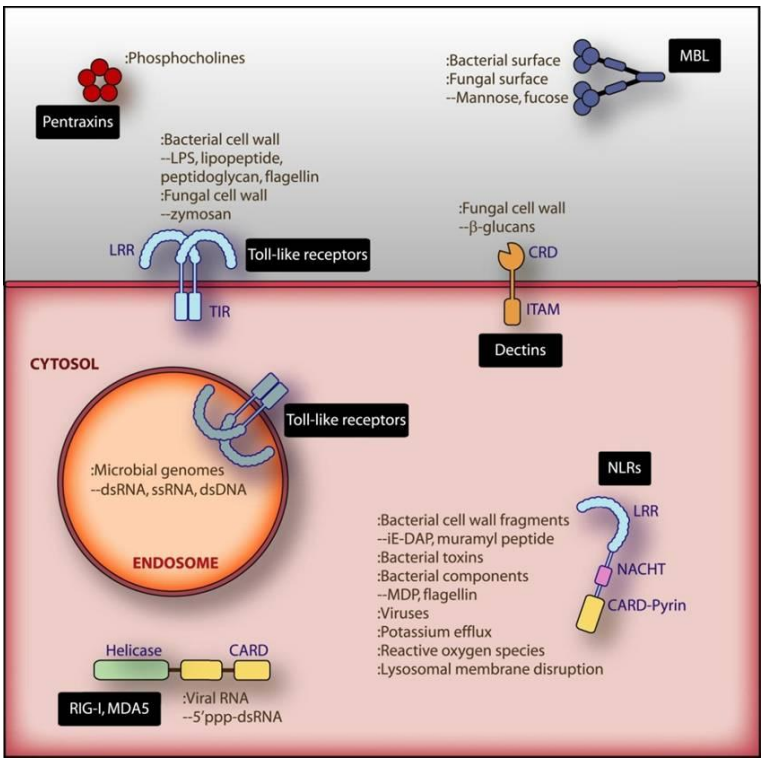
basophils, eosinophils and neutrophils, and Natural Killer cells (NK) are all innate cells, whereas complement proteins represents the only "non-cellular" component. In addition, γδ T cells and NKT cells, which express some adaptive-associated receptors together with innate-typical receptors, can be considered to some extent innate cells. On the other hand, B and T cells compose adaptive immunity. Although they are characterized by different features, innate and adaptive immunity are strictly linked, as a complete response can be mount only by the co-operation between the two arms of immune system.



Danoff G et al., Nat. Rev. Cancer 2004

Figure 1. Innate and adaptive immune cells. Innate immunity is composed by numerous cell types of myeloid (macrophages, dendritic cells, mast cell, granulocytes) and lymphoid (NK cells) origin, whereas adaptive immunity comprises B and T lymphocytes. γδ T cells and NKT cells place in between.

Innate immune cells are characterized by the expression of Pattern Recognition Receptors (PRRs), which recognize molecular structures as the Lipopolysaccharide (LPS) (Pathogen Associated Molecular Patterns, PAMPs) that are broadly shared by pathogens. PPRs are localized in different cellular compartments: on the cell surface, as Toll-like receptor 4 (TLR4) and Dectins, on the endosomes, as TLR9, in the cytosol, as NOD and RIG-like receptors, or can be also secreted, as pentraxin (PTX) and Mannose Binding Ligands (MBLs). PRRs can be divided in three main families of molecules: Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Figure 2) (1).



Medzhitov R., Immunity 2009

Figure 2. Component of PRRs family and their localization. PRRs are composed by three main families: TLRs, localized both at the cell surface and in the endosomal compartment, NLRs and RLRs, localized in the cytosol. In addition, also dectins associated to the membrane and soluble MBL and pentraxin belong to PRRs.

The toll-like receptor family is composed by 13 different TLRs, which localize in different cell compartments and recognize different ligands. They can be divided into two main groups: TLRs associated to the plasma membrane, as TLR1, TLR2, TLR4, TLR5 and TLR6, and TLRs associated to intracellular compartments, mainly associated to the endosomes, as TLR3, TLR7, TLR8, TLR9, TLR11 and TLR13. Toll-like receptors, together with Interleukin-1 receptors, give rise to a receptor superfamily associated by a TIR intracellular receptor domain, which signalizes mainly through MyD88 adaptor molecules (1).

The NOD-like receptors are intracellular sensors of PAMPs, which are activated by previously phagocytized pathogens. NLRs are composed by 3 domains: a C-terminal leucine-rich repeat (LRR) domain, which senses the ligands, a central NATCH domain, which is common to all NLRs and mediates ATP-dependent self-oligomerization, and a variable effector N-terminal domain, which can be composed by caspase recruitment domains (CARDs), pyrin domains (PYDs) or baculovirus inhibitor repeats domains (BIRs). The N-terminal domain is responsible for NLR interactions with

adapter molecules and mediates the signal transduction. NLRs family is composed by at least 20 different NLRs, conferring a high level of complexity. To simplify, NLRs are divided in 3 sub-classes on the basis of their molecular structures: NLRC sub-family, characterized by a CARD domain, the NLRP sub-family, carrying a PYD domain, and the NAIP sub-family, which presents three BIRs (2).

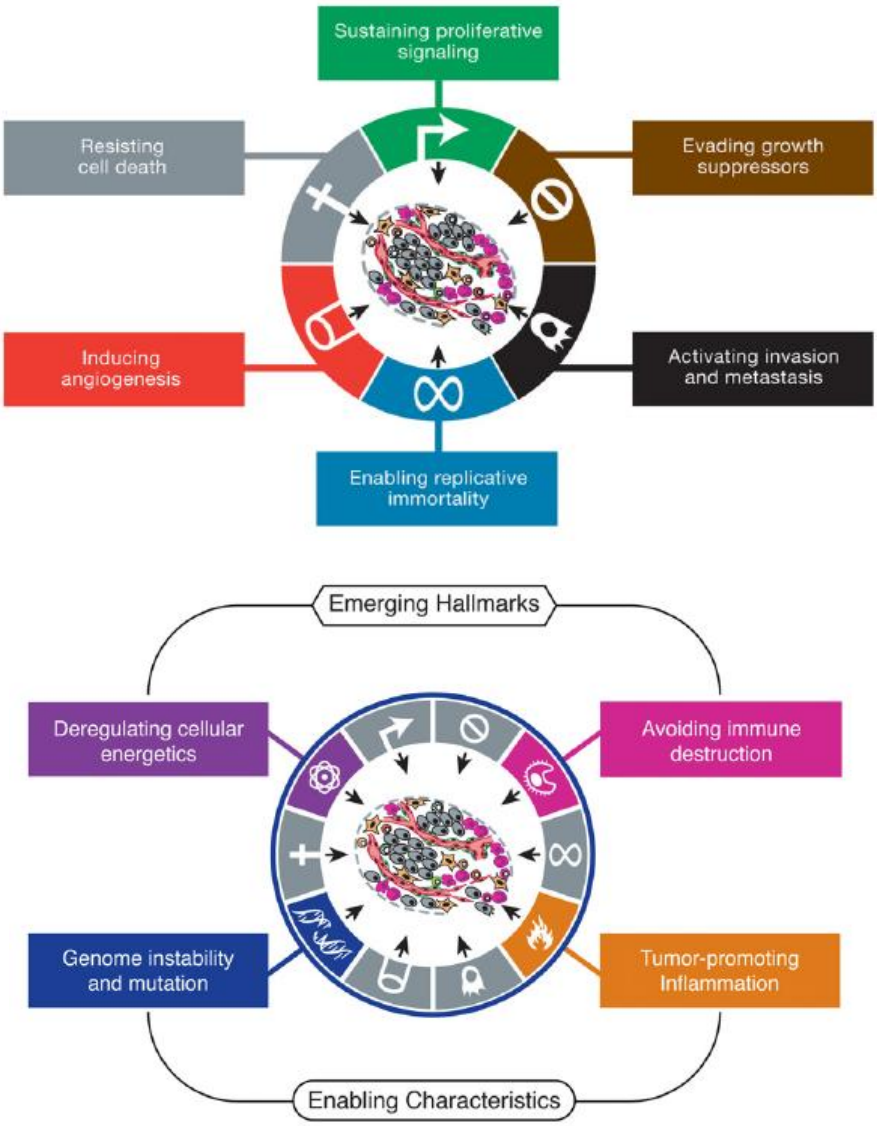
The RIG-I-like receptors are intracellular sensors of double stranded RNA. Together with the Melanoma Differentiation Associated factor-5 (MDA-5) and the Laboratory of Genetics and Physiology-2 (LGP2) proteins, RLRs belong to the RNA helicases superfamily-2. As NLRs, RLRs share a similar molecular organization. They are composed by 2 main domains: a C-terminal domain (CTD), responsible for the RNA binding, and a central DExD/h-BOX helicase domain (Hel) (3) .

TLRs, NLRs and RLRs collaborate together to guarantee the recognition of a wide range of pathogens and to cover all molecular pattern associated to them, supplying the lack of specific variant receptors by innate immune cells.

1.2 Immune cells and cancer: cancer-related inflammation and immuno-editing

Cancer is commonly defined as the result of “uncontrolled” growth of cells. These cells are normal cells, which go through a series of genetic mutations and alterations leading to the inhibition of checkpoints of the cell cycle. As a consequence, these cells acquire the capability to rapidly grow and expand, favoring the formation of proliferating clones, and give rise to tumor masses. Through the years, distinctive and complementary abilities responsible for tumor growth and metastatic dissemination have been identified and named as “hallmarks of cancer” by Hanahan D. and Weinberg R.. Hallmarks of cancer are represented by: the maintenance of proliferative signals, evasion of growth suppression, activation of invasion capability and metastatization, resistance to cell death, in addition to the induction of replicative immortality and angiogenesis (Figure 3). More recently, two “enabling characteristics”, crucial for the acquisition of the six hallmarks capabilities, and two “emerging hallmarks” have been described (Figure 3). Deregulating cellular energetics and avoiding immune destruction are the emerging hallmarks, whereas genome instability, mutation and tumor-promoting inflammation represent the enabling characteristics (Figure 3). It is

interesting to note that one emerging hallmark and one enabling characteristic are linked by the immune system (4).



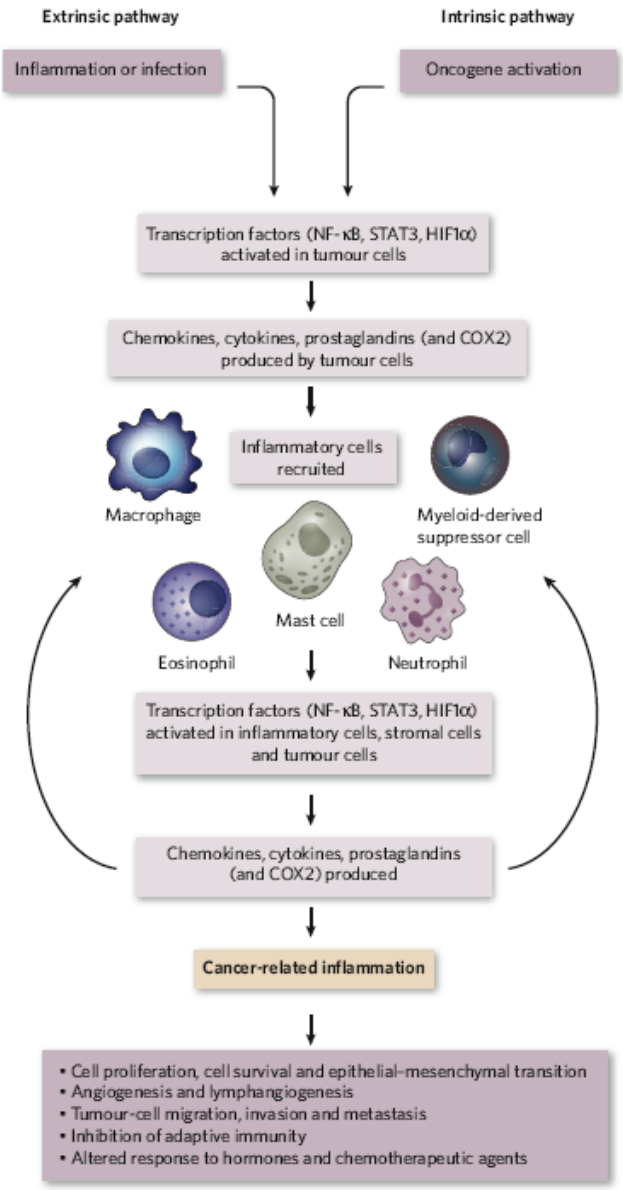
Modified from Hanahan D. and Weinberg R.A., Cell Review 2011

Figure 3. Classical and new cancer hallmarks. Classical six hallmarks of cancer are depicted in the upper panel. They are: sustaining proliferative signals, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. Below, the two enabling characteristics (genome instability and mutation and tumor-promoting inflammation) and the two emerging hallmarks (deregulating cellular energetics and avoiding immune destructions) are shown.

More in details, the inflammatory state of pre-tumoral or tumoral lesions, driven by immune cells, play an active and important role in sustaining tumor growth. The importance of the so called cancer-related inflammation (CRI) in tumor development is more and more accepted, and therefore it has been proposed as a new hallmark of cancer. If the presence of inflammation at the cancer site has been associated with cancer promotion, cancer cells have to develop strategies to evade immune cell attacks. This paradox highlights the dichotomic role of the immune system, that on one hand antagonizes and on the other hand enhances tumor development and progression (5).

First evidences of the relationship between cancer and inflammation came from the nineteenth century and derived from two main clinical observations. First of all, it has been observed that tumors often raised in proximity of a site of inflammation. Secondly, it has been possible to identify immune cells in tumor biopsies. These evidences were supported by epidemiological studies showing that individuals affected by chronic inflammation resulted predisposed to develop different types of cancer. This increased risk to cancer was sustained by different inducers of chronic inflammation, as microbial infections, viral infections and autoimmune diseases, but also by inflammatory conditions originated by unknown sources. Further confirming a strict link between cancer and inflammation, non-steroidal anti-inflammatory drugs, such as cyclo-oxygenase 2 (COX-2) inhibitors, decreased cancer risk and mortality. These observations were followed by several experimental approaches that clarified the role of inflammation in cancer development and progression (5).

Briefly, what emerged was that the relationship between cancer and inflammation is mainly regulated by two principal pathways: an intrinsic pathway and an extrinsic pathway (Figure 4). The intrinsic pathway is triggered by oncogene activation, leading to oncogene mutation, chromosomal amplification or rearrangement and/or the inactivation of onco-suppressor genes. On the contrary, the extrinsic pathway is induced by inflammation or infections. Both pathways converged in transcription factor activation as NF-kB, STAT3 and HIF-1α by tumor cells, inducing the production of cytokines, chemokines and prostaglandins. These molecules promote the recruitment and activation of innate immune cells, mainly belonging to myelomonocytic lineage.

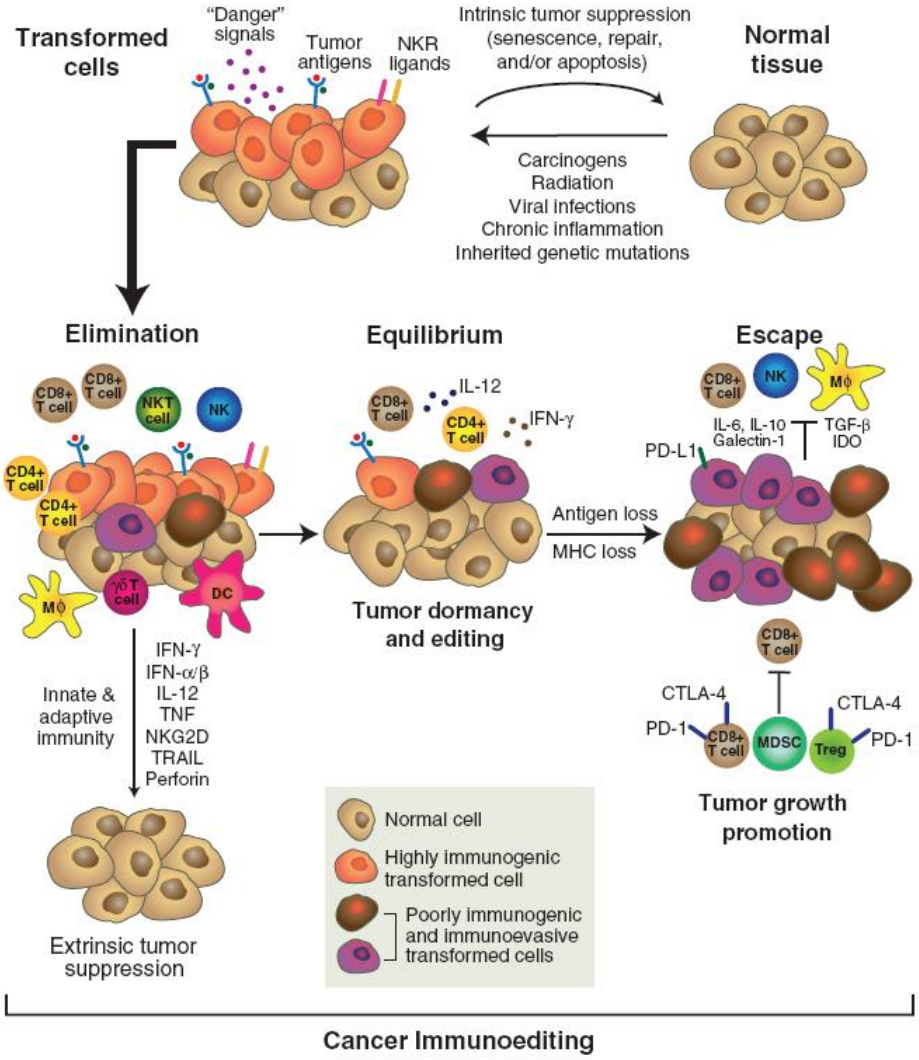


Mantovani A. et al., Nature Review 2008

Figure 4. Inflammation and Cancer are connected by two converging pathways. The intrinsic and the extrinsic pathway collaborate to induce and sustain a loop of auto-maintenance between cancer and inflammation, contributing to the generation of cancer-related inflammation.

Innate immune cells, through the activation of the same transcription factors which were induced in tumor cells, are able to release cytokines and chemokines. These immune cell-derived cytokines and chemokines, in turn, sustain tumor cell activation, allowing the generation of a loop. The final outcome is the instauration of an auto-maintained inflammatory tumor micro-environment. On one hand it promotes tumor progression by favoring cell proliferation and survival, angiogenesis and lymphogenesis, cell migration and evasion. On the other hand, it interferes with adaptive immunity and negatively modulates responses to hormones and chemotherapeutic reagents (5). It

further confirms the fact that the generation of tumor-promoting inflammation is strictly correlated with cancer cell strategies to avoid immune destruction. Indeed, tumor cells express specific antigens, which can activate immune cells. The existence of tumor associated antigens has been first demonstrated by the fact that mice immunized with homogenates of chemically induced tumors resulted protected when re-challenged with the same tumor (6). The existence of tumor antigens demonstrates that tumor cells can be recognized and eliminated by immune cells, contributing to the generation of a cancer immuno-editing hypothesis (Figure 5). The cancer immuno-editing process is composed by three main phases. The first phase is represented by the elimination phase, also known as cancer immuno-surveillance. Components of innate and adaptive immunity co-operate together to contain and eliminate a raising tumor clone, before it becomes clinically apparent. This process is mediated by antigen presenting cells, which sense danger signals, and, in turn, activate innate and adaptive cytotoxic cells. In addition, the expression of tumor antigens by cancer cells further sustains NK cell and T cell activation. The second phase consists in the equilibrium phase: the expansion of tumor clone survived to the elimination phase is controlled by adaptive immune cells, which induce a sort of tumor dormancy. If adaptive immune cells result efficient in regulating tumor dormancy, the cancer immuno-editing process is concluded. On the contrary, when tumor cells, as a consequence of the continuous pressure of the immune selection, develop strategies to circumvent immune recognition and destruction, then the escape phase begins. This gives rise to visible tumor masses. The mechanisms which are responsible of tumor cells escape are different. Frequently, cancer cells downmodulate the expression of tumor antigens, but they can also accumulate mutations in the anti-apototic pathway, leading to persistent activation of oncogenes, or they can directly activate anti-apototic molecules. In result of all these alterations, probably possible by the genetic instability that characterized tumor cells, adaptive immune cells lose the capability to recognize cancer cells. In addition, not only tumor cells become insensible to immune effector mechanisms but also actively participate to generate an immune-suppressive micro-environment, by manipulating immune cells, as occurs for tumor associated macrophages (6).



Schreiber R., Science Review 2010

Figure 5. The different phases of the cancer immuno-editing theory. The three stages of cancer immuno-editing are depicted. Once transformed cells arised, immune cells eliminate them (elimination phase). Then, an equilibrium phase in which immune cells limit tumor cell growth occurs (equilibrium phase). However, if their strategies to avoid immune cell control are predominant, tumor cells go through an escape phase.

1.3 Innate immunity and cancer

According to the cancer immuno-editing theory, innate immune cells play a pivotal role in early stages of cancer development, particularly in the elimination phase. Although all immune cells participate to the control of tumor growth, included the "hybrid" γδ T cells and NKT cells, the first line of defense against tumors is composed by NK cells, dendritic cells and macrophages, mast cells and granulocytes, or PMNs (polymorphonuclear cells).

NK cells represent the most important immune cell type that mediate the recognition and killing of non-MHC expressing cancer cells. NK cell-dependent elimination of tumor cells is mainly determined by the release of the cytotoxic molecules, perforins and granzymes. Since NK cells are characterized by the expression of a cohort of activating receptors, they are able to sense stress-induced ligands and activating receptor ligands expressed by tumor cells, leading to the activation of NK cell cytotoxicity. In addition, NK cells largely express TNF family ligands, as the TNF-related apoptosis-inducing ligand (TRAIL) or Fas ligands, which induce tumor apoptotic cell death. Finally, through immunoglobulin receptors, also called Fc Receptors, NK cells can recognize and kill antibodies-coated tumor cells (7).

The role of macrophages in controlling tumor progression is not linear: on one hand, they contribute to tumor elimination, and on the other hand, they support tumor escape and progression. In general, during the early phases of tumor development, macrophages display a pro-inflammatory phenotype, leading to phagocytosis of apoptotic tumor cells, recruitment and activation of adaptive immune cells and bacterial clearance. Moreover, as NK cells, macrophages express Fc receptors, which induce macrophage-mediated elimination of tumor cells. During the escape phase, cancer cells induce macrophage switching to an anti-inflammatory phenotype, thus sustaining tumor growth, angiogenesis, metastasis and recruiting immune-suppressive cells. The so called Tumor Associated Macrophages (TAMs) represent the most important tumor-promoting cells (7).

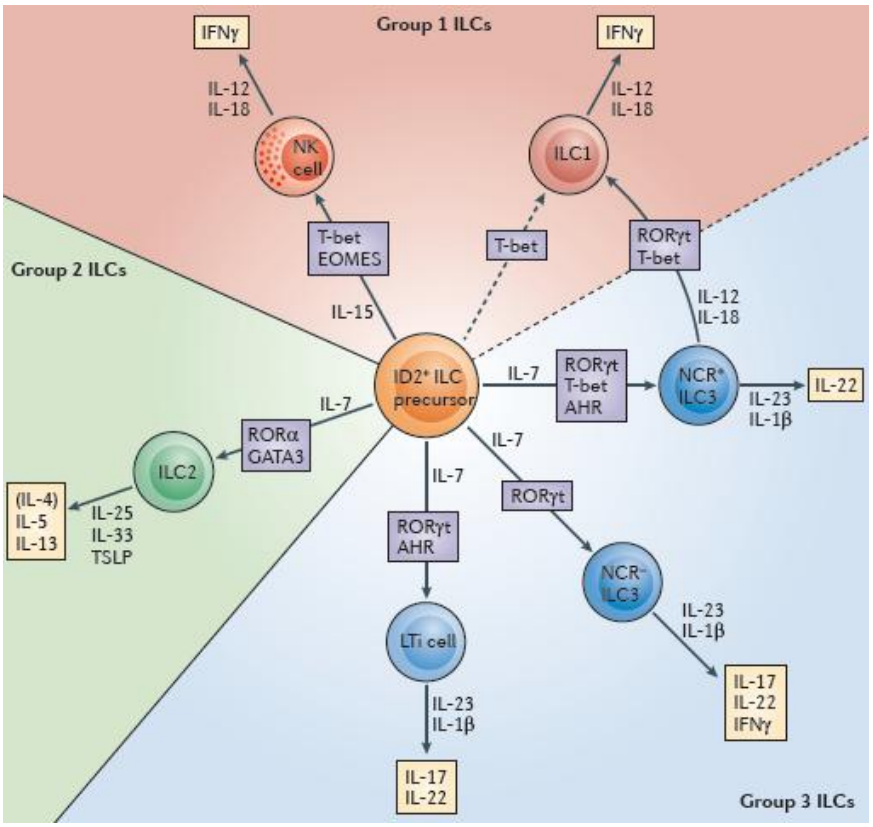
Dendritic cells (DCs) are the principal antigen presenting cells and represent the most important link between innate and adaptive immunity. DCs, together with macrophages, orchestrate the adaptive immune response by presenting tumor associated antigens to T cells. As a consequence, T cell receptor rearrangement is induced, leading to recognition and elimination of tumor cells. DCs play a fundamental role in the elimination phase, and, through the release of pro-inflammatory cytokines, not only maintain an anti-tumor micro-environment, but also participate to activate innate immune cells (7).

PMNs and mast cells have a significant role in tumorigenesis and metastasis. The interactions between tumor cells and PMNs and mast cells are not completed depicted, but it is known that neutrophils, through Fc receptors, recognize antibody-coated antigens on tumor cells, leading to cytokines and chemokines secretion. The release of these soluble molecules induces the recruitment and the activation of DCs and macrophages in the tumor site. In parallel, mast cells, by

the stimulation of inhibitory Fc receptors, decrease their Ig-E mediated release of IL-4 and histamine, triggering inflammation within the tumor (7).

2. NK cells

NK cells were first discovered in 1975 by Kiessling and Herberman (8). They were classical defined as innate immune cells, due to their capability to recognize and respond rapidly to cell targets without a prior sensitization (9). Recently, NK cells were classified as members of a specialized cohort of leukocytes, namely Innate Lymphoid Cells (ILCs) (10). The major feature that allows ILCs discrimination from B and T lymphocytes is represented by their lack of immunoglobulin and T cell receptors, which are the result of somatic gene rearrangement. Since ILCs share transcriptional factors involved in their development, it has been hypothesized that they derive from a common ILC precursor. Moreover, to simplify their recognition, ILCs have been sub-divided in three main groups, characterized by different phenotypes and effector functions: IFN-γ producing cells are defined as ILC1, type 2 cytokine producing cells which need GATA-binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor-α (RORα) for their development and functions are classified as ILC2, whereas RORγt dependent cells, which produce IL-17 and/or IL-22, compose ILC 3 group (Figure 6) (10). NK cells belong to one of the two subsets described to give rise to ILC 1 group. Indeed, NK cells are characterized by IFN-γ production upon activation, which, in addition to their cytotoxic capability, represents the main NK cell effector function. These effector functions give to NK cell important role in bacterial and viral infection resolution, in transplantation and pregnancy, and in the early phases of cancer elimination.



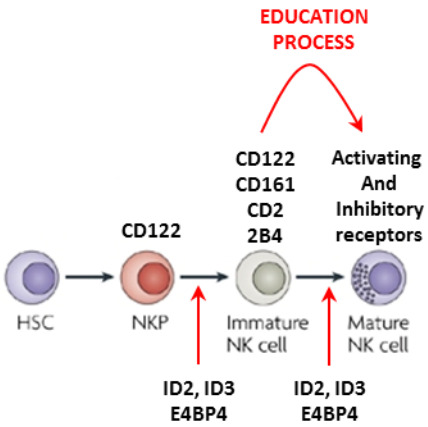
Spitz H. et al., Nature Review Immunology 2013

Figure 6. ILCs family: different groups for different functions. The complex ILC population is divided in three groups, on the basis of their functions. Each group is composed by different cells, identified by different transcription factors.

2.1 NK cell origin and development

Similarly to all other leukocytes, NK cell development is characterized by a series of differentiation and maturation steps, leading on one hand to progressive restriction towards NK cell lineage and on the other hand to acquisition of a functional competency. NK cell originate from $CD34^+$ hematopoietic progenitor cells (HPCs) located in the bone marrow. Indeed, NK cell development is completely abrogated in the presence of bone marrow ablation or destruction in experimental models (11, 12). However, recent data support the idea that NK cell development is not totally restricted to bone marrow. A small population of NK cells that express CD127 has been found in the thymus (13) and it has been described that $CD56^{bright}$ NK cells originate from hematopoietic precursor cells in the lymph nodes (14). In addition, also the maternal decidua contains hematopoietic precursors, suggesting that this district could generate NK cells (15). Moreover, it has been proposed that NK cells could develop in the liver and this phenomena could explain the

presence of immature NK cells in the adult liver (9). In any case, up to date, it is not completely clear whether NK cell population derived from thymus, lymph nodes and liver can be considered as distinct lineages or peripheral cells with immature phenotype originated from the bone marrow. To further complicate the picture, a recent study suggest that NK cells could also arise from a myeloid precursors (16). For simplicity, we assume that NK cells develop only from a common lymphoid progenitor (CPL). CLPs generate NK, B and T cells. The first step of differentiation consists in the formation of a bipotent NK/T progenitor, which originates specifically NK cells and T cells. The event that characterizes the generation of NK cell precursors is the acquisition of the β subunit of IL-15R, also called CD122, which confers to the cells the capability to sense IL-2 and/or IL-15. The fact that the main feature of NK cell precursors is the expression of IL-15R reveals that IL-15 is fundamental for NK cell differentiation, maturation and survival (17). The passage from NK cell precursors to immature NK cells is characterized by the decrease of some growth factor receptors, as FMS-like tyrosine kinase 3 (FLT3) and IL-7Rα, and the increase of IL-2Rβ, CD2 and 2B4. Moreover, immature NK cells express high levels of CD161 (18). The acquisition of NK activating and inhibitory receptors determines NK cell maturation. Of course, not only receptors but also transcription factors drive NK cell development. Indeed, ID2, ID3 and in particular E4BP4 activation underlies the progression to immature and then mature NK cells (18). During the last phases of maturation, NK cells are educated to recognize *self* molecules. In particular, the education process enables NK cells to distinguish between MHC class I bearing cells and altered cells, conferring them the license to kill (Figure 7) (18).



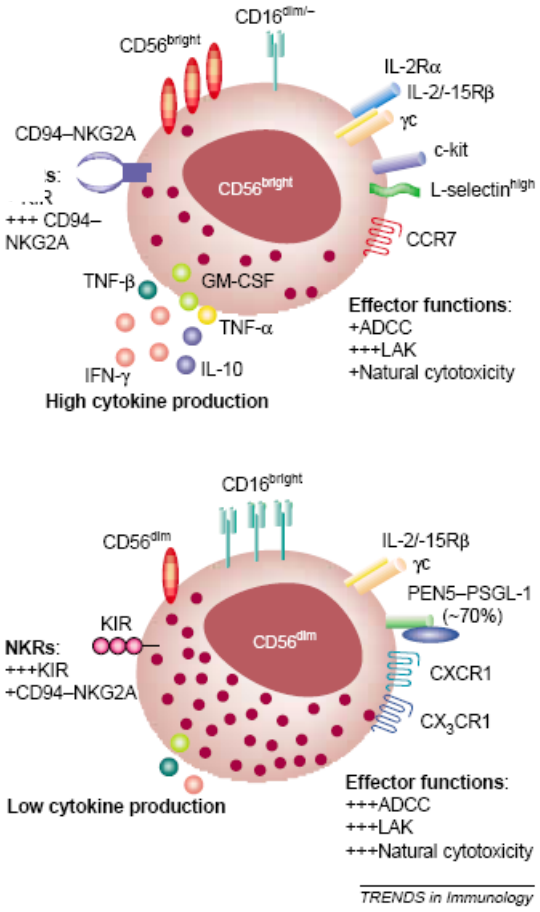
Modified from Nicholas D. et al., Nature Review Immunology 2007

Figure 7. Schematically phases of NK cell development in the bone marrow. NK cells originate from a NK cell precursor (NKP). Through the acquisition of typical NK cell markers, NK cell precursors generate immature NK cells and then mature NK cells. During NK cell maturation the education process occurs.

2.2 Human NK cell subsets

Human NK cells represent 5-15% of peripheral blood lymphocytes. In general, human NK cells are defined by the expression of CD56 and CD16, in absence of CD3. The levels of CD56 determine two different NK cell subsets. $CD56^{dim}$ NK cells constitute the majority (around 90%) of circulating NK cells and are characterized by intermediate levels of CD56 and high levels of CD16. On the opposite, $CD56^{bright}$ NK cells highly express CD56 and express low or even null levels of CD16 (19). CD56 represents an adhesion molecule, in particular an isoform of the human neural cell adhesion molecule. Its expression on NK cells has not been yet associated with some NK cell function (20). As a consequence, it is impossible to define whether different levels of expression of CD56 correlate with different activities of NK cells. However, it has been observed that different NK cell populations are associated with different functional properties. It means that other receptors differently expressed confer unique features to each subsets (19). One of them is CD16. CD16 is the low affinity FcγRIII receptor, that binds immunoglobulin-coated targets, inducing a mechanism of antibody dependent cellular cytotoxicity (ADCC). First functional studies performed by Lanier revealed that different expression of these receptors corresponds to different capabilities of ADCC by NK cells. $CD56^{dim}$ are $CD16^{bright}$, thus resulting more cytotoxic compared to $CD56^{bright}$, which almost totally lack CD16 expression (21). Concerning inhibitory receptors, $CD56^{dim}$ NK cells express high levels of KIRs (Killer-cell Immunoglobulin-like Receptors) but low levels of CD94/NKG2 receptors. On the contrary, $CD56^{bright}$ NK cells are characterized by low to absent expression of KIRs and largely express CD94/NKG2A receptors (22). In regard to activating receptors, although both $CD56^{bright}$ and $CD56^{dim}$ NK cells are comparable in terms of NKG2D expression (23), they differ in terms of NKp46 expression, as NKp46 density results higher on the $CD56^{bright}$ population (24). Furthermore, if only IL-2Rαβγ is selective expressed by $CD56^{bright}$ NK cells (25, 26), it is possible to observed that NK cell subsets display a unique repertoire of chemokine receptors (27). This evidence, together with the fact that different NK cell populations express different adhesion molecules, confers to NK cell subsets divergent migratory properties and tissue distribution (22, 28). For example, thanks to their expression of CCR7 and CXCR3, $CD56^{bright}$ homing is preferentially directed to the lymph nodes, whereas $CD56^{dim}$ migrate preferentially to acute site of infections (22, 28). Focusing on their functional responses, $CD56^{dim}$ NK cells are considered more cytotoxic than $CD56^{bright}$ NK cells. However, upon *in vitro* stimulation with IL-2 or IL-12, $CD56^{bright}$ NK cells reach $CD56^{dim}$'s capability to kill target cells (29). On the contrary, $CD56^{bright}$ NK cells display higher

capability to produce immuno-regulatory cytokines, in particular IFN-γ, compared to CD56^{dim} population (Figure 8) (19). Importantly, recent works highlight the fact that CD56^{dim} NK cells could produce similar amount of IFN-γ compared to CD56^{bright} population upon activating receptor triggering or short-term cytokine stimulation (30, 31). All together, these evidences suggest to revisit the idea that NK cell subsets are characterized by peculiar effector functions. Indeed, it seems that not different expression of CD56 but rather tissue-specific factors, immune cell-derived factors or other influences strictly linked to the microenvironment have the ability to modulate the activity of NK cell subsets (22).



Cooper M.A. et al., TRENDS in immunology 2001

Figure 8. Different features of NK cell subsets. CD56^{bright} and CD56^{dim} markers and functional features are depicted. Historically, CD56^{bright} population is characterized by high cytokine production, whereas CD56^{dim} population by high cytotoxic potential. However, these characteristics start to be revisited.

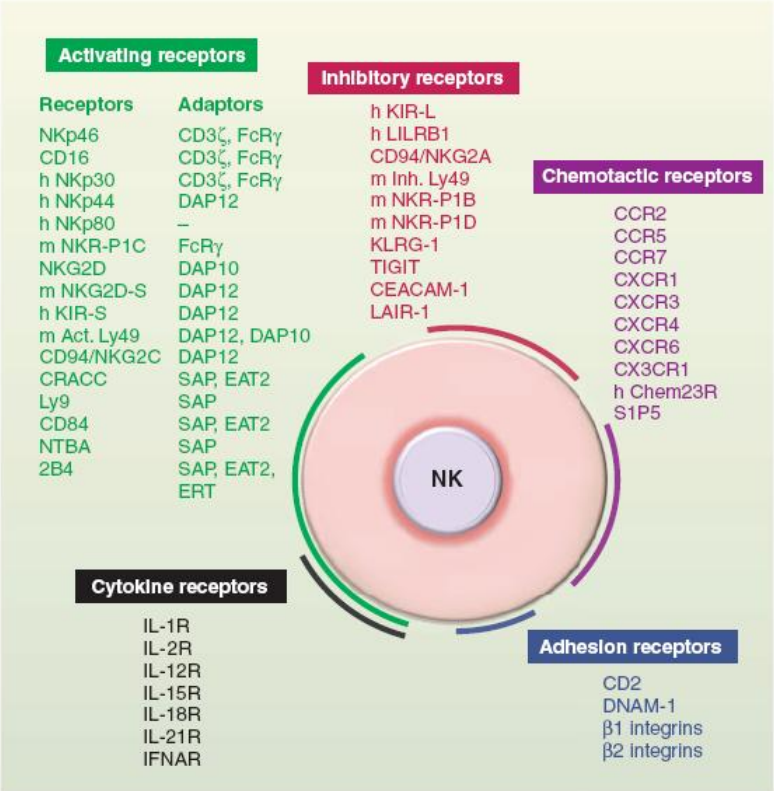
2.3 NK cell activation and effector functions

Upon activation, NK cells start to release Th1 typical cytokines, as Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α), Granulocyte-Macrophage Colony Stimulator Factor (GM-CSF) or Th2 typical cytokines, as Interleukin-10 (IL-10) and Interleukin-13 (IL-13). The production of Th1 or Th2 typical cytokines is determined by the maturation level of NK cells: more immature cells secrete type 2 cytokines, whereas more mature cells release type 1 cytokines. In addition, NK cells also produce many chemokines, including CCL-2, CCL-3, CCL-5, XCL-1 and CXCL-8. The fact that NK cells secrete inflammatory cytokines and chemokines confers to them not only the capability to recruit hematopoietic cells, as dendritic cells, but also the capability to regulate immune responses (32). Concerning NK cell cytotoxicity, it is mainly mediated by the release of cytoplasmic granules, also defined as "secretory lysosomes". They consist in complex organelles which are isolated from the cytosol by a bi-layer membrane. NK cell lytic granules contain enzymes typically active in the lysosomes as well as specialized molecules with degradative functions, selectively stored in cytotoxic granules (33). These specialized molecules are perforins and granzymes. Perforins are multi-domain proteins able to create pores on the plasma membrane of target cells, acting similarly to the C9 component of the complement system. They are constitutively expressed by NK cells. On the other hand, granzymes are serine proteases able to activate caspase-dependent and independent apoptotic pathways. Although 5 different isoforms (A, B, M, H, K) of granzymes codified from different gene cluster have been identified, granzyme A and granzyme B are the main studied. In accordance with the fact that perforins are strictly required for granzymes entry into the cytoplasm of target cells, they are essential for NK cell cytotoxicity. Indeed, it has been observed that even small inhibition of perforin expression leads to a marked decrease of NK cell killing capability (34-36). Concerning granzymes delivery to target cells, two different theories have been developed, which are still on debate. The so called "classical model" assumes that granzymes diffuse into the cytoplasm of target cells through perforin-made pores. On the contrary, a second theory supports the idea that perforins and granzymes are simply internalized through a mechanism of endocytosis: once delivered into the endosomes, perforins exert their action, creating pore in the endosome membrane and allowing the distribution of granzymes into the cytosol. Independently from the mechanism that regulates its delivery, granzymes are able to trigger either the caspase-dependent pathways, by direct cleavage of caspases, or the caspase-independent pathway, through the generation of reactive oxygen species, the induction of

mitochondrial outer membrane permeabilization and the initiation of DNA damage response (33). However, NK cell cytotoxicity is not totally restricted to granzymes and perforins action. Indeed, by the expression of TNF family ligand, NK cells can induce also TRAIL or FASL mediated apoptosis of target cells. Finally, NK cells are also able to generate ADCC via CD16 engagement (37).

In order to contain and regulate their inflammatory and cytotoxic potential, NK cells developed sophisticated mechanism to strictly control the initiation of their effector functions, thus avoiding tissue damage. As a consequence, in order to better understand NK cell biology and to manipulate their activities in pathological diseases, many studies were performed to dissect these mechanisms of NK cell effector function regulation. One of the main mechanism by which NK cell functions are controlled consists in receptor-ligand interactions: the engagement of NK cell receptors can stimulate or dampen NK cell activity. It is possible by the fact that NK cells express two main groups of receptors with opposite functional properties. Activating receptors trigger NK cell effector functions, whereas inhibitory receptors are responsible for the dampening of NK cell effector functions. As a consequence, NK cell activation consists in the result of a balance between activating versus inhibitory signals. When activating receptor triggering overcomes inhibitory receptor one, activation of NK cell functionality is predominant. Vice versa, when inhibitory receptors are more engaged and dominate activating signals, NK cell effector functions are silenced. Importantly, NK cell receptor repertoire is totally encoded by the genome and not generated by somatic recombination, as occurs for B and T cells (32).

NK cell activating receptors are generally defined as trans-membrane receptors able to recognize stress-ligand expressed by infected or altered cells, included tumor cells. However, some NK cell activating receptors bind also *self* antigens, and this is the reason why a panel of inhibitory receptors have been developed. It is possible to divide activating receptors in four classes: Natural Cytotoxicity Receptors (NKp30, NKp44 and NKp46), NKG2D, KIRs with activation activity, and co-stimulatory molecules (2B4, DNAM-1 and NKp80) (Figure 9). In regard to their signaling properties, only few activating receptors have been completely described.



Vivier E. et al., Science 2011

Figure 9. NK cell receptor repertoire. Five classes of NK cell receptors have been identified: activating receptors, cytokine receptors and adhesion receptors are involved in NK cell activation, whereas chemotactic receptors favor NK cell recruitment to inflamed tissues. Inhibitory receptors mediate NK cell dampening.

In general, activating receptors signal through transmembrane adaptor proteins, as DAP12, FcR γ and CD3 ζ, carrying Immunoreceptor Tyrosine-based Activating (ITAM) motifs. If DAP12 contains only one ITAM motif and works as an homodimer, FcR γ and CD3 ζ are able to form both homo and heterodimers. For example, NKp46 and NKp30 are linked to FcR γ and/or CD3 ζ, whereas NKp44 is associated to DAP12 (38). Src kinases are responsible for ITAM tyrosines phosphorylation, inducing the formation of ZAP70 and Syk kinases binding site. Once bound on ITAMs motif, ZAP70 and Syk kinases initiate the intracellular signaling cascade, which leads to the activation of NK cell effector functions (39). On the contrary, NKG2D utilizes a different adaptor protein, a short-transmembrane molecule called DAP10. DAP10 contains a tyrosine-based motif (YxxM) which is different from the canonical ITAM motif (40). Once phosphorylated, DAP10 can recruit either phosphoinositide 3-kinase (PI3K) or a complex composed by the small protein Grb2 and the guanine nucleotide exchange factor (GEF) Vav1 (41, 42). NKG2D triggering correlates with activation of NK effector functions. It is important to underline that, due to the interactions between many receptors and ligands, the identification of individual activating receptor

contribution to NK cell functionality is really difficult. In addition, activating receptors tend to co-operate, inducing synergistic activation signals. Up to date, the only receptors sufficient to trigger a functional response by NK cells are NKG2D and NCRs, which engagement gives rise to the "natural" degranulation of NK cells (Figure 10). In support to that, NK cell cytotoxicity towards tumor cell lines can be totally blocked by the presence of neutralizing antibodies against these receptors. On the contrary, others receptors, such as 2B4 and DNAM-1, have been considered co-stimulatory, as they are able to induce NK cell degranulation only in combination with others. Concerning the contribution of NK cell receptors to the induction of cytokine and chemokine production, the picture is more complex. It has been described that 2B4 engagement is sufficient to trigger IFN-γ secretion. Nevertheless, when it co-operates with other activating receptors the release of IFN-γ is more pronounced and the production of additional cytokines and chemokines is induced (39). Furthermore, several evidences suggested that NKG2D engagement could also correlate with IFN-γ production by NK cells (43, 44).

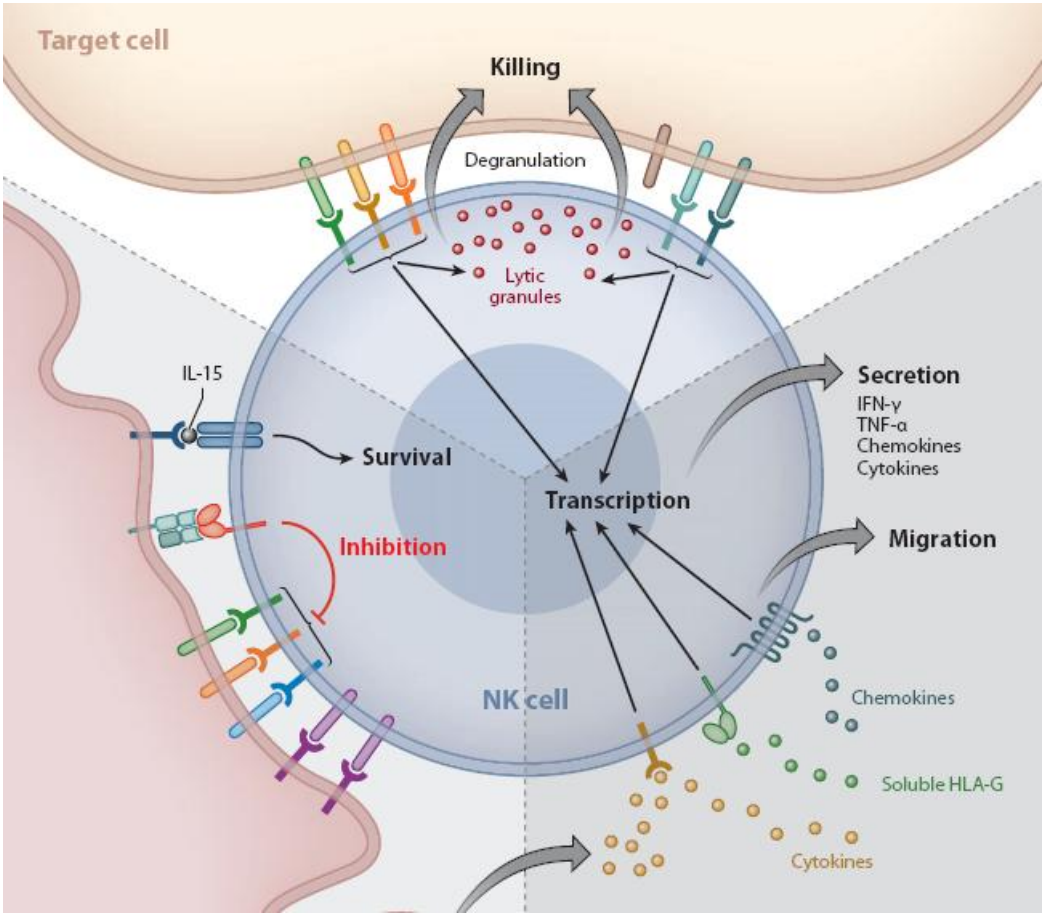
Cytokine receptors are able to fully activate NK cells, both in terms of cytolitic activity and cytokine production (Figure 10). NK cells express either cytokine receptors coupled with the common γ chain, such as IL-2R, IL-15R and IL-21R, or cytokine receptors coupled with MyD88 adaptor, as IL-1R and IL-18R (32). In addition, they express also IFNAR (Figure 9). Therefore, NK cells are responsive to a panel of inflammatory cytokines present in the tissue micro-environment as IL-2, IL-12, IL-15, IL-18, IL-21 and type I interferons (37). Each of the previously mentioned cytokines is sufficient to induce NK cell cytotoxicity or cytokine secretion or both, and, in particular, they determine the intensity and the quantity of these effector functions. Importantly, since MHC class I molecules bind their receptors in the context of an immunological synapses, NK cell activation by cytokines, which do not require direct interactions with target cells, is not subjected to the control of inhibitory receptors (Figure 10) (39). An exception is represented by IL-15. Indeed, thanks to a mechanism of trans-presentation, it activates NK cells through the formation of a immuno-synapse with IL-15Rα expressing cells (39). NK cell capability to respond and consequently produce pro-inflammatory cytokines assigns them an important role during innate and adaptive immune cell networking. Indeed, pro-inflammatory cytokines secreted by innate immune cells enhance NK cell production of IFN-γ, which, in turn, sustains a pro-inflammatory micro-environment, acting both on innate cells and adaptive cells. In addition, since cytotoxicity can be activated by cytokine receptors, in the presence of a pro-inflammatory micro-environment, NK cell can exert their killing activities. Interestingly, a considerable fraction of human circulating NK cells

can be defined resting, thus displays poor effector functions (45). As a consequence, it has been proposed that NK cells, like T cells, require a priming to be fully activated. Up to date, the capability to induce a NK cell priming has been associated to IL-15, through a mechanism of trans-presentation by dendritic cells and macrophages (46, 47), to IL-12 (48) and to IL-18 (49). However, processes of NK cell priming are not completely known. In any case, these evidences confer further importance to NK cell-innate immune cell cross-talk in the control of NK cell activities. Another interesting open question concerns NK cell capability to generate a sort of memory. It has been demonstrated that, upon *ex vivo* stimulation with cytokines, NK cell transferred into naïve mice could develop memory-like functions (50, 51). It regards only IFN-γ production, as cytotoxicity can not be re-triggered after a second stimulation. Furthermore it has been demonstrated only in mouse models. Again, whether it can occur also in human or the mechanisms which regulate this process have not yet been defined (39).

Finally, also adhesion molecules can be included in the list of activating receptors (Figure 9). Indeed, by interacting with adhesion molecules on target cells, they can initiate perforins and granzymes release (52). The best example of adhesion activating receptor is DNAM-1.

To conclude, a brief overview on inhibitory receptors. Inhibitory receptors have been classically defined as receptors able to recognize MHC class I molecules. They can be divided into two big families: inhibitory receptors that bind classical MHC class I molecules, as human KIRs (Killer Immunoglobulin-like Receptors), or inhibitory receptors that bind non-classical MHC class I molecules, as CD94/NKG2A (Figure 9). The non-classical MHC class I molecules, also called MHC class 1b glycoprotein, differ from classical MHC class I in terms of transcription patterns, protein expression and functionality. The KIR family count 16 types of transmembrane molecules in the Ig superfamily, which are encoded by a gene cluster, whereas CD94/NKG2 family is composed by C-type lectin receptors (52). Inhibitory receptors signal through Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs), localized in their cytoplasmic domain. Upon phosphorylation, ITIMs recruit tyrosine phosphates belonging to the Src family, as SHP1 or SHP2. The mechanism by which inhibitory signal interferes with the activating one is still unclear. However, it has been suggested that ITIM-mediated signaling correlates with both dephosphorylation or specific phosphorylation of molecules involved in cell signaling (53). Interestingly, some inhibitory receptors belonging to KIRs and CD94/NKG2 behave as activating receptors, since they are able to interact with DAP12

adaptors (39). NK cell inhibitory receptors are the major players of NK cell tolerance towards *self* cells and have a key role in NK cell education, which occurs during NK cell development.



Modified from Long E. O. et al., Ann. Rev. Immunol. 2013

Figure 10. NK cell receptors regulate NK cell functions. Each NK cell receptor triggering activates a precise intracellular pathway, aimed to modulate NK cell responses to micro-environmental stimuli.

2.4 NCR, NKG2D, 2B4 and DNAM-1 contribution on NK cell functions

NKp46, NKp30 and NKp44 compose the NCR family. NCRs are activating receptors belonging to the IgG superfamily that were discovered in the late 1990s. In humans, NKp46 and NKp30 are expressed by almost all NK cells, both activated or not, whereas NKp44 appears only on activated NK cells (53-56). In addition, if NKp46 and NKp30 share their signaling machinery, NKp44 utilizes different adaptor proteins (54). NCR engagement is considered as one of the major mechanism

involved in NK cell cytotoxicity, in particular towards tumor cells. This is supported by the fact that *in vivo* the deletion of a single NCR gene correlates with reduced NK cell tumor killing (39). For example, NKp46 not only recognizes viral hemagglutinins, playing important roles in viral infections, but also heparan sulfate proteoglycans (HSPG), which are widely distributed in mammalian tissue and are involved in processes related to malignancy insurgence, conferring to NK cells the capability to sense tumor antigens (53). Nevertheless, it is important to underline that when primary resting NK cells are stimulated with NKp46 cross-linking antibodies, they do not degranulate. It suggests that NKp46 *per se* is not sufficient to fully activate NK cell cytotoxicity, but requires "co-stimolatory" signals (45). Since NKp30 has several features in common with NKp46, it acquires more and more importance in NK cell anti-tumoral activities. Indeed, NKp30 not only recognizes HSPGs as NKp46, but it is also able to bind nuclear factor HLA-B associated transcript 3 (BAT-3) (57), which is released from tumor cells (53). Recently, it has been demonstrated that B7-H6 represents a NKp30 ligand (58). B7-H6 is a transmembrane protein expressed by several tumor cell lines and is considered a specific tumor antigen (58). The mechanism that regulates NKp30 triggering by B7-H6 is the topic of several ongoing studies. In addition, NKp30 plays a key role during human NK cell interactions with dendritic cells. Dendritic cells represent one of the most important cell type involved in the activation of NK cell anti-tumoral effector functions (54, 59). On the other hand, NKp44 represents the first NK cell activation marker, as it is selectively expressed by activated NK cells. Its expression by resting NK cells is triggered, *in vitro*, by IL-2 stimulation and correlates with increased capability to exert cytolitic activities towards sensible target cells. It suggests that, upon activation, NK cells amplify their receptor repertoire in order to increase their capability to recognize antigens on target cells (55). Apart of viral hemagglutinins, NKp44 ligands remain largely unknown. However, recently it has been published that an isoform of the Mixed-Lineage Leukemia 5 gene (MLL5), named NKp44L, could activate NK cell cytotoxicity via NKp44, suggesting that it represents a ligand for NKp44. Importantly, NKp44L is expressed by blood and tumor cells (60). An important aspect that has to be considered for the study of NCR activities is that, in general, the surface density of NCRs is variable from individual to individual. However it clearly correlates with NK cell capability to eliminate tumor cells: the more NCR are expressed the more NK cells recognize tumor antigens (61). It means that stimuli which are able to increase NCR levels on NK cells could directly trigger NK cell cytotoxicity. In addition, the activation mediated by NCR can be amplified by the fact that NCRs can work in synergy. Interestingly, it has been shown that NCR cross-linking by monoclonal antibodies induces tyrosine phosphorylation

not confined to a single signaling cascade, but that can involve signaling molecules associated to other NCRs. It suggests that NK cell cytotoxicity can be strongly triggered by the simultaneous engagement of different NCRs (56). Finally, since it has been shown that NKp46 and NKp44 cross-linking induce IFN-γ and TNF-α production, whereas interactions with autologous iDCs trigger different NK cell cytokine secretion via NKp30, NCR activities are not confined to the enhancement of NK cell cytotoxicity, but comprise also the induction of NK cell cytokine production.

NKG2D is a C-type lectin receptor associated with the adaptor protein DAP10. It is expressed by almost all NK cells, independently from their activation state. Nevertheless its expression can be further induced upon cytokine stimulation, in particular upon IL-15 treatments (53, 62). NKG2D engagement correlates with both NK cell cytotoxicity and cytokine secretion (40, 62-65). NKG2D ligands are well known and largely described. They comprise MHC class I related proteins as stress-inducible MHC class I chain-related gene A (MIC-A) and B (MIC-B), in addition to UL-16 binding protein 1 (ULBP-1), 2 (ULBP-2), 3 (ULBP-3) and 4 (ULBP-4). The expression of these molecules is associated to DNA damage response or heat-shock response pathway, which are generally induced in tumor cells (66, 67). The fact that NKG2D specifically recognizes molecules upregulated by altered and stressed cells confers to NKG2D a predominant role in NK cell mediated-monitoring of tumor cells. Tumor cells have developed strategies to evade NKG2D recognition. For example, it has been observed that tumor cells release soluble MIC-A and MIC-B, which work as decoy molecules, interfering with NKG2D binding to membrane associated ligands. In addition, since the level of NKG2D expression correlates with the intensity of NK cell cytotoxicity, the modulation of NKG2D levels on NK cells represents a good strategy to avoid their capability to recognize tumor antigens. In accordance with that, tumor cells are able to induce NKG2D downregulation on NK cells through the release of Tumor Growth Factor β (TGF-β) (68).

2B4 is a member of the CD2 immunoglobulin family. It is expressed constitutively by all NK cells and preferentially binds CD48, which belongs to the same family of receptors. CD48 has been first identified on B cell lymphoblast but it is expressed also by other immune cells, as dendritic cells and macrophages. The contribution of 2B4 to NK cell activation is still not clear. Indeed, 2B4 was first defined as an activating receptor able to trigger NK cell cytotoxicity, as its cross-linking correlates with enhancement of NK cell degranulation (55). However, it has been observed that 2B4 can act also as inhibitory receptor, participating to the dampening of NK cell activities. These opposing results can be partially explained by the fact that 2B4 is characterized by a functional

heterogeneity: on the basis of the adaptor proteins recruited, 2B4 can initiate activating signals as well as inhibitory ones (69-71). To further confirm that, it has been demonstrated that 2B4 counts different isoforms, which could recruit different adaptor protein and consequently initiate different signaling pathways. For example, in humans, 2B4 can acquire two isoforms, but only one of them has been associated to activation signals (72). Finally, another explanation for these conflicting functions could be that 2B4 is expressed by immature NK cells. Therefore, the engagement of 2B4 during different stages of NK cell maturation could explain different functional outcomes (73). Focusing on 2B4 as activating receptor, it has been demonstrated that 2B4 engagement by CD48 is sufficient to trigger IFN-γ secretion by NK cells (74). Furthermore, it has been described that 2B4, by recognition of CD48 expressed by melanoma cells, plays an important role in tumor rejection (75).

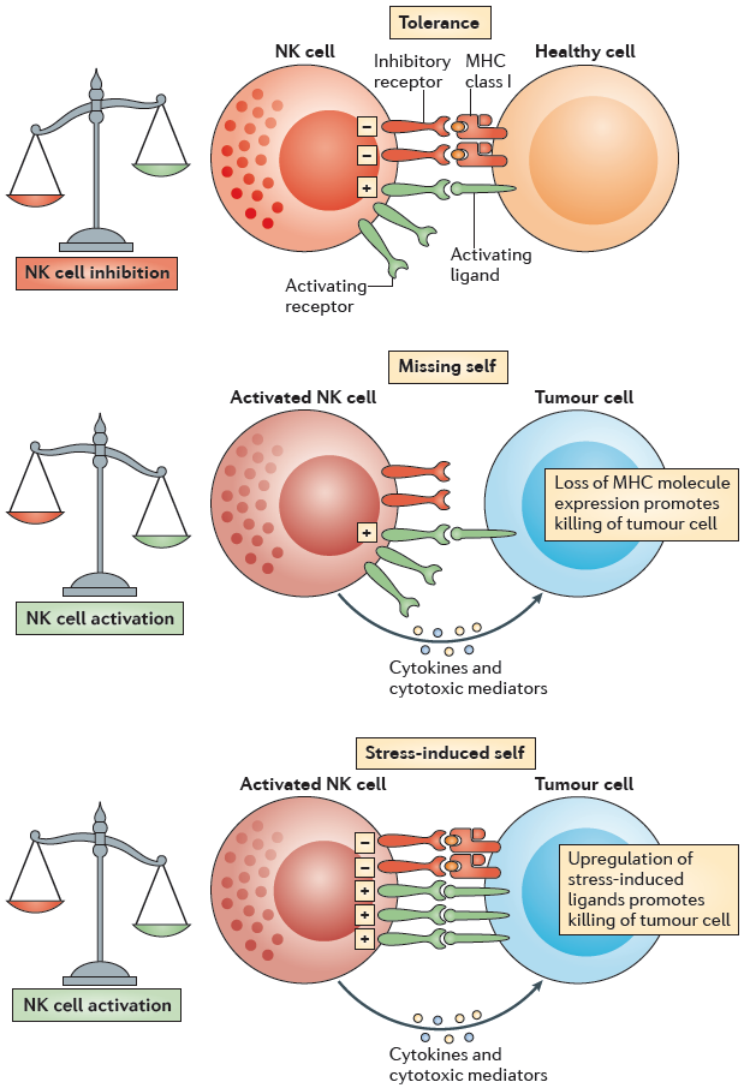
DNAM-1 belongs to the IgG superfamily and represents an adhesion receptor. It is present constitutively on the 50% of NK cells and it has been defined as a co-stimulatory receptor (76). DNAM-1 binds preferentially CD155 (polio-virus receptor, PVR) and CD112 (Nectin-1). CD155 has a role in the establishment of adherens junctions between epithelial cells, whereas CD112 is a component of the adherens junctions. Both of them can be strongly upregulated in tumor cells, conferring to DNAM-1 a role in tumor recognition (53, 77). In addition, DNAM-1 activation has been associated to the induction of Lymphocyte Function-associated Antigen 1 (LFA-1), an adhesion molecule characterized by the ability to bind intracellular adhesion molecule 1 (ICAM-1), participating to actin polymerization and activation (53). As a consequence, DNAM-1 can strongly sustain the formation of stable interactions between NK cells and target cells, promoting NK cell recognition of tumor antigens (78). Recently, it has been observed that DNAM-1 could trigger by its own NK cell cytotoxicity towards target cells which express DNAM-1 ligand but no other activating receptor ligands (79). It suggests that DNAM-1 can be considered as an activating receptor and not only as a co-stimulatory molecule.

2.5 NK cells and cancer

Several *in vitro* and *in vivo* studies demonstrated that NK cells play a role in tumor control. In general, it has been observed that the impairment of NK cell activities is correlated with an increased risk to develop cancer. Indeed, patients affected by lung, gastric or colorectal cancer which display high number of tumor infiltrating NK cell generally have a better prognosis (80). In particular, focusing on gastric carcinoma, abundant NK cell infiltration is associated to a reduction of tumor invasion and lymph node metastasis (81). In addition, it is now evident that NK cells are particularly involved in tumor metastasis control. Indeed, NK cell anti-tumoral activities impact on pulmonary metastasis, melanoma metastasis and colorectal adenocarcinoma and peritoneal dissemination (82).

Since NK cells actively participate to tumor elimination, several studies were performed to widely elucidate mechanism underlying NK cell recognition of tumor cells. As described before, NK cells express several inhibitory receptors that bind to MHC class I molecules. Therefore NK cells can discriminate *self* and *non-self* cells. During maturation phases, NK cell inhibitory receptor repertoire is adapted to the MHC class I molecules bearded by the host, guaranteeing further NK cell tolerance towards *self* cells. This process was defined as NK cell education. However, up to date, the driving mechanism of NK cell education is still poorly understood. On the other hand, NK cell can be activated by the presence of an activating receptor repertoire. In the presence of healthy cells, activation signals mediated by activating receptors are minimal, thus the binding of inhibitory receptors to MHC class I molecules is sufficient to induce NK cell tolerance (Figure 11). On the contrary, altered cells, including tumor cells, can activate NK cells by two different mechanism. The first consists in the "missing *self*" process: tumor cells which lose MHC class I molecules fail to trigger inhibitory signals, thus favoring the predominance of activation signals mediated by activating receptors. On the other hand, tumor cells can upmodulate stress-induced ligands, generally absent on healthy cells. These ligands are efficiently recognized by activating NK cell receptors. Therefore, even if tumor cells bear MHC class I, activating signals overcome the inhibitory ones, resulting in NK cell activation (Figure 11) (83). It is important to underline that NK cell anti-tumoral activities are not restricted to NK cell cytolitic granules release, but include also cytokine production, and in particular IFN-γ secretion. Indeed, it has been demonstrated that IFN-γ plays a role in the dampening of angiogenesis and tumor vascularization and in favoring antigen

presentation (84). Furthermore, IFN-γ can control cell proliferation and sensibility to apoptosis (37). Finally IFN-γ participates to the tumor micro-environment modulation.



Vivier E. et al., Nature Review 2012

Figure 11. Tumor cell-mediated activation of NK cells. Three are the main mechanisms by which NK cells recognize healthy or transformed cells. Healthy cells are protected by the presence of MHC class I molecules, inducing NK cell tolerance. On the contrary, the expression of stress-induced ligands together with the lacking of MHC class I molecules by tumor cells favor NK cell activation.

Unfortunately, it has been observed that the frequency of NK cell which infiltrate the tumor is low. In addition, studies on colon and lung cancers showed that NK cells are preferentially found around blood vessels and do not co-localize with tumor cells (85, 86). However, the treatment with therapeutic monoclonal antibodies or pro-inflammatory cytokines, as IL-2, IL-12 and IL-21, increase both numbers and functions of peripheral blood NK cells recruited to the tumor site (87).

Therefore, inducing NK cell recruitment and activation in tumor tissues could really improve NK cell anti-tumoral effects (81). Of note, NK cells that reach the tumor are preferentially $CD56^{bright}$. It can be due to the fact that the bright population express higher levels of CXCR3 than the dim counterpart, which together with CX3CR1, is the main chemokine receptor involved in NK cell migration to the tumor site (81). Up to date, it has been proposed that tumor cells could actively participate to the inhibition of NK cell recruitment and it may represents one of the escape strategies aimed to impair NK cell anti-tumoral activities. By preventing NK cell accumulation, tumor cells result protected from NK cell effector functions. However, it represents only one mechanism by which tumor cells could escape from NK cell-mediated elimination. Indeed, tumor cells could also modulate the expression of NK cell activating receptors, inducing their downregulation, altering their functions or promoting their desensitization. This process can be valid also for NK cell adhesion molecules. Finally, tumor cells could release immuno-suppressive cytokines, as TGF-β and IL-10, or apoptotic mediators, in order to dampen NK cell activation (37). In support to these hypothesis, many studies demonstrated that the activity of NK cells isolated from tumor tissue resulted impaired (88, 89). Nevertheless, NK cells still represent a valuable target for several immuno-therapies. For examples, strategies for the enhancement of endogenous NK cell activation or of NK cell-mediated ADCC have been proposed. In addition, protocols for NK cell-based donor lymphocyte infusions have been develop, as *ex vivo* NK cell expansion followed by adoptive transfers (90, 91).

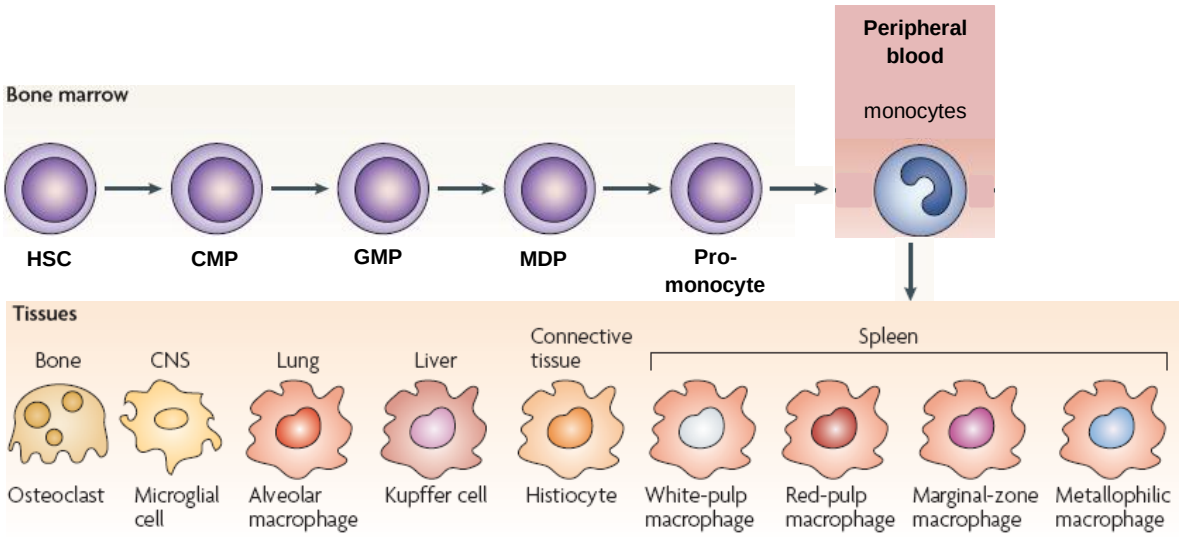
3. Macrophages

Macrophages are important effector of the innate immunity. They represent resident phagocytic cells located in lymphoid and non-lymphoid tissue where they play an important role under homeostatic and inflammatory conditions. They are implicated in the removal of cellular debris and apoptotic cells. At the same time, they are antigen presenting cells and are important producers of pro-inflammatory cytokines and chemokines. All these functions are mediated in part by the expression of a broad-range of pathogen recognition receptors (PRRs), which allow macrophages to sense a very wide range of danger signals (92, 93). One of main the feature of macrophages consists in plasticity. Macrophages undergo several types of activation depending on the nature of the stimuli they encounter.

3.1 Macrophage origin

Macrophages differentiate from circulating monocytes that are recruited to the tissue. Monocyte migration to tissues is mediated by the expression of a panel of chemokine receptors and adhesion molecules. Since they are able to produce inflammatory cytokines and to internalize toxic molecules, monocytes can be considered as immune effector cells and not only precursors of tissue macrophages (92). Monocytes originate from hematopoietic stem cells (HSCs) through several commitment steps. Each of these step is strictly regulated by a defined combination of transcription factors, which synergize or compete determining the fate decisions (94). In the bone marrow, HSCs generate a common myeloid progenitor (CMPs), which gives rise to a granulocyte/macrophage progenitor (GMP). Different myeloid-linages originate from GMP, thus it generates granulocytes, mast cells and monocytes. However, in the presence of macrophage colony stimulating factors (M-CSF), GMP is driven specifically into a MDP, that represents the macrophage/dendritic cell precursor. MDP gives rise to pro-monocytes and finally to monocytes. MDP can further develop in dendritic cell progenitor, which lose monocyte/macrophage potential. It is important to underline that each commitment step correlates with restriction of developmental potential of the respective progenitor cell and it is controlled by several lineage specific transcription factors. For example, PU.1 represents the master transcription factor that regulate macrophage and dendritic cell differentiation. Its expression is increased during the

differentiation phases and regulated by other transcription factors, as C/EBP-α and Runx1 (94). Once released in the blood stream, monocytes are recruited to tissues, where they differentiate into a wide spectrum of tissue-associated macrophages (Figure 12) (93).



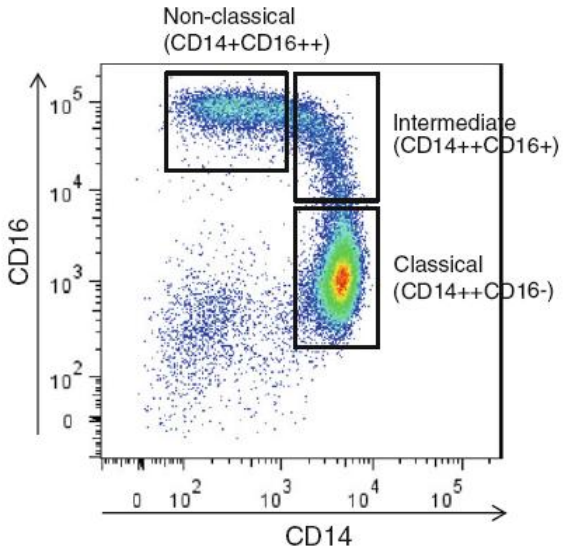
Modified from Mosser D.M. et al., Nature Review 2008

Figure 12. Macrophage generation: from bone marrow to tissues. Steps of monocytes differentiation from HSC in the bone marrow are depicted. Mature monocytes reach the circulation and are recruited to tissues, where give rise to a wide spectrum of tissue-specific macrophages.

3.2 Human monocyte subsets

Monocytes in the blood stream are heterogeneous. They are composed by different subsets and several evidences suggest that these subsets exert different functions and generate different phenotypes of tissue-macrophages. Human peripheral blood monocytes represent the 5-10% of all circulating leukocytes. They were historically identified by CD14 expression. CD14 is the co-receptor of TLR4 involved in LPS signaling pathway. However, on the basis of their expression of CD16, also known as the FcγRIII, it is possible to distinguish three different monocyte populations: the "classical subset", characterized by high levels of CD14 and negative for CD16 ($CD14^{++}CD16^{-}$), the "intermediate subset", determined by high levels of CD14 and intermediate levels of CD16 ($CD14^{++}CD16^{+}$) and the "non-classical subset", which express high levels of CD16 but low levels of CD14 ($CD14^{+}CD16^{++}$) (Figure 13). $CD14^{++}CD16^{-}$ are the more abundant population, which cover 80-

90% of all circulating monocytes, whereas $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ represent the remaining 10%. Monocyte subsets can be further discriminated through their different expression of chemokine, immunoglobulin, adhesion and scavenger receptors. Indeed, $CD14^{++}CD16^{-}$ present high levels of CCR1, CCR2 and CXCR2, but low levels of CX3CR1. On the contrary, $CD14^{+}CD16^{++}$ express high levels of CX3CR1 and low levels of CCR2 (95). In accordance, through transendothelial migration assays, it has been observed that $CD14^{++}CD16^{-}$ sense CCL2, whereas $CD14^{+}CD16^{++}$ respond to CX3CL1 (96). In regard to their adhesion molecules, $CD14^{++}CD16^{-}$ are characterized by higher levels of L-selectin (CD62L) and CD11b and lower levels of MHC class II compared to $CD14^{+}CD16^{++}$ (97). Recently, it has been identified a third monocyte population, defined as "intermediate subset", which is characterized by high expression of CD14 and intermediate expression of CD16 ($CD14^{++}CD16^{+}$).

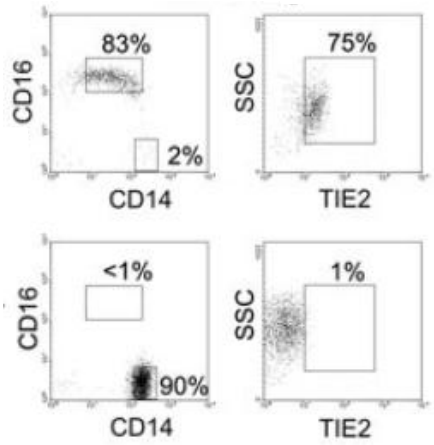


Wong K. L. et al, Immunol. Res. 2012

Figure 13. CD14 and CD16 expression by human monocyte subsets. Three human monocyte subsets are characterized by different levels of expression of CD14 and CD16, as represented by this FACS plot.

The relationship between monocyte subsets is far to be completely depicted in human, as it is still unknown whether they represent three different states of differentiation or three different phenotypes. By gene expression profiles, it has been found that genes associated to non-classical monocytes cluster separately from gene associated to classical or intermediate monocytes (98). In opposite, other reports assign to the intermediate subset closer relationship with the non-classical one (99, 100). In line with these discordances, some studies suggested that isolated non-classical

monocytes do not produce cytokines (as TNF-α, IL-1β, IL-10 and IL-6) and chemokines (as CCL-2, CCL3 and CXCL-8) in response to LPS stimulation, whereas they are able to strongly secrete them when they are activated by TRL7/8 ligands (98, 101). On the contrary, other studies indicated that isolated non-classical monocytes upon LPS stimulation are able to secrete higher amount of TNF-α and IL-1β than other monocyte populations (99). These opposite results give an idea of how difficult it is to assign specific functions of heterogeneous human monocyte subsets in experimental settings. To further complicate the picture, recently, two other "new" subsets of human monocytes have been identified: one population characterized by the expression of the angiopoietin receptor 2 (Tie-2) and another population characterized by the expression of M-DC8. Importantly, the expression of these markers does not correlate with different levels of CD14 and CD16 expression, meaning that they can not be included in the previous classification (102). Tie-2 expressing monocytes represent the 20% of all monocytes (103). They are located preferentially in the intermediate population, even if 2-7% of Tie-2 positive cells are placed in the non-classical one (Figure 14) (103, 104). Tie-2 monocytes are further characterized by the expression of CD115, CD11b, CD33 and CCR5. They are considered as a distinct functional subset, as they show peculiar pro-angiogenic activities, in particular in the context of cancer (102).

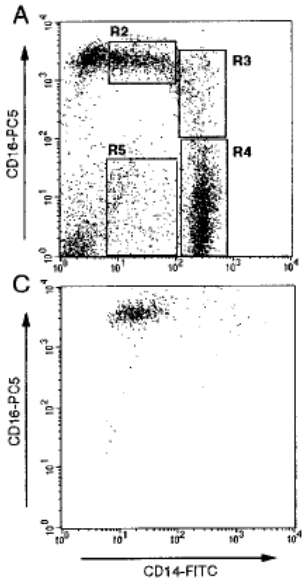


Modified from Venneri M.A. et al., Blood 2007

Figure 14. Tie-2 expression by classical and non-classical monocytes. TIE-2 expression by human monocytes sorted for their expression of CD16 (upper panels) or CD14 (lower panel) was assessed by FACS.

On the other hand, the M-DC8 or slan positive population represents a rare population, covering only the 0,5-1% of all circulating leukocytes (105). Slan monocytes constitute the 30-50% of the non-classical monocytes (Figure 15) (102). From a functional point of view, slan monocytes are phagocytic cells able to stimulate CD8 T cells and NK cells (102, 106). Since they are able to present

antigens to T cells, it has been proposed that M-DC8 monocytes represent circulating dendritic cells (slan DCs).



Modified from de Baey etal., Eur. J. Immunol 2001

Figure 15. M-DC8 positive monocyte localization between human monocyte subsets. CD14 and CD16 expression by human monocytes is plotted in the upper panel. Below, it is represented CD14 and CD16 expression by M-DC8 positive monocytes (gated on M-DC8$^+$ cells).

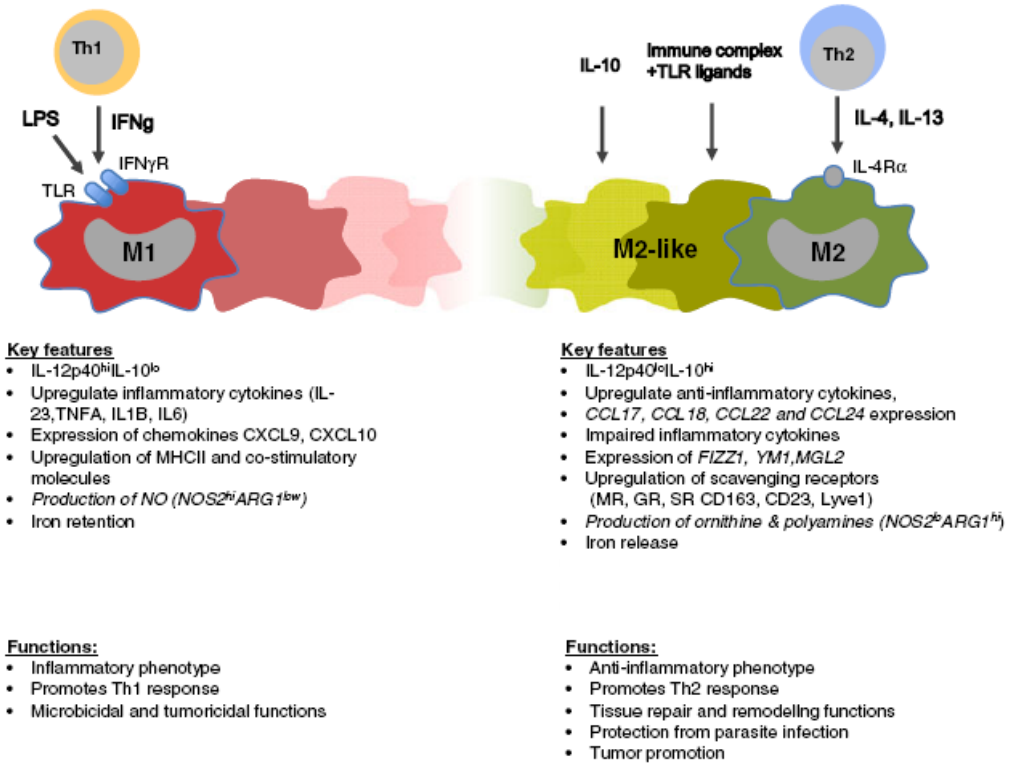
In conclusion, the classification of human monocyte subsets is difficult. Indeed, discordant results can occur when different protocol of isolation are used. In addition, after the isolation procedure, the lack of a physiological micro-environment can alter monocyte behavior. Therefore, *in vivo* studies result more informative and consequentially are preferentially performed in murine models. However, it is still on debate whether murine populations can totally recapitulate the human ones, and *vice versa*. At the moment, although they express very different markers, functions displayed by different murine monocyte subsets seem to mimic the ones observed in humans: the so called murine pro-inflammatory subset can be associated to the human classical one, whereas the murine patrolling subset can be associated to the human non-classical one.

3.3 Macrophage activation

On the basis of their effector functions, macrophages can be generally divided into two big groups: classically-activated macrophages and alternatively-activated macrophages. Classically-activated macrophages consist in pro-inflammatory macrophages, thus associated with inflammatory responses. They are activated by the presence of IFN-γ or TNF-α, and/or bacterial moieties, as LPS, which enhance their anti-bacterial and anti-tumoral capacities. Since IFN-γ released by NK cells is sufficient to trigger classical activation but it is generally transient, Th1 T cells are responsible for the maintenance of the classical phenotype in inflamed tissues. In addition, TNF-α and IFN-β, produced by TLR stimulated antigen presenting cells, including macrophages, sustain classical activation and can even replace NK cell and T cell derived IFN-γ. Classically-activated macrophages are characterized by the production of pro-inflammatory cytokines, superoxide anions and toxic nitrogen and oxygen radicals, leading to their killing abilities (107). Since classically-activated macrophages are strong pro-inflammatory cytokine producers, they play an important role not only in the generation of inflammation but also in immune response modulation. Indeed, they are able to secrete IL-1β and IL-18, IL-6, IL-12 and IL-23, TNF-α, and also type I interferons, triggering NK and T cell responses. In addition, classically-activated macrophages secrete high amounts of inflammatory chemokines, as CXCL-9 and CXCL-10, displaying the ability to recruit inflammatory immune cells, as NK cells and Th1 T cells (108, 109). Since they express high levels of MHC class II and co-stimulatory molecules, they are efficient antigen presenting cells, maintaining Th1 T cell activation. The production of toxic nitrogen and oxygen radicals, together with their capability to retain iron, confers to classically-activated macrophages an important role in pathogens elimination. As a consequence, classical macrophage activation has to be strictly regulated, to avoid host-tissue damage (93, 109). According to all these functional properties, classically-activated macrophages, thanks to their inflammatory phenotype, promote Th1 responses and exert microbicidal and tumoricidal functions (Figure 16).

Alternatively-activated macrophages are generated upon IL-4 stimulation (110). They have been defined by Mosser and Edward as wound-healing macrophages. Indeed, wound-healing represents one of their main features. Interestingly, they play also a key role in helminthes infection eradication and in immune cell inhibition (111). Both innate immune cells (i.e. basophils and mast cells) and adaptive immune cells (Th2 T cells) can release IL-4 required to activate wound-healing macrophages. However, IL-4 is not the only stimulus involved in their generation, as IL-13, mainly

produced by Th2 cells, is sufficient to induce their phenotype (93, 108). Recently, it has been also described that helminthes infections could trigger alternative activation of macrophages *in vivo* (112). In turn, alternatively-activated macrophages secrete extracellular matrix components and polyamines, which dampen inflammatory immune responses, and highly express IL-1 decoy receptor and IL-1RA, which prevents IL-1β signaling pathway (108). In addition, alternatively-activated macrophages are characterized by anti-inflammatory cytokine expression (i.e. IL-10), participating to the formation of immuno-suppressive micro-environment. In parallel, they release also anti-inflammatory chemokines, as CCL-17, CCL-18, CCL-22 and CCL-24, which sustain the recruitment of immune cells involved in the resolution of inflammation (113). Alternatively-activated macrophages express a wide panel of scavenging receptors (i.e. mannose receptor and CD163) which are involved in tissue repair and remodeling. Finally, they allow iron release, facilitating pathogens survival. All these features confer to alternatively-activated macrophages a predominant role in Th2 response promotion, protection from parasite infections and tissue repair. Since Tumor Associate Macrophages (TAMs) resemble alternative phenotypes, alternatively-activated macrophages are involved in tumor promotion (Figure 16) (93, 109). Mirroring the Th1/Th2 balance, Mantovani A. et al. suggested another nomenclature to identify macrophage functional phenotypes: classically-activated macrophages have been defined as M1, whereas alternatively-activated macrophages have been defined as M2 (114). It is important to underline that M1 and M2 macrophages represent only two extremes of a broad range of macrophage activation states. Indeed, in the presence of IL-10, glucocorticoids or LPS in combination with immune complexes, macrophages can acquire M2-like phenotypes. This generates a third group of macrophage population in terms of functionality (115). Mosser and Edwards classified these macrophages as “regulatory macrophages”. In the presence of glucocorticoids, mainly released by adrenal cells in response to stress, macrophage inflammatory functions are inhibited together with their contribution to host defense, allowing the generation of a regulatory phenotype more similar to M2. The same situation can occur when, upon apoptotic cell phagocytosis, macrophages release TGF-β, or, in the presence of immune complexes together with LPS, they secrete IL-10, promoting anti-inflammatory responses (93). In addition, although regulatory macrophages are more similar to M2 in terms of functions, they are unable to produce extracellular matrix components and they express co-stimulatory molecules as CD80 and CD86, typically present in M1 (116).



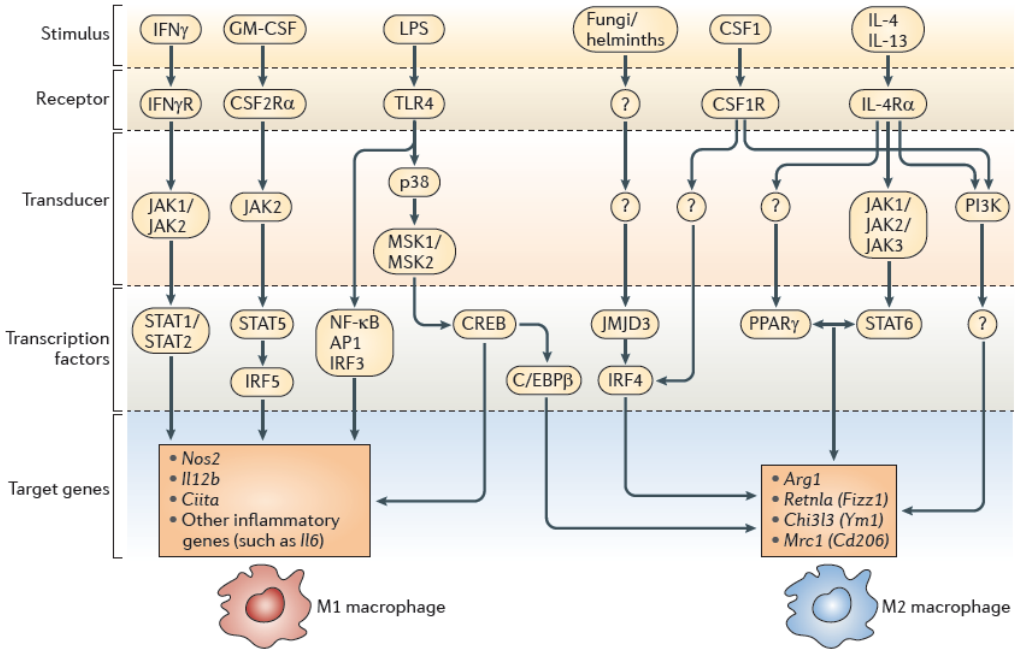
Modified from Biswas S. et al., Semin. Immunopathol. 2013

Figure 16. Macrophage spectrum of activation states. Macrophages are characterized by a wide spectrum of activation states, which extremes are represented by M1 and M2 macrophages. On the basis of their activation, macrophages display very different effector functions.

3.4 Molecular mechanisms of macrophage activation

Macrophages can be defined on the basis of phenotypic markers and their effector functions. Several studies have shown that specific molecular mechanisms lead to specific macrophage phenotypes. M1 polarization is driven by IFN-γ, which is associated to the transcription factor STAT1 signaling pathways. STAT1 activity correlates with the upregulation of transcription factors belonging to the IRF family (more specifically IRF1, IRF2, IRF3 and IRF8), leading to cytokine production. In parallel, TLR4 engagement by LPS induces NF-kB pathway, sustaining inflammatory cytokine transcription (Figure 17). On the other hand, M2 polarization, induced by IL-4 and IL-13, is characterized by STAT6 activation. STAT6 is a transcription factor able to trigger the expression of PPARγ, another transcription factor that works in synergy with STAT6 in inducing the expression of M2-associated genes. KLF4, the Krüppel-like factor 4, not only co-operates with STAT6 in the enhancement of M2 genes but inhibits also the expression of M1 genes (Figure 17) (117). Recently, it has been published that alternative activation of macrophages requires the

transcription factor c-MYC (118). Importantly, transcription factors induce specific and/or overlapping gene expression programs that can discriminate the phenotypes and have a functional impact because it is the "genetic" output of a cell in response to specific challenge. In addition, macrophage polarization is regulated also by epigenetic factors. For example, JMJD3 is an histone demethylase which decreases H3K27 methylation on gene promoters. Its activity has been associated to M2 polarization, since it activates the expression of IRF4, thus sustaining M2 gene expression. In addition, it has been reported that JMJD3 directly reduces the transcription of M1 associated genes (Figure 17) (108, 117, 119). However, JMJD3 expression has been correlated to the presence of pro-inflammatory stimuli, even if its contribution to M1 phenotype is restricted to fine-tuning the expression of few genes in response to LPS (120). It is important to underline that epigenetic regulation of macrophage polarization is continuously revisited, since the understanding of the molecular machinery underlying is still in evolution. However, it is a matter of fact that epigenetic factors play a pivotal role in macrophage polarization.

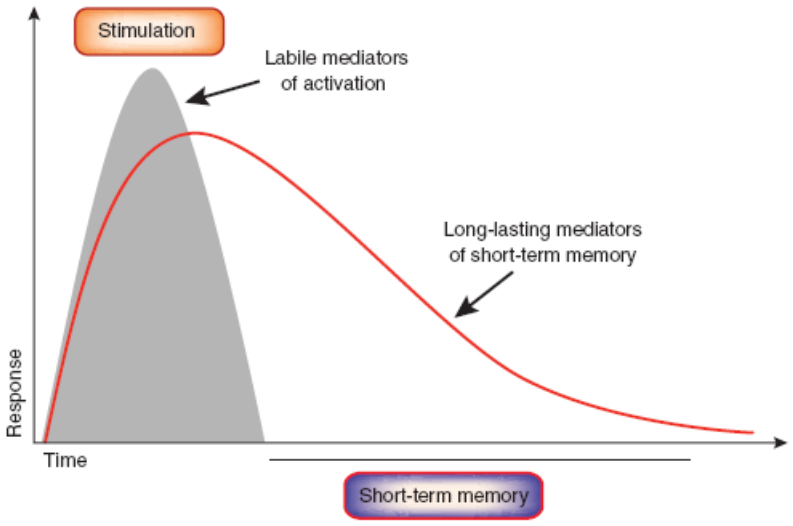


Lawrence T. and Natoli G., Nature Review Immunology 2010

Figure 17. Molecular regulation of macrophage polarization. Molecular regulation of macrophage polarization is composed by different steps, which involve different categories of molecular factors.

3.5 Macrophage plasticity

Macrophages are characterized by a repertoire of invariant receptors which allow them to rapidly sense a wide range of micro-environmental stimuli, leading to an appropriate immune response. One of the main macrophage features is plasticity (108, 121). Upon different stimulations, macrophages can acquire different phenotypes which are correlated with different effector functions. Interestingly, macrophages seem to retain their plasticity, since they can still respond to environmental signals upon activation, switching from one to another phenotype. For this reason, over all *in vivo*, it is quite difficult to distinguish and to identify markers associated to an individual macrophage population (93). The mechanisms that regulate macrophage plasticity remain largely unknown. However, several studies are emerging on macrophage plasticity and on their capability to generate a sort of "memory". For example, Natoli G. et al. proposed that the generation of a short-term memory leads to increased capability to respond better and stronger to new stimuli on the basis of previous experiences. This "short-term" memory is mediated by long-lasting mediators, which are usually epigenetic factors, as microRNA and chromatin modifications. These modifications remain active for a short period of time after the first stimulation, favoring faster and more intense responses to further stimuli (Figure 18). Importantly, in order to avoid possible dangerous consequences, the short-memory process is controlled by the time, as it is restricted to a short window (122).



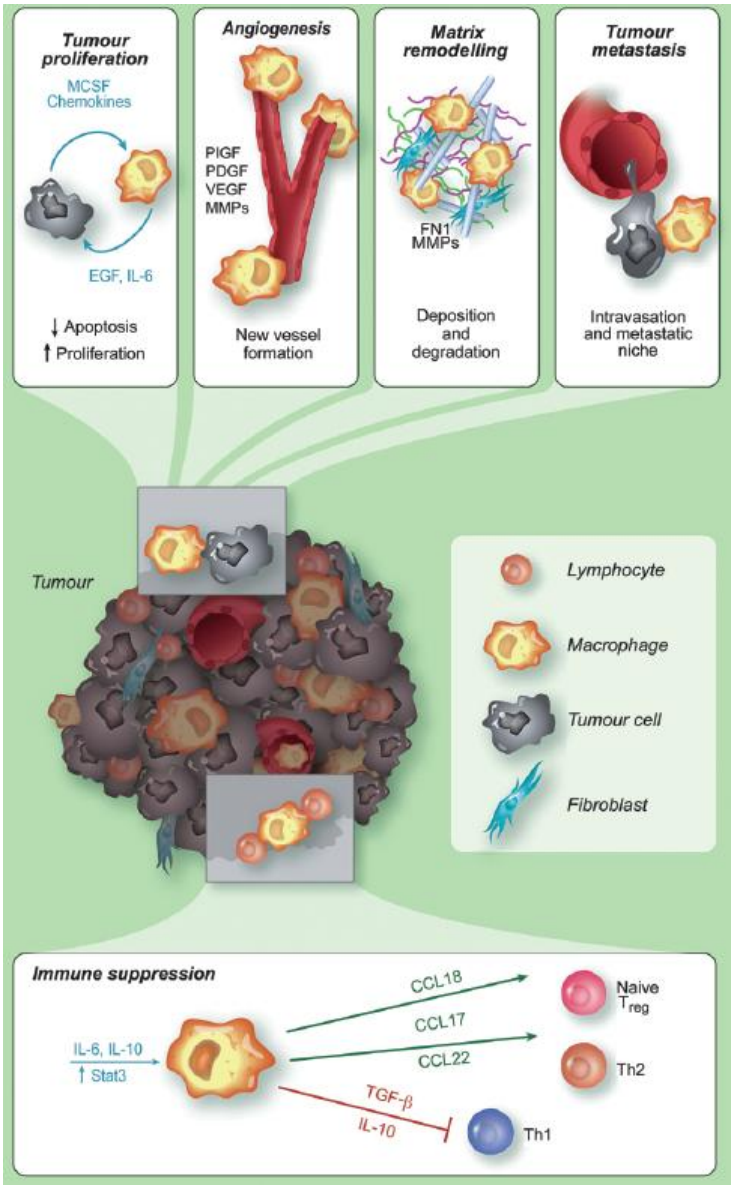
Monticelli S. and Natoli G., Review Nature Immunology 2013

Figure 18. Short-memory is mediated by long-lasting mediators. Through the expression of long-lasting mediators it is possible to generate a short-term memory, responsible for faster and stronger cell responses upon a second stimulation.

3.6 Macrophages and Cancer

In line with the relationship between cancer and inflammation, monocytes and macrophages are recruited to tumor tissues and represent a huge percentage of total immune cells accumulated within the tumor. As other innate immune cells, macrophages infiltrate cancer lesions from the very beginning, even before the recruitment of lymphocytes. Macrophages that participate to the cancer elimination phase can be defined as M1 macrophages, as they display anti-tumoral and tissue destruction features. M1 contribute to sustain an inflammatory micro-environment, by the release of pro-inflammatory cytokines and chemokines which recruit innate and adaptive cytotoxic cells, as NK cells and $CD8^+$ T cells. In addition, they behave as antigen presenting cells, stimulating T cell activation. However, macrophages isolated from metastatic tumors, both in human and mice, generally display an M2-like phenotype. It means that they have low tumoricidal potential but high capability to stimulate extracellular matrix remodeling and angiogenesis. In addition, they dampen adaptive immune responses and generate an anti-inflammatory micro-environment, recruiting Th2 T cells. These features confer to M2-like cells, also defined as Tumor Associated Macrophages (TAMs), a role in the promotion of tumor growth and progression (108, 117, 123). Their development is correlated with the absence of M1 polarization signals, such as IFN-γ, and with the presence of Th2 lymphocytes, which are the main source of IL-4 and IL-13 (124). Cancer cells also participate to the generation of TAMs. Indeed, together with stromal cells, they are able to recruit monocytes from the blood vessels through the release of chemokines, such as CCL-2 (125, 126). Once recruited, monocytes sense the presence of M-CSF secreted by cancer cells and thus differentiate into macrophages. Due to the presence of a tumoral micro-environment, macrophages are polarized towards TAMs (123). TAMs participate to the instauration of chronic inflammation processes, favoring cancer-related inflammation, and thus promoting tumor cell progression. TAMs express active forms of the nuclear factor NF-kB (117, 123, 124). Indeed, TAMs preferentially accumulate into hypoxic region of tumor masses. Under hypoxic conditions, the transcription factor HIF-1α is induced, leading to the initiation of a transcriptional program that sustain NF-kB expression (127, 128). It indicates that TAMs can continuously produce cytokines. In particular, low but persistence doses of TNF correlate with tumor growth, angiogenesis and activation of matrix degrading enzymes (129). In parallel, IL-6 production by TAMs enhances cell growth and inhibits apoptosis (130-132). In addition, TAMs represent a source of CCL-2, that, as previously mentioned, is involved in monocytes recruitment

to the tumor, and contributes to positive angiogenesis regulation (133-135). In parallel, by the production of IL-10 and TGF-β, TAMs exert immuno-suppression, further sustaining tumor progression (123). In regard to their chemokine pattern, if macrophages in the first stages of cancer development secrete inflammatory chemokine as CXCL-9 and CXC-10 recruiting cytotoxic lymphocytes, TAMs release anti-inflammatory chemokines, that exert immuno-suppression. Indeed, TAMs are able to secrete CCL-17 and CCL-22, which are involved in Th2 T cells and Treg cells recruitment (115, 135), and CCL-18, which allows T naïve cell migration into the tumor (Figure 19) (136, 137). Once infiltrated the tumor mass, T naïve cells become anergic cells, due to the presence immature DCs and TAMs, which sustain anti-inflammatory micro-environment (138, 139). Treg cells recruited to the tumor are sources of IL-10, they thus actively contribute to maintain immune suppression and TAM-proned environment. Indeed, the presence of IL-10 and TGF-β secreted by T regs (and TAMs) not only maintain a M2-like polarization of TAMs, but also strongly inhibits Th1 responses, promoting tumor progression (Figure 19) (108). However, cytokine and chemokine production is not the only mechanism by which TAMs sustain tumor biology. Through the release of pro-tumoral growth factors (i.e. Epithelial Growth Factor (EGF) and Fibroblast Growth Factor (FGF)), TAMs favor on one hand tumor cell proliferation and on the other hand pro-apopototic pathway inhibition (Figure 19). In addition, TAMs promote extracellular matrix remodeling, as they are the main source of proteolytic enzymes, such as metalloproteases (i.e. MMP-9) (123). Extracellular matrix degradation mediated by TAMs strongly contributes to tumor cell invasiveness, helping their penetration into the blood vessels and the consequent dissemination in the organism, giving rise to metastasis (Figure 19) (140). Furthermore, TAMs positively influence angiogenesis. Indeed, they allow the predominance of pro-angiogenic factors (i.e. VEGF and CXCL8) on anti-angiogenetic ones, switching the balance through new vessel formation (Figure 19) (141). Interestingly, recent studies identified a particular subsets of human macrophages characterized by the expression of the angiopoietin receptor 2 (Tie-2) (142). Similarly, it has been observed that a population of macrophages associated to the tumor, further called TEMs, express Tie-2 and, by transcriptional analysis, it emerged that they have a M2-like phenotype (143). It suggests that TEMs could represent a TAM population that actively participate to the formation of new vessels. In addition, very recent reports evidenced that the presence of TAMs with pro-angiogenic features correlates with failure of anti-tumor therapies. Indeed, angiogenesis is strongly required for tumor cell growth and the presence of pro-angiogenic cell directly sustains tumor promotion (144, 145).



Allavena P. and Mantovani A., Journal of Translational Immunology 2012

Figure 16. Tumor Associated Macrophage contribution to tumor progression. Immuno-suppression, tumor proliferation, angiogenesis and matrix remodeling are TAM features that allow tumor promotion. By exerting their pro-tumoral functions, TAMs also contribute to the formation of metastasis, further sustaining tumor progression.

It is important to underline that the majority of the studies focused on TAMs were performed in murine models. However, epidemiological studies revealed that the presence of TAMs within human tumors mainly correlates with poor prognosis (109). Up to date, the impact of TAMs on solid tumor biology was largely described for ovarian cancer, hepatocellular carcinoma and breast cancer. It has been observed for example that TAM infiltration within ovarian cancers is huge (146) and that this infiltration maybe due to the recruitment of monocytes mediated by CCL-2 produced

by tumor cells. In addition, *in vitro* studies demonstrated that the co-culture between ovarian cancer cells and resting macrophage is sufficient to determine a M2-like phenotype (147). TAMs regulate progressive stages of human hepatocellular carcinoma formation. In these tumors, TAMs acquire different phenotype on the basis of their localization. In particular, TAMs within cancer nest maintain their typical immuno-suppressive phenotype, which trigger Treg expansion and immuno-suppression, contributing to bad prognosis (148). Similarly to ovarian cancers, breast cancers are characterized by considerable CCL-2 dependent infiltration of monocytes which then differentiate and polarized into TAMs. These TAMs are characterized by high levels of M-CSF receptor expression, that correlate with poor prognosis (149). Importantly, the only tissue in which TAMs accumulation is not associated to bad prognosis is colon. Surprisingly the *in vitro* characterization of TAM isolated by colon cancer spheroid revealed the presence of an anti-tumoral phenotype. These TAMs not only are able to dampen the proliferation of cancer cells, but are also able to secrete pro-inflammatory cytokines, which sustain Th1 responses (150). The presence of different clinical outcomes indicates that also TAMs are composed by heterogeneous phenotypes. Indeed, their functional properties are strongly determined by the type, the stage and the location of the tumor masses (109).

In order to counteract their positive modulation on tumor biology, different strategies to target TAMs have been proposed. The first aim consists in blocking monocyte recruitment to cancer tissues. Since the expression of M-CSF receptor is confined to the monocyte-macrophage lineage, M-CSF receptor inhibitors have been developed and are under clinical studies (123). In parallel, it has been observed that the delivery of anti-CCL-2 together with chemotherapeutic drugs favors the regression of prostate-cancer and colitis-associated cancerogenesis in murine models. Another approach consists in TAMs depletion. Indeed, experimental data demonstrated that macrophage elimination correlates with reduction of tumor growth and metastasis. In addition, TAM depletion allows better outcomes of canonical chemotherapeutic drugs or therapies aimed to inhibit angiogenesis. If in murine model macrophage depletion is mainly mediated by clodronate encapsulated lyposomes, bisphosphonates represent the strategy utilized for clinical studies (123). Finally, the most recent approach is represented by “TAM re-education”. TAM re-education is mediated by the conversion of the TAM pro-tumoral phenotype into an anti-tumoral one. The better way to do it is to induce a TAM molecular re-programming, aimed at driving them from M2 to M1-like cells. In this way, it would be possible to induce an inflammatory micro-environment, which could sustain immune cell responses towards cancer cells. According to the re-education

approach, it has been described that M2 re-education can be efficiently induced by the administration of anti-CD40 monoclonal antibodies (151). Similar positive results were obtained when plasma proteins HRG were delivered (152). Importantly, it is also possible to influence macrophage polarization by acting on the tumor microenvironment. IL-12 is an important regulator of Th1 cell activities and favors IFN-γ production. On the contrary, IL-6 represents one of the main TAM-provided cytokines involved in tumor cell growth regulation. In line with that, the delivery of exogenous IL-12 or the inhibition of IL-6 can contribute to M2 re-programming (123). In particular, in murine models, it has been observed that specific treatments with IL-12 reduced M2 typical chemokines, enhancing the production of M1 typical ones, and induced IL-15 secretion, which represents a stronger NK cell and Th1 cell activator (153, 154). Moreover, NF-kB inhibitors can be considered as therapeutic tools, since by inhibiting NF-kB, TAMs can acquire a more cytotoxic phenotype, finally leading to tumor regression (155). Considering that M1 polarization is triggered by the presence of bacterial moieties together with IFN-γ, another possibility to revert M2 polarization can be the treatment with TLR agonist. In particular, it has been shown that *in vitro* treatment of M2 macrophages with the TLR7 agonist imiquimod induces the downmodulation of M2 markers, even if it is not sufficient to re-direct their functions (156). In parallel, it has been observed that, upon *in vitro* treatment with recombinant IFN-γ, human TAMs started to release IL-12 and express the co-stimulatory molecule CD86 on their membrane. Moreover, they reduced their secretion of CCL18, VEGF and MMP9 (157). To directly increase the levels of IFN-γ in the tumor micro-environment is necessary to activate NK cells and T cells, which are endogenous sources of IFN-γ. Up to date, the development of strategies aimed to trigger NK cell and T cell production of IFN-γ during tumor progression are still under evaluation.

According with the fact that NK cell and macrophage functions have a strong impact on tumor cell biology, the study of the cross-talk between these two innate immune cells could represent an interesting field of research. Indeed, since it is known that NK cells and macrophages interact one with each other, the understanding of the cellular mechanisms underlying could provide new approaches aimed to interfere with cancer progression.

4. The cross-talk between human macrophages and NK cells

NK cells are, together with T cells, the main producers of IFN-γ and thus are able to drive M1 polarization (108). However, the cross-talk between human NK cells and polarized macrophages is still elusive for several aspects. Indeed, if some studies have been published on human NK cell and macrophages interactions during bacterial, viral and parasitic infections, the knowledge in regard to reciprocal regulation between NK cells and macrophages in a tumor micro-environment is almost totally absent.

The first evidence that human NK cells and macrophages can co-operate to generate immune responses was given by Baratin M. et al in 2005. IFN-γ secretion results fundamental for viral infection resolution and, to some extent, also for tumor growth regulation. Therefore, they asked whether human macrophages were able to trigger NK cell production of IFN-γ in parasitic infections, contributing to infection eradication. In particular, they focused on an *in vitro* model of human malaria. They observed that, upon incubation with erythrocytes infected with *Plasmodium Falciparium*, NK cells increased their expression of CD69 and augmented their capability to secrete IFN-γ. As expected, macrophage-derived IL-12 was strictly required for full NK cell activation, conferring to macrophages a pivotal role in the orchestration of NK cell effector functions towards parasitic infections (158). Few years later, Nedvetski S. et al. observed that LPS-activated macrophages could induce NK cell proliferation, IFN-γ secretion and cytotoxic activity towards target cells (myeloid leukemia cell line K562 and EBV-transformed B cell line 721.221). Furthermore, activating receptors, as NCRs and NKG2D, were positively modulated by LPS-activated macrophages. All these evidences indicated that macrophages seemed to trigger NK cell functions during bacterial infections. In addition, they were able to demonstrate that enhanced IFN-γ secretion was regulated by the interaction between 2B4 and CD48. Indeed, 2B4 is constitutively expressed by NK cells and CD48 is expressed on the membrane of LPS-activated macrophages. Finally, they found that when they treated macrophages with “high” doses of LPS (200 ng/ml), NK cells recognized over-activated macrophages and eliminated them through the formation of an immuno-synapse mediated by F-actin mobilization. It emphasized the fact that bi-directional interactions occurred during bacterial infections (74). Similarly, Lapaque N. et al. showed that *Salmonella*-infected macrophages were able to trigger CD69 upregulation and IFN-γ production by NK cells. They demonstrated that ICAM-1/LFA-1 interactions were responsible for IFN-γ enhancement. The presence of IL-12 and IL-18 was furthermore strongly required. Although

they detected a positive modulation of NKG2D, it was not sufficient to trigger neither IFN-γ and CD69, nor NK cell degranulation, which resulted again IL-12 and IL-18 dependent. These results further confirmed that NK cell-macrophage cross-talk was important to generate anti-bacterial responses (159). It is well known that macrophages are tissue-associated cells, which acquire different name and features on the basis of their localization. On this line, a couple of studies were focused on the interaction between human NK cells and microglia, central nervous system macrophages, and between uterine NK cells and uterine macrophages. In 2008, Lünemann A. et al. published that IL-2 activated NK cells were able to recognize and kill autologous microglia, by the formation of an immuno-synapse. NK cell mediated killing of microglia was induced by NKG2D and NKp46 engagement. Interestingly, they found that LPS-activated microglia was protected from NK cell cytotoxicity. It could be explained by the fact that they observed higher levels of NKG2D ligands on resting microglia compared to the activated counterpart. These data suggested that NK cells were important modulator of CNS-associated microglia (160). Basu S. et al. observed that upon poly I:C stimulation, uterine NK cells (uNK) started to secrete IFN-γ. This IFN-γ induction resulted more pronounced when uterine macrophages were present. Since uterine macrophages increased the expression of the NKG2D ligand MIC-A after TLR3 triggering by poly I:C, they hypothesized that NKG2D could be responsible for this process. Indeed, in the presence of neutralizing antibodies against NKG2D the enhancement of IFN-γ production was almost abolished. It suggested that NKG2D engagement by macrophages was involved in NK cell IFN-γ pathway. It indicated also that, macrophages were able to activate NK cell responses also in human uterus (161). Data concerning the interactions between human NK cells and polarized macrophages (M1 and M2) were available only a couple of years ago. Bellora F. et al. observed that, although resting NK cells were not able to kill autologous macrophages, once activated by IL-12, they developed cytotoxicity preferentially towards resting (M0) or M2 macrophages. This mechanism was almost totally mediated by NKp46 and DNAM-1 activation. M1 resulted protected from NK cell killing thanks the expression of higher levels of HLA-I compared to M0 and M2. Nevertheless, when M0 or M2 macrophages were triggered by LPS, also resting NK cells acquired the capability to eliminate them. On the contrary, M1 macrophages re-challenged with LPS still maintained their protection from NK cell killing. They found that NK cell co-cultured with LPS-activated M0 or M2 macrophages displayed higher level of CD69 expression and secrete higher amount of IFN-γ compared to LPS-re-challenged M1. If IL-12, IL-15 and IL-18 neutralization did not affected CD69 modulation, in the presence of anti-IL-18 antibody NK cell secretion of IFN-γ was

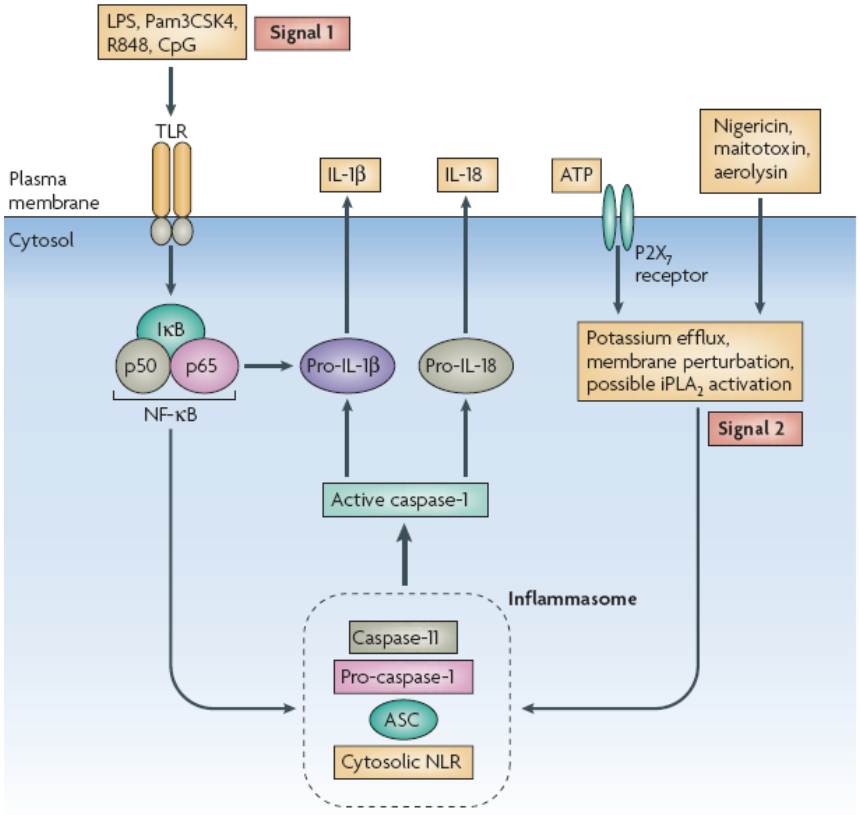
reduced, suggesting a pivotal role of this cytokine in IFN-γ enhancement (162). To further complete these data, two years later the same authors published another paper in which they demonstrated that IL-18 dependent NK cell activation can be attributed to LPS-induced secretion of a membrane associated form of IL-18 by M0 and M2. To better characterize this membrane associated IL-18 (mIL-18), they followed its expression during monocyte-to-macrophage differentiation in the presence of M-CSF or GM-CSF. They observed that only in the presence of M-CSF mIL-18 was detectable on M0 and M2 macrophages, revealing that M-CSF was needed to induce mIL-18 expression. Furthermore, they evaluated mIL-18 levels on macrophages differentiated through M-CSF but derived from different monocyte subsets. Since they did not see any differences, they concluded that the expression of CD14 and CD16 was not involved in mIL-18 modulation. In accordance with these observations, M0 and M2 derived from both monocyte subsets, once activated by LPS, maintained their comparable capability to activate NK cell IFN-γ production. In conclusion, they hypothesized that caspase-1 could be responsible for mIL-18 cleavage and subsequent release. Indeed, caspase-1 inhibitors strongly reduced IL-18 secretion by LPS-activated M0 or M2. Independently from the mechanism which regulated mIL-18 secretion, both studies indicated that different polarized macrophages could have different effects on resting NK cells, suggesting a possible implication in tumor micro-environment modulation (163). Finally, Romo N. et al. characterized the influence of polarized macrophages infected with human cytomegalovirus (HCMV) on NK cell effector functions. NK cell degranulation was triggered by both infected M1 and M2 macrophages in a NKp46, DNAM-1 and 2B4 dependent manner. In opposite, IFN-γ secretion by NK cells was induced only in the presence of HCMV infected M1 and it resulted partially mediated by IL-12. It suggested that, in contrast to bacterial infections, the re-challenge of M1 macrophages by HCMV favored the instauration of an inflammatory micro-environment in the site of infections mediated by NK cells, which, in turn, could sustain M1 polarization (164).

In conclusion, although these recent studies started to dissect human NK cells and polarized macrophages cross-talk, the understanding of the cellular mechanisms underlying are far to be completely depicted. Our results suggest that cytokines released by macrophages and in particular IL-1β, IL-15 and IFN-β have an impact on NK cell activities. This is totally in line with what it has been described before for NK cell activation (NK cell chapter). In the further paragraphs we summarize the role of these cytokines in immune responses and in particular in NK cell activation.

5. IL-1β

IL-1β represents the prototypic pro-inflammatory cytokine. Indeed it is able to affect a wide range of immune responses by the induction of a panel of different genes generally not expressed in homeostatic conditions. IL-1β is involved not only in cyclo-oxygenase (COX-2) and inducible nitric oxide synthase (iNOS) activation, but it contributes also to the enhancement of pro-inflammatory cytokines (i.e. IL-6 and TNF-α) or chemokines released by immune cells. In addition, by the promotion of tissue proteases and metalloproteases expression and by the inhibition of proteoglycan synthesis, IL-1β participates to tissue remodeling. Finally, it works as growth factor in the bone marrow, through the induction of myeloid progenitor cell proliferation and neutrophils trafficking from the bone marrow to the periphery, favoring neutrophilia. Of note, in contrast with others pro-inflammatory cytokines, IL-1β is not involved in cell killing (165). IL-1β was originally defined as endogenous fever-producing molecule and was first cloned in 1984, together with another endogenous pyrogen carrying an acidic isoelectric point discovered soon after and named IL-1α. Since it has been demonstrated that IL-1β and IL-1α share the same receptor, the IL-1R I, they are completely comparable in terms of biological functions. However, if IL-1α can also behave as transcription factors, IL-1β displays its functions only when secreted, having a key role in the generation of systemic inflammation (165).

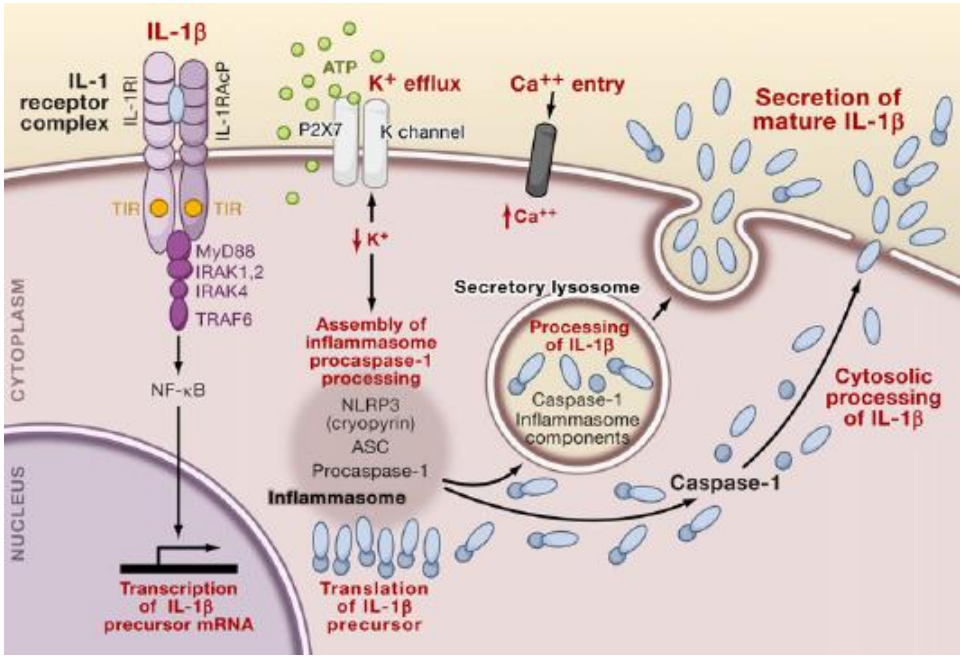
One of the most important feature of IL-1β consists in its mechanism of secretion, which represents a first step of regulation (Figure 20). Indeed, as IL-18, IL1β mRNA transcription is triggered upon TLR stimulation and gives rise to an immature form of IL-1β protein, the so called pro-IL-1β, which is totally inactive. Then, pro-IL-1β is retained in the cytosol, whereas a little fraction is loaded into specialized secretory lysosome, where co-localized with pro-caspase-1. Only in the presence of a "second stimulus" able to activate NLRP3 inflammasome (i.e. ATP derived from death cells which generate a K^+ efflux), pro-caspase-1 is converted into active caspase-1. The active form of caspase-1 mediates pro-IL-1β cleavage into mature IL-1β, which can be secreted (166). As a consequence, IL-1β processing is strictly related to IL-1β secretion, representing a mechanism of IL-1β regulation.



Mariathasan S. et al., Nature Review Immunology 2007

Figure 17. Mechanism of IL-1β production. IL-1β is produced in an immature form upon TLR stimulation. Then, the presence of a "second stimulus" allows the generation of the active form of IL-1β, which is released.

It has been also reported that IL-1β can auto-sustain its secretion, as its binding on the IL-1R I expressed by monocytes, macrophages or dendritic cells is sufficient to induce a signaling cascade aimed to trigger NF-kB activity, leading to pro-IL-1β synthesis (Figure 21) (167). In addition, it has been also proposed that pro-IL-1β cleavage by caspase-1 can occur not only in the lysosomes but also in the cytosol (168). In this case, a component of the GTP-ase protein family Rab39a is responsible for IL-1β trafficking from the cytosol to the vesicular compartment (169), where it is released either by exocytosis (170) or thanks a lack of membrane integrity (Figure 21) (171).



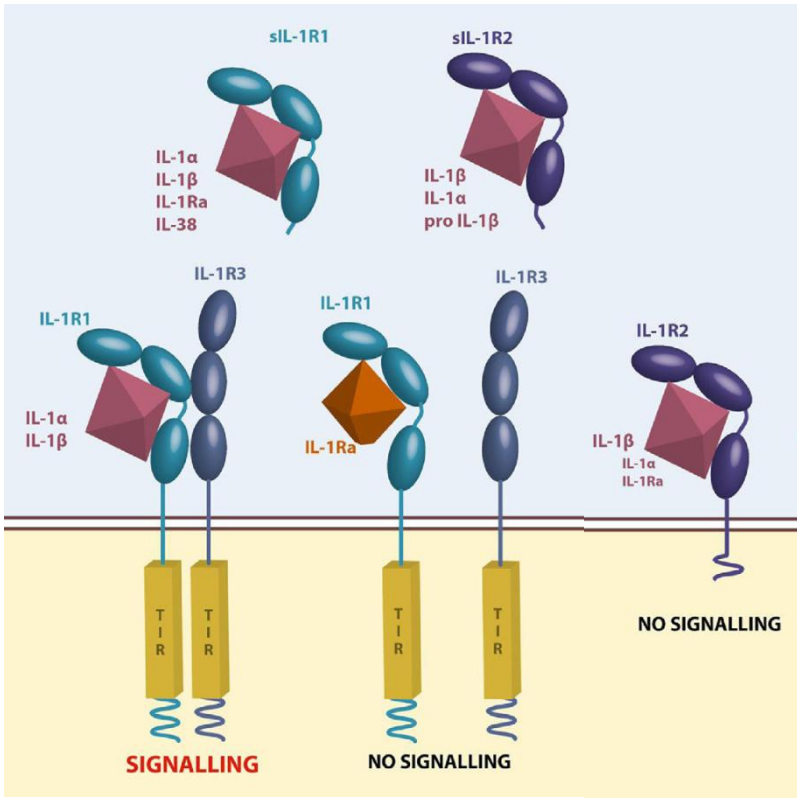
Dinarello C., Cell Review 2010

Figure 18. Mechanisms of IL-1β auto-maintenance and alternative secretion. IL-1β production could be sustained by the presence of IL-1R I triggering. In addition, caspase-1 mediated cleavage of pro-IL-1β is not restricted to the lysosomes but can occur also in the cytosol.

5.1 IL-1 receptors

IL-1 receptors constitute a family composed by 10 members and belong to the TIR superfamily. Different members of the family recognize different ligands. In particular IL-1R1 and IL-1R2 are responsible for IL-1β (and IL-1α), IL-1R5 and IL1R7 for IL-18, and IL-1R4 and IL-1R6 respectively for IL-33 and IL-36 signaling pathway. The other receptors act as regulatory receptors: IL-1R3, also named IL-1RaP, represents the accessory chain of IL-1R1, IL-1R2, IL-1R4 and IL-1R6, whereas IL-1R8, also known as TIR8, is a IgG domain receptor localized in the cytosol and involved in IL-1R signaling dampening. Finally, IL-1R9 and 10 are considered as orphan receptors and exert a negative regulatory function. To be active, IL-1R has to form heterodimeric complexes with the so called accessory proteins (IL-1RaP). Indeed, IL-1RaP is responsible for the intracellular signaling cascade initiation, mediated by MyD88 adaptor proteins. The final outcome consists in NF-k-B activation (172, 173). IL-1 R I was the first receptor of the IL-1 family discovered in 1988 (174) and selectively binds to 3 different ligands: IL-1α, IL-1β and IL-1RA. IL-1RA represents an antagonist characterized by molecular structure similar to IL-1β. However, once bound on IL-1R I, IL-1RA prevents IL-1RaP recruitment, inhibiting its signaling pathway (174, 175). In particular, the crystal

structure of IL-1R I revealed that the extracellular domain is composed by two N-terminal region which are rigid, named D1 and D2, and one flexible region called D3, which is connected to D2. These portions of the extracellular domain are involved in IL-1β binding. In particular, IL-1β binding sites are represented by two domains, one located between D1 and D2, and the other located in D3 (Figure 22). The same is assumed for IL-1α. On the contrary, IL-1RA interacts only with the region between D1 and D2 but not with D3. Therefore it occupies IL-1R I impending its correct folding (Figure 22) (176). The affinity displayed by IL-1R I for IL-1β and IL-1α is high, but comparable to the one observed for IL-1RA (177). This receptor antagonism represents one of the mechanisms that strictly regulates IL-1β/IL-1R I axis. Indeed, given the potent inflammatory activity of IL-1β, several mechanism aimed to control its signaling pathway have been developed. For example, the extracellular domain of IL-1R I can be cleaved by metalloproteases and released, generating a soluble form of the receptor. This soluble form can be considered as a decoy receptor: it sequestrates IL-1β, preventing its interaction with membrane associated IL-1R I (Figure 22) (178, 179). However, at the same time, soluble IL-1R I binds also IL-1RA, decreasing its inhibitory activity (177, 178).



Modified from Boraschi D. and Tagliabue A., Seminar in Immunology 2013

Figure 19. IL-1R I and IL-1R II binding leads to opposite effects. IL-1β signaling is controlled by the opposite action of its receptors, IL-1R I, IL-1R II and their soluble forms.

IL-1R II presents high homology with the extracellular domain of IL-1R I, and thus it can efficiently bind IL-1β. However, differently from IL-1R I, it is unable to initiate IL-1β signaling pathway, but sequestrates IL-1RaP, limiting its availability for IL-1 R I (Figure 22) (175). As a consequence, IL-1R II acts as decoy receptor and represents an additional IL-1R I signaling regulator. As IL-1R I, IL-1R II can also be secreted, exerting its function of soluble decoy receptor (Figure 22). Therefore, IL-1R II sequestrated IL-1β at the cell surface, whereas sIL-1R II in the cellular micro-environment, guaranteeing a double step of control. Importantly, the affinity displayed by IL-1R II for IL-1β is higher than the one reserved for IL-1RA, thus indicating that it preferentially binds IL-1β (174). A peculiar feature of sIL-1R II is that it is able to bind also pro-IL-1β. It means that sIL-1R II is characterized by a further mechanism of control of IL-1β activity. Indeed, it prevents the cleavage of pro-IL-1β released after cell necrosis mediated by other proteases than caspase-1, impeding its biological action (177, 180). Importantly, if anti-inflammatory cytokines and chemokines positively regulate IL-1R II expression, pro-inflammatory cytokines tend to reduce its expression. Furthermore, pro-inflammatory cytokines enhance the expression of the signaling complex, such as they would prepare the cell to sense IL-1β (175).

Finally, the last mechanism involved in the control of IL-1R I signaling pathway is represented by the action of TIR8. TIR8 presents an intracellular domain able to intercalate between IL-1R I and IL-1RaP, preventing first their dimerization and then the induction of the intracellular signaling cascade (175).

5.2 IL-1β and human NK cells

The first evidence that IL-1β can contribute to activation of human NK cells came from a paper published in 2001 by M.A. Cooper et al. They observed that recombinant IL-1β, if delivered together with IL-12, was able to trigger IFN-γ secretion by human isolated $CD56^{bright}$ NK cells, even better than IL-15 combined with IL-12. On the contrary, $CD56^{dim}$ NK cells seemed to be not responsive. In addition, IFN-γ production by $CD56^{bright}$ NK cells upon IL-1β and IL-12 stimulation was further confirmed by RT-PCR and by flow cytometry. Importantly, in the presence of IL-1β neutralizing antibody, IFN-γ secretion by $CD56^{bright}$ NK cells was selectively reduced. In order to be responsive to IL-1β, NK cells have to express IL-1R I and its accessory protein. By q-PCR, they were

able to show that IL-1R I and IL-1RaP were constitutively expressed by resting NK cells, even if $CD56^{bright}$ presented a more intense band compared to the dim counterpart. Furthermore this expression was not affected by the treatment with IL-12 and IL-1β. Importantly, both NK cell subsets did not express IL-1R II, suggesting that IL-1β action on NK cells was not inhibited. All together, these evidences demonstrated that when IL-1β collaborated with other activating cytokines it was able to modulate NK cell functions, at least in the $CD56^{bright}$ population. Moreover, NK cells could sense the presence of IL-1β thanks to the constitutive expression of IL-1R I but not of IL-1R II (181). Later on, in 2009, van de Wetering D. et al. showed that also IL-23 sustained IL-1β enhancement of IFN-γ secretion by $CD56^{+}$ cells. Indeed, they first demonstrated that macrophages stimulated with TLR ligand or infected with *Salmonella* selectively secreted IL-23 and IL-1β, but not IL-12. Then, when they cultured $CD56^{+}$ cells with activated macrophage-derived supernatants, they observed that NK cells efficiently increased IFN-γ production. These data were further confirmed by the fact that $CD56^{+}$ cells treated with recombinant IL-23 and IL-1β augmented their capability to secrete IFN-γ, indicating that IL-1β together with IL-23 played a role in the enhancement of IFN-γ production by $CD56^{+}$ cells (182). The same year, Cella M. et al. identified a new subset of human NK cells, selectively associated with MALTs (Mucosal Associated Lymphoid Tissues). These cells were characterized by high expression of NKp44, which was thus proposed as a marker to recognize this NK cell population. In addition, they produced IL-22 in response to IL-23 (183). This work was crucial as it opened a new field of research leads to the definition of ILCs. On year later, the same group was also able to shown that IL-1β not only strongly collaborated with IL-23 in the induction of IL-22 production by these NK cells, but it also participated to their *in vitro* expansion, together with IL-7. It conferred to IL-1β an important role in MALT associated NK cell sustainment and activation (184). In parallel, the same year Hughes T. et al. published that IL-β was responsible for the sustainment and the expansion of immature IL-22 producing NK cells located in secondary lymphoid tissues. In particular, they demonstrated that these immature NK cells expressed high levels of IL-1R I, and this was the reason why they were particularly responsive to IL-1β stimulation. On the contrary, they lose the expression of IL-1R II. Furthermore, they showed that IL-1β triggered IL-22 production by immature NK cells in secondary lymphoid tissues, inducing their activation. However, IL-1β also impaired their differentiation in mature cells. Finally, they attributed to DCs the source of IL-1β (185). Again, by these evidences, IL-1β importance in NK cell biology was underlined. Finally, very recently, Glatzer T. et al. demonstrated that in ILCs characterized by RORγt expression NKp44 not only represented a marker but actively

participated to their effector functions. Indeed, NKp44 engagement, synergizing with IL-1β, IL-7 and IL-23, strongly boosted inflammatory cytokines production by these ILCs. It suggested a relationship between this activating receptor, historically defined as cytotoxic receptor, and pro-inflammatory cytokines (186). The presence of a synergism between NKp44 and IL-1β could also reveal a molecular link between NKp44 and IL-1R I, which is highly expressed by RORγt^{+} cells. Of note, on the basis of the new nomenclature recently proposed by Spits H. et al. the canonical human NK cells, which are sources of IFN-γ, were selectively excluded from the IL-1R I expressing ILCs (10). Although previous results attributed to IL-1β a functional effect on NK cells, this new nomenclature excludes it. IL-1β involvement on NK cell biology is therefore still a matter of debate.

6. IL-15

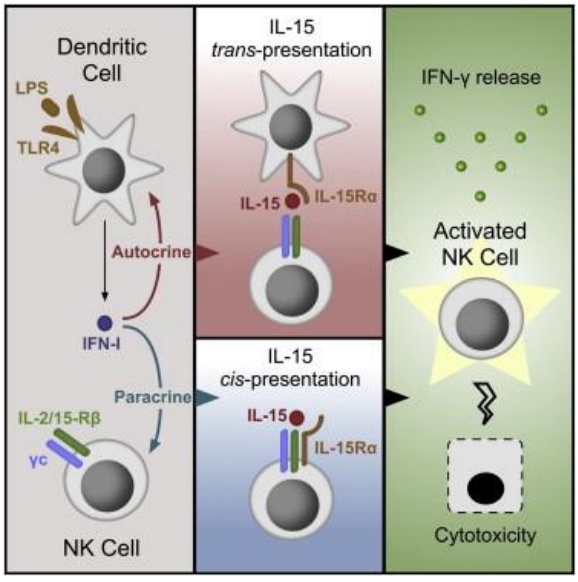
IL-15 is a pro-inflammatory cytokine discovered in 1994 simultaneously by two different groups (187, 188) and defined as T cell growth factor. It signals through the same receptor of IL-2. In particular, IL-15 shares with IL-2 not only the β (CD122) and the γ (CD132) chain of its receptor, but also the JAK/STAT signaling pathway, which comprises JAK1/JAK3 and STAT5. As a consequence, IL-15 action can overlap the IL-2 one in the induction of T cell proliferation, cytotoxic T cell formation and NK cell generation and persistence. However, IL-15 can also act differently from IL-2 and sometimes also competing with it. For example, IL-15 results more appropriated for cancer immuno-therapies than IL-2, as it seems less cytotoxic for the host (189). IL-15 is a protein of 14-15 kDa, which is encoded by a gene localized on the chromosome 4 in humans. IL-15 transcription generates two different isoforms, characterized by different length, which are the result of alternative splicing mechanisms. The long form (LSP) is composed by 48 amino acids, whereas the short form (SSP) only by 21. Although both isoforms give rise to the same protein, they undergo to different intracellular trafficking processes. Indeed, IL-15LSP, once leaved the Golgi apparatus through early endosomes, reaches the endoplasmic reticulum secretory pathway. In opposite, IL-15SSP is not secreted and does not leave the nucleus, suggesting that it may

contribute to transcriptional regulation (189). Although mRNA transcripts of IL-15 can be detected into a wide range of hematopoietic and non-hematopoietic cells (190), IL-15 protein expression is restricted to hematopoietic cells. In particular, it is expressed by monomyelocitic cells, as dendritic cells and macrophages. It denotes that IL-15 is subjected to huge post-transcriptional regulation and it can be due to the fact that IL-15 works as a potent inducer of inflammatory responses. Indeed, IL-15 stimulates the production of TNF-α, IL-1β and IFN-γ by immune cells, thus requiring to be strictly controlled, in order to avoid dangerous reactions (189). However, recently, it has been reported that also T lymphocytes and NK cells, upon activation, can translate low levels of IL-15 protein (191, 192). IL-15 exerts different biological effects. Starting from the evidences that it is involved in T and NK cell development and activation, as IL-15 KO animals display reduced numbers of both cell types (189, 193), it has been shown that IL-15 functions as growth factor for neutrophils and mast cells, by blocking the apoptotic pathway (194, 195). In addition, IL-15 enhances macrophage phagocytosis and cytokine (IL-12, IL-6, TNF-α) and chemokine (MCP-1 and IL-8) production (196). In parallel, when incubated with IL-15, dendritic cells display higher levels of co-stimulatory molecules and MHC class II, suggesting that it ameliorates their antigen presenting ability (197). Focusing on NK cells, it has been demonstrated that IL-15 not only sustains NK cell development, but it represents a strong NK cell activator. Indeed, in the presence of IL-15, NK cells start to proliferate and produce cytokines. IL-15 further enhances their cytotoxic activities (198). In particular, IL-15 induces proliferation of $CD56^{bright}$ NK cells similarly to IL-2 and triggers both ADCC and cytotoxicity by $CD56^{bright}$ and $CD56^{dim}$ NK cells towards NK-resistant target cells (199). Finally, together with IL-12, IL-15 participates to the enhancement of IFN-γ secretion by NK cells. Of note, IL-15 alone is sufficient to trigger GM-CSF production by NK cells (200).

6.1 IL-15 trans and cis-presentation

The first evidence that IL-15 displays an unconventional mechanism of action arose in 2001, when Ma and al. published that IL-15 responsiveness of T cells required the presence of IL-15Rα expression by surrounding cells in mice treated with poly I:C (201). IL-15Rα represents the third chain of IL-2/IL-15 receptor and its expression is completely independent from IL-15Rβγ (202). In addition, if the β and the γ chain are responsible for the recognition of both IL-2 and IL-15 proteins and for the initiation of both IL-15 and IL-2 induced signaling pathway, the α chain displays specific affinity for IL-15 (203). IL-15Rα is encoded by a gene located on the human chromosome 10 and counts 8 different splicing isoforms (204). It is characterized by a very high affinity (Kd $<10^{-11}$ M) for IL-15, such that IL-15/IL-15Rα complexes can be found in the endoplasmic reticulum, before the complex is delivered to the membrane (189, 193). It has been demonstrated that the presence of membrane associated IL-15/IL-15Rα correlated with enhanced T cell proliferation (205, 206). This mechanism by which IL-15Rα retains IL-15 to the plasma membrane without altering its action was defined as IL-15 trans-presentation and represented also a good explanation for the difficulties to detected IL-15 in biological solution. Indeed, IL-15/IL-15Rα expressing cells form an immuno-synapse with IL-15 responsive cells limiting the exposure of circulating IL-15 and allowing a better control of IL-15 inflammatory action (189). Indeed, as just mentioned before, IL-15 trans-presentation preserves IL-15 capability to interact with IL-15Rβγ chain, which recruited JAK1/JAK3 and then STAT3/STAT5. STATs protein phosphorylate kinases of the Syk family and phospholipase γ (PLCγ), permitting PI3K/AKT or MAPK dependent activation of Bcl-2, Myc, Fos-Jun and NF-kB (196). In addition, IL-15/IL-15Rα complexes can recycle for many days through endosomes, guaranteeing a persistence of membrane-bound IL-15 (205). Although IL-15 trans-presentation seems to be an efficient mechanism to control IL-15 availability, the study of its regulation and impact on immune cell biology results particularly tricky, over all in humans. If in animal models is possible to create IL-15, IL-15Rα or double-knock out, the only way to investigate the contribution of IL-15 trans-presentation *in vitro* human models may rely on the usage of blocking antibodies. Indeed, although IL-15 is never detected as free protein suggesting that it is always bound to IL-15Rα, IL-15/IL-15Rα complexes associated to the membrane are still difficult to visualize. It seems only to be possible to detect separately IL-15 and IL-15Rα on the membrane, assuming that they form complexes. Nevertheless, focusing on NK cell activation, it has been clearly demonstrated in mouse model that IL-15 trans-presentation by antigen presenting cells is really required for full NK

cell activation. IL-15 induces the expression of IFN-γ, by targeting T-bet, and granzyme B, by activating Blimp-1 (46, 207-209). Furthermore, it has been observed that not only activated DCs represent the mediators of IL-15 trans-presentation to NK cells, but also monocytes and macrophages are involved (47, 210). In parallel, the importance of IL-15 trans-presentation by monocytes and dendritic cells in human NK cell survival, maintenance and activation has been proposed (211-213). Importantly, at the moment, it is not possible to exclude that other mechanisms of IL-15 presentation by IL-15Rα than trans-presentation occur. Indeed, it remains the fact that also lymphocytes can express IL-15Rα. Since now a mechanism of IL-15 cis-presentation is ruled out. Indeed, murine studies indicated that IL-15Rα expression by $CD8^+$ T cells and NK cells was not needed to allow IL-15 signaling through IL-15Rβγ. In addition, neither NK cells nor $CD8^+$ T cells are able to produce IL-15 (188, 205). In contrast, recently, it has been demonstrated by Zanoni I. et al. that, at least in murine models, IFN-β secreted by activated dendritic cells led to IL-15 expression and IL-15Rα induction on NK cells. Moreover, it resulted sufficient to allow a process of cis-presentation. Importantly, this NK cell-mediated cis-presentation played a key role in the enhancement of NK cell effector functions at early time point of activation (Figure 23). In their model, LPS-activated DCs represent the source of IFN-β (192).



Zanoni I. et al., Cell Reports 2013

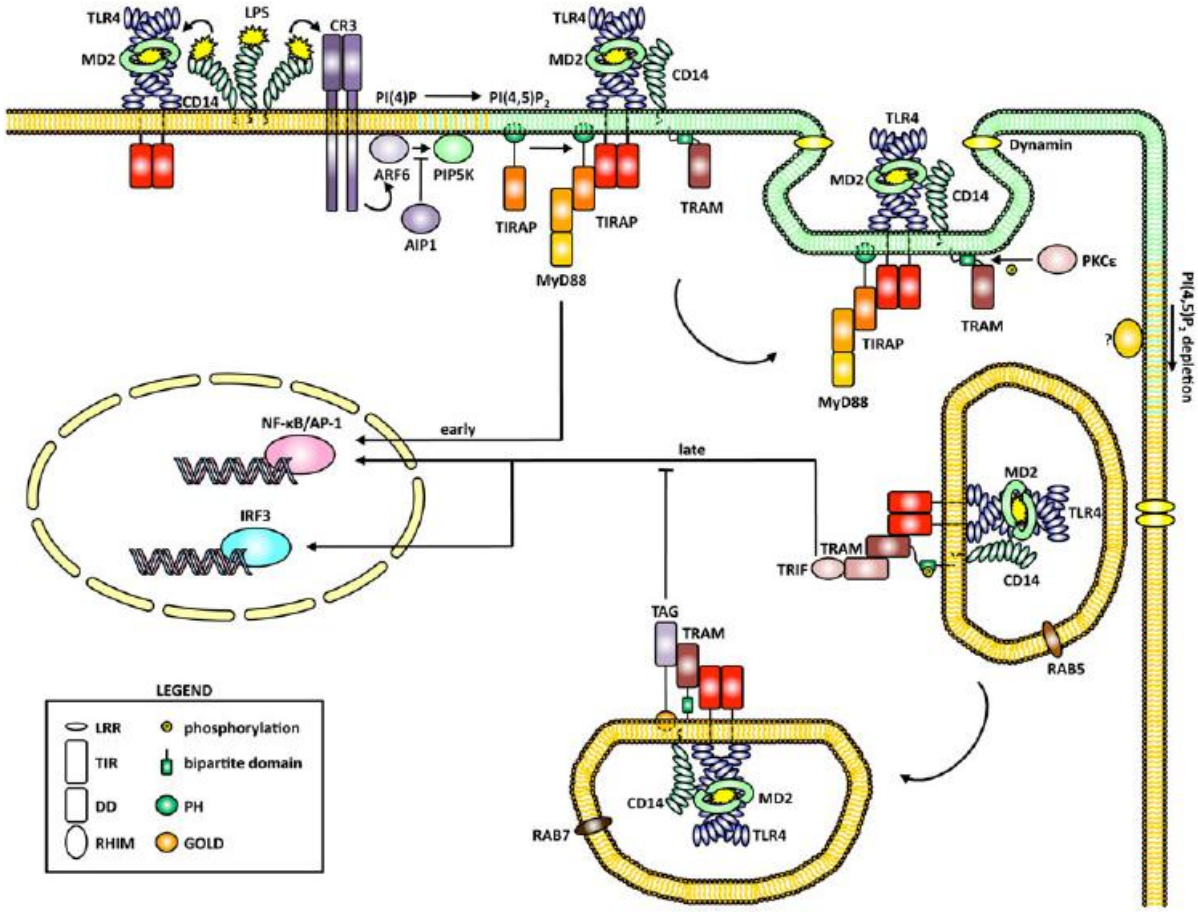
Figure 20. IFN-β mediated IL-15 cis-presentation correlates with NK cell activation. Both IL-15 cis and trans-presentation participate to the induction of NK cell effector functions mediated by DCs.

Nevertheless, even if they were able to detect IL15 and IL15RA transcripts upon recombinant IFN-β treatments also in human NK cells, up to date the involvement of IL-15 cis-presentation in the induction of human NK cell activities has to be defined.

In parallel to IL-15 cis-presentation, it has been also proposed that IL-15/IL-15Rα complexes can be released by presenting cells, in order to allow a sort of soluble trans-presentation. IL-15/IL-15Rα soluble complexes can be generated upon metallo-protease cleavage (214) or by the formation of alternative splicing isoforms of IL-15Rα that lack the transmembrane domain (215, 216). The presence of soluble IL-15/IL-15Rα complexes can represent a mechanism to deliver IL-15 in a controlled way but without the requirement of immuno-synapse. Indeed, it has been observed that soluble IL-15/IL-15Rα complexes, generated *in vitro* through recombinant proteins, displayed the same agonist action of IL-15/IL-15Rα complexes anchored to the membrane (217-220). However, once isolated from murine serum, soluble complexes resulted less active in NK cells and T cells stimulation compared to the membrane associated form. In addition, only recently it was possible to detect the presence of soluble IL-15/IL-15Rα complexes in serum of cancer patients (221). Although its reduced activity when associated to soluble IL-15Rα than to membrane IL-15Rα, it remains a matter of fact that IL-15 bound on IL-15Rα results more stable than the free cytokine. As a consequence, the IL-15Rα-dependent stability of IL-15 could be used for the development of cancer therapies. Indeed, if its ability to stimulate NK cell and T cell proliferation can sustain some hematological malignancies, it has been observed that IL-15 participates to trigger immuno-surveillance of non-hematological solid tumors. However, although its direct administration correlates with anti-tumoral effects in pre-clinical murine models, IL-15 *per se* is not sufficient to induce immune cell responses towards tumor cells (189). The co-administration with anti-programmed cell death-1 ligand (PD-L1) or anti-cytotoxic lymphocyte antigen 4 (CTLA-4) results in better anti-tumoral activities of IL-15 (222). In these context, it has been observed that either anti-CD40 administration, which triggered IL-15Rα expression by antigen presenting cells, or the covalent binding of IL-15 to IL-15Rα strongly ameliorated the efficiency of IL-15 as anti-tumoral protein (217, 223).

7. Type I interferons

First evidences of the existence of interferons (IFNs) came from 1957, when they were identified as new antiviral molecules. Later, their action was not only restricted to anti-viral responses but also to cell growth and differentiation processes. Therefore they were further classified as cytokines. Indeed, due to their capability to modulate immune responses, interferons are considered as active members of the cytokine family. Interferons can be subdivided into two main groups: type I interferons, which included IFN-α and IFN-β, and type II interferon, that is IFN-γ. The two subfamilies are characterized by similar structure, which consists in 15-25 kDa single chain polypeptides (224). Focusing on IFN-β, human IFN-β is encoded by a single gene, which does not present introns, thus excluding the presence of polymorphisms. It is produced by different cell types, as endothelial cells, epithelial cells and over all immune cells (225). In particular, it is released by monomyelocitic cells upon TLRs engagement. It is well known that IFN-β production is triggered by viral RNA and DNA sensors, as TLR3, TLR7 and TLR9. Relatively recently it has been demonstrated that also TLR4 engagement is able to induce IFN-β transcription. Indeed, in addition to the canonical MyD88 mediated pathway, TLR4 can signal through a TRIF dependent pathway. Upon internalization, TLR4 can initiate TRIF pathway by the endosomes. The activation of TRIF signaling correlates with “late” gene expression, included type I interferons (226). In particular, the protein adaptor TRAM allows the co-localization of TLR4 with TRIF, initiating a signaling pathway which not only sustains NF-kB but also induces IRF3 activation, favoring IFN type I transcription (Figure 24) (227). More recently, it has been observed that CD14 is strictly required for TLR4 internalization, and consequently for IFN-β expression (228).



Ostuni R. et al., Cell. Mol. Life Sci. 2010

Figure 21. TLR4 signaling pathway. Both MyD88 and TRIF pathways of TLR4 signaling are depicted.

On the other hand, once released, IFN-β binds to its receptor on the membrane of IFN responsive cells. IFN-β receptor is characterized by two different chains, one involved in signaling, IFNAR1, and one involved in binding, IFNAR2. Although IFN-β is recognized only by IFNAR2 chain, the presence of IFNAR1 is strictly needed. Indeed, the affinity displayed by IFN-β for IFNAR2 results higher when IFNAR1 is present (229, 230). Experiments performed with KO animals revealed that both chain were required for the generation of an IFN-β-mediated response (231, 232). More in details, upon IFN-β binding to IFNAR2 extracellular domain, IFNAR2 recruits IFNAR1, leading JAK1 and Tyk2 inducement and subsequently STATs activation. STATs protein activation allows the formation of a complex, which includes other cytoplasmatic proteins, that reaches the nucleus. Once in the nucleus, the complex induces the transcriptional control regions named Interferon Sensitive Response Elements (ISRE), which regulates several IFN-responsive genes, such as some cytokine and chemokine genes (224).

Since IFN-β, and more in general, type I IFNs can trigger a wide range of immune modulating functions, it has been considered an important inducer of both innate and adaptive immune responses. IFN-β is involved in the enhancement of IL-15, in addition to its capability to sustain its own production. Indeed, IFN-β not only sustains IRF3 activation, but also induces IRF7 expression, which are needed for IL-15/IL-15Rα expression (233). Focusing on NK cells, type I interferons are strongly involved in the regulation of NK cell cytotoxicity, *in vitro* and *in vivo* (234). Several evidences identified dendritic cells as the major source of type I IFNs which activate NK cell cytotoxicity (235). Up to date, it is well accepted that DC derived IFN-β can trigger also IFN-γ production by NK cells (236-238). Since the cross-talk between human NK cells and macrophages is poorly studied, no evidences are available on the influence of human macrophage-derived IFN-β on NK cells, even if macrophages, as dendritic cells, are sources of IFN-β (239). However, one recent paper published by Zhou Z. et al. highlighted the fact that IFN-β secreted by murine macrophages, together with IL-15, induced NKG2D expression on murine NK cells. It suggested that IFN-β not only participated to NK cell effector functions as IFN-γ secretion and cytotoxicity, but it also modulated NKG2D expression, facilitating their recognition of NKG2DL (240). As a consequence, macrophage derived IFN-β effects on human NK cell has to be determined. Finally, it has been demonstrated that IFN-β is involved in the regulation of IL-15 and IL-15Rα production by monomyelocitic cells. Lucas M. et al. in 2007 observed that NK cell priming mediated by DCs in murine model of viral infections was IL-15 trans-presentation dependent, but required also type I IFNs, suggesting a connection between IFN-β signaling and IL-15 trans-presentation (46). A couple of year later, Mortier E. et al. demonstrated a role of murine macrophage mediated IL-15 trans-presentation in NK cell activation (47). Last year Soudja S. et suggested that IFN-β controlled IL-15 and IL-15Rα expression, as IRF3, IFNs type I and IFNs type I signaling were strictly required for IL-15 trans-presentation by murine inflammatory monocytes (210). All these evidences argue for the existence of an indirect IFN-β effect on NK cell functions. Indeed, IFN-β directly triggers NK cell IFN-γ secretion and cytotoxicity and in parallel induces IL-15 trans-presentation, which represents a mechanism strongly involved in NK cell activation. Finally, few months ago, Zanoni I. et al. published that not only DC derived IFN-β sustained a mechanism of IL-15 trans-presentation, but it represented the main inducer of IL-15 cis-presentation by NK cells. Indeed, only in the presence of IFN-β NK cells could activate a molecular machinery leading to IL-15 and IL-15Rα expression. It further denoted a strict relation between IFN-β and IL-15/IL-15Rα complexes during myeloid-mediated NK cell activation (192). However, it is important to underline that all these data were

obtain in murine models. Up to date any studies were performed to determine whether IFN-β can trigger IL-15 cis or trans-presentation in humans.

AIM OF THE STUDY

NK cells are lymphocytes of the innate immune system able not only to infiltrate tumor masses but also to exert anti-tumoral activities. These anti-tumoral functions are mainly mediated by the release of IFN-γ, which contributes to the formation of inflammatory micro-environment, and cytolitic granules, which directly kill tumor cells. In parallel, macrophages are the main immune cell type involved in tumor progression. During early phases of tumor development, macrophages behave as effector cells, participating to tumor cell elimination. As a consequence, similar to dendritic cells, they could actively impact on NK cell anti-tumoral activities. However very few studies analyzed the effect of polarized macrophages on NK cell functional properties.

We therefore focused our work on the understanding of human NK cell-macrophage cross-talk in a reconstituted tumor micro-environment model. To this end, we set up an autologous system of co-culture of NK cells and polarized macrophages and analyzed the outcome of the co-culture on cancer cell lines. We particularly sought to decipher the contribution of soluble mediators and direct contact interactions between resting NK cells and different polarized macrophages and to which extent this could impact on NK cell activities in the presence of tumor cells. We sought to perform the most exhaustive analysis possible on both sides of the partners of the cross-talk and thus analyzed the contribution of a wide range of cytokines and NK cell activatory receptors.

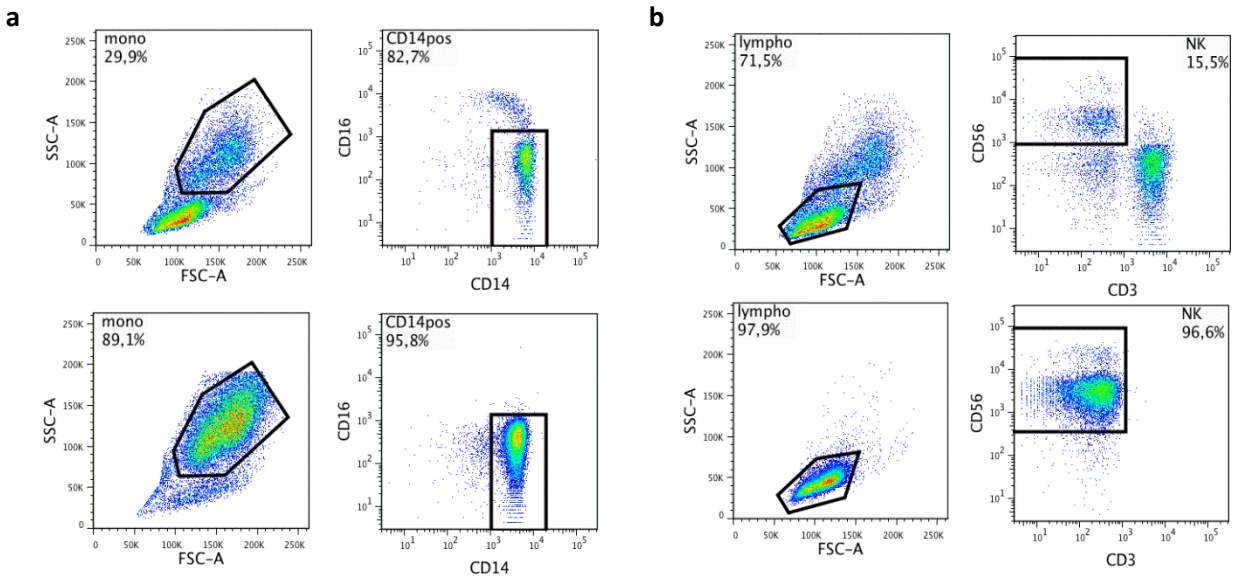
With the aim to better appreciate a potential re-education of macrophages by NK cells, we also analyzed the impact of NK cell-derived IFN-γ on macrophage polarization. In other words, the scope of the second part of this work was to evaluate whether NK cell-derived IFN-γ was sufficient to re-direct M2 macrophages towards M1. The idea behind this was that by reverting TAMs to a M1-like phenotype, we not only could avoid TAM mediated promotion of tumor growth, but we could also convert them into anti-tumoral cells actively fighting against the tumor. Moreover, since both TAM formation and re-education are mainly driven by the tumor micro-environment, the study of the cross-talk between macrophages and immune cells able to shape tumoral milieu, as NK cells, resulted mandatory. These mechanisms of re-polarization in response to NK cell-derived IFN-γ were appreciated by analyzing the expression of a panel of genes and proteins characteristic of M2 and M1 macrophages.

MATERIALS AND METHODS

1. Isolation and culture of monocyte-derived macrophages and NK cells

Human peripheral mononuclear cells (PBMCs) were obtained from buffy coats of healthy donors signed consent forms in accordance with clinical protocols approved by the Institutional Review Board of Desio Hospital, Milan, Italy. PBMCs were isolated over Ficoll density gradient centrifugation (GE Health Care Biosciences). Then, upon platelet elimination, PBMCs were divided: 1/3 was used for monocytes isolation, whereas 2/3 were utilized for NK cell isolation. We adopted a negative selection protocol through an automatic magnetic cell-sorting technique (Robosep Cell Separator, Stem Cell Technologies). Briefly, PBMCs were incubated with a cocktail of antibodies against all (CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A) but CD14 for monocytes (Human Monocyte Enrichment kit, Stem Cell Technologies) and all (CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA-DR, glycophorin A) but CD56 for NK cells (Human NK cell Enrichment kit, Stem Cell Technologies). Then, PBMCs were incubated with magnetic beads, that, through a dextran-mediated binding, were attached to the antibodies. As a consequence, when flowed through a magnetic field, all stained PBMCs were retained, whereas unstained cells which are monocyte and NK cells were retrieved. In this way, collecting the negative fraction, we obtained pure $CD14^+$ monocytes and $CD56^+$ NK cells. The purity of the isolated cells was always checked by FACS. $CD14^+$ monocytes resulted more than 90% pure, whereas $CD56^+$ NK cells contained ≤ 5% contamination with other PBMC populations (Panel 1).

To generate resting macrophages (M0), monocytes were cultured for 7 days with RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutammine, 1% Pen/Strept (Lonza) and 100 ng/ml M-CSF (R&D System). M0 macrophages were then incubated for 24 hours with 100 ng/ml of LPS purified from *Escherichia Coli 055:B5* (Sigma) and 20 ng/ml of rhIFN-γ (Peprotech) to induce M1 polarization or with 20 ng/ml of rhIL-4 (Peprotech) to induce M2 polarization. On the contrary, fresh NK cells were frozen in 10% DMSO (Sigma) supplemented FBS immediately after their isolation from PBMCs. NK cells were thawed 24h before performing the experiments.



Panel 1. Monocyte (a) and NK cell (b) purity after magnetic particle isolation from the same donor. On the upper panel are figured total PBMCs staining, whereas in the lower panel are represented the respective purified monocytes and NK cells. The plots are represented of one donor.

2. NK cell treatment with autologous macrophage-derived soluble factors or recombinant cytokines

NK cells were thawed and cultured for 24h at a density of $3x10^6$ cells/ml in complete medium (RPMI 1640, 10% FBS, 1% Pen-Strep, 1% L-Glu), in absence of any stimuli. The viability of these cells was assessed by trypan blue exclusion, and further checked by flow cytometry, through LIVE/DEAD AQUA fluorescent-reactive dye (Life Technologies) staining and resulted ≥ 90%. Then, $2.5\text{-}5x10^5$ NK cells were incubated with 30% or 50% conditioned media from M0, M1 or M2 macrophages, previously centrifuged to eliminate any cellular debris, or with 200 U/ml of rhIL-2 (Peprotech), 200 U/ml rhIFN-β (R&D Systems), 10 or 20 ng/ml rhIL-15 (Peprotech) or 20 or 100 ng/ml rhIL-18 (R&D Systems). To evaluate a potential effect of the LPS and IFN-γ carried over from M1-conditioned medium, we treated resting NK cells with 30% of a medium supplemented with 100 ng/ml of LPS and 20 ng/ml of rhIFN-γ, the same doses used to polarize M1. Twenty four hours later, cells were harvested and analyzed for receptors expression (activating receptors, CD69, IL-1 R I, IL-1R II, IL-15Rα), by FACS or by immunofluorescence, or effector functions, IFN-γ transcription (RT-PCR) or secretion (ELISA) and degranulation (CD107a degranulation assay).

3. Co-culture of autologous NK cells with polarized macrophages

After thawing, NK cells were cultured in complete medium as described before. Then, $1x10^5$ NK cells were co-cultured at 1:1 cell ratio with resting or polarized macrophages, previously washed to avoid any modulation of NK cell phenotype and functions by cytokines or soluble factors produced during their maturation and polarization. Then, in order to analyze activating receptor expression, NK cells were harvested and stained. On the contrary, for IFN-γ detection, we simply collected the supernatant after a brief incubation with K562 (see Detection of cytokines and chemokines). CD107a assay was performed directly into the plate where macrophages and NK cells were co-cultured, to interfere as little as possible with the micro-environment generated during direct interactions.

4. Blocking experiments

The effect of cytokines or their receptors on NK cell phenotype and functions was blocked through saturating concentrations of commercial neutralizing antibodies, enlisted in Table 1. NKp30, NKp44 and 2B4 blocking antibodies produced from hybridomas (clones F252, KS38 and C054 respectively) were kindly provided by Prof. Alessandro Moretta and Dr.ssa Emanuela Marcenaro (Istituto Gianna Gaslini, Genova). Macrophage-conditioned media were incubated with blocking antibodies against cytokines at RT for 45 minutes prior NK cell stimulation. IL-1R I or IL-15Rα were blocked at RT for 45 minutes directly on NK cells during macrophage-conditioned medium treatment, whereas activating receptors were blocked at RT for 45 minutes after treatments and before targets addition. On the other hand, for macrophage-NK cell direct interactions, activating receptors were masked at RT for 45 minutes before NK cells were co-cultured with polarized macrophages. Similarly, macrophages were incubated RT for 45 minutes with blocking antibodies before NK cell addition. Concerning IL-15Rα, in order to be able to distinguish cis from trans-presentation of IL-15, we selectively blocked IL-15Rα on macrophages by incubating them RT 1 hour with saturating concentration of anti-IL-15Rα. Then, macrophages were washed and NK cells were added to the culture. Moreover, each neutralizing conditions were compared to an isotype control, from the same company and used at the same concentration than the relative blocking antibody.

Table 1. Blocking antibodies

Antigen	Concentration	Company
NKp44	10 µg/ml	A. M. and E. M.
NKp30	10 µg/ml	A. M. and E. M.
NKG2D	1 µg/ml	Biolegend
2B4	10 µg/ml	A. M. and E. .M
IL-1β	5 µg/ml	BD Pharmigen
IL-15	0,5 µg/ml	R&D Systems
IL-18	5 µg/ml	MBL International Corporation
IL-23p19	0,8 µg/ml	R&D Systems
IFN-β	0,2 µg/ml	R&D Systems
IL-1R I	2 µg/ml	R&D Systems
IL-15Rα	5 µg/ml	R&D Systems

A. M. (Alessandro Moretta), E. M. (Emanuela Marcenaro)

5. Macrophage treatment with autologous NK cell-derived soluble factors

2.5-3x10^5 $CD14^+$ monocytes were cultured for 6 days with a medium supplemented with 50 ng/ml of hM-CSF, to induce their differentiation in resting macrophages. Then, one part of resting macrophages were polarized to M1, through LPS and rhIFN-γ. 50% of their conditioned medium was utilized to treat autologous resting NK cells (24h), previously thawed. In parallel, the remaining part of resting macrophages were polarized in M1 (LPS+rhIFN-γ) or M2 (rhIL-4). Eighteen hours later, M0, M1 and M2 macrophages were extensively washed and treated with 100% of resting, M0-primed or M1-primed NK cell-conditioned media, collected after a brief incubation with K562, or with complete medium as control. After 24h, macrophages were detached through Acutase (Millipore) and analyzed for receptors, cytokines and chemokines expression (RT-PCR or FACS analysis) or secretion (ELISA).

6. Detection of cytokines and chemokines

Cytokines and chemokines considered (Table 2) were dosed by commercial ELISA kits (Duoset, R&D Systems). Generally, supernatants derived from polarized macrophages or NK cell-treated macrophages were collected, centrifuged in order to avoid any cellular debris, and analyzed by ELISA. For IFN-γ detection, NK cells were washed after treatment with autologous macrophage-conditioned media to eliminate any rhIFN-γ carry-over and then stimulated with K562 (E-T ratio 2:1) for 4 h. In this way, we triggered NK cell release of cytokines, including IFN-γ, stored into the granules, allowing its detection by ELISA. Indeed, in absence of a degranulation inducer, the levels of IFN-γ secreted by NK cells resulted below the sensibility of the kit. This procedure was adopted also for direct co-cultures. Data were analyzed by SoftMaxPro 5.3 software.

Table 2. Cytokine and Chemokine dosed by ELISA kit

Cytokine/chemokine	Reference	Company
IFN-γ	DY285	R&D Systems
IL-1β	DY201	R&D Systems
IL-15	DY247	R&D Systems
IL-15/IL-15Rα complex	DY6924	R&D Systems
CCL-5	DY278	R&D Systems
CCL-17	DY364	R&D Systems
CCL-18	DY394	R&D Systems
CCL-22	DY336	R&D Systems
CXCL-9	DY392	R&D Systems
CXCL-10	DY266	R&D Systems

7. Flow cytometry

For direct multi-color flow cytometry analysis (FACS Canto II, BD Bioscience), purified NK cells, monocytes and polarized macrophages were stained with directly conjugated monoclonal antibodies labeled with different fluorochromes. Table 3 presents the list of anti-human antibodies we used. For each antibody, the fluorescence was set on its appropriate isotype control. The staining procedure consisted in cell incubation with labeled antibodies, protected from light, for 20 minutes at 4° C in FACS buffer (HBSS 2% FBS, Lonza), followed by a wash. Cells were always acquired within one hour, thus were never fixed. For indirect staining, purified NK cells were incubated for 45 min at room temperature (RT) with primary unlabeled antibodies. Then, they were stained with secondary labeled antibody (RT, 30 minutes, dark) (Table 4). The same protocol was used for isotype controls. Again, cells were not fixed. The data were analyzed by using FACS Diva and Flow Jo Software.

Table 3. Monoclonal anti-human labeled antibodies for FACS analysis

Antigen	Clone	Fluorochrome	Company
CD3/CD56	-	FITC/PC5	Beckman Coulter
CD3	HIT3a	FITC	BD Pharmigen
CD14	M5E2	FITC	BD Pharmigen
CD16	3G8	PeCy7	BD Pharmigen
CD48	Tü145	PE	BD Pharmigen
CD56	HCD56	BV 421	Biolegend
CD69	FN50	PeCy7	BD Pharmigen
CD80	L307.4	PE	BD Pharmigen
CD107a	H4A3	PE	BD Pharmigen
CD119	92101	PE	R&D Systems
CD206	19.2	FITC	BD Pharmigen
CD209	DCN46	PE	BD Pharmigen
NKp46	BAB281	PE	Beckman Coulter
NKp44	Z231	PE	Beckman Coulter
NKp30	Z25	PE	Beckman Coulter
NKG2D	ON72	PE	Beckman Coulter

DNAM-1	KRA236	PE	Beckman Coulter
2B4	-	PE	R&D Systems
IL-15Rα	JM7A4	PE	Biolegend
IL-1R II	34141	PE	R&D Systems

Table 4. Primary and secondary antibodies for indirect FACS staining

Primary Antibody	Primary Antibody specie	Company	Secondary Antibody specie	Secondary Antibody fluorochrome	Company
IL-1R I	Goat anti-human	R&D Systems	mouse anti-goat	AF647	Invitrogen

8. CD107a degranulation assay

NK cell treated with macrophage-conditioned media were cultured with K562 (erytroleukemia cell line) at 2:1 effector-target ratio, HEK-293T (embryonic kidney cell line) at 5:1 effector-target ratio or JAR (choriocarcinoma cell line) at 5:1 effector-target ratio. Cells were incubated with K562 in a U-bottom 96 well plate, with HEK-293T in a 48 well plate or with JAR in a 48 well plate for 4 hours, in the presence of a PE-labeled CD107a antibody (BD Pharmigen), or its isotype control (mouse $IgG1_k$-PE BD Pharmigen). CD107a is a lysosomal-associated membrane protein 1, also called LAMP-1. It is expressed at the inner membrane of NK cell cytolotic granules and it is exposed on the plasma membrane after NK cell degranulation. Consequently, the presence of CD107a positivity on the surface of NK cells is indicative of cell degranulation and cytoxicity. Concerning NK cell-macrophage direct co-cultures, 2:1 effector-target ratio K562, JA3 (Jurkat leukemia cell line), MOLT-4 (acute lymphoblastic leukemia cell line), Raji (Burkitt's lymphoma cell line) or 221 (B-lymphoblastoid cell line) were incubated for 4 hours directly into NK cell-macrophage co-cultures, together with PE-labeled CD107a antibody, or its isotype control. Thereafter, cells were washed, stained with CD56 and CD3 antibodies (to gate on NK cells) and incubated in FACS buffer for 20 minutes at 4°C. Cells were then washed and analyzed by flow cytometry, without fixing them. Importantly, for each assay and for both approaches, we evaluated NK cell degranulation in

absence of target cells, as negative control.

9. Immunofluorescence analysis

NK cell treated with macrophage-conditioned media or cytokines were attached to poly-L-lysin solution (Sigma) coated cover glasses (Thermo-Scientific). Then, they were fixed using 4% Paraformaldehyde (Merck) $PBS^{+/+}$ (with Ca^{2+} and Mg^{2+}, Biosera) for 10 min and washed. A blocking solution containing 2% Bovin Serum Albumin (Sigma) and 5% donkey serum (Sigma) were next added. After 1 hour, NK cells were incubated with unlabeled primary antibody for 1h (Table 5), or its corresponding isotype control. NK cells were then washed with 0,05% Tween 20 (Merck) $PBS^{+/+}$ and stained with labeled secondary antibody (Table 5). One hour later, NK cells were extensively washed and further stained with the nuclear dye DAPI (Invitrogen) for 10 minutes. Finally, after repeated washes, cover glasses where mounted on glasses (Waldermar Knittel) with Fluor-preserve Reagent (Calbiochem) and analyzed by Olympus FV1000 confocal microscopy. Concerning macrophage immunofluorescence analysis, $CD14^{+}$ monocytes were plated directly on cover glasses, differentiated into resting macrophages and then polarized, as described before. They were extensively washed before being fixed and stained, following the same protocol used for NK cells. Table 5 lists primary and secondary antibodies we utilized. It is important to underline that both NK cells and macrophages were not permeabilized, in order to detect only membrane associated protein.

Table 5. Primary and secondary antibodies for immunofluorescence analysis

Primary Antibody	**Primary Antibody specie**	**Company**	**Secondary Antibody specie**	**Secondary Antibody fluorochrome**	**Company**
IL-1R I (2,5 µg/ml)	Goat anti-human	R&D	Donkey anti-goat	AF488	Invitrogen
IL-15Rα (5 µg/ml)	Goat anti-human	R&D	Donkey anti-goat	AF488	Invitrogen

10. Real-time PCR

Total RNA was extracted from NK cells treated with macrophage-conditioned media, polarized macrophages treated or not with NK cell-derived supernatants and HEK-293T or JAR (miRNeasy mini kit, Qiagen). RNA was dosed (Nanodrop) and it resulted always of good quality according to the A260/A280 ratio (max ratio of 2). Then, it was converted in cDNA (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Genes analyzed were IFNγ, IL1R1, IL15 and IL15RA for NK cells (Table 6 a and b). Macrophages were analyzed for the level of expression of IL1β, IL15, IL15RA, CD80, CD206, ALOX15, CCL5, CCL17, CCL18, CCL22, CXCL9 and CXCL10 mRNA (Table 6 a and b). HEK-293T and JAR were evaluated for MLL5 and NKp44L expression (Table 6 a). Results were normalized on the amount of s18 for NK cell analysis, on the amount of GAPDH for macrophage analysis and on the amount of β-actin for HEK-293T and JAR analysis. Each of these genes represented the best housekeeping founded for each cell type. Reactions were performed following the recommended protocols for SYBR green Master Mix (Applied Biosystems) or Taq-man Fast Advanced Master Mix (Applied Biosystems). Data were analyzed by SDS 2.4 Software when 7900HT Fast Real Time PCR System (Applied Biosystems) was used or by Opticon Monitor 3 when we utilized PTC-200 PCR (MJ Research). Results were expressed as $2^{-(\Delta Ct)}$. Ct represents the intersection between an amplification curve and a threshold line. It is the relative measure of the concentration of target in a PCR reaction. ΔCt is the difference between the Ct of the gene of interest and the Ct of an housekeeping expressed by the same sample. IFN-γ transcription during time-course experiments, was defined as fold change versus untreated NK cells. It means that we plotted the $2^{-(\Delta\Delta Ct)}$. ΔΔCt represents the difference between the ΔCt of IFN-γ expressed by treated NK cells and the ΔCt of IFN-γ expressed by untreated NK cells. As a consequence, we set to 1 the level of IFN-γ expression by untreated NK cells. Each reaction was performed in triplicate.

Table 6 a. Primers for sybr green RT-PCR

Gene	forward	reverse
CD80	GGGAAAGTGTACGCCCTGTA	GCTACTTCTGTGCCCACCAT
CD206	GGGCAGTGAAAGCTTATGGA	CCTGTCAGGTATGTTTGCTCA
ALOX15	CTTGCTCTGACCACACCAGA	GCTGGGGCCAAACTATATGA
IL1β	AGTCTGCCCAGTTCCCCAAC	GTTATATCCTGGCCGCCTTTG
IFNγ	CTCTTGGCTGTTACTGCCAGG	CTCCACACTCTTTTGGATGCT
CCL5	TATTCCTCGGACACCACACC	ACACACTTGGCGGTTCTTTC
CCL17	CACCCCAGACTCCTGACTGT	CCCTCACTGTGGCTCTTCTT
CCL18	GTGGAATCTGCCAGGAGGTA	CCCAGCTCACTCTGACCACT
CCL22	GCCGTGATTACGTCCGTTAC	CGGCACAGATCTCCTTATCC
CXCL9	TTTTCCTCTTGGGCATCATC	TCAATTTTCTCGCAGGAAGG
CXCL10	AGCCAATTTTGTCCACGTGT	TGATGGCCTTCGATTCTGGA
MLL5	GGTTCAAGGACCTCAGCAGG	ATGCCACCCTGACCCATGGTA
NKp44L	GCTTCCGAATAAGTGAGTCAA	GCTACTACATTGCTTCCCACA
18s	CCGCAGCTAGGAATAATGGAATA	CGAAAACCAACAAAATAGAACCG
GAPDH	GATCATCAGCAATGCCTCCT	TGTGGTCATGAGTCCTCCCA
βactin	CCCAAGGCCAACCGCGAGAAGAT	GTCCCGGCCAGCCAGGTCCAG

Table 6 b. Taq-man Assays

Gene	Reference	Company
IL1β	Hs01555410_m1	Applied Biosystems
IL2	Hs00174114_m1	Applied Biosystems
IL15	Hs01003716_m1	Applied Biosystems
IL1R1	Hs00991002_m1	Applied Biosystems
IL15RA	Hs00542604_m1	Applied Biosystems
GAPDH	Hs99999905_m1	Applied Biosystems
18s	Hs99999901_s1	Applied Biosystems

11. Statistical analysis

Results were expressed as mean ± SEM from multiple independent experiments. n is referred to the numbers of independent donors. Statistical analysis were performed by using Prism (Graphpad) and/or Excel (Microsoft) software. Two tailed Student t test were performed with 95% confidence intervals and p values of < 0,05 were considered to be significant. *= p<0,05; **=p<0,01; ***=p<0,001.

RESULTS

1. Thawed NK cell characterization and macrophage polarization analysis

In order to set up an autologous system, we isolated human $CD14^+$ monocytes and human NK cells from the same buffy coat of an healthy donor. To obtain resting macrophages, monocytes were cultured for 7 days with M-CSF supplemented medium and then polarized to M1, through LPS and IFN-γ stimulation, or to M2, through IL-4 stimulation (24h). We controlled macrophages polarization by FACS, evaluating CD80, CD206 and CD209 expression. CD80 is a co-stimulatory molecule of MHC class II, strongly associated with M1 phenotype. CD206 is the mannose receptor 1 and CD209 is a C-type lectin receptor. Both of them are induced in M2 phenotype. As expected, our M1 macrophages increased CD80 levels (Figure 1 A) compared to M0 or M2, whereas M2 macrophages were characterized by higher expression of CD206 (Figure 1 B) and CD209 (Figure 1 C) compared to M0 and M1.

Fresh NK cells were frozen immediately after their isolation and were thawed 24h before performing the experiments. The viability of NK cells after thawing resulted around 90% (Figure 1 D). Importantly, fresh and thawed NK cells displayed similar expression of NKG2D (Figure 1 E), which is an activating receptor constitutively expressed by NK cells, and CD69 (Figure 1 F), a well-established activation marker. Moreover, either NKG2D or CD69 levels were much higher on short-term activated NK cells than fresh or thawed NK cells. NK cells we used in the experiments thus displayed a very good viability and were not activated by the “freeze-thaw” procedure. We therefore considered them as resting NK cells.

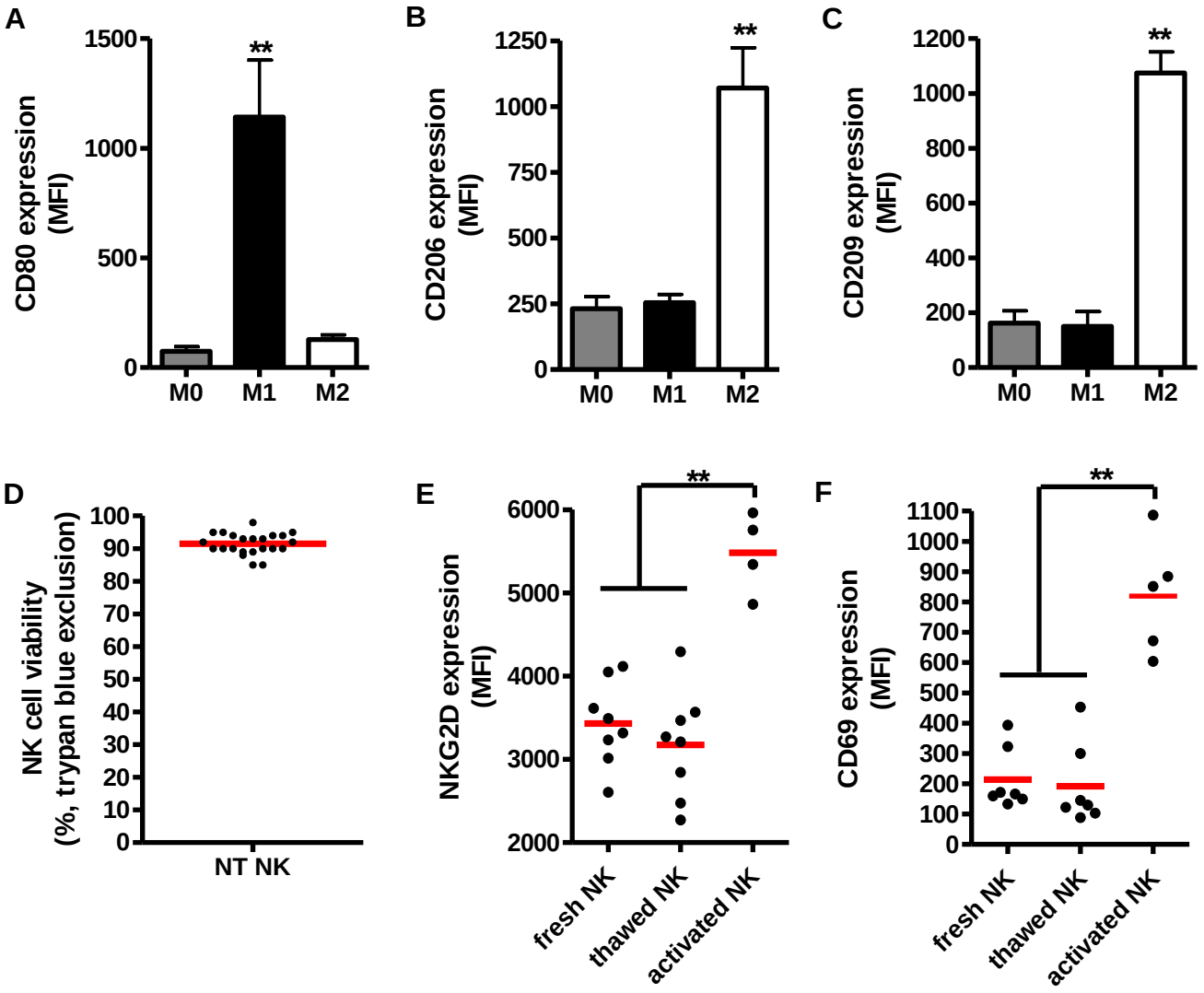


Figure 1. Characterization of human polarized macrophages and thawed NK cells

Isolated $CD14^+$ monocytes were differentiated into resting macrophages by 7 days of M-CSF treatment. Then, they were polarized towards M1 through LPS (100 ng/ml) and rhIFN-γ (20 ng/ml) or towards M2 via IL-4 (20 ng/ml). The expression of CD80 (A), CD206 (B) and CD209 (C) was evaluated by FACS. Data were expressed as Mean Fluorescence Intensity (MFI); n=4. The viability of thawed NK cells were evaluated through trypan blue exclusion (D) and plotted as percentage; n=23. Then, NKG2D (E) and CD69 (F) expression by fresh, thawed and activated (10 ng/ml rhIL-15 and 100 ng/ml rhIL-18 o.n. stimulation) NK cells was assessed by FACS. Results were expressed as MFI; n=4-8. Statistical analysis: Student t test; *= p<0,05; **=p<0,01.

2. Human macrophage-dependent enhancement of NK cell effector functions and activation requires M1 polarization

In order to study the relevance of different polarized macrophages on resting NK cell activities, we set up two different experimental approaches, which allow us to distinguish the effect of macrophage-derived soluble mediators from macrophages-mediated cellular interactions on NK cell effector functions.

First, we treated resting NK cells with 30% conditioned medium from resting macrophages (M0-primed NK), M1 macrophages (M1-primed NK) and M2 macrophages (M2-primed NK). We evaluated IFN-γ production by macrophage-primed NK cells, in terms of mRNA by real-time PCR, and protein secretion by ELISA, in addition to NK cell degranulation by CD107a degranulation assay. In time course-experiments, the transcript levels of IFN-γ were significantly increased in M1-primed NK cells compared to M0 or M2-primed NK cells. The peak of M1-mediated increment of IFN-γ mRNA was observed after 24 hours (Figure 2 A). We then assessed IFN-γ secretion by NK cells. Of note, before collecting the supernatant, we performed a short incubation (4h) of macrophage-primed NK cells with K562 tumor cell line, in order to allow the mobilization of NK cell intracellular granules and the consequent release of stored cytokines (30). In line with what we observed for mRNA, our results showed that the amount of secreted IFN-γ was significantly higher only when NK cells were primed by M1 (Figure 2 B). Finally, we determined whether macrophage soluble mediators modulated also NK cell cytotoxicity. To this end, we performed CD107a degranulation assay in the same above-mentioned experimental setting, using K562 cell as target. Again, we observed that a significant increment of CD56/CD107a positive NK cells occurred only after incubation with M1 conditioned medium compared to that of M0 and M2 (Figure 2 D). This effect was also observed when we used HEK 293T as targets (Figure 2 C), further confirming our previous results.

Next, we evaluated the ability of resting and polarized macrophages to regulate NK cell functions through direct cellular interactions. Similar to what we obtained with macrophage-conditioned media, we found that only the co-culture with M1 macrophages, and not with M0 and M2, induced a significant increase of IFN-γ secretion by autologous NK cells (Figure 3 A). Furthermore, CD107a degranulation assay revealed that autologous NK cells co-cultured with M1 macrophages were more cytotoxic not only towards K562 (Figure 3 B), but also against other tumor target cells,

as JA3, Raji, 221 and Molt4 (Figure 3 C-D-E-F, respectively). Of note, we never detected NK cell degranulation in absence of tumoral target cells (Figure 3 G).

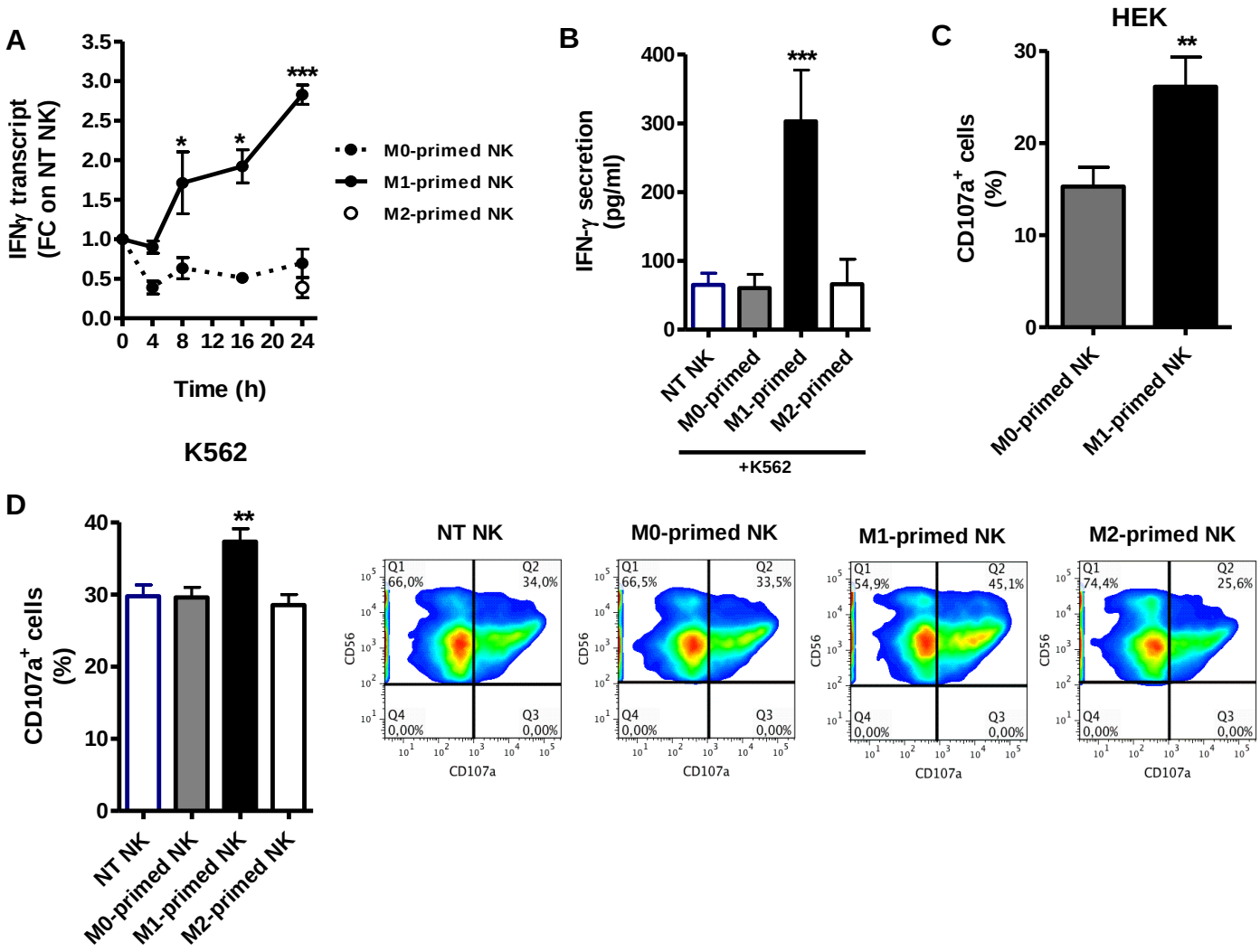
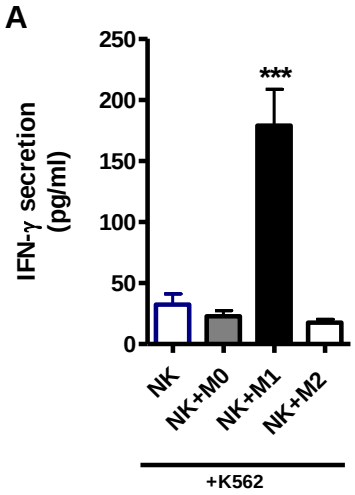
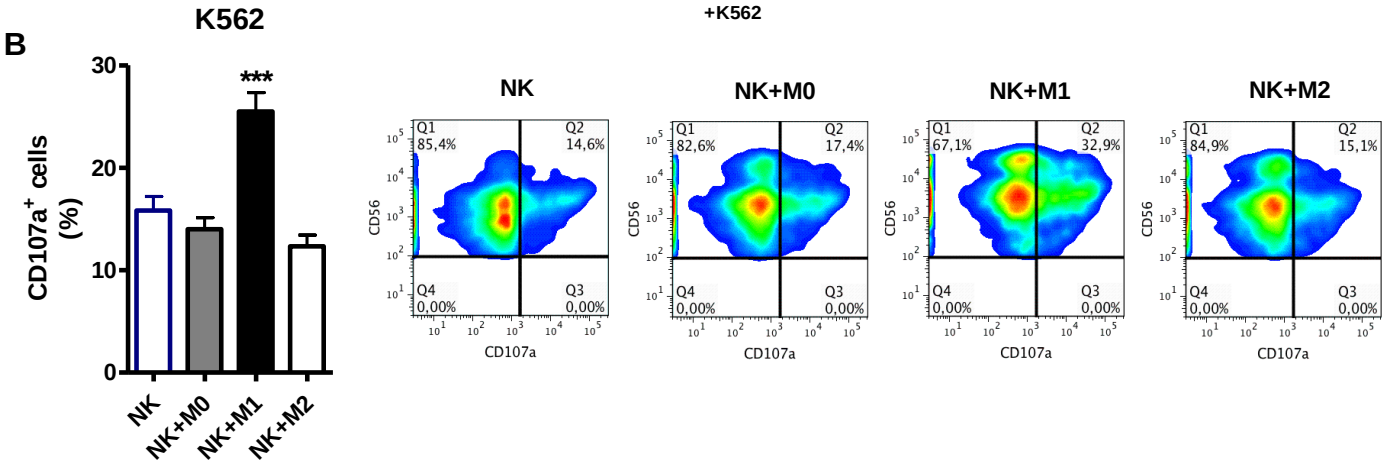


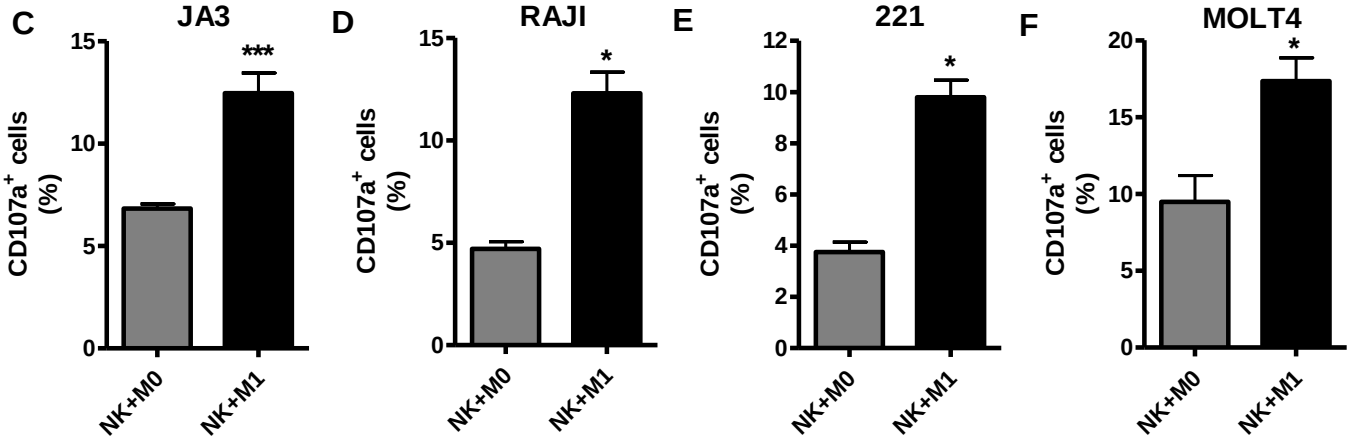
Figure 2. Influence of macrophage-derived soluble factors on NK cell effector functions.

Resting NK cells were treated for 24h with M0 (M0-primed NK), M1 (M1-primed NK)or M2 (M2-primed NK)-conditioned media. Then, NK cell IFN-γ transcription (A) and secretion (B) or NK cell degranulation towards HEK293T (C) and K562 (D) were evaluated. Not treated NK cells (NT NK) represent the negative control. A) IFN-γ mRNA was quantified by RT-PCR in a time-course experiment. Data were expressed as Fold Change versus Not Treated NK (FC vs NT NK) through $2^{-(\Delta\Delta Ct)}$ calculation; n=4. B) IFN-γ secretion by NK cells in the presence of K562 (4h co-culture) was dosed by ELISA. Results were expressed as pg/ml; n=11-20. NK cell degranulation against HEK293T (E:T ratio 5:1, 4h incubation) (C) or K562 (E:T ratio 2:1, 4h incubation) (D) was assessed via CD107a degranulation assay. Data were expressed as percentage (%) of CD56/CD107a positive cells; n=7-18. Statistical analysis: Student t test; *= p<0,05; **=p<0,01; ***=p<0,001.



(Legend on next page)





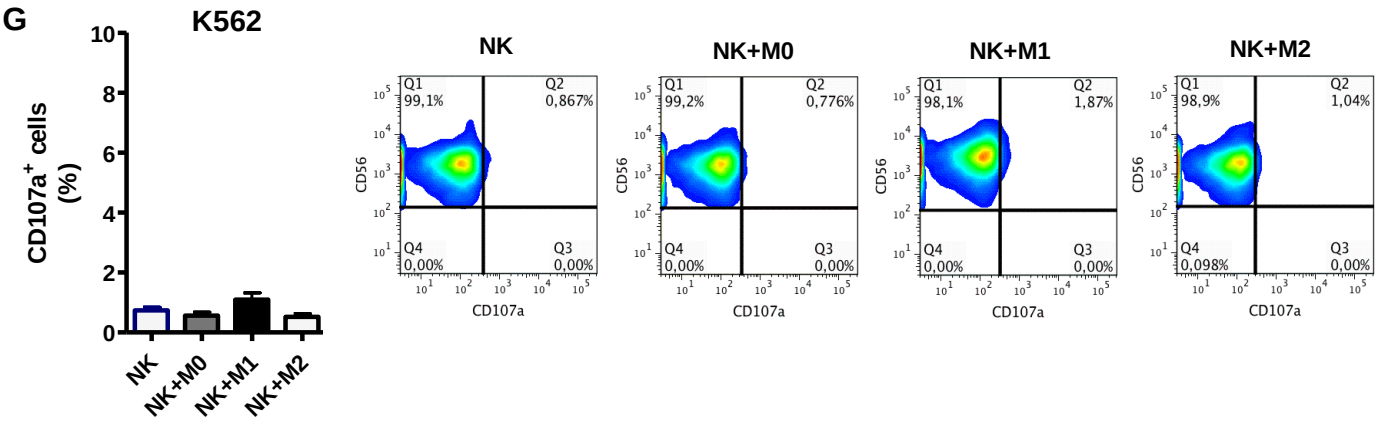


Figure 3. Effects of direct contact interactions between NK cells and macrophages on NK cell activities.

Resting NK cells were co-cultured for 24h with M0 (NK+M0), M1 (NK+M1)or M2 (NK+M2). Then, NK cell IFN-γ secretion (A) or NK cell degranulation either in the presence (B-C-D-E-F) or in absence (G) of target cells were evaluated. NK cells cultured with RPMI (NK) represent the negative control. A) IFN-γ secretion by NK cells in the presence of K562 (4h co-culture) was dosed by ELISA. Results were expressed as pg/ml; n=10. NK cell degranulation against K562 (E:T ratio 2:1, 4h incubation) (B), JA3 (E:T ratio 2:1, 4h incubation) (C), RAJI (E:T ratio 2:1 , 4h incubation) (D), 221 (E:T ratio 2:1, 4h incubation) (E) and MOLT4 (E:T ratio 2:1, 4h incubation) (F) was assessed via CD107a degranulation assay. Data were expressed as percentage (%) of CD56/CD107a positive cells; n=4-20. G) NK cell degranulation in absence of target cells was determined via CD107a degranulation assay. Results were expressed as percentage (%) of CD56/CD107a positive cells; n=4. Statistical analysis: Student t test; *= p<0,05; ***=p<0,001.

Finally, as expected, we found that surface levels of CD69 was significantly increased either on M1-primed NK cells or NK cell co-cultured with M1 macrophages compared to M0-primed NK cells and NK cell co-cultured with M0 macrophages (Figure 4 A and B respectively). We noted also that CD69 up-regulation was more pronounced on M1-primed NK cells, suggesting that soluble mediators were stronger inducers of CD69 expression than cell-to-cell interactions.

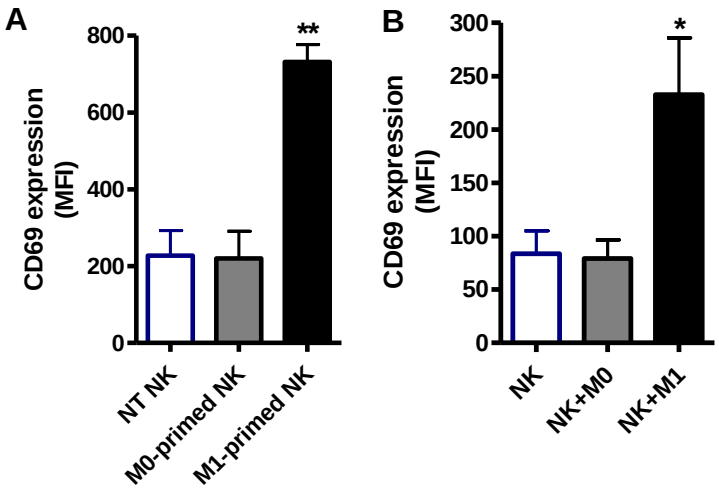


Figure 4. M1 macrophages induce CD69 expression by resting NK cells.

The expression of CD69 by resting NK cells treated with M0 and M1-conditioned media (A) or direct co-cultured with M0 and M1 macrophages (B) was evaluated by FACS. Results were expressed as Mean Fluorescence Intensity (MFI); n=4. Statistical analysis: Student t test; *= p<0,05; **=p<0,01.

Overall we demonstrated that, in the context of the macrophage-NK cell crosstalk, the triggering of resting NK cell activation (CD69 expression) and functions (IFN-γ secretion and degranulation) was mainly mediated by the pro-inflammatory macrophages M1. Furthermore, NK cell activation required either the secretion of soluble mediators or a cell-to-cell contact.

3. M1-released soluble factors modulate NKp44 and NKG2D expression, sustaining NK cell degranulation

Given the ability of M1 macrophages to trigger NK cell degranulation and secretion of IFN-γ, we focused our attention on the cellular mechanisms that may be involved in M1-induced NK cell activation. First, we evaluated whether M1 were able to modulate NK cell activating receptor repertoire. Indeed, once engaged, NK cell activating receptors overcome the signals coming from the inhibitory receptors, favoring NK cell activation. In particular, we focused our attention on NCRs (NKp30, NKp44 and NKp46), and NKG2D, which are the most important activating receptors involved in the recognition of tumor cells. We also considered 2B4, which acts as a co-receptor and participates to IFN-γ secretion, and DNAM-1 which plays a role in metastasis control (53, 55). We analyzed by FACS the expression of these NK cell activating receptors either following incubation with macrophage-conditioned media or following direct co-culture with macrophages. We observed that NKp46, NKp30, DNAM-1 and 2B4 levels were not affected by polarized macrophages (Figure 5 A, B, C, D, E, F, G, H), in both experimental approaches.

On the contrary, NKp44 and NKG2D were upregulated only in M1-primed NK cells (Figure 6 A and C). We did not observe any modulation of these receptors during M1-NK cell co-culture (Figure 6 B and D). These results suggested that cytokines secreted by M1 were involved in the regulation of NKp44 and NKG2D expression. Moreover, we observed comparable levels of induction in NK cell primed with LPS-activated macrophages (M0+LPS-primed NK) (Figure 6 A and C, hatched bars), indicating that LPS-dependent cytokines may mainly be involved in this process. Of note, we can totally exclude a role of exogenous IFN-γ and LPS on NK cells (Figure 6 E and F, green bars) and we further observed that M1-mediated induction of NKp44 or NKG2D was almost comparable to the one observed in IL-2 short-term activated NK cells (Figure 6 E and F, red bars). IL-2 is a classical stimuli which activate NK cells and it is not produced by macrophages, as we assessed by RT-PCR (Figure 6 G and H), representing an informative positive control.

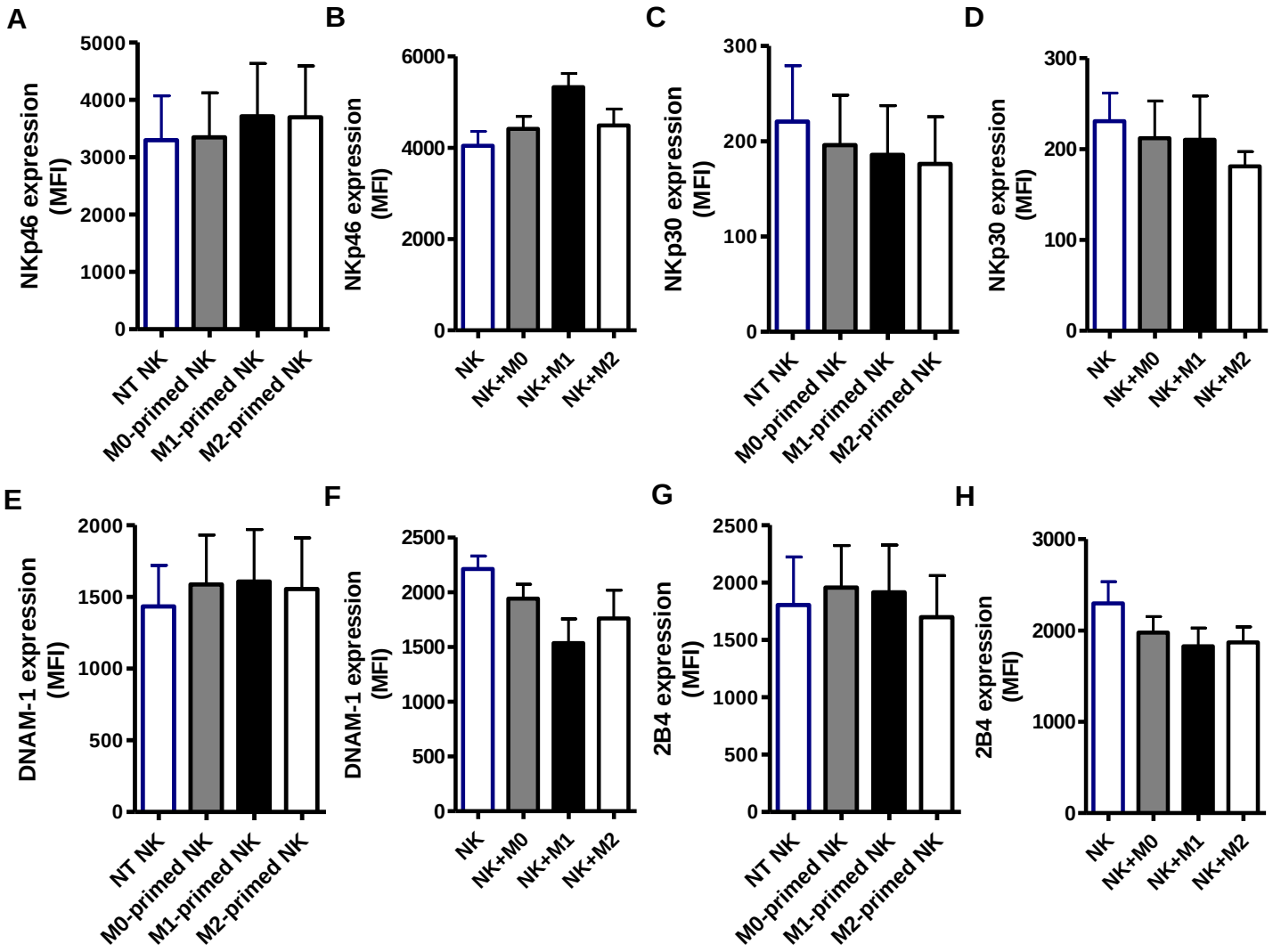
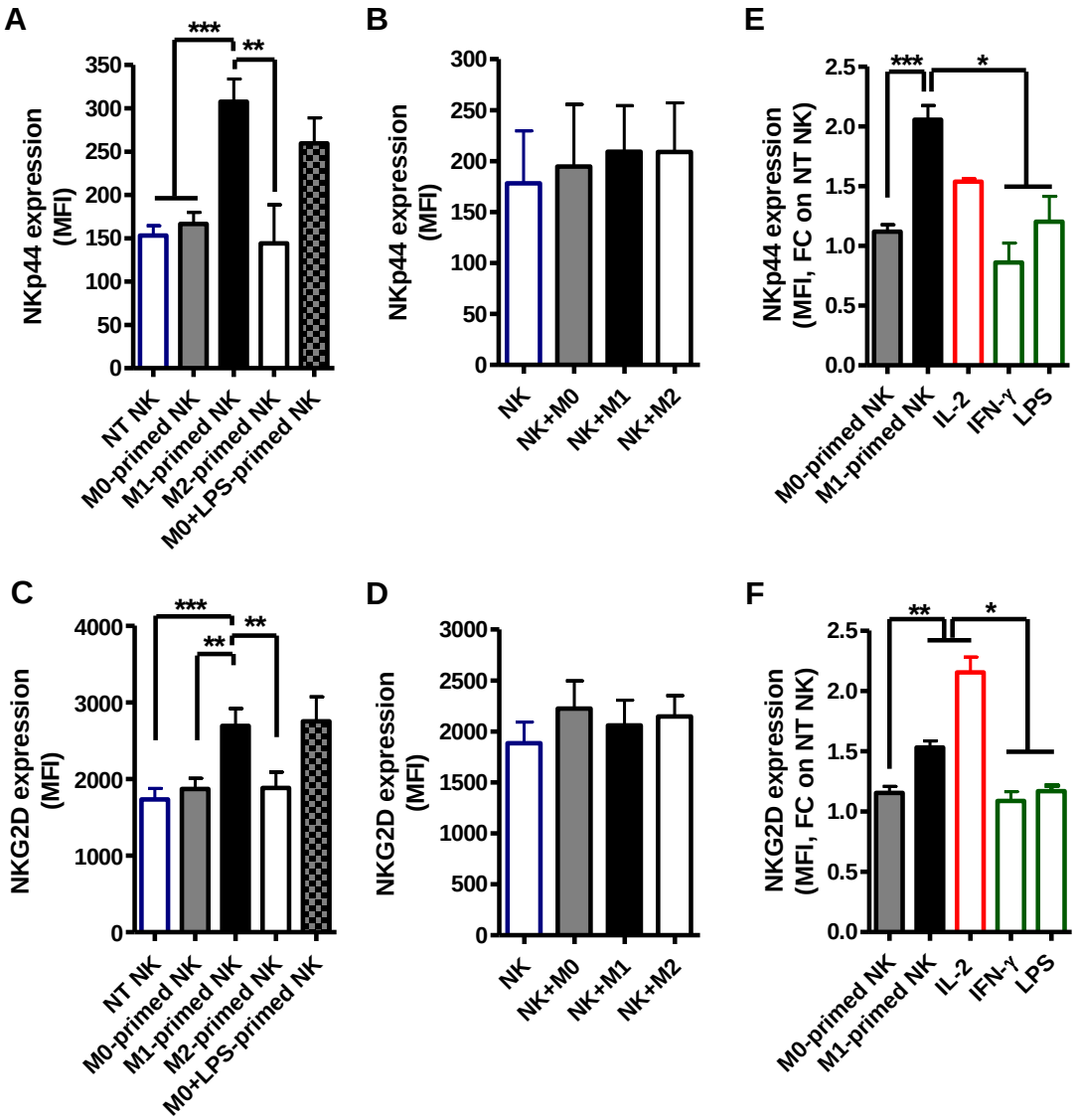
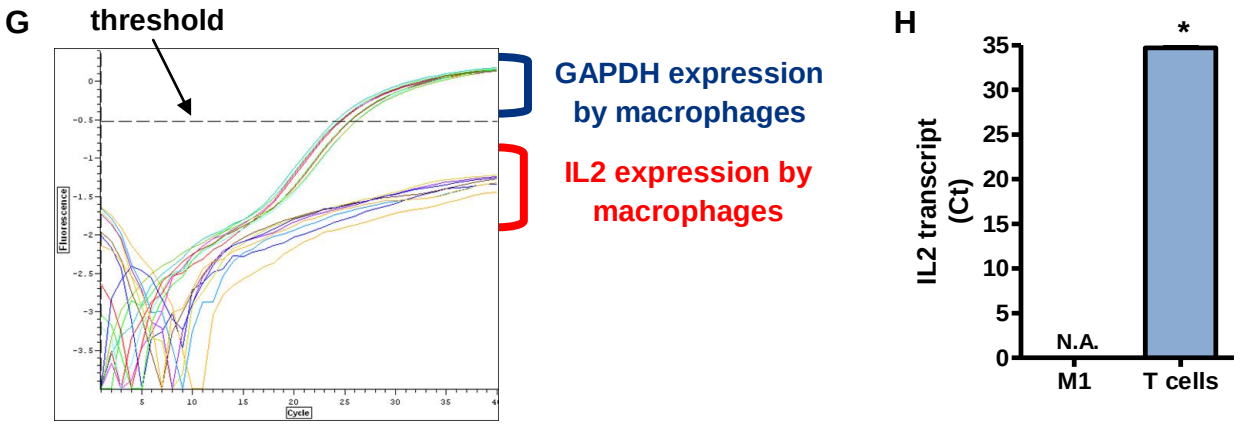


Figure 5. NKp46, NKp30, DNAM-1 and 2B4 expression by NK cells is not modulated by polarized macrophages.

Activating receptor expression by resting NK cells treated with M0, M1 and M2-conditioned media (A, C, E, G) or direct co-cultured with M0, M1 and M2 macrophages (B, D, F, H) was evaluated by FACS. NKp46 expression by macrophage-primed NK cells (A) or NK cells co-cultured with macrophages (B). NKp30 expression by macrophage-primed NK cells (C) or NK cells co-cultured with macrophages (D). DNAM-1 expression by macrophage-primed NK cells (E) or NK cells co-cultured with macrophages (F). 2B4 expression by macrophage-primed NK cells (G) or NK cells co-cultured with macrophages (H). Results were expressed as Mean Fluorescence Intensity (MFI); n=4-5. Statistical analysis: Student t test, no statistic differences were observed.





(Legend on next page)

Figure 6. NKp44 and NKG2D expression is upregulated only in M1-primed NK cells, whereas their expression during direct co-cultured is not modulated.

NKp44 (A) and NKG2D (C) expression by resting NK cells treated with M0 and M1-conditioned media was evaluated by FACS. In parallel, NKp44 (B) and NKG2D (D) expression by NK cells direct co-cultured with M0 and M1 macrophages was evaluated by FACS. Results were expressed as Mean Fluorescence Intensity (MFI); n=6-20. NKp44 (E) and NKG2D (F) expression by M0 (grey bar) and M1-primed NK cells (black bar) were compared to the one of IL-2 activated (200 U/ml, 24h) NK cells (red bar) and LPS or IFN-γ treated (30% of 100 ng/ml LPS and 20 ng/ml IFN-γ supplemented medium) NK cells (green bars). Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values; n=3-14. G) IL2 and GAPDH expression by M1 macrophages was assessed by RT-PCR. Threshold was set to 0,3; n=4. H) IL2 expression by M1 was compared to IL2 expression by T cells, which represents a positive control. Results were plotted as Ct; n=3. Statistical analysis: Student t test; *= p<0,05; **=p<0,01; ***=p<0,001.

In order to establish whether increased levels of NKp44 and/or NKG2D correlated with increased NK cell capability to recognize and kill tumor cells, we searched for target cells bearing ligands for NKG2D and/or NKp44. If NKG2D ligands are well described and are widely expressed by tumor cell lines, NKp44 ligands identity remains elusive. Recently, it has been published that a novel isoform of the mixed-lineage leukemia-5 protein (MLL-5), called NKp44L, preferentially expressed by leukemia cells, represented one NKp44 ligand (60). Interestingly, NKp44L was also found on solid tumor cells, such as the choriocarcinoma cell line JAR, or embryonic cells, such as HEK-293T. Starting from these evidences, we first assessed the expression of MLL5 and NKp44L mRNAs in JAR and HEK-293T cell lines (Figure 7 A and B). JAR or HEK expressed both genes, even if they were significantly more expressed by HEK. We thus hypothesized that HEK were more sensible to NKp44 mediated killing than JAR. It was confirmed by CD107a degranulation assay. Indeed, M1-primed NK cells were not favored to recognize and kill JAR, compared to M0-primed NK cells, even if they expressed higher levels of NKp44 (Figure 7 C). Only when we further induced NKp44 expression by increasing the percentage of M1 conditioned medium to 50% (Figure 7 D), M1-primed NK cells resulted more cytotoxic towards JARs (Figure 7 E, white bars). However, the blocking of NKp44 by a neutralizing antibody revealed that the increment of NK cell degranulation was totally NKp44 independent (Figure 7 E, black bars). It suggested that the level of expression of MLL5 and NKp44L seemed to be crucial to trigger NKp44 mediated killing, convincing us to repeat these experiments on HEK. Of interest, it has been described that HEK expressed NKG2D ligands, in particular the ULBPs family (241). It allowed us to assess at the same time NKp44 and NKG2D involvement in NK cell degranulation. We then proceeded by neutralizing NKp44, NKG2D or both, during macrophage-conditioned medium treatment. We observed that NK cell degranulation was reduced in the presence of NKp44 blocking (Figure 7 F, black bars) and NKG2D blocking (Figure 7 F, gray bars), but the highest reduction was observed when both NKp44 and NKG2D were blocked (Figure 7 F, hatched bars), arguing for a co-operation of these receptors in the recognition of HEKs.

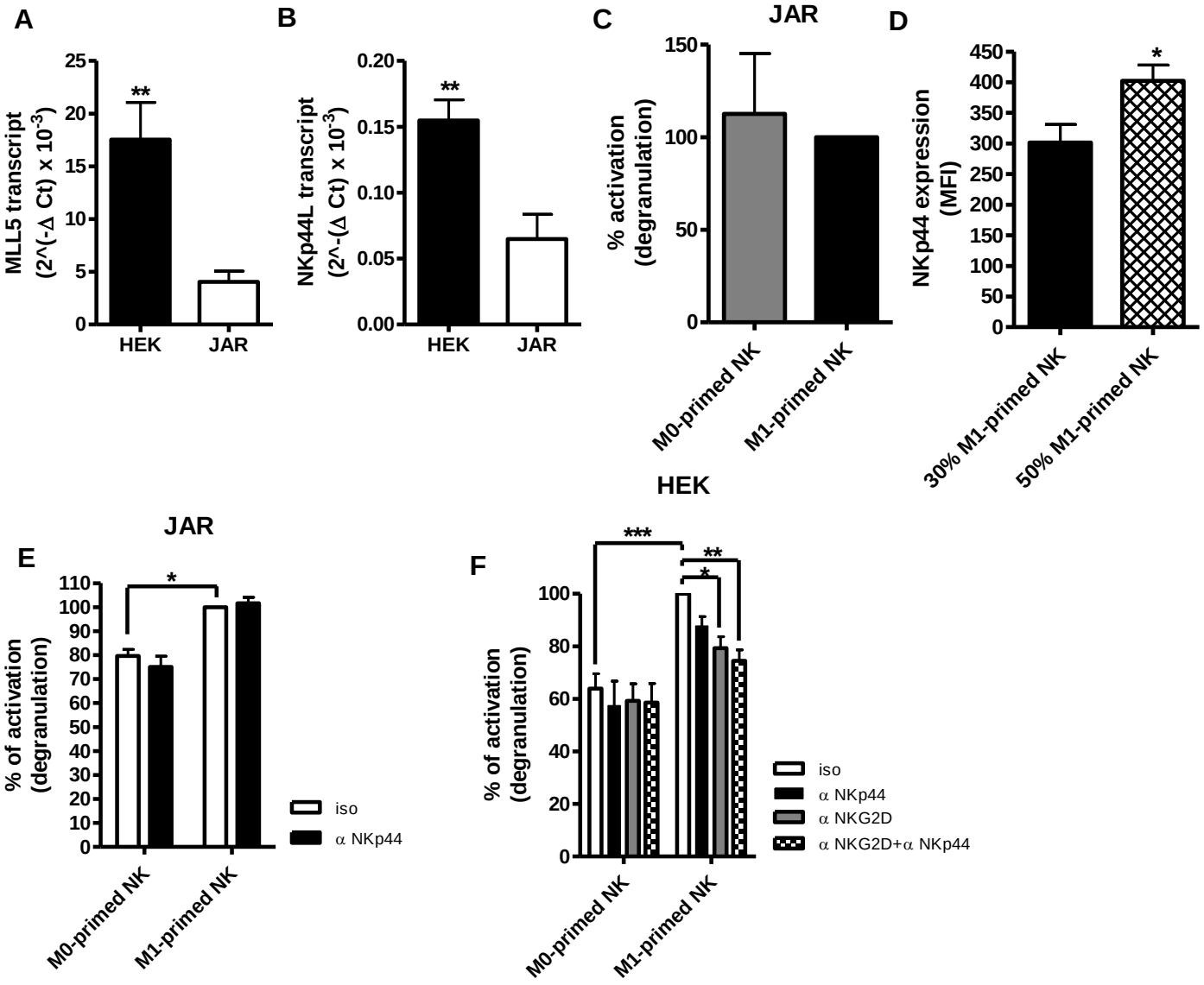


Figure 7. NKp44 and NKG2D upregulation correlates with increased degranulation by M1-primed NK cells.

MLL5 (A) and NKp44L (B) expression by JAR and HEK293T cell lines was determined by RT-PCR. Data were plotted as $2^{-(\Delta Ct)}$; n=3-5. C) Degranulation of 30% M0 and M1-primed NK cells towards JAR (E:T ratio 5:1, 4h incubation) was assessed through CD107a degranulation assay. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=3. D) NKp44 expression by NK cell treated with 30% (black bar) and 50% (hatched bar) M1-conditioned media was detected by FACS. Data were plotted as Mean Fluorescence Intensity (MFI); n=4. E) Degranulation of 50% M0 and M1-primed NK cells towards JAR (E:T ratio 5:1, 4h incubation) assay in the presence of NKp44 blocking antibodies (black bars) or isotype controls (white bars) was assessed through CD107a degranulation. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=4. F) Degranulation of 30% M0 and M1-primed NK cells towards HEK293T (E:T ratio 5:1, 4h incubation) in the presence of blocking antibodies against NKp44 (black bars), NKG2D (grey bars) and both (hatched bars) or isotype controls (white bars) was assessed through CD107a degranulation assay. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=5-10. Statistical analysis: Student t test; *= p<0,05; **=p<0,01; ***=p<0,001.

Taken together these results demonstrate that LPS-inducible cytokines, secreted by M1, are required to modulate NKp44 and NKG2D expression by NK cells, whereas cell-to-cell interactions are not needed. The upregulation of both receptors is similar to the one observed after IL-2 stimulation and it is specifically regulated by M1-derived factors. It is important to underline that NKp44 is expressed at very low levels by resting circulating NK cells and its increase correlates with NK cell activation. As a consequence, NKp44 induction, together with CD69 and NK cell effector function triggering, strongly supports the fact that M1 macrophages are able to fully activate resting NK cells. In addition, we demonstrate that augmented levels of NKp44 and NKG2D correlates with increased degranulation. Indeed, not only they individually contribute to NK cell recognition of altered cells, but they synergize, conferring to NK cell higher cytotoxic potential. In conclusion, M1 macrophages, by inducing NKp44 and NKG2D up-regulation, strongly improve NK cell capability to recognize and kill tumor cells.

4. M1-produced IL-1β regulates NKp44 expression by $CD56^{dim}$ NK cells, which express higher level of IL-1R I compared to their $CD56^{bright}$ counterpart

Having shown that M1 macrophages activate NK cells, we next sought to identify which soluble factors were responsible for NKp44 up-regulation. We screened a panel of candidate cytokines produced by M1 macrophages: IL-18 and IL-23, which have been described to have a role in NK cell activation (162, 182) and IL-15, together with IL-15Rα. IL-15 is a potent regulator of NK cell activities. It displays a very high affinity for its receptor α chain and forms a complex with IL-15Rα, usually anchored to the plasma membrane of IL-15 producing cells. Recently, it has been reported that membrane associated IL-15/IL-15Rα complexes can be cleaved by metallo-proteases and secreted as soluble complexes, also detected in human serum (221). In addition, we considered also IL-1β, that is produced and secreted by M1 macrophages, as we assessed by RT-PCR (Figure 8 A) and ELISA (Figure 8 B). It has been reported that IL-1β plays a role in the expansion of innate lymphoid subsets associated to MALT, that are characterized by high levels of NKp44 expression (183, 184, 186). Considering that M1-primed NK cells expressed relatively high levels of NKp44, we wondered whether IL-1β could be involved, as observed in human tonsils *in vivo*. Interestingly, in

the presence of neutralizing antibodies against all cytokines considered (IL-18, IL-23, IL-15/IL-15Rα), we observed that only IL-1β blocking reduced NKp44 expression on M1-primed NK cells (Figure 8 C), supporting our previous hypothesis. In addition, IL-1R I neutralization and the simultaneous blocking of IL-1β and IL-1R I diminished NKp44 levels, further confirming that the IL-1β/IL-1R I axis took part in this process. Surprisingly, although M1-primed $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets upregulated NKp44 to similar extent, we found that only $CD56^{dim}$ NK cells responded to IL-1β and IL-1R I neutralization (Figure 8 D and E). It appeared that NKp44 modulation on $CD56^{dim}$ NK cells was totally regulated by IL-1β/IL-1R I. It is important to underline that $CD56^{dim}$ is more abundant than $CD56^{bright}$ population, which represents 80-90% of all circulating NK cells. This is the reason why we were able to appreciate a sensibility to IL-1β also when we considered all $CD56^{positive}$ cells.

To further extend these results, we verified whether NK cells, treated or not with macrophage-conditioned media, effectively expressed IL-1R I. Indeed, up to date, circulating NK cells are considered almost totally insensitive to IL-1β, since they have been evaluated as IL-1R negative cells. On the contrary, we were able to show that resting NK cells express IL-1R I at the mRNA level (Figure 9 A), and also on their surface, by FACS (Figure 9 B) and confocal microscopy (Figure 9 D). In addition, we also observed an induction of IL-1R I expression in M1-primed NK cells (Figure 9 A and B, black bars). Of note, neither untreated NK cells nor M0 or M1-primed NK cells expressed IL-1 R II, which plays an antagonistic role respect to IL-1R I, contributing to switching off the signal mediated by IL-1β (Figure 9 C). Furthermore, when we distinguished $CD56^{bright}$ NK cells from $CD56^{dim}$ NK cells, we observed that only the $CD56^{dim}$ population significantly upregulated IL-1R I in the presence of M1-conditioned medium (Figure 10 B and D), since the levels of IL-1R I in the $CD56^{bright}$ subset remained stable during the treatment (Figure 10 A and C).

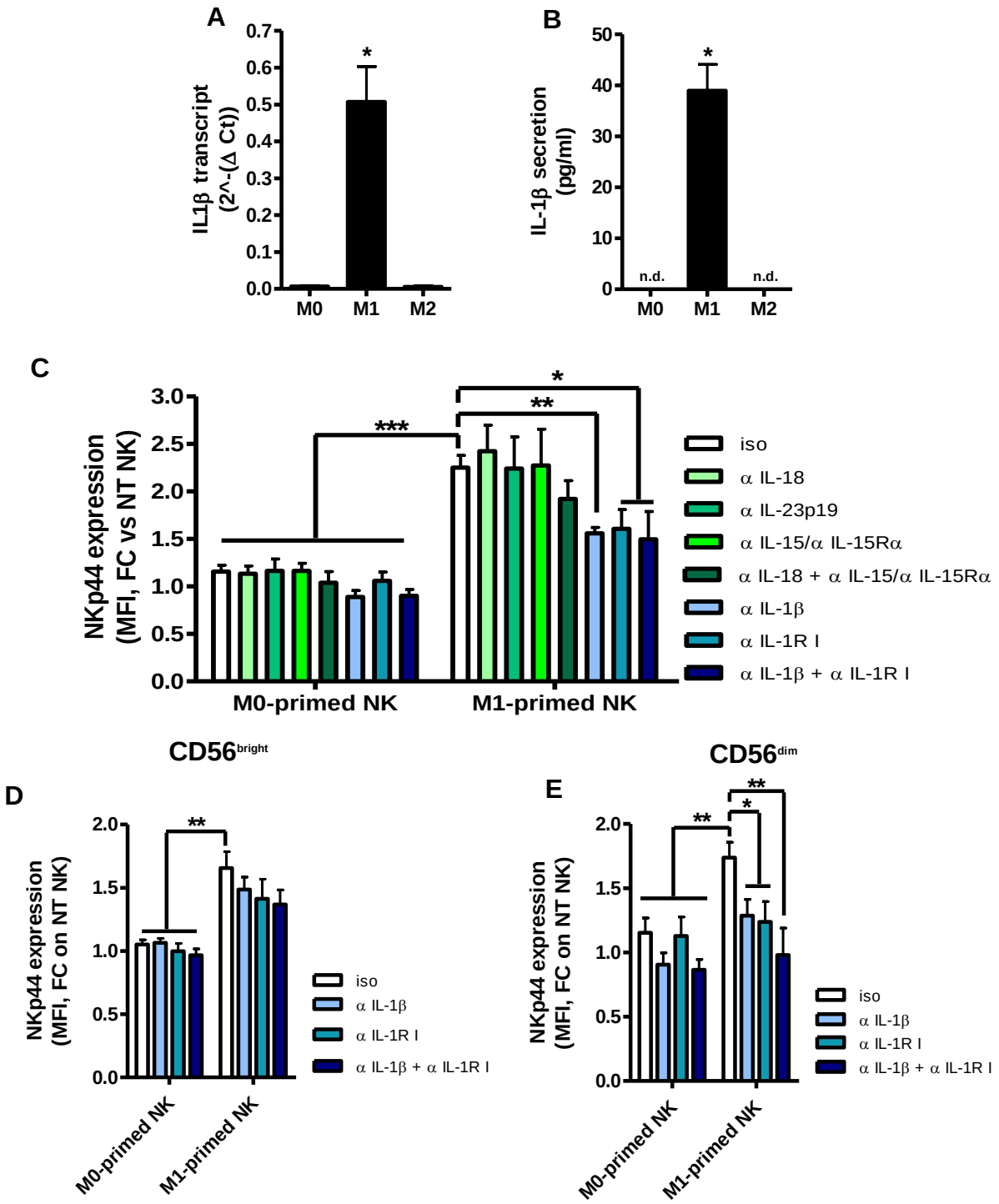
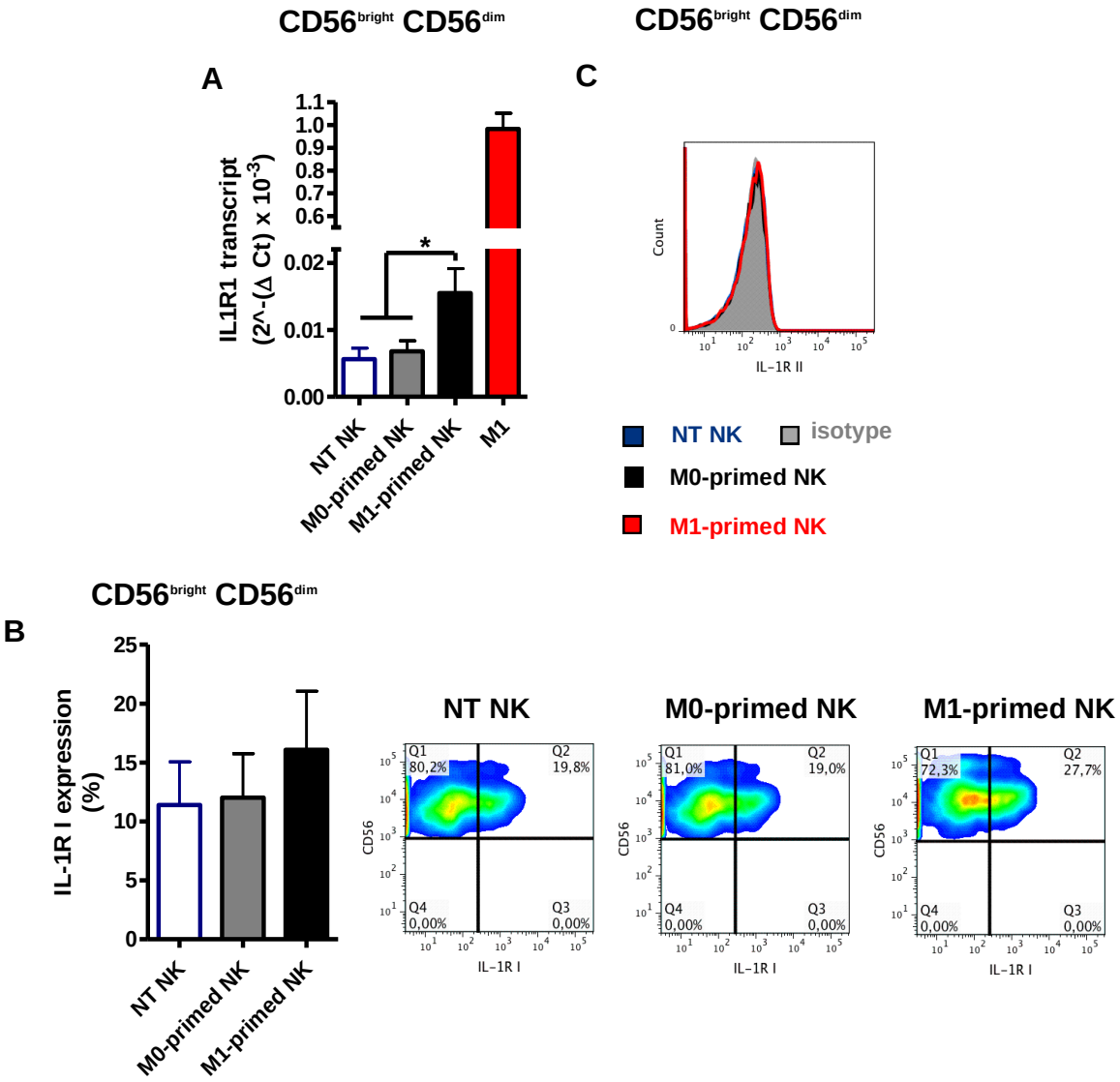


Figure 8. IL-1β secreted by M1 regulates NKp44 expression by CD56^{dim} NK cells.

IL-1β mRNA levels (A) and IL-1β secretion (B) by M1 macrophages were evaluated through RT-PCR and ELISA. Data were plotted as $2^{-(\Delta Ct)}$ or as pg/ml respectively; n=4. C) NKp44 expression by CD56^{positive} M0 and M1-primed NK cells in the presence of IL-18, IL-23p19, IL-15/IL-15Rα, IL-18+ IL-15/IL-15Rα,IL-1β, IL-1R I and IL-1β+IL-1R I blocking antibodies (colored bars) or isotype controls (white bar) was assessed by FACS. Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values; n=4-10. NKp44 expression by CD56^{bright} M0 or M1-primed NK cells (D) and CD56^{dim} M0 or M1-primed NK cells (E) in the presence of blocking antibodies against IL-1β (light blue bars), IL-1R I (sky blue bars) and both (blue bars) or isotype controls (white bars) was assessed by FACS. Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values; n=7. Statistical analysis: Student t test; *= p<0,05; **=p<0,01; ***=p<0,001.

To summarize, we demonstrate that IL-1β secreted by M1 macrophages acts on CD56^{dim} NK cells, induces their activation and leads to NKp44 upregulation. The increment of NKp44 expression mediated by IL-1β on the CD56^{dim} population, historically defined as the "cytotoxic subset", favors NK cell capability to recognize tumoral cells, increasing their degranulation. CD56^{dim} NK cells result more sensitive to M1-produced IL-1β than CD56^{bright}, because they express higher levels of IL-1R I in response to M1-conditioned medium treatment. We evidence that circulating NK cells express IL-1R I and become sensitive to IL-1β, thanks to IL-1R I up-regulation.



(figure continued on next page)

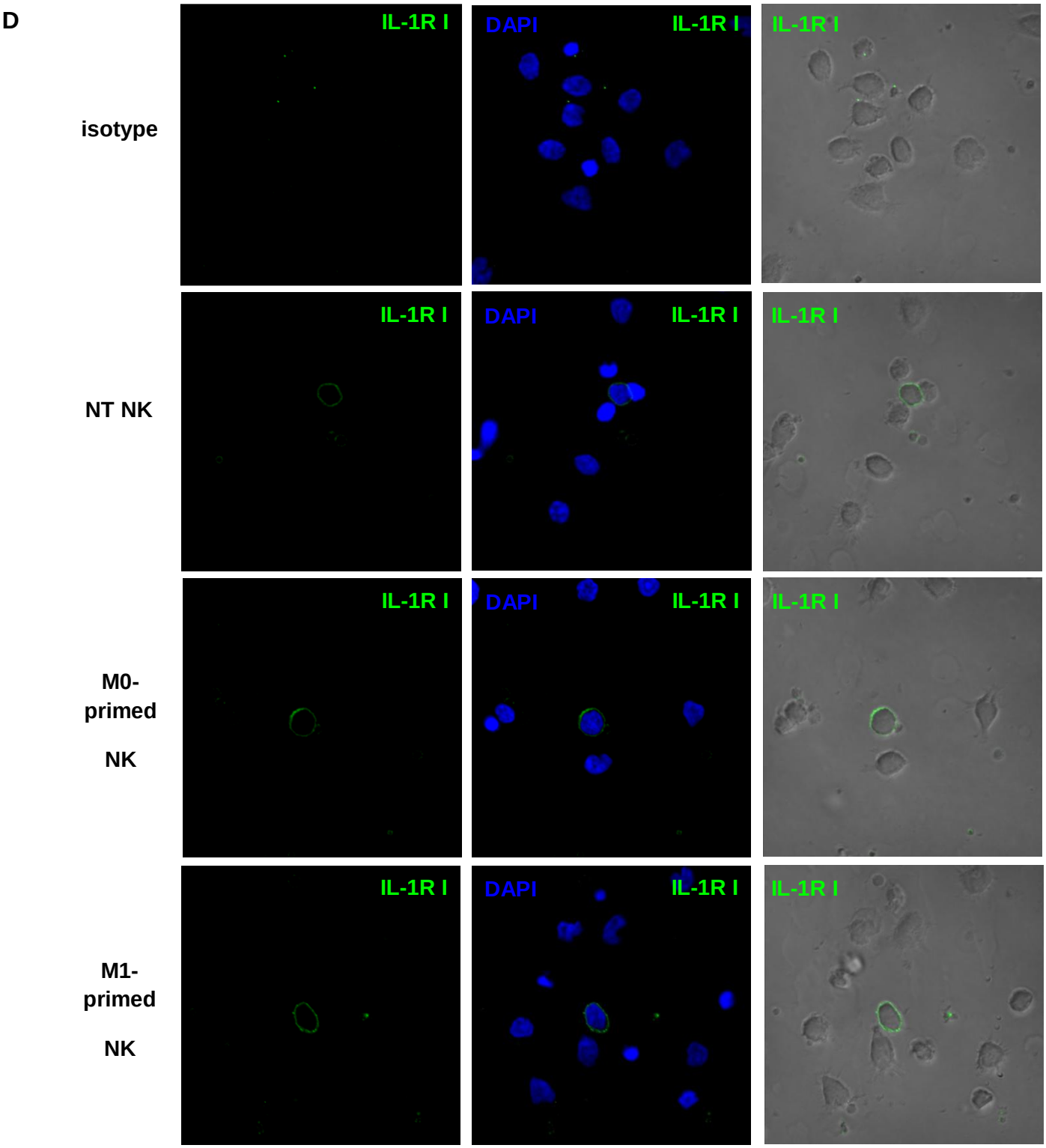


Figure 9. Human blood NK cells treated or not with macrophage-conditioned media express IL-1R I but not IL-1R II.

A) IL-1R I mRNA levels in resting, M0 or M1-primed NK cells and M1 macrophages were quantified by RT-PCR. Data were expressed as $2^{-(\Delta Ct)}$; n=4-6. B) IL-1R II expression by $CD56^{positive}$ resting, M0 and M1-primed NK cells were assessed by FACS. The expression of IL-1R II is representative of one donor between three performed. C) IL-1R I expression by $CD56^{positive}$ resting, M0 and M1-primed NK cells was assessed by FACS. Results were plotted as percentage (%) of CD56/IL-1R I positive cells ; n=5. D) IL-1R I surface expression by $CD56^{positive}$ resting, M0 and M1-primed NK cells was determined by immunofluorescence analysis. Voltages were set on isotype control, performed on M1-primed NK cells. Plots were representative of one donor between three performed. Statistical analysis: Student t test; *= p<0,05.

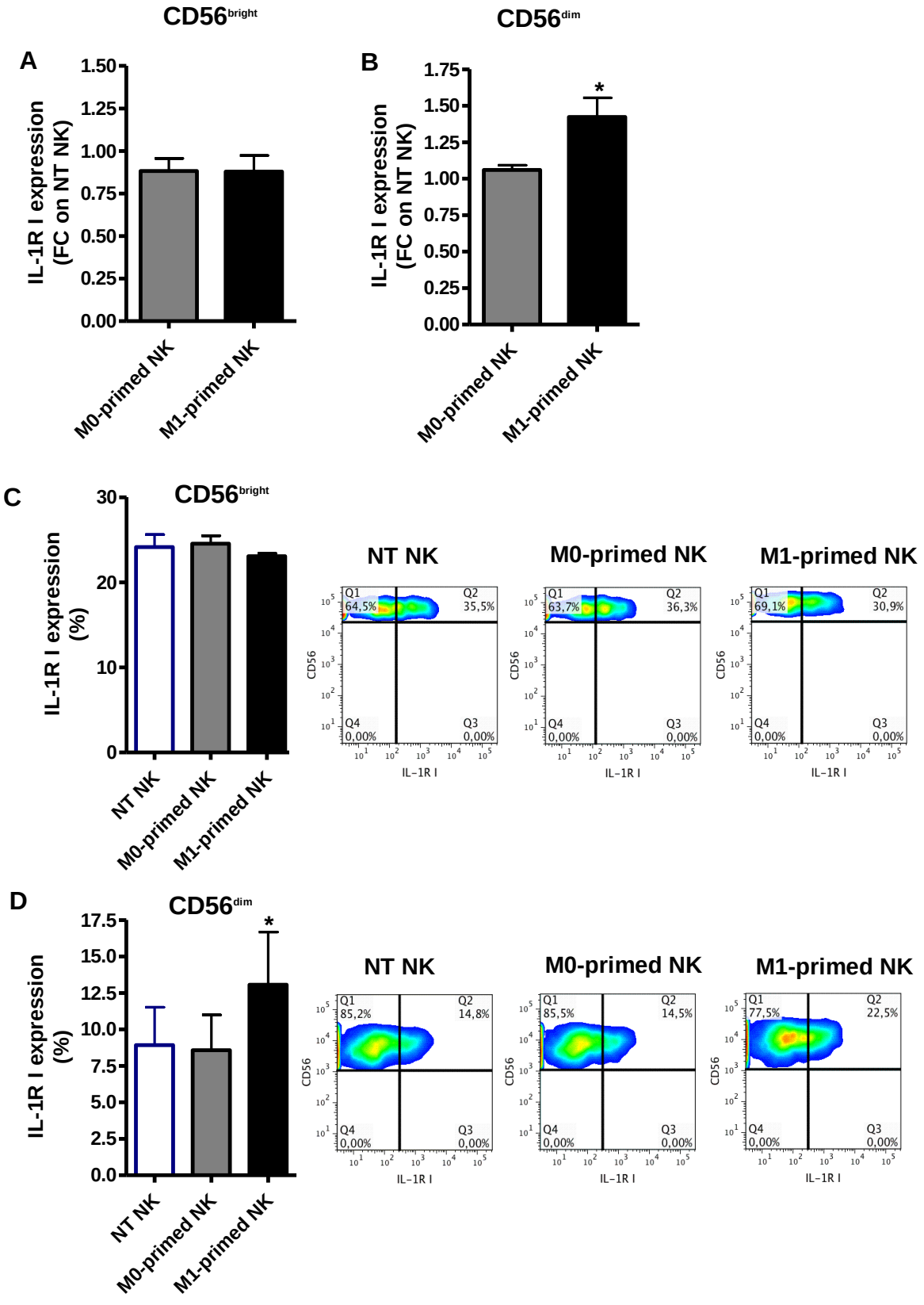


Figure 10. IL-1R I expression by CD56^{dim} NK cells is induced in the presence of M1-conditioned medium.

IL-1R I expression by CD56^{bright} (A and C) and CD56^{dim} (B and D) resting, M0 or M1-primed NK cells was assessed by FACS. Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values (A and B) or percentage (%) of CD56^{bright} or CD56^{dim}/ IL-1R I^{positive} cells (C and D); n=4. Statistical analysis: Student t test; *= p<0,05.

5. M1 macrophages induce IL-15Rα and NKG2D expression on NK cells through IFN-β release

We next addressed which soluble factors could regulate NKG2D induction. Again, we short-listed cytokines described to modulate NKG2D expression on NK cells and we found that exogenous recombinant IL-15 and IL-18 are the major responsible of NKG2D upregulation on resting NK cells (Figure 11 A). They have been described to be both produced by M1 macrophages (113, 162), but, differently from IL-18, IL-15 displays a peculiar mechanism of action. As we underlined previously, IL-15 barely works as a free cytokine, but is rather presented to responsive cells loaded on IL-15Rα. In line with what it has been reported recently (221), we hypothesized that IL-15/IL-15Rα complexes anchored to M1 membrane could be cleaved and released in order to allow a soluble trans-presentation to NK cells, favoring NKG2D induction. However, we were never able to detect IL-15/IL-15Rα complexes in the supernatant of M1 macrophages through commercial ELISA kits (Figure 11 B and C). We obtained the same results when we boiled the supernatant in order to disrupt IL-15 binding to IL-15Rα before measuring free IL-15 protein by ELISA (Figure 11 D). This strongly suggested that M1 macrophages did not secrete IL-15/IL-15Rα complexes.

On the other hand, a very recent work published by Zanoni I. et al. highlighted that murine NK cells, after dendritic cell-derived IFN-β stimulation, start to express IL-15 and IL-15Rα on their membrane (192). It leads to an IL-15 cis-presentation to the IL-15Rβγ chain, that permits NK cell activation. On this basis, we wondered whether IL-15 and IL-15Rα expression by NK cells could occur also in our experimental settings. Indeed, as murine dendritic cells, human M1 macrophages are able to produce IFN-β (239). To better understand whether IL-15 cis-presentation occurred in our settings, we assessed IL-15 and IL-15Rα expression by M1-primed NK cells by RT-PCR. We observed that NK cells expressed IL-15 and IL-15Rα transcripts, although at lower levels than M1. Importantly, M1-primed NK cells expressed higher levels of IL-15 and IL-15Rα genes than untreated or M0-primed NK cells (Figure 12 A and B, respectively). Moreover, we were also able to detect IL-15Rα only on the surface of M1-primed NK cells by FACS (Figure 12 C, white bars) and by confocal microscopy (Figure 12 D), further confirming our hypothesis. In addition, we found that IL-15Rα expression was strictly correlated with IFN-β stimulation, as IFN-β blocking by a neutralizing antibody totally prevented IL-15Rα expression by M1-primed NK cells (Figure 12 C, black bars). We additionally observed that resting NK cells stimulated with rhIFN-β expressed IL-15Rα on their membrane, similar to M1-primed NK cells (Figure 12 E).

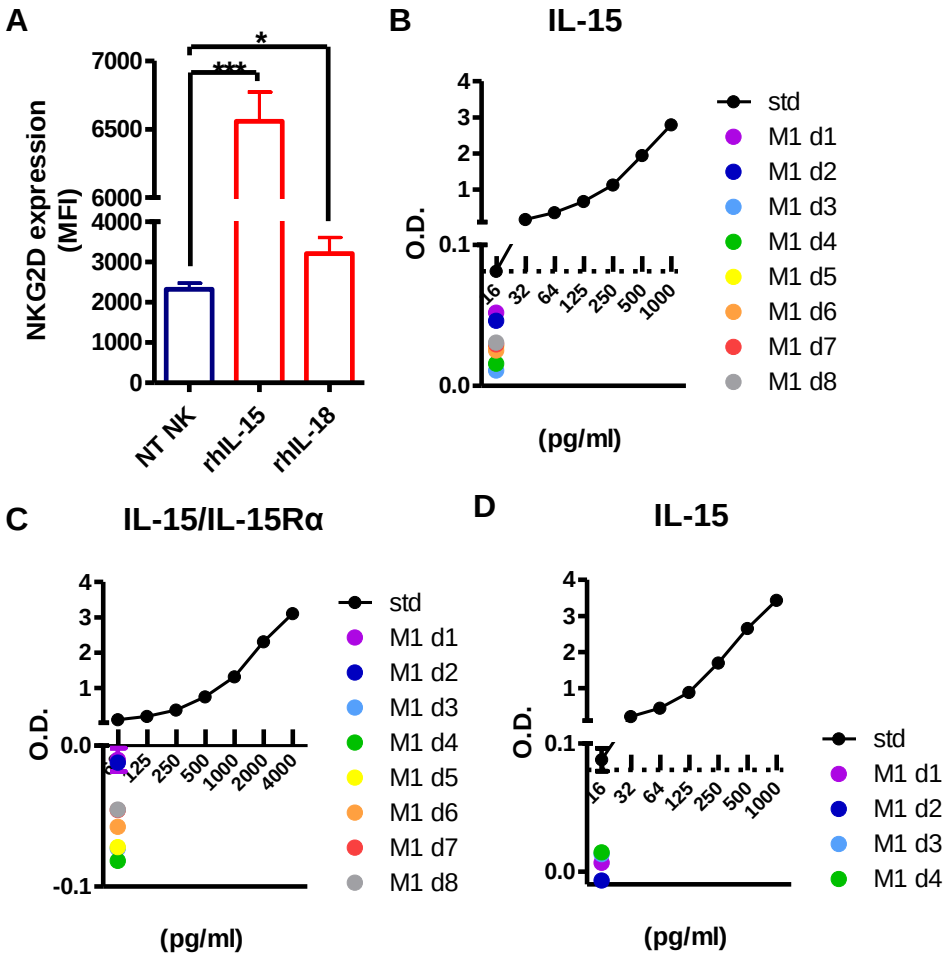
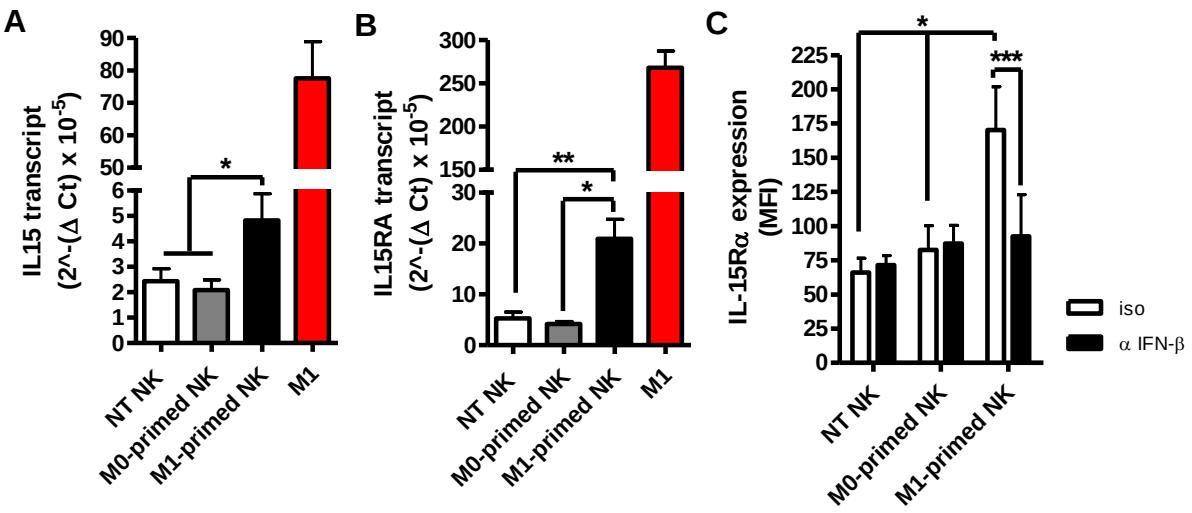
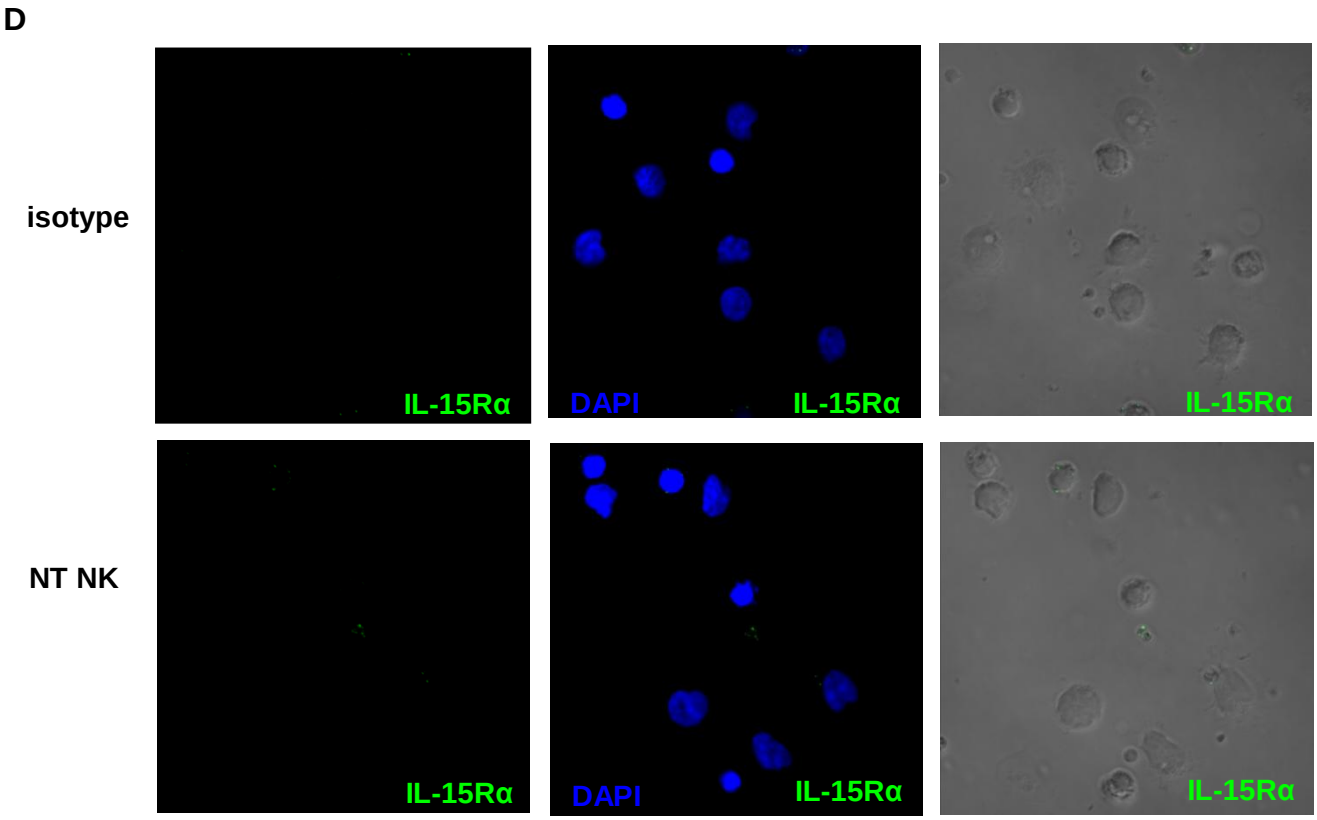


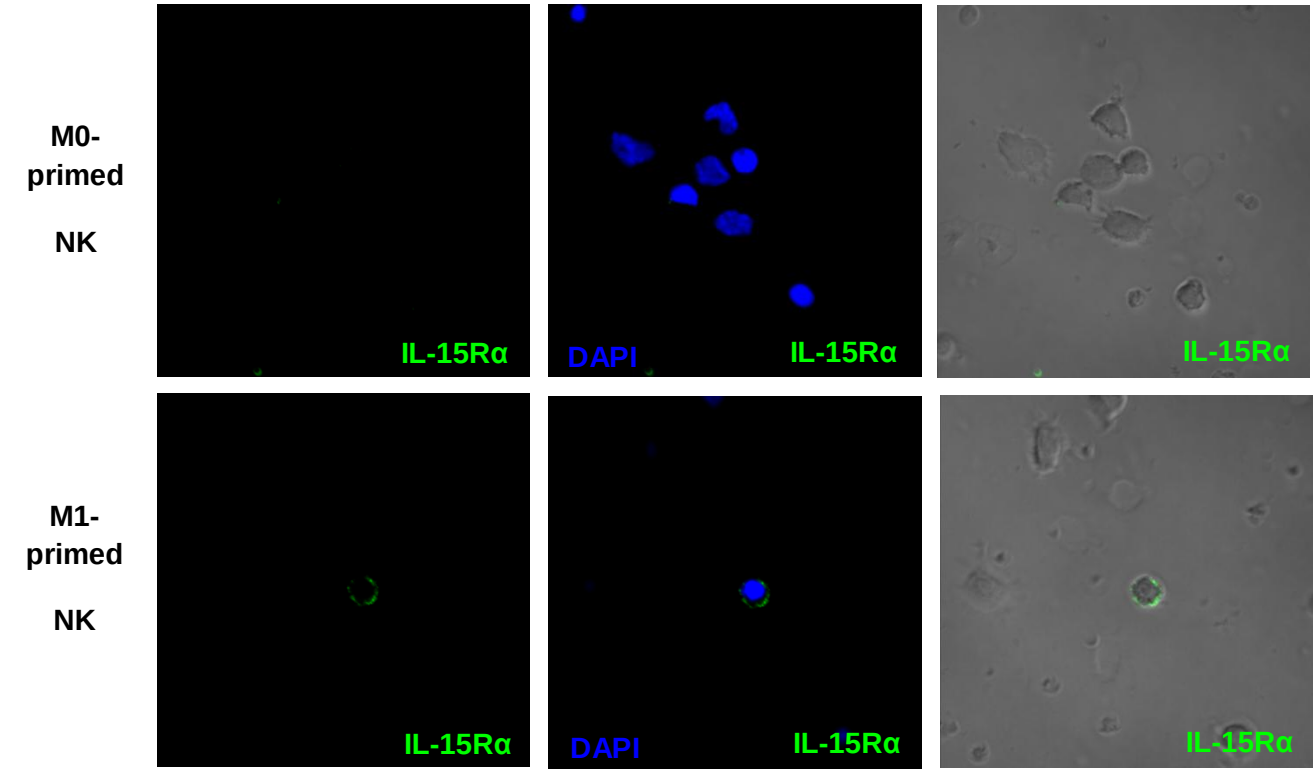
Figure 11. IL-15 is the best NKG2D inducer, however M1 macrophages are not able to secrete it.

A) NKG2D expression by resting NK cells (blue bar) treated with recombinant IL-15 (20 ng/ml) or IL-18 (100 ng/ml) (red bars) was assessed by FACS. Results were expressed as Mean Fluorescence Intensity (MFI); n=4. B) IL-15 was dosed by ELISA in 30% M1-conditioned medium. Data were expressed as Optical Density (O.D.); n=8. C) IL-15/IL-15Rα was dosed by ELISA in 30% M1-conditioned medium. Data are expressed as Optical Density (O.D.); n=8. D) IL-15 was quantified by ELISA in 30% M1-conditioned medium previously incubated 30 min at 37° C. Data were expressed as Optical Density (O.D); n=4. Statistical analysis: Student t test; *= p<0,05; ***=p<0,001.

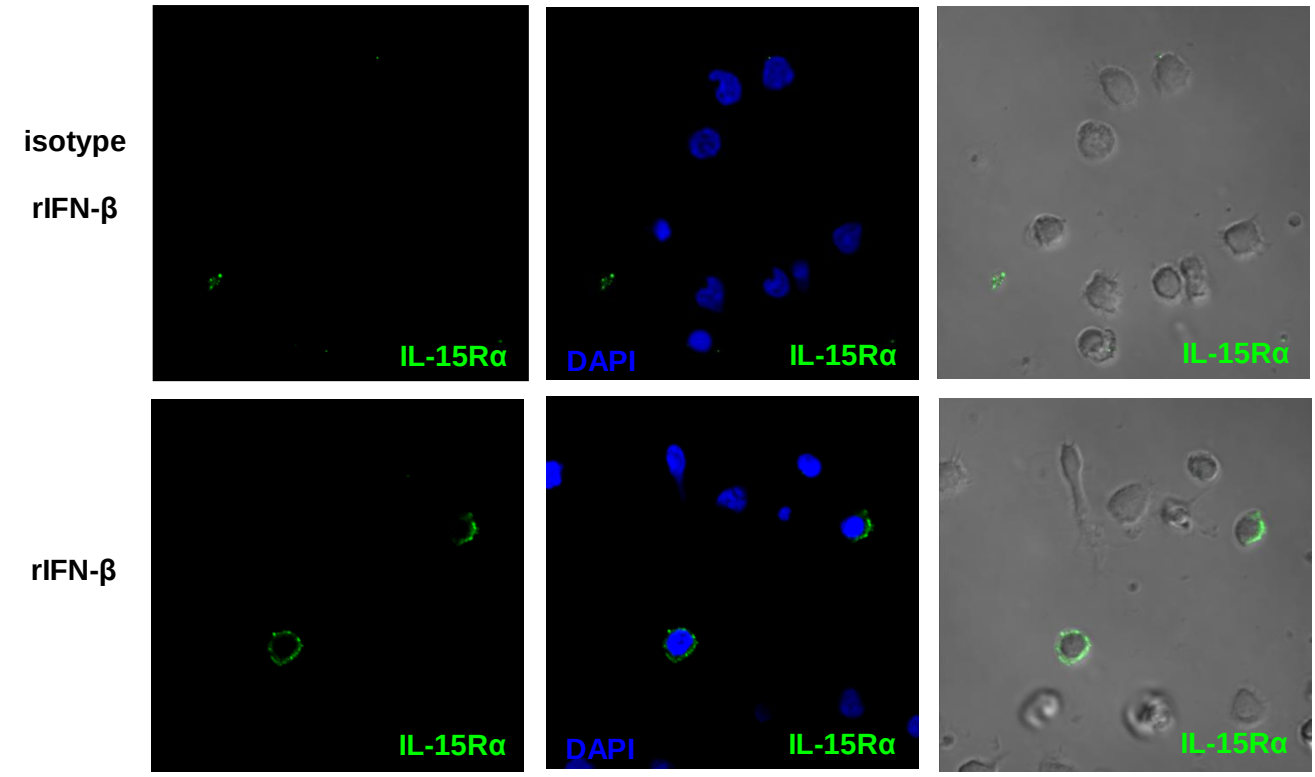




(figure continued on next page)



E



(figure continued on next page)

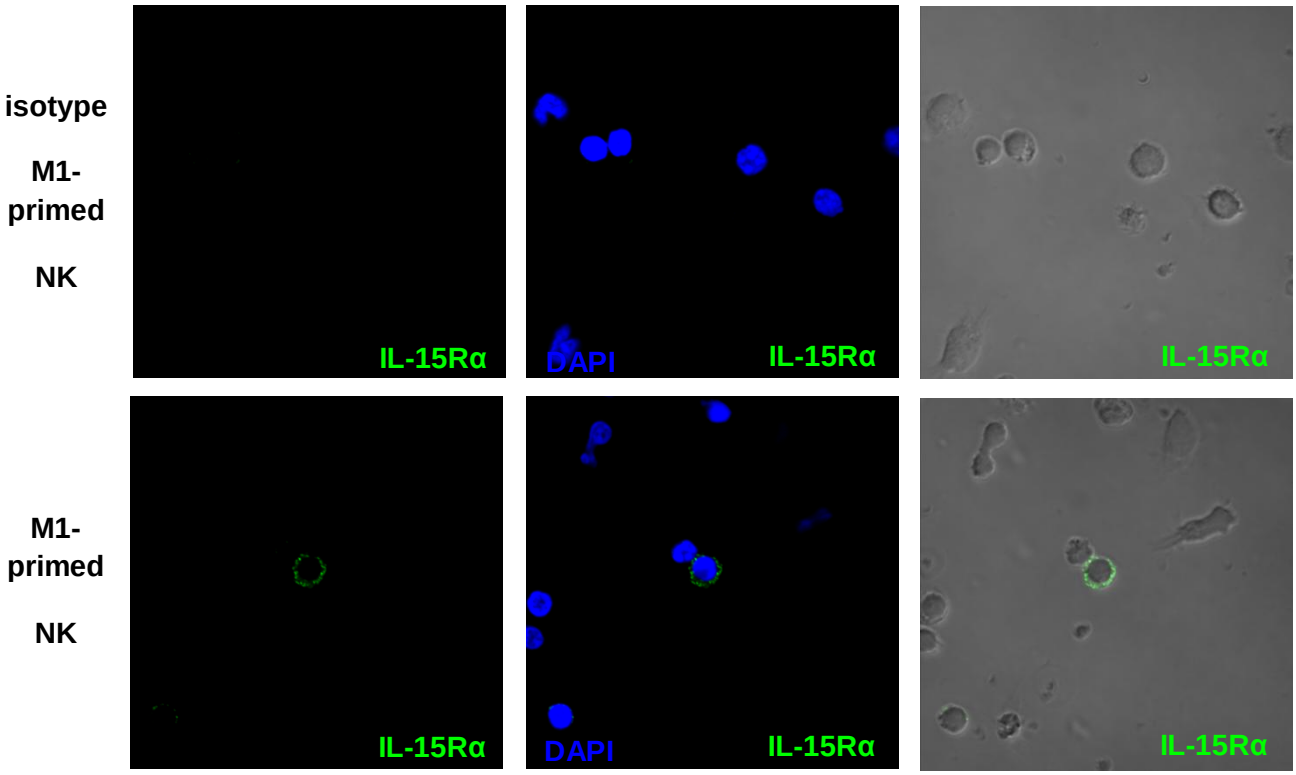


Figure 12. M1-released IFN-β induces IL-15 and IL-15Rα by resting NK cells.

IL15 (A) and IL15RA (B) mRNA levels in resting, M0 or M1-primed NK cells and M1 macrophages were quantified by RT-PCR. Data were expressed as $2^{-(\Delta Ct)}$; n=4. C) IL-15Rα expression by resting, M0 and M1-primed NK cells in the presence of IFN-β blocking antibody (black bars) or isotype control (white bars) was evaluated by FACS. Results were expressed as Mean Fluorescence Intensity (MFI); n=4. D) IL-15Rα surface expression by resting, M0 and M1-primed NK cells was determined by immunofluorescence analysis. Voltages were set on isotype control, performed on M1-primed NK cells. Plot are representative of one donor between three performed. E) IL-15Rα surface expression by rhIFN-β (200 U/ml, 24h) or M1-primed NK cells was determined by immunofluorescence analysis. Voltages were set on isotype controls. Plot are representative of one donor between three performed. Statistical analysis: Student t test; *= p<0,05; **=p<0,01; ***=p<0,001.

We next investigated whether IL-15 cis-presentation correlated with NKG2D induction. To this end, we analyzed NKG2D expression by FACS in the presence of neutralizing antibodies against IL-15 and IL-15Rα, thus preventing IL-15 cis-presentation, and to IFN-β, which represents the upstream molecule needed for IL-15Rα expression by NK cells. Unexpectedly, if IL-15 and IL-15Rα blocking did not affect NKG2D levels on M1-primed NK cells, IFN-β neutralization slightly but significantly reduced it (Figure 13 A). It suggested that, although M1-induced IL-15 cis-presentation did not contribute to NKG2D modulation, IFN-β itself was sufficient to induce NKG2D upregulation. In addition, when we separated our analysis of NK cells into $CD56^{dim}$ and $CD56^{bright}$, we could appreciate that IFN-β blocking principally regulated NKG2D expression on the $CD56^{bright}$ subset (Figure 13 C). The reduction of NKG2D levels on $CD56^{dim}$ population in the presence of IFN-β neutralization resulted less pronounced and not significant (Figure 13 D). It justified also the fact

that NKG2D decrease was not huge when we considered NK cells in their totality, since CD6^{bright} population represents only the 10-20% of circulating NK cells. However, it is important to underline that NKG2D upregulation occurred on both CD56^{dim} and CD56^{bright} M1-primed NK cells. Of note, by neutralizing IL-18 we did not see any NKG2D modulation, confirming a predominant role of IFN-β in this process (Figure 13 B).

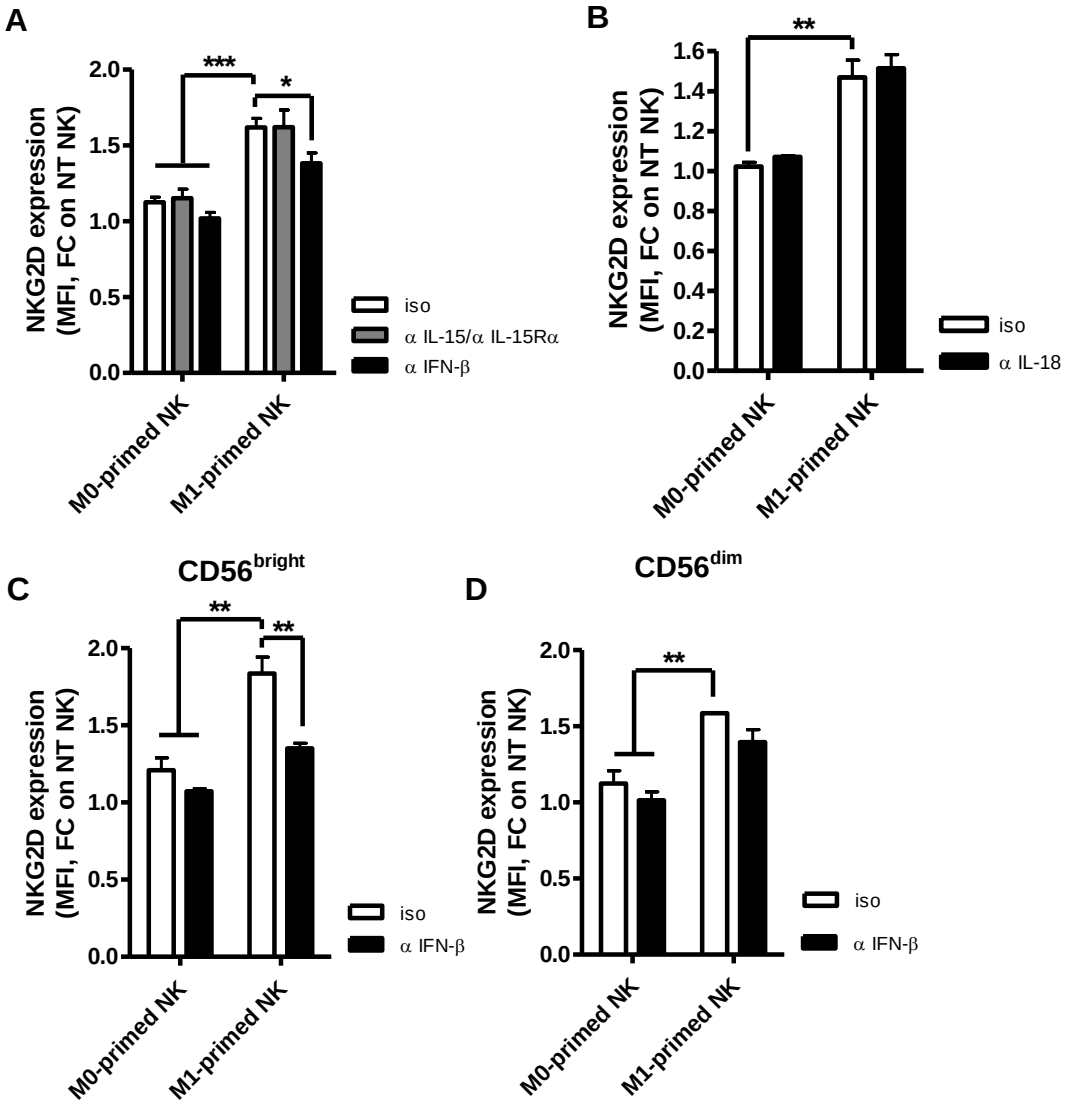


Figure 13. M1-released IFN-β regulates NKG2D expression by CD56^{bright} NK cells.

A) NKG2D expression by M0 and M1-primed NK cells in the presence of IL-15/IL-15Rα (gray bars) and IFN-β blocking antibodies (black bars) or isotype control (white bars) was evaluated by FACS. Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values; n=3-5. B) NKG2D expression by M0 and M1-primed NK cells in the presence of IL-18 blocking antibody (black bars) or isotype control (white bars) was evaluated by FACS. Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values; n=3. NKG2D expression by M0 and M1-primed CD56^{bright} NK cells (C) or M0 and M1-primed CD56^{dim} NK cells (D) in the presence of IFN-β blocking antibody (black bars) or isotype control (white bars) was evaluated by FACS. Results were expressed as Fold Change (FC) versus Not Treated NK cells (NT NK) calculated on Mean Fluorescence Intensity (MFI) values; n=4.Statistical analysis: Student t test; *= p<0,05; **=p<0,01; ***=p<0,001.

Taken together, our data demonstrate that M1 macrophages through IFN-β secretion induce IL-15 and IL-15Rα expression by NK cells, allowing NK cell IL-15 cis-presentation. In parallel, M1-derived IFN-β is responsible of NKG2D up-regulation, at least in the $CD56^{bright}$ population, favoring NK cell recognition of tumor cells. In conclusion, M1-secreted IFN-β has a double effect on NK cells: on one hand it renders NK cells able to auto-present IL-15 to IL-15Rβγ chain through IL-15Rα, and on the other hand, it triggers the expression of one of the most important NK cell activating receptor (NKG2D), thereby increasing their capability to sense and eliminate tumor cells.

6. M1-induced IL-15 cis-presentation by NK cells results in increased IFN-γ secretion

We next aimed to establish whether IL-15 cis-presentation by M1-primed NK cells correlates with enhancement of NK cell effector functions. First, we focused on IFN-γ. We measured IFN-γ secretion by M0 or M1-primed NK cells when IL-15 and IL-15Rα or IFN-β were blocked. We observed that it was largely reduced by both IL-15/IL-15Rα and IFN-β neutralization (Figure 14 A). Since IFN-β blocking resumed the IL-15/IL-15Rα one, we concluded that IL-15 cis-presentation, mediated by IFN-β, triggered IFN-γ secretion by NK cells. Next, we investigated whether NKG2D, induced by IFN-β, played a role in this process, also considering that it has been reported that NKG2D can positively impact the IFN-γ pathway (43, 44). We performed the same experiment in the presence of NKG2D neutralizing antibody but we did not observed any modulation of IFN-γ secretion by M1-primed NK cells (Figure 14 B). This result was further confirmed by RT-PCR (Figure 14 C), allowing us to definitively exclude an NKG2D dependency.

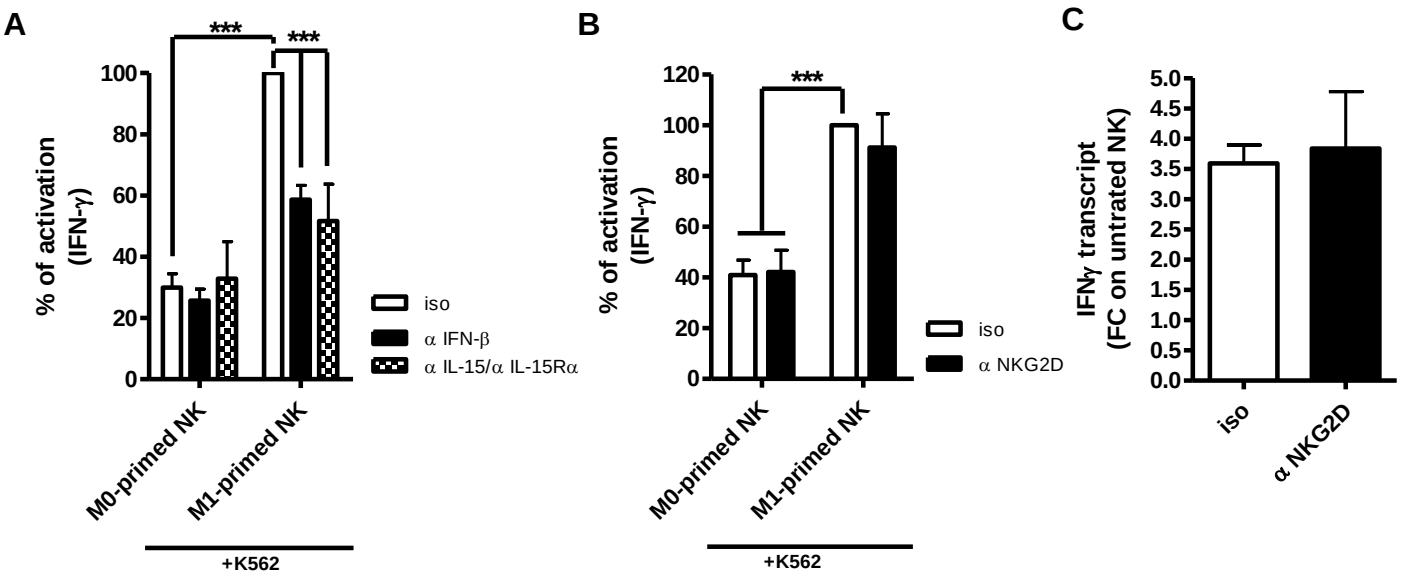


Figure 14. IL-15 cis-presentation induces IFN-γ secretion by M1-primed NK cells.

A) IFN-γ secretion by M0 and M1-primed NK cells in the presence of IFN-β (black bars) and IL-15/IL-15Rα (hatched bars) blocking antibodies or isotype controls (white bars) was detected by ELISA. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were pg/ml; n=6-9. B) IFN-γ secretion by M0 and M1-primed NK cells in the presence of NKG2D blocking antibody (black bars) or isotype controls (white bars) was detected by ELISA. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were pg/ml; n=6. C) IFNγ mRNA levels expressed by M1-primed NK cells in the presence of NKG2D blocking antibody (black bars) or isotype controls (white bars) were quantified by RT-PCR. Data were expressed as Fold Change versus Not Treated NK (FC vs NT NK) through $2^{-(\Delta\Delta Ct)}$ calculation; n=4. Statistical analysis: Student t test; ***=p<0,001.

Although the mechanism underlying the enhancement of IFN-γ in M1-primed NK cells was found, it still remained to be determined which mechanism regulated the induction of IFN-γ in direct NK cell-M1 co-cultures. It is essential to underline that, to eliminate the large part of macrophage-derived cytokines released during the polarization period, we extensively washed the macrophage layer before adding resting NK cells. By doing this we could exclude the soluble component by the co-culture and could thus appreciate the real contribution of cell-to-cell interactions mediated by membrane-associated receptors. To begin, we asked whether M1-mediated IL-15 cis-presentation could sustain IFN-γ secretion also during NK cell-M1 co-cultures. We observed that neither IL-15/IL-15Rα nor IFN-β neutralization affected IFN-γ release by NK cell co-cultured with M1 macrophages (Figure 15 A). It suggested, first, that IL-15 cis-presentation did not regulate IFN-γ secretion during direct NK cells and M1 co-culture. It is probably due to the fact that, by washing M1 macrophages, the majority of M1-secreted IFN-β was eliminated, preventing IL-15Rα induction on NK cells, and thus IL-15 cis-presentation.

Secondly, since IL-15/IL-15Rα neutralization was irrelevant, we can also exclude a role of IL-15 trans-presentation. Indeed, by co-culturing NK cells with M1, we allowed IL-15 trans-presentation by M1 macrophages. Having determined that IL-15 cis and trans-presentation did not contribute to IFN-γ modulation during direct NK cell-macrophage co-cultures, we focused our attention on NK cell activating receptors. In particular, we considered NK cell receptors described to have a role in IFN-γ secretion. Since Nedvetzki S. et al. reported a relationship between 2B4 engagement and IFN-γ secretion (74) and considering that 2B4 is constitutively expressed by NK cells (Figure 5 G and H), we evaluated IFN-γ release in the presence of 2B4 neutralizing antibody. We observed a significant reduction of IFN-γ secretion by NK cell co-cultured with M1 (Figure 15 B) when 2B4 was blocked, arguing for 2B4 involvement. Interestingly, we found that M1 macrophages highly upregulated CD48, which is the major ligand of 2B4, in response to both LPS and IFN-γ stimulation (Figure 15 C). It further supported the evidence that IFN-γ secretion by NK cell co-cultured with M1 was mainly modulated by 2B4-CD48 interactions.

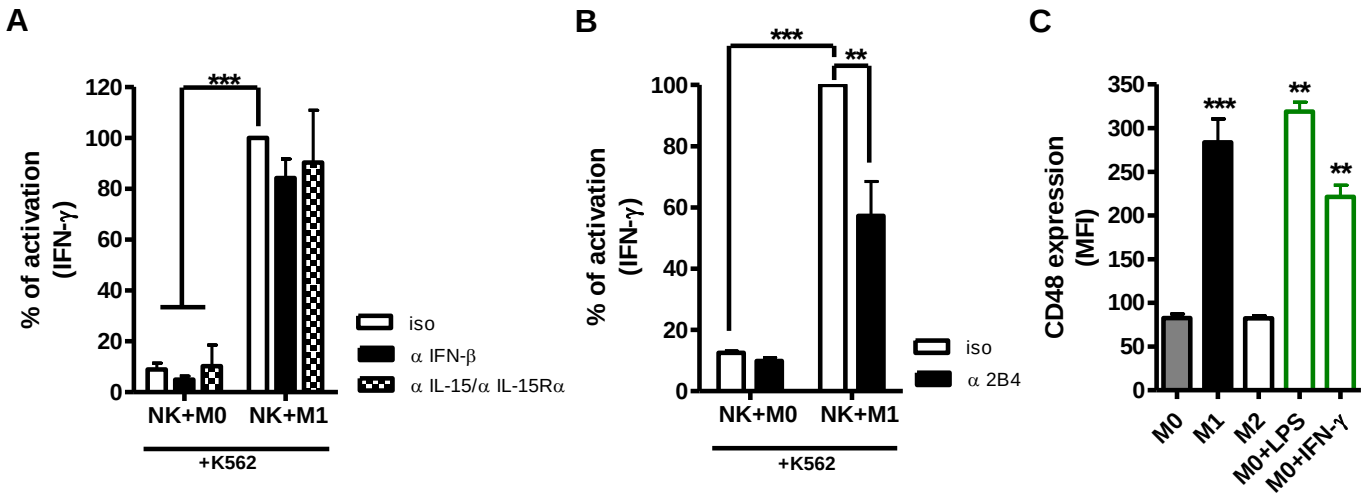


Figure 15. 2B4-CD48 interactions are responsible for increased IFN-γ secretion by NK cells co-cultured with M1.

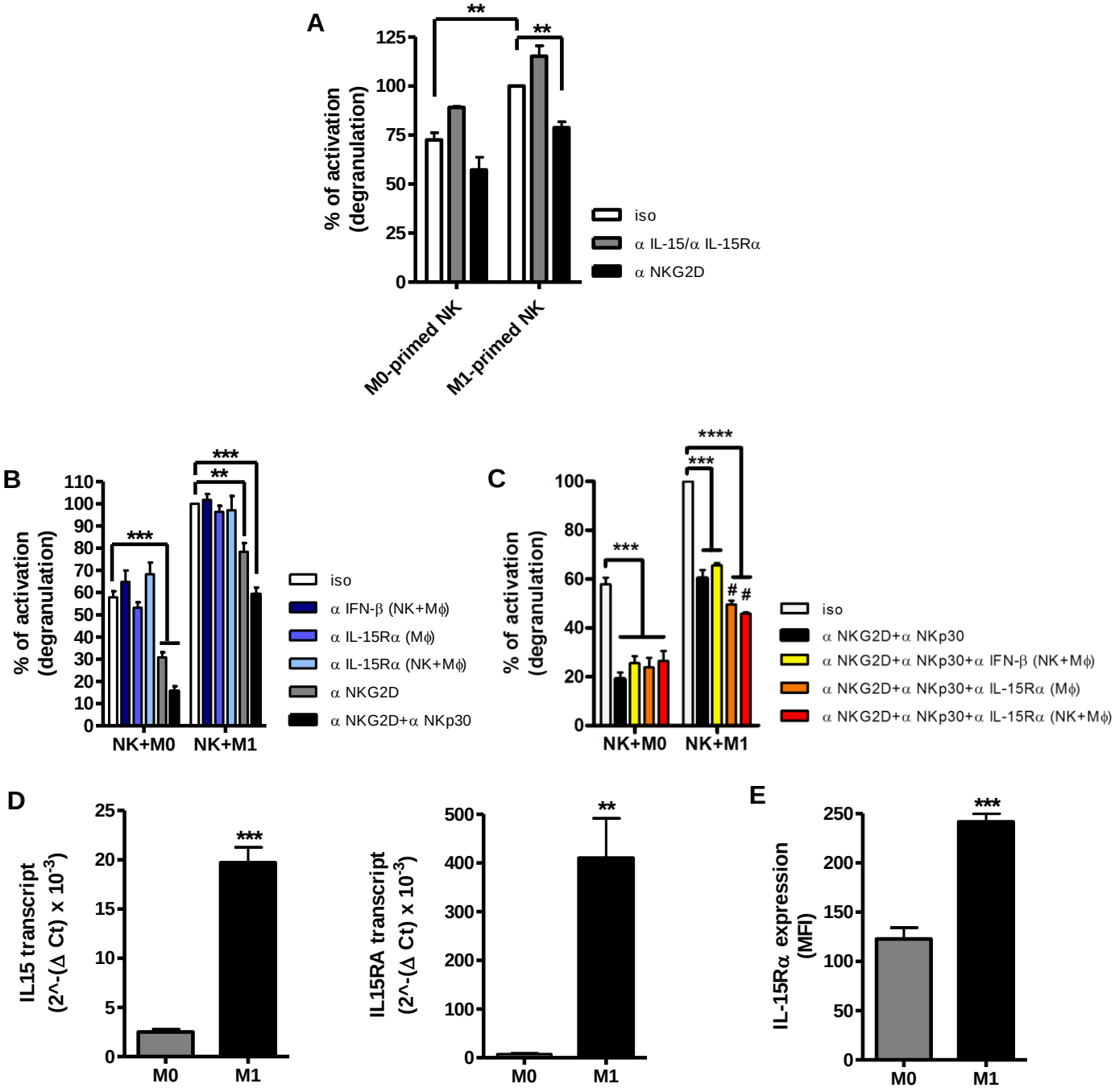
A) IFN-γ secretion by NK cells co-cultured with M0 and M1 in the presence of IFN-β (black bars) and IL-15/IL-15Rα (hatched bars) blocking antibodies or isotype controls (white bars) was detected by ELISA. Results were expressed as percentage (%) of activation, setting M1-co-cultured NK cell values to 100%. Values utilized were pg/ml; n=9. B) IFN-γ secretion by NK cells co-cultured with M0 and M1 in the presence of 2B4 blocking antibody (black bars) or isotype controls (white bars) was detected by ELISA. Results were expressed as percentage (%) of activation, setting M1-co-cultured NK cell values to 100%. Values utilized were pg/ml; n=5. C) CD48 expression by different polarized macrophages was evaluated by FACS. Data were plotted as Mean Fluorescence Intensity (MFI); n=6: Statistical analysis: Student t test; **=p<0,01; ***=p<0,001.

To summarize this part, we demonstrate that IL-15 cis-presentation strongly favors IFN-γ secretion by NK cells and it represents a mechanism completely mediated by M1-released IFN-β. These evidences prove not only that IFN-β is strictly needed to induce IL-15 and IL-15Rα expression by NK cells, but also that IL-15 cis-presentation has a functional effect, at least in terms of IFN-γ production. We can also affirm that NKG2D displays a predominant role in NK cell cytotoxicity towards tumor cells, since, in our hands, it does not contribute to IFN-γ secretion. Furthermore, we can speculate that IL-15 cis-presentation does not occur during NK cell-M1 co-culture, probably due to the elimination of IFN-β by the wash procedure. We further demonstrate that 2B4-CD48 binding is responsible for the enhancement of IFN-γ production by M1-co-cultured NK cells, highlighting the fact that both soluble mediators and receptor interactions play an important role during NK cell-macrophages cross-talk.

7. IL-15 trans-presentation by M1 macrophages, together with NKG2D, triggers NK cell degranulation towards tumor cells

Having determined that IL-15 cis-presentation enhances IFN-γ production by NK cells, we next asked which mechanism drives the increased degranulation by M1-primed NK cells. To this aim, we assessed whether IL-15 cis-presentation could have a role in this process. We treated resting NK cells with M0 or M1-conditioned media in the presence of IL-15 and IL-15Rα neutralizing antibodies, then we detected NK cell degranulation towards K562, through CD107a degranulation assay. Since we were not able to see any reduction of M1-primed NK cell degranulation (Figure 16 A, grey bars), we excluded an IL-15 cis-presentation contribution. This result strongly suggested that NK cell degranulation was totally NKG2D dependent. Indeed, we demonstrated that M1-primed NK cells killed HEK through a mechanism of co-operation between NKp44 and NKG2D, which were both upregulated. In addition, it is well known that K562, our target cells, express high levels of NKG2D ligands and, consequently, are mainly killed by NKG2D (242). To confirm our hypothesis, we blocked directly NKG2D and we observed that NK cell degranulation towards K562 was significantly diminished in M1-primed NK cells (Figure 16 A, black bars). It definitely demonstrated an NKG2D dependency. Of note, the fact that we could appreciate a slight and not significant reduction also in M0-primed NK cells fits with the evidence that NK cell express

constitutively NKG2D (Figure 6 D). It means that M0-primed NK cells, through NKG2D, are able to recognize K562, which express NKG2D ligands, and it is sufficient to stimulate their degranulation. However, M1-primed NK cells presented higher levels of NKG2D, that explained their increased cytotoxicity towards K562 compared to M0-primed NK cells.



(figure continued on next page)

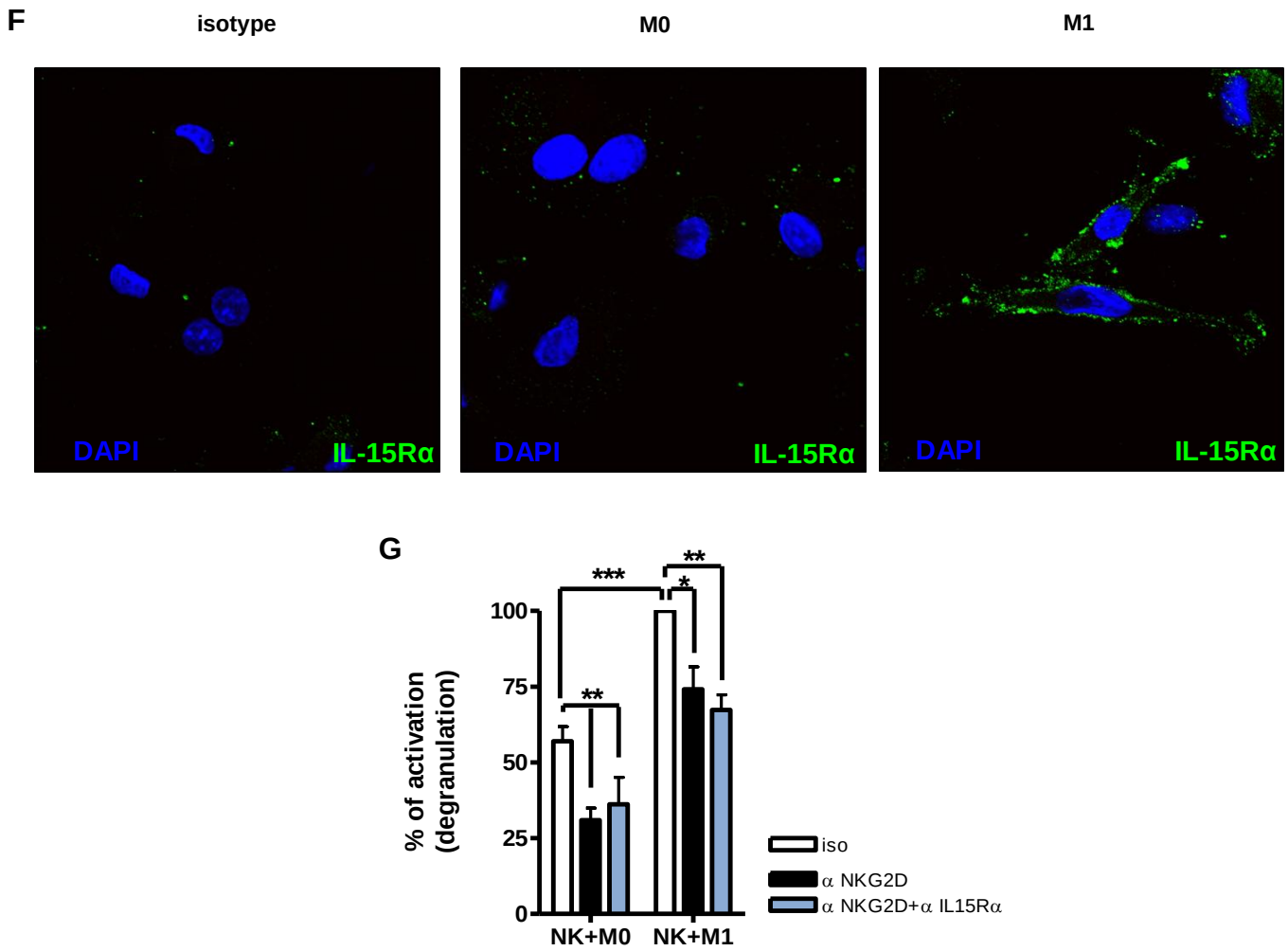


Figure 16. NKG2D engagement strongly enhances NK cell degranulation, which is further induced by IL-15 trans-presentation during direct co-cultures.

A) Degranulation of M0 and M1-primed NK cells towards K562 (E:T ratio 2:1, 4h incubation) in the presence of IL-15/IL-15Rα (gray bars) and NKG2D (black bars) blocking antibodies or isotype controls (white bars) was assessed through CD107a degranulation assay. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=4-6. B) M0 and M1 co-cultured NK cell degranulation towards K562 (E:T ratio 2:1, 4h incubation) in the presence of IFN-β (blue bars), IL-15/IL-15Rα (light blue bars), NKG2D (grey bars) and NKG2D+NKp30 (black bars) blocking antibodies or isotype controls (white bars) was assessed through CD107a degranulation assay. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=6-10. C) M0 and M1 co-cultured NK cell degranulation towards K562 (E:T ratio 2:1, 4h incubation) in the presence of NKG2D+NKp30 (black bars), NKG2D+NKp30 IFN-β (yellow bars) and NKG2D+NKp30+IL-15/IL-15Rα (specific on macrophages, orange bars, or on NK cells and macrophages, red bars) blocking antibodies or isotype controls (white bars) was assessed through CD107a degranulation assay. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=3-10; #=p<0,05 compared to αNKG2D+αNKp30. IL15 (D left) and IL15RA (D right) mRNA levels M0 and M1 macrophages were quantified by RT-PCR. Data were expressed as $2^{-(\Delta Ct)}$; n=4. E) IL-15Rα expressed by M0 and M1 macrophages was evaluated by FACS. Results were plotted as Mean Fluorescence Intensity (MFI); n=6. F) IL-15Rα surface expression by M0 and M1 macrophages was determined by immunofluorescence analysis. Voltages were set on isotype control, performed on M1 macrophages. Plots are representative of one donor between three performed. G) M0 or M1 co-cultured NK cell degranulation towards JA3 (E:T ratio 2:1, 4h incubation) in the presence of NKG2D (black bars) and NKG2D+IL-15Rα (light blue bars) blocking antibodies or isotype controls (white bars) was assessed through CD107a degranulation assay. Results were expressed as percentage (%) of activation, setting M1-primed NK cell values to 100%. Values utilized were the percentage of CD56/CD107a positive cells; n=4. Statistical analysis: Student t test; *=p<0,05; **=p<0,01; ***=p<0,001.

We next focused on NK cell-M1 co-culture. Since we did not see any modulation of NK cell activating receptors in this context, we first evaluated IL-15 cis and trans contribution to NK cell mediated killing of K562. We observed that neither IFN-β (Figure 16 B, dark blue bars) nor IL-15Rα blocking (Figure 16 B, light blue bars) interfered with NK cell degranulation. It is important to keep in mind that, by co-culturing NK cells with M1 macrophages, we permitted also a mechanism of IL-15 trans-presentation. Indeed, as we assessed by RT-PCR, M1 macrophages are characterized by high levels of IL15 and IL15RA mRNA transcription compared to M0 (Figure 16 D), and, consequently, by increased levels of IL-15Rα membrane expression, as we measured by FACS and confocal microscopy (Figure 16 E and F). Since we and others demonstrated that IL-15 cis-presentation is totally IFN-β dependent, we can assume that when we blocked IFN-β (αIFN-β (NK+MΦ)) we specifically inhibited IL-15 cis-presentation, whereas when we blocked IL-15Rα during the all the period of the co-culture (αIL-15Rα (NK+MΦ)) we neutralized both cis and trans-presentation. On the contrary, when we blocked IL-15Rα specifically on the macrophage side, followed by a wash before NK cells addition (αIL-15Rα (MΦ)), we inhibited only IL-15 trans-presentation. In that way, comparing all conditions (αIFN-β (NK+MΦ), αIL-15Rα (NK+MΦ) and αIL-15Rα (MΦ)), we were able to distinguish cis from trans presentation during direct NK cell-macrophage co-cultures. Since IFN-β neutralization did not affected NK cell degranulation, IL-15 cis-presentation *per se* was not sufficient to trigger NK cell cytolitic activity. It is further confirmed by the blocking of IL-15Rα during all the period of the co-culture (IL-15Rα (NK+MΦ)). At the same time, the inhibition of IL-15 trans-presentation *per se* (αIL-15Rα (MΦ)) did not altered NK cell cytotoxicity towards tumor cells, letting us to hypothesize that also trans-presentation was not sufficient to induce NK cell degranulation. These evidences suggested a predominant role of NK cell activating receptors, as we observed during NK cell treatment with M1-conditioned medium, in this process. To verify that, we determined the contribution of NKG2D. NK cell degranulation was reduced in the presence of NKG2D blocking antibody (Figure 16 B, gray bars) and, since NKp30 neutralization further reduced NK cell capability to degranulate, we can appreciate a synergy between NKG2D and NKp30 mediated killing (Figure 16 B, black bars). Of note, as before, NK cell degranulation was diminished also during NK cell-M0 co-culture, arguing for an a-specific mechanism. Again, it is probably due to the fact that K562 express NKG2D and NKp30 ligands and are sufficient to induce NK cell degranulation. As decreased NK cell degranulation by NKG2D and NKp30 neutralization was not confined to M1, we still searched for a M1 specific mechanism that explained increased NK cell degranulation. We tried to exclude NKG2D and NKp30 ligands

participation, hoping that possible M1-specific contribution previously covered by activating receptor activity could emerge. To this end, we performed the same experiment described before, combining NKG2D, NKp30 and IFN-β or IL-15Rα neutralization. We observed that, by adding IFN-β blocking, NK cell degranulation did not further reduced (Figure 16 C, yellow bars), whereas by blocking IL-15Rα, either on macrophages (Figure 16 C, orange bars) or during the co-culture (Figure 16 C, red bars), NK cell degranulation resulted more diminished and statistically different than either NKG2D and NKp30 or NKG2D, NKp30 and IFN-β neutralization. Importantly, it occurred specifically when NK cells were co-cultured with M1, and not during M0 co-culture. Moreover, the level of inhibition mediated by anti-IL15Rα on macrophages and the level of inhibition observed when anti-IL-15Rα was leaved during all the period of co-culture were comparable. All together, these observations suggested that, once NKG2D and NKp30 contribution was excluded, IL-15 trans-presentation, and not cis-presentation, favored NK cell degranulation. It was further confirmed by target switching, from K562 to JA3. Indeed, NK cells co-cultured with M1 were more cytotoxic towards JA3, which were mainly killed via NKG2D, as NKG2D blocking partially reduced NK cell degranulation. Interestingly, when we further added IL-15Rα neutralization M1-induced NK cell degranulation was specifically reverted to the basal level (Figure 16 G).

Taken together, these data demonstrate that the enhancement of NK cell degranulation mediated by M1 is totally independent from IL-15 cis-presentation. On the contrary, NKG2D induction correlates with increased NK cell degranulation by M1-primed NK cells, whereas M1-dependent IL-15 trans-presentation contributes to trigger NK cell cytotoxicity during NK cell-M1 co-culture. Summarizing, IL-15 cis-presentation and NKG2D upregulation, both mediated by IFN-β, are needed to enhance NK cell IFN-γ secretion and degranulation, respectively. On the other hand, when the soluble component is almost eliminated, M1 still maintain the capability to activate NK cell effector functions through CD48-2B4 engagement or IL-15Rα trans-presentation, which regulate respectively NK cell IFN-γ production and degranulation.

To conclude, we finally define a series of M1 dependent mechanisms that trigger NK cell activation and effector functions. We first demonstrate that M1 macrophages induce resting NK cell activation, that is supported by CD69 and NKp44 upregulation, with the consequent induction of NK cell IFN-γ secretion and degranulation. We could correlate NKp44 and NKG2D increased expression on M1-primed NK cells with higher capability to recognize and eliminate tumor cells. Furthermore, we show that IL-1β secreted by M1, acting on IL-1R I, which is more expressed by

$CD56^{dim}$ NK cells, is responsible of NKp44 upregulation, whereas M1-released IFN-β induces not only NKG2D expression, preferentially on $CD56^{bright}$ NK cells, but also IL-15 and IL-15Rα levels, allowing a mechanism of IL-15 cis-presentation by NK cells. In cis IL-15 presentation strongly boosts NK cell capability to secrete IFN-γ, which is mainly mediated by 2B4-CD48 interactions once NK cells are in contact with M1 macrophages. On the contrary, IL-15 trans-presentation by M1 macrophages represents the specific mechanism by which M1 macrophages enhance NK cell degranulation, whereas, once upregulated, NKG2D acts as the major inductor of NK cell cytotoxicity towards tumor cells.

These results describe the strategies that M1 macrophages use to orchestrate NK cell ability to recognize and eliminate tumor cells, possibly participating to limit tumor expansion. In addition, mediating the production of IFN-γ, they contribute to maintain a pro-inflammatory micro-environment, that not only retard tumor cell growth, but can also be used as re-education strategy to re-direct TAM polarization.

8. Alternatively-activated macrophages sense NK cell-derived IFN-γ and downmodulate the expression of CD206 and ALOX15

Although we well described the effects of macrophage polarization on resting NK cells, the characterization of the cross-talk between NK cells and macrophages in a tumor micro-environment is far to be completely depicted. For sure, one still open question consists in understand whether NK cell-derived IFN-γ could modulate macrophage polarization, and, in particular, whether it can be sufficient to drive macrophages from a M2 to a M1 phenotype. Indeed, tumor associated macrophages partially resume M2 pro-tumoral features and their re-education to an anti-tumoral phenotype represents one of the new approaches for immunotherapy.

To this aim, we built up an autologous system to study the influence of M1-activated NK cells on polarized macrophages. We collected the supernatant of resting, M0-primed and M1-primed NK cells, which contain the highest amount of IFN-γ, and we used it to treat autologous M0, M1 or M2. Of note, in order to further improve NK cell capability to secrete IFN-γ, we increased the

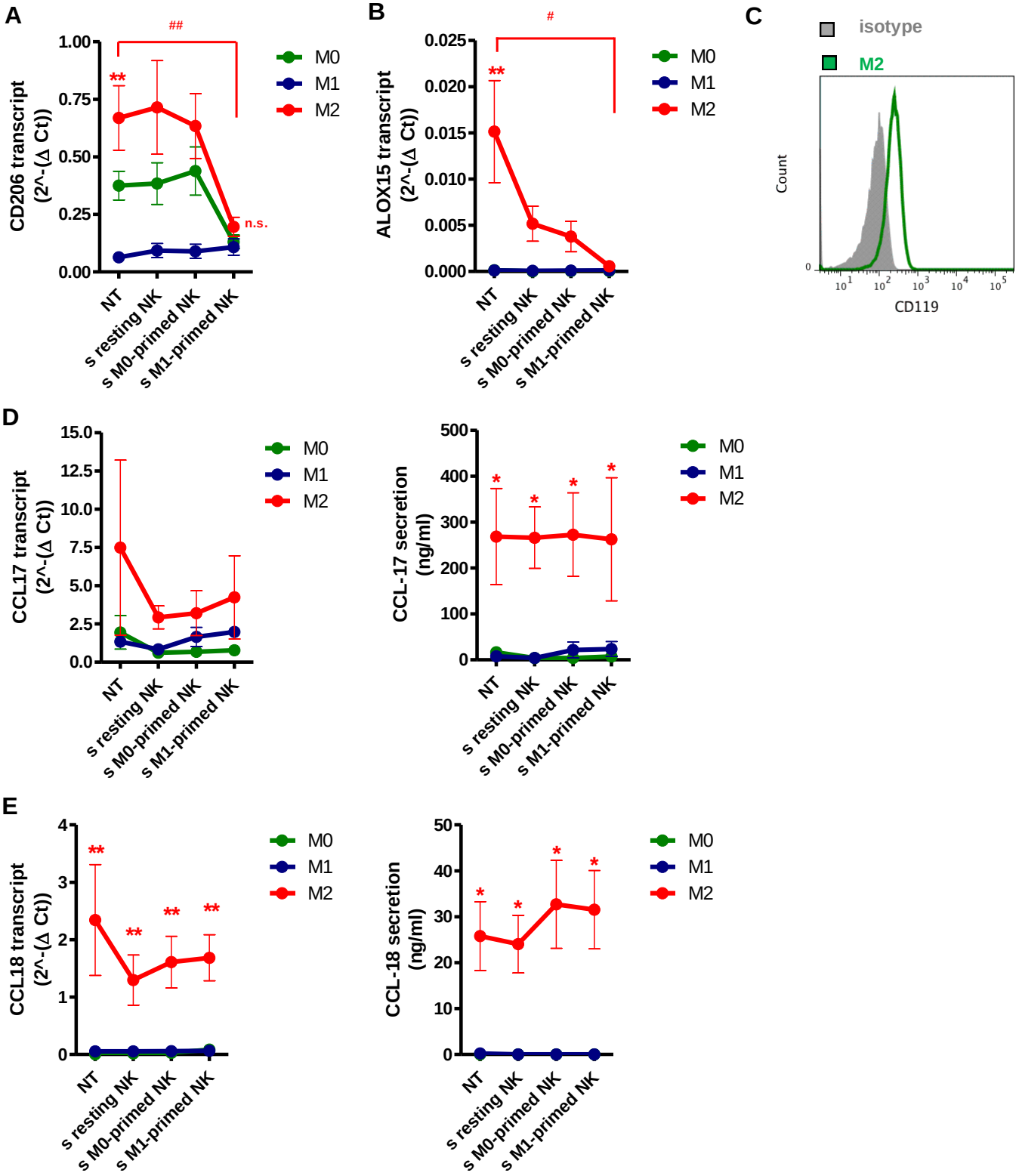
percentage of M0 or M1-conditioned medium, reaching 50%. Moreover, NK cell supernatants, obtained after a brief co-culture with K562, were not diluted, in order to not alter the quantity of IFN-γ.

After 24h of incubation with resting, M0-primed or M1-primed NK cell supernatants (s resting NK, s M0-primed NK and s M1-primed NK, respectively) macrophages were analyzed for the expression of M1 or M2 associated molecules, both in terms of mRNA and protein secretion. In parallel, we performed the same analysis in untreated M0, M1 and M2, as control. Considering all conditions together, we can compare, at the same time, not only the levels of M1 or M2 genes between different polarization states, but also between different treatments.

We started by analyzing M2 genes. We chose two well established M2 markers, CD206 and ALOX15, both IL-4 dependent (110, 118, 243, 244). CD206 is the mannose receptor 1, a C-type lectin receptor involved in macrophage phagocytosis and resolution of inflammation (110); whereas ALOX15 is a arachidonate15-lypoxigenase important for lipid metabolism, promoting anti-inflammatory effects through lipid mediator synthesis (243). Both have M2 specificity, as they resulted upregulated specifically in untreated M2. We observed that both CD206 and ALOX15 transcription was strongly downmodulated after M1-primed NK cell supernatant treatment (Figure 17 A and B, red lines), reaching M1 expression levels (Figure 17 A and B, blue lines). It suggested that M2 macrophages were able to sense IFN-γ, which, in turn, dampened the expression of CD206 and ALOX15. M2 sensibility to IFN-γ is further confirmed by the fact that they express CD119, the IFN-γ receptor (Figure 17 C), as we assessed by FACS. Curiously, if CD206 reduction was specifically associated to the treatment with M1-primed NK cell supernatant, ALOX15 was differently modulated. Indeed, it was sufficient to treat M2 macrophages with “NK cell-related” supernatants to see a decreased of ALOX15 mRNA copies. Nevertheless, it is important to underline that all NK cells were briefly incubated with K562, in order to allow the release of IFN-γ stored into the granules. It means that also resting and M0-primed NK cell supernatants contain IFN-γ, even if far less than M1-primed NK cells. As a consequence, we can hypothesize that ALOX15 resulted more susceptible to IFN-γ stimulation than CD206, by sensing even very low doses of IFN-γ. Importantly, higher doses of IFN-γ correlated with more pronounced downregulation of the gene, which reached M1 levels only in the presence of M1-primed NK cell supernatant.

To extend these results, we evaluated the expression of chemokines usually produced by M2 macrophages, such as CCL-17, CCL-18 and CCL-22, by RT-PCR and through commercial ELISA kits. These chemokines are all involved in the recruitment of type-2 cells, favoring the formation of a tumor-promoting micro-environment. In particular, CCL-17 and CCL-22 are responsible for Th2 and Treg cell recruitment, whereas CCL-18 leads to naïve T cell mobilization (115). Surprisingly, we did not observed neither specific modulation of CCL17 and CCL18 genes (Figure 17 D left and E left) nor CCL-17 and CCL-18 protein secretion (Figure 17 D right and E right). On the contrary, although protein secretion was not affected (Figure 17 F right), we were able to appreciate a slight downregulation of CCL22 gene in the presence of M1-primed NK cell supernatant (Figure 17 F left). These evidences further confirmed that M2 associated genes were only partially modulated by NK cell-derived IFN-γ, as different genes were controlled by different mechanism.

All together these data sustain the idea that, once a M2 program is undertaken, some genes can be quite easily manipulated but others are strongly determined. It means that the main macrophage feature, that is plasticity, can be only partially modulated. These aspects open innumerable questions concerning the mechanisms underlying these processes. For sure, we need to complete our analysis including more M2 associated genes. Nevertheless, in order to have a clearer picture, the study of M1 genes modulation in M2 macrophages during NK cell-derived IFN-γ treatments can be informative.



(figure continued on next page)

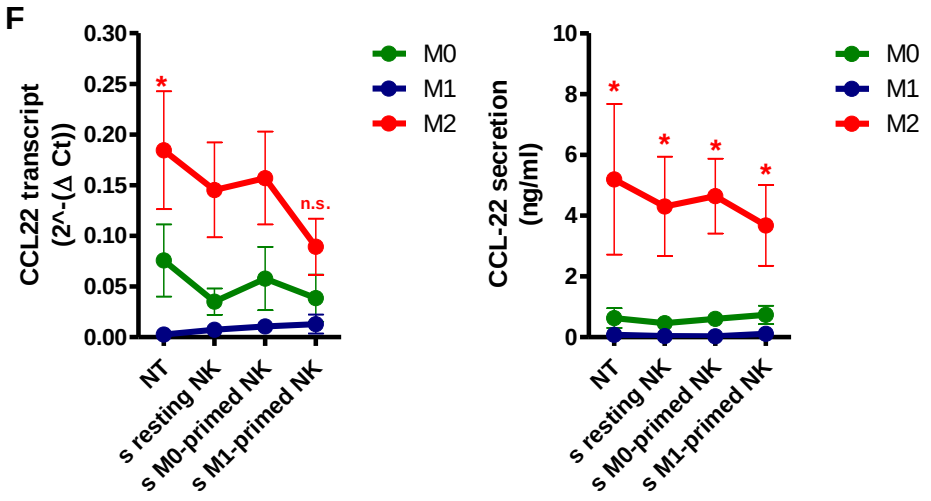


Figure 17. NK cell derived IFN-γ is sufficient to downregulate CD206 and ALOX15 expression by alternatively-activated macrophages.

CD206 (A), ALOX15 (B), CCL17 (D left), CCL18 (E left) and CCL22 (F left) mRNA levels expressed by untreated M0, M1 and M2 or M0, M1 and M2 treated with resting NK cell supernatant (s resting NK), M0-primed NK cell supernatant (s M0-primed NK) and M1-primed NK cell supernatants (s M1-primed NK) were quantified by RT-PCR. Data were plotted as $2^{-(\Delta Ct)}$; n=5. C) CD119 expression by M2 macrophages was evaluated by FACS. The expression of CD119 is representative of one donor between three performed. CCL-17 (D right), CCL-18 (E right) and CCL-22 (F right) secretion by untreated M0, M1 and M2 or M0, M1 and M2 treated with resting NK cell supernatant (s resting NK), M0-primed NK cell supernatant (s M0-primed NK) and M1-primed NK cell supernatants (s M1-primed NK) were detected by ELISA. Results were expressed as ng/ml; n=5. Statistical analysis: Student t test; macrophage phenotypes treated with a given primed NK cell media were compared between each other (the comparison reads in a "vertical" way). *=p<0,05; **=p<0,01. Macrophage from a given phenotype treated with different primed NK cell media were compared (the comparison reads in a "horizontal" way). #=p<0,05; ##=p<0,01.

9. NK cell-derived IFN-γ induces the expression of pro-inflammatory molecules by alternatively-activated macrophages

Having determined that NK cell-derived IFN-γ partially downregulate M2 typical genes, we asked whether, in opposite, it could induce the expression of M1 genes by M2 macrophages. To this aim, we focused our attention on three classes of genes. First, we analyzed M1 surface markers, as CD80 and CD48. CD80 is one of the two co-receptor of MHC class II and plays an important role during antigen presentation to T cells, sustaining Th1 responses; whereas CD48 is the major ligand of 2B4 and, as we demonstrated before, it favors NK cell IFN-γ secretion. Both of them resulted upregulated in M2 macrophages during the treatments (Figure 18 A left and B). More in details,

we observed an induction of CD80 mRNA expression by M2 only in the presence of M1-primed NK cell supernatant**.**

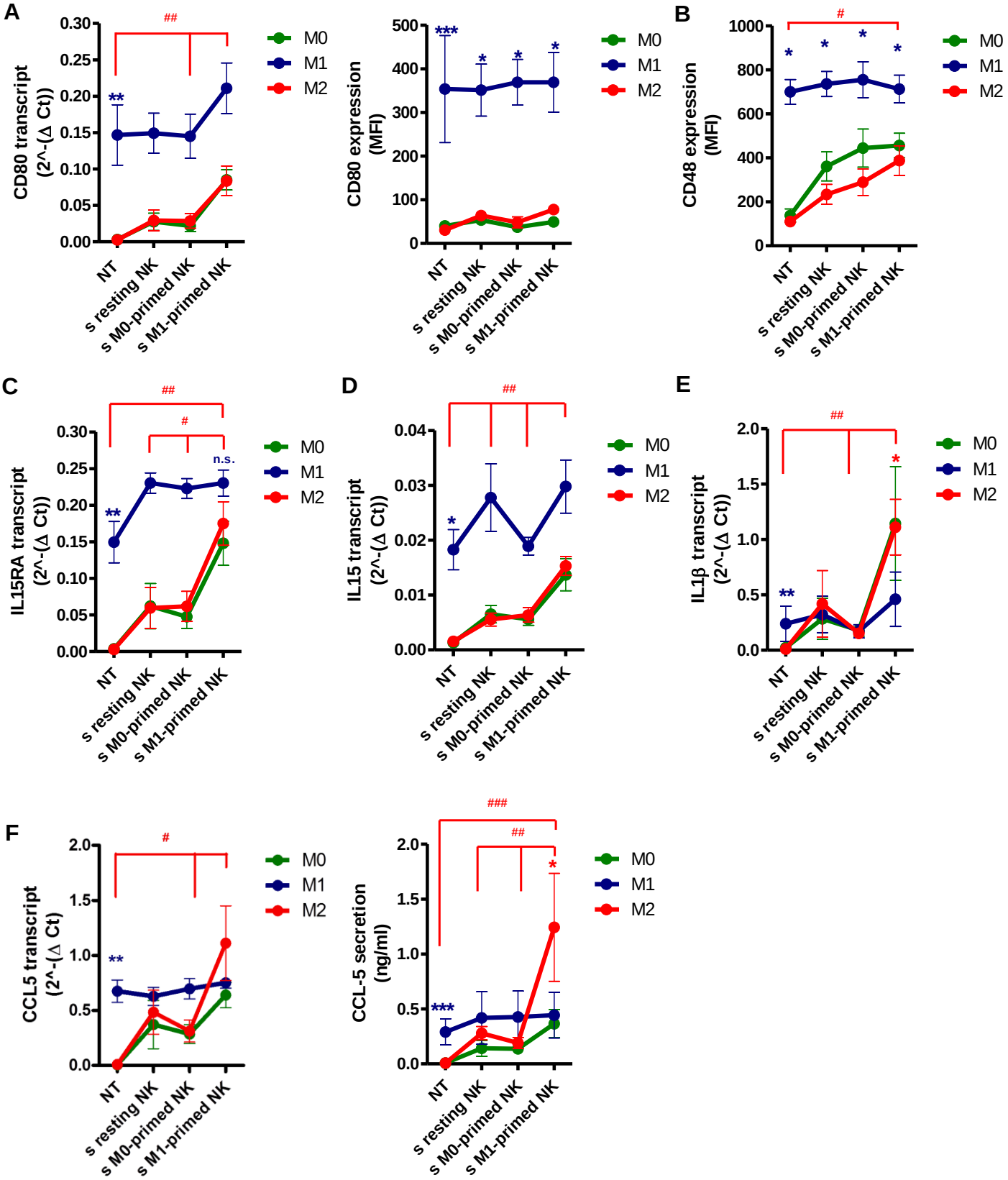
Interestingly, M1 macrophages re-challenged with NK cell-derived IFN-γ further increased CD80 mRNA levels, meaning that CD80 gene still responded to additional doses of IFN-γ. Consequently, M2 expression of CD80 mRNA did not reached M1 levels. Although mRNA resulted upregulated, surface levels of CD80 were not significantly altered, as we assessed by FACS (Figure 18 A right). It suggested that probably other mechanisms interfere with the protein expression, or simply more time was required to modulate the protein level. Concerning CD48, FACS analysis was sufficient to evidence an IFN-γ responsiveness, with a peak in M2 treated with M1-primed NK cell supernatant (Figure 18 B). It is quite relevant if we consider NK cell-macrophage cross-talk, because we can hypothesize that CD48 expression by M2 could engage 2B4, resulting in the enhancement of IFN-γ secretion, which, in turn, sustains CD48 expression by M2.

The second class of molecules analyzed was pro-inflammatory cytokines. In particular, we evaluated the mRNA expression of IL15, its receptor IL15RA and IL1β. IL-15 is one of the most important cytokine involved in T and NK cell activation. On the other hand, IL-1β is one of the most potent pro-inflammatory cytokine. Its production is regulated by the inflammasome. Briefly, the activation of inflammasome-associated proteins leads to the transcription of IL1β, which is then translated into an immature form of IL-1β, called pro-IL-1β. Only with the persistence of a "second stimulation", pro-IL-1β is converted to its mature form, IL-1β, and secreted. By RT-PCR, we observed that M2 transcription of IL15, IL15RA and IL1β mRNA were strongly and selectively induced by M1-primed NK cells (Figure 18 C, D and E). If we focused on IL15RA, we can appreciate that not only it was upregulated only in M2 macrophages treated with M1-primed NK cell supernatant, but the levels of expression were comparable with the ones observed in M1 macrophages, even if they further increased it when re-triggered with NK cell-derived IFN-γ. Interestingly, at least in this context, IL15RA seemed to be totally IFN-γ dependent and, more importantly, easily inducible in M2 macrophages. In parallel, although its expression by M1 macrophages during the treatments was less clear, IL15 resulted significantly upmodulated by M1-primed NK cells, allowing us to hypothesize that whether mRNA expression is followed by protein translation, M2 macrophages are able to produce IL-15/IL-15Rα complexes. Again, it can be really relevant for NK cell-macrophage cross-talk. As a consequence, the measurement of IL-15RA surface expression by M2 macrophages is mandatory. Finally, IL1β displayed a particular tendency.

Indeed, the mRNA levels in M2 macrophages treated with resting or M0-primed NK cell supernatants reached M1 levels, whereas M2 macrophages stimulated with M1-primed NK cell supernatant even overcame the expression by M1 in the same condition. It gave us two informations: first, IL1β is not more inducible by IFN-γ. It means that M1 macrophages resulted "tolerant" towards a second stimulation with IFN-γ in terms of IL1β transcription. Secondly, IL1β can be strongly upregulated on M2 macrophages after a single dose of IFN-γ, revealing that, in this context, IL1β seemed to be IFN-γ dependent. Again, from a functional point of view, it could be really important to determine whether M2 macrophages stimulated with NK cell-derived IFN-γ are able not only to produce but also to secrete IL-1β. Indeed, in addition to its peculiar inflammatory features, IL-1β actively participates to NK cell activation, as we demonstrated before. Importantly, the synthesis of IL1β transcript does not always correlate with secretion of mature IL-1β. The cleavage of pro-IL-1β is needed, and thus a second stimulation. In any case, more than their capability to secrete it, it is quite intriguing to see that M2 macrophages can potentially transcript higher levels of IL1β than M1.

Finally, the last class of molecules considered was represented by inflammatory chemokines, as CCL-5, CXCL-9 and CXCL-10. All these chemokines are strongly required to recruit cytotoxic T and NK cells at the tumor site, promoting anti-tumoral responses. As previous analysis of M2 related chemokines, we evaluated mRNA transcription in parallel with protein secretion. In line with the results obtained for inflammatory cytokines, CCL5, CXCL9 and CXCL10 were upregulated in M2 macrophages treated with M1-primed NK cell supernatants (Figure 18 F left, G left and H left). In addition, mRNA induction was followed by increased secretion of the relative protein (Figure 18 F right, G right and H right). If we analyzed chemokine by chemokine, we can affirm that CCL5 mRNA, as observed for IL1β, cannot be further induced by a second stimulation with IFN-γ in M1 macrophages. However, it resulted very responsive to a first dose of IFN-γ, as CCL5 mRNA was significantly enhanced in M2 macrophages treated with NK cell-derived IFN-γ. Importantly, the major induction occurred after M1-primed NK cell stimulation. Protein secretion completely mirrored mRNA tendency. Concerning CXCL9 and CXCL10 mRNA, their IFN-γ-mediated modulation was comparable. We observed a huge increase even in M2 treated with resting and M0-primed NK cell supernatants, which was further extend during M1-primed NK cell stimulation. The same occurred for the protein secretion. It confirmed a strong IFN-γ responsiveness of these chemokines, as largely reported in literature (115, 133). Of note, here it is possible to observe a slight increase of mRNA transcription also in M1 macrophages. From a functional point of view,

M2 macrophages stimulated with NK cell-derived IFN-γ secreted CCL-5, CXCL-9 and CXCL-10, even better than M1 macrophages.



(figure continued on next page)

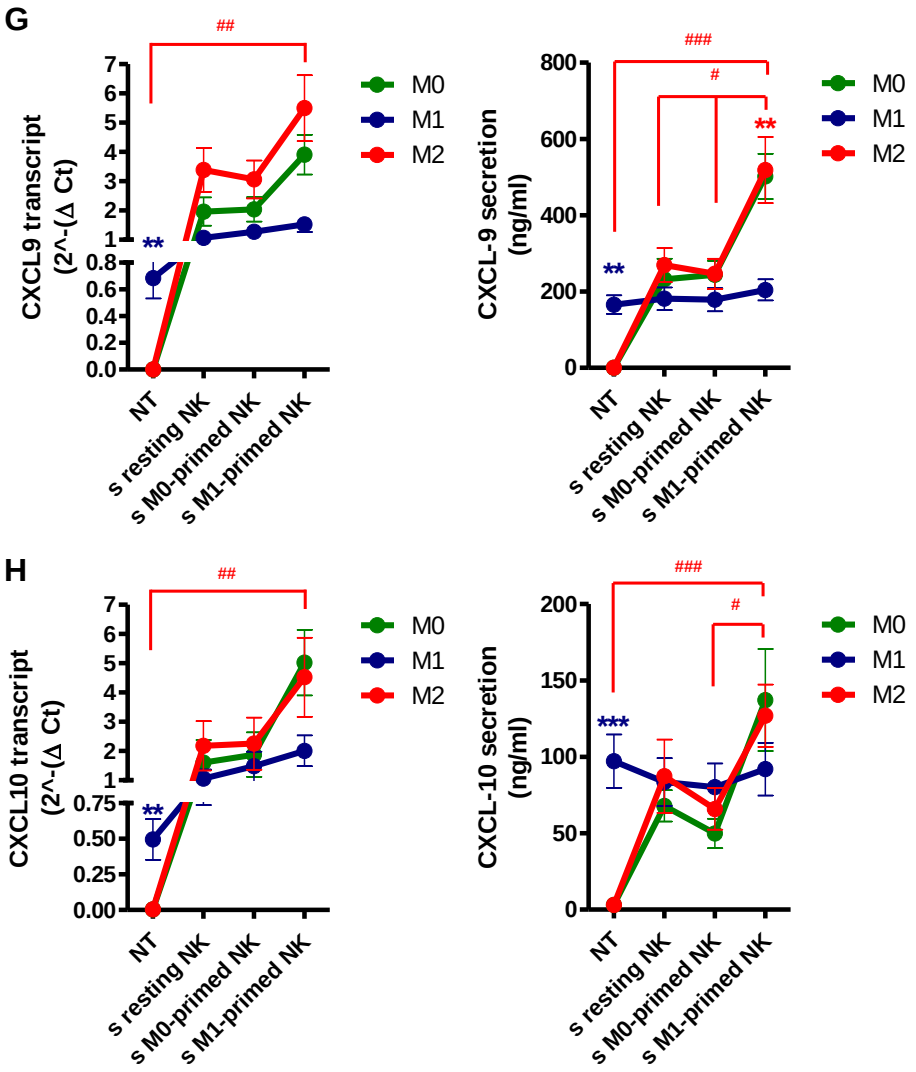


Figure 18. NK cell-derived IFN-γ is sufficient to upregulate CD80, CD48, IL-15Rα, pro-inflammatory cytokine and chemokine expression by alternatively-activated macrophages.

CD80 (A left), IL1β (C), IL15 (D), IL15RA (E), CCL5 (F left), CXCL9 (G left) and CXCL10 (F left) mRNA levels expressed by untreated M0, M1 and M2 or M0, M1 and M2 treated with resting NK cell supernatant (s resting NK), M0-primed NK cell supernatant (s M0-primed NK) and M1-primed NK cell supernatants (s M1-primed NK) were quantified by RT-PCR. Data were plotted as $2^{-(\Delta Ct)}$; n=6-8. CD80 (A right) and CD48 (B) expression by untreated M0, M1 and M2 or M0, M1 and M2 treated with resting NK cell supernatant (s resting NK), M0-primed NK cell supernatant (s M0-primed NK) and M1-primed NK cell supernatants (s M1-primed NK) was evaluated by FACS. Results were expressed as Mean Fluorescence Intensity (MFI); n=4-6. CCL-5 (F right), CXCL-9 (G right) and CXCL-10 (G right) secretion by untreated M0, M1 and M2 or M0, M1 and M2 treated with resting NK cell supernatant (s resting NK), M0-primed NK cell supernatant (s M0-primed NK) and M1-primed NK cell supernatants (s M1-primed NK) was detected by ELISA. Results were expressed as ng/ml; n=8.Statistical analysis: Student t test; macrophage phenotypes treated with a given primed NK cell media were compared between each other (the comparison reads in a "vertical" way). *=p<0,05; **=p<0,01; ***=p<0,001. Macrophage from a given phenotype treated with different primed NK cell media were compared (the comparison reads in a "horizontal" way). #=p<0,05; ##=p<0,01; ###=p<0,001.

Taken together these results demonstrate that IFN-γ produced by M1-educated NK cells is sufficient to boost the expression of pro-inflammatory molecules by M2 macrophages, which, in turn, partially downmodulated their typical markers. These are only preliminary data and a deeper analysis, in addition to a functional validation, is required. Furthermore, it remains to be determined whether this holds true when NK cells are in direct co-culture with M2 macrophages. In any case, we can speculate that NK cells, once activated, can play an important role in macrophage re-education, opening the concrete possibility to use them to drive M2 macrophages into M1 macrophages. In addition, if we focused on the macrophage side, it could be really interesting to study why M2 macrophages seem to respond even better to IFN-γ than M1 macrophages and which is the molecular machinery that regulates M1 or M2 patterns. Moreover, we can contextualize all these *in vitro* studies in a model of pathology, working with real tumor associated macrophages to better define whether our observations can be translated in effective anti-tumoral properties.

DISCUSSION

According to the fact that *in vivo* studies are limited in humans, we set up an *in vitro* autologous tumor micro-environment model, in order to evaluate first whether different human polarized macrophages could modulate NK cell anti-tumoral activities, and then whether activated NK cells could influence macrophages polarization. In particular, we focused on NK cell derived IFN-γ effect on M2 macrophages, investigating whether it could be sufficient to re-direct their polarization to a more cytotoxic and pro-inflammatory phenotype, closer to "anti-tumoral" M1. The aim was to elucidate cellular mechanisms underlying NK cell-macrophage interactions hoping to find new potential targets for the enhancement of anti-tumoral activities. It is important to underline that although recently at least two works were published on the crosstalk between human NK cells and polarized macrophages (162, 164), the authors utilized polarized macrophages that were re-stimulated. Therefore the effect of M1 and M2 polarization *per se* on NK cell effector functions was not considered, despite it represents an important aspect in cancer micro-environment. Indeed, the presence of pathogens within a tumor tissue is not frequent, whereas M1 and over all M2 polarization are strongly sustained. In addition, it remains totally elusive whether, on the basis of their polarization, M1 macrophages can be sufficient to recruit NK cells from blood vessels and subsequently be able to trigger their anti-tumoral activity. Furthermore, all these studies were focused on the role of polarized macrophages in the modulation of NK cell effector functions, but the influence of NK cell IFN-γ production on M1 and over all on M2 polarization has to be determined, together with the role of NK cell-derived IFN-γ in TAMs re-education.

First, we demonstrated that M1 polarization was required to trigger resting NK cell effector functions, in terms of IFN-γ secretion, degranulation towards tumor cells and CD69 expression. In addition, we observed that the M1-mediated induction of NK cell anti-tumoral activities occurred both in the presence of direct cell contact and when NK cells were simply stimulated with M1-derived soluble factors. On the contrary, resting (M0) or M2 macrophages seemed to not affect NK cell activities. This evidence highlights three important points. First, according with their pro-inflammatory phenotype, M1 macrophages act as NK cell activator. This is in line with what it has been just published, as LPS-activated macrophages are really close to M1 (74). However, up to date, the use of resting NK cells was not frequent. Indeed, in order to appreciate stronger responses, human NK cells were generally stimulated with cytokines before use, such as treatments with IL-2 for 7 days. Although this protocol facilitates cytokine detection and

degranulation from a technical point of view, it is less physiologic. Indeed, circulating NK cells are almost totally “naïve”, whereas they go through rapid activation only once recruited to inflamed tissues (45). Considering that our goal was to evaluate whether macrophages were sufficient to trigger NK cell anti-tumoral activities, IL-2 treatment could really alter the outcome. As a consequence, the fact that M1 macrophages are effectively required to trigger resting NK cell activities is not yet be determined, and, over all, was not obvious. Secondly, even if NK cells do not place close to them, M1 macrophages could stimulate their effector functions. Indeed, macrophage-conditioned media were totally sufficient to fully activate NK cells. It means that M1 action on NK cells is not restricted nearby them, but can be extended far from them. Finally, the fact that resting NK cells in the presence of M2-conditioned medium do not alter their phenotype suggests that they do not sense M2 anti-inflammatory cytokines, and thus potentially their effector functions are not dampened by M2. In addition, M2 macrophages seem to not express molecules aimed to interfere with NK cell recognition of tumor cells.

Having determined that M1 macrophages are required for NK cell activation, we investigated which cellular mechanisms underlying this process. Since NK cell activation is regulated by the expression of activating receptors and considering that the level of expression of these receptors strictly correlates with NK cell activities, we evaluated whether M1 could modulate NK cell activating receptor repertoire. In particular, we focused on NCRs, NKG2D, 2B4 and DNAM-1, which are considered the main receptors involved in tumor antigen recognition. We observed that M1 induced the upregulation of NKp44 and NKG2D only. Furthermore, it occurred exclusively in the presence of M1-derived soluble factors, as direct interactions did not affect the expression of these receptors. It means that M1-produced cytokines enhance NK cell effector functions through NKp44 and NKG2D modulation. Indeed, importantly, NKp44 and NKG2D upregulation correlated with increased capability to recognize altered cells. Indeed, in the presence of neutralizing antibodies against NKp44, NKG2D or both, NK cell degranulation was decreased. Interestingly, the best reduction occurred when both activating receptors were blocked, highlighting that they co-operated to trigger NK cell degranulation. It is totally in line with the fact that often activating receptors synergize to guarantee activating signals overcoming of the inhibitory ones (39, 56). Of note, NKp44 is selectively expressed by activated NK cells, since it is almost absent in resting NK cells. It further confirms M1 capability to activate NK cells. Therefore, M1 macrophages through the release of inflammatory cytokines increase NK cell expression of NKp44 and NKG2D, enhancing NK cell capability to kill target cells.

Surprisingly, we were able to demonstrate that IL-1β secreted by M1 was responsible for NKp44 upregulation, as its blocking reduced NKp44 expression by M1-primed NK cells. This result is particularly intriguing. Indeed, IL-1β is not described as a NK cell activating cytokine. In murine models NK cells were considered almost insensitive to IL-1β and in humans only a couple of works attributed to IL-1β a role in NK cell activation. Importantly, in one case IL-1β amplified the action of IL-12, and in the other case the one of IL-23 (181, 182). Therefore, up to date, IL-1β *per se* does not mediate any effect on human NK cells. However, since ILCs associated to MALT were discovered, IL-1β has been described as a fundamental cytokine not only for ILCs survival and expansion, but also for the enhancement of IL-22 production (183, 184). Interestingly, these population of ILCs is characterized by very high expression of NKp44 and, as recently reported by Glatzer T. et al., NKp44 triggering collaborates with IL-1β in IL-22 induction (186). These evidences suggest that NKp44 could somehow be connected to IL-1β signaling pathway, as we observed in our model. However, to sense IL-1β, NK cells need to express IL-1R I. According with the new nomenclatures proposed by Spits H. et al., circulating NK cells were included in ILC1 group and defined as IL-1R I negative, at least *in vivo* (10). In our hands, both resting $CD56^{bright}$ and $CD56^{dim}$ NK cells express IL-1R I, even if the $CD56^{bright}$ population seems to be slightly more positive. On the contrary, they are totally negative for IL-1R II. Of note, it is completely in line with what published by Cooper M. A. in 2001, before the ILCs coming (19). In addition, we observed that IL-1R I expression was selectively induced in $CD56^{dim}$ population upon M1 treatment and it correlated with the fact that by blocking IL-1β, IL-1R I or both, NKp44 reduction was more pronounced in the $CD56^{dim}$ population compared to the $CD56^{bright}$ counterpart. Therefore, we can affirm that M1, through the release of IL-1β, induce NKp44 upregulation preferentially on $CD56^{dim}$ NK cells, which express increased levels of IL-1R I. These evidences confer an important role to IL-1β in inducing NK cell activation and open the possibility of a strict relationship between NKp44 and IL-1R I pathways.

In parallel, we investigated which M1-derived cytokine could be responsible for NKG2D upregulation. Once excluded IL-15 and IL-18, a possible candidate was IFNs type I. Indeed, in a recent paper aimed to study the cross-talk between NK cells and macrophages in an *ex vivo* murine model it has been defined that IFN-β was able to induce NKG2D expression (240). By blocking IFN-β, we observed a reduction of NKG2D expression specifically in the $CD56^{bright}$ population. Therefore, we can affirm that NKG2D enhancement is totally IFN-βdependent, at least for $CD56^{bright}$ NK cells. The rational to evaluate cytokine effects on NK cell subsets come from the

fact that for long time $CD56^{bright}$ and $CD56^{dim}$ were considered different in terms of effector functions. The $CD56^{bright}$ population has been associated to cytokine production, whereas the $CD56^{dim}$ counterpart to cytolitic granules release (19). However, up to date, several evidences denote that NK cell subsets share comparable functional potential, which is strongly regulated by the micro-environment and, in particular, by pro-inflammatory cytokines (22). In line with that, we were able to demonstrate that different subsets respond in different ways to environmental cytokines.

In regard to M1-produced IFN-β effect on NK cells, we demonstrated that it was sufficient to induce IL-15 and IL-15Rα expression. Indeed, if both proteins were almost absent in resting NK cells, the treatment with M1 soluble factors strongly induced not only the mRNAs expression, but also the presence of membrane associated IL-15Rα. The production of IL-15 and IL-15Rα by NK cells has a big impact on NK cell biology. Indeed, since now, lymphocytes were considered unable to produce IL-15 and over all to present IL-15 (188, 205). The fact that NK cells, in addition to IL-15Rβγ chain, express also IL-15 and IL-15Rα leads us to hypothesize a possible IL-15 cis-presentation, that, since now, has been totally excluded. According with the recent work published from Zanoni I. et al. concerning the cross-talk between murine NK cells and dendritic cells (192), M1 macrophages, by releasing IFN-β, confer to NK cells the capability to cis-present IL-15. More importantly, we demonstrated that thanks to a mechanism of IL-15 cis-presentation IFN-γ secretion by NK cells was strongly triggered. Therefore, M1 not only induce IL-15 and IL-15Rα production by NK cells, but also a cis-presentation process, that impacts NK cell IFN-γ secretion. On the contrary, NK cell cytotoxicity was not affected by IL-15 cis-presentation. It can be explained by the fact that in our settings NK cytotoxicity was totally NKG2D dependent. Indeed, we demonstrated that NKG2D upregulation was responsible for increased NK cell degranulation. This is in line with the important role that NKG2D engagement plays in the induction of NK cell cytotoxicity reported in literature (37, 39, 55). We demonstrated on one hand that IL-15 cis-presentation could occur in human NK cells and on the other hand that human M1 macrophages were fundamental for the induction of this process. These results may have an important impact on tumor immuno-therapies. Indeed, IL-15 administration is one of the clinical strategies to boost immune cell activities towards tumors (189). However, IL-15 itself is not sufficient to generate a real clinical reduction of tumor masses, also due to the fact that it resulted instable. For this reason, new approaches suggest the use of IL-15/IL-15Rα complexes rather than IL-15 alone, which would guarantee a better IL-15 stability and activity. Although soluble IL-15/IL-15Rα

complexes were found in human serum (221), it has been demonstrated that IL-15 presented by soluble complexes generated *in vitro* exerted a less potent action compared to the ones associated to the membrane (193). For this reason, the study of the possible mechanisms that allow IL-15 presentation by IL-15Rα, which are still partially unknown in humans, results really mandatory, in particular whether new innovative and efficient immuno-therapies would be developed. The fact that NK cells, *per se*, could auto-trigger anti-tumoral activities by a mechanism of IL-15 cis-presentation can open new perspectives, over all if we consider that NK cells are often utilized for immuno-therapies (91). However, the big drawback of NK cell mediated immuno-therapies consists in the fact that they have to be continuously activated to maintain their effector functions. Overall, we can speculate that by recombinant IFN-β stimulation or directly by M1-conditioned medium treatment, we could trigger IL-15 cis-presentation by NK cells. This is therefore the only mechanism available at the moment to allow auto-sustainment of NK activation, maybe ameliorating NK cell anti-tumoral activities.

It is important to underline that IL-15 cis-presentation is not the only mechanism by which M1 increase IFN-γ secretion by NK cells. Indeed, in absence of soluble factors, the binding between CD48, that is selectively expressed by M1 macrophages, and 2B4, constitutively express by NK cells, was sufficient to trigger IFN-γ secretion by NK cells. It indicates that NK cell activation is a multi-faceted process. Indeed, it is mediated either by cytokine receptors, as IL-1R I and IL-15Rβγ, and activating receptors triggered upon ligand binding. We can speculate that *in vivo*, all these mechanisms co-operate to generate better NK cell responses. In line with these results, we demonstrated that, when NK cells were strictly in contact with M1 and when soluble mediator activity was excluded, M1 macrophages still maintained their capability to trigger NK cell degranulation. Indeed, although the absence of modulation of activating receptor able to recognize tumor antigens, M1 macrophages enhanced NK cell killing activities through IL-15 trans-presentation. M1 macrophages are characterized by high levels of IL-15 and IL-15Rα transcripts and express IL-15Rα on their membrane, allowing a mechanism of IL-15 trans-presentation. Similarly to what we observed, macrophage-mediated IL-15 trans-presentation has been associated to increased production of granzyme B and increased CD69 expression by NK cells in murine models (47). In general, we can conclude that different mechanisms of IL-15 presentation lead to different NK cell functions. Indeed, when IFN-β produced by M1 trigger IL-15 cis-presentation, NK cell IFN-γ secretion is enhanced. On the other hand, when soluble factors are excluded and NK cells direct interact with M1, IL-15 trans-presentation favors NK cell

degranulation. These results underline the importance to really dissect IL-15 presentation processes, in order to further understand IL-15 mechanisms of action and then allowing the generation of focused anti-tumoral therapies.

In conclusion, in line with the fact that M1 macrophages contribute to orchestrate anti-tumoral responses during the early phases of tumor appearance and considering our data, we can speculate that M1 not only behave, together with dendritic cells, as Antigen Presenting Cells able to trigger adaptive immunity, but they can also induce innate anti-tumoral responses. Through activation of resting NK cells, they favor the elimination of tumor cells and they sustain a pro-inflammatory micro-environment.

Although it is well known that NK cells are sources of IFN-γ and therefore they contribute to M1 polarization, it is not yet determined whether NK cell-derived IFN-γ can be sufficient to re-educate alternatively-activated macrophages. Since they are often associated with bad prognosis and represent the main immune cell responsible for cancer promotion, several strategies have been proposed to target TAMs (123, 156). One of them is represented by TAM re-education. This mechanism is aimed to modulate the tumor micro-environment in order to drive TAMs, which resemble M2 phenotype, to M1-like phenotypes. This mechanism not only should inhibit the pro-tumoral features of macrophages within the tumor, but should also directly limit tumor growth. Indeed, M1 are characterized by anti-tumoral activities and pro-inflammatory features. Considering that macrophages are important regulators of innate and adaptive immune, switching to an M1 phenotype could positively impact on other immune cells functions, favoring the generation of an hostile milieu for tumor cells.

In order to study the effect of NK cell-derived IFN-γ on M2 macrophages, we took advantage of the same *in vitro* autologous system we utilized before. Of note, we exploited the fact that M1 macrophages triggered NK cell IFN-γ secretion to mimic as close as possible an *in vivo* context. Indeed, we can assume that, in the early phases of tumor development, macrophages associated to the tumor display a M1 phenotype. As a consequence, they can be able to recruit resting NK cells from the circulation, leading to their activation. Then, since cancer cells actively determine the switch from M1 to M2 phenotype, we asked whether M1-activated NK cells could be able to revert, and in some extent to prevent, M2-like phenotype typical of TAMs, generating a sort of functional loop. We therefore treated M2 macrophages with autologous M1-primed NK cell-conditioned medium, containing high amounts of IFN-γ. To start, we determined whether NK cell-

derived IFN-γ was sufficient to downmodulate M2 associated genes. We observed that CD206 and ALOX15 transcripts were strongly downregulated upon the treatment with NK cell-derived IFN-γ. It suggests that, in line with one of their main feature that is plasticity, macrophages could rapidly respond to micro-environmental stimuli leading to different genetic program induction. In confirm to that, we observed that M1 typical markers, as CD80 and CD48, were upregulated in M2 macrophages treated with NK cell-derived IFN-γ. According with that, they displayed also induced expression of pro-inflammatory cytokines as, IL-1β and IL-15 (together with IL-15Rα), or chemokines, as CCL-5, CXCL-9 and CXCL-10. It suggests that NK cell-derived IFN-γ is sufficient not only to reduce M2 markers but also to trigger M1 associated molecules. However, two aspects have to be considered. First, the downmodulation of anti-inflammatory chemokines typically produced by M2 macrophages as CCL-17 and CCL-18 did not occur in M2 macrophages treated with NK cell-derived IFN-γ. Only CCL-22 was slightly decreased. These results suggest that if the expression of some M2 genes can easily be reverted, other M2 genes seem to be strictly determined. Secondly, we were surprised to see that the level of pro-inflammatory cytokines and chemokines in M2 macrophages triggered with NK cell-derived IFN-γ was even higher than the one observed in M1 in the same conditions. It indicates that M2 macrophages are paradoxically more prone to respond to IFN-γ than M1. Both observations lead us to hypothesize that a deeper analysis has to be done, in order to better understand the mechanisms underlying this processes. Considering the recent work published by Ostuni R. et al. (245), we can speculate that this macrophage plasticity is regulated by epigenetic mechanisms which confer to them a kind of “short-memory”. It means that upon a first stimulation, epigenetic modification occurr, leading to the acquisition of a different chromatin configuration. In the presence of a second stimuli, given relatively soon after, these epigenetic modifications “prime” or “shut-down” the macrophage epigenome. The genes that are in a “open” configuration, once re-stimulated, could generate faster and stronger responses compared to the ones that were “shut-down”. This hypothesis has to be demonstrated in our settings, but represents an intriguing mechanism of multi-step regulation of macrophage plasticity. M2 macrophages upon IFN-γ stimulation could produce pro-inflammatory cytokines that can be involved in the sustainment of NK cell effector functions. As a consequence, not only NK cells can be considered as good inducers of TAM re-education, but their IFN-γ secretion can be further sustained by re-educated TAM, amplifying the cross-talk loop.

By studying the cross-talk between NK cells and macrophages in an *in vitro* re-constituted tumor micro-environment model, we highlighted new mechanisms that regulate innate immune responses to cancer cells, giving the basis to develop new immunotherapeutic approaches. For sure, we have to extend this study *in vivo*, trying to work closer to the physiological and pathological conditions. In any case, we can at least affirm that, before adaptive immunity is required and macrophages are manipulated by the tumor, innate immunity can be armed to fight against cancer cells. By our studies, we expanded our knowledge on innate immune cell networking. In line with the mechanisms we highlighted, new therapeutically approaches can emerge. Indeed, innate immunity represents a source easily available to rapidly contain pathological malignancies. In conclusion, if we improve our tools to manipulate the tumor-microenvironment using the players that are just there, we could be pretty confident to give raise to new innovative and incisive approaches for cancer therapies.

REFERENCES

1. Medzhitov R (2009) Approaching the asymptote: 20 years later. *Immunity* 30(6):766-775.

2. Carneiro LA & Travassos LH (2013) The Interplay between NLRs and Autophagy in Immunity and Inflammation. *Frontiers in immunology* 4:361.

3. Zinzula L & Tramontano E (2013) Strategies of highly pathogenic RNA viruses to block dsRNA detection by RIG-I-like receptors: Hide, mask, hit. *Antiviral research* 100(3):615-635.

4. Hanahan D & Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646-674.

5. Mantovani A, Allavena P, Sica A, & Balkwill F (2008) Cancer-related inflammation. *Nature* 454(7203):436-444.

6. Schreiber RD, Old LJ, & Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331(6024):1565-1570.

7. Liu Y & Zeng G (2012) Cancer and innate immune system interactions: translational potentials for cancer immunotherapy. *J Immunother* 35(4):299-308.

8. Luevano M, Madrigal A, & Saudemont A (2012) Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. *Cellular & molecular immunology* 9(4):310-320.

9. Sun JC & Lanier LL (2011) NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nature reviews. Immunology* 11(10):645-657.

10. Spits H*, et al.* (2013) Innate lymphoid cells--a proposal for uniform nomenclature. *Nature reviews. Immunology* 13(2):145-149.

11. Kumar V, Ben-Ezra J, Bennett M, & Sonnenfeld G (1979) Natural killer cells in mice treated with 89strontium: normal target-binding cell numbers but inability to kill even after interferon administration. *J Immunol* 123(4):1832-1838.

12. Seaman WE*, et al.* (1978) beta-Estradiol reduces natural killer cells in mice. *J Immunol* 121(6):2193-2198.

13. Vosshenrich CA*, et al.* (2006) A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nature immunology* 7(11):1217-1224.

14. Freud AG*, et al.* (2005) A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* 22(3):295-304.

15. Vacca P*, et al.* (2011) CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proceedings of the National Academy of Sciences of the United States of America* 108(6):2402-2407.

16. Grzywacz B, Kataria N, Blazar BR, Miller JS, & Verneris MR (2011) Natural killer-cell differentiation by myeloid progenitors. *Blood* 117(13):3548-3558.

17. Becknell B & Caligiuri MA (2005) Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Advances in immunology* 86:209-239.

18. Yu J, Freud AG, & Caligiuri MA (2013) Location and cellular stages of natural killer cell development. *Trends in immunology* 34(12):573-582.

19. Cooper MA, Fehniger TA, & Caligiuri MA (2001) The biology of human natural killer-cell subsets. *Trends in immunology* 22(11):633-640.

20. Lanier LL, Testi R, Bindl J, & Phillips JH (1989) Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *The Journal of experimental medicine* 169(6):2233-2238.

21. Lanier LL, Le AM, Civin CI, Loken MR, & Phillips JH (1986) The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol* 136(12):4480-4486.

22. Inngjerdingen M, Kveberg L, Naper C, & Vaage JT (2011) Natural killer cell subsets in man and rodents. *Tissue antigens* 78(2):81-88.

23. Bauer S*, et al.* (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285(5428):727-729.

24. Vitale M*, et al.* (2002) Analysis of natural killer cells in TAP2-deficient patients: expression of functional triggering receptors and evidence for the existence of inhibitory receptor(s) that prevent lysis of normal autologous cells. *Blood* 99(5):1723-1729.

25. Caligiuri MA*, et al.* (1990) Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *The Journal of experimental medicine* 171(5):1509-1526.

26. Nagler A, Lanier LL, & Phillips JH (1990) Constitutive expression of high affinity interleukin 2 receptors on human CD16-natural killer cells in vivo. *The Journal of experimental medicine* 171(5):1527-1533.

27. Campbell JJ*, et al.* (2001) Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol* 166(11):6477-6482.

28. Poli A*, et al.* (2009) CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology* 126(4):458-465.

29. Robertson MJ*, et al.* (1992) Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *The Journal of experimental medicine* 175(3):779-788.

30. Fauriat C, Long EO, Ljunggren HG, & Bryceson YT (2010) Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 115(11):2167-2176.

31. De Maria A, Bozzano F, Cantoni C, & Moretta L (2011) Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on

activation. *Proceedings of the National Academy of Sciences of the United States of America* 108(2):728-732.

32. Vivier E*, et al.* (2011) Innate or adaptive immunity? The example of natural killer cells. *Science* 331(6013):44-49.

33. Krzewski K & Coligan JE (2012) Human NK cell lytic granules and regulation of their exocytosis. *Frontiers in immunology* 3:335.

34. Keefe D*, et al.* (2005) Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity* 23(3):249-262.

35. Portales P*, et al.* (2003) Interferon-alpha restores HIV-induced alteration of natural killer cell perforin expression in vivo. *AIDS* 17(4):495-504.

36. Thiery J*, et al.* (2011) Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nature immunology* 12(8):770-777.

37. Smyth MJ, Hayakawa Y, Takeda K, & Yagita H (2002) New aspects of natural-killer-cell surveillance and therapy of cancer. *Nature reviews. Cancer* 2(11):850-861.

38. Moretta L & Moretta A (2004) Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *The EMBO journal* 23(2):255-259.

39. Long EO, Kim HS, Liu D, Peterson ME, & Rajagopalan S (2013) Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annual review of immunology* 31:227-258.

40. Wu J*, et al.* (1999) An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285(5428):730-732.

41. Graham DB*, et al.* (2006) Vav1 controls DAP10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. *J Immunol* 177(4):2349-2355.

42. Upshaw JL*, et al.* (2006) NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nature immunology* 7(5):524-532.

43. Horng T, Bezbradica JS, & Medzhitov R (2007) NKG2D signaling is coupled to the interleukin 15 receptor signaling pathway. *Nature immunology* 8(12):1345-1352.

44. Zwirner NW, Fuertes MB, Girart MV, Domaica CI, & Rossi LE (2007) Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system. *Cytokine & growth factor reviews* 18(1-2):159-170.

45. Bryceson YT, March ME, Ljunggren HG, & Long EO (2006) Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107(1):159-166.

46. Lucas M, Schachterle W, Oberle K, Aichele P, & Diefenbach A (2007) Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* 26(4):503-517.

47. Mortier E*, et al.* (2009) Macrophage- and dendritic-cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8+ T cell subsets. *Immunity* 31(5):811-822.

48. Guia S*, et al.* (2008) A role for interleukin-12/23 in the maturation of human natural killer and CD56+ T cells in vivo. *Blood* 111(10):5008-5016.

49. Chaix J*, et al.* (2008) Cutting edge: Priming of NK cells by IL-18. *J Immunol* 181(3):1627-1631.

50. Cooper MA*, et al.* (2009) Cytokine-induced memory-like natural killer cells. *Proceedings of the National Academy of Sciences of the United States of America* 106(6):1915-1919.

51. Cooper MA & Yokoyama WM (2010) Memory-like responses of natural killer cells. *Immunological reviews* 235(1):297-305.

52. Cooley S & Weisdorf DS (2010) Natural killer cells and tumor control. *Current opinion in hematology* 17(6):514-521.

53. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, & Kershaw MH (2011) Activating and inhibitory receptors of natural killer cells. *Immunology and cell biology* 89(2):216-224.

54. Hudspeth K, Silva-Santos B, & Mavilio D (2013) Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. *Frontiers in immunology* 4:69.

55. Moretta A*, et al.* (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annual review of immunology* 19:197-223.

56. Biassoni R*, et al.* (2001) Human natural killer cell receptors and co-receptors. *Immunological reviews* 181:203-214.

57. Pogge von Strandmann E*, et al.* (2007) Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* 27(6):965-974.

58. Brandt CS*, et al.* (2009) The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *The Journal of experimental medicine* 206(7):1495-1503.

59. Ferlazzo G*, et al.* (2002) Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *The Journal of experimental medicine* 195(3):343-351.

60. Baychelier F*, et al.* (2013) Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. *Blood* 122(17):2935-2942.

61. Sivori S*, et al.* (1999) NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *European journal of immunology* 29(5):1656-1666.

62. Raulet DH (2003) Roles of the NKG2D immunoreceptor and its ligands. *Nature reviews. Immunology* 3(10):781-790.

63. Sutherland CL*, et al.* (2002) UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J Immunol* 168(2):671-679.

64. Zompi S*, et al.* (2003) NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. *Nature immunology* 4(6):565-572.

65. Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, & Leibson PJ (2003) NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nature immunology* 4(6):557-564.

66. Jolly C & Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of the National Cancer Institute* 92(19):1564-1572.

67. Groh V*, et al.* (1996) Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proceedings of the National Academy of Sciences of the United States of America* 93(22):12445-12450.

68. Castriconi R*, et al.* (2003) Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America* 100(7):4120-4125.

69. Garni-Wagner BA, Purohit A, Mathew PA, Bennett M, & Kumar V (1993) A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol* 151(1):60-70.

70. Lee KM*, et al.* (2004) 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *The Journal of experimental medicine* 199(9):1245-1254.

71. McNerney ME, Lee KM, & Kumar V (2005) 2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8+ T cells. *Molecular immunology* 42(4):489-494.

72. Mathew SO, Rao KK, Kim JR, Bambard ND, & Mathew PA (2009) Functional role of human NK cell receptor 2B4 (CD244) isoforms. *European journal of immunology* 39(6):1632-1641.

73. Lanier LL (2005) NK cell recognition. *Annual review of immunology* 23:225-274.

74. Nedvetzki S*, et al.* (2007) Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. *Blood* 109(9):3776-3785.

75. Vaidya SV*, et al.* (2005) Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD244) in the rejection of B16 melanoma cells. *J Immunol* 174(2):800-807.

76. Shibuya A*, et al.* (1996) DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* 4(6):573-581.

77. Ksienzyk A*, et al.* (2011) IRF-1 expression is essential for natural killer cells to suppress metastasis. *Cancer research* 71(20):6410-6418.

78. Tassi I, Klesney-Tait J, & Colonna M (2006) Dissecting natural killer cell activation pathways through analysis of genetic mutations in human and mouse. *Immunological reviews* 214:92-105.

79. Gilfillan S*, et al.* (2008) DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *The Journal of experimental medicine* 205(13):2965-2973.

80. Imai K, Matsuyama S, Miyake S, Suga K, & Nakachi K (2000) Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 356(9244):1795-1799.

81. Stojanovic A & Cerwenka A (2011) Natural killer cells and solid tumors. *Journal of innate immunity* 3(4):355-364.

82. Langers I, Renoux VM, Thiry M, Delvenne P, & Jacobs N (2012) Natural killer cells: role in local tumor growth and metastasis. *Biologics : targets & therapy* 6:73-82.

83. Vivier E, Ugolini S, Blaise D, Chabannon C, & Brossay L (2012) Targeting natural killer cells and natural killer T cells in cancer. *Nature reviews. Immunology* 12(4):239-252.

84. Qin Z & Blankenstein T (2000) CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity* 12(6):677-686.

85. Esendagli G*, et al.* (2008) Malignant and non-malignant lung tissue areas are differentially populated by natural killer cells and regulatory T cells in non-small cell lung cancer. *Lung Cancer* 59(1):32-40.

86. Sandel MH*, et al.* (2005) Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Molecular immunology* 42(4):541-546.

87. Terme M, Ullrich E, Delahaye NF, Chaput N, & Zitvogel L (2008) Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nature immunology* 9(5):486-494.

88. Konjevic G, Jurisic V, & Spuzic I (2001) Association of NK cell dysfunction with changes in LDH characteristics of peripheral blood lymphocytes (PBL) in breast cancer patients. *Breast cancer research and treatment* 66(3):255-263.

89. Hersey P, Edwards A, & McCarthy WH (1980) Tumour-related changes in natural killer cell activity in melanoma patients. Influence of stage of disease, tumour thickness and age of patients. *International journal of cancer. Journal international du cancer* 25(2):187-194.

90. Ljunggren HG & Malmberg KJ (2007) Prospects for the use of NK cells in immunotherapy of human cancer. *Nature reviews. Immunology* 7(5):329-339.

91. Levy EM, Roberti MP, & Mordoh J (2011) Natural killer cells in human cancer: from biological functions to clinical applications. *Journal of biomedicine & biotechnology* 2011:676198.

92. Geissmann F*, et al.* (2010) Development of monocytes, macrophages, and dendritic cells. *Science* 327(5966):656-661.

93. Mosser DM & Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* 8(12):958-969.

94. Molawi K & Sieweke MH (2013) Transcriptional control of macrophage identity, self-renewal, and function. *Advances in immunology* 120:269-300.

95. Weber C*, et al.* (2000) Differential chemokine receptor expression and function in human monocyte subpopulations. *Journal of leukocyte biology* 67(5):699-704.

96. Ancuta P*, et al.* (2003) Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *The Journal of experimental medicine* 197(12):1701-1707.

97. Ziegler-Heitbrock HW*, et al.* (1993) The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages. *European journal of immunology* 23(9):2053-2058.

98. Cros J*, et al.* (2010) Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 33(3):375-386.

99. Wong KL*, et al.* (2011) Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood* 118(5):e16-31.

100. Zawada AM*, et al.* (2011) SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. *Blood* 118(12):e50-61.

101. Rossol M, Kraus S, Pierer M, Baerwald C, & Wagner U (2012) The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis and rheumatism* 64(3):671-677.

102. Wong KL*, et al.* (2012) The three human monocyte subsets: implications for health and disease. *Immunologic research* 53(1-3):41-57.

103. Murdoch C, Tazzyman S, Webster S, & Lewis CE (2007) Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J Immunol* 178(11):7405-7411.

104. Venneri MA*, et al.* (2007) Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 109(12):5276-5285.

105. Schakel K*, et al.* (1998) A novel dendritic cell population in human blood: one-step immunomagnetic isolation by a specific mAb (M-DC8) and in vitro priming of cytotoxic T lymphocytes. *European journal of immunology* 28(12):4084-4093.

106. Costantini C*, et al.* (2011) Human neutrophils interact with both 6-sulfo LacNAc+ DC and NK cells to amplify NK-derived IFN{gamma}: role of CD18, ICAM-1, and ICAM-3. *Blood* 117(5):1677-1686.

107. Dale DC, Boxer L, & Liles WC (2008) The phagocytes: neutrophils and monocytes. *Blood* 112(4):935-945.

108. Biswas SK & Mantovani A (2010) Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature immunology* 11(10):889-896.

109. Biswas SK, Allavena P, & Mantovani A (2013) Tumor-associated macrophages: functional diversity, clinical significance, and open questions. *Seminars in immunopathology* 35(5):585-600.

110. Stein M, Keshav S, Harris N, & Gordon S (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine* 176(1):287-292.

111. Wilson MS*, et al.* (2007) Immunopathology of schistosomiasis. *Immunology and cell biology* 85(2):148-154.

112. Jenkins SJ & Allen JE (2010) Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes. *Journal of biomedicine & biotechnology* 2010:262609.

113. Martinez FO, Gordon S, Locati M, & Mantovani A (2006) Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 177(10):7303-7311.

114. Mantovani A, Sozzani S, Locati M, Allavena P, & Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in immunology* 23(11):549-555.

115. Mantovani A*, et al.* (2004) The chemokine system in diverse forms of macrophage activation and polarization. *Trends in immunology* 25(12):677-686.

116. Edwards JP, Zhang X, Frauwirth KA, & Mosser DM (2006) Biochemical and functional characterization of three activated macrophage populations. *Journal of leukocyte biology* 80(6):1298-1307.

117. Sica A & Mantovani A (2012) Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation* 122(3):787-795.

118. Pello OM*, et al.* (2012) Role of c-MYC in alternative activation of human macrophages and tumor-associated macrophage biology. *Blood* 119(2):411-421.

119. Lawrence T & Natoli G (2011) Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nature reviews. Immunology* 11(11):750-761.

120. De Santa F*, et al.* (2009) Jmjd3 contributes to the control of gene expression in LPS-activated macrophages. *The EMBO journal* 28(21):3341-3352.

121. Gordon S & Taylor PR (2005) Monocyte and macrophage heterogeneity. *Nature reviews. Immunology* 5(12):953-964.

122. Monticelli S & Natoli G (2013) Short-term memory of danger signals and environmental stimuli in immune cells. *Nature immunology* 14(8):777-784.

123. Allavena P & Mantovani A (2012) Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. *Clinical and experimental immunology* 167(2):195-205.

124. Solinas G, Germano G, Mantovani A, & Allavena P (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *Journal of leukocyte biology* 86(5):1065-1073.

125. Bottazzi B*, et al.* (1983) Regulation of the macrophage content of neoplasms by chemoattractants. *Science* 220(4593):210-212.

126. Zachariae CO*, et al.* (1990) Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. *The Journal of experimental medicine* 171(6):2177-2182.

127. Rius J*, et al.* (2008) NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* 453(7196):807-811.

128. Fang HY*, et al.* (2009) Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood* 114(4):844-859.

129. Balkwill F (2009) Tumour necrosis factor and cancer. *Nature reviews. Cancer* 9(5):361-371.

130. Mancino A & Lawrence T (2010) Nuclear factor-kappaB and tumor-associated macrophages. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16(3):784-789.

131. Lamb J*, et al.* (2003) A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. *Cell* 114(3):323-334.

132. Liu Y, Li PK, Li C, & Lin J (2010) Inhibition of STAT3 signaling blocks the anti-apoptotic activity of IL-6 in human liver cancer cells. *The Journal of biological chemistry* 285(35):27429-27439.

133. Mantovani A*, et al.* (2010) The chemokine system in cancer biology and therapy. *Cytokine & growth factor reviews* 21(1):27-39.

134. Lazennec G & Richmond A (2010) Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends in molecular medicine* 16(3):133-144.

135. Balkwill F (2004) Cancer and the chemokine network. *Nature reviews. Cancer* 4(7):540-550.

136. Adema GJ*, et al.* (1997) A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* 387(6634):713-717.

137. Schutyser E*, et al.* (2002) Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *The Journal of biological chemistry* 277(27):24584-24593.

138. Steinman RM, Hawiger D, & Nussenzweig MC (2003) Tolerogenic dendritic cells. *Annual review of immunology* 21:685-711.

139. Banchereau J & Palucka AK (2005) Dendritic cells as therapeutic vaccines against cancer. *Nature reviews. Immunology* 5(4):296-306.

140. Wyckoff JB*, et al.* (2007) Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer research* 67(6):2649-2656.

141. Kessenbrock K, Plaks V, & Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 141(1):52-67.

142. De Palma M*, et al.* (2005) Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer cell* 8(3):211-226.

143. Pucci F*, et al.* (2009) A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood* 114(4):901-914.

144. De Palma M, Murdoch C, Venneri MA, Naldini L, & Lewis CE (2007) Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends in immunology* 28(12):519-524.

145. Welford AF*, et al.* (2011) TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *The Journal of clinical investigation* 121(5):1969-1973.

146. Negus RP, Stamp GW, Hadley J, & Balkwill FR (1997) Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *The American journal of pathology* 150(5):1723-1734.

147. Hagemann T*, et al.* (2006) Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. *J Immunol* 176(8):5023-5032.

148. Zhou J*, et al.* (2009) Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. *International journal of cancer. Journal international du cancer* 125(7):1640-1648.

149. Scholl SM*, et al.* (1994) Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *Journal of the National Cancer Institute* 86(2):120-126.

150. Ong SM*, et al.* (2012) Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *European journal of immunology* 42(1):89-100.

151. Beatty GL*, et al.* (2011) CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science* 331(6024):1612-1616.

152. Rolny C*, et al.* (2011) HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. *Cancer cell* 19(1):31-44.

153. Watkins SK, Egilmez NK, Suttles J, & Stout RD (2007) IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages in vitro and in vivo. *J Immunol* 178(3):1357-1362.

154. Watkins SK*, et al.* (2009) Rapid release of cytoplasmic IL-15 from tumor-associated macrophages is an initial and critical event in IL-12-initiated tumor regression. *European journal of immunology* 39(8):2126-2135.

155. Hagemann T*, et al.* (2008) "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *The Journal of experimental medicine* 205(6):1261-1268.

156. Heusinkveld M*, et al.* (2011) M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. *J Immunol* 187(3):1157-1165.

157. Duluc D*, et al.* (2009) Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *International journal of cancer. Journal international du cancer* 125(2):367-373.

158. Baratin M*, et al.* (2005) Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to Plasmodium falciparum. *Proceedings of the National Academy of Sciences of the United States of America* 102(41):14747-14752.

159. Lapaque N, Walzer T, Meresse S, Vivier E, & Trowsdale J (2009) Interactions between human NK cells and macrophages in response to Salmonella infection. *J Immunol* 182(7):4339-4348.

160. Lunemann A*, et al.* (2008) Human NK cells kill resting but not activated microglia via NKG2D- and NKp46-mediated recognition. *J Immunol* 181(9):6170-6177.

161. Basu S*, et al.* (2009) Human uterine NK cells interact with uterine macrophages via NKG2D upon stimulation with PAMPs. *Am J Reprod Immunol* 61(1):52-61.

162. Bellora F*, et al.* (2010) The interaction of human natural killer cells with either unpolarized or polarized macrophages results in different functional outcomes. *Proceedings of the National Academy of Sciences of the United States of America* 107(50):21659-21664.

163. Bellora F*, et al.* (2012) M-CSF induces the expression of a membrane-bound form of IL-18 in a subset of human monocytes differentiating in vitro toward macrophages. *European journal of immunology* 42(6):1618-1626.

164. Romo N*, et al.* (2011) Natural killer cell-mediated response to human cytomegalovirus-infected macrophages is modulated by their functional polarization. *Journal of leukocyte biology* 90(4):717-726.

165. Dinarello CA (2005) Interleukin-1beta. *Critical care medicine* 33(12 Suppl):S460-462.

166. Andrei C*, et al.* (2004) Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. *Proceedings of the National Academy of Sciences of the United States of America* 101(26):9745-9750.

167. Bocker U*, et al.* (2001) Expression and localization of IL-1beta mRNA is interrelated with cytoskeletal rearrangement in monocytes stimulated by adherence: a light microscopy in situ hybridization study. *Immunology and cell biology* 79(5):444-453.

168. Brough D & Rothwell NJ (2007) Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *Journal of cell science* 120(Pt 5):772-781.

169. Becker CE, Creagh EM, & O'Neill LA (2009) Rab39a binds caspase-1 and is required for caspase-1-dependent interleukin-1beta secretion. *The Journal of biological chemistry* 284(50):34531-34537.

170. Qu Y, Franchi L, Nunez G, & Dubyak GR (2007) Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J Immunol* 179(3):1913-1925.

171. Laliberte RE, Eggler J, & Gabel CA (1999) ATP treatment of human monocytes promotes caspase-1 maturation and externalization. *The Journal of biological chemistry* 274(52):36944-36951.

172. O'Neill L (2000) The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. *Biochemical Society transactions* 28(5):557-563.

173. Martin MU & Wesche H (2002) Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. *Biochimica et biophysica acta* 1592(3):265-280.

174. Boraschi D & Tagliabue A (2013) The interleukin-1 receptor family. *Seminars in immunology*.

175. Garlanda C, Riva F, Bonavita E, & Mantovani A (2013) Negative regulatory receptors of the IL-1 family. *Seminars in immunology*.

176. Schreuder H*, et al.* (1997) A new cytokine-receptor binding mode revealed by the crystal structure of the IL-1 receptor with an antagonist. *Nature* 386(6621):194-200.

177. Symons JA, Young PR, & Duff GW (1995) Soluble type II interleukin 1 (IL-1) receptor binds and blocks processing of IL-1 beta precursor and loses affinity for IL-1 receptor antagonist. *Proceedings of the National Academy of Sciences of the United States of America* 92(5):1714-1718.

178. Burger D, Chicheportiche R, Giri JG, & Dayer JM (1995) The inhibitory activity of human interleukin-1 receptor antagonist is enhanced by type II interleukin-1 soluble receptor and hindered by type I interleukin-1 soluble receptor. *The Journal of clinical investigation* 96(1):38-41.

179. Kopf M, Bachmann MF, & Marsland BJ (2010) Averting inflammation by targeting the cytokine environment. *Nature reviews. Drug discovery* 9(9):703-718.

180. Hazuda DJ, Strickler J, Kueppers F, Simon PL, & Young PR (1990) Processing of precursor interleukin 1 beta and inflammatory disease. *The Journal of biological chemistry* 265(11):6318-6322.

181. Cooper MA*, et al.* (2001) Interleukin-1beta costimulates interferon-gamma production by human natural killer cells. *European journal of immunology* 31(3):792-801.

182. van de Wetering D, de Paus RA, van Dissel JT, & van de Vosse E (2009) Salmonella induced IL-23 and IL-1beta allow for IL-12 production by monocytes and Mphi1 through induction of IFN-gamma in CD56 NK/NK-like T cells. *PloS one* 4(12):e8396.

183. Cella M*, et al.* (2009) A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457(7230):722-725.

184. Cella M, Otero K, & Colonna M (2010) Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proceedings of the National Academy of Sciences of the United States of America* 107(24):10961-10966.

185. Hughes T*, et al.* (2010) Interleukin-1beta selectively expands and sustains interleukin-22+ immature human natural killer cells in secondary lymphoid tissue. *Immunity* 32(6):803-814.

186. Glatzer T*, et al.* (2013) RORgammat(+) innate lymphoid cells acquire a proinflammatory program upon engagement of the activating receptor NKp44. *Immunity* 38(6):1223-1235.

187. Burton JD*, et al.* (1994) A lymphokine, provisionally designated interleukin T and produced by a human adult T-cell leukemia line, stimulates T-cell proliferation and the induction of lymphokine-activated

killer cells. *Proceedings of the National Academy of Sciences of the United States of America* 91(11):4935-4939.

188. Grabstein KH*, et al.* (1994) Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* 264(5161):965-968.

189. Steel JC, Waldmann TA, & Morris JC (2012) Interleukin-15 biology and its therapeutic implications in cancer. *Trends in pharmacological sciences* 33(1):35-41.

190. Anderson DM*, et al.* (1995) Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes. *The Journal of biological chemistry* 270(50):29862-29869.

191. Waldmann TA & Tagaya Y (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annual review of immunology* 17:19-49.

192. Zanoni I*, et al.* (2013) IL-15 cis presentation is required for optimal NK cell activation in lipopolysaccharide-mediated inflammatory conditions. *Cell reports* 4(6):1235-1249.

193. Stonier SW & Schluns KS (2010) Trans-presentation: a novel mechanism regulating IL-15 delivery and responses. *Immunology letters* 127(2):85-92.

194. Kennedy MK*, et al.* (2000) Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *The Journal of experimental medicine* 191(5):771-780.

195. Pelletier M, Ratthe C, & Girard D (2002) Mechanisms involved in interleukin-15-induced suppression of human neutrophil apoptosis: role of the anti-apoptotic Mcl-1 protein and several kinases including Janus kinase-2, p38 mitogen-activated protein kinase and extracellular signal-regulated kinases-1/2. *FEBS letters* 532(1-2):164-170.

196. Budagian V, Bulanova E, Paus R, & Bulfone-Paus S (2006) IL-15/IL-15 receptor biology: a guided tour through an expanding universe. *Cytokine & growth factor reviews* 17(4):259-280.

197. Anguille S*, et al.* (2009) Short-term cultured, interleukin-15 differentiated dendritic cells have potent immunostimulatory properties. *Journal of translational medicine* 7:109.

198. Fehniger TA & Caligiuri MA (2001) Interleukin 15: biology and relevance to human disease. *Blood* 97(1):14-32.

199. Carson WE*, et al.* (1994) Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *The Journal of experimental medicine* 180(4):1395-1403.

200. Fehniger TA*, et al.* (1999) Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* 162(8):4511-4520.

201. Lodolce JP, Burkett PR, Boone DL, Chien M, & Ma A (2001) T cell-independent interleukin 15Ralpha signals are required for bystander proliferation. *The Journal of experimental medicine* 194(8):1187-1194.

202. Marks-Konczalik J*, et al.* (2000) IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 97(21):11445-11450.

203. Giri JG*, et al.* (1995) Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *The EMBO journal* 14(15):3654-3663.

204. Dubois S*, et al.* (1999) Natural splicing of exon 2 of human interleukin-15 receptor alpha-chain mRNA results in a shortened form with a distinct pattern of expression. *The Journal of biological chemistry* 274(38):26978-26984.

205. Dubois S, Mariner J, Waldmann TA, & Tagaya Y (2002) IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity* 17(5):537-547.

206. Musso T*, et al.* (1999) Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15. *Blood* 93(10):3531-3539.

207. Minagawa M*, et al.* (2002) Enforced expression of Bcl-2 restores the number of NK cells, but does not rescue the impaired development of NKT cells or intraepithelial lymphocytes, in IL-2/IL-15 receptor beta-chain-deficient mice. *J Immunol* 169(8):4153-4160.

208. Koka R*, et al.* (2004) Cutting edge: murine dendritic cells require IL-15R alpha to prime NK cells. *J Immunol* 173(6):3594-3598.

209. Kallies A*, et al.* (2011) A role for Blimp1 in the transcriptional network controlling natural killer cell maturation. *Blood* 117(6):1869-1879.

210. Soudja SM, Ruiz AL, Marie JC, & Lauvau G (2012) Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity* 37(3):549-562.

211. Huntington ND*, et al.* (2009) IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *The Journal of experimental medicine* 206(1):25-34.

212. Mortier E, Woo T, Advincula R, Gozalo S, & Ma A (2008) IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation. *The Journal of experimental medicine* 205(5):1213-1225.

213. Kobayashi H*, et al.* (2005) Role of trans-cellular IL-15 presentation in the activation of NK cell-mediated killing, which leads to enhanced tumor immunosurveillance. *Blood* 105(2):721-727.

214. Castillo EF & Schluns KS (2012) Regulating the immune system via IL-15 transpresentation. *Cytokine* 59(3):479-490.

215. Budagian V*, et al.* (2004) Natural soluble interleukin-15Ralpha is generated by cleavage that involves the tumor necrosis factor-alpha-converting enzyme (TACE/ADAM17). *The Journal of biological chemistry* 279(39):40368-40375.

216. Mortier E, Bernard J, Plet A, & Jacques Y (2004) Natural, proteolytic release of a soluble form of human IL-15 receptor alpha-chain that behaves as a specific, high affinity IL-15 antagonist. *J Immunol* 173(3):1681-1688.

217. Dubois S, Patel HJ, Zhang M, Waldmann TA, & Muller JR (2008) Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. *J Immunol* 180(4):2099-2106.

218. Rubinstein MP*, et al.* (2006) Converting IL-15 to a superagonist by binding to soluble IL-15R{alpha}. *Proceedings of the National Academy of Sciences of the United States of America* 103(24):9166-9171.

219. Stoklasek TA, Schluns KS, & Lefrancois L (2006) Combined IL-15/IL-15Ralpha immunotherapy maximizes IL-15 activity in vivo. *J Immunol* 177(9):6072-6080.

220. Mortier E*, et al.* (2006) Soluble interleukin-15 receptor alpha (IL-15R alpha)-sushi as a selective and potent agonist of IL-15 action through IL-15R beta/gamma. Hyperagonist IL-15 x IL-15R alpha fusion proteins. *The Journal of biological chemistry* 281(3):1612-1619.

221. Bergamaschi C*, et al.* (2012) Circulating IL-15 exists as heterodimeric complex with soluble IL-15Ralpha in human and mouse serum. *Blood* 120(1):e1-8.

222. Yu P, Steel JC, Zhang M, Morris JC, & Waldmann TA (2010) Simultaneous blockade of multiple immune system inhibitory checkpoints enhances antitumor activity mediated by interleukin-15 in a murine metastatic colon carcinoma model. *Clinical cancer research : an official journal of the American Association for Cancer Research* 16(24):6019-6028.

223. Zhang M*, et al.* (2009) Interleukin-15 combined with an anti-CD40 antibody provides enhanced therapeutic efficacy for murine models of colon cancer. *Proceedings of the National Academy of Sciences of the United States of America* 106(18):7513-7518.

224. Rudick RA & Goelz SE (2011) Beta-interferon for multiple sclerosis. *Experimental cell research* 317(9):1301-1311.

225. Taniguchi T & Takaoka A (2002) The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Current opinion in immunology* 14(1):111-116.

226. Zanoni I & Granucci F (2013) Role of CD14 in host protection against infections and in metabolism regulation. *Frontiers in cellular and infection microbiology* 3:32.

227. Ostuni R, Zanoni I, & Granucci F (2010) Deciphering the complexity of Toll-like receptor signaling. *Cellular and molecular life sciences : CMLS* 67(24):4109-4134.

228. Zanoni I*, et al.* (2011) CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 147(4):868-880.

229. Arduini RM*, et al.* (1999) Characterization of a soluble ternary complex formed between human interferon-beta-1a and its receptor chains. *Protein science : a publication of the Protein Society* 8(9):1867-1877.

230. Uze G, Schreiber G, Piehler J, & Pellegrini S (2007) The receptor of the type I interferon family. *Current topics in microbiology and immunology* 316:71-95.

231. Cleary CM, Donnelly RJ, Soh J, Mariano TM, & Pestka S (1994) Knockout and reconstitution of a functional human type I interferon receptor complex. *The Journal of biological chemistry* 269(29):18747-18749.

232. Kumaran J, Colamonici OR, & Fish EN (2000) Structure-function study of the extracellular domain of the human type I interferon receptor (IFNAR)-1 subunit. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 20(5):479-485.

233. Biron CA (2001) Interferons alpha and beta as immune regulators--a new look. *Immunity* 14(6):661-664.

234. Biron CA, Nguyen KB, Pien GC, Cousens LP, & Salazar-Mather TP (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual review of immunology* 17:189-220.

235. Chijioke O & Munz C (2013) Dendritic cell derived cytokines in human natural killer cell differentiation and activation. *Frontiers in immunology* 4:365.

236. Pontiroli F*, et al.* (2012) The timing of IFNbeta production affects early innate responses to Listeria monocytogenes and determines the overall outcome of lethal infection. *PloS one* 7(8):e43455.

237. Walzer T, Dalod M, Robbins SH, Zitvogel L, & Vivier E (2005) Natural-killer cells and dendritic cells: "l'union fait la force". *Blood* 106(7):2252-2258.

238. Zanoni I, Granucci F, Foti M, & Ricciardi-Castagnoli P (2007) Self-tolerance, dendritic cell (DC)-mediated activation and tissue distribution of natural killer (NK) cells. *Immunology letters* 110(1):6-17.

239. Porta C*, et al.* (2009) Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proceedings of the National Academy of Sciences of the United States of America* 106(35):14978-14983.

240. Zhou Z, Zhang C, Zhang J, & Tian Z (2012) Macrophages help NK cells to attack tumor cells by stimulatory NKG2D ligand but protect themselves from NK killing by inhibitory ligand Qa-1. *PloS one* 7(5):e36928.

241. Suarez-Alvarez B*, et al.* (2010) Epigenetic mechanisms regulate MHC and antigen processing molecules in human embryonic and induced pluripotent stem cells. *PloS one* 5(4):e10192.

242. Bae DS, Hwang YK, & Lee JK (2012) Importance of NKG2D-NKG2D ligands interaction for cytolytic activity of natural killer cell. *Cellular immunology* 276(1-2):122-127.

243. Wuest SJ, Crucet M, Gemperle C, Loretz C, & Hersberger M (2012) Expression and regulation of 12/15-lipoxygenases in human primary macrophages. *Atherosclerosis* 225(1):121-127.

244. Porcheray F*, et al.* (2005) Macrophage activation switching: an asset for the resolution of inflammation. *Clinical and experimental immunology* 142(3):481-489.

245. Ostuni R*, et al.* (2013) Latent enhancers activated by stimulation in differentiated cells. *Cell* 152(1-2):157-171.

RINGRAZIAMENTI

Vorrei ringraziare per primi il Prof. Massimo Locati e il Dott. Domenico Mavilio per avermi permesso di svolgere il dottorato nei loro laboratori e per avermi proposto un progetto interessante in un ambito ancora tutto da scoprire.

Grazie a Matthieu. Tu che hai sempre creduto in me, da quando "piccolina" ti guardavo lavorare sotto cappa. Grazie per il tuo sostegno, per esserci sempre stato. Per avermi saputa ascoltare e soprattutto consigliare. Per avermi fatta crescere, a suon di musica alta e litigate. Per aver capito quando era il momento di lasciarmi camminare con le mie gambe, ma per essere sempre stato presente, dietro l'angolo. Grazie per le pause caffè, per i pranzi a parlare di "scienza" (povero Paolo), grazie per avermi insegnato a parlare inglese e grazie per aver imparato l'italiano. Grazie per avermi insegnato a fare l'indice e avermi salvato da End Note. Grazie per avermi fatto sentire sempre al tuo livello, e mai inferiore a te (a parte quando si parla di introduzione e di macrofagi). Grazie per avermi confermato che si può lavorare insieme ed essere amici. Non avrei potuto farne a meno.

Grazie a Raffaella, Elena, Chiara, Benedetta, Graziella, Alessandro, Tiziana, Federica, Grazia e ai più giovani Sabrina, Manuel, Mariaelvy del gruppo Locati. Grazie alle mie fide compagne di dottorato, Nicoletta e Cinzia, per avermi fatto sentire parte del gruppo da subito. Data la mia "postazione" ho potuto condividere con voi meno di quello che avrei voluto.

Grazie a Silvia, Enrico, Aska, Francesca, Kelly, Elena, Alessandra, Alessandra, Monica, Elena, e ai più giovani Veronica, Marco, Tonia, Federica, Max, Silvia del gruppo Mavilio. Grazie per avermi accolta e per avermi "sopportata" in laboratorio.

Grazie a Paolo, compagno di studi da sempre. Grazie per essere entrato in questo percorso e per aver migliorato il cammino. Grazie per la tua saggezza e per la tua tranquillità. Grazie per avermi capita (e grazie per lasciarmi più di metà scrivania!!!).

Grazie a Martina. Grazie per avermi dato la possibilità di insegnare. Grazie per avermi veramente aiutata. Grazie per i successi e per gli errori, dai quali ho capito i miei sbagli ma con i quali ho potuto crescere. Io ho cercato di fare del mio meglio. Ora tocca a te.

Grazie alla mia mamma e al mio papà. È solo merito vostro se sono arrivata fin qui. Grazie per avermi permesso di studiare e per avere rispettato la mia scelta. Grazie per avermi sostenuto anche in questa scelta. È stato difficile e sono cambiata, ma ora guardandomi indietro, sono orgogliosa di me. Grazie al mio papà, perchè la mamma è sempre la mamma, con lei è più facile, ma vedere che sei orgoglioso di me è impagabile.

Grazie al mio fratellino. Grazie per non essere geloso e per aver dimostrato di sapere fare meglio. Grazie per la fiducia che riponi in me e per la considerazione che hai di me. Tu sei appena

“arrivato”, ma io ne sono già fiera. L’ho sempre detto io che tu sei il più intelligente tra i due, oltre che il più alto, il più bello, il più simpatico...

Grazie a Mauro. Sempre tu. Sempre con me. Grazie per aver cambiato idea. Grazie per avermi resa felice. Grazie per avermi ascoltata e consolata e per essere stato felice con me. Grazie per aver tentato di imbucarti all’ICI. Grazie per non esserci riuscito. Grazie per essere sempre fiero di me. Ma soprattutto grazie per questi ultimi mesi. Grazie per avermi lasciato spazio, per i sabati in lab e le domeniche al PC. Grazie per le cene alle 22 e per lavare i piatti. Grazie per rispettare me e il mio lavoro. Grazie per essere cresciuto con me. Grazie per essere come sei.

Questa tesi è dedicata te, che con le tue canzoni mi vieni sempre a cercare...