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"Molecular characterization and immunomodulatory properties of MM cell-derived exosomes on NK cell-mediated functions: role of Toll-like receptor 2"

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INTRODUCTION

1. Natural Killer cells

Natural Killer (NK) cells are large granular lymphocytes of innate immune system involved in many processes in the immune response, including direct recognition of infected, tumor and damaged cells and play an important role in determining the outcome of adaptive immune responses through cytokine and chemokine secretion and the direct interaction with other immune cells.

Despite NK cells derive from the common **$CD34^+$ hematopoietic progenitor cell** (HPC), as T and B lymphocytes, they are distinct from those cells because of their different morphologic, phenotypic and functional features, and pass through some stage before the complete maturation. The NK-cell precursor (NKP) is generated from HPC and develop into immature NK cell and then into mature NK cells, acquiring the expression of NK-cell-specific markers. In addition to the maturation process, NK cells are "educated" and "licensed" to prevent the formation of autoreactive NK cells through mechanisms still not completely known. Thus, licensed and educated NK cells have a mature phenotype, are fully responsive to activating receptors triggering and are tolerant to self. (Huntington et al., 2007). Increasing evidence show the importance of both bone marrow (BM) and lymph nodes (LN) for NK cell development and maturation (Freud and Caligiuri, 2006; Caligiuri, 2008), thanks to soluble factors present in that microenvironment, even if the necessary factors and molecular mechanisms underlying this process are still unknown.

BM has a crucial role in driving NK cell functional maturation thanks to stromal cell production of cytokines, such as interleukin 15 (IL-15) that promotes the HPC differentiation in cytolytic NK cells (Mrozek et al., 1996) and NK cell expansion due to the effect of IL-15 in combination with c-kit ligand, also known as stem cell factor (SCF) and flt3 ligand. Moreover, IL-15 plays a pivotal role also in peripheral organ NK cell homeostasis thanks to IL-15 expression on monocytes, macrophages and dendritic cells (DC), that could be *trans*-presented to NK cells (Koka et al., 2003). The current theory of IL-15 *trans*-presentation proposes that intracellular IL-15 binds to a high affinity IL-15R (i.e. IL-15Rα) that is shuttled to the cell surface where it stimulates IL-15 signaling components on NK cells through the IL-15R complex containing the β and γ subunits (Dubois et al., 2002). Transcription and expression of both IL-15 and IL-15Rα within the same cell seems to be important for *trans*-presentation, and, while the expression of IL-

15Rα is ubiquitous, IL-15 expression is stringently controlled (Blauvelt et al., 1996). Moreover, increasing evidence supports the crucial role of IL-15 *trans*-presentation as main mechanism underlying the effect of IL-15 (Stonier and Schluns, 2010). Other cytokines, such as IL-12, IL-18, IFN-α (Vivier et al., 2011) and IL-2, are important for NK cell survival and for their full acquisition of effector functions, and their role will be discussed below.

Mature NK cell localization and phenotype

Mature human NK cells are circulating cells in blood, where they represent 5-15% of peripheral blood lymphocytes in adult healthy individuals and can be detected at variable levels in lymphoid organs and in peripheral tissues such as lungs, liver, gut and deciduas (Cerwenka and Lanier, 2001; Shi et al., 2011; Gregoire et al., 2007).

Human NK cells are surface phenotypically characterized as $CD3^{-}CD56^{+}CD16^{+/-}$ cells (Caligiuri, 2008) and depending on the level of CD56 surface expression they can be classified in two different of NK cell subsets: the **$CD56^{dim}$** and the **$CD56^{bright}$**, also expressing high and low levels of CD16, respectively. These two subsets also differ functionally, in fact $CD56^{dim}$ NK cells are mainly cytotoxic and are more represented in blood (90% of peripheral NK cells) while those $CD56^{bright}$ are specialized cytokine producers and are more abundant in LN (85% of total LN NK cells) (Cooper et al., 2001; De et al., 2011). Some evidence suggest that these two different subpopulations only represent two stages of NK cells differentiation, where $CD56^{bright}$ NK cells are precursor or "developmental intermediates" of $CD56^{dim}$ ones (Freud and Caligiuri, 2010) but it is also true that these different subsets could be either terminally differentiated and have crucial functions in humans.

Furthermore, considering the surface expression of CD16, NK cells could be further divided in three different subsets based on the expression levels of both CD56 and CD16: (i.e: $CD56^{low}CD16^{high}$, $CD56^{low}CD16^{low}$ and $CD56^{high}CD16^{-}$). Stabile and colleagues have recently shown that the $CD56^{low}CD16^{low}$ NK cell subset is capable to either produce high levels of IFN-γ and to possess a strong cytotoxic potential (Stabile et al., 2015).

Regulation of NK cell activity

NK cells sense target cells through a panel of activating and inhibitory receptors expressed on their surface and their functions are regulated through the resulting integration of the opposing signals transduced by their engagement. Thus, the amount of activating and inhibitory receptors on NK cells and the amount of ligands on target cells, as well as the

qualitative differences in the signals transduced, determine the extent of the NK cell response and the lysis of "abnormal" cells (Lanier, 2005). In the classical model of NK cell activation, NK cells are defined to respond to target cells with a reduced expression of MHC class I molecules typical of virus-infected or transformed cells, a concept termed the **'Missing-self'** hypothesis (Karre et al., 1986). In physiological conditions a balance between activating and inhibitory signals is crucial to avoid an accidental NK cell stimulation, but in condition of cellular stress, due to infection or malignant transformation, a loss of inhibitory signals and/or upregulation of ligands for activating receptors **("Induced self"**) lead to NK cell activation.

Moreover, NK cell activation can be triggered by several pro-inflammatory cytokines. IL-2 produced during infection by $CD4^{+}$T cells, together with IL-12, IL-15, IL-18 and IFN-α released by DCs, promote the proliferation of NK cells and "prime" their cytolitic activity and IFN-γ release. Also, IL-15 presented by DC in *trans* through surface IL-15Rα is crucial for NK cell development, survival and effector functions as mentioned before. Pre-activation of NK cells by the cytokine combination of IL-12/IL-15/IL-18 triggers the high-affinity IL-2Rα (CD25) up-regulation on NK cells, usually absent on resting mature human NK cells, and thus their responsiveness to IL-2. In this way, these cytokines improve and prolong the cytolitic and cytokine producing potential of NK cells (Leong et al., 2014).

Upon activation, NK cells are ready to initiate their effector functions, such as the release of cytoplasmic granules containing cytotoxic proteins, such as perforin and granzymes, by exocytosis into the synapse at the NK-target interface (Krzewski and Strominger, 2008) and secretion of both cytokines and chemokines.

NK cell-mediated functions

NK cell-mediated functions include cytotoxicity and release of a wide variety of soluble factors.

Cytotoxicity consists in a rapid response after NK cell activation with the release of pre-formed granules containing perforin and granzymes leading to target cell lysis (Orange, 2008; Trapani et al., 2000). This mechanism could be activated through the engagement of activating receptors or with a process named Antibody Dependent Cellular Cytotoxicity (ADCC) based on the recognition of IgG-opsonized targets by the low-affinity receptor for IgG, FcgRIIIA (CD16) (Trinchieri and Valiante, 1993). Moreover, NK cells can induce apoptosis in target cells through a perforin-indipendent mechanism mediated by death

receptors including Fas and TNF-related apoptosis-inducing ligand (TRAIL) (Takeda et al., 2005; Smyth et al., 2005).

For what concerning the release of soluble factors, it is widely described NK cell production of regulatory **cytokines,** such as IFN-γ and TGF-β, haematopoietic factors such as TNF-α, GM-CSF, IL-3 (Newman and Riley, 2007), and also IL-5, IL-8, IL-13, together with some **chemokines** such as CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES (Robertson, 2002; Loza et al., 2002; Dorner et al., 2004). Of note, IFN-γ plays an important role in activating both innate and adaptive immunity, stimulating macrophages, Th1 immune response and $CD8^+$ priming, as well as in initiating anti-viral responses and tumor immunosurveillance.

1.1 NK cell receptors and their ligands

Inhibitory receptors

Inhibitory NK cell receptors recognize the presence of MHC class I molecules on target cells (Bryceson and Long, 2008). These receptors contain in their cytoplasmic tails immunoreceptor tyrosine-based inhibitory motifs (ITIM), able to recruit tyrosine phosphatases SHP-1 and SHP-2 that in turn dephosphorylate some signaling molecules crucial for the activation cascade (Campbell et al., 1996). This recognition mode prevents NK cell cytotoxic lysis of normal self cells expressing MHC class I molecules. This class of receptors includes the killer cell immunoglobulin-like receptors (KIR) family specific for the recognition of allotypes HLA-B and HLA-C, the leukocyte immunoglobulin-like receptors (LIR) family, and CD94/NKG2 lectin-like receptor family that binds HLA-E (Long, 2008).

Activating receptors

Activating NK cell receptors are divided in three groups: receptors associated with immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits, such as FcγRIIIA (CD16) that mediates the ADCC and natural cytotoxicity receptors (NCR); the DAP10-associated receptor NKG2D; other receptors with different signaling pathways, such as 2B4 that has both activatory and inhibitory properties, DNAM-1 and CD2.

NKG2D is a homodimeric C-type lectin-like NK receptor that is also expressed on $CD8^+$ αβ T cells, γδ T cells, and Natural Killer T cells (NKT) in humans. NKG2D has charged residues in its transmembrane (TM) domain that mediates the interaction with complementary-charged aminoacids in the signal-transducing adaptor proteins: DAP10 in humans (Wu et al., 1999) and either DAP10 or DAP12 in mice (Gilfillan et al., 2002; Diefenbach et al., 2002). In the human system, NKG2D recognizes two families of ligands:

MHC class I chain-related protein A/B (MICA/B) and the UL16-binding proteins (ULBP1-6) generally absent on normal cells but expressed on transformed, infected and damaged or stressed cells. In 2005, Gasser and collegues reported that the DNA damage response pathway (DDR) represented a major signaling pathway implicated in the induction and upregulation of NKG2DL expression (Gasser et al., 2005). DDR, also called as genotoxic stress response, is related to a complex series of cellular stress-induced pathways that detect DNA damage and that are involved in the maintenance of genome integrity and avoidance of mutated DNA duplication (Sancar et al., 2004). Several studies show that NKG2DL expression can be regulated at both transcriptional, post-transcriptional and post-translational level, through protein modifications, trafficking and release as soluble forms in the extracellular space (Raulet et al., 2013). In fact, in addition to being displayed on the cell surface, NKG2D ligands can also be shed or excreted from cells. In some cases, the ligands are cleaved from the plasma membrane by proteinases, or are found associated with membrane vesicles that are excreted from cells, such as exosomes (Chitadze et al., 2013).

DNAM-1 (CD226) is an Ig-like glycoprotein formed of an extracellular Ig-like domain, a transmembrane region and a cytoplasmic tail containing three putative phoshorylation sites crucial for signaling cascade. The engagement of this receptor results in an increased NK cell-mediated cytotoxicity and IFN-γ production. DNAM-1 recognizes the polio virus receptor, PVR (CD155) and nectin-2 (CD112) (Bottino et al., 2003). These ligands are frequently overexpressed on human tumor cells including multiple myeloma derived plasma cells (Soriani et al., 2009a). Similarly to NKG2DL, it has been shown that also DNAM-1 ligands expression can be induced/upregulated through a mechanism involving the activation of DDR in different cellular models (Cerboni et al., 2014). In NK cells, DNAM-1 can be detected in a complex containing LFA-1 (CD11a/CD18), and the ligation of LFA-1 causes Fyn-dependent phosphorylation of tyrosine residues in the cytoplasmic domain of DNAM-1 that are necessary for DNAM-1-dependent NK cell functions (Shibuya et al., 1999). Of note, NK cells also express CD96 (also called Tactile) capable of recognizing CD155 thus promoting NK cell adhesion and activation (Fuchs et al., 2004). It should be considered that recently TIGIT a new inhibitory receptor expressed by T and NK cells binds to PVR and inhibits NK cell cytotoxicity directly through its ITIM domain (Stanietsky et al., 2009).

NCRs are potent activating receptors and in human this family comprises of NKp30, NKp44 and NKp46, NKp80; they are structurally formed of one or two Ig-like extracellular domains

with charged residues in their transmembrane regions necessary for the association with ITAM-bearing signaling molecular adaptors, such as FcεRI-γ and CD3-ζ for NKp30 and NKp46, or DAP12 for NKp44 (Bryceson and Long, 2008; Moretta et al., 2006). Their engagement leads to induction of cytotoxicity and cytokine production. Nowadays the identity of NCRs ligands still remains poorly defined.

NKp30 is expressed both on resting and activated human NK cells. Two ligands for this receptor have been characterized to date: B7-H6 detectable on the cell surface of transformed cells (Brandt et al., 2009) and the nuclear factor HLA-B-associated transcript 3 (BAT3 also known as BAG-6) released by tumor cells (Pogge von et al., 2007) or by immature DCs and associated to exosomes (Simhadri et al., 2008). **NKp46** has been described to bind viral structural motifs (Arnon et al., 2004). Recently, a novel isoform, of the mixed-leukemia-5 (MLL5) nuclear protein was proposed as a cancer cell-expressed ligand for NKp44 (Baychelier et al., 2013). **NKp44** is found on activating NK cells and is constitutively expressed on specialized subsets of NK cells in the deciduas (Cantoni et al., 1999).

Other receptors

Moreover, in addition to activating and inhibitory receptors, NK cells express many other receptors such as Toll-like receptors, that will be discussed later, cytokine and chemokine receptors, including CCR2, CCR5, CX3CR1, and CXCR3, together with receptors for adhesion molecules, for example LFA-1 and LFA-2, to enhance the adhesion to target cells.

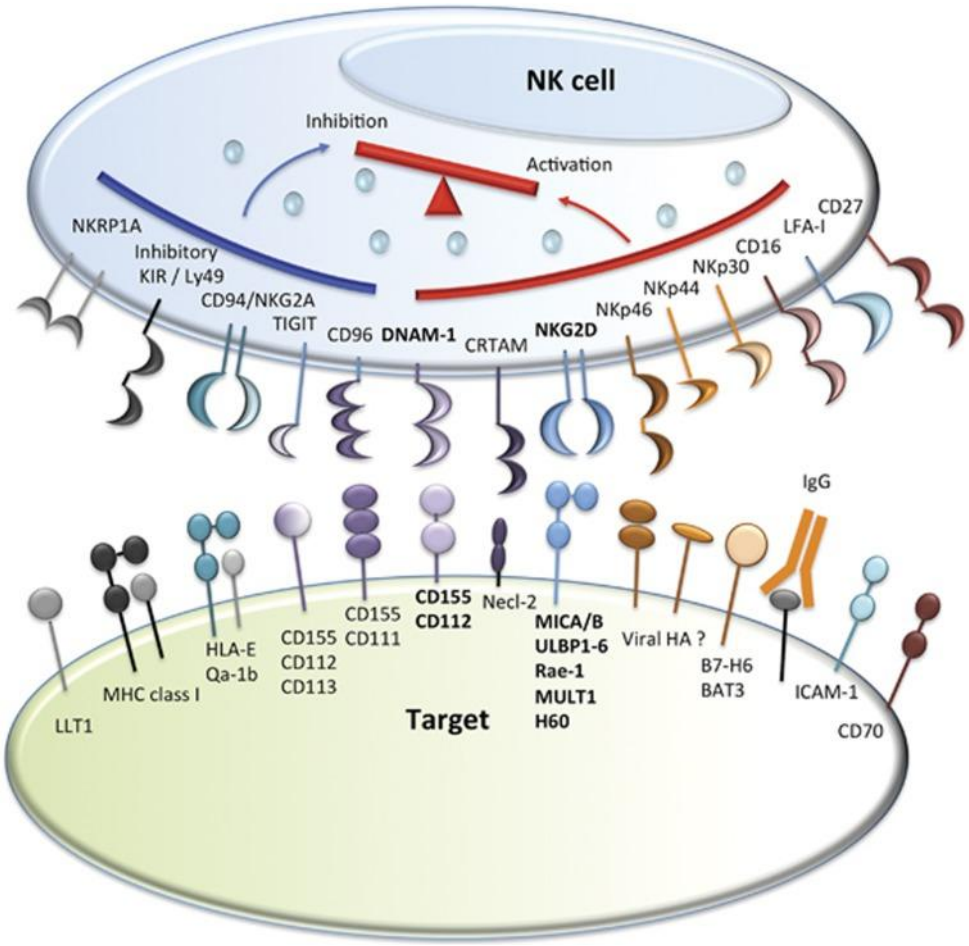

Figure 1. NK cell interaction with target cell. ***From Chan at al., 2014.*** NK cell repertoire of both activating and inhibitory receptors with their ligands expressed on target cells. The main receptors are shown.

1.2 Toll-like receptors

Toll-like receptors (TLR) have long been known for their ability to initiate immune responses upon exposure to conserved microbial components named ***"pathogen-associated molecular pattern molecules" (PAMPs)***. More recently, this family of pattern recognition receptors (PRR) has been attributed a critical role in the elicitation of anticancer immune responses.

TLR represent a conserved family of pattern-recognition receptors (PRRs) best known for their ability to detect *PAMPs*. Currently 10 members of functional TLRs have been identified, but only nine of these have been well characterized in human (O'Neill et al., 2013). Some of these receptors are expressed on the cell surface and recognize microbial membrane components. In particular TLR4, the first mammalian TLR identified, senses mainly but not only lipopolysaccharide (LPS) in complex with MD2 (Park et al., 2009); TLR5 binds to bacterial flagellin (Hayashi et al., 2001); TLR2 recognizes a wide range of

microbial lipopeptides and other non-lipopeptidic PAMPs derived from bacteria, fungi, parasites and viruses. TLR2 forms heterodimers with both TLR1 (Jin et al., 2007) and TLR6 (Kang et al., 2009) recognizing triacylated and diacylated lipopetides respectively. Another member of this family, TLR10 heterodimerizes with TLR2 probably because of its similarity to TLR1. Other TLRs, localized in intracellular compartements, such as endosomes, lysosomes and endoplasmic reticulum (ER), are able to sense nucleic acid associated to viral or bacterial infection and require the internalization of their ligands. In particular, TLR9 binds unmethylated CpG-rich DNA motifs usually associated to virus and bacteria DNA but almost rare in mammalian cells (Hemmi et al., 2000); TLR3 recognizes double-stranded (ds) viral RNA (Liu et al., 2008), while TLR7/TLR8 sense single-stranded (ss) RNA from virus and bacteria (Heil et al., 2004).

Several TLRs have been recently shown to sense also endogenous danger signals which are released in response to tissue damage known as ***"damage-associated molecular patterns" (DAMPs)***, produced mainly during cell death and injury or transformation. They include several heat shock proteins (i.e: Hsp60, Hsp70), uric acid, the non-histone chromatin binding protein high mobility group 1 (HMGB1), surfactant protein A all of which function as TLR2 or TLR4 agonists. Moreover, degraded components of extracellular matrix which mainly activate TLR4 (Kawai and Akira, 2010; Harris and Raucci, 2006) and mitochondrial DNA have been shown to bind also TLR9.

Interestingly recent reports demonstrate a possible interaction between TLRs and some molecules expressed either on the exosome surface or inside these vesicles as demonstrated for TLR2 able to bind palmytoilated proteins associated to exosomes (Chow et al., 2014a) or as in the case of TLR8 recognition of exosomal microRNAs (Fabbri et al., 2012). This concept will be fully explained later.

The release of specific DAMPs by death cells has been proposed to constitute the essence of immunogenic cell death (ICD), a peculiar type of apoptosis that activates adaptive immune responses. To date, only few inducers of ICD have been identified: specific chemotherapeutic agents (i.e: doxorubicin), the endoplasmic reticulum calreticulin, ATP, Hsp70 and HMGB1. Importanly, Hsp70 and HMGB1 appear to exert immunostimulatory functions by activating TLR4 on the surface of antigen-presenting cells.

In summary TLRs appear to play a prominent role not only in the orchestration of innate immune responses against infectious pathogens but also in anti-cancer immunity, be it spontaneous or elicited by (chemo)therapeutic interventions.

TLR signaling

TLRs are structurally formed by an N-terminal domain enriched in leucine repeat mediating the recognition of their ligands, a transmembrane domain (TM) and a cytosolic signaling tail, known as Toll/IL-1R (TIR) that recruit the signaling adaptors MyD88 and TRIF, and also the sorting adaptors TIRAP and TRAM (Kawai and Akira, 2010), leading to downstream activation of NF-kB, IRFs (IRF3 and IRF7), MAPK, p38 and ERKs pathways (Kawasaki and Kawai, 2014), that results in the production of several chemokines and cytokines. TLRs differ in their signal transduction pathways depending on either their cellular localization and the type of adaptor molecules involved. In particular, these different signaling pathways can be classified as MyD88- or TRIF-dependent suited to elicit different responses. For instance, the triggering of cell surface TLRs (i.e: TLR1, 2, 4, 5, 6) induce mainly inflammatory cytokine production, through the usage of the adaptor molecule MyD88 leading to the activation of NF-kB and MAPKs through the IL-1 receptor-associated kinases (IRAK)1,2,4, that form an activated complex with TRAF6 leading to the activation of the kinase TAK1. This kinase activates some MAPKs, such as Erk1,2, p38 and Jnk, which in turn activate downstream transcription factors CREB and AP1. TAK1 also phosphorylates the IKK complex determining NF-kB activation (Figure 2). On the contrary, TLR3 and TLR4, through the usage of TRIF adaptor, and the engagment of TRAF3 can either induce two different pathways leading to NF-kB activation or IRF3 activation leading to proinflammatory or type I interferon production, respectively as represented in Figure 2. Endosomal TLRs, TLR7/8 and TLR9, require the adaptor MyD88 to activate IRF7 and to induce type I interferon production, and are potentially able to stimulate the NF-kB pathway associated to inflammatory cytokine production by using the TRAF6 adaptor (Kawai and Akira, 2008) A schematic representation of signaling transduction pathways activated by TLRs are shown in Figure 2 .

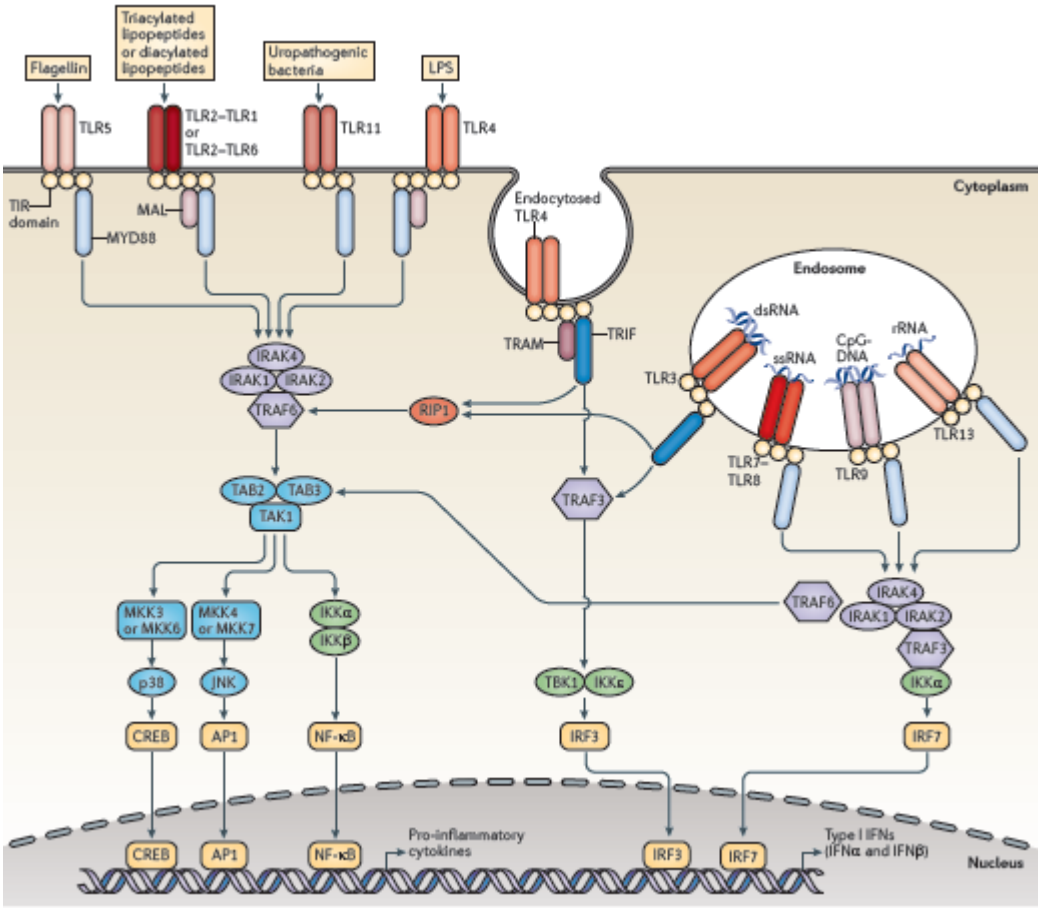

Figure 2. Toll-like receptor family and signaling. ***From O'Neill et al., 2013*****.** TLRs can be classified on the basis of their cellular localization, so TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 are expressed on the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 are localized to the endosomes. The engagement of these receptors leads to the activation of different signaling pathways as indicated, determining the final activation of some transcription factors and the induction of pro-inflammatory cytokines or type I interferons.

Role of TLR in NK cell mediated functions

NK cells express TLR1 (He et al., 2013), TLR2 (Marcenaro et al., 2008), TLR3 (Schmidt et al., 2004), TLR5 (Chalifour et al., 2004), TLR7/8 (Hart et al., 2005) and TLR9 (Sivori et al., 2004) independently from their activation state and the TLR triggering occurs in both resting and cytokine activated NK cells. Many evidence demonstrate the involvement of different TLRs in stimulating NK cell-mediated functions alone or in combination with suboptimal doses of cytokines such as IL-12 (Chalifour et al., 2004; Marcenaro et al., 2008; Sivori et al., 2004; Hart et al., 2005). For example, TLR3 triggering in highly purified human NK cells leads to enhanced NK cell-mediated cytotoxicity, up-regulation of activation marker CD69, and production of several cytokines like IL-6, IL-8 and IFN-γ (Schmidt et al., 2004). Moreover, He and colleagues have recenltly demonstrated in both human and mouse model that some microRNAs, in particular miR-15b and miR-122, in combination with low doses

of IL-12, are able to activate the NF-kB pathway with the induction of CD69 expression, IFN-γ production and expression of the degranulation marker CD107, through a mechanism mediated by TLR1 (He et al., 2013). It has also been reported that PSK a TLR2 agonist can activate human NK cells to secrete IFN-γ and exert enhanced cytolytic activity in sensitive K562 target cells and trastuzumab-coated breast cancer cells (Lu et al., 2011).

2. Extracellular vesicles and exosomes: general features

Cellular cross-talk is a crucial event in multicellular organisms, where cells communicate through direct cell-cell contact or through secreted molecules, such as chemokines and cytokines, proteins, ormons, lipids and nucleic acids. Currently, increasing evidence describe the extracellular vesicle release as an additional mechanism for intercellular communication (Bobrie et al., 2011) that provide an autocrine, paracrine and endocrine signals to target cells thanks to vesicle ability to move through body fluids.

A wide range of extracellular vesicles have been described to date and their classification depends on their size and on the cellular compartment they derive from. In fact, extracellular vesicles are called microvesicles or ectosomes, when are shed from the plasma membrane and have a size of 100-1000 nm, apoptotic bodies, generated during apoptotic cell death with size falling in the range of 100-5000 nm, and exosomes (Bobrie et al., 2011).

Exosomes are nano-sized (30-100 nm) couple-shaped (the round-shaped and the couple-shaped morphology seems to be dependent only on exosome dehydratation during the procedure necessary for ultrastructural analysis (Robbins and Morelli, 2014)) membrane-bound vesicles secreted from most types of living cells into extracellular environment, under both normal and pathologic conditions. Exosomes were firstly described in 1987 and at the beginning they were considered as a mechanism to eliminate unneeded cellular proteins and organelles during the maturation of reticulocytes (Johnstone et al., 1987; Vidal and Stahl, 1993), but during the last two decades these vesicles have become a central topic in current research.

2.1 Biogenesis, molecular sorting and secretion of exosomes

Exosomes are formed in the late endosomal compartment for inward budding of multivescicular bodies (MVBs) and then released through the fusion of this compartment with plasma membrane. Many evidence show two possible “destiny” for multivescicular endosomes (MVEs) depending on their cholesterol composition: cholesterol-poor population are addressed for degradation and cholesterol-rich vesicles are committed to secretion

(Mobius et al., 2002). The formation of MVEs directed to secretion, as well as exosomes cargo sorting, seems to be dependent on various mechanisms including endosomal sorting complex required for transport (ESCRT) machinery, lipid-raft microdomains and tetraspanins enriched domains (Raposo and Stoorvogel, 2013). The ESCRT machinery is important for protein sorting and for intraluminal vesicles (ILVs) formation. Moreover, it has been observed that some post-translational protein modifications, such as monoubiquitylation, glycosylation, oxidation and phosphorylation, seem to be signals for this sorting machinery, even if the real mechanism underlying this process remains elusive (Moreno-Gonzalo et al., 2014). Briefly, ESCRT0 is recruited by the presence of Phosphatidyl-inositol 3-phosphate (PI3P) on endosomal membrane that binds ubiquitylated proteins; this binding allows the recruitment of both ESCRTI and II components initiating the reverse budding of MVB membrane. Then, ESCRTII engages ESCRTIII which promotes exosome cleavage; during this process many proteins of this complex, such as tumor susceptibility gene 101 protein (Tsg101) and ALG2-interacting protein X (Alix), are recovered in exosomes (Robbins and Morelli, 2014). An alternative pathway for exosome biogenesis involves lipid-raft affinity of tetraspanins (Trajkovic et al., 2008) or protein interactions with Hsp70 and transferrin receptor (TfR) (Hemler, 2001). Furthermore, ceramides are thought to induce aggregation of lipid microdomains into larger ones and this event seems to be important for generation of ILVs that are addressed for secretion as exosomes rather than for lysosomal degradation.

Nowadays, the mechanism underlying exosomes release from parent cells is still not completely clear but it seems to be a very rapid process. Some evidence propose two different mechanisms of exosome secretion: constitutive, via the Trans-Golgi Network (TGN), and inducible, depending on the cell type and the activation state of the cell (Thery et al., 2009).

Constitutive secretion from the Golgi

Proteins destinated to the cell surface or to be secreted into the extracellular space can be routed from the TGN by an ubiquitary constitutive pathway that does not require a specific stimulus, albeit controlled according to cell activity (intracellular signalling, cell growth, differentiation, etc.). Some members of small GTPases Rab family, as Rab11, Rab8 and Rab13, or heterotrimeric G-protein and protein kinase D (PDK1-2) have been identified in the regulation of vesicle trafficking from TGN to the plasma membrane (Record et al.,

2011). During this constitutive secretion, exosomes are transported within vesicles containing only one or two exosomes, and appeared not to transit via MVB.

Inducible secretion from MVB

Inducible secretion pathway is regulated by different activation processes. For example, modification of intracellular levels of Ca^{2+} in mast cells and in the human erythroleukemia cell line K562 (Savina et al., 2003) or K^{+}- induced depolarization in neurons are both stimuli for exosome secretion. Cross-linking of IgE receptor in mast cells or CD3 in T cells produce exosome-induced secretion.

The involvement of several members of the small Rab GTPase associated with the endocytic system might occur also in this scenario (Thery et al., 2009; Hsu et al., 2010; Ostrowski et al., 2010). For example, Rab27 isoform has been shown to be important in MVE docking at the plasma membrane (Ostrowski et al., 2010).

Moreover, it has been described that stress conditions, such as senescence (Lehmann et al., 2008), radiation and other events inducing DNA damage, lead to tumor suppressor-activated pathway 6 (TSAP6)-dependent increase of exosome secretion. In this study, TSAP6 is shown to colocalize with TGN compartment and its absence produces a reduction in exosome formation and secretion while its p53-induced up-regulation causes an enhancement in exosome release (Lespagnol et al., 2008).

Finally, very little is known about the fusion of MVB with plasma membrane, and it is just described the contribution of soluble N-ethylmaleimide–sensitive factor attachment proteins (SNAREs) in this process. The mechanisms implicated in exosomes formation and secretion are illustrated in Figure 3.

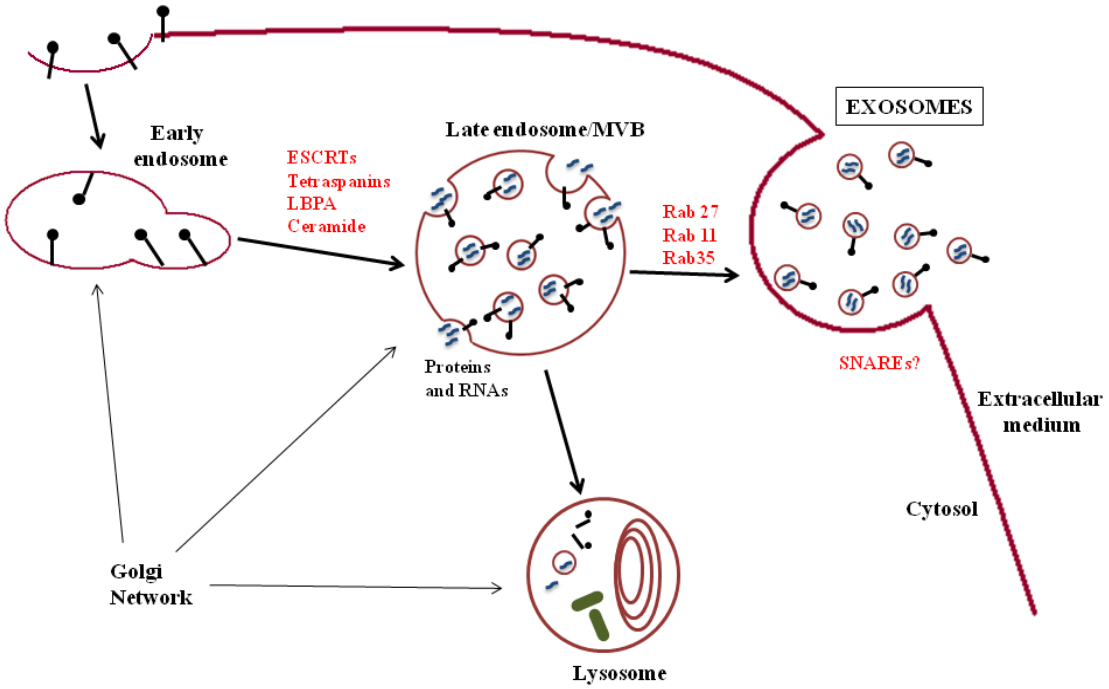

Figure 3. Exosome formation and secretion (*Adapted from Bobrie et al., 2011*). Cellular machinery for exosome formation in early endosome, maturation in the late endosomal compartment and secretion. In the first step of exosome formation, ESCRT complex, tetraspanins, LBPA and ceramide are involved; in regard to the vesicular trafficking Rab proteins play a crucial role, while for what concerning exosome release in extracellular space, the contribution of SNARE proteins seems to be necessary.

2.2 Molecular composition of exosomes

The structure of exosomes consists of a lipid bilayer membrane, enriched in cholesterol, sphingomyelin and ceramide (Simpson et al., 2008) and contains a typical pattern of proteins, lipids and nucleic acids derived from their parental cell, thanks to the sorting process of these molecules.

In regard to the proteins contained in exosomes, they are mainly derived from cellular cytosol and plasma membrane and usually not from nucleus, mitochondria or endoplasmic reticulum (Mears et al., 2004). In particular, exosomes abundantly express on their surface some proteins belonging to the tetraspanin family (CD9, CD63 and CD81) and some adhesion molecules, including integrins, involved in the exosome internalization into target cells, as for example, the inter cellular adhesion molecule 1 (ICAM-1), the leukocyte function-associated antigen-1 (LFA-1). In addition, they contain some proteins involved in

exosome biogenesis and secretion (Rab GTPases, Alix, Tsg101), molecular chaperones as heat-shock proteins (Hsp70 and Hsp90), transmembrane molecules, metabolic enzymes (peroxidases, fatty acid syntase, glyceraldeide-3-phosphate dehydrogenase, pyruvate kinase), cytoskeleton and motility proteins (tubulin, actins, myosin, etc.) and also proteins implicated in signal transduction (kinases, RhoA, GTPase Hras, etc.). The relative abundance of some molecules might also depends on the exosomes source, as in the case of MHC class I and II and costimulatory molecules (i.e: CD86) associated to exosomes derived from antigen-presenting cells (APCs) (Tran et al., 2015; Record et al., 2011).

As mentioned above, exosomal membrane contains a large amount of lipids that reflects lipid raft composition with an enrichment in sphingomyelins, that could be hydrolysed into ceramide by sphingomyelinases in cholesterol, in particular in B-cell-derived exosomes, in phosphatidylserine, only in DC-derived exosomes (Subra et al., 2007) and in lyso-phosphatidilcholine in mast cell-derived exosomes (Laulagnier et al., 2004). Moreover, lipids present in exosomes are described to be enriched in saturated fatty acid compared to those of parental cells (Luketic et al., 2007).

Regarding RNA cargo, the first study showing the presence of mRNA and microRNA in exosomes was published in 2007 (Valadi et al., 2007) and nowadays it is well known that these RNAs are functional and able to modulate recipient cell protein production (Record et al., 2011). Moreover exosomes, as vehicles for RNAs, can protect these nucleic acids and deliver them to specific target cells. Interestingly, some mRNAs and microRNAs could be exclusively present in exosomes and not in parental cells. The description of exosome microRNA cargo will be depicted more in depth in the following paragraph.

2.3 Exosomes as vehicle of miRNA

As mentioned above, exosomes have been reported to contain significant amount of microRNA (miRNA) and their transport gives them protection against circulating RNAases and allows their delivery to target cells.

MiRNAs are small (19-25 nucleotides) non-coding single-stranded RNAs transcribed in nucleus by polymerase II into primary miRNAs (pri-miRNAs) and then cleaved by a complex composed of two RNAses III, Drosha and Pasha, into precursor miRNAs (pre-miRNAs). Exportin-5 transfers pre-miRNAs in cytoplasm where they are processed in a series of steps mediated by exonuclease Dicer into mature miRNAs. Their role, when they are incorporated into the RNA-induced silencing complex (RISC), is to regulate gene

expression impairing mRNA translation through the binding of specific target mRNAs leading to a repression of translation or mRNA degradation.

As described before, exosomes exhibit a typical miRNA signature that is more similar to exosomes derived from different cell types than to their parental cells, suggesting a specific mechanism for miRNA sorting in exosomes (Mittelbrunn et al., 2011). The loading into exosomes has been proposed to be dependent on the binding of a specific miRNA motif to the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), and this process seems to be regulated by sumoylation of hnRNPA2B1 (Villarroya-Beltri et al., 2013).

Moreover, an alternative mechanism is linked to the amount of a single miRNA in parental cells where the overexpression of cellular miRNAs facilitates their loading in the exosomal cargo (Squadrito et al., 2014). For example, B lymphocytes express miR-155 and the expression level of this miRNA is enhanced in monoclonal B-cell lymphocytosis (MBL) and chronic lymphocytic leukemia (CLL) patients compared to healthy individuals, therefore circulating exosomes, isolated from MBL and CLL patients, contain high level of miR-155 (Ferrajoli et al., 2013).

Many evidences show the presence of specific miRNAs in exosomes derived from different cells of immune system or not, as miR-150 contained in exosomes secreted from T cell, B cell (de et al., 2013) and macrophage, where the level of this miRNA directly correlate with the efficiency of human microvascular endothelial cell line HMEC-I migration in atherosclerosis patients compared to healthy donors (Zhang et al., 2010). Macrophage-derived exosomes contain also miR-223 and miR-191, and it has been observed the ability of miR-223 to induce complete macrophage maturation in recipient monocytes (Ismail et al., 2013).

The miRNA delivery to recipient cells through exosomes allows the modulation of a large variety of different functions in target cells. For example, mesenchymal stromal cells produce functional exosomal miRNAs, such as miR-133b able to induce neurite growth when is delivered in neurons and astrocytes (Xin et al., 2012).

For what concerning pregnancy, human placental cell-derived exosomes and also exosomes contained in breast milk carry immunomodulatory miRNAs, such as miRNA cluster located in chromosome 19 (C19MC) that is enriched in embryonic stem cells and placenta (Bullerdiek and Flor, 2012), or miR-155 in breast milk-derived exosomes (Melnik et al., 2014).

DC-derived exosomes has been described to have an immunomodulatory role in many studies because of their importance as APCs, but it still unknown the functions of their miRNAs in recipient cells (Fernandez-Messina et al., 2015), and likewise, Valadi *et al*., demonstrate the transfer of exosomal miRNAs from mast cells to target cells but the functional role of these miRNAs remains unknown (Valadi et al., 2007).

In the context of viral infection, EBV-infected B-cells could release exosomes containing viral miRNAs that could repress specific mRNA translation in recipient cells (Pegtel et al., 2010). Also in the course of HCV infection, HCV RNA is packed into exosomes and transferred to plasmacytoid DC (pDC) leading to IFN-α production (Dreux et al., 2012).

Several examples of tumor cell-derived exosomes containing miRNAs have been reported to date. It has been recently described that an oncomiR, miR-135b, is enriched in hypoxic multiple myeloma cell-derived exosomes and this small RNA increases angiogenesis targeting factor–inhibiting hypoxia-inducible factor 1 (FIH-1) mRNA and thus facilitating hypoxia-inducible factor 1 (HIF-1) transcriptional activity (Umezu et al., 2014). Another study, supporting the classical role of exosomal miRNAs in recipient cells, has demonstrated the down-regulation of TLR4 in DC caused by direct targeting of miR-203 that is overexpressed in pancreatic cancer cell-derived exosomes. This reduction in TLR4 expression was shown to inhibit normal DC cytokine production (Zhou et al., 2014). Moreover, it has been shown that miR-433 up-regulation induces senescence in ovarian cancer cells and the exosomal transfer of this miRNA between these cancer cells leads to the senescence bystander effect (Weiner-Gorzel et al., 2015).

Beside the classical role of miRNAs in modulating mRNA translation, increasing evidence propose a direct binding of miRNA to Toll-like receptors (TLRs) leading to a direct immunomodulatory effect. Recently, Fabbri and colleagues have reported that lung cancer cell-derived exosomes containing high levels of miR-21 and miR-29a, can act as ligand for human TLR8 in macrophages, leading to NF-kB pathway activation with the consequent induction of CD69 expression and the production of several inflammatory cytokines, including IL-6 and TNF-α (Fabbri et al., 2012). Likewise, another study performed in a murine model has demonstrated that let-7, an highly abundant regulator of gene expression in the CNS, binds to TLR7 expressed in neurons and activates signals involved in neurodegeneration (Lehmann et al., 2012). Moreover, Yu and coworkers provided another evidence of TLR engagement *via* miRNA. In particular, they demonstrated that TLR1 is able to recognize some circulating miRNAs, including miR-122, miR-155, miR-21 and

miR-15b, with a consequent enhanced CD69 surface expression and IFN-γ production on human NK cells in combination with low doses of IL-12, and similar results were obtained with these miRNAs contained in exosomes (He et al., 2013).

2.4 Exosome uptake

Many evidence support exosome uptake in recipient cells, and this process can be directly visualized by confocal microscopy or flow cytometry after exosome labeling with a lipid membrane fluorescent dye.

Exosomes can be taken up through different possible mechanisms including endocytosis (clathrin-mediated or caveolin-dependent), phagocytosis, macropinocytosis and fusion with plasma or endosomal membrane; moreover, it should be considered that exosome internalization pathways could vary depending on target cell (Mulcahy et al., 2014).

Protein interactions involved in the exosome uptake

The exosome uptake mechanism can involve protein-protein interactions thus facilitating subsequent endocytosis and this event may probably confer a selective interaction with recipient cells. Indeed, proteinase K treatment of exosomes was shown to significatively reduce their uptake by ovarian cancer cells (Escrevente et al., 2011). Many different proteins associated to exosomes have been described to interact with membrane receptors on target cells, including tetraspanins (Hemler, 2005), integrins, immunoglobulins, proteoglycans and lectins.

Tetraspanins are membrane proteins which have numerous functions including cell adhesion, motility, activation and proliferation, and are highly abundant on the exosome surface. As already mentioned, the tetraspanins CD63, CD9 and CD81 are well-established markers of exosomes and it is reported that the treatment of recipient cells with antibodies against CD81 or CD9 can reduce exosome uptake by DC (Morelli et al., 2004). Tumor cells over expressing tetraspanin Tspan8 release exosomes carrying a Tspan8-CD49d complex which plays a crucial role in the exosome uptake by rat aortic endothelial cells (Nazarenko et al., 2010).

Several reports suggest that ***integrins***, usually involved in a wide range of functions, may also partecipate in the exosome uptake by different types of immune cells. Thus, for example, antibodies that mask the binding site of CD11a or its ligand ICAM-1 can reduce exosome uptake by DC (Morelli et al., 2004). Similar results were obtained after blocking the integrins αv (CD51) and β3 (CD61) on the DC cell surface. Naïve T cells have been

shown to internalize exosomes through a mechanism requiring the participation of T cell receptor (TCR), CD28 and LFA-1 (Hwang et al., 2003). In another study, Nolte-'t Hoen and coworkers have observed that T cells can recruit DC-derived exosomes containing major histocompatibility complex class II (MHCII) molecules during cognate DC-T cell-cell interaction. Recruitment of these exosomes required T-cell activation and was LFA-1-dependent. Interestingly, the induction of a high-affinity state of LFA-1 on resting T cells was sufficient to improve exosome binding (Nolte-'t Hoen et al., 2009). These results highlight the emerging roles of integrins in vesicles uptake, particularly in immune cells. ***Lectins*** have also been involved in exosome uptake as shown for DC-SIGN, a C-type lectin receptor able to recognize and internalize glycoprotein ligands. The recruitment of exosomes derived from breast milk by monocyte-derived DC was strongly reduced by neutralizing antibodies specific for DC-SIGN (Naslund et al., 2014). Another interesting molecule, galectin-5, has also been detected within exosomes. Galectin is a type of lectin that shows affinity for β-galactoside. Extracellular galectins cross-link cell surface and extracellular glycoproteins and may thereby modulate cell adhesion and induce intracellular signals. It was observed that galectin-5 positive exosomes were phagocytosed into rat peritoneal macrophages and J774 macrophages with a mechanism galectin-5 dependent (Barres et al., 2010).

Endocytosis

Endocytosis, as mentioned above, is one of the possible mechanisms for exosome uptake and many studies demonstrate that is an active, rapid, endoergonic process requiring a functional cytoskeleton. In particular, Franzen and colleagues performed experiments on exosome uptake by bladder cancer cells, evidentiating a direct correlation between vesicle internalization and incubation time, with saturation point after 14 hours; in addition, they have demonstrated that the exosome uptake was dose dependent and that was abrogated at 4°C, suggesting that energy was necessary for this process (Franzen et al., 2014). Further evidence that internalization is not a passive process is provided by observations that vesicles are not taken up by cells fixed in paraformaldehyde. Endocytosis comprises more than one internalization pathways, such as macropinocytosis, phagocytosis and endocytosis mediated by clathrin, caveolin and lipid-raft. ***Macropinocytosis*** is not the main pathway involved in exosome uptake, and is probably specific for some cell types, as well as phagocytosis is typical of macrophages and other specialized cells. During ***phagocytosis*** membrane invaginations progressively surround exosomes or other materials to internalize,

while in macropinocytosis membrane extensions perform the encapsulation of extracellular components (Mulcahy et al., 2014). Phosphatidylserine located on exosome surface, was described to play a crucial role in both macropinocytosis and phagocytosis, engaging T-cell Immunoglobulin-Mucin domain containing molecules 4 (TIM4) (Feng et al., 2010) and TIM1 on Th2 cells (Zakharova et al., 2007). Instead, during internalization mediated by clathrin, caveolin or lipid-raft, the mechanisms consist in invaginations of domains enriched in those molecules with subsequent surrounding of exosomes or other material and transfer into cytosol of target cells.

Cell surface membrane fusion

Finally, another mechanism for exosome uptake consists in the direct fusion of vesicle membrane with the cell plasma membrane through a lipid-lipid interaction (Parolini et al., 2009). Several protein families, including SNAREs and Rab proteins, participate in this process (Jahn and Sudhof, 1999) and the fusion of the membranes can be observed via fluorescent lipid dequenching. Furthermore, exosome fusion with plasma or endosomal membrane is suggested by the evidence of exosome delivery of miRNA and luciferin to the cytosol of the recipient cells. A schematization of different ways of exosomes uptake is depicted in Figure 4.

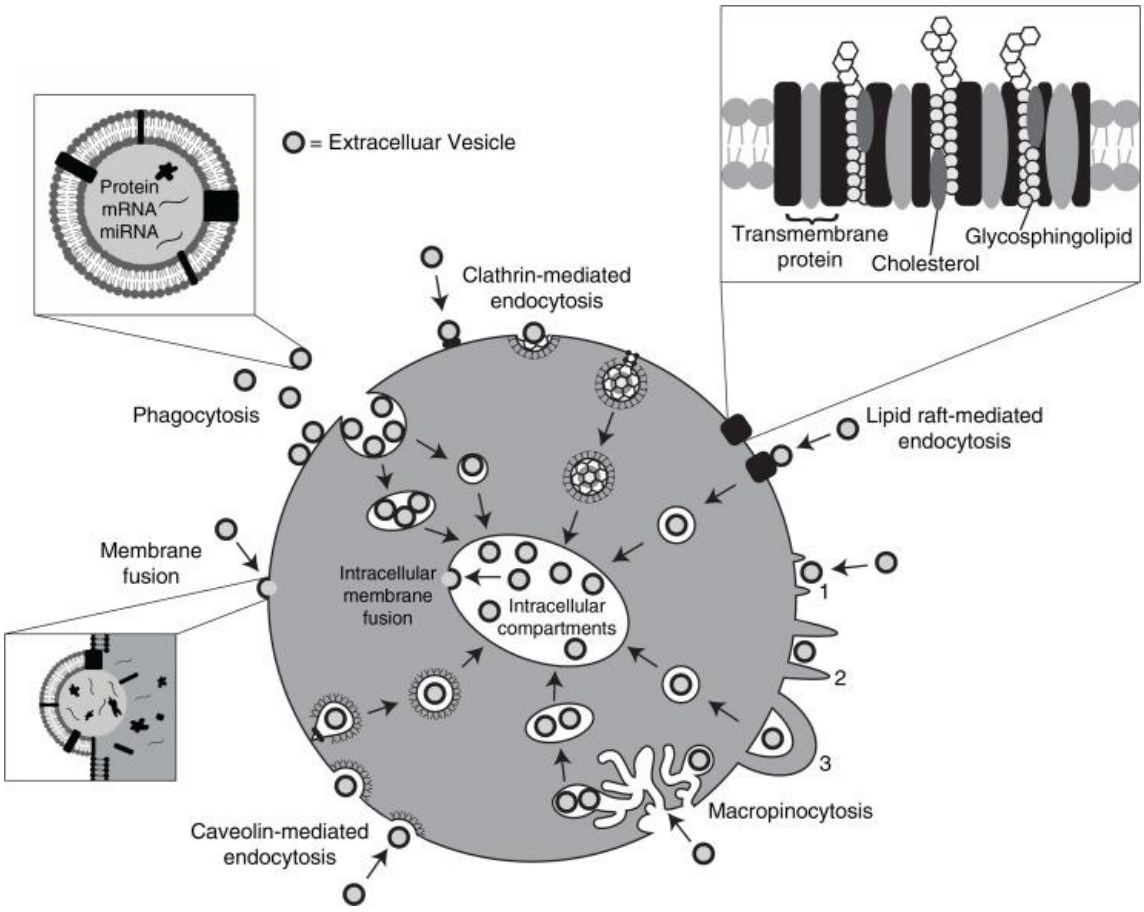

Figure 4. Mechanisms for exosome uptake. ***From Mulcahy et al., 2014*****.** Exosomes are taken up by target cells through different mechanisms. Exosome internalization can be mediated by caveolin, lipid-raft or clathrin dependent endocytosis, macropinocytosis, phagocytosis and the direct fusion with plasma membrane, and eventually with the endosomal membrane.

2.5 Exosome modulation of immune response

Exosomes are reported to be important in both physiological and pathological conditions, and their biological effects seem to be mainly dependent on their producing cells. In 1996, Raposo and coworkers demonstrated for the first time a role for exosomes in antigen presentation, showing that exosomes secreted by B lymphoblastoid cells carry MHC class II –antigen complexes able to induce antigen-specific MHC class II-restricted T cell responses. (Raposo et al., 1996). At the same way, two years later, another study reported that DC-derived exosomes, expressing MHC class I-antigen complexes, were efficaceous in the stimulation of antigen specific $CD8^+$ T cells (Zitvogel et al., 1998). Since these discoveries, several papers came out describing a central role of DCs in the activation of adaptive immunity, through an indirect antigen presentation mechanism mediated by these nanovesicles (Morelli et al., 2004). In another scenario, there are several evidence describing

DCs uptake of exosomes derived from many cell types bringing specific antigens, such as tumor-antigens or pathogen-derived antigens from infected macrophages (Giri and Schorey, 2008) and also virus-derived ones derived from cytomegalovirus infected endotelials cells (Walker et al., 2009). After the uptake of antigen-carrying exosomes, DCs acquire the ability to present these molecules to T lymphocytes initiating the adaptive immune response. In another interesting work, it has been reported that mast cell-derived exosomes induced immature DCs to up-regulate MHC class II, CD80, CD86, and CD40 molecules and to acquire potent Ag-presenting capacity to T cells (Skokos et al., 2003), thus evidentiating a collaborative interaction between mast cells and DCs leading to the elicitation of specific immune responses. All together, these observations show that exosomes can stimulate T-cell mediated immune reponses through two different ways, indirectly by affecting the maturation process of DCs or directly by presenting antigen/MHC complexes to T lymphocytes.

In regard to the effects of tumor cell-derived exosomes (Tex) on immune cells, different consequences have been described from immune stimulation or immune suppression, depending mainly on the molecules expressed on exosome surface and their molecular cargo.

Several Tex-mediated immune suppressive mechanisms have been reported to date. In some circumstances, Tex restrain tumor immune surveillance by promoting Myeloid-Derived Suppressor Cells (MDSC) or regulatory T cells (Treg) functions. For example, exosomes derived from melanoma and colorectal carcinoma cells have the capability to impair monocyte differentiation into DCs promoting at the same time the generation of MDSC with a consequent production of IL-6 and transforming growth factor-β (TGF-β) which suppress T cell proliferation and cytolytic functions and promote tumor growth (Valenti et al., 2006). Another study performed in both mice and humans reported that Hsp72 associated to Tex triggered Stat3 activation in MDSCs in a TLR2/MyD88-dependent manner through autocrine production of IL-6 (Chalmin et al., 2010). Moreover mesothelioma cell-derived exosomes, expressing TGF-β1, impair IL-2 dependent T cell proliferation and promote Treg cells functions leading to a switch towards an immunosuppressive phenotype (Clayton et al., 2007). An alternative mechanism of immune escape mediated by Tex might be linked to the expression of Fas ligand (FasL), which could engage its receptor on T cells inducing apoptosis (Huber et al., 2005; Andreola et al., 2002). Likewise, it should be noted that

TRAIL- and FasL-bearing exosomes, released by human placenta, are able to induce T cell apoptosis conferring immune privilege for the fetus (Stenqvist et al., 2013).

In the matter of immunostimulatory effects of Tex, a recent study described macrophage activation after incubation with breast cancer cell-derived exosomes. In particular, the presence of palmitoylated proteins on exosome surface triggers TLR2 on macrophages and thus activates the NF-kB pathway with a consequent enhanced production of pro-inflammatory cytokines (Chow et al., 2014b). Even in the context of autoimmunity, exosomes could play an activating role; for example, a membrane form of TNF-α was found to be associated to exosomes derived from the synovial fibroblasts of rheumatoid arthritis patients and was responsible to stimulate the activation of NF-kB and AKT pathways in $CD4^+$ T cells (Zhang et al., 2006).

The immunomodulatory role of exosomes is summarized in table 1 and 2, while their effects on NK cell-mediated functions will be discussed later.

Source of exosomes	Recipient cells	Mechanism	Effects
Dendritic cell	$CD8^+$ T cell	- MHCI-TCR interaction - Antigen transfer	Direct or indirect antigen presentation
	Primed $CD4^+$ T cell	MHCII	Direct antigen presentation
	Naïve $CD4^+$ T cell	MHC and antigen transfer	Indirect antigen presentation
	NK cell	- IL-15 transpresentation - NKG2D-NKG2DL interaction	Activation
B cell	Primed $CD4^+$ T cell	MHCII	Direct antigen presentation
Infected macrophage	Immature DC	Pathogen	Antigen transfer and maturation
Tumor cell	DC	---	Antigen tranfer
	NK cell	Hsp	Activation
	Macrophage	Hsp	
Fibroblast	Primed $CD4^+$ T cell	TNF-TNFRI interaction	Resistance to AICD

Table 1. Activating functions of exosomes on immune cells (*Adapted from Thery et al., 2009*).

Source of exosomes	Recipient cells	Mechanism	Effects
Dendritic cell	Primed $CD4^+$ T cell	MHCII	Inhibition of activation
Tumor cell	$CD8^+$ T cell	?	Inhibition of cytotoxic activity
	NK cell	NKG2D-NKG2DL interaction	
	Regulatory $CD4^+$ T cell	TGFβ-TGFβR interaction	Promotion of regulatory activity
	Monocyte	?	Inhibition of differentiation into DCs
	MDSC	?	Differentiation into MDSCs
	$CD4^+$ T cell	Galectin9-TIM3 interaction	T cell killing
	Effector $CD4^+$ T cell	CD95-CD95L interaction	
Primed $CD4^+$ T cell	Effector $CD4^+$ T cell	CD95-CD95L interaction	T cell killing
Placenta	Effector $CD4^+$ T cell	CD95-CD95L interaction	T cell killing

Table 2. Inhibitory functions of exosomes on immune cells (*Adapted from Thery et al., 2009*).

2.6 Role of exosomes in the modulation of NK cell functions

Exosomes could be divided on the basis of their effect on NK cells in immunostimulatory and immunosuppressive, considering those derived from APCs in the first group and those originating from placenta or normal epitelium in the second one (Mincheva-Nilsson and Baranov, 2014), even if the real functional consequences of exosome-NK cell interaction are not completely understood. In particular the role of Tex on NK cells is controversial and seems to be dependent on exosome molecular cargo and the source of these vesicles.

Many studies report that NK cell-mediated functions can be modulated by exosomes carrying NK cell activating ligands on their surface. Ashiru and colleagues have demonstrated that MICA*008, one allelic form of the NKG2DL MICA, released mainly in association with exosomes (Ashiru et al., 2013) caused NKG2D downmodulation and a concomitant reduction of NK cell cytotoxicity, suggesting an alternative pathway to induce immune suppression on NK cells (Ashiru et al., 2010). Similarly, exosomes derived from mesothelioma and prostate cancer cells strongly induce NKG2D dowmodulation on NK cells with a mechanism mediated by TGFβ1 and MICA/B molecules associated to these nanovesicles (Clayton et al., 2008). The immunosuppressive effect of exosomes on NK

cells, mediated by NKG2D-NKG2DL interaction has also been described for placenta-derived exosomes. Indeed, these vesicles express either ULBPs and MIC molecules able to engage NKG2D and impair NKG2D-mediated cytotoxicity on NK cells (Hedlund et al., 2009).

NKp30 represents another NK cell activating receptor whose ligand named BAG6/BAT3 has been shown associated to exosomes. Simhadri and collegues observed that DC-derived exosomes expressing high levels of BAG6/BAT3 molecule stimulate IFN-γ and TNF-α release in NK cells (Simhadri et al., 2008). Interestingly, soluble BAG6 was detected in the plasma of chronic lymphocytic leukemia (CLL) patients, with the highest levels at the advanced disease stages. Incubation of NK cells, with the soluble form of BAG6/BAT3 or with exosome-bearing this ligand, resulted in the suppression or activation of NK cell cytotoxicity respectively (Reiners et al., 2013), thus suggesting that a dysregulated balance of exosomal vs soluble BAG6/BAT3 expression may cause immune evasion of tumor cells (Reiners et al., 2013).

Other mechanisms of exosome-mediated modulation of NK cells functions have been described over the last years. The presence of IL-15Rα on DC-derived exosomes surface can increase IL-15-mediated NK cell proliferation (Viaud et al., 2009). These authors have also noticed that NKG2DL expressed on Dex can have a stimulatory role on NK cell functions (Viaud et al., 2009), evidentiating that the contribute of different molecules determines the final immunomodulatory effect.

One of the major components of exosomes is Hsp70, a molecular chaperone belonging to the heat-shock protein family, which can be localized in the cytosol as well as associated to the exosome membrane. Interestingly, only exosomes with membrane Hsp70, produced by Hsp70-positive tumor cells, stimulated either NK cell migration and lytic activity against tumor cells through granzyme release (Gastpar et al., 2005). Similarly, another group demonstrated NK cell activation mediated by Hsp-bearing exosomes secreted by human hepatocellular carcinoma cells, with a resulting increase in NKG2D, NKp44 and CD94 surface expression, and NK cell cytotoxicity associated to an upregulation of granzyme B release (Lv et al., 2012). These studies emphasize an immune stimulatory effect of exosomal Hsp70.

In several different models, it has been observed the expression of TGFβ1 molecule on exosomes. In general, TGFβ1 positive exosomes have an inhibitory role on NK cell mediated functions as shown for exosomes derived from the sera of acute myeloid leukemia

(AML) patients which have the capability to strongly downmodulate NKG2D expression and NK cell cytotoxicity with a mechanism dependent on the formation of SMAD-DNA complexes (Szczepanski et al., 2011). A summary of exosome-mediated modulatory effects on NK cell-mediated functions is reported in Table 3.

Source of exosomes	Organism	Effect on NK cells	Mechanism	References
Dendritic cell	Human/ mouse	Increase cell proliferation and IFN-γ production	IL-15Rα and NKG2DLs (ULBP1) on exosomes	Viaud S et al. , 2009
	Mouse	Increase IFN-γ production	TNF-α on exosomes	Munich et al., 2012
	Human	Increase IFN-γ and TNF-α production	BAT3 on exosomes	Simhadri et al., 2008
Tumor cells	Human	Reduction of NKG2D-mediated functions: IFN-γ production and cytotoxicity	TGFβ and NKG2DLs on exosomes	Clayton et al., 2008
		Reduction of cytotoxicity	NKG2DLs on exosomes	Hedlung et al., 2011
		Increase Granzyme B production and cytotoxicity	HSP70 on exosomes	Gastpar et al., 2005
		Increase cytotoxicity and Granzyme B production	HSPs (60,70,90) on exosomes	Lv et al., 2012
	Mouse	Inhibition of cytotoxicity. Reduction of perforin and IL-2 dependent NK cell proliferation	Block of Jak3 and Cyclin D3 activation	Liu et al., 2006
Placenta	Human	NKG2D downmodulation and cytotoxicity	NKG2DLs (ULBP1-5, MIC) on exosomes	Hedlung et al., 2009

Table 3. Modulatory effects of exosomes on NK cell-mediated functions.

3. Multiple Myeloma

Multiple Myeloma (MM) is a very common neoplastic plasma cell (PC) disease characterized by a multifocal proliferation of clonal long-lived PC with an accumulation in the bone marrow (BM) (over 10% by definition) and less frequently in extramedullary tissues (Kyle and Rajkumar, 2004; Kyle, 1985). Several symptoms are typically associated with this malignancy including serum monoclonal gammopathy, skeletal destruction, immune suppression/immune deficiency, renal failure, anemia and hypercalcemia that arise during tumor progression.

MM can be considered a disease with an high level of molecular heterogeneity, thus its etiological genetic baground is characterized by different structural and numerical chromosomal aberrations such as loss and gain of whole chromosomes as well as mutations in oncogenes and tumor-suppressor genes together with translocation and a wide range of mutation (Anderson and Carrasco, 2011). For example, some translocation juxtaposed cyclin genes to a stronger Ig enhancer leading to cyclin D1 or D3 overexpression and a consequent increase in tumor growth and survival (Shaughnessy, Jr. et al., 2001; Gabrea et al., 1999; Bergsagel and Kuehl, 2001). Furthermore, mutation in TP53 or PTEN together with other mutations are described to have a role in MM pathogenesis through the constitutive activation of the noncanonical NF-kB pathway (Keats et al., 2007).

Of note, increasing evidence demonstrate the importance of the mutual interaction between MM cells and BM microenvironment in tumor progression. Thus both extracellular matrix proteins, fibronectin, collagen, laminin and osteopontin, and several cell types, such as hematopoietic stem cells, progenitor and precursor cells, immune cells, endothelial cells, erythrocytes, adipocytes, osteoclasts and osteoblasts, could influence and facilitate tumor growth through the secretion of IL-6, TNF-α and other paracrine factors (Caers et al., 2008; Ribatti et al., 2006). This evidence can provide the basis for the development of novel therapeutic strategies for MM targeting MM-bone marrow stromal cells (BMSC) interaction.

MM procedes by an age-progressive premalignant condition termed *monoclonal gammopathy of undetermined significance* (MGUS), present in 1% of adults over the age of 25, and then can evolve in active MM. Recent screening studies demonstrate that all the diagnosed MM patients had previously been diagnosed with MGUS (Anderson and Carrasco, 2011). Moreover, MGUS patients display abnormal secretion of monoclonal Ig without any organ dysfunction and in theory their malignant PCs can produce all classes of immunoglobulin, even if IgG are the most common followed by IgA and IgM while those

producing IgD and IgE are usually very rare. In some patients an asymptomatic intermediate and more advanced premalignant stage named *smoldering MM* (*SMM*) is clinically recognized and characterized by a stable intramedullary tumor-cell content reaching about 30% and the risk of evolvement to symptomatic MM is 10-20% per year (Kyle et al., 2007). The progression to the terminal stage of MM is associated with increasingly severe secondary features. MM has a greater intramedullary tumor-cell content which causes bone lesion and subsequent bone fracture and pain and interferes with normal hematopoiesis. The breakdown of bones also leads to calcium release into the blood, resulting in hypercalcemia and its associated symptoms already mentioned. Furthermore, the presence in blood and urine of a monoclonal immunoglobulin called "Bence Jones protein" is considered a typical feature of MM patients. This protein is responsible of the onset of kidney injuries onset together with the hypercalcemia. PCs infiltration into the bone marrow and renal dysfunction often cause severe anemia in these patients (Palumbo and Anderson, 2011). Furthermore in some patients, the tumor can acquire the ability to grow in extramedullary sites such as blood, pleural fluid and skin thus generating a more aggressive disease.

Two methods are used to classify patients considering the complication associated with diferent stages of disease and many different parameters such as age, performance status and renal function, even if these classification have several limitations because this malignancy is characterized by a broad molecular heterogeneity (Bataille et al., 2013). Thus, Durie and Salmon devised a staging system to predict the outcome by evaluating haemoglobin, serum calcium levels, monoclonal immunoglobulin levels in serum and urine, extent of bone lesions (Durie and Salmon, 1975) and since 2005, the MM International Staging System (ISS) has recognized the combination of beta-2 microglobulin (β_2M) with serum albumin (SA) concentrations as the most simple and potent combination to determine the prognosis in MM patients (Greipp et al., 2005).

Nowadays MM is still an incurable disease even if some chemotherapeutic strategies are in use. Considering the heterogeneity underlying this pathology, several different approaches have been developed including autologous stem cell transplantation or subministration of different drugs that can be divided in "old drugs" and "new generation drugs". The first class includes alkylators, such as Melphalan, corticosteroids and anthracyclines, such as Doxorubicin, while the second class is represented by proteasome inhibitors, such as Bortezomib, and immunomodulatory drugs (IMiDs) that induce an increase in immune response against tumor cells. Nowadays, the most common strategies in MM therapy are

based on multidrug-combinations that maximize their synergism while minimizing toxicities (Kaiser et al., 2010; Lamottke et al., 2012).

3.1 Role of NK cells in the immunosurveillance of MM: implication for immune-chemotherapy

Current insights into the molecular specificities that regulate NK cell-mediated functions suggest that it might be possible to design NK-cell-based immunotherapeutic strategies against human cancer because of the NK prominent role in immune response toward tumors including MM. In fact, several studies show a significative increase in NK cell number in patients with MGUS and MM in the early stages compared to normal individuals (Osterborg et al., 1990; Gonzalez et al., 1992) and this number is further enhanced during tumor progression while NK cell cytotoxic activity decrease (Ogmundsdottir, 1988), suggesting that these cells may initially contribute to the control of malignant PCs but this effect is attenuated in the course of tumor progression. NK cells can also contribute to graft-versus-myeloma responses in haploidentical hematopoietic stem cell transplantation (HSCT) (Shi et al., 2008) and importantly these cells can elicit potent allogenic and autologous response to myeloma cells *in vitro* and in patients (Shi et al., 2008; Ruggeri et al., 2007).

Within the tumor microenvironment NK cells recognize and lyse MM cells through a mechanism dependent on the engagement of different activating receptors, such as NKG2D, DNAM-1 and NCRs (El-Sherbiny et al., 2007; Carbone et al., 2005). In this regard, several studies demonstrated the presence of DNAM-1 and/or NKG2D ligands in MM cell lines and in primary malignant PCs derived from MM patients (El-Sherbiny et al., 2007; Soriani et al., 2009b).

Given the importance of NK cells in immune response toward MM, combination therapies that enhance NK cell functions are showing promise in treating this malignancy. A pivotal strategy to improve NK cell-mediated anti-tumor activity involves the use of several chemotherapeutic agents such as genotoxic drugs or inhibitors of proteasome, histone deacetylases, GSK3 or of the Hsp-90 molecular chaperone, that are able to induce the up-regulation of NKG2D and DNAM-1 ligands on tumor cell surface leading to a better NK cell-mediated recognition and lysis (Fionda et al., 2009; Wu et al., 2012; Jinushi et al., 2008; Soriani et al., 2009b). In addition to their ability to activate specific transduction pathways associated to the up-regulation of different NK cell activating ligands on the tumor cells, these compounds can also affect tumor survival.

In particular, the induction of DDR in response to genotoxic agents Doxorubicin and Melphalan (Mel) strongly enhanced NKG2DL and DNAM-1L on MM cells increasing their susceptibility to NK cell-mediated killing (Soriani et al., 2009b).

Moreover, in our laboratory we found that different drugs targeting the GSK3 kinase can up-regulate both MICA protein surface and mRNA expression in MM cells, with little or no effects on MICB and DNAM-1L expression (Fionda et al., 2013); furthermore, exposure to GSK3 inhibitors renders myeloma cells more susceptible to NK cell-mediated cytotoxicity with a mechanism mostly dependent on NKG2D (Fionda et al., 2013).

Other alternative strategies used in MM therapy consist in stimulation of NK cell-mediated function through the administration of some 'new generation' drugs including Thalidomide and other Immunomodulatory drugs (IMiDs®), such as lenalidomide (CC-5013, Revlimid®) and pomalidomide (CC-4047, Actimid®), that are able to activate NK cells directly or indirectly, through T cell production of IFN-γ and IL-2 or DC activation (Bartlett et al., 2004). We have recently shown that these drugs can strongly enhance the expression of the ligands for NKG2D and DNAM-1 both in human MM cell lines and in primary malignant PCs. We found that the transcription factors IKZF1/3 and IRF4 play a crucial role in this regulatory mechanism through the ability to repress the basal transcription of *mica* and *pvr* gene expression. Lenalidomide-induced downregulation of these transcription factors leads to de-repression of *mica* and *pvr* promoter activity, and consequently to increased gene transcription. Thus, we identified IKZF1/3 and IRF4 as "druggable" transcriptional repressors of NK cell-activating ligand expression in MM cells (Fionda et al., 2015).

The release of soluble NKG2D ligands has been suggested to be a major mechanism of tumor cell evasion from NKG2D mediated immunosurveillance. As a matter of fact, soluble forms of NKG2DL are present in the serum of MM patients and other types of malignancies and their levels correlate with tumor stage and metastasis and with reduced expression of NKG2D on NK cells and other cytotoxic lymphocytes. Soluble NKG2DLs can be released through metalloproteinase-mediated cleavage, exosome secretion, or alternative splicing. We have recently studied the effect of genotoxic drugs on NKG2D ligands shedding. Our data demonstrate that genotoxic agents stimulate the shedding of MIC molecules sensitive to proteolytic cleavage with a mechanism mediated by ADAM10 protease. Interestingly, we found that the combined use of chemotherapeutic drugs and metalloproteinase inhibitors enhances NK cell-mediated recognition of MM cells preserving MIC molecules on the cell surface (Zingoni et al., 2015). Our results stronlgy suggest that targeting of

metalloproteinases in conjunction with chemotherapy could be exploited for NK cell-based immunotherapeutic approaches, thus contributing to avoid the escape of malignant cells from stress-elicited immune responses.

3.2 Role of exosomes in the progression of Multiple Myeloma

The release and the reciprocal exchange of exosomes from both stromal and MM cells can be considered an important mechanism of cross-talk between MM and BM microenvironment and could facilitate directly or indirectly tumor growth and progression.

Roccaro and colleagues have recently demonstrated that exosomes derived from BMSCs of patients affected by MM could be transferred to MM cells inducing MM cell growth and promoting dissemination, whereas exosomes derived from BMSCs of healthy donors didn't have the same effects (Roccaro et al., 2013). In another study, exosomes obtained from BMSCs induced survival, proliferation, migration and drug resistance of human MM cells through the transport of bioactive molecules, such as miRNAs, mRNAs and proteins (Wang et al., 2014).

On the other hand, a very recent study has provided the first evidence that MM cell-derived exosomes directly influence bone-resorbing osteoclasts (OCs) differentiation and function, such as migration and secretion of factors involved in bone resorption activity thus facilitating tumor growth. Moreover, it has been shown that these vesicles could enhance survival and anti-apoptotic gene expression in OCs through a mechanism mediated by the activation of AKT pathway (Raimondi et al., 2015). Other studies have reported the ability of exosomes derived from MM cells to stimulate angiogenesis. In particular, Liu and colleagues demonstrated the crucial role of MM cell-derived exosomes in the induction of endothelial cell proliferation and secretion of both IL-6 and vascular endothelial growth (VEGF) (Liu et al., 2014), while Umezu et al, showed that these vesicles could enhance angiogenesis through exosomal miR-135b-mediated HIF-1 signaling. They also demonstrated that MM cells in chronic hypoxic conditions were able to secrete more exosomes than the parental cells under normoxia (Umezu et al., 2014). Interestingly, Di Noto and colleagues recently observed that MM patients released an higher amount of exosomes compared to MGUS and healthy donors, suggesting a direct correlation between exosome secretion and MM progression. They also observed the involvement of exosomes in tumor survival and expansion because of their ability to transport mRNA, miRNA and several proteins, such as cytokines and growth factors (Di et al., 2015). Overall, these recent

studies denote an emerging role of exosomes in the intercellular communication between MM and stromal cells but little is known about the effects of MM-cell derived exosomes on immune cells.

AIM

NK cells are reported to be an important component of the innate immunity response against stressed, infected and transformed cells. They can either sense damaged cells through the recognition of different activating ligands and at the same time they are able to detect DAMPs released by abnormal cells trough Toll-like receptors. Multiple myeloma represents a clonal B-cell malignancy characterized by the expansion of transformed plasma cells in the bone marrow. Several studies show that NK cells participate in the surveillance of MM. In the cross-talk between NK and malignant cells, tumor cells can release a wide range of soluble factors and extracellular vesicles that could modulate NK cell-mediated functions. In this context, we focused our attention on a specific class of nanovesicles named exosomes. These vesicles can carry proteins, lipids and nucleic acid, as mRNAs and miRNAs, and are considered key players of intercellular communication.

My PhD project was aimed at investigating the molecular and biochemical features as well as the immunomodulatory properties of exosomes released from MM cells in steady state conditions and after treatment with Melphalan, a genotoxic agent used in MM therapy. We found that MM-cell derived exosomes have an immunostimulatory role on NK cell in terms of proliferation, CD69 induction and IFN-γ production. Subsequently, we analyzed the molecular mechanisms underlying these events and focused our attention on Toll-like receptor family. We identified the TLR2/NF-kB axis as one of the mechanisms mediating exosome-induced IFN-γ production.

We envisage that a better understanding of exosome molecular phenotype and immunomodulatory properties will provide new insight into their importance in cancer therapy and their possible use as biomarkers.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were from BD Biosciences (San Jose, CA): anti-CD3/APC-H7 (clone SK7), anti-CD3/APC, anti-CD56/PE (clone NCAM16.2), anti-CD14/FITC (clone HCD14), anti-IFN-γ/APC (clone B27). Anti-TNF-α/APC (clone cA2) was from Miltenyi Biotec (Cologne, Germany). Anti-human IL15Rα from R&D Systems (Minneapolis, MN). Anti-human Ki-67 antigen/FITC (clone MIB-1) was from DAKO (Golspug, Denmark). Anti-CD63 (H-193), anti-Hsp70/Hsc70 (W27), anti-CD81 (H-121), NFkB p65 (C-20), anti-Tsg101 (M-19) were purchased from Santa Cruz Biotechnology (CA). Phospho-NF-kB p65 (Ser 536) (clone 7F1) was from Cell Signaling (Danvers, MA). Anti-calreticulin was from Thermo Fisher Scientific (Rockford, USA). Anti-MHC I (clone HC10) was kindly provided from Dr. P. Giacomini, Regina Elena Cancer Institute, Rome, Italy. The following antibodies were from BioLegend (San Diego, CA): control mouse IgG1 (clone MOPC-21), anti-human CD69/APC (clone FN50), anti-human IL-12/IL-23p40/Alexa Fluor 647 (clone C11.5), anti-human CD63/PE (clone H5C6). F(ab)2 fragments of APC conjugated goat-anti-mouse (GAM-APC or GAM-PE) IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-β-actin (clone AC-74, IgG2a) was from Sigma-Aldrich (St Louis, MO); anti-hTLR2-IgA neutralizing antiboby (clone B4H2) was from InvivoGen (San Diego, CA) and anti-human CD282 (TLR2) APC (clone TL2.1) was from eBioscience (California, USA). Enzyme-linked immunosorbent assay (ELISA) for IFN-γ and TNF-α were from R&D Systems. The NF-kB SN50 inhibitor peptide was from Calbiochem (Los Angeles, USA). Other reagents used were as follows: bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT), brefeldin A (BFA), saponin, paraformaldehyde (PFA), propidium iodide (PI), trypan blue, dimethylformamide (DMF), puromycin, Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE), Melphalan, all from Sigma-Aldrich (St Louis, MO). In addition, hygromycin, blasticidin, R848, Pam3CSK4, ODN 2216, Poly (I:C), LPS were from InvivoGen (San Diego, CA). Recombinant Human IL-15 and IL-2 were purchased from Peprotech (Rocky Hill, NJ).

Cell lines

The human MM cell lines SKO-007(J3) and ARK, were provided by P. Trivedi (“Sapienza” University of Rome). The cell lines were maintained at 37°C and 5% CO2 in RPMI 1640

(Life Technologies, Gaithersburg, MD) supplemented with 10% FCS. All cell lines were mycoplasma free (EZ-PCR Mycoplasma test kit; Biological Industries).

Exosome purification

Exosome-free medium was obtained as follows: FCS was centrifuged at 100.000 g for 3 hours in a Beckman ultracentrifuge (Beckman Coulter, Brea, CA) in order to remove microvesicles-like exosomes. RPMI 1640 was supplemented with 10% of FCS-exosome free and antibiotics.

ARK and SKO-007(J3) cell lines were cultured at 1 x 10^6 cells/ml in exosome-free medium for 24-48 hours. In some experiments, cells were treated with a sublethal dose of Melphalan (Mel) determined by the assay as previously described (Soriani et al., 2009a). Concentrations 10 times lower than IC_{50} values were used to treat the different cell lines as follows: ARK (3μM) and SKO-007(J3) (5μM). After 48 hours of drug treatment, cells were washed and plated at 1,5 x 10^6 cells/ml in fresh exosome-free medium and incubated for additional 24 hours at 37°C and 5% CO_2 .

Exosome purification protocol consists of different sequential centrifugations as previously reported (Thery et al., 2006) and it is schematically represented in Figure 5. Cells were harvested by centrifugation at 300 g for 10 minutes and supernatants were collected. Cell-free supernatants were then centrifuged at 2.000 g for 20 minutes followed by centrifugation at 10.000 g for 30 minutes to remove cells debris. Supernatants were filtered using a 0.22 μm filter and centrifuged at 100.000 g for 70 minutes at 4°C in a Beckman ultracentrifuge in order to pellet exosomes. The resulting pellet was washed in a large volume of cold PBS and again centrifuged at 100.000 g for 70 minutes at 4°C. Finally, exosomes were resuspended in PBS for further analyses and functional studies.

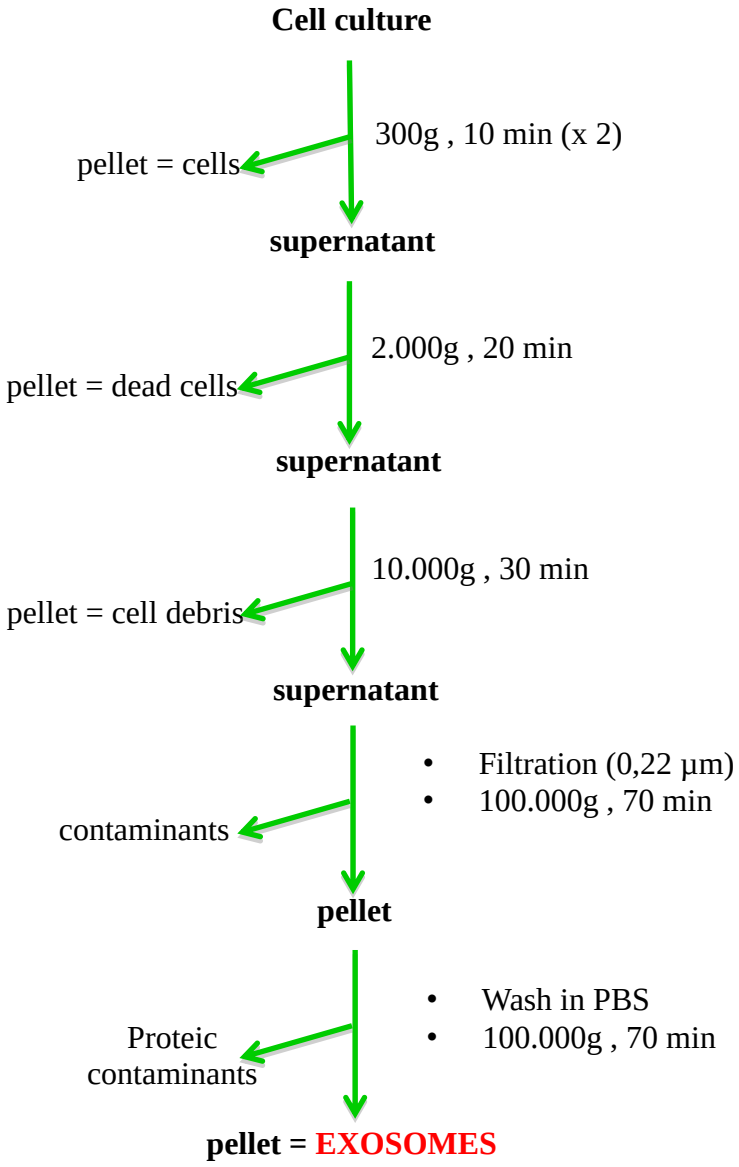

Figure 5. Exosome isolation protocol. Schematic representation of exosome isolation protocol based on a series of differential centrifugation and ultracentrifugation. Every step allows to remove both alive and death cells, cell debris, apoptotic bodies and vesicular or proteic contaminants to obtain a final pellet enriched in exosomes.

Flow cytometric analysis of exosomes

Flow cytometric analysis of $CD63^+$ exosomes was performed by positive magnetic selection using $CD63^+$ dynabeads (Invitrogen). About 5-10 µg of exosome preparation were diluted with Isolation buffer (PBS/0.1% BSA) in a final volume of 100 µl, then 20 µl of magnetic beads conjugated with anti-CD63 antibodies were added. The suspension was incubated with gentle tilting and rotation for 18-22 hours at 4°C and then washed twice with isolation buffer. The beads-bound exosomes were resuspended in 200 µl of isolation buffer, labeled

with cIg/PE or anti-CD63/PE (BioLegend). Samples were acquired using a FACSCanto (BD Biosciences, San Jose, CA) and data analysis was performed using the FlowJo program.

RNA isolation, RT-PCR, and real-time PCR

Total RNA from human primary purified NK cells or monocytes was extracted using Total RNA Mini Kit (Geneaid, New Taipei City, Taiwan) after different times of exosome treatment. Total RNA (100-800 ng) was used for cDNA first-strand synthesis using oligo-dT (Promega, Madison, WI) in a 25μL reaction volume. Real-time PCR was performed using the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA). cDNAs were amplified in triplicate with primers for IFN-γ (Hs00989291_m1), TNF-α (Hs01113624_m1), CD69 (Hs00934033_m1) and IL-12B (Hs01011518_m1), and human β-actin (Hs99999903_m1) all conjugated with fluorochrome FAM (Applied Biosystems). The cycling conditions were: 50°C for 10 min, followed by 40 cycles of 95°C for 30 sec, and 60°C for 2 min. Data were analyzed using the Sequence Detector v1.7 analysis software (Applied Biosystems). The level of gene expression was measured using threshold cycle (Ct). The Ct was obtained by subtracting the Ct value of the gene of interest from the housekeeping gene (β-actin) Ct value. In the current study, we used Ct of the untreated sample as the calibrator. The fold change was calculated according to equation $2^{-\Delta\Delta Ct}$, where ΔΔCt was the difference between Ct of the sample and the Ct of the calibrator (according to the formula, the value of the calibrator in each run is 1).

Exosomal RNA isolation

100 μg of exosomes were used for exosomal-RNA isolation with Total Exosome RNA and Protein Isolation Kit according to the manufacturer's instructions (Invitrogen). Briefly, exosome pellet was resuspended in 200 μl of-PBS and then one volume of preheated (37°C) 2X Denaturing Solution, was added, gently mixed and incubated on ice for 5 minutes. One volume of Acid-Phenol:Chloroform was added and mixed by vortexing for 30-60 seconds. The samples were centrifuged for 5 minutes at 12.000 g at room temperature, the upper acqueous phase was collected in a fresh tube and 1.25 volume of 100% ethanol was added, mixed and loaded into the Filter Cartridge (supplied by the kit) to allow the RNA binding. The column was washed three times with washing solutions containing ethanol, the flow-through was discarded and the column was transferred into a fresh collection tube. RNA was

eluted with 30 µl of preheated (95°C) nuclease-free water and then quantified using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific).

Exosome miRNA profiling

Exosome microRNAs (miRNA) expression profiling was performed by using Megaplex Pools cards A and B containing up to 380 miRNAs for each (Applied Biosystems). About 600 ng of total exosome-RNA was used for the synthesis of single-stranded cDNA in a final volume of 7.5 µl. The cycling conditions were: 40 cycles at 16°C for 2 min, 42°C for 1 min, and 50°C for 1 sec, followed by 85°C for 5 min and 4°C for 5 min. 6 µl of RT product was mixed with the TaqMan Universal PCR Master Mix and nuclease-free water in a final volume of 900 µl. 100 µl of this mixture was dispensed into each port of the microRNA Array card A and B. Data were analyzed using the Sequence Detector v1.7 analysis software (Applied Biosystems). Thermal-cycling conditions according to the manufacturer's instructions (Applied Biosystems).

Expression analysis of single microRNA was performed by using the TaqMan Small RNA assay (Applied Biosystems). 10 ng of exosomal-total RNA was utilized for RT reaction and as RT primers: miR -15b, miR-21, miR-29a, miR-122, miR-155, RNU-44, RNU-48 and U6 snRNA were used (Applied Biosystems). The cycling conditions were: 16°C for 30 min, 42°C for 30 min, followed by 85°C for 5 min and 4°C for 5 min. RT products were used to prepare qPCR reaction mix; real-time PCR specific miRNA primers were all conjugated with fluorochrome FAM. The cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. Real-time PCR was performed using the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA). Data were analyzed using the Sequence Detector v1.7 analysis software (Applied Biosystems).

SDS-PAGE and Western blot analysis

For Western blot analysis, SKO-007(J3) and ARK cells or exosome preparations were lysed in 1X RIPA lysis buffer (1% NP-40, 0.1% SDS 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1 mM EDTA in water) plus complete protease inhibitor mixture and phosphatase inhibitors sodium orthovanadate and sodium fluoride (Sigma-Aldrich). The lysate was incubated 20 min on ice and then centrifuged at 12.000 rpm for 20 min at 4°C, and the supernatant was collected as whole-cell and -exosome extract. Protein

concentration was determined with the Bio-Rad Protein Assay. 40 to 50 µg of cell or exosome extract was run on 8% or 10% denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were then electro-blotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NJ) and blocked in 5% milk in TBST buffer for 1 hours. Immunoreactive bands were visualized on the nitrocellulose membranes, using Horseradish Peroxidase (HRP)-coupled goat anti-rabbit or goat anti-mouse Igs and the enhanced chemiluminescence kit (ECL) detection system (GE Healthcare Amersham), following the manufacturer's instructions.

Human NK cell isolation

Highly purified primary NK cells were obtained from human peripheral blood mononuclear cells (PBMCs) by negative selection using magnetic beads (Miltenyi Biotec). Briefly, 100 x 10^6 PBMCs were resuspended in 400 µl of isolation buffer (PBS without Ca^{2+} and Mg^{2+} supplemented with 0,5% bovine serum albumin BSA, and 2mM EDTA, pH 7.2) and incubated with 100 µl of "Biotin-Antibody Cocktail", containing human antibodies against antigens not expressed by NK cells, for 5 minutes at 4°C and then with 300 µl of isolation buffer and 200 µl of "MicroBead Cocktail" for 10 minutes at 4°C. The cell suspension was applied onto LS column placed in the magnetic field of MACS Separator (Miltenyi Biotec) and the unlabeled cells, enriched in NK cells were collected. NK cell purity was more than 95% $CD56^+CD3^-$ as assessed by immunofluorescence and flow cytometry analysis (Figure 6). In some experiments, purified NK cell subsets, $CD56^{bright}$ and $CD56^{dim}$, were sorted by FACSARIA (BD).

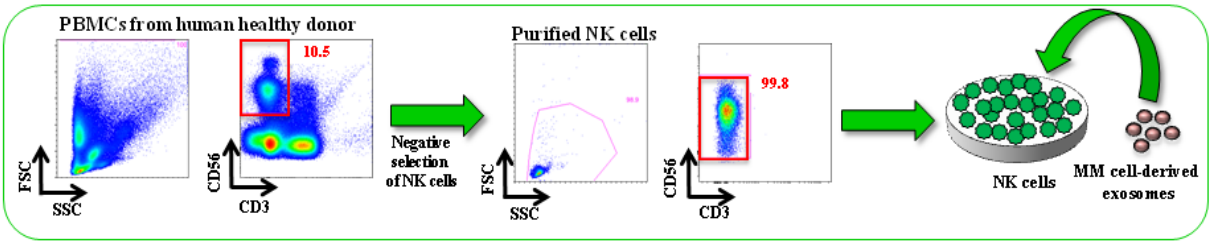

Figure 6. NK cell isolation and purification procedure. Primary human NK cells were purified from PBMCs derived from healthy donor through immunomagnetic negative selection. The purified cells were incubated in exosome-free medium in the presence of MM cell-derived exosomes**.**

Cytokine production and cell proliferation

For intracellular cytokine detection, NK cells were seeded at 2-3 x 10^6 /ml in exosome-free medium, and incubated with different doses of MM cell-derived exosomes (1-20 µg/ml) for 24 hours. BFA was added at 5 µg/ml and left for additional 24 hours.

For evaluating cell proliferation, NK cells were labelled with CFSE as previously described (Cerboni et al., 2007). Briefly, cells were extensively washed with PBS and then resuspended in PBS at 50 x 10^6 cells/ml and labeled with 2,5µM of CFSE for 10 minutes at 37°C. Cell labeling was blocked by adding complete RPMI 1640 medium. Cells were washed twice before been cultured for five days in the presence of 20µg/ml of MM cell-derived exosomes.

In some experiments, NK cells were treated with different doses of recombinant human IL-15 (5-50 ng/ml), or with 500 U/ml of human recombinant IL-2. Sometimes, before exosome treatment, NK cells were pre-incubated with the NF-kB peptide inhibitor SN50 (10-15µM) for 1 hour at 37°C.

Human Monocyte purification

Monocytes were purified from human PBMCs by negative selection using magnetic beads (Dynal Invitrogen, Carlsbad, CA). 100 x 10^6 PBMCs were resuspended in 1 ml of isolation buffer; 200 µl of blocking reagent (aggregated gamma globulin in 0.9% NaCl) plus 200 µl of antibody mix (containing biotinylated mouse IgG antibodies against CD3, CD7, CD16, CD19, CD56, CDw123 and CD235a) were added to the cells. The cell suspension was incubated for 20 minutes at 4°C and then washed with isolation buffer before being mixed with 1 ml of pre-washed magnetic beads for 15 minutes at 4°C with gentle tilting and rotation. The mixture containing cells and magnetic beads was placed in a magnet for 2 minutes and the supernatant containing $CD14^+$ monocytes was transferred to a new tube for functional studies. Monocyte cell purity was about 80-90% $CD14^+$ as assessed by immunofluorescence and flow cytometry analysis.

Monocytes were seeded at 2-3 x 10^6/ml in exosome-free medium and incubated with 20 µg/ml of MM cell-derived exosomes for 48 hours. For intracellular cytokine detection, after 24 hours BFA was added at 5 µg/ml and left for additional 24 hours. In some experiments monocytes were pre-incubated with the NF-kB peptide inhibitor SN50 (10-15µM) for 1 hour at 37°C.

Immunofluorescence and FACS analysis

For intracellular cytokine detection, human primary NK cells and monocytes were labeled with anti-CD56/PE and anti-CD14/FITC or anti-CD14/PerCP respectively for 25 minutes at 4°C. Cells were washed with PBS and fixed in 1% PFA for 20 minutes at room temperature (RT). Cells were washed with PBS and then incubated with PBS containing 2% BSA for 15 minutes at RT and then permeabilized with PBS containing saponin 0.5% and FCS 1% for 20 minutes at RT. NK cells and monocytes were washed with PBS/1%FCS, and labeled with anti-IFN-γ/APC or anti-TNF-α/APC respectively for 25 minutes at 4°C. Samples were washed in PBS- 0.5% saponin-1% FCS. Cellular pellets were resuspended in PBS/1%FCS.
In other experiments, NK cells were stained with anti-CD69/APC for 20 minutes at 4°C and washed with PBS. All the samples were acquired using a FACSCanto (BD Biosciences, San Jose, CA) and data analysis was performed using the FlowJo program.

Electron microscope and immunogold-labeling

A total of 20 to 30 µl prepared exosome samples for electron microscopy were fixed in 2% formaldehyde in PBS, pH 7.4, at 48 °C. They were then washed in PBS and postfixed in 1.33% osmium tetroxide for 2 h at 48 °C. After several washes in PBS, the samples were dehydrated in graded alcohol, transferred into toluene, and embedded in Epon 812 resin. The resin was allowed to polymerize in a dry oven at 60 °C for 24 h. Thin sections were cut with a glass knife on a Reichert microtome, stained with toluidine blue, and examined on Axioscope microscope (Zeiss Jena GmbH, Germany). Ultrathin sections were cut on a Richert microtome using a diamond knife, stained with uranyl acetate–lead citrate and evaluated on a Philips electron microscope Morgagni 268D (Philips, Endhoven, The Netherlands). Each observation was carried out independently 6–7 times per sample.
For immunogold labeling, exosomes were applied to formvar carbon-coated copper grids and incubated with rabbit anti-human CD81 monoclonal antibody and mouse anti-human Tsg101 antibody (Santa Cruz Biotechnology) at room temperature for 1 hour. Incubation with PBS served as a blank control. Following washing in PBS, exosomes were incubated with 20 µl of protein A immunogold (SPA) (1:15 diluted) at room temperature for 30 minutes. A negative dye containing 15 µl uranyl acetate was performed at room temperature for 30 seconds. Positively labeled exosomes were seen as vesicles containing black colloidal gold particles under the transmission electron microscope. The numbers of exosomes were

counted in 10 random fields (1000 nm × 700 nm). Counting was repeated five times and the average was calculated for each of the specimens.

Exosome uptake

100 μg of exosomes diluted in PBS were incubated with the red fluorescent dye PKH26 for 10 minutes (Sigma-Aldrich) and then exosomes were washed with PBS by ultracentrifugation at 100.000g for 1 hour. PKH26-labelled exosomes were diluted with PBS and used for uptake experiments.

Primary NK cells cells were plated on poly-L-lysine-coated multichamber glass plates in complete medium and incubated with PKH26-labelled exosomes (20 μg/ml) for different times. Medium was removed and the cell monolayer was gently washed with PBS, and fixed with 4% paraformaldehyde. Cover slips were mounted using SlowFade Gold reagent (Life Tecnologies) and acquired at room temperature using an ApoTome Observer Z.1 microscope with an Axiocam MR equipped with AxioVision Version 4.6.3 software for image acquisition. In some experiments, PKH26-labelled exosomes were incubated with highly purified NK cells for different times. Cells were collected, washed twice with PBS and then samples were acquired using a FACSCanto (BD Biosciences, San Jose, CA). Data analysis was performed using the FlowJo program.

ELISA

Detection of IFN-γ or TNF-α in supernatants (SNs) collected after exosome stimulation of primary NK cells or monocytes was performed using a sandwich ELISA procedure according to the manufacturer's instructions (R&D Systems). Plates were developed using a peroxidase substrate system (R&D Systems), and then read with the Victor3 multilabel plate reader (Model # 1420-033, Perkin Elmer, Santa Clara, CA) capable of measuring absorbance in 96-well plates using dual wavelength of 450-540 nm. Results were expressed as picograms per milliliter (pg/ml) and referred to a standard curve obtained by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve through the points on the graph.

Moreover, detection of proinflammatory cytokines and chemokines, fractalkine, IFN-γ, IL-15, IL-6, IL-8, IP-10, MIP1-β and Rantes, in the same supernatants was performed with a Milliplex ^{MAP} Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex

Assay according to the manufacturer's instructions (Millipore). Plates were read with Bio-Plex MAGPIX Multiplex Reader (BIO-RAD).

NF-κB Luciferase Reporter Assay

TLR-specific activation assays were performed using human embryonic kidney 293 (HEK293) cells expressing luciferase under control of the NF-κB promoter and stably transfected with either TLR4, MD2, and CD14 (TLR4-HEK293), TLR2 (TLR2-HEK293), TLR3 (TLR3-HEK293), TLR7 (TLR7-HEK293), TLR8 (TLR8-HEK293) and TLR9 (TLR9-HEK293). HEK293-transfected cells were maintained in DMEM supplemented with 4.5 g/liter glucose and 10% FCS, 1% penicillin/streptomycin solution (Invitrogen), and specific antibiotics for the different cell lines were added as shown in Table 4.

Cell line	***Antibiotics***	***Agonist***
HEK293-Luc	Puromycin 5 μg/ml	---
TLR2-HEK293	Puromycin 5 μg/ml Hygromycin 250 μg/ml	Pam3SCK4 1μM
TLR3-HEK293	Puromycin 2 μg/ml Blasticidin 10 μg/ml	Poly(I:C) 1 μg/ml
TLR4-HEK293	Puromycin 5 μg/ml Blasticidin 10 μg/ml Hygromycin 250 μg/ml	LPS 10 μg/ml
TLR7-HEK293	Puromycin 5 μg/ml Blasticidin 10 μg/ml	R848 10μM
TLR8-HEK293	Puromycin 5 μg/ml Blasticidin 10 μg/ml	R848 10μM
TLR9-HEK293	Puromycin 5 μg/ml Blasticidin 10 μg/ml	CpG 50μM

Table 4. HEK239 reported cell culture conditions

All the HEK293-transfected cells were kindly provided by Dr U. D'Oro (Novartis, Siena).
For the NF-κB luciferase assay 30000 cells/well were seeded in 100 μl of complete DMEM without antibiotics in 96-well plates and incubated for 18 hours at 37°C. Different concentrations of exosomes (1-20 μg/ml) were added and left for different times (2-18 hours). As positive control for each transfectant specific TLR agonists were used as indicated in Table 4. After incubation, supernatants were aspired from each well, cells were washed with PBS and then were lysed for 15 minutes at room temperature using 100 μl/well of 1:5 diluted "passive lysis buffer" (Promega, Madison WI). Protein concentration was

evaluated by Bio-Rad Protein Assay. 3μg of total proteins for each sample were diluted in 50 μl of PBS and 50 μl of luciferase assay substrate (Promega, Madison WI) was added. Emitted light was immediately quantified using a luminometer GloMax-Multi Detection System (Promega, Madison WI).

Statistics

Error bars represent SD or where indicated SEM. Statistical analysis was performed with the Student paired test; $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$.

RESULTS

Structural and biochemical characterization of exosomes derived from Multiple Myeloma cells upon Mel treatment

To isolate and characterize exosomes produced by MM cells, we utilized a classic exosome isolation protocol that exploits their differential sedimentation properties. Exosomes were isolated from the conditioned media of ARK and SKO-007(J3) cell lines in steady state conditions or upon treatment with Melphalan (Mel), a genotoxic agent used in MM therapy. Interestingly, we found that drug-treated cells released a higher amount of exosomes either in SKO-007(J3) and ARK cell lines (Figure 7).

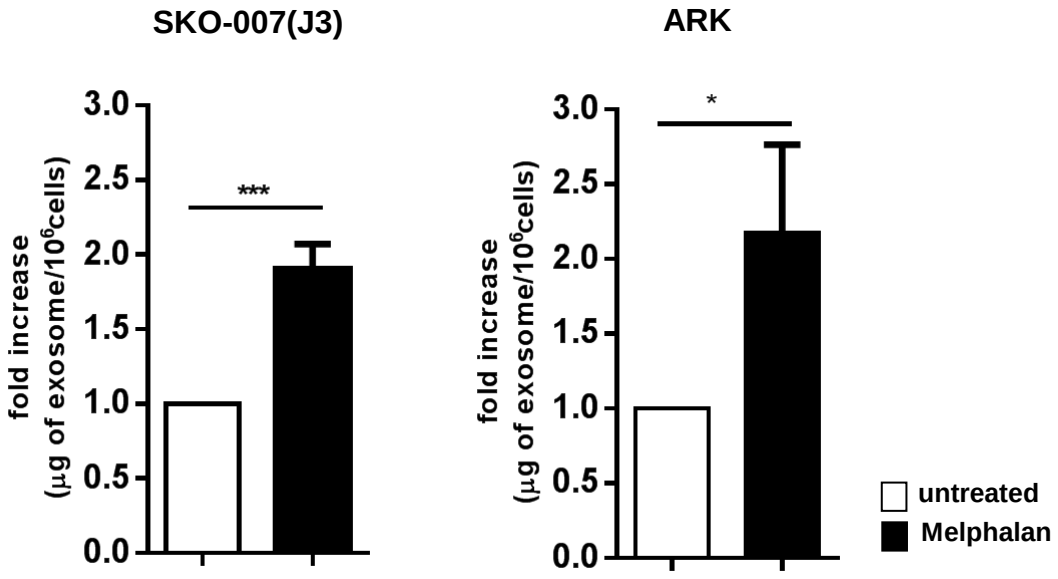

Figure 7. Melphalan-treated MM cells release a higher amount of exosomes. The exosome amount was measured by BCA protein assay and was normalized per 10^6 cells. Data are expressed as fold increase of the μg/10^6 cells values obtained from Melphalan treated MM cells divided by μg/10^6 cells of untreated MM cells. The mean of ten (for SKO-007(J3) exosomes) or five (for ARK exosomes) independent experiments +/- SEM is shown. Statistical analysis was performed with paired t-test,* $p \leq 0.05$ and *** $p \leq 0.001$.

Morphological analysis of the MM-purified exosomes is shown in Figure 8A, revealing that these vesicles were couple-shaped with a size comprised between 50-80 nm. In addition, immunogold labeling showed the expression of exosome markers like CD81 and Tsg101 on the outer layer of MM vesicles (Figure 8B). Western blot analysis of MM isolated exosomes confirmed the presence of other "accepted" exosome markers including Tsg101, CD63, MHC I and Hsp70. Importantly, calreticulin, which is exclusively associated to endoplasmic reticulum (RE) was not found in exosome preparations (Figure 8C). A similar pattern of expression was found in both untreated and drug-treated MM cells.

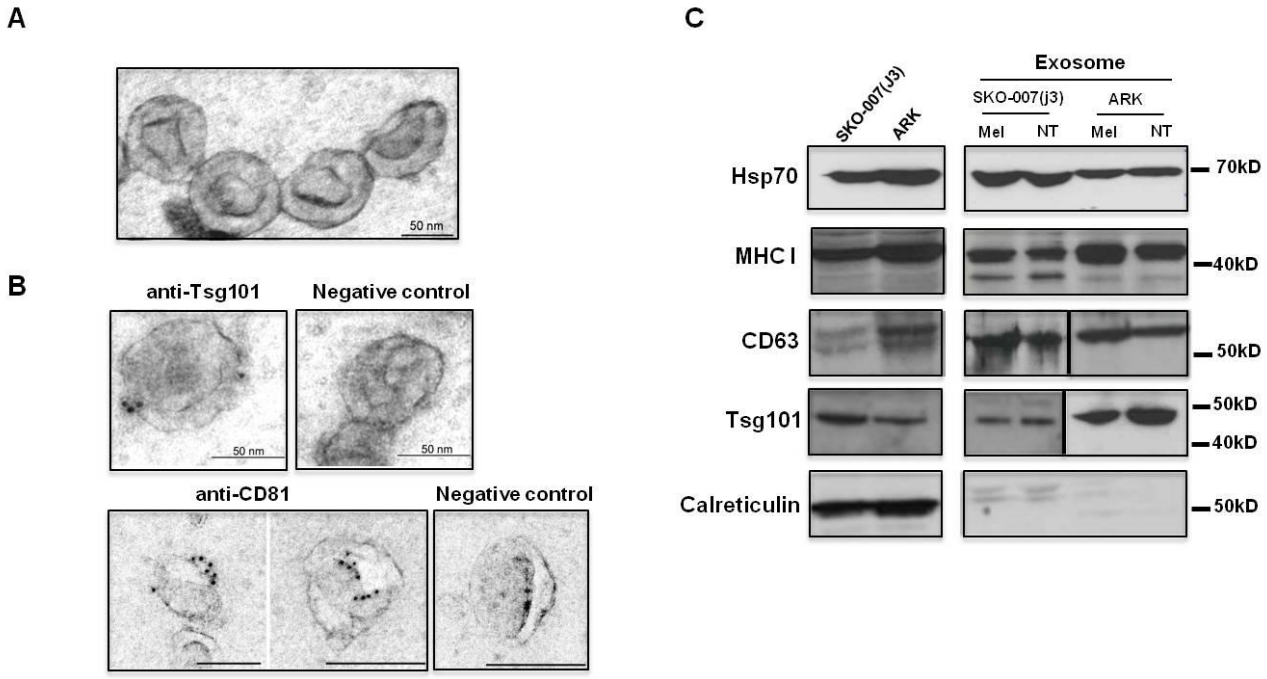

Figure 8. Characterization of MM cell-derived exosomes. (A) Electron microscope analysis of exosome morphology and size. A representative picture of SKO-007(J3)-derived exosomes is shown. **(B)** Immune-gold labeling for Tsg101 and CD81 of SKO-007(J3)-derived exosomes. **(C)** Western blot analysis was performed on lysates derived from exosome fractions or from cell pellet, using anti-Hsp70, anti-CD63, anti-Tsg101, anti-MHC I and anti-calreticulin antibodies.

CD63 expression on the surface of exosomes was also evaluated by immunofluorescence and FACS analysis using magnetic beads conjugated to exosomes as shown in Figure 9 A-B. All together these data show that MM cells secrete exosomes expressing the traditional exosomes markers and that drug treatment determines an increased release of exosomes.

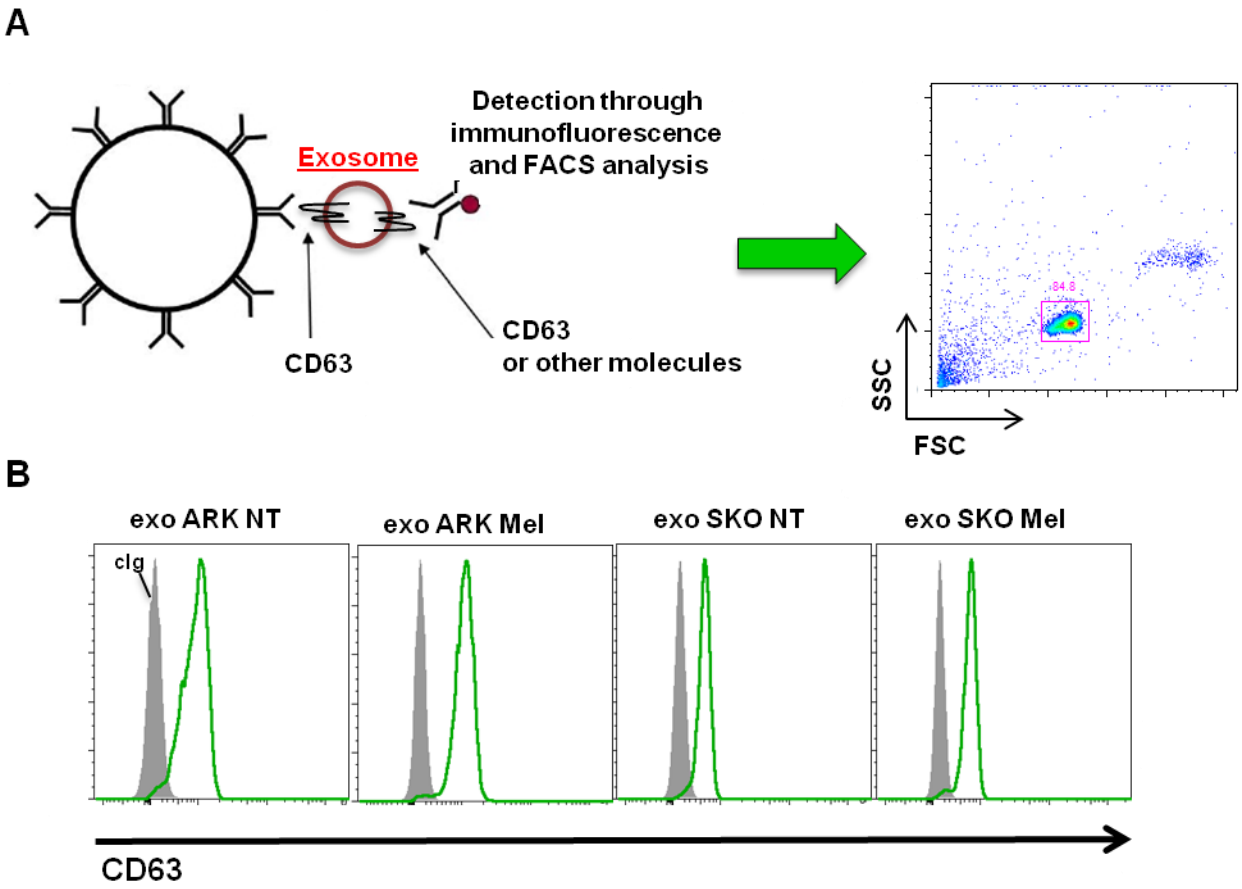

Figure 9. CD63 tetraspanin is expressed on exosome surface. (A) Schematic representation of CD63-conjugated beads binding of exosomes and their visualization through immunofluorescence and FACS analysis. **(B)** CD63 expression on the surface of exosomes was assessed by immunofluorescence and FACS analysis of CD63 conjugated beads coated with exosomes. After overnight incubation of exosomes with beads, exosomes were stained with anti-CD63 mAb (thin histograms) or control isotypic Ig (filled histograms).

MicroRNA profiling of MM cell-derived exosomes

We next investigate the exosome microRNA profiling. To this aim, the expression of 754 miRNAs and 4 controls was evaluated either on SKO-007(J3) cells and on SKO-007(J3)-derived exosomes. As shown in the Venn Diagram, we found that a portion of these miRNAs was exclusively present in the cell, whereas other miRNAs were expressed only in exosomal cargo, while the great amount of these was in common between SKO-007(J3) cells and SKO-007(J3)-derived exosomes (Figure 10). Moreover, we checked for the presence of some microRNAs that were described to have immunomodulatory properties on NK cell mediated functions, for example through their binding of TLR1 on NK cells, leading to enhancing of CD69 surface expression and IFN-γ production (He et al., 2013) and also acting as TLR8 ligands (Fabbri et al., 2012). Considering this evidence, we analyzed, through a Real-Time qPCR, the expression of: miR-15b, miR-29a, miR122 and miR-155, normalized with U6snRNA, observing an enrichment of these specific miRNAs in the exosomal cargo compared to cellular ones (data not shown).

These data demonstrate a difference between cellular and exosomal miRNA cargo, supporting the presence of a specific mechanism for the sorting of miRNAs in the exosomes, as described in 2013 by Sanchez-Madrid and colleagues (Villarroya-Beltri et al., 2013).

Cells		Common					Exosomes
hsa-let-7c	hsa-miR-652	hsa-let-7e	hsa-miR-138	hsa-miR-331	hsa-miR-708	hsa-miR-340#	hsa-miR-126
hsa-let-7f	hsa-miR-744	hsa-let-7g	hsa-miR-139-5p	hsa-miR-340	hsa-miR-885-5p-	hsa-miR-190b	hsa-miR-127
hsa-miR-23a	hsa-miR-384	hsa-miR-9	hsa-miR-140-3p	hsa-miR-155	hsa-miR-886-3p	hsa-miR-425#	hsa-miR-145
hsa-miR-32	hsa-miR-520e	hsa-miR-15b	mmu-miR-140	hsa-miR-342	hsa-miR-886-5p	hsa-miR-34a#	mmu-miR-379
hsa-miR-34a	hsa-miR-520f	hsa-miR-16	hsa-miR-142-3p	hsa-miR-362	hsa-miR-376c	hsa-miR-106b#	hsa-miR-383
hsa-miR-95	dme-miR-7	hsa-miR-17	hsa-miR-142-5p	hsa-miR-365	hsa-miR-30a-3p	hsa-miR-550	hsa-miR-410
mmu-miR-96	hsa-miR-206	hsa-miR-19a	hsa-miR-146a	hsa-miR-374	hsa-miR-30a-5p	hsa-miR-18a#	hsa-miR-433
hsa-miR-128a	hsa-miR-567	hsa-miR-19b	hsa-miR-146b	mmu-miR-374-5p	hsa-miR-30d	hsa-miR-19b-1#	mmu-miR-451
hsa-miR-133b	hsa-miR-616	hsa-miR-20a	hsa-miR-148a	hsa-miR-376a	hsa-miR-30e-3p	hsa-miR-625#	hsa-miR-487b
hsa-miR-148b	hsa-miR-766	hsa-miR-21	hsa-miR-150	hsa-miR-422a	hsa-miR-378	hsa-miR-338-5P	hsa-miR-539
hsa-miR-184	hsa-miR-505#	hsa-miR-24	hsa-miR-181a	hsa-miR-425-5p	hsa-miR-432#	hsa-miR-590-3P	hsa-miR-618
hsa-miR-194	hsa-miR-130b#	hsa-miR-25	hsa-miR-186	hsa-miR-454	rno-miR-7#	hsa-miR-1225-3P	hsa-miR-220
hsa-miR-200a	hsa-miR-148a#	hsa-miR-26a	hsa-miR-191	hsa-miR-484	hsa-miR-550	hsa-miR-1233	hsa-miR-346
hsa-miR-203	hsa-miR-92a-1#	hsa-miR-26b	hsa-miR-192	hsa-miR-489	hsa-miR-629	hsa-miR-1227	hsa-miR-584
hsa-miR-216a	hsa-miR-148b#	hsa-miR-27a	hsa-miR-193b	mmu-miR-491	hsa-miR-638	hsa-miR-1290	hsa-miR-770-5p
hsa-miR-324-3p	hsa-miR-942	hsa-miR-28	hsa-miR-195	hsa-miR-494	hsa-miR-643	hsa-miR-1208	hsa-miR-135b#
hsa-miR-324-5p	hsa-miR-183#	hsa-miR-29a	hsa-miR-197	hsa-miR-518f	hsa-miR-650	hsa-miR-1274A	hsa-miR-541#
hsa-miR-330	hsa-miR-30d#	hsa-miR-29c	hsa-miR-199a-3p	hsa-miR-532-3p	hsa-miR-571	hsa-miR-1274B	hsa-miR-7-2#
hsa-miR-337-5p	hsa-miR-193b#	hsa-miR-30b	hsa-miR-210	hsa-miR-532	hsa-miR-572	hsa-miR-720	hsa-miR-409-3p
hsa-miR-339-5p	hsa-miR-15a#	hsa-miR-30c	hsa-miR-218	hsa-miR-545	hsa-miR-573	hsa-miR-1260	hsa-miR-144
hsa-miR-342-5p	hsa-miR-628-3p	hsa-miR-92a	hsa-miR-222	hsa-miR-574-3p	hsa-miR-34b	hsa-miR-664	hsa-miR-1303
hsa-miR-345	hsa-miR-20a#	mmu-miR-93	hsa-miR-223	hsa-miR-590-5p	hsa-miR-93#		hsa-miR-1264
hsa-miR-361	hsa-miR-21#	hsa-miR-103	hsa-miR-299-5p	hsa-miR-598	hsa-miR-15b#		hsa-miR-1184
hsa-miR-362-3p	hsa-miR-27a#	hsa-miR-106a	hsa-miR-301	hsa-miR-625	hsa-miR-939		hsa-miR-1262
hsa-miR-502-3p	hsa-miR-1254	hsa-miR-106b	hsa-miR-301b	hsa-miR-628-5p	hsa-miR-9#		hsa-miR-1289
hsa-miR-502	hsa-miR-320B	hsa-miR-132	hsa-miR-320	hsa-miR-636	hsa-miR-378		hsa-miR-1247
hsa-miR-642		hsa-miR-133a	hsa-miR-328	hsa-miR-660	hsa-miR-151-3p		

Figure 10. miRNA profiling of MM cell-derived exosomes. The total amount of RNA was isolated from both SKO-007(J3) cells and SKO-007(J3)-derived exosomes. About 600 ng of total RNA was used to perform microRNA expression profiling through the use of Megaplex Pool cards A and B, as described in Materials and Methods. The Venn Diagram was performed using an online program (http://www.bioinformatics.lu/venn.php) with the data reported in the lower table. In the left column of the table are represented miRNAs expressed only in SKO-007(J3) cells, in the central column miRNAs present both in SKO-007(J3) cells and SKO-007(J3)-derived exosomes and in the right column those expressed only in SKO-007(J3)-derived exosomes. A representative experiment is shown.

Exosomes can be taken up by NK cells and induce CD69 expression

To investigate the effects of exosomes on NK cell-mediated functions, we first explore whether these nanovesicles could be taken up by NK cells. To this aim, highly purified primary NK cells were obtained from PBMCs by negative immunomagnetic selection and NK cell purity was more than 95% $CD3^{-}CD56^{+}$ as assessed by immunofluorescence and flow cytometry analysis (Figure 6). Exosomes were labelled with the red fluorescent dye PKH26 and then incubated for different times with purified NK cells in the presence or not of IL-15. As shown in Figure 11A, exosomes can be internalized by both resting and IL-15 activated NK cells and a peak after 3 hours of incubation was observed. To discern whether exosomes were internalized or just bounded to the cell surface, exosome:cell conjugates were treated with trypsin before FACS analysis and we found that trypsin just marginally reduced the intensity of fluorescence showing that the majority of exosomes were inside the cells (Figure 11B). We also incubated NK cells with an excess of unlabelled exosomes and we obtained a complete abrogation of $PKH26^{+}$ cells thus proving that exosome uptake was a specific process (Figure 11B). Fluorescence microscopy analysis on NK cells incubated 3 hours with PKH26-labelled exosomes further confirmed exosome internalization (Figure 11C).

Different doses of exosomes were incubated with primary NK cells for 48 hours and than the expression of the activation marker CD69 was evaluated by FACS analysis. Interestingly, CD69 was induced on exosome-treated NK cells in a dose dependent manner (Figure 11D). Similar effects were observed using exosomes from ARK or SKO-007(J3) cells and no significant differences were noted between exosomes derived from untreated or Mel-treated MM cells (Figure 11E).

Collectively, our results show that MM cells derived-exosomes are internalized by both resting and IL-15 activated NK cells and stimulate the induction of the activation marker CD69.

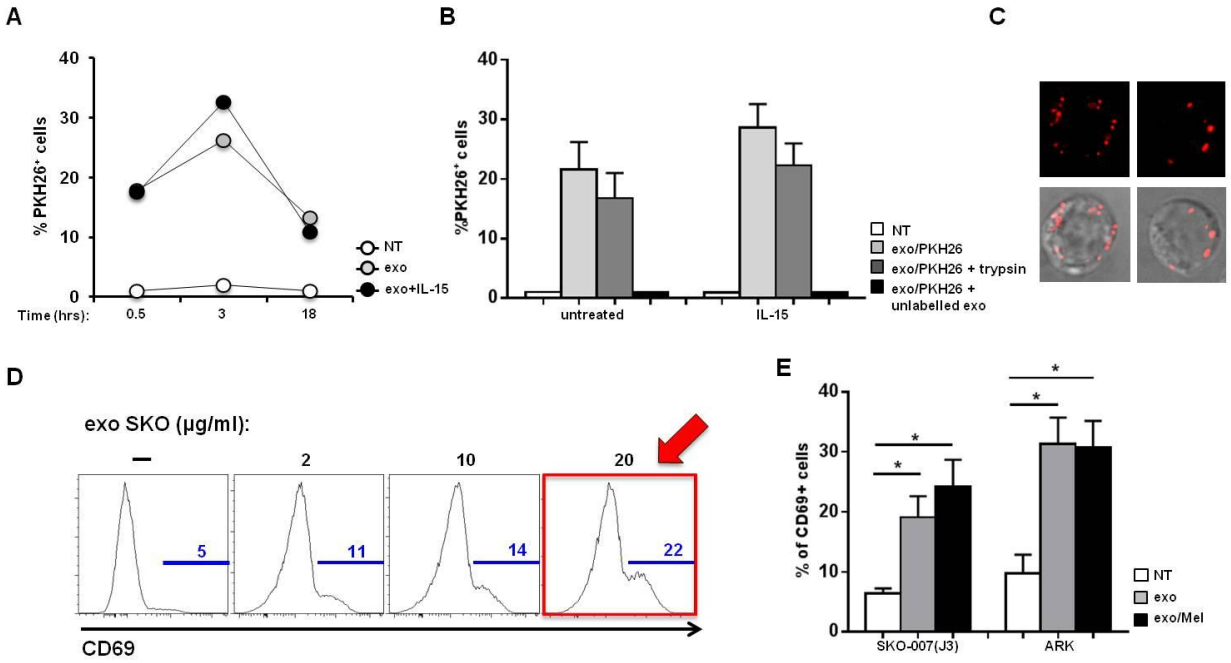

Figure 11. MM cell-derived exosomes are taken up by primary NK cells and induce CD69 expression. (A) MM cell-derived exosomes were labeled with the red fluorescent dye PKH26. Primary human NK cells were incubated for different times with 20 µg/ml of PKH26 labeled exosomes with or without IL-15 (50 ng/ml). The fluorescence of internalized exosomes was evaluated by immunofluorescence and FACS analysis and measured as the percentage of $PKH26^+$ cells. One representative experiment is shown. **(B)** NK cells were cultured for 3 hours in presence of PKH26-labeled exosomes and IL-15 as described in panel A, or with a combination of PKH26-labeled exosomes and trypsin, or PKH26-labeled and unlabeled exosomes at 1:3 ratio. The mean of two indipendent experiments +/- SEM is shown. **(C)** NK cells were incubated for 3 hours with PKH26-labelled exosomes (20 µg/ml), washed and plated on poly-L-lysine-coated multichamber glass plates and fixed. Images were acquired using an ApoTome Observer Z.1 microscope with a 60x/1.4 NA Plan-Neofluar objective. Upper panels: Representative images of single cells are shown as maximum intensity projection (3 Z sections with 0.2 µm spacing). Lower panels: differential interference cotrast overlay the fluorescence images. **(D)** NK cells were incubated for 48 hours with different amounts of SKO-007(J3)-derived exosomes. CD69 expression was evaluated by immunofluorescence and FACS analysis. A representative experiment is shown. **(E)** NK cells were incubated with 20 µg/ml of SKO-007(J3) and ARK–derived exosomes as described in panel D. Where indicated exosomes were prepared from Melphalan-treated cells (exo Mel). Data were represented as mean values of the percentage of $CD69^+$ NK cells of four (for SKO-007(J3)-derived exosomes) or five (for ARK-derived exosomes) independent experiments +/- SEM. Statistical analysis was performed with the paired Student test, *$p \leq 0.05$.

Exosomes express IL-15Rα and increase IL-15 induced NK cell proliferation

We next extended the analysis of the effects of exosomes on NK cell proliferation. To this aim, NK cells were labelled with CFSE and cultured with exosomes in the presence or not of IL-15. Our data show that exosomes alone are not able to stimulate NK cell proliferation but they can significantly increase IL-15 induced NK cell proliferation. Similarly to the data obtained with the CD69 marker, we didn't observe any significant difference between exosomes derived from untreated of Mel-treated MM cells (Figure 12A).

Since IL-2 and trans-presentation of IL-15 by IL-15Rα are required for NK cell proliferation (Koka et al., 2003), we asked whether exosome-mediated NK cell proliferation was dependent only from the presence of exogenous IL-15 or could be mediated also by IL-2. Interestingly, the augmentation of NK cell proliferation, measured with the Ki67 marker, was observed only with IL-15 but not with IL-2 strongly suggesting that this effect could be mediated by IL-15 *trans*-presentation (Figure 12B). Indeed, it has been shown that IL-15Rα is constitutively expressed on several MM cell lines (Tinhofer et al., 2000) but it is not known whether this molecule could be associated to MM cell-derived exosomes although a prior study had reported the presence of IL-15Rα on exosomes produced by DCs (Viaud et al., 2009). Thus, we investigated whether MM cell-derived exosomes expressed IL-15Rα molecules. As shown in Figure 12C, IL-15Rα was detected in exosome preparations derived from both ARK and SKO-007(J3) cells.

These data highlight that IL-15Rα harbored by MM-cell derived exosomes is functional, leading to NK cell proliferation when associated to exogenous IL-15.

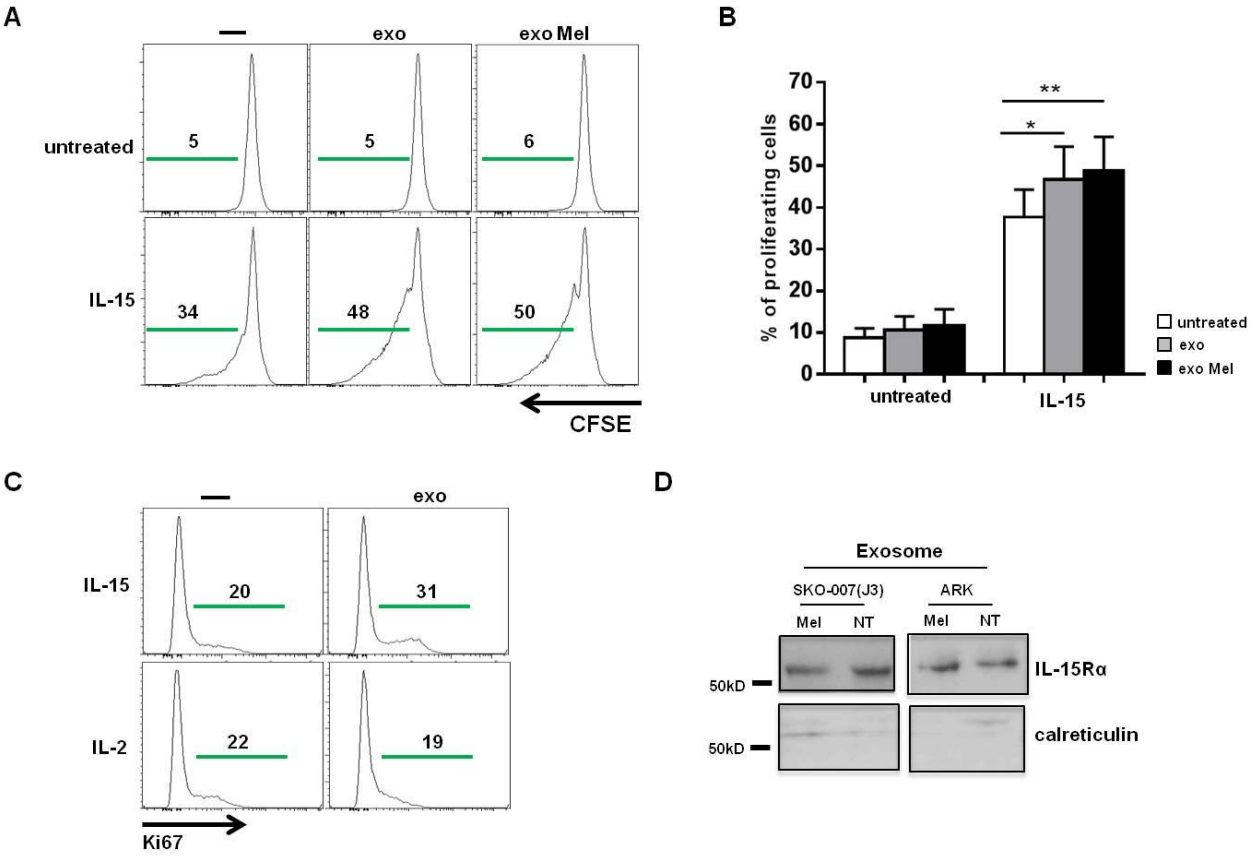

Figure 12. Exosomes express IL-15Rα and increase IL-15 induced NK cell proliferation. (A) CFSE-labeled NK cells were incubated with 20 μg/ml of SKO-007(J3) cell-derived exosomes in the presence of IL-15 (50 ng/ml). After five days cell divisions were evaluated by immunofluorescence and FACS analysis by measuring CFSE reduction and expressed as percentage. One representative experiment is shown. **(B)** CFSE-labeled NK cells were incubated with 20 μg/ml of MM cell-derived exosomes in the presence of IL-15 as described in panel A. The mean values of six independent experiments is shown. Statistical analysis was performed with the paired t-test, **p < 0.01 and *p < 0.05. **(C)** NK cells were incubated for five days with 20 μg/ml of SKO-007(J3) cell-derived exosomes in the presence of IL-15 (50 ng/ml) or IL-2 (500 U/ml). The percentage of $Ki67^+$ cells was evaluated through immunofluorescence and FACS analysis. A representative experiment is shown. **(D)** Western blot analysis was perfomed on lysates derived from exosome fractions derived from both SKO-007(J3) and ARK cells using anti-IL-15Rα and anti-calreticulin antibodies.

Exosomes stimulate IFN-γ production through a mechanism mediated by Nuclear factor (NF)-kB signaling pathaway

High expression of the activation marker CD69 on NK cells is usually coupled with functional activation (Borrego et al., 1993)**.** We therefore investigated the effect of MM cell-derived exosomes on NK cell-mediated IFN-γ production. NK cells were incubated with exosomes for 48 hours and IFN-γ production was evaluated both at mRNA and protein level. As shown in Figure 13A-B, exosomes alone significantly stimulated IFN-γ production in resting NK cells. Cytokine-induced IFN-γ production occurs mainly through the JAK-STATs, T-BET, MAPK, or NF-kB signaling pathways (Schoenborn and Wilson, 2007). Transcription factors in these signaling pathways associate with corresponding binding sites in the regulatory elements of the IFNG gene, subsequently enhancing IFNG mRNA synthesis. Thus, we explored whether NF-kB signaling pathway was activated in NK cells after exosome treatment. Our results showed that althought the total level of p65 protein, a transactivation component of NF-kB signaling, was unchanged, the treatment with exosomes induced an increase in the phosphorylation of p65 in primary NK cells (Figure 13C). To further confirm the involvement of NF-kB in the exosome-induced IFN-γ production, NK cells were pre-treated with SN50, a cell permeable peptide which inhibits translocation of the NF-kB active complex into the nucleus, and then incubated with exosomes. As shown in Figure 13D, SN50 treatment blocks exosome-induced IFN-γ production. In addition, we found that the combined stimulation of NK cells with IL-15 and exosomes further increase IL-15 induced IFN-γ production with no differences between exosomes derived from untreated or Mel-treated MM cells (Figure 13D). Remarkably, exosomes didn't affect NK cell degranulation against the K562 cell line (data not shown).

These data demonstrate that exosomes stimulate NK cell IFN-γ production with a mechanism dependent on NF-kB signaling pathway without affecting NK cell degranulation.

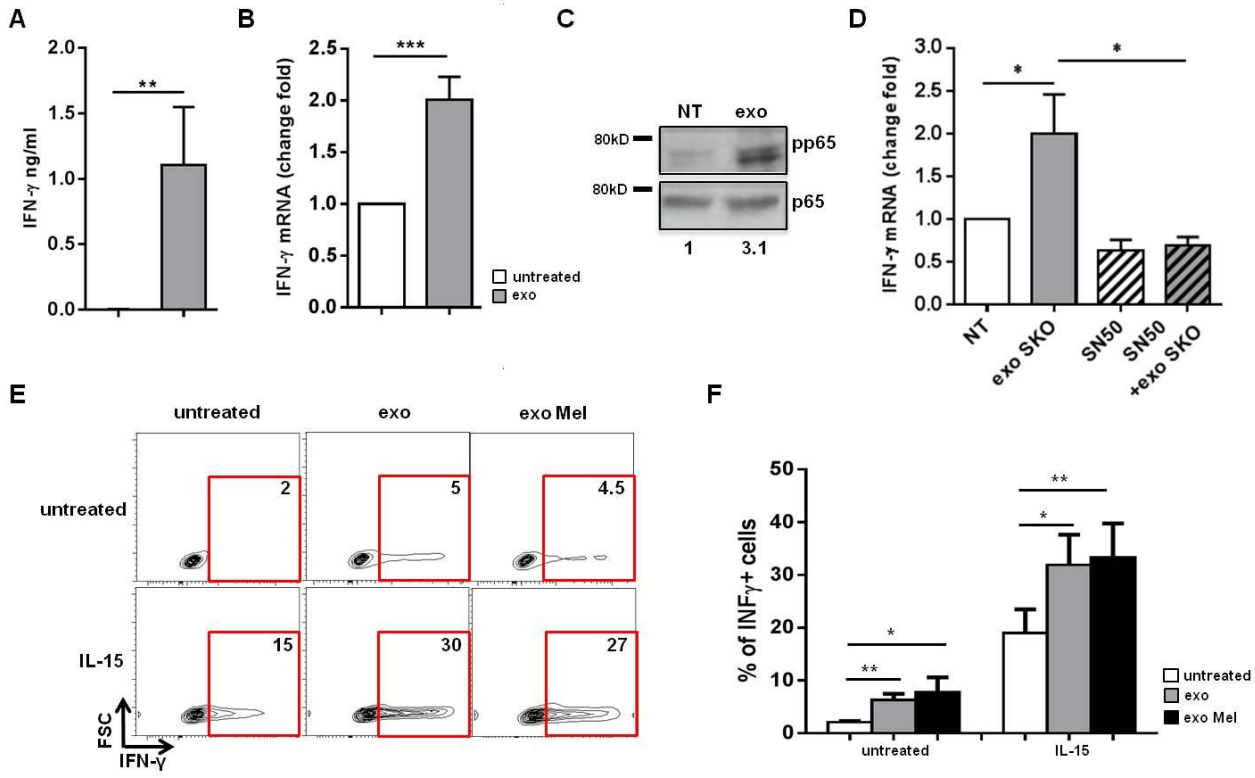

Figure 13. Exosomes induce IFN-γ production through a mechanism mediated by NF-kB. **(A-B)** NK cells were incubated with 20 μg/ml of SKO-007(J3)-derived exosomes for 48 hours. **(A)** The levels of soluble IFN-γ were determined in the supernatants by a specific sandwich ELISA. The mean of four experiments is shown. **(B)** Real-time PCR analysis of IFN-γ mRNA. Data, expressed as fold change units, were normalized with β-actin and referred to the untreated cells considered as calibrator. Values reported represent the mean of six independent experiments +/- SEM. Statistical analysis was performed with the paired Student test, **$p \leq 0.01$ and ***$p \leq 0.001$. **(C)** NK cells were incubated with 20 μg/ml of SKO-007(J3)-derived exosomes in the presence of a suboptimal concentration of FCS (2%) for 48 hours. Western blot analysis was performed on total cell lysates using p65 and phospho-p65 (p-p65) Abs. Numbers beneath each line represent quantification of p-p65 by densitometry normalized with p65. **(D)** NK cells were pre-treated for 1 hour with the NF-kB inhibitor, SN50 (10μM), and then incubated with 20 μg/ml of SKO-007(J3)-derived exosomes for 48 hrs. Real-time PCR analysis of IFN-γ mRNA was performed as described in panel B. The mean of three independent experiments is shown. **(E)** NK cells were cultured with 20μg/ml of SKO-007(J3) cell-derived exosomes in the presence of IL-15 (50 ng/ml). After 24 hours, Brefeldin A (5 μg/ml) was added and left for additional 24 hours. Intracellular IFN-γ expression was evaluated by immunofluorescence and FACS analysis. One representative experiment is shown. **(F)** Data were represented as mean values of the percentage of IFN-γ^+ cells of seven independent experiments (using both SKO-007(J3)- and ARK-derived exosomes) +/- SEM. Statistical analysis was performed with the paired t-test, * $p \leq 0.05$ and ** $p \leq 0.01$.

Exosomes stimulate IFN-γ production with a mechanism dependent on Toll-like receptor 2 (TLR2)

Some studies show that different type of exosomes have the capability to trigger immune cell functions through a mechanism requiring receptors belonging to toll like receptor family including TLR 7, 8, 1 and 2 (Chow et al., 2014a; Chalmin et al., 2010; He et al., 2013; Fabbri et al., 2012). Since downstream TLR pathway involves NF-kB activation, we asked whether the exosome-induced NK cell activation was mediated by one or more TLR expressed on NK cells. With this rationale, cells stably co-expressing a TLR gene and an NF-κB-inducible luciferase reporter gene were used to test the capability of MM cell-derived exosomes to engage a specific TLR. Interestingly, exosome treatment of reporter cells was found to induce luciferase activity only in those cells expressing TLR2 whereas no effect on luciferase reporter activity was observed in cells expressing TLR3, TLR4, TLR7, TLR8 and TLR9 or cells transfected with the NF-κB-inducible luciferase reporter gene alone (Figure 14A-C).

These data suggest that MM cell-derived exosomes can selectively engage TLR2 receptor.

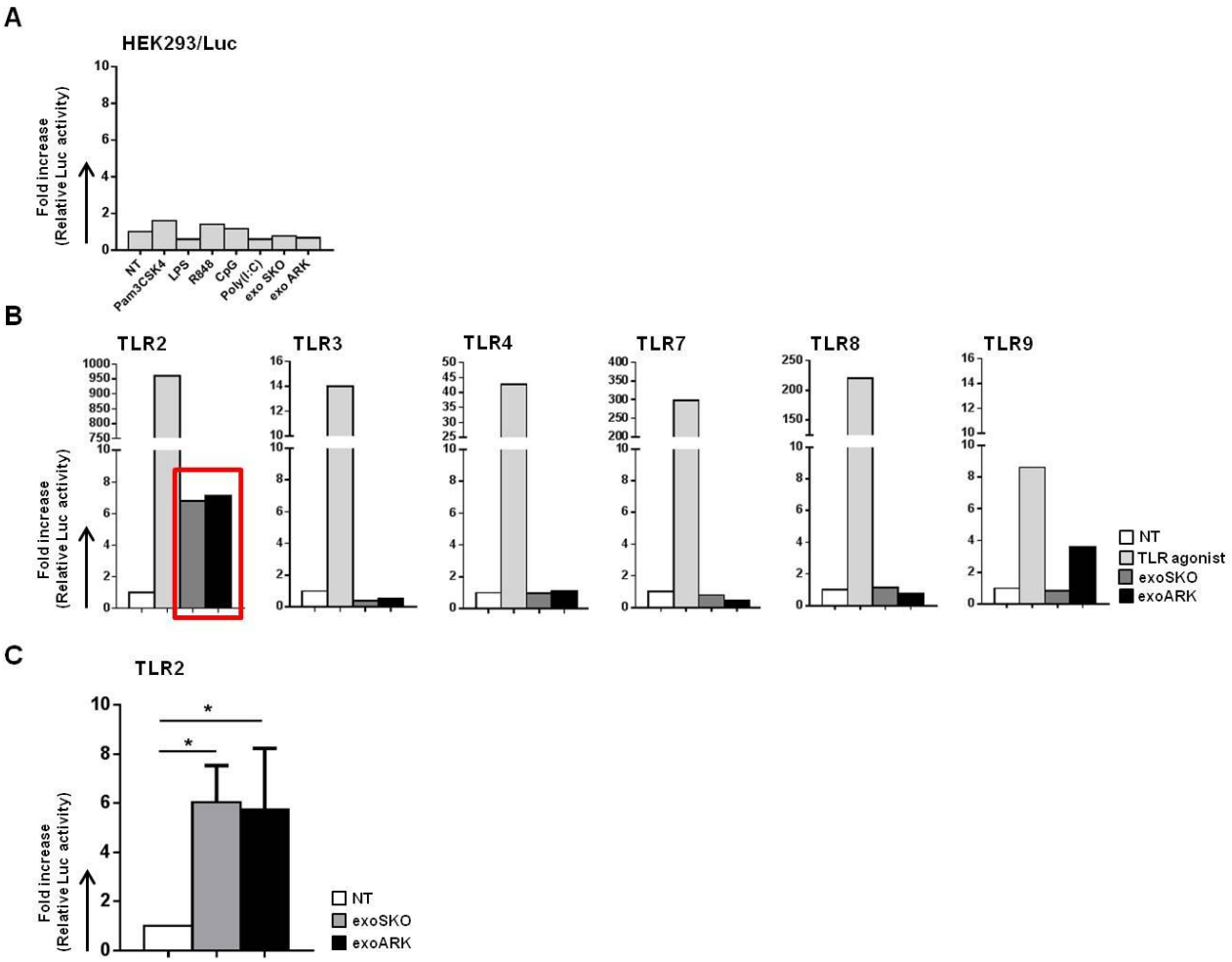

Figure 14. Exosomes derived from SKO-007(J3) and ARK cell lines stimulate NF-kB activity through TLR2 in 293/Luc reporter cells. (A) HEK293/Luc reporter cells transfected with a vector containing NF-kB/Luc alone were incubated with with 20 µg/ml of SKO-007(J3) cell-derived exosomes, ARK cell-derived exosomes and different TLR agonists, as indicated, for 24 hours. Data are expressed as fold increase of the Relative Luc activity of cells treated with TLRs agonists, compared to untreated cells. A representative experiment is shown. **(B)** HEK293/Luc reporter cells transfected with a single TLR were incubated with exosomes, as described in panel A, and with the specific TLR agonists, as described in Materials and Methods. Data are expressed as fold increase of the Relative Luc activity, as in panel A. **(C)** TLR2 transfected cells were incubated with 20 µg/ml of SKO-007(J3) cell-derived exosomes, ARK cell-derived exosomes and 1µM of Pam3CSK4 for 18 hours. The mean values of five independent experiments +/- SEM is shown. Statistical analysis was performed with the paired t-test, $*p \leq 0.05$.

Since we define an important role for TLR2, the effect of its agonist on NK cells was promptly examined. A previous work has been shown that IFN-γ production in response to PSK2, one of the known TLR2 agonists, was mainly associated to the NK $CD56^{bright}$ subset (Lu et al., 2011)**.** Thus, highly purified NK cells were treated with different doses of TLR2 agonist, Pam3CSK4, or exosomes and IFN-γ production was evaluated by immunofluorescence and FACS analysis on both $CD56^{bright}$ and $CD56^{dim}$ cells. Interestingly, Pam3CSK4 treatment determines a strong increase of IFN-γ production in the majority of $CD56^{bright}$ cells whereas just a small percentage of $CD56^{dim}$ cells were able to respond to TLR2 agonist. Remarkably, exosome-induced IFN-γ production was prevalent in $CD56^{bright}$ cells (Figure 15A). To further confirm that $CD56^{bright}$ NK cell subset was more responsive to both exosomes and Pam3CSK4, $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets were sorted, and IFN-γ mRNA was measured upon exosome or Pam3CSK4 treatment. As shown in Figure 15B, an increase of IFN-γ mRNA in response to exosome or TLR2 agonist was observed only in the $CD56^{bright}$ NK cell subset. The different response to TLR2 agonist was not attributable to different levels of cell surface TLR2 on NK cell subsets as shown in Figure 15C, in which TLR2 was measured on NK cells derived from 10 different healthy donors.

Finally, to investigate the contribute of TLR2 in the exosome-mediated IFN-γ production by NK cells, a neutralizing antibody against TLR2 was used to pre-treat NK cells before exosome stimulation. As shown in Figure 16, anti-TLR2 treatment blocks either Pam3CSK4 and exosome-mediated IFN-γ induction in NK cells.

Collectively, our results indicate that MM-cell derived exosomes stimulate IFN-γ production by human primary NK cells through the engagement of TLR2 and that $CD56^{bright}$ NK cell subset is more responsive to TLR2 stimulation.

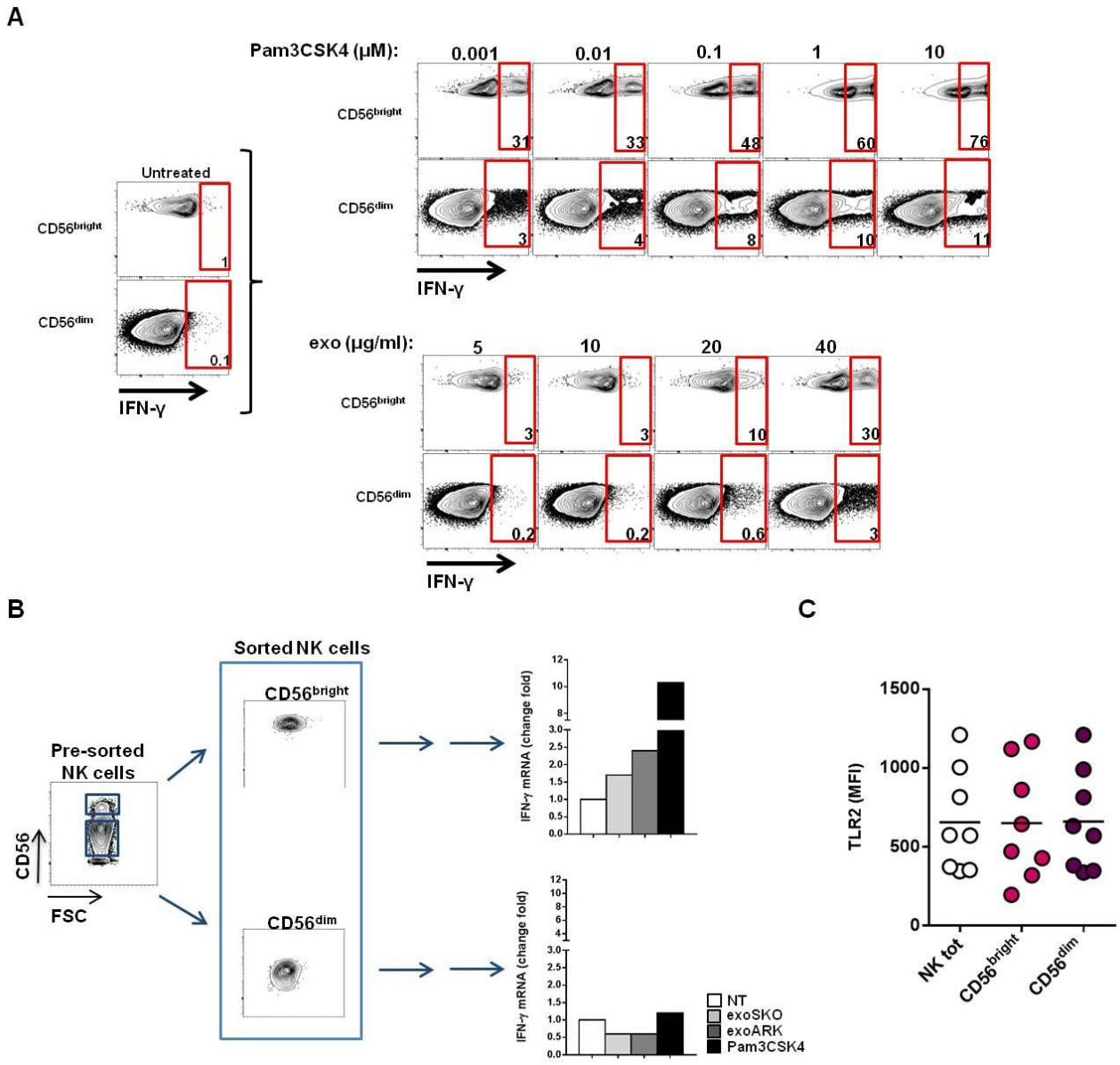

Figure 15. TLR2 agonist and MM cell-derived exosomes stimulate IFN-γ production mainly in the CD56^{bright} NK cell subset. (A) NK cells were incubated with increasing doses of Pam3CSK4 or ARK cell-derived exosomes, as indicated. After 24 hours, Brefeldin A (5 μg/ml) was added and left for additional 24 hours. Intracellular IFN-γ expression was evaluated by immunofluorescence and FACS analysis. The gating strategy used consists in separating CD56^{bright} cells from CD56^{dim} NK cells. A representative experiment is shown. **(B)** NK cells were purified by immunomagnetic negative selection (pre-sorted), and then CD56^{bright} and CD56^{dim} cells were sorted by FACSARIA (BD) and incubated with 20 μg/ml of SKO-007(J3) cell-derived exosomes, ARK cell-derived exosomes and 1μM of Pam3CSK4 for 48 hours. Real-time PCR analysis of IFN-γ mRNA was performed and the data, expressed as fold change units, were normalized with β-actin and referred to the untreated cells considered as calibrator. One representative experiment is shown. **(C)** Cell surface expression of TLR2 was evaluated on CD56^{+}CD3^{-} total NK cells and on CD56^{bright} and CD56^{dim} NK cell subsets of total PBMCs derived from ten different healthy donors. Values represent the mean fluorescence intensity (MFI) of TLR2 subtracted from the MFI value of the isotype control Ig.

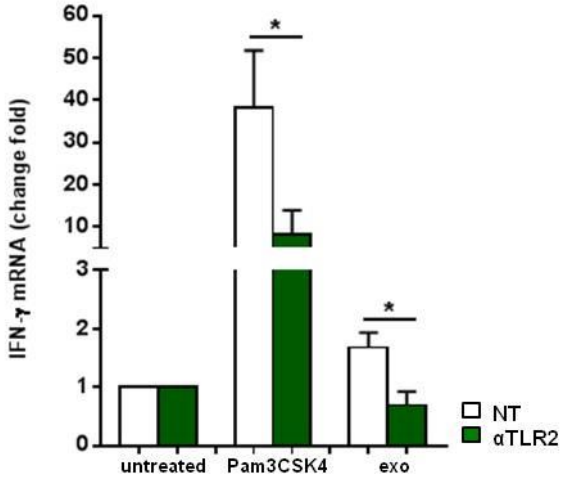

Figure 16. Exosome-induced IFN-γ production is dependent on TLR2. Primary NK cells were pre-treated with 2 µg/10^6cells of α-TLR2 for 20 minutes, washed and then incubated with 20 µg/ml of ARK cell-derived exosomes and 1µM of Pam3CSK4 for 48 hours. Real-time PCR analysis of IFN-γ mRNA was performed and the data, expressed as fold change units, were normalized with β-actin and referred to the untreated cells considered as calibrator. The mean values of three independent experiments +/- SEM is shown. Statistical analysis was performed with the paired t-test, *$p \leq 0.05$.

DISCUSSION

Exosomes are not still considered as “garbage bags” but currently are thought to be an important mechanism for intercellular communication, thanks to their ability to convey many proteins, lipids, miRNA and mRNA (Valadi et al., 2007; Tran et al., 2015). Several studies describe the involvement of exosomes in the modulation of both innate and adaptive immune response through different mechanisms (Thery et al., 2009).

Our results show that MM cells produce exosomes with a immunostimulatory role on NK cells functions. We have used two different MM cell lines as a model for analyzing exosomes molecular features and their immunomodulatory properties. Previously, it has been described the ability of MM cells to secrete these nanovesicles (Umezu et al., 2014). In our study, we observed that MM cells release nanovesiscles that we further characterized as exosomes through the evaluation of the typical size and morphology by electron microscopy and the expression analysis of some characteristic exosomal markers such as CD63, CD81 and Tsg101. We also performed a miRNA expression profiling by analyzing more than 700 miRNA and we found that some miRNAs were exclusively present into exosomes, thus indicating a specific mechanism of miRNA sorting into these vesicles as previously shown (Villarroya-Beltri et al., 2013).

Exosomes could interact with and taken up by target cells through different mechanisms. May be an initial ligand-receptor binding can occur at the beginning of cell-exosome interaction (Nolte-'t Hoen et al., 2009; Hwang et al., 2003) and then comes the internalization through many possible mechanisms including endocytosis (Mulcahy et al., 2014). Interestingly, a role for adhesion molecules and tetraspanins has been reported to mediate exosome internalization either in T cells and DC (Mulcahy et al., 2014). In this study we observed that human purified NK cells internalize, and not only bind, MM cell-derived exosomes with a rapid kinetics and a specific process. Further experiments are necessary to define the mechanism underlying the NK cell exosome uptake.

Our data show that MM cell-derived exosomes increased NK cell proliferation only in combination with IL-15 but not with IL-2. It is possible that IL-15Rα, highly expressed on MM cell derived exosomes could mediate IL-15 *trans*-presentation to NK cells. In line with these results, Viaud and colleagues had previously showed that IL-15Rα expressed on exosomes derived from DC could t*rans*-presents IL-15 to NK cells thus stimulating cell proliferation (Viaud et al., 2009). It shoud be also considered that MM cells express a

functional IL-15R (Tinhofer et al., 2000; Soriani et al., unpublished observations), thus indicating that these cells are potentially responsive to IL-15 *trans*-presented by exosomes. Further experiments are needed to better elucidate the role exosome-mediated IL-15 *trans*-presentation to MM cells.

We found that exosome treatment of human primary NK cells strongly induced the expression of CD69 activation marker on the cell surface and stimulated the production of IFN-γ with a mechanism dependent on the NF-kB pathway. Indeed, exosomes induced an increase in the phosphorylation of p65, a transactivation component of NF-kB signaling pathway. In addition, NK cells pre-treated with SN50, a cell permeable peptide which inhibits translocation of the NF-kB active complex into the nucleus, blocked exosome-induced IFN-γ production.

Currently, the immunomodulatory role of exosomes on NK cell-mediated functions is a controversial question and seems to be strictly dependent on exosomal molecular cargo and cell source.

It is widely described that the NF-kB pathway could be activated by the engagement of different Toll-like receptors by their specific agonists (Kawai and Akira, 2008; Deng et al., 2014). Increasing evidence suggest that, in addition to PAMPs, TLRs could also be triggered by DAMPs derived from stressed, damaged, apoptotic and tumor cells (Kawai and Akira, 2010; Harris and Raucci, 2006). Interestingly, some studies have been shown that PAMPs can be also transported in association with extracellular vesicles (Yi et al., 2012; Liu et al., 2006). Our results strongly indicate that the exosome-induced IFN-γ production on NK cells was mediated by TLR2 engagement. Through the usage of reporter cells either expressing different TLRs in combination with NF-kB inducible luciferase reporter gene, we found that MM-cell derived exosomes were able to selectively activate the luciferase activity only in cells expressing TLR2. Consistent with these results, a neutralizing antibody against TLR2 blocked the exosome-induced IFN-γ production on primary NK cells.

Human NK cells can be divided into two main subsets, $CD56^{dim}$ and $CD56^{bright}$, on the basis of their cell surface density of CD56 (Cooper et al., 2001; De et al., 2011). The $CD56^{dim}$ NK cell subset is more naturally cytotoxic whereas $CD56^{bright}$ NK cells promptly produce cytokines in response to different stimuli thus suggesting that these subsets may have distinct roles in immune response. Accordingly, our findings show that TLR2 agonist as well as exosomes mainly stimulate IFN-γ production in the $CD56^{bright}$ cell subset. The different response to TLR2 agonist was not attributable to different levels of cell surface

TLR2 expression on NK cell subsets and we also exclude a possible contribution of innate cytokines derived from monocytes since NK cell subsets were highly purified following FACS sorting. In line with these results, it was previously shown that IFN-γ production in response to PSK2, one of the known TLR2 agonists, was mainly associated to the NK $CD56^{bright}$ subset (Lu et al., 2011). Because of the importance of NK cells in tumor surveillance, many efforts have been made to improve this process. In this regard, TLR2 agonist treatment was proposed as adjuvant in cancer immunotherapy (Seya et al., 2015) and it has demonstrated that TLR2 agonist treatment strongly activated human NK cells potentiating ADCC against trastuzumab-coated breast cancer cells (Lu et al., 2011).

Interestingly, a recent study has proposed a model of TLR2 mediated NF-kB activation and consequent cytokine production in human macrophages in response to breast cancer cell-derived exosomes. In particular, they have shown that palmitoylated proteins expressed on tumor exosomes but not on exosomes derived from healthy cells were able to trigger TLR2 (Chow et al., 2014a). In the present study, we observed that MM exosomes can also stimulate TNF-α production in monocytes, at both protein and mRNA level (data not shown). Of note, several studies have shown a disregulation of palmitoylation process in cancer cells (Yeste-Velasco et al., 2015). Further experiments are needed to establish the role played by palmitoylated proteins on the cell surface of MM-cell derived exosomes in the regulation of TLR2 triggering.

TLR2 can recognize different types of DAMPs including heat shock proteins (Hsps) (Jin et al., 2007; Kang et al., 2009). In general, these molecular chaperones are usually expressed intracellularly and support the folding and the transport of a great variety of proteins. In contrast, membrane-bound and extracellular located Hsps act as potent danger signals (Multhoff, 2007). Several evidences demonstrate that extracellular located HSPs can be associated to extracellular vesicles (Chalmin et al., 2010; Gastpar et al., 2005; Lv et al., 2012). In this regard, Chalmin et colleagues demonstrated that Hsp72, expressed on the cell surface of various types of tumor exosomes, was able to engage TLR2 expressed on MDSCs (Chalmin et al., 2010). In addition, Hsp70-bearing exosomes were described to stimulate different NK cell functions (Gastpar et al., 2005; Lv et al., 2012). Interestingly, our results show that exosomes derived from both ARK and SKO-007(J3) cell lines express high levels of Hsp70, suggesting a possible role of this molecule as “DAMP” through the engagement of TLR2 on NK cells.

Although we demonstrate that MM cell-derived exosomes stimulate NK cells through TLR2-mediated NF-kB activation, it should be considered that in tumor microenviroment other types of immune cells, expressing TLR2 are present including MDCSs that could impair anti-tumor immune response (Valenti et al., 2006; Clayton et al., 2007). Thus MM cell derived exosomes *in vivo* could impair or stimulate the immune system depending on the differential contribution of innate immune cells expressing TLR2 localized in the bone marrow microenvironment.

Exosome release is a process that occurs in both physiological and pathological conditions. Interestingly, several studies showed an increase of exosomes release during malignant transformation. In this regard, a recent report has shown that MM patients produce about four folds more exosomes than MGUS and healthy individuals (Di et al., 2015). It has been observed that exosome release in tumor cells was augmented in response to hipoxia (Di et al., 2015; Umezu et al., 2014), and to agents inducing DDR, including etoposide (Lv et al., 2012) and Doxorubicin (Yang et al., 2015). In this regard, Telerman and collaborators have demonstrated a correlation between tumor suppressor-activated pathway 6 (TSAP6), a direct p53 transcriptional target gene, and exosome nonclassical secretory pathway. In particular, they shed light on the involvement of TSAP6 both in the sorting of exosomal molecular cargo and in exosome secretion (Amzallag et al., 2004). They also demonstrated that DDR-induced p53-dependent exosome secretion was completely abrogated in TSAP-6 null cells (Lespagnol et al., 2008). Consistent with these observations, we found that genotoxic agents like Melphalan or Doxorubicin (data not shown) stimulate an increased release of exosomes from MM cells. In terms of modulatory effects on NK cells, no differences were observed between exosomes derived from untreated and Melphalan treated cells. Moreover, since we found a dose dependent correlation between the exosome amount and NK cell activation, chemotherapy could play a crucial role in the induction of a stronger NK cell-mediated immune response associated to the enhancement of exosome release. On the other hand, it will be crucial to investigate whether exosomes derived from drug-treated cells could affect the growth rate of MM cells through an autocrine process.

In conclusion, we have demonstrated that MM cell-derived exosomes have a stimulatory role on NK cells in terms of proliferation, CD69 induction and IFN-γ production (Figure 17). We also identified the TLR2/NF-kB axis as one of the mechanisms mediating exosome-induced IFN-γ production. Bridging our understanding of exosome molecular phenotype and

immunomodulatory properties will provide key insight into their importance in cancer therapy and their possible usage as biomarkers.

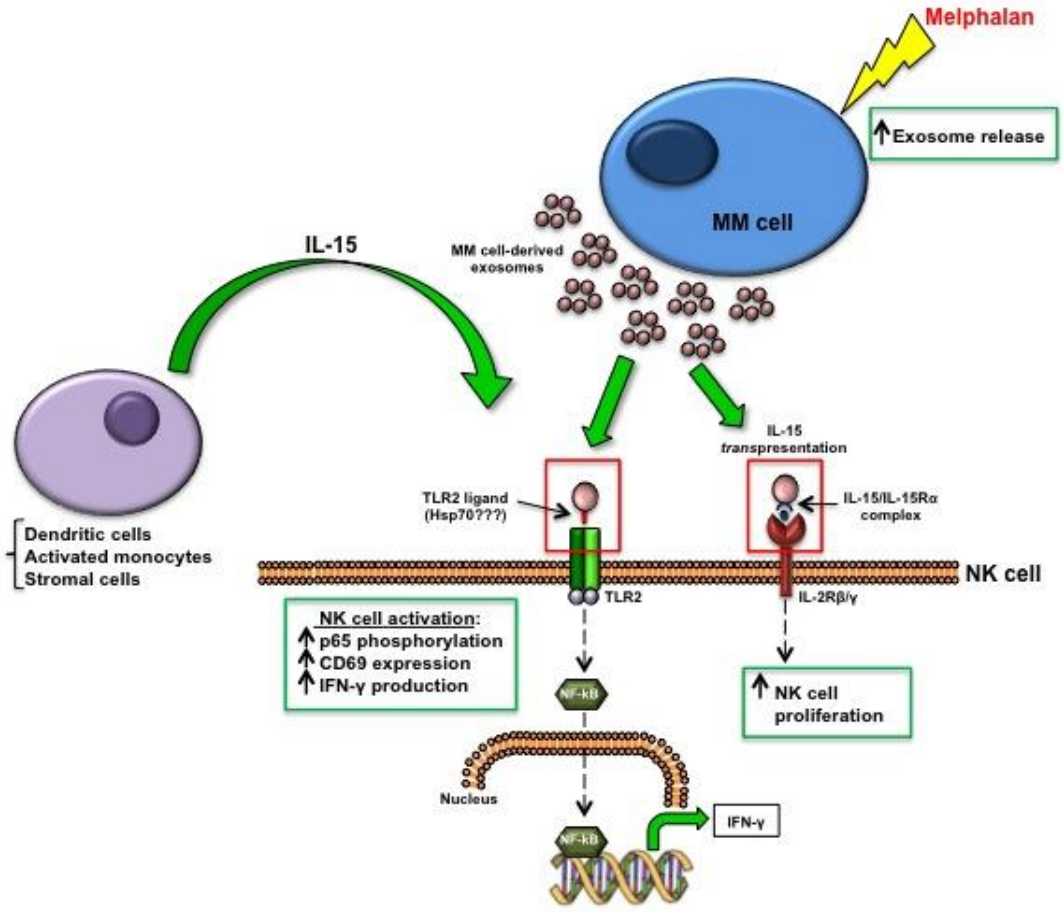

Figure 17. Model of exosome immunomodulatory role on NK cell-mediated functions. Exosome release from MM cells is enhanced after Melphalan treatment. IL-15Rα expressed on exosomes can mediate IL-15 *trans*-presentation, leading to increased NK cell proliferation. Moreover, exosome stimulate CD69 expression, p65 phoshorylation and IFN-γ production through a mechanism mediated by TLR2/NF-kB pathway. Finally, exosomes further enhance IL-15-mediated IFN-γ production.

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Abbreviations used in this thesis:

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity

AML: Acute Myeloid Leukemia

APC: Antigen presenting cell

Alix: ALG2-interacting protein X

B_2M: beta-2-microglobulin

BM: Bone Marrow

BMSC: Bone Marrow Stromal Cell

DAMP: Damage-Associated Molecular Pattern

DC: Dendritic Cell

Dex: Dendritic cell-derived exosomes

DNAM-1: DNAX Accessory Molecule 1

DDR: DNA Damage Response

ELISA: Enzyme-Linked ImmunoSorbent Assay

ESCRT: Endosomal Sorting Complex Required for Transport

FACS: Fluorescence-Activated Cell Sorter

HLA: Human Leukocyte Antigen

HMGB1: High Mobility Group Box 1

hnRNPA2B1: heterogeneous nuclear ribonucleoprotein A2B

Hsp: Heat-shock protein

HSCT: Haploidentical hematopoietic stem cell transplantation

ICAM-1: Inter Cellular Adhesion Molecule 1

ICD: Immunogenic Cell Death

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

ILVs: IntraLuminal Vesicles

IMiDs: ImmunoModulatory Drugs

ISS: International Staging System

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor Tyrosine-based Inhibitory Motif

KIR: Killer cell Immunoglobulin-like Receptors

LFA-1: Leukocyte Function-associated Antigen-1

LN: Lymph Nodes

LIR: Immunoglobulin-Like Receptors

MDSC: Myeloid-Derived Suppressor Cells

Mel: Melphalan

MGUS: Monoclonal gammopathy of undetermined significance

MHC: Major Histocompatibility Complex

MIC: MHC class I polypeptide-related Chain

MM: Multiple Myeloma

MVBs: Multi Vescicular Bodies

MVEs: Multivescicular Endosomes

NCR: Natural Cytotoxicity Receptor

NF-kB: Nuclear Factor kappa B

NK: Natural Killer

NKG2D: Natural Killer cell Group 2 D

NKG2DL: NKG2D Ligand

OCs: Osteoclasts

PBMC: Peripheral Blood Mononuclear Cell

PAMP: Pathogen-Associated Molecular Pattern

PC: Plasma Cell

PRR: Pattern-Recognition Receptors

SA: Serum Albumin

SN: Super Natant

SNAREs: Soluble N-ethylmaleimide–sensitive factor attachment proteins

TLR: Toll-like receptor

TGF-β: Transforming Growth Factor β

TGN: Trans-Golgi Network

Tex: Tumor cell-derived exosomes

TLR: Toll-like Receptor

TNF: Tumor Necrosis Factor

TRAIL: TNF-Related Apoptosis-Inducing Ligand

Treg: regulatory T cells

TSAP6: Tumor Suppressor-Activated Pathway 6

Tsg101: Tumor susceptibility gene 101 protein

ULBP: U-16 Binding Protein

VEGF: Vascular endothelial growth