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Role of redox-activated DNA damage response in the regulation of drug-induced NKG2D and DNAM-1 ligand expression on human senescent multiple myeloma cells

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1. INTRODUCTION

1.1. NK cells

1.1.1. The NK cells in the immune system

The immune response rises from the activation of two broad systems that provide innate and adaptive immunity and work together to efficiently fight the wide range of pathogens. While adaptive immune cells provide long-lasting specific immunity, the first line of defence against pathogens is the innate immune system. The innate immune system precedes adaptive immunity from a phylogenetic standpoint and is present in both plants and animals **(Janeway & Medzhitov 2002)**. At first sight innate immunity might appear primitive, but recently the innate immune system and its cellular components have been recognized to be more complex and sophisticated than previously thought **(Cooper 2009).**

In particular, in the last years the idea of the Innate Lymphoid Cell (ILC) family is coming out. This is a family of hematopoietic effectors that have protective roles in innate immune response to infectious microorganisms, in lymphoid tissue formation, in tissue remodeling after damage and in the homeostasis of tissue stromal cells. Among the different components, Natural Killer (NK) cells are the prototypes of the ILC family **(Spits 2011).**

Since their identification in 1975 **(Herberman 1975, Kiessling 1975)**, NK cells have been classified as lymphocytes on the basis of their morphology, expression of many lymphoid markers, and origin from a common lymphoid progenitor cell in the bone marrow (BM). NK cells, still today, constitute the third major lymphocyte subset and represent approximately 10-15% of circulating lymphocytes in the peripheral blood (PB). *In vivo*, NK cells have a limited life span and hence must be continuosly replenished to maintain homeostasis **(Yokoyama 2004)**.

NK cells were initially described as cytolytic effector lymphocytes, which, as their name suggests, can directly, induce the death of tumor cells and virus-infected cells in

the absence of specific immunization. Subsequently, NK cells have been recognized as major producers of proinflammatory and immunosuppressive cytokines, and growth factors, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin (IL)-10, GM-CSF (granulocyte macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), and IL-3, in many physiological and pathological conditions. NK cells can also secrete many chemokines, including CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), XCL1 (lymphotactin), and CXCL8 (IL-8), the secretion of which is crucial for their colocalization with other hematopoietic cells in areas of inflammation **(Fauriat 2010, Vivier 2011)**. Moreover, in tissue such as uterus and pancreas, NK cells have roles unrelated to protection against pathogens and instead promote vascular remodeling **(Ashkar 2000)** or tissue-specific pathologies **(Gur 2010)**.

NK cell development primarily occurs within the BM microenvironment **(Yokoyama 2004, Colucci 2003)**. Selective BM ablation studies in mice provided the first evidence supporting an important role of the BM in NK cell maturation **(Haller 1977, Hackett 1986)**. These studies demonstrated that NK cells mature within the BM, and that functional maturation of NK cells requires an intact BM microenvironment. Therefore, although these early data indicated that the BM is important for mouse NK cell development, they neither proved or disproved whether terminal steps in NK cell maturation occur specifically within this tissue. It is now clear that, although NK cell precursors are primarily found in the BM, NK cells can also develop in peripheral organs, including the thymus **(Vosshenrich 2006)**, and possibly in human lymph nodes **(Freud 2005)**. There is also evidence that murine NK cells continue to mature after leaving the BM, as splenic NK cell populations consist of cells displaying varying degrees of maturation (**Hayakawa 2006; Huntington 2007, Chiossone 2009)**.

Human NK cell maturation and differentiation is characterized by CD56 acquisition **(Yokoyama 2004, Colucci 2003)**. Two major populations of peripheral blood $CD3^{-}CD56^{+}$ NK cells can be distinguished based on the cell surface density of CD56: $CD56^{bright}$ NK cells that have an enhanced capacity of cytokine production and low or absent CD16 expression, and $CD56^{dim}$ NK cells that are characterized by a higher cytotoxic potential and are $CD16^{+}$ **(Jacobs 2001, Cooper 2001, Nagler 1989)**. Only a minority (5-15%) of peripheral blood NK cells is $CD56^{bright}$ while they are predominant among the NK cells resident in the lymph nodes **(Fehniger 2003)**. Although the expression of CD56 (in combination with the absence of other lineage-specific markers such as CD3) identifies cells of the NK cell lineage, additional antigens

are needed to distinguish functionally mature NK cells from immature intermediates *in vivo*. Freud *et al.* recently used the combination of CD34, CD117, and CD94 antigens to discriminate four functionally distinct stages of human $CD56^{bright}$ NK cell development within the secondary lymphoid tissue: stage 1, $CD34^{+}CD117^{-}CD94^{-}$ (pro-NK); stage 2, $CD34^{+}CD117^{+}CD94^{-}$ (pre-NK); stage 3, $CD34^{-}CD117^{+}CD94^{-}$ (iNK); and stage 4, $CD34^{-}CD117^{+/-}CD94^{+}$ **(Freud 2006)**. Unlike stage 1 pro-NK and stage 2 pre-NK, stage 3 iNK cells seem to be definitively committed to the NK cell lineage but they do not present the hallmark features of mature NK cells: the ability to produce IFN-γ and to induce killing of target cells **(Freud 2006)**.

IL-2, IL-15, and, to a lesser extent, IL-7, which all signal through the γ_c **(Ma 2006)**, can support the generation of $CD3^{-}CD56^{bright}$ NK cells in the absence of additional cytokines or stroma *in vitro* **(Miller 1994, Mrozek 1996, Mingari 1997, Barao 2003)**. *In vivo*, IL-15 seems to be the most central and critical cytokine supporting NK cell development because mice and/or humans lacking IL-15 or components of its receptor show deficiencies more severe compared to those observed in the absence of IL-2 or IL-7 signaling **(Kennedy 2000, Kundig 1993, von Freeden-Jeffry 1995)**.

The activation of NK cell effector functions is coordinated by a hierarchy in terms of strength of the activating stimuli: adhesion between NK cell and target cell needs a low threshold, induction of chemokine production requires stronger activating stimuli, whereas degranulation and production of cytokines display the most stringent requirements for induction **(Bryceson 2005, Fauriat 2010)**.

NK cells kill infected or transformed cells via both a perforin-dependent and a death receptor-dependent pathway. The death receptor pathway leads to killing of repeatedly stimulated T and B cells by ligation of Fas (CD95), TNFRs (tumor necrosis factor receptors) or TRAILRs (TNF-related apoptosis inducing ligand receptors) with their cognate ligands (FASL, TNF and TRAIL, respectively) **(Siegel 2000)**. The perforin-dependent pathway involves the secretion of cytotoxic granules that contain perforin and granzymes towards the target cell.

NK cells use several types of integrins, namely LFA-1 (αLβ2, CD11a/CD18) and VLA-4 (α4β1, CD49d/CD29), to mediate the initial contact and adhesion between NK cell and target cell **(Watzl 2010)**. LFA-1 activation, in particular, has a key role in promoting granule polarization. NK cells actively crawl over surfaces with activating ligands and an immunological synapse rises between NK cell and target cell. Granules polarize towards the interaction site in an LFA-1-dependent manner, through actin

cytoskeleton remodeling, and microtubule-dependent transport **(Bryceson 2005, Barber 2004, Mentlik 2010)**. Lytic granules are hybrid organelles that are specialized for the secretion of the cytotoxic effector molecules **(Burkhardt 1990)**. Perforin and the serine protease granzymes are the main effectors of the cytotoxic activity. Perforin is a pore-forming protein required for delivery of all granzymes (GzmA, GzmB, GzmH, GzmK and GzmM in humans) to the target cells **(Voskoboinik 2006)**. How perforin achieves this is currently a matter of debate. GzmB, the most abundant and best studied members of the human granzyme family, is an efficient inducer of caspase-dependent apoptosis through direct processing and activation of caspase-3 and caspase-7. The other member of this family are less represented but it was demonstrated that they efficiently induce apoptosis in a caspase-independent manner through generation of single stranded DNA nicks, production of reactive oxygen species (ROS) and loss of mitochondrial potential **(Heutinck 2010)**. Lysosomal membrane glycoprotein-1 (LAMP-1 or CD107a) molecules are delivered to NK cell surface during the degranulation making it a useful degranulation marker detected by immunostaining **(Alter 2004)**. Recently, it was reported that LAMP-1 cell surface expression has a key role in protecting degranulating-NK cells from perforin and granzyme attack **(Watzl SIICA 2011)**.

1.1.2. 'Leading actors' of NK cell activation

NK cells, unlike T and B cells, do not express receptors that require somatic gene rearrangements to generate receptor diversity and specificity. NK cell functions are controlled by a wide array of germline-encoded inhibitory and activating receptors, many of which are expressed in a stochastic and variegated pattern.

The understanding of NK cell activation mechanisms began in 1986 when Kärre *et al.* observed that NK cells attack cells exhibiting 'missing self', i.e. lacking Major Histocompatibility Complex (MHC) class I molecules. The 'missing self' hypothesis supposed that an NK cell has inhibitory receptors that, by binding to MHC class I molecules (expressed on all healthy cells), prevent NK cell activation in normal healthy condition **(Kärre 1986)**, while trigger NK cell cytotoxicity towards cell lacking MHC-I, a situation that can occur when cells are perturbed by viral infection or neoplastic

transformation. Some years later, Yokoyama provided the first molecular evidence for the missing self hypothesis by identifying an inhibitory Ly49 receptor on mouse NK cells that specifically recognizes MHC class I and suppresses NK cell function **(Karlhofer 1992)**. Afterwards, several groups identified and cloned the genes encoding human inhibitory NK cell receptors recognizing different HLA class I family members **(Colonna 1995, D'Andrea 1995, Gumperz 1995, Wagtmann 1995)**. All the inhibitory receptors in humans and mouse contain one or more intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM; I/VxYxxL/V) **(Orr 2010)**.

The human killer cell immunoglobulin (Ig)-like receptors (KIR; also known as CD158) compose a family of transmembrane glycoproteins expressed on NK cells and on a subset of T cells and are encoded by 14 polymorphic genes. Human KIR contain either two (KIR2D) or three (KIR3D) Ig-like domains in the extracellular domain, and are called KIR2DL or KIR3DL when they possess a long ITIM-containing cytoplasmic domain. By contrast, KIR2DS and KIR3DS have short cytoplasmic domains lacking ITIM, but possess charged residues in their transmembrane region to allow the association with DAP12 that contains immunoreceptor tyrosine-based activation motifs (ITAM; Yxx(L/I/V)x6–8Yxx(L/I/V)) and triggers activating signaling through the recruitment of Syk/ZAP-70 tyrosine kinases **(Campbell 2011)**. The only exception to this short/long-tailed rule is KIR2DL4, which is the unique activating KIR with a long cytoplasmic domain. KIR2D receptors typically recognize human leukocyte antigen-C (HLA-C) alleles, whereas KIR3D receptors recognize HLA-B, or some HLA-A alleles **(Campbell 2011)**; also non-MHC-I molecules may serve as KIR ligands **(Sivori 2010)**. KIR acquisition is cumulative and stable, occurs as a late event during NK cell maturation **(Freud 2006)** and has a key role in NK cell education processes **(Kim 2005, Gasser 2006)**.

In addition to KIR family, human NK cells express other ITIM-containing receptors. Human CD94-NKG2A binds to the non classical MHC molecule HLA-E. LILR (also known as ILT, LIR, and CD85) is the third family of ITIM-containing receptors on human NK cells that bind to MHC class I, and display permissive binding to several different MHC class I molecules. Morevover, NK cells express several other inhibitory receptors non MHC-specific: NKR-P1 (CD161) binds to human LLT, Siglec-3,7,9 (CD328) to sialic acid, LAIR-1 (CD305) to collagen, KLRG1 to cadherins, CEACAM-1 (also known as BGP and CD66a) to itself, PILRα to CD99, and finally CD300a (also known as IRp60) which has no known ligand at present **(Long 2008)**.

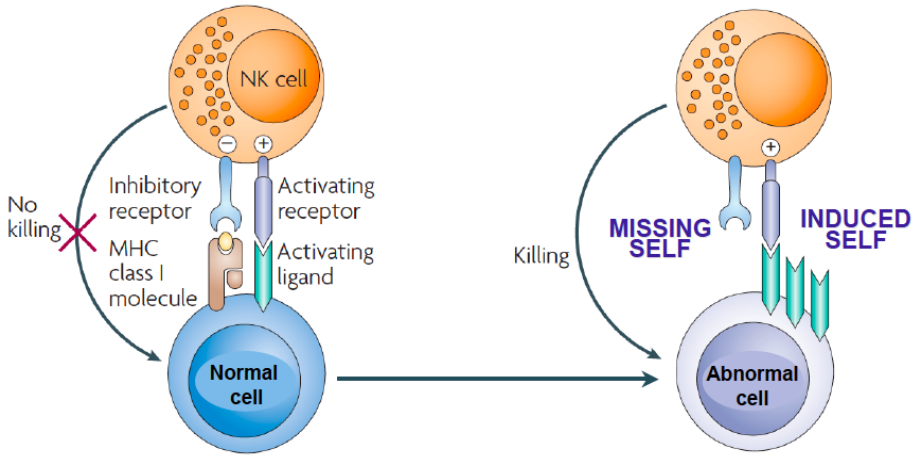


Figure 1. NK cell recognition of altered cells (adapted from *Ljunggren et al. 2007*).
NK cell activation is the result of a balance between inhibitory signals from MHC class I molecules and activating signals from induced self molecules. When the activating signals overcome the inhibitory signals, the NK cell carries out its cytotoxicity against the abnormal target cell.

Today is largely accepted that NK cell activation is the result of a balance between inhibitory signals and activating signals. NK cell activation, in fact, does not occur in the absence of MHC class I molecules but requires the engagment of NK cell activating receptors i.e. by stress-induced self molecules expressed on abnormal cells.

The most important NK cell activating receptors are the Natural Cytotoxicity Receptors (NCR), NKG2D, DNAM-1, 2B4, NTB-A, CRACC and CD16. Many of the activating receptors lack intracellular signaling motifs but are non-covalently associated via a charged residue in the receptor transmembrane domain with the ITAM-containing adapters DAP12, FcεRIγ, or CD3ζ, which recruit and activate the Syk or ZAP70 tyrosine kinases, or in some cases the DAP10 adapter which induces phosphatidylinositol 3-kinase (PI3K) signaling.

The NCRs NKp30, NKp44, and NKp46 were among the first identified activating receptors in NK cells but they can also be found on subsets of T cells. **(Pessino 1998, Cantoni 1999, Pende 1999)**. NCR are Ig-like type I transmembrane glycoproteins that are expressed as monomers. NKp46 and NKp30 associate with disulfide linked homodimers or heterodimers of the TCRζ and/or the FcRγ chains, whereas NKp44 associates with the disulfide-linked homodimer DAP12. Several viral ligands for the NCR have been identified **(Arnon 2006)** but the cellular ligands for

these receptors remain poorly defined. B7-H6 was recently identified as a ligand of NKp30 **(Brandt 2009)**.

2B4, NTB-A, and CRACC are monomeric Ig-like type I transmembrane glycoproteins members of the SLAM-related receptors, expressed by NK cells and also by many other cells of the immune system. Under certain circumstances 2B4 and NTB-A can also inhibit NK cell functions **(Veillette 2006)**. It seems that, in mouse, the amount of 2B4 expression, the strength of cross-linking, and the expression level of SAP adapter molecule determine if 2B4 is activating or inhibitory **(Chlewicki 2008)**.

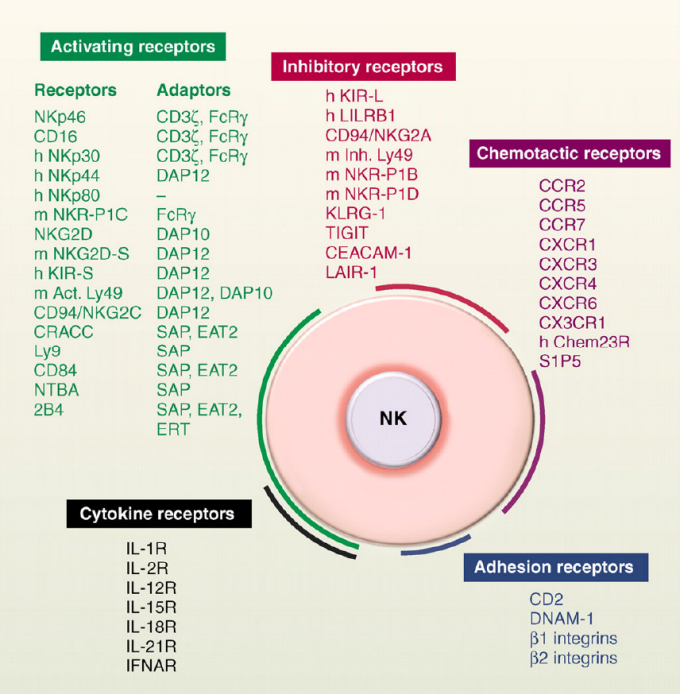


Figure 2. The numerous receptors expressed by NK cells (from *Vivier et al. 2011*)**.**
Here, are reported all the surface receptors that an NK cell can express. They can be grouped in activating (green), inhibitory (red), of adhesion (blue), for cytokine (black), and chemotactic (purple) receptors.
Together with activating receptors, adaptor molecules responsible of the signaling are reported. The list comprises both human (h) and mouse (m) receptors; receptors without any indication are conserved in both species.

The $CD56^{dim}$ NK cells are able to detect antibody-coated cells through the FcγRIIIA (CD16) cell surface receptor and trigger antibody-dependent cellular cytotoxicity (ADCC). CD16 associates with disulfide-linked homodimers or heterodimers of the TCRζ and/or the FcεRγ chains **(Lanier 1991)**, which in turn activate the classical ITAM pathway **(Ting 1995).** Engagement of CD16 on NK cells is sufficient to induce NK cell degranulation **(Bryceson 2005)**. Other NK cell receptors have been termed 'coactivation receptors', as engagement of each receptor alone is insufficient to induce activation in freshly isolated peripheral blood NK cells, whereas

engagement of specific pairwise combinations of receptors can induce synergistic intracellular Ca^{2+} mobilization and degranulation or cytokine production. The most synergistic combinations are activating receptors with different types of motifs **(Bryceson 2006)**.

In conclusion, potent NK cell effector functions activation requires dynamic integration of signals derived from multiple receptors.

1.1.3. NKG2D and its ligands

Among all the receptors designed to recognize self-molecules on cells under conditions of stress (‘induced-self ’ or ‘stressed-self ’ recognition), NKG2D (Natural Killer group 2, member D) is one of the most important and powerful activating immunoreceptor. Initially described as an NK cell receptor, it is now known to be expressed by a number of immune effector cells such as $CD8^+$ T cells and $\gamma\delta^+$ T cells **(Raulet 2003)**.

NKG2D was first identified in a screen for genes that are expressed preferentially by human NK cells **(Houchins 1990, Houchins 1991)**. NKG2A, NKG2C and NKG2E complementary DNAs were isolated in the same screen and, although originally given a common name, subsequent analysis showed that NKG2D should be considered as a distinct receptor: NKG2A, NKG2C and NKG2E proteins are all highly related in sequence, while NKG2D differs markedly **(Houchins 1991)**.

The receptor NKG2D belongs to the family of C-type lectin-like receptors, therefore NKG2D has been called *klrk1*/KLRK1 (killer cell lectin-like receptor subfamily K, member 1) but, as the gene encoding this receptor resides in the NKG2 (natural killer group 2) complex, the gene product was also named *nkg2d*/NKG2D. The NKG2 complex is located on chromosome 12 in humans **(Glienke 1998)**.

NKG2D is a type II transmembrane-anchored C-type lectin like receptor expressed as a disulfide-linked homodimer on the cell surface **(Garrity 2005)**. NKG2D has no signaling motif within its short intracellular domain **(Houchins 1991)** and, similar to many others activating receptors, associates with signal-transducing proteins via charged residues in its transmembrane domain. In humans, each NKG2D

homodimer associates with two homodimers of DAP10 (DNAX-activating protein of 10kDa), hence forming a hexameric structure **(Wu 1999, Garrity 2005) (Fig. 3)**. The short cytoplasmic tail of DAP10 contains a tyrosine-based signaling motif (YINM), which can be phosphorylated by Src-family kinases and bind either to the p85 subunit of PI3K or to the adapter molecule Grb2 (growth factor receptor-bound protein 2) **(Chang 1999, Wu 1999)**. Binding of both molecules is essential for NKG2D mediated Ca^{2+} flux and cytotoxicity **(Upshaw 2006)**.

The most remarkable trait of the NKG2D receptor system is the diversity of ligands that can bind to this single invariant receptor. Activating receptors that recognize self-proteins are very specific and usually have no more than one or two different binding partners; for example 2B4 only binds to CD48. NKG2D is a notable exception to this rule; it shows promiscuous interactions with a wide range of self-proteins.

NKG2D ligands are distantly related homologs of MHC class I proteins and new members of this family continue to be discovered. The first evidence for a protein that binds to NKG2D came from a study by Bauer *et al.* **(Bauer 1999)** showing that a soluble form of MHC class-I-chain-related protein A (MICA) - a non-classical class I molecule first described as cell stress induced proteins expressed in gastrointestinal epithelium **(Groh 1996)** - binds to various lymphocyte subsets. A monoclonal antibody blocking the interaction was prepared, and the antibody was subsequently shown to bind to NKG2D **(Bauer 1999)**. Further analysis showed that MICB - a close relative of MICA - also binds to NKG2D **(Groh 1996, Steinle 2001)**. Subsequently, two human cell surface glycoproteins that bound to the human cytomegalovirus (HCMV) UL16 glycoprotein were described and named UL16-binding protein 1 and 2 (ULBP1 and ULBP2) **(Cosman 2001)**. UL16 was studied because it was a candidate virus protein that was involved in evasion of the immune response. Similar to MICA and MICB, ULBP1 and ULBP2 also bound to the NKG2D receptor and stimulated human NK cells. Other researchers named the same molecules human RAET1 proteins, in recognition of their relatedness to the mouse Raet1 proteins **(Radosavljevic 2002, Steinle 2001, Onda 2001)**. Based on sequence homology with ULBP1 and ULBP2, four additional human ULBP family members were described: ULBP3, ULBP4 (or RAET1E), ULBP5 (or RAET1G), and ULBP6 (or RAET1L) **(Radosavljevic 2002, Bacon 2004, Chalupny 2003, Eagle 2009)**.

Mouse Raet1 proteins include three subfamilies of proteins: the Rae1α-ε molecules, the histocompatibility 60 (H60) protein, and the Mult-1 protein. Human ligands show around 25–30% amino acid identity to murine ligands **(Raulet 2003)**.

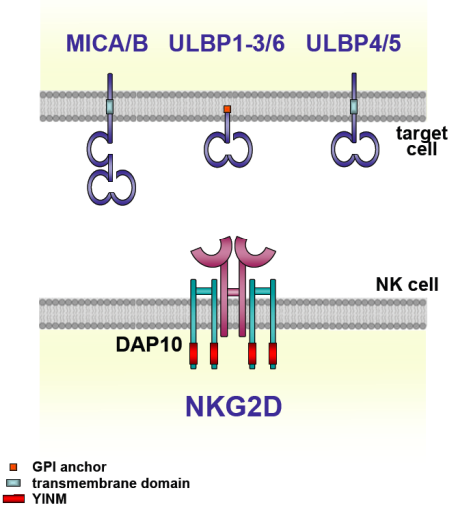


Figure 3. The human NKG2D receptor and its ligands.
Human NKG2D is a homodimeric type II-transmembrane protein with a short cytoplasmic tail and, hence, associated with the signaling molecule DAP10 forming a hexameric structure.
Among the human NKG2D ligands, MIC molecules are characterized by three Ig-like domains, while ULBP proteins are characterized by two Ig-like domains. MICA, MICB, ULBP4, and ULBP5 are transmembrane proteins, while ULBP1, 2, 3, and 6 are GPI-linked protein, feature that makes them more strongly associated with lipid rafts. The various ligands have different affinity for NKG2D binding.

The *MIC* gene family members are localized within the HLA gene complex and are expressed in most mammalian species except mice. In contrast, both mice and humans express the *RAET1* gene family. The *ULBP/RAET1* gene family is not part of the MHC but maps on the long arm of human chromosome 6 **(Radosavljevic 2002)**. Human RAET1 proteins share only 25% or less of their amino acids with MIC proteins. Analyzing the alignment of *MIC* and *ULBP/RAET* promoter regions, Eagle *et al.* showed that *MICA* and *MICB* were the most similar members of the cluster, sharing 85% identity. *RAET1E* was the most divergent, sharing <8% identity with the other genes. Other members of the *ULBP/RAET* gene family shared between 23 and 67% identity **(Eagle 2006)**.

NKG2D ligands are polymorphic, in particular for *MICA* and *MICB* genes have been described 70 and 31 alleles, respectively. There is also evidence for human *RAET1* genes and promoter sequence variants **(Romphruk 2009)**. Interestingly, allelic variants of these ligands have been shown to bind with variable affinity to NKG2D **(Champsaur 2010)**; moreover, polymorphisms within promoter sequences have been associated also with several human diseases **(Eagle 2006)**.

NKG2D ligands are distant relatives of MHC class I proteins, but unlike class I

proteins, they do not associate with β_2-microglobulin or bind to antigenic peptides. All have Ig-like α1 and α2 domains, the region that binds to NKG2D, and MICA/B have an additional α3 domain **(Bahram 1994, Groh 1996)**. NKG2D ligands differ in the way they are anchored to the cell surface: ULBP1-3, 6 are GPI linked to the cell membrane, whereas MICA/B, ULBP4, and ULBP5 have transmembrane and cytoplasmic tail domains **(Fig. 3)**. This has effects on the way that ligands interact with the plasma membrane suggesting that GPI-linked proteins are more strongly associated with lipid rafts **(Eleme 2004)**.

Notably, most of the receptor aminoacidic residues that dominate binding to the different ligands are the same, and several of the contact residues on the ligands are conserved **(McFarland 2003a)**. Moreover, crystal structures of NKG2D receptor in the soluble form and bound to ligands suggest that NKG2D binds to its ligands through 'rigid adaptation' recognition, allowing binding to a wide variety of ligands. Therefore, despite the marked differences in their aminoacid sequences, the different ligands interact with NKG2D similarly, and the receptor does not seem to undergo marked conformational changes to accommodate different ligands **(McFarland 2003a, McFarland 2003b)**.

The number of known ligands for NKG2D continues to grow, raising the question of why is there such diversity and why so many are needed. One explanation may be that an evolutionary pressure applied by certain viruses to inhibit NKG2D ligand expression in virus-infected cells is the driving force for ligand diversification **(Bahram 2005)**. Another explanation is that engagement of NKG2D by different NKG2D ligands, their tissue-specific expression and their differential induction may fine-tune the extent of activation and allow the system to answering to a greater range of cellular distress. NKG2D ligands, infact, are not all functionally equivalent, but rather can have unique, tissue-specific roles, suggesting that there might be additional driving forces for this diversity. Although there is no evidence that the different ligands induce qualitatively distinct biological effects in responding cells, this remains a clear possibility. The various ligands could differ quantitatively in their effects based on the marked differences in their affinity for NKG2D: it has been shown that the sequence variation in the α1α2 domains of NKG2D ligands in mice translates into significantly different binding affinities for NKG2D and that some ligand interactions with NKG2D are also temperature dependent **(O'Callaghan 2001)**.

There is evidence that some NKG2D ligands have evolved specific adaptations that allow them to signal to the immune system in highly specialized tissues. The cytoplasmic tail of MICA contains a signal sequence that allows this ligand to be targeted to the basolateral surface of human intestinal epithelium, where immune cells can interact with epithelial cells. This polarization allows epithelial cells that become stressed, for example by the presence of pathogenic bacteria in the gut, to upregulate MICA at the specific location required to alert the immune system. However not all MICA variants have this capacity **(Suemizu 2002)**. Other NKG2D ligands may have specialized functions at other immune interfaces, for example ULBP4 has been reported to be expressed in the skin **(Chalupny 2003)**.

Of note, NKG2D ligands not always function to generate cytotoxic immune responses: MICA has also been shown to be expressed in the trophoblast during normal pregnancy **(Mincheva-Nilsson 2006)**.

Contrary to its ligands, the NKG2D receptor is highly conserved. The human and mouse NKG2D receptors are 70% identical. Strikingly, NKG2D from a given species can bind NKG2D ligands from other species; for example, mouse NKG2D can bind to human ULBP1 and ULBP2 **(Eagle 2007)**.

NKG2D signal is strong enough to overcome, in some cases, the inhibitory signaling by MHC-specific receptors. In contrast to the results obtained with T cells where NKG2D function as a co-stimulator receptor **(Groh 2001)**, crosslinking of NKG2D on human NK cells with recombinant ligands was sufficient to trigger cytokine release from NK cells **(Kubin 2001, André 2004)**. However, exposure of purified NK cells to NKG2D ligand-expressing targets *in vitro* results only in low levels of target cell killing **(Bryceson 2006)**, indicating that for optimal triggering of NKG2D-mediated effector functions, additional signals are required. In fact, *in vitro* culture of NK cells in the presence of IL-15 or high doses of IL-2 significantly increases NKG2D-mediated killing of tumor targets. Moreover, Horng *et al.* demonstrated that the IL-15 receptor pathway couples to NKG2D signaling in NK cells. Triggering of the IL-15 receptor resulted in phosphorylation of DAP10 by janus kinase 3, which facilitated downstream signaling and NKG2D-mediated effector **(Horng 2007)**.

1.1.4. NKG2D ligand expression

The various NKG2D ligands have distinct patterns of expression, suggesting that they cannot be considered functionally redundant. Although much remains to discover about ligand expression and regulation, a common theme is that their surface protein expression by normal cells in adults is generally absent or present at low levels in certain tissue, but in pathological conditions is often upregulated. In humans, MICA and MICB are expressed only by intestinal epithelial cells, likely as a consequence of stimulation by the neighbouring bacterial flora **(Groh 2006)**. Despite that, total tissue scans of MICA and MICB of healthy individuals revealed that with the exception of the central nervous system both genes are widely transcribed **(Schrambach 2007)**. Rae1-encoding mRNA, especially the Rae1β and Rae1γ isoforms, is expressed diffusely throughout early embryos, and in particular in the brain but, upon 18 days of gestation, expression of the transcripts is down-regulated and remains so in all of the normal adult tissues that were examined **(Zou 1996, Nomura 1996)**. Similarly, in humans, NKG2D ligands are expressed at low levels on $CD34^+$ hematopoietic stem cells and expression of the ULBP proteins is enhanced upon differentiation into myeloid progenitors **(Nausch 2008)**. Various normal cells significantly expressed at the mRNA level some of the ULBP molecules in humans, and Mult1 in mice but cell surface expression by normal cells is low or has not been documented. In humans, ULBP transcripts are detectable in different healthy tissues, including kidney, prostate, uterus, tonsil and lymph nodes **(Cosman 2001)**. In addition, ULBP4 is specifically transcribed in the skin **(Chalupny 2003)**.

These findings suggest that transcription of the genes encoding the human and mouse NKG2D ligands have been detected in numerous normal healthy tissues in the adult and in the normal mouse embryo. However, post-transcriptional and post-translational mechanisms exist to prevent translation and expression of these ligands in the healthy individual, presumably to avoid autoimmunity.

Although NKG2D ligands are rarely expressed on the cell surface of adult healthy cells, their expression is associated with malignant transformation because they are markedly upregulated and expressed at functional levels on the cell surface of numerous tumor cell lines and primary tumors. Cell transformation by certain oncogenes including K-ras, c-myc, Akt, E1A or Ras V12, or their combinations, does

not directly force the expression of NKG2D ligands. These data suggest that additional events are required for NKG2D ligand expression **(Gasser 2005)**.

Prior studies have established that expression of NKG2D ligands on tumors renders them susceptible to killing by NK cells *in vitro* **(Bauer 1999, Cerwenka 2000, Diefenbach 2000)** and results in the *in vivo* rejection of transplantable tumors expressing these ligands **(Cerwenka 2001, Diefenbach 2001)**. Moreover, NKG2D ligands have been reported on a wide variety of human and mouse tumors. In general, the proportions of ligand-positive tumors, the levels of ligand expression and the types of ligands expressed are heterogeneous among different tumor types and among individual samples of the same tumor.

MICA and MICB are expressed on a subset of human hepatocellular carcinoma tissues and are involved in hepatoma cell sensitivity to NK cells **(Jinushi 2003a)**. Tumor cells extracted from patients with different types of leukemia, including acute myeloid leukemia (AML), acute lymphatic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphatic leukemia (CLL), express heterogeneous levels of NKG2D ligands being MIC the most expressed **(Salih 2003)**. MIC proteins are expressed by a broad range of tumors and were detected also in various carcinomas (breast, lung, colon, kidney, ovary and prostate), gliomas, neuroblastomas and melanomas **(Groh 1999, Pende 2002, Vetter 2002, Friese 2003, Watson 2006, Castriconi 2007)**.

In addition, using the human NK cell line NKL as effector cells, Salih *et al.* **(Salih 2003)** showed that expression of NKG2D ligands on patient-derived AML and CML cells rendered them susceptible to NK cell mediated lysis in an NKG2D-dependent manner. In colorectal cancer patients, high levels of MICA were associated with a good prognosis **(Watson 2006)**. Human melanoma cells express high amounts of NKG2D ligands **(Vetter 2002)**, and NKG2D ligand expression was lost during the progression of uveal melanoma **(Vetter 2004)**.

It is possible that tumor cells often express insufficient levels of NKG2D ligands to stimulate tumor cell rejection, either because expression of the ligands is not sufficiently upregulated early in the development of the tumor, or because tumor cells with lower levels of ligand expression are selected by the immune system *in vivo* as the tumor evolves. In fact, tumors induced in NKG2D-deficient mice expressed higher amounts of NKG2D ligands, strongly supporting the hypothesis that immunoselection by NKG2D favors the loss of NKG2D ligand expression by primary tumors **(Guerra**

2008).

Ligand expressing tumors might also evolve mechanisms to evade NKG2D-mediated immunity, as indicated by the evidence that human tumors that express MICA often produce a soluble version of MICA that reaches high levels in the serum and causes a systemic desensitization of NKG2D in T cells (and possibly other immune system cells) **(Groh 2002, Salih 2003, Jinushi 2008)**. However, the existence of tumors that evade NKG2D does not detract from the possibility that many other tumors are successfully eliminated by NKG2D mediated immune activation, because such evasion mechanisms are unlikely to be universally effective.

The expression of NKG2D ligands is also upregulated by cells that are infected with pathogens. Infection with cytomegalovirus (CMV) leads to the upregulation of expression of NKG2D ligand transcripts, including MICA, MICB, and ULBPs in humans, and Rae1 in mice **(Groh 2001, Welte 2003)**. To the contrary, both HCMV and MCMV encode proteins that interfere with ligand expression at the cell surface: HMCV-encoded UL16 protein retains MICB, ULBP1 and ULBP2 in an internal compartment **(Welte 2003, Lodoen 2003, Krmpotic 2002, Dunn 2003)**. Human immunodeficiency virus (HIV) infection of primary $CD4^+$ T cell blasts induced the expression of ULBP1, ULBP2, and ULBP3 **(Ward 2007)**. On the other hand, there are evidences that the HIV protein Nef can downregulate NKG2D ligands upon infection **(Cerboni 2007a)**. MICA expression was also upregulated as a result of Mycobacterium tuberculosis infection **(Das 2001)** and of the binding of Escherichia coli adhesin AfaE to cellular CD55 **(Tieng 2002)**. By contrary, herpes simplex virus (HSV) infection does not seem to upregulate NKG2D ligand expression suggesting that their upregulation requires specific triggers by some pathogens and is not simply associated with viral infection, genome integration, and replication.

NKG2D ligands are also upregulated on rapidly proliferating normal cells. Zwirner *et al.* first reported that phytohemagglutinin (PHA) induced the expression of MICA protein in $CD4^+$ and $CD8^+$ T cells **(Zwirner 1997)**. TCR/CD3 engagement and costimulation via CD28 induced a sustained increased expression of MICA on activated $CD4^+$ and $CD8^+$ T cells **(Molinero 2002)**. In addition, Cerboni *et al.* described the induction of MICA and ULBP1-3 on a fraction of dividing $CD4^+$ and $CD8^+$ T cells activated with the superantigen Staphylococcus aureus enterotoxin B **(Cerboni 2007b)**.

1.1.5. Who or what does manage NKG2D ligand expression?

The induction of NKG2D ligands on transformed, infected, or proliferating cells suggests that cells have sensing mechanisms that recognize changes associated with a 'stress' and activate pathways that upregulate cell surface expression of NKG2D ligands. Therefore, NKG2D ligands act as molecular flags that say to the immune system that something is wrong. The mechanisms that coordinate the expression of NKG2D ligands are only partially understood but it was widely accepted that, depending on the cell type and stress stimulus, NKG2D ligand expression is regulated at transcriptional and post-transcriptional levels.

Transformation *per se* does not induce ligand expression because cell transformation by overexpression of oncogenes is insufficient to induce expression of NKG2D ligands **(Gasser 2005)**.

In NKG2D ligand promoters there are some binding sites that share a common function and a similar relative position within the promoters; they are probably evolutionarily conserved because of their function. The heat shock elements are some of them because putative heat shock transcription factor (HSF1) binding sites were identified in four of the NKG2D ligand promoters (*MICA, ULBP1, ULBP2* and *ULBP6*) suggesting that some of the MIC and ULBP molecules are involved in shock responses **(Groh 1996, Eagle 2006, Venkataraman 2007)**. Other transcription factor binding sites identified in the region of homology of *ULBP1-3, 5* and *6* genes were the binding site for the myeloid zinc finger 1 (MZF1) protein, a factor known to be induced by retinoic acid and proposed to have a role in the hematopoietic development **(Hromas 1991, Hamerman 2004)**. The basonuclin (BNC) sites found in all *ULBP/RAET* gene promoters are also interesting. BNC is a transcription factor found mainly in cells of the basal layer of stratified squamous epithelia, and expression of some NKG2D ligands has been observed in normal epithelial cell layers, with overexpression seen in epithelial tumors **(Groh 1998)**. Moreover, interferon regulatory factor (IRF) sites were identified in most *ULBP/RAET* promoters. Most of these were IRF7 sites, as shown in *ULBP3, 4* and *6* promoters. NF-kB has been proposed to be important in *MICA* expression and there is an NF-kB binding site in intron 1 of the gene **(Molinero 2004)**. An NF-kB binding site, similar to that identified in *MICA*, was located in an aligned stretch of *ULBP1-3* and *5*. Finally, the transcription factor AP-1, which is involved in

tumorigenesis and cellular stress response, was found to regulate *Raet1* through the JunB subunit **(Nausch 2006)**.

Despite the presence of the same binding site in several NKG2D ligand promoters, the promoter regions of *ULBP2*, *3*, and *4* are polymorphic. Eagle *et al.* suggested that polymorphisms in the promoter regions of NKG2D ligand genes have the potential to modulate gene expression by altering the binding of transcription factors **(Eagle 2006)**.

The DNA Damage Response (DDR) has been shown to be highly involved in NKG2D ligand regulation. DDR, also called as genotoxic stress response, is a stress pathway involved in the maintenance of the genome integrity and activated in normal cells subjected to DNA damage with the aim of arresting the cell cycle, promoting DNA repair functions, and, in highly damaged cells, inducing apoptosis **(Sancar 2004)**. Recent studies have shown that DDR is often constitutively activated in human cancer cells and in cells infected by certain viruses suggesting that tumorigenesis and infection can damage DNA or stress the genome **(Bartkova 2005, Gorgoulis 2005, Norman 2011)**.

Gasser *et al.* provided evidence that this pathway actively regulates NKG2D ligand transcription **(Gasser 2005)**. Both mouse and human cells upregulated NKG2D ligands following treatment with DNA-damaging agents. This effect was dependent on ATM/ATR function, as inhibitors of ATR and ATM kinases prevented ligand upregulation in a dose-dependent manner. These findings provide a link between the constitutive activity of the DNA damage response in tumors and the frequent upregulation of NKG2D ligands in transformed cell supporting the idea that constitutive ligand expression is maintained by persistent genotoxic stress in tumor cell lines and suggesting that either ATM or ATR may be predominantly responsible of NKG2D ligand expression maintenance. The exact molecular events linking the ATM/ATR-dependent recognition of DNA damage and the transcription of NKG2D ligands remain elusive. DNA damage in tumor cells could be the responsible of the induction of NKG2D ligands and increases the sensitivity of the damaged cells to NK cell- or T cell-mediated lysis, possibly imposing an immune-mediated barrier to tumorigenesis **(Gasser 2005, Hoglund 2006)**.

It is not yet well established if DDR is also involved in NKG2D ligands expression induced by pathogen infection. Recently it was demonstrated that the HIV viral protein R (Vpr), through the interaction with a uracil glycosylase and the

subsequent inhibition of the host antiviral factor APOBEC3G, activates the DDR-dependent NKG2D ligand expression **(Norman 2011)** suggesting that the DDR could be a common mechanisms by which cells answer to and protect from a wide range of danger.

About the role of p53, one of the main effector molecule of the DDR, the debate is still open and there are controversial evidences. Gasser *et al.* ruled out p53 from the mechanisms at the basis of genotoxic drug induced-NKG2D ligand up-regulation because it occurred also in $p53^{-/-}$ knock-out fibroblasts **(Gasser 2005)**. By contrast, recently new evidences demonstrating p53 involvement have been provided **(Textor 2011, Li 2011)**. Textor *et al.* showed that the induction of wild-type (wt) p53, but not mutant (mut)p53, in a p53-null non small cell lung cancer cell line, resulted in ULBP1 and -2 upregulation and dependent NK cell activation. Furthermore, they demonstrated that ULBP1 and -2 are direct p53 target genes and that the treatment of certain cancer cells with RITA, a small molecular compound that reactivates wtp53, resulted in the upregulation of ULBP2 expression **(Textor 2011)**. At the same time, Li *et al.* showed that RITA induces a p53-dependent upregulation of ULBP2 in human colon carcinoma and human breast cancer cell lines and that this induction is not DDR-dependent because is not affected by caffeine pretreatment or ATM depletion. Moreover, they demonstrated that *ULBP2* is a direct target gene of p53 and that p53 can bind to the first intron of *ULBP2* gene **(Li 2011)**.

It was demonstrated that Toll-like receptor (TLR) signaling can result in NKG2D ligand transcription. Treatment of peritoneal macrophages with TLR agonists *in vitro* and injection of LPS *in vivo* resulted in Rae1 upregulation on peritoneal macrophages **(Hamerman 2004)**. TLR signaling on DCs also results in NKG2D ligand expression **(Draghi 2007, Ebihara 2007)**.

The cytokines are able to influence NKG2D ligand expression. In particular, interferons have pleiotropic effects on NKG2D ligand expression. In humans, IFN-α induces to the expression of MICA on DCs **(Jinushi 2003)**. By contrast, Bui *et al.* showed that IFN-α and IFN-γ treatment led to the selective downregulation of H60 on certain mouse sarcoma cells at the transcript level **(Bui 2006)**. In accordance with this study, treatment of human melanoma cells with IFN-γ resulted in decreased MICA mRNA levels **(Schwinn 2009)**. Moreover, transforming growth factor-β (TGF-β) also decreases the transcription of MICA, ULBP2, and ULPB4 on human malignant gliomas **(Friese 2004, Eisele 2006)**. Therefore, cytokines and interferons can differentially

affect NKG2D ligand expression in different cell types and environments.

Various mechanisms are also responsible for the post-transcriptional regulation of NKG2D ligands: inhibition of mRNA translation by cellular or viral microRNAs (miRNAs), ubiquitin-dependent proteasomal degradation, and shedding from cell surface.

Different groups identified several endogenous cellular miRNAs that bound to the 3'-UTR of MICA and MICB and repressed their translation **(Stern-Ginossar 2008, Yadav 2009)**. In accordance with these findings, silencing of Dicer, a key protein in the miRNA processing pathway, leads to the upregulation of MICA and MICB **(Tang 2008)**. Interestingly, HCMV was found to encode a viral miRNA, hcmv-miR-UL112, that competed with endogenous miRNA for binding to MICA and MICB 3'-UTR, thus repressing the translation of these ligands **(Stern-Ginossar 2007)**.

In particular, Stern-Ginossar *et al.* identified a group of endogenous cellular microRNAs that suppress MICA and MICB expression by binding to *MICA* and *MICB* 3' UTR sites that overlap those bound by hcmv-miR-UL112. They showed that these cellular microRNAs are ubiquitously expressed in various human tissues to avoid autoimmunity but, when MICA and MICB mRNA expression was induced after short-term stress, the threshold determined by miRNAs was overcome and MICA and MICB protein expression was upregulated at the cell surface. Of note, a large portion of the identified cellular microRNAs is overexpressed in various tumors determining the increment of this threshold level, the subsequent inhibition of NKG2D ligand upregulation and the immune evasion **(Stern-Ginossar 2008)**.

Recently, Nice *et al.* showed that MULT1 protein undergoes ubiquitination dependent on the lysines in its cytoplasmic tail, resulting in its rapid degradation. Moreover they demonstrated that this ubiquitination was reduced in response to heat shock or ultraviolet irradiation, allowing cell surface expression of MULT1, but was not affected by genotoxic stress, suggesting that different stimuli regulate NKG2D ligands differently **(Nice 2009)**. Post-transcriptional regulation of MICA was also recently documented by a study showing that virus-encoded proteins can ubiquitinate and down-regulate MICA from the cell surface **(Thomas 2008)**. Whether other ligands with long cytoplasmic tails are similarly regulated has not yet been investigated. The presence of multiple lysines in the cytoplasmic tail of H60a, H60b, MICB, and ULBP5 suggests that this translational control mechanism might be used by other NKG2D ligands.

In addition to membrane-bound NKG2D ligands, secreted forms of the ligands have been described in humans. In 2002, two independent groups reported secretion of MICA proteins from tumor cells and the presence of high amounts of soluble MICA (sMICA) in cancer patient sera **(Groh 2002, Salih 2002)**. The presence of sMICA was subsequently described in patients suffering from a variety of cancers, including leukemia **(Salih 2003)**, pancreatic carcinomas **(Marten 2006)**, hepatocellular cancer **(Jinushi 2005)**, colon carcinoma **(Doubrovina 2003)**, and multiple myeloma **(Jinushi 2008)**. Since sMICA and sMICB are found in substantial amounts in the sera of patients affected by different cancers, these proteins were suggested as diagnostic markers for cancer progression (**Nausch 2008**). Furthermore, soluble forms of ULBP2 and ULBP4 have been detected **(Waldhauer 2006, Cao 2007)**.

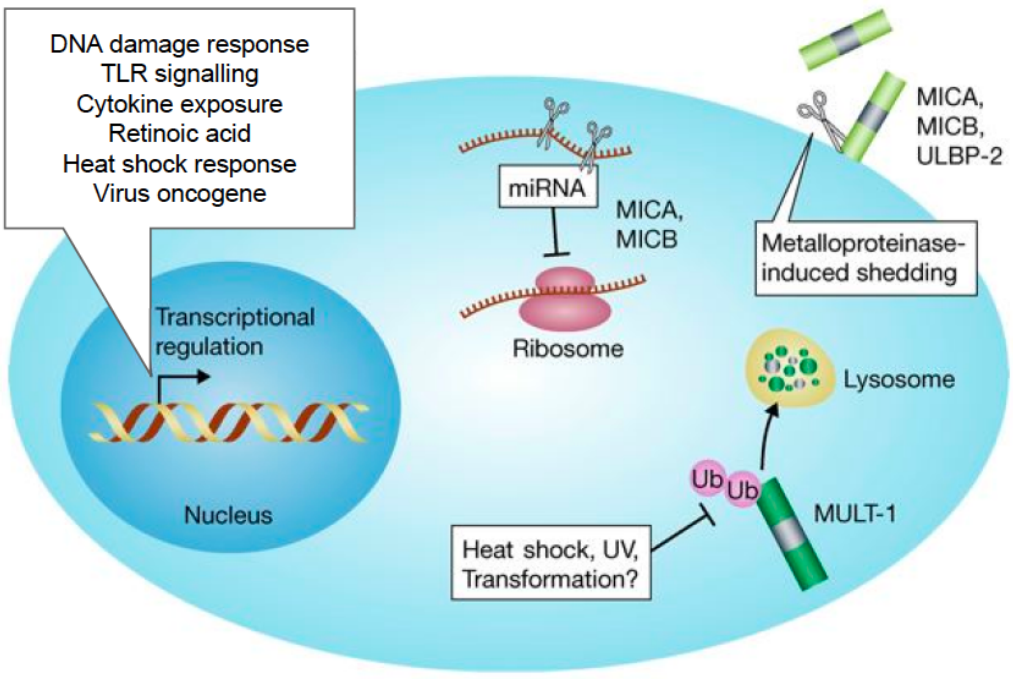


Figure 4. Transcriptional and post-transcriptional regulation of NKG2D ligands (adapted from *Cerwenka 2009*).
Always more possibilities join the wide group of mechanisms by which NKG2D ligand expression is regulated. Here are reported the known molecular mechanisms underlying NKG2D ligand expression at the moment.

Two distinct mechanisms of generating soluble NKG2D ligands have been described. The first mechanism involves the cleavage of ligands from the cell surface by

proteases. Prior studies reported that a broad-range metalloprotease inhibitor reduced the levels of sMICA detected in tumor cell supernatants and increased the levels of surface MICA on these tumors **(Salih 2002)**. Subsequently, metalloproteases were also found to be responsible for the shedding of both sMICB and sULBP2 **(Salih 2006, Waldhauer 2006)**. Recently, two groups have reported the involvement of members of the 'a disintegrin and metalloproteinase' (ADAM) family in the shedding of NKG2D ligands **(Waldhauer 2008, Boutet 2009)**. In addition, a recent report demonstrated that cell surface endoplasmic reticulum protein 5 (ERp5) is required for MICA shedding **(Kaiser 2007)**. A second mechanism to generate soluble NKG2D ligands is by alternative RNA splicing. Two groups have demonstrated the existence of alternative RNA splicing of the ULBP family of human ligands **(Bacon 2004, Cao 2007)**.

Of note, shedding of NKG2D ligands has multiple consequences on NKG2D-mediated responses: reduction of ligand density on the tumor cell surface, downmodulation of the receptor on effector cells and blocking of the NKG2D-binding site for surface expressed NKG2D ligands.

In summary, the mechanisms that govern NKG2D ligand expression are numerous and very different from each other and it is also possible that multiple mechanisms cooperate in this function. In some cells or circumstances, these levels of regulation may serve as serial checkpoints, in response to distinct stimuli, to ensure that ligands are induced only in diseased cells and not in normal cells. Some ligands could show a specialization in the type of stress that regulates them, providing broad innate immune 'coverage' for numerous stresses associated with various diseases.

Although in the last year many new evidence emerged about NKG2D ligand regulation, much remains to be understood.

1.1.6. DNAM-1 and its ligands

The activating receptor DNAM-1 (DNAX accessory molecule-1) (also called CD226) was firstly described by Burns and colleagues as a T lineage-specific activation antigen (TLiSA) involved in the differentiation of cytotoxic lymphocytes **(Burns 1985)**. Subsequently, Scott *et al.* described it as a molecule involved in the adhesion of platelet

to endothelium and re-named it PTA1 for platelet and T cell activation antigen 1 **(Scott 1989)**. Some years later, Shibuya characterized an adhesion molecule responsible for CTL and NK cell cytotoxicity and proposed the name DNAM-1 **(Shibuya 1996)**.

This receptor is encoded by a gene mapped to human chromosome 18 and in peripheral blood is expressed on the majority of TCR$\alpha\beta^+$ T cells, TCR$\gamma\delta^+$ T cells, NK cells, monocytes, and on a subset of B cells **(Shibuya 1996)**. DNAM-1 is a type I-transmembrane glycoprotein belong to the Ig-superfamily of receptors, containing two Ig-like domains in its extracellular portion **(Shibuya 1996)**. The cytoplasmic domain of DNAM-1 contains a PDZ-binding motif characterized by a conserved sequence (X-Ser/Thr-X-Val), that binds the afadin PDZ motif (an actin-interacting cytoskeletal linker), or members of the membrane-associated guanylate kinase homolog (MAGUK) family and a binding motif for protein of the band 4.1 family **(Fig. 5)**. The band 4.1 family proteins and MAGUK family proteins are necessary for DNAM-1 association with the integrin LFA-1 (CD11a/CD18) **(Ralston 2004)**. There is a functional relationship between DNAM-1 and LFA-1; LFA-1 association, in fact, is key for DNAM-1 downstream pathway, as suggested by the observation that NK cells and T cells from leukocyte adhesion deficiency (LAD) patients, who lack LFA-1 expression, have functional defects in DNAM-1 signaling **(Shibuya 1999)**. The cytoplasmic tail of DNAM-1 contains three tyrosine residues that can be phosphorylated by the Src-family kinases Fyn upon receptor engagement, and recruits actin-binding proteins **(Shibuya 1996, Shibuya 1999)** strongly triggering NK cell cytotoxicity. LFA-1 is responsible for Fyn activation and subsequent Fyn-dependent phosphorylation of DNAM-1 **(Shibuya 1999)**. Moreover, DNAM-1 intracellular domain contains two phosphorylation sites for PKC and it was demonstrated that PKC plays an important role for DNAM-1 adhesion to its ligands and DNAM-1-mediated signaling **(Shibuya 1998)**.

Bottino and colleagues, not many years ago, identified the human ligands for DNAM-1 among the members of nectin/nectin-like family: the poliovirus receptor (PVR or CD155 or Necl-5) and the Nectin-2 (Nec-2 or CD112 or PRR2 from poliovirus receptor related) **(Bottino 2003)**. DNAM-1 ligands are very closely related molecules encoded by genes located in chromosome 19.

Nectins and nectin-like molecules are a group of Ig superfamily proteins involved in Ca^{2+}-independent cell-cell adhesion and in the formation of adherens junctions between neighboring epithelial cells **(Fuchs 2006)**. Both PVR and Nec-2 are

expressed on epithelial and endothelial cells and are overexpressed by several tumors, suggesting that these proteins may provide an advantage for growth, spreading or metastasis of tumor cells **(Fuchs 2006, Bottino 2003, Tahara-Hanaoka 2004)**. DNAM-1, hence, while in monocytes and other immune cells plays a role in transendothelial migration facilitating adherence to endothelial cells and migration between cell junctions **(Reymond 2004)**, in NK cells can act as a tumor surveillance receptor **(Fuchs 2006)**.

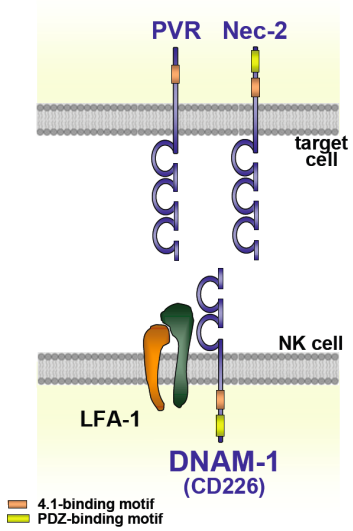


Figure 5. Human DNAM-1 and its ligands.
DNAM-1 and its ligands are transmembrane proteins belonging to the Ig superfamily. In particular, the receptor DNAM-1 presents two Ig-like domains and is characterized by the association with the integrin LFA-1, necessary for signal transduction.
PVR and Nec-2 have very similar structures, with three Ig-like domains. Despite their similarity, they bind to DNAM-1 with different affinity.

PVR is firstly described as the receptor for poliovirus, because of the observation that the anti-PVR antibodies were able to block viral entry into cells **(Mendelsohn 1989, Nobis 1985)**. PVR, like other nectin members, is a transmembrane protein and presents three Ig-like domains in the extracellular portion but, unlike other members of its family, lacks the binding motif for afadin in the intracellular tail **(Fig. 5)**. PVR can also bind the extracellular matrix protein vitronectin, suggesting a role in cell-matrix interaction and cell migration **(Lange 2001)**. PVR contains also an ITIM motif in the cytoplasmic tail that triggers inhibitory signals, and thus promoting cell detachment from the extracellular matrix and migration of tumor cells **(Sloan 2004, Oda 2004)**. PVR can also bind to CD96 receptor (also named Tactile for T cell activation increased late expression) expressed on NK cells, although with less affinity compare to DNAM-1. However, it appears to be involved mostly in cell-cell adhesion rather than in promoting NK cell cytotoxicity **(Fuchs 2004)**.

In addition to the most common transmembrane form of PVR, this ligand could be also expressed as a soluble form. PVR mRNA, in fact, can be spliced into four different isoforms: α and δ variants encode membrane-bound isoforms, while β and γ isoforms encode soluble proteins constituted by only the extracellular portion **(Koike 1990, Baury 2003)**.

Nec-2 is a transmembrane protein characterized by an extracellular portion with three Ig-like domains and a cytoplasmic tail that can bind to afadin **(Fig. 4)**. Like many other proteins of its family, Nec-2 serves as a viral-entry receptor for alpha-herpesvirus **(Fuchs 2006)**.

The murine DNAM-1 (mDNAM-1) binds to murine Nec-2 and the PVR mouse homolog Tage4 **(Tahara-Hanaoka 2005)**.

1.1.7. Expression and regulation of DNAM-1 ligands

Unlike most of the ligands for other NK cell activating molecules, DNAM-1 ligands are expressed on normal cells. PVR is expressed at low levels by cell of epithelial origin and at significant levels on peripheral blood monocytes, where is physical associated with the hyaluronan receptor CD44 **(Freistadt 1997)**, likely enhancing cell migration through both receptors. Nec-2 is ubiquitously expressed in cells of several origins, mostly in epithelial cells, neurons and fibroblasts **(Fuchs 2006)**.

Upon engagement by its specific ligands, DNAM-1 cooperates with other activating NK cell receptors in triggering NK cell cytotoxicity. Although several normal cells express PVR or Nec-2, the inhibitory signals triggered by normal levels of MHC class I overcome the DNAM-1 activating signal. By contrary, in most tumor cells DNAM-1 ligands are upregulated and, at the same time, MHC class I expression is reduced increasing the possibility of recognition as altered cells by NK cells **(Bottino 2003)**.

PVR and Nec-2 are expressed by several tumor cell lines of epithelial or neuronal origin such as carcinomas, melanomas, and neuroblastomas **(Masson 2001, Bottino 2003)**. Castriconi *et al.* showed that freshly isolated primary neuroblastoma cells express PVR and that the PVR expression level correlates with their susceptibility to NK cell lysis suggesting that recognition of PVR by DNAM-1 strongly contributes to

the NK cell killing of neuroblastoma cells **(Castriconi 2004)**. Moreover, high expression of PVR and Nec-2 was, also, detected on myeloid leukemia cells and it was demonstrated to be necessary in triggering an efficient *in vitro* NK cell lysis of AML cells **(Pende 2005)**. Nec-2 was found highly expressed on human gastric and colon cancer and in the same paper it was demonstrated that ectopic expression of DNAM-1 ligands in tumor cells triggered a strong and rapid *in vivo* anti-tumor immune response and, consequently, tumor rejection **(Tahara-Hanaoka 2006)**. Moreover, Sloan *et al.* demonstrated that PVR is highly expressed in primary glioblastoma cells and that PVR expression confers to these cells the ability to migrate and invade other tissues, thus, contributing to tumorigenesis **(Sloan 2004)**.

Anti-tumor activity of DNAM-1 is confirmed also by data obtained with DNAM-1 deficient mice in which tumor surveillance is significantly impaired and in which DNAM-1 ligand expressing fibrosarcoma and papilloma cells developed significantly more as compared to WT mice (in response to chemical carcinogens) **(Gilfillan 2008, Iguchi-Manaka 2008)**.

The mechanisms regulating DNAM-1 ligand expression are almost completely unknown. The only evidences about the transcriptional machinery involved in PVR expression are by Solecki *et al.* that analyzed PVR transcription with a particular attention to the embryogenesis and to the nervous system development. The authors proposed that nuclear respiratory factor-1 (Nrf-1) is involved in regulating PVR expression at specific stages of central nervous system development **(Solecki 2000)**. Later, they also analyzed the relation between PVR and Sonic hedgehog (Shh), co-localized during embryonic development, and demonstrated that *PVR* gene is a transcriptional target of the Shh signaling cascade and *PVR* promoter contains a GLI binding site. Moreover, they suggest a role for Shh activation in inducing PVR expression on medulloblastoma and glioblastoma cells **(Solecki 2002)**.

The possibility of producing soluble ligands is another important mechanism regulating PVR expression. Soluble PVR was found in human serum, likely produced mostly by the liver, but its function remains unknown **(Baury 2003)**. Soluble PVR binds to DNAM-1 with the same affinity of transmembrane PVR, but does not induce receptor crosslinking and signal transduction. Soluble PVR, similar to soluble NKG2D ligands, could be produced by alternative splicing **(Baury 2003)** or by proteolytic cleavage by members of ADAM family, but currently the knowledge about this and

other mechanisms of DNAM-1 ligand regulation is still very poor and many other studies need to be performed.

1.2. Chemo-immunotherapy

Conventional chemotherapeutic drugs act mostly by disrupting regulatory pathways essential for tumor growth and survival but their activity is too often limited by mechanism of drug resistance and toxic side effects directed to normal host tissue. Moreover, conventional chemotherapy does not take care of effects that could induce in tumor microenvironment established by the host-tumor interaction. At present, it is largely accepted that the host-tumor interaction is the major determinant of clinical course and treatment outcomes of cancer. Among the host elements that respond to cellular transformation, stromal fibroblasts, endothelial cells, tumor-associated macrophages, NK cells, and tumor-specific lymphocytes are critical for tumor growth and progression. It is now emerging the idea that several common used chemotherapeutic drugs are able to modulate these cells favoring their anti-tumor effects **(Emens 2010, Zitvogel 2008)**.

Standard and high dose chemotherapy regimens are often immunosuppressive and induce lymphopenia, but the effects of their immunomodulatory potential could be changed by using different doses and schedule. For an instance, a single low dose of cyclophosphamide 1-3 days before antigen exposure promotes humoral and cellular immunity, while cyclophosphamide given at the same time or subsequently to antigen exposure induces immune tolerance **(Emens 2010, Zitvogel 2008)**.

Current data suggest that chemo-immunotherapy regimens have great potential for optimizing the clinical outcomes of cancer patients and in last years the scientific community is making headway in this direction.

1.2.1. NK cell-based strategies against tumors

The better knowledge of NK cell and NK cell mechanisms of activation permits

to design and improve chemo-immunotherapeutic protocols finalized to 'wake up' NK cell cytotoxicity against tumor cells.

All along NK cells are described for their ability to kill tumor cells in the absence of previous stimulation, but their manipulation to create NK cell-based therapies was very difficult until the mechanisms of their activation remained unknown **(Ljunggren 2007)**. In the early 1980s, Grimm *et al.* showed the possibility, by stimulation of the PBMC with IL-2, of generating lymphokine-activated killer (LAK) cells able to lyse human fresh tumor cells **(Grimm 1982)**, phenomenon mediated mostly by NK cells. Unfortunately, ten years later, all the studies published about clinical trials using LAK therapy in cancer showed a clinical response rate of about 15-20%, a rate not superior to IL-2 monotherapy or IL-2 combined with IFN-α. The clinical results did not improve either using a large number of LAK cells nor using highly purified activated NK cells **(Rosenberg 1985, Law 1995, Lotze 1986)**.

NK cell-based adoptive transfer therapies are widely studied but several issues need to be still addressed before the development of a successful protocol. The choice of a specific NK cell subset to be infused or of the *ex vivo* NK cell activation protocol and the KIR genotypes. The activating receptor haplotypes of the donor may also affect the outcome **(Terme 2008)**.

Antibodies targeting tumor antigen in combination with adjuvants is a useful strategy, mostly against tumors in which susceptibility to NK cell lysis is reduced (ALL, CLL, lymphoma), to activate antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity **(Carter 2006)**. Dimeric or trimeric bispecific antibodies that recognize both tumor antigen and an activating NK cell receptor could also be used to trigger a stronger response **(Shahied 2004)**.

Vaccines based on dendritic cell preparations **(Chaput 2004)**, on molecules activating NK cell response (for example heat shock proteins or TLRs) **(Sivori 2004)**, or on therapeutic compounds that indirectly enhance NK cell response (such as thalidomide in multiple myeloma therapy) **(Davies 2001)** could also be prepared to boost NK cell activity in patients.

Several mediators (for example TGF-β, soluble NKG2D and DNAM-1 ligands, HLA-G molecules, regulatory T cells) negatively affect NK cell functions; it could be important to neutralize them. In addition, at the same time, the development of protocols aimed at prompting NK cell trafficking towards tumor or secondary lymphoid organs could be an efficient strategy.

Moreover, for any NK cell-based strategy it could be also very important to analyze NK cell effects on adaptive immune responses.

Many tumors express NK cell activating ligands, but why are they not rejected by NK cells *in vivo*? NK cells might not be in sufficient amount in the transformed tissue, or not efficiently recruited to the primary tumor site to have a significant effect or, often, tumors develop several strategies that allow them to escape from NK cell recognition and killing. NK cell activating receptors can be modulated or desensitized after interaction with ligand-expressing tumors or with soluble ligands released by tumor cells **(Groh 2002, Baury 2003)**. Loss of adhesion molecules on NK cell or on tumor cell might be an important mechanism of tumor escape because the conjugate formation between NK cell and tumor cell is a crucial step for NK cell activation **(Maki 1998)**. Moreover, some tumors secrete immunosuppressive cytokines, such as TGF-β and IL-10, or produce apoptotic mediators on their own, such FASL and NO.

Once the tumor is established, its growth rate might exceed the ability of NK cells to respond, and tumor cells might become resistant to NK cell effector mechanisms. So, the immunomodulatory capacity of the chemo-immunotherapy could be a necessary strategy for overcoming the mechanisms of tumor escape from immune cell system and reawaking the natural immune system ability of fighting tumors.

1.2.2. Chemotherapy-induced senescence

Cellular senescence is an *in vivo* mechanism for arresting the proliferation of potential cancer cells. Senescent cells are viable cells that irreversibly stop to synthesize DNA and that present characteristic morphological features (flattening, granularity, and vacuole-rich cytoplasm), biochemical changes (senescence associated-β-galactosidase activity, senescence and DNA damage marker expression), and chromatin remodelling **(Schmitt 2007)**.

There are several evidences demonstrating the wide presence of senescent cells in premalignant lesions while the scarce presence in developed tumors **(Braig 2005, Chen 2005, Collado 2005)**. The senescence program, in fact, depends critically on the p53 and Rb/$p16^{INK4a}$ tumor suppressor pathways and is involved in oncogene

repression. Defects in tumor suppressor pathways compromise cellular ability to undergo senescence, and greatly increase susceptibility to cancer **(Collado 2006, Rodier 2011)**.

Interestingly, some tumor cells retain the ability to senesce and could do so in response to chemotherapy. Anticancer drugs, in fact, can trigger an acutely inducible form of cellular senescence, morphologically and biochemically related to classical replicative senescence, termed premature senescence.

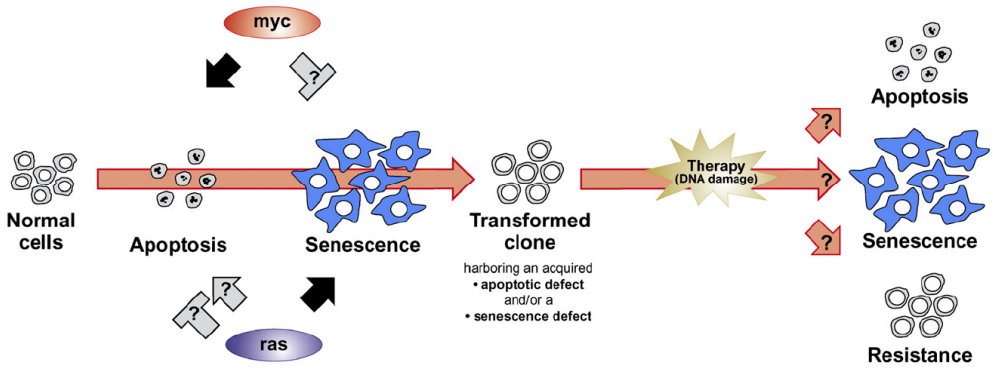


Figure 6. Chemotherapy-induced senescence (from *Schmitt 2007*).
Chemotherapeutic treatments could reawake the anti-tumor mechanism of cellular senescence that was overcome by malignant transformation.

A better knowledge of drug-induced senescence comes from genetic analysis of oncogene-induce senescence, because it is likely that both mechanisms act trough genotoxicity. DDR has been shown to play a critical role in chemotherapy-induced senescence **(Schmitt 2007)**. Among the several chemotherapeutic drugs, the topoisomerase I inhibitor camptothecin **(Hayward 2003)**, the topoisomerase II inhibitor doxorubicin **(Elmore 2002)**, the DNA-crosslinker cisplatin **(Chang 1999)**, γ-irradiation **(Mirzayans 2005)**, and the anti-metabolite cytarabine **(Rosenbeck 2011)** are promising DNA damaging agents for inducing senescence. Schwarze *et al.* showed that lower doses of chemotherapeutic agents are more efficient in inducing the senescent phenotype in cancer cells whereas higher concentrations were associated with apoptosis **(Schwarze 2005)**. Interestingly, te-Poele and colleagues, analyzing sections from breast tumors of patients treated with doxorubicin, 5-fluorouracil, and cyclophosphamide, observed senescence associated-β-galactosidase staining in tumor cells but not in

normal tissue, suggesting that chemotherapy-induced senescence is a specific response of tumor cells **(te Poele 2002)**. Oxidative stress is also a mediator of cellular senescence. Several entrinsic or intrinsic stimulus producing a rise in intracellular ROS levels trigger senescence entry. Oxidants may directly activate redox-sensitive pathways linked to senescence or, alternatively, may induce a spectrum of damage to cellular components, such as the DNA damage, that directly leads to senescence **(Lu 2008)**.

In addition to the effect of tumor cell proliferation arrest, the senescence response triggers an inflammatory status that stimulates the immune cells to eliminating the senescent cells. Krizhanovsky and colleagues found that, in response to a liver damage to produce liver fibrosis in mouse, hepatic stellate cells entered in a senescence program. Moreover, the accumulation of senescent cells triggered an enhanced immune surveillance and NK cells preferentially killed senescent stellate cells facilitating the resolution of fibrosis **(Krizhanovsky 2008)**. Recently, with a model of murine hapatocellular carcinoma, it was demonstrated that oncogene-induced senescence occurs in pre-malignant hepatocytes making them subjected to an immune-mediated clearance mediated by $CD4^+$ T cell and monocytes/macrophages. It was, also, shown that an impaired immune surveillance of pre-malignant senescent hepatocytes results in the development of the full-blown tumor **(Kang 2011)**.

Of note, it is now emerging the concept that cellular senescence, in addition to suppress tumorigenesis, is also involved in tumor promotion, tissue repair, and aging **(Rodier 2011)**. So, when developing protocols of chemotherapy-induced senescence, it could be very important to consider each aspect of this phenomenon.

1.3. Multiple Myeloma

Multiple Myeloma (MM) is a malignant plasma cell (PC) disorder that usually evolves from an asymptomatic premalignant stage characterized by the proliferation of clonal PCs and designated as ‘monoclonal gammopathy of undetermined significance’ (MGUS). MM is a very common neoplasia among the adults; infact, more than 3% of the population over 50 years old is affected by MGUS, and 1% per year progresses to MM or related malignancies. Although MM is a very long studied malignancy (the first

documented case was in 1844; **Fig. 7**), is still incurable and, with the conventional treatments, the prognosis is 3-4 years, which may be extended to 5-7 years with newer treatments **(Kyle 2008, Palumbo 2011)**.

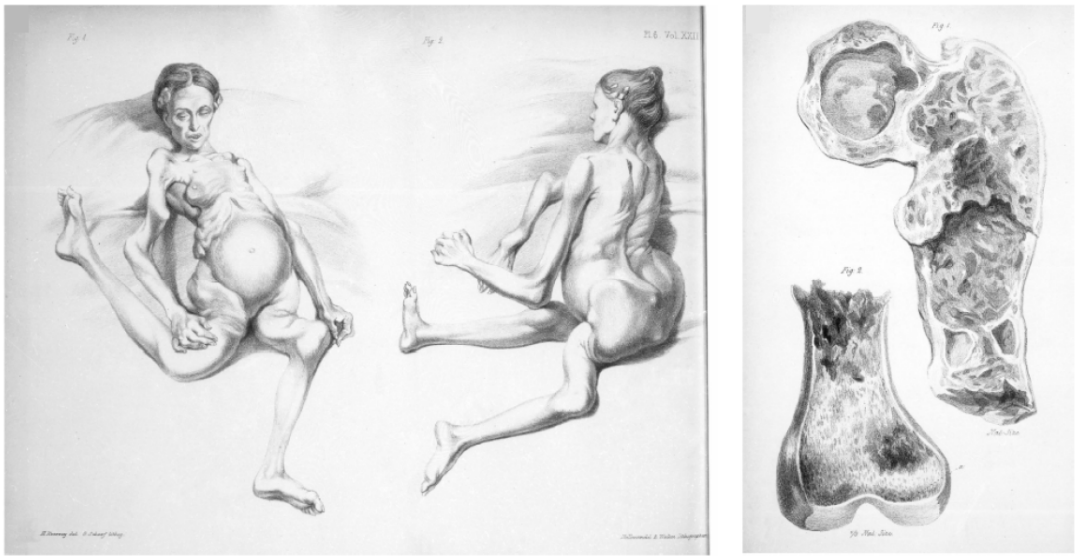


Figure 7. The first reported patient with multiple myeloma (from *Kyle et al. 2008*).

At the end of 1800, PCs were well described as cells with blocked chromatin, eccentric position of the nucleus, a perinuclear pale area, and a spherical or irregular cytoplasm. Later, with the introduction of bone marrow aspiration, the recognition of MM, and also the amount of reported cases, increased **(Kyle 2008)**.

Malignant PCs accumulate in bone marrow, causing bone lesion and subsequent bone fracture and pain **(Fig. 7)**, and interfering with normal hematopoiesis. The breakdown of bones also leads to release of calcium into the blood, resulting in hypercalcemia and its associated symptoms. The presence, in blood and urine, of a monoclonal immunoglobulin called 'Bence Jones protein' is a typical feature of MM patients. This protein is responsible, together with the hypercalcemia, of the onset of kidney injuries. In theory, malignant PCs can produce all classes of immunoglobulin, but IgG are most common, followed by IgA and IgM. IgD and IgE myeloma are very rare. PCs infiltration into the bone marrow and renal dysfunction often cause severe anemia in these patients **(Palumbo 2011)**.

The pathogenesis of bone disease involves an increase in RANKL (receptor activator of NF-κB ligand) accompanied by a reduction in the level of its decoy receptor osteoprotegerin and by the action of macrophage inflammatory protein 1α (MIP1α), resulting in sustained osteoclast activation **(Rajkumar 2011)**.

In the cellular bone marrow compartment, MM cells interact with hematopoietic and non-hematopoietic cells. Bone marrow stromal cells send signals (directly through cell-cell contact or indirectly through secretion of soluble factors), which affect malignant PC growth, survival, migration, and drug resistance. Moreover, integrin-mediated adhesion of MM cells to bone marrow cells induces upregulation of cell cycle regulatory proteins, antiapoptotic proteins, and telomerase activity in PCs, and NF-κB-dependent transcription and secretion of IL-6 in bone marrow cells. IL-6 secreted by bone marrow cells enhances the production and secretion of VEGF by myeloma PCs, and viceversa **(Palumbo 2011)**.

Malignant PCs are terminally differentiated cells but the tumor is maintained by a very small subset of MM stem cells, which reside in either the osteoblastic or the endothelial niche, and can self-renew and differentiate **(Peacock 2007)**.

Patient survival depends on many different parameters such as age, performance status, renal function, and disease stage. Two methods are used to classified MM patients, the Durie-Salmon Stage **(Durie 1975)** and the International Staging System **(Greipp 2005)**, but both have several limitations because this malignancy is characterized by a broad molecular heterogeneity, both among patients and in each patient over time. Although is considered to be a single disease, it consists of at least six non-overlapping cytogenetic subtypes, evident early in the course of the disease, based on cytogenetic abnormality observed in PCs. So, to define the outcome for each individual patient, it is fundamental also to consider the individual cytogenetic abnormalities **(Rajkumar 2011)**.

All MM patiens, also many years before the diagnosis of full-blown myeloma, have the prolonged premalignant asymptomatic phase MGUS or a more advanced indeterminate stage termed smoldering MM **(Kyle 2007)**. MGUS is characterized by abnormal immunoglobulins detectable in patient peripheral blood and/or urine, as well as clonal PCs present in the bone marrow. Smoldering MM is characterized by a much higher risk of progression to MM: the first 5 years after smoldering MM diagnosis, the risk of progression to MM is 10% per years but decrease significantly over time. All

MGUS and smoldering MM patients are clinically followed without treatment until progression to save them from the toxicity of chemotherapeutic drugs **(Rajkumar 2011)**. However, in the last years, many efforts have been made to better understanding the molecular mechanisms and the probability of progression of these premalignant PC disorders with the aim to identify those patients who may benefit from early treatments **(Korde 2011, Landgren 2011)**.

Actually, the most common strategies in MM therapy are protocols based on the combination of 2-3 chemotherapeutic drugs such as bortezomib, dexamethasone, thalidomide, vincristine, doxorubicin, melphalan, and prednisone. Only few patients are candidates for allogeneic transplantation because of age, availability of a HLA-matched sibling donor, and adequate organ function **(Rajkumar 2011)**. In the last years, the overall survival of MM patients is increased, but however almost all patients relapse. So, it is still necessary to find new powerful strategies to contrast this tumor.

1.3.1. NK cell activity against multiple myeloma

The anti-tumor action of NK cells is widely accepted and, of note, bone marrow is one of the anatomic districts where the highest number and activity of NK cells are present. Moreover, in the last years, several new evidences are emerged about the role of NK cells against MM tumor cells suggesting the possibility of developing new chemo-immunotherapeutic approaches to fight this malignancy.

Carbone and colleagues demonstrated that bone marrow-derived PCs from MM patients were more susceptible to NK cell lysis as compared to myeloma cells from pleural effusion, a very late stage of the malignancy. Susceptibility to NK cell lysis of bone marrow-derived MM cells was dependent to low MHC class I molecule expression and to the presence of the NK cell activating ligand MICA on the surface of these cells. They suggested that pleural effusion-derived MM cells were the result of a selection process based on NK cell activity. In MM the initial tumor population is controlled by NK cells but, during tumor progression, a clonal variant with defective MICA transcription could emerge and escape from NKG2D-mediate immunosurveillance **(Carbone 2005)**. Recently, Jinushi *et al.* provided futher explanations to the immune-escape phenomen. He showed that alterations in MICA expression are also associated

with the progression from MGUS to MM demonstrating that MM patients have a lower level of MICA expression on PCs compare to MGUS patients because they express large amount of the disulfide isomerase Erp5, responsible of MICA shedding, and hence of sMICA in serum. In contrast, MGUS patients produce high-titer of anti-MICA antibodies that antagonize the immune suppressive activity of sMICA **(Jinushi 2008)**. Another group found that the DNAM-1 ligands PVR and Nec-2 are often expressed by bone marrow-derived MM cells and that these malignant PCs can be killed by NK cells in a DNAM-1-dependent manner **(El-Sherbiny 2007)**. Moreover, Davies *et al.* showed that the anti-myeloma effect of thalidomide and its immunomodulatory derivatives mights be due, at least in part, to a modulation of NK cell number and activity **(Davies 2001)**. All together, these findings strongly support the idea that NK cells efficiently, but not always, kill MM cells and try to counter the onset of this tumor.

2. AIM

It is, now, widely accepted the key role of NK cells as effector cells against tumors. Among the several receptors by which NK cells recognize transformed cells, NKG2D and DNAM-1 have a relevant role. Expression of NKG2D or DNAM-1 ligands by tumor cells results in immune destruction *in vivo*. Moreover, tumor surveillance is strongly impaired in NKG2D or DNAM-1 deficient mice. Tumor cells naturally express NKG2D or DNAM-1 ligands but, often, they do not trigger a sufficient NK cell response to eliminate the tumor or they evolve mechanisms to evade from the immune system. In this context, it could be a useful strategy developing protocols aimed at increasing the density of NKG2D and DNAM-1 ligands on tumor cells to efficiently activate antitumor NK cell response.

One of the pathways mostly involved in the induction of NKG2D ligands is the DNA damage response; there are, instead, little information on the mechanisms controlling the induction of DNAM-1 ligands. Of note, several agents commonly used in classical chemotherapy carry out their effects causing DNA damage.

All together, these findings encourage to research the right way of using the chemotherapeutic drugs for enhancing the NK cell response against MM cells and to understand how they might exert their activity.

This research was aimed at investigating new chemo-immunotherapeutic approaches based on the exposure of MM cells to not toxic doses of chemotherapeutic drugs to up-regulate NKG2D and DNAM-1 ligands, in order to render them more susceptible to the NK cell lysis. We tested several chemotherapeutic agents in order to detect a drug that, used at low doses, could induce NK cell activating ligand up-regulation on MM cell lines and malignant PCs from bone marrow aspirates of MM patients and could increased NK cell degranulation towards ligand expressing cells.

In addition, we dissected the molecular events underlying chemotherapy-induced ligand expression. In particular, we analyzed the role played by the several components of the DNA damage pathway. Moreover, given the role of the DNA damage response in controlling cellular proliferation and senescence, we investigated the presence of senescent cells among the MM cells upon the treatment with low doses of chemotherapeutic drugs. Finally, in accordance with the evidences on the induction of oxidative stress by several chemotherapeutic agents, we evaluated the redox status in

drug-treated MM cells and the involvement of oxidative stress production in drug-induced NKG2D and DNAM-1 ligand expression.

With these findings, we provided new informations on the regulation of the expression of NKG2D and DNAM-1 ligands and we suggested new approaches to stimulate their expression on MM cells.

3. MATERIALS AND METHODS

3.1. Cell lines and clinical samples

The human MM cell lines ARK, LP-1, OPM-2, RPMI-8226, SKO-007(J3), and U266 were kindly provided by Prof P. Trivedi ('Sapienza' University of Rome). The cell lines were maintained at 37°C and 5% CO_2 in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 15% Fetal Calf Serum (FCS). All cell lines were mycoplasma-free (EZ-PCR Mycoplasma Test Kit; Biological Industries, Haemek, Israel).

Peripheral blood and bone marrow samples from untreated patients with MM were managed at the Institute of Hematology ('Sapienza' University of Rome). Informed consent was obtained from all patients, and approval was obtained from the Ethics Committee of the 'Sapienza' University of Rome. Patients were classified according to Durie-Salmon and International Staging System **(Table 1)**. The bone marrow aspirates were lysed to obtain Bone Marrow Mononuclear Cells (BMMCs) using a buffer composed of 1.5M NH_4Cl, 100mM $NaHCO_3$, and 10mM ethylenediaminetetraacetic acid (EDTA).

Patient no.	Sex/Age	Clinical Stage	Monoclonal Ig	%PC in BM
1	F/78	III/onset	micro-κ	40
2	F/76	smoldering	IgG-λ	20
3	M/65	smoldering	IgG-κ	10
4	F/74	smoldering	IgG-κ	18
5	F/71	smoldering	IgG-κ	16
6	M/79	II/onset	IgA-κ	19
7	F/80	I/onset	IgA-κ	20
8	M/75	I/onset	IgG-κ	24
9	M/72	I/onset	IgG-κ	21
10	F/58	I/onset	IgG-κ	8
11	M/82	III/onset	IgG-κ	31
12	F/64	I/smoldering	IgG-λ	33
13	F/61	III/onset	micro-κ	64
14	M/66	I/smoldering	IgA-κ	33
15	F/77	III/relapse	IgG-κ	79
16	M/68	III/onset	IgG-κ	50
17	F/73	I/onset	IgE-λ	37
18	F/43	III/onset	IgG-κ	30

Table 1. Patient characteristics.

In some experiments, and anti-CD138 magnetic beads (Miltenyi Biotec, Auburn, CA) were used to separate malignant myeloma PCs from BMMCs. More than 95% of the purified cells were $CD138^+$. Bone marrow-derived mononuclear cells or separated $CD138^+$ PCs were maintained at 37°C and 5% CO_2 in complete medium supplemented with 20ng/mL human recombinant IL-3 and 2ng/mL human recombinant IL-6 (Pepro-Tech, Rocky Hill, NJ).

3.2. Antibodies and reagents

The following unconjugated monoclonal antibodies (mAbs) were used for immunostaining: anti-MICA (MAB159227), anti-MICB (MAB236511), anti-ULBP1 (MAB170818), anti-ULBP2 (MAB165903), anti-ULBP3 (MAB166510), and anti-NKG2D (149810) from R&D Systems (Minneapolis, MN); anti-Nec-2 (R2.525) from BD Pharmingen (San Diego, CA); anti-DNAM-1 (DX11) from Serotec (Oxford, United Kingdom); anti-PVR (46.31 or SKII.4) kindly provided by Prof M. Colonna (Washington University, St Louis, MO); MHC class I (W6/32) from ATCC (Manassas, VA); anti-ATMSer1981, anti-p85 (10H11.E12) were purchased from Millipore (Billerica, MA); anti-p53Ser15 and phospho-Chk1/2 Ab Sampler Kit were purchased from Cell Signaling Technology (Danvers, MA); anti-p53 (DO-1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-β-actin (AC-15) and anti-mouse IgG1 (MOPC-21) were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated Goat affinity purified $F(ab')_2$ fragment to Mouse IgG (GAM) was purchased from MP Biomedicals (Solon, OH), allophycocyanin (APC)-conjugated GAM and R-phycoerythrin (PE)-conjugated GAM were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-γH2AX FITC (JBW301) was purchased from Millipore. APC-conjugated anti-CD38 (HIT2), anti-CD138/FITC (MI15), anti-CD56/FITC (NCAM16), anti-CD16/PE (332779), anti-CD107a/FITC (H4A3), and anti-CD138/PerCP-Cy5.5 (MI15) were purchased from Becton Dickinson. Anti-CD3/APC (HIT3a), anti-CD56/PE (HCD56), anti-mouse IgG1/FITC, IgG1/PE, or IgG1/APC (MOPC-21) were purchased from BioLegend (San Diego, CA).

Propidium Iodide (PI), Bafilomycin A1, the ATM/ATR pharmacologic inhibitor Caffeine and KU-55933 and the ROS scavenger N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich. 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) was from Invitrogen (Frederick, MD). The Chk1/2 pharmacologic inhibitors SB218078 and UCN-01 were purchased from Calbiochem, EMD Chemicals (Darmstadt, Germany). The p53 pharmacologic inhibitor Pifithrinα (PFTα) was purchased from Biomol, Enzo Life Sciences (Farmingdale, NY).

3.3. Drug treatment

MM cell lines were cultured for a maximum of 72h in U-bottom 96-well tissue culture plates at 37°C and 5% CO_2 at different cellular densities in the absence or presence of different drug concentrations. The following therapeutic drugs were tested: cisplatin, doxorubicin, melphalan, etoposide, and bortezomib. On the day of the assay, 10μL MTT (5mg/mL) (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) was added to each well, and cells were incubated for 3h at 37°C and 5% CO_2. After blocking the reaction and making the crystals soluble with isopropanol/HCl 0.04N, samples were moved into a flat-bottom 96-well tissue culture plate, and the absorbance was read with an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. Dose-response curves were calculated and an IC50 value (concentration of drug resulting in 50% inhibition of cell growth) was obtained. IC50 values or concentrations 10 times lower were used to treat the different cell lines, as follows: doxorubicin: ARK (0.06μM), LP-1 (0.06μM), OPM-2 (0.08μM), RPMI-8226 (0.05μM), SKO-007(J3) (0.05μM), U266 (0.1μM); etoposide: ARK (0.4μM), LP-1 (3.5μM), OPM-2 (0.7μM), RPMI-8226 (0.1μM), SKO-007(J3) (0.3μM); melphalan: ARK (7μM), LP-1 (21.5μM), OPM-2 (1.6μM), RPMI-8226 (1.5μM), SKO-007(J3) (22μM), U266 (15μM); bortezomib: ARK (1.4nM), LP-1 (0.95nM), OPM-2 (0.75nM), RPMI-8226 (1nM), SKO-007 (J3) (4.9nM); cisplatin: ARK (14μM), LP-1 (1μM), OPM-2 (9.4μM), RPMI-8226 (7μM), SKO-007(J3) (9.4μM). Patient-derived PCs were incubated with melphalan (20μM), doxorubicin (0.05μM), or bortezomib (5nM) for 48h at 37°C and 5% CO_2. Cell lines and patient-derived PCs were cultured in tissue culture plates at a density of $3x10^5$ and $5x10^5$, respectively.

In some experiments, cells were pre-treated for 1h with the ROS scavenger NAC (10mM for cell lines and 2 mM for patient-derived PCs) or the following pharmacologic inhibitors: caffeine (1mM), KU-55933 (10μM), SB218078 (1μM), UCN-01 (20nM), and PFTα (30μM). A dose-response curve was performed with these pharmacological inhibitors to identify the doses that did not affect cell viability.

3.4. Immunofluorescence and flow cytometry

The expression of the DNA damage marker γH2AX on SKO-007(J3) cell line was evaluated upon drug treatment through the staining with FITC-conjugated anti-γH2AX Ab and FACS analysis. After washing, cells were fixed and permeabilized with 70% ethanol, and incubated with anti-γH2AX mAb. NKG2D and DNAM-1 ligand surface expression on MM cells and patient-derived PCs was analyzed by immunofluorescence staining using anti-MICA, anti-MICB, anti-ULBP1/2/3, anti-PVR or anti-Nec2 unconjugated mAbs, followed by secondary GAM-FITC or GAM-APC for cell line or GAM-PE mAb for patient PCs. In some experiments, cells were stained with PI (1μg/μL) to assess cell viability. The analysis of ligand expression on patient-derived PCs, when treated without performing magnetic separation, was performed by gating on the $CD38^{+}CD138^{+}$ PC population. Samples were analyzed using a FACS Calibur (BD Biosciences, San Jose, CA).

Samples from experiments performed with MM cell lines were analyzed using a FACS Calibur and samples from experiments with patient PCs with a FACS Canto II (BD Biosciences, San Jose, CA). Flow cytometric analysis was performed using the FlowJo software version 8.8.7 (TreeStar, Ashland, OR).

NKG2D and DNAM-1 ligand expression was also evaluated at different cell cycle phases. SKO-007(J3) cell cycle distribution was analyzed by PI staining after 72h drug treatment. Cells were incubated, firstly, with anti-MICA, anti-MICB or anti-PVR unconjugated mAbs and, then, with FITC-conjugated GAM secondary Ab. Cells were washed in PBS with 0.1% sodium azide and fixed for 2h at 4°C in cold 70% ethanol. Thereafter, cells were incubated for 30 min at room temperature with 50μg/mL PI in PBS containing 40μg/mL RNAse (Sigma-Aldrich) and immediately analyzed using a

FACS Calibur. Flow cytometric analysis was performed using the FlowJo software version 8.7.7.

3.5. Degranulation assay

NK cell-mediated cytotoxicity was evaluated using the lysosomal marker CD107a as previously described **(Bryceson 2005)**. As source of effector cells, we used peripheral blood mononuclear cells (PBMCs) isolated from healthy donors by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and then cocultured for 10 days with irradiated (30 Gy) Epstein-Barr virus (EBV)-transformed B-cell line RPMI 8866 at 37°C in a humidified 5% CO_2 atmosphere, as previously described **(Mainiero 2000)**. On day 10, the cell population was routinely more than 90% $CD56^+CD16^+CD3^-$, as assessed by immunofluorescence and flow cytometry analysis. NK cells were activated overnight with 200U/mL human recombinant IL-2 (R&D Systems). When patient-derived PCs were used as targets, autologous PBMCs were cultured for 2 days in complete medium supplemented with 100U/mL IL-2. Drug-treated MM cell lines or patient-derived PCs were incubated with activated NK cells at different effector-target (E/T) ratios, from 10:1 to 1:1, in a U-bottom 96-well tissue culture plate in complete medium at 37°C and 5% CO_2 for 2h. Thereafter, cells were washed with PBS and incubated with anti-CD107a/FITC (or cIgG/FITC) for 45 min at 4°C. Cells were then stained with anti-CD3/APC, anti-CD56/PE (or anti-CD16/PE), and anti-CD138/PerCP-Cy5.5 to gate the $CD3^-CD56^+CD16^+CD138^-$ NK population. In some experiments, cells were pretreated for 20 min at room temperature with anti-NKG2D or anti-DNAM-1 neutralizing mAbs and with anti-CD56 or anti-MHC I as control Abs. Analyses were performed using a FACS Calibur.

3.6. Analysis and isolation of senescent cells

We performed the Senescence Associated β-Galactosidase assay (SA-βGal) by both microscope and flow cytometer. Forty-eight hour melphalan- or doxorubicin-

treated MM cells were cultured for further 24h without drug before performing the classic microscope assay. Cells were then fixed for 5 min at room temperature in 3.6% formaldehyde and incubated overnight at 37°C without CO_2 with fresh SA-βGal stain solution: 1mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), 150mM NaCl, 2mM MgCl2, 40mM citric acid, 5mM sodium phosphate (pH 6.0), 5mM potassium ferrocyanide, and 5mM potassium ferricyanide. Senescent cells were identified as blue-stained cells by standard light microscopy. Cells were acquired and analyzed by an Olympus BX51 microscope (Hamburg, Germany) and IAS 2000 software (Biosystem, Rome, Italy) using original magnification 200X/0.50.

We performed the assay using the fluorogenic substrate C_{12}FDG to measure β-galactosidase activity by flow cytometry **(Kurz 2000)**. This compound is a membrane-permeable, nonfluorescent substrate of β-galactosidase, which after hydrolysis of the galactosyl residues emits green fluorescence and remains confined within the cell. High levels of acid lysosomal β-galactosidase present in MM cells of all replicative ages would mask the detection of senescence-dependent activity in live cells. Therefore, drug-treated cells were incubated 1h at 37°C and 5% CO_2 with 100nM bafilomycin A1 in culture medium to induce lysosomal alkalinization at pH 6 and, then, for 1h with 33μM C_{12}FDG. The specific activity of the enzyme at pH 6 is low in proliferating cells, whereas senescent cells show highly positive staining. Subsequently, surface staining for MICA, MICB, and PVR was performed as previously described and samples were immediately analyzed using a FACS Calibur. The C_{12}-fluorescein signal was measured on the FL-1 detector, and β-galactosidase activity was estimated using the median fluorescence intensity (MFI) of the population.

In some experiments 72h doxorubicin-treated SKO-007(J3) cells were incubated with C_{12}FDG as previously described, and βGal^{low} from βGal^{high} MM cells were isolated by a FACS Aria cell sorter (BD Biosciences) through FL-1 fluorescence emission.

3.7. RT-PCR

One microgram of total RNA was isolated using TRIzol reagent (Invitrogen) and used for cDNA first-strand synthesis in a 25μL reaction volume; 1μL of the resulting

cDNA was used in a 25μL PCR reaction in the presence of FastStart Taq DNA polymerase (Roche). Forward and reverse primers for polymerase chain reaction (PCR) amplification were, respectively: 5'-TGCTTCTGGCTGGCATCTTCC-3' and 5'-TAGTTCCTGCAGGCAGTC-TGC-3' for MICA; 5'-TCTCGCTGAGGGACATCTGGA-3' and 5'-CAGGTCTGGTAGGTTCCATTC-3' for MICB; 5'-GAGGTGACGCATGTGTCACAG-3', 5'-TCTTGCCGTCCACCTGGCTTG-3' for PVR; 5'-ACCTGACTGACTACCTCATG-3' and 5'-GCAGTGATCTCCTTCTGCAT-3' for β-actin.

PCR conditions were as follows: 94°C for 50 seconds, 58°C for 50 seconds, and 72°C for 50 seconds for 28-32 cycles.

3.8. Real Time PCR

MICA, MICB and PVR mRNA expression was analyzed by Real Time PCR. Total RNA from MM cell lines or from patient malignant PCs was extracted using Trizol (Invitrogen) after 24h of drug treatment. Total RNA (1μg) was used for cDNA first-strand synthesis using oligo-dT (Promega, Madison, WI) in a 25μL reaction volume.

Real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA). To analyze ligand mRNA expression, the cDNA was amplified in triplicate with the following primers: Hs00792952_m1 for MICA, Hs00792952_m1 for MICB, Hs00197846_m1 for PVR, and 4326315E for β-actin all conjugated with fluorochrome FAM, excepted for the β-actin conjugated with fluorochrome VIC (Applied Biosystems, Foster City, CA).

3.9. SDS-PAGE and Western Blot

Drug-treated SKO-007(J3) and U266 cells were lysed for 20 min at 4°C in ice-cold lysis buffer containing 0.2% Triton X-100, 0.3% NP40, 50mM Tris HCl pH 7.6, 1mM EDTA, 150mM NaCl, 10μg/mL leupeptin, 1mM PMSF, 10μg/mL aprotinin,

10mM NaF and 1mM Na_3VO_4 to detect phospho-ATM and p85 or in ice-cold lysis buffer containing 1% Triton X-100, 0.5% DOC, 0.1% SDS, 50mM Tris HCl pH 7.5, 1mM EDTA, 1mM EGTA, 50 mM $Na_4P_2O_7$, 150mM NaCl, 5 mM $MgCl_2$, 10μg/mL leupeptin, 1mM PMSF, 10μg/mL aprotinin, 100mM NaF and 1mM Na_3VO_4 to detect p21, phospho-p53, p53, phospho-Chk1/2, and β-actin. In experiments directed to detect ATM phosphorylation, cells were mantained in RPMI 1640 with 2% FCS 24h at 37°C before drug treatment. The Bio-Rad Protein Assay (Bio-Rad Laboratories; Hercules, CA) was used to measure protein concentration. Eighty μg of total lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore) or nitrocellulose membranes (Whatman GmbH; Dassel, Germany). After blocking with BSA, membranes were probed with specific purified antibodies. A Horseradish Peroxidase (HRP)-conjugated secondary antibody and an enhanced chemiluminescence kit (Amersham, GE Healthcare; Buckinghamshire, UK) were used to reveal immunoreactivity.

4. RESULTS

4.1. The DNAM-1 and NKG2D ligands are up-regulated on human MM cell lines by low doses of therapeutic agents

It has been recently shown that the expression of NKG2D ligands on human fibroblasts can be up-regulated by chemotherapy agents through the activation of the DNA damage response **(Gasser 2005)**. Thus, we wanted to investigate whether low doses of different therapeutic agents with well-documented clinical activity in the treatment of MM could modulate the expression of the ligands for NKG2D and DNAM-1 activating receptors on a panel of MM cell lines. To this aim, we evaluated the expression of NKG2D (MICA, MICB, ULBP1-3) and DNAM-1 (PVR and Nec-2) ligands on ARK, LP-1, OPM-2, RPMI-8226, SKO-007(J3), and U266 MM cell lines upon treatment with doxorubicin, etoposide, melphalan, bortezomib, and cisplatin at the doses described in 'Drug treatment', and not affecting cell viability as assessed by PI staining (data not shown). Immunofluorescence and fluorescence-activated cell sorting (FACS) analysis revealed that 3 (RPMI-8226, SKO-007(J3) and U266) of 6 MM cell lines constitutively expressed PVR, MICA, and MICB; 3 (ARK, LP-1, and OPM-2) expressed only PVR; whereas Nec-2, ULBP1, ULBP2, and ULBP3 were undetectable on all the MM cell lines (data not shown). Fortyeight hour-treatment with low doses of pharmacologic drugs differently modulated NKG2D and DNAM-1 ligands on the MM cell lines, with up-regulation of the already expressed ligands. A dose-response curve was performed to select the dose that did not affect cell viability and induced ligand up-regulation **(Fig. 8A)**. Of note, we found that doxorubicin and melphalan were particularly effective in enhancing both MICA and PVR expression on RPMI-8226, SKO-007(J3) and U266 myeloma cells. Thus, we focused our attention on the SKO-007(J3) MM cell line since in addition to MICA and PVR, MICB was also up-regulated on these cells and not on the cells of the other MM cell lines **(Fig. 8B)**.

We then tested by Real Time PCR whether PVR, MICA, and MICB surface expression on melphalan- or doxorubicin-treated SKO-007(J3) cells was accompanied by a corresponding increase in mRNA levels, and we found an augmentation of PVR, MICA, and MICB transcripts at 24h after treatment **(Fig. 8C)**.

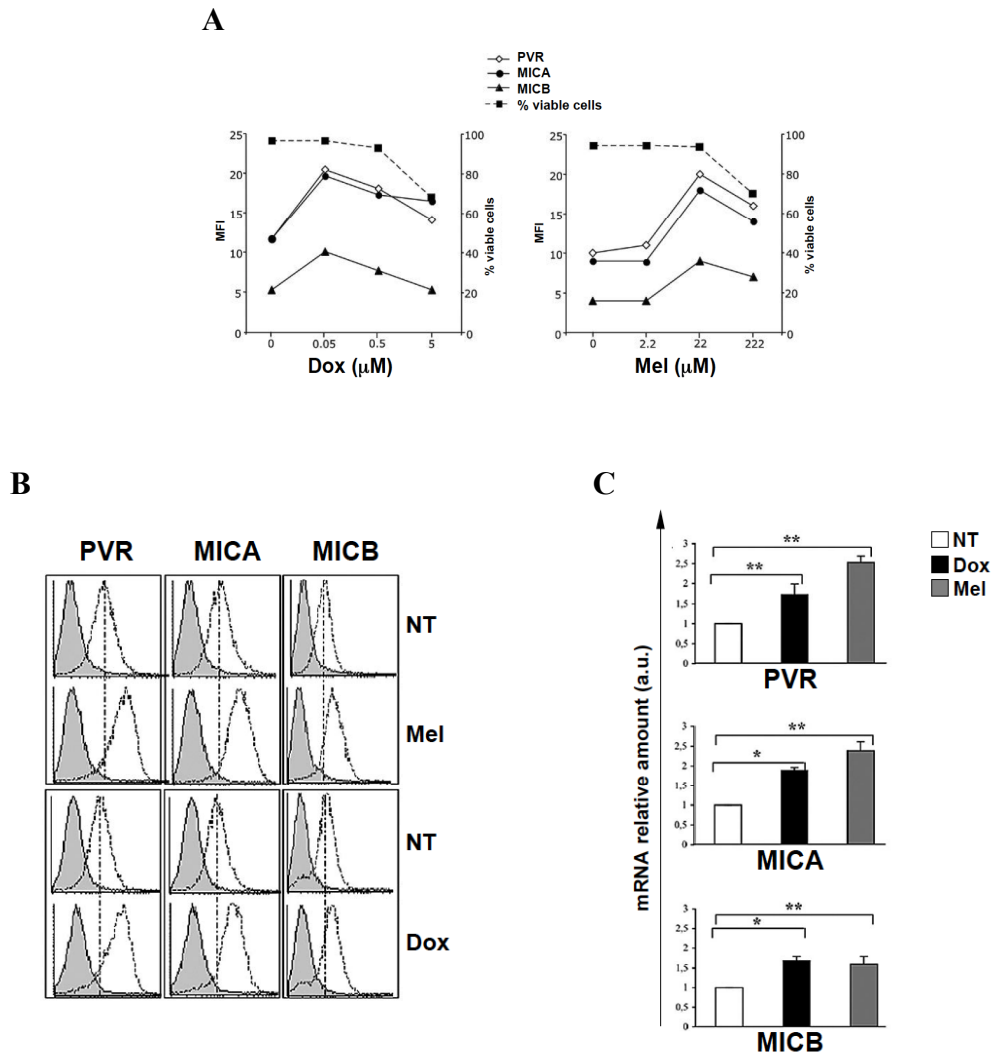


Figure 8. Modulation of PVR, MICA, and MICB expression on the SKO-007(J3) cell line after doxorubicin or melphalan treatment.
(A) A dose-response curve was performed to choose the drug dose that combines the better ligand up-regulation with the less toxicity. **(B)** PVR, MICA, and MICB surface expression was analyzed by flow cytometry on SKO-007(J3) cells treated with doxorubicin (0.05μM) or melphalan (22μM) for 48h. The gray histogram represents the isotype control antibody, whereas dashed lines represent the specific ligand. Data are representative of 1 of 4 independent experiments. **(C)** The corresponding increase in mRNA levels has been tested after 24h by Real Time PCR performed as described in 'Real Time PCR'. Data, expressed as arbitrary units, were normalized with β-actin, and referred to untreated cells considered as calibrator. Data are presented as the means plus or minus SD of triplicates. Significant differences as calculated by paired Student *t* test are indicated: **P* < .01; ***P* < .05.

4.2. Doxorubicin or melphalan treatment of SKO-007(J3) MM cells increase NK cell degranulation in a NKG2D- and DNAM1-dependent manner

The enhanced expression of NKG2D and DNAM-1 ligands on drug-treated

SKO-007(J3) myeloma cells prompted us to test whether they were able to trigger NK-cell degranulation. The expression of the lysosomal marker CD107a, which correlates with NK cell cytotoxicity **(Bryceson 2005)** was evaluated by immunofluorescence and FACS analysis by gating on NK cells upon their interaction with doxorubicin- or melphalan-treated, or untreated, SKO-007(J3) cells used as targets. The up-regulation of NKG2D and DNAM-1 ligands was verified before the degranulation assay (data not shown). As shown in **Fig. 9**, expression of CD107a on NK cells contacting SKO-007(J3) target cells indicates that NK cell degranulation is induced and that increases after drug treatment. The assay was performed at the E/T ratio of 2.5:1 and similar results were obtained using different E/T ratios (data not shown).

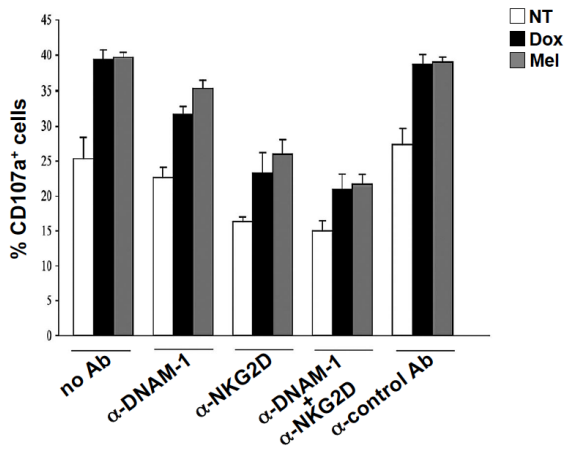


Figure 9. Doxorubicin or melphalan treatment of SKO-007(J3) cells increases NK-cell degranulation in an NKG2D- and DNAM1-dependent manner.
NK cells derived from PBMCs of healthy donors, preactivated with 200 U/mL IL-2 for 12h, were incubated with SKO-007(J3) cells, untreated or treated as described in the legend of Figure 8, and used as target cells in a degranulation assay. The assay was performed at the effector-target (E/T) ratio of 2.5:1. After 2h at 37°C, cells were stained with anti-CD56, anti-CD3, and anti-CD107a mAbs. Cell surface expression of CD107a was analyzed on $CD56^{+}CD3^{-}$ cells. To evaluate the role of NKG2D and DNAM-1, the assay was performed also treating NK cells with blocking anti-DNAM-1, anti-NKG2D, or anti-CD56 mAb used as control. Results are expressed as the percentage of $CD107a^{+}$ cells obtained by subtracting the percentage of isotype control antibody, and are representative of 1 out of 4 independent experiments. Data are presented as the means plus or minus SD of triplicates. Antibody blocking on drug-treated cells always showed a statistically significant increase in CD107a expression, compared with drug-treated cells with no Ab or control Ab ($P < .05$ or $P < .005$). A statistically significant difference was also observed between NT versus drug-treated samples for no Ab ($P < .05$). All other combinations were not significant.

Based on these findings, we evaluated the role of NKG2D and DNAM-1 in MM

cell recognition by performing the degranulation assay in the presence of anti-NKG2D and/or anti-DNAM-1 blocking mAbs. Treatment of NK cells with NKG2D and DNAM-1 blocking antibodies decreased CD107a expression, whereas no changes were observed upon treatment with a control mAb **(Fig. 9)**. In addition, NKG2D and DNAM-1 blocking antibodies partially affected basal degranulation, leading to the conclusion that constitutive NK-cell degranulation also involves these activating receptors.

Our results demonstrate that SKO-007(J3) cells treated with low doses of chemotherapeutic agents enhance NK-cell degranulation by promoting their recognition by NKG2D and DNAM-1 activating receptors.

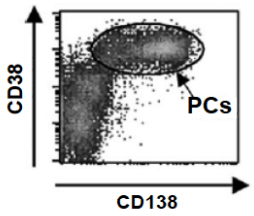
4.3. Therapeutic drug-induced up-regulation of NKG2D and DNAM-1 ligands on patient-derived malignant PCs contributes to the degranulation of autologous NK cells

We next investigated whether our findings could be extended to patient-derived myeloma cells obtained from bone marrow samples. We studied 4 patients affected by smoldering MM, and 6 patients affected by an active MM according to Durie and Salmon's staging system, prior to treatment **(Table 1**, Pt. 1-10**)**. We first characterized by flow cytometry the cell surface expression of NKG2D and DNAM-1 ligands gating on $CD38^{+}CD138^{+}$ PCs **(Fig. 10A)**, and we found that patient-derived PCs displayed different levels of both NKG2D and DNAM-1 ligands independently of the clinical stage and/or the percentage of malignant PCs **(Fig. 10B)**.

In light of the results obtained with MM cell lines *in vitro*, we assessed whether treatment of malignant PCs with melphalan could up-regulate the expression of NKG2D and DNAM-1 ligands. In addition, we tested the proteasome inhibitor bortezomib commonly used in MM therapy. Depending on the amount of PCs obtained from the different patients, samples were treated with one (P1, P3, and P4) or both (P2, P5, and P6) drugs **(Fig. 11)**. Consistent with the data obtained with *in vitro* cell lines, drug-treated *ex vivo* PCs expressed higher levels of surface NKG2D and DNAM-1 ligands, with considerable variations observed among different patients not related to the stage of disease **(Fig. 11)**. PCs from patient P2 were the best responders to drug treatment as shown by the marked increase of MICA, MICB, and Nec-2 expression. It is worth noting that unlike MM cell lines, the expression of Nec-2 and ULBP1-3 on patient-

derived PCs was also enhanced by drug treatment.

A



B

	MFI						
Patient no.	ULBP1	ULBP2	ULBP3	MICA	MICB	PVR	Nec-2
1	13	9	3	16	1	4	1
2	3	10	3	1	3	17	3
3	1	8	2	1	2	8	1
4	5	3	5	2	1	2	2
5	1	1	1	1	2	1	8
6	1	7	1	20	20	3	35
7	27	30	12	1	1	6	18
8	13	10	3	1	1	17	1
9	9	8	2	2	2	8	2
10	3	3	2	5	1	8	2

Figure 10. Patient characteristics.
(A) Malignant PCs obtained from BM samples of MM patients were selected by gating on $CD38^+CD138^+$ cells and an example is shown (P2). **(B)** NKG2D and DNAM-1 ligand expression was evaluated on malignant PCs from BM sample by gating on the $CD38^+CD138^+$ population. The mean fluorescence intensity (MFI) of each specific ligand subtracted from the MFI of isotype control antibody is reported.

Furthermore, some patients (P7, P8, P9, and P10) were also tested by Real Time PCR to investigate whether ligand surface expression on melphalan- or bortezomib-treated PCs was accompanied by a corresponding increase in mRNA levels at 24h after drug treatment **(Fig. 12)**.

When a sufficient number of PCs (P1, P2, P3, and P5) were isolated, we examined autologous NK-cell degranulation, to assess the functional role of ligand up-regulation. The degranulation assay was performed by analyzing the expression of CD107a on autologous $CD3^-CD16^+CD56^+CD138^-$ NK cells. The percentage of peripheral blood NK cells from the different patients was comparable, as was the expression of NKG2D and DNAM-1 activating receptors (data not shown). NK cells derived from the MM patients expressed CD107a upon their interaction with the autologous malignant PCs and this expression was increased upon melphalan or bortezomib treatment. In addition, for 2 patients (P2 and P5) we performed the degranulation assay in the presence of anti-NKG2D or anti-DNAM-1 blocking mAbs and we found that NK cell degranulation upon their interaction with drug-treated but not

with untreated malignant PCs was dependent on both NKG2D and DNAM-1 receptors **(Fig. 13)**.

Thus, the results with patient-derived malignant PCs confirm our findings on MM cell lines and strongly indicate that low doses of therapeutic agents increase NK-cell degranulation by promoting cognate interaction of both NKG2D and DNAM-1 with their respective ligands.

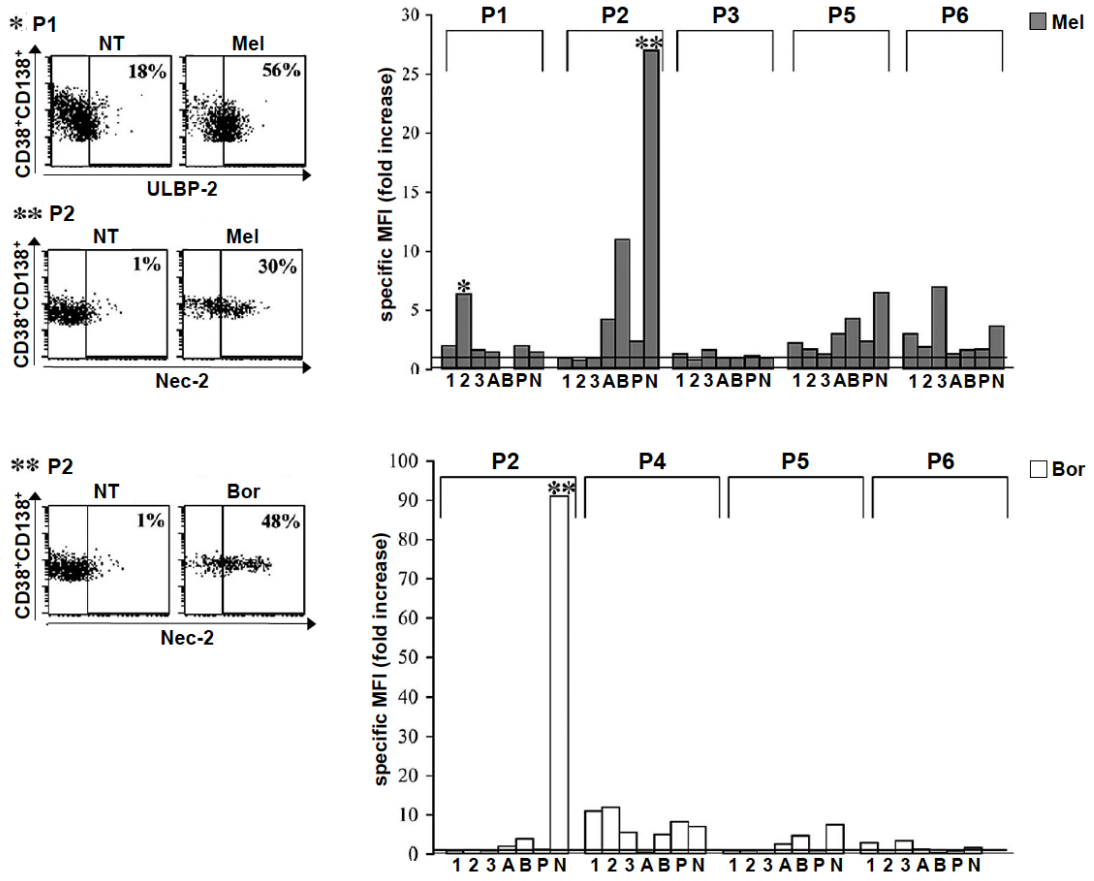


Figure 11. DNAM-1 and NKG2D ligands are upregulated on patient-derived PCs after therapeutic treatment.
Mononuclear cells were cultured with melphalan (20μM) or bortezomib (5nM) in complete medium supplemented with 20ng/mL IL-3 and 2ng/mL IL-6. Upon 48h-treatment, the expression of ULBP1 (1), ULBP2 (2), ULBP3 (3), MICA (A), MICB (B), PVR (P), and Nec-2 (N) was analyzed by flow cytometry gating on $CD38^{+}CD138^{+}$ PCs. Three examples of the typical FACS analysis performed are represented in the squares. *=P1; **=P2. Data are expressed as fold increase between the MFI of specific ligand subtracted for MFI of isotype control of treated cells divided by MFI of specific ligand subtracted for MFI of isotype control of untreated cells.

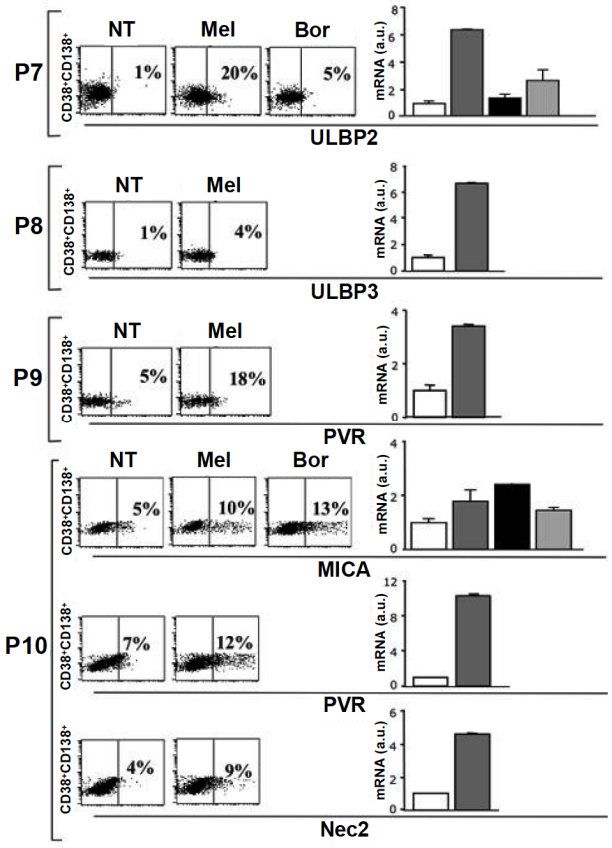


Figure 12. Modulation of DNAM-1 and NKG2D ligand expression on patient-derived PCs following therapeutic treatment at protein and mRNA level.
Malignant PCs from BM samples were treated and ligand surface expression was analyzed as previously described. The percentage of $CD38^{+}CD138^{+}$ cells expressing the indicated ligand before and after the drug-treatment is shown. The corresponding increase in mRNA levels has been tested after 24h by Real Time PCR performed as described in the Materials and Methods. The relative mRNA amount for the different ligands was evaluated on untreated (white) and melphalan (dark grey) (20μM) or bortezomib (black) (5nM) treated PCs. The mRNA levels have been also assessed on PCs (P7 and P10) treated for 24h with doxorubicin (light grey) (0.05μM). Data expressed as arbitrary units were normalized with β-actin, and referred to untreated cells considered as calibrator. Data are presented as the means plus or minus standard deviations from triplicates.

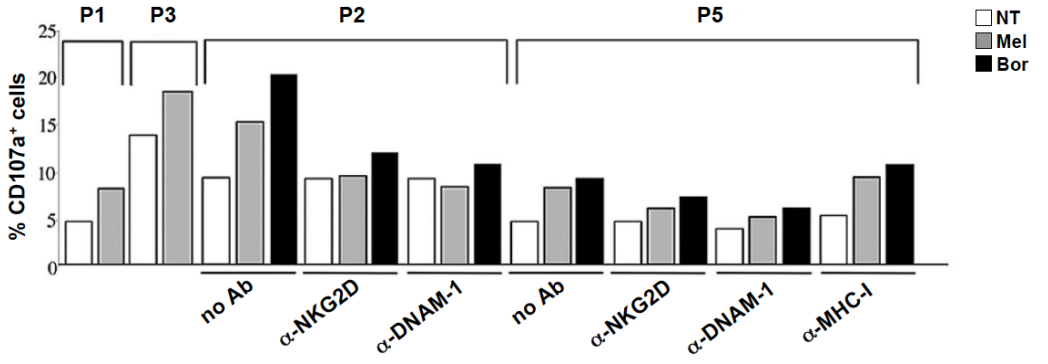


Figure 13. Therapeutic drug-induced up-regulation of NKG2D and DNAM-1 ligands on patient-derived malignant PCs contributes to the degranulation of autologous NK cells.
Mononuclear cells derived from the BM of the patients were prepared as described in 'Cell lines and clinical samples', treated with melphalan (20μM) or bortezomib (5nM) for 48h, then compared with untreated cells for their capability to enhance NK cell degranulation. Myeloma cells were exposed for 2h to autologous IL-2–activated PBMCs and cell surface expression of CD107a on NK cells was analyzed. The assay was performed at the effector-target (E/T) ratio of 2.5:1. Significant differences, as calculated by paired Student *t* test, were found comparing NT versus melphalan-treated samples ($P < .05$). Statistical analysis on bortezomib-treated samples was not performed since 2 patients (P2 and P5) were studied. To evaluate NKG2D and DNAM-1 contribution, we performed the degranulation assay by preincubating PBMCs with the anti-NKG2D, anti-DNAM-1, or anti-MHC I neutralizing mAbs before the assay (P2 and P5).

4.4. Low doses of doxorubicin and melphalan chemotherapeutic drugs trigger the DDR and induce ATM/ATR- and Chk1/2-dependent MICA, MICB and PVR expression on myeloma cells

Gasser *et al.* demonstrated that genotoxic stress-induced NKG2D ligand up-regulation on human fibroblasts and mouse tumor cell lines was dependent on DDR pathway triggering **(Gasser 2005)**. In addition, many of the chemotherapeutic drugs used in clinical trials today, including doxorubicin or melphalan, have the capability of inducing ATM activation **(Kurz 2004, Bozko 2005)**. Thus, we decided to investigate whether treatment with low doses of doxorubicin and melphalan, were able to initiate the DDR signaling cascade in order to identify some of the molecular events underlying the increased expression of NK cell receptor activating ligands.

To this aim, we firstly evaluated the ability of low doses of doxorubicin and melphalan to trigger ATM kinase activation on SKO-007(J3) cells by Western Blot analysis employing a phospho-specific antibody directed against Ser1981 that recognizes the activated form of this kinase. Doxorubicin- or melphalan-treatment resulted in enhanced ATM phosphorylation that was evident at 5h of drug stimulation and still persisted at 24h **(Fig. 14A** and data not shown**)**.

The activation of the components of the ATM/ATR-dependent signaling cascade such as H2AX, considered a main marker of DNA damage, Chk1/2 cell cycle checkpoint kinases, and p53, were also analyzed. ATM-induced histone phosphorylation on residue serine 139 (γ-H2AX) in response to low doses of melphalan and doxorubicin was examined by immunofluorescence and flow cytometry and was observed to reach its maximum at 24h **(Fig. 14B** and data not shown**)**. Evaluation of Chk1 and Chk2 phosphorylation on multiple aminoacidic residues by Western Blot analysis revealed a more prominent phosphorylation for Chk1 on Ser296 and Ser345 residues and for Chk2 on Thr68 and Ser516 residues on drug-treated cells **(Fig. 14C)**. We then examined whether drug treatment was also able to induce ATM dependent-p53 activation. ATM-dependent p53 phosphorylation was evaluated by Western Blot analysis using a phospho-specific antibody directed against p53-Ser15, in the presence or absence of ATM/ATR inhibitor caffeine. The increase of total p53 expression was also established. As shown in **Fig. 14D**, increased p53 phosphorylation was already evident at 3-5h after drug treatment, and was reduced by caffeine further indicating its dependence on ATM kinase activation. Similar results were obtained with U266 MM cell line (data not shown).

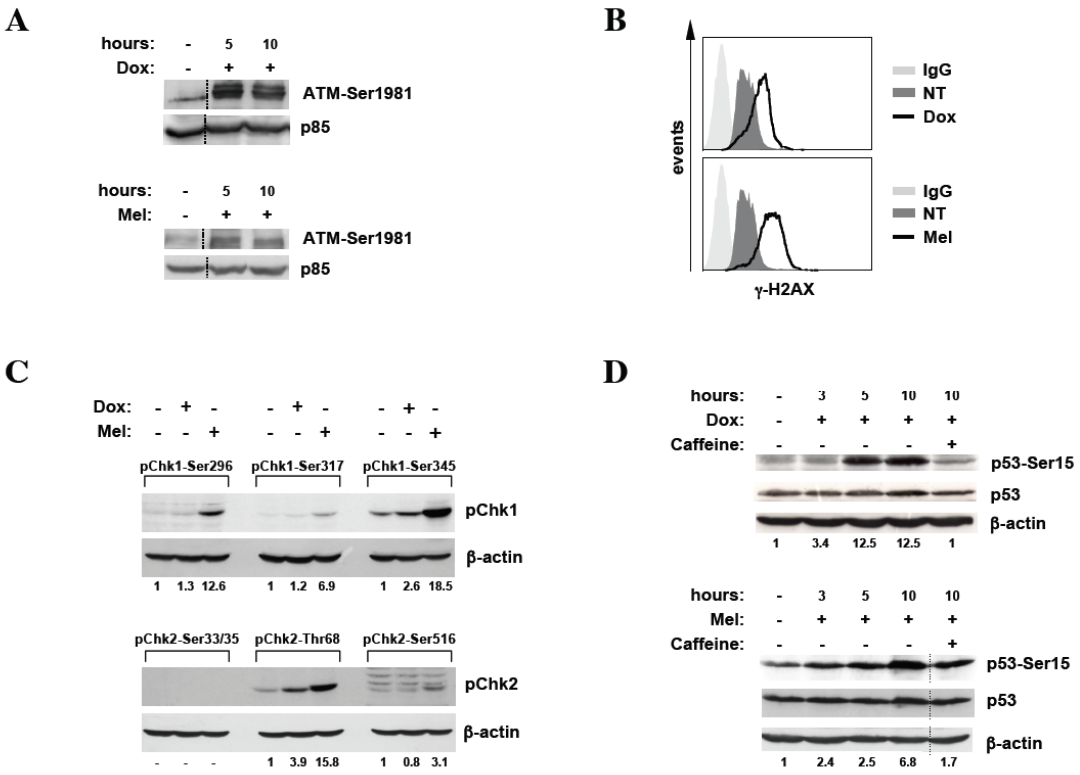


Figure 14. Low doses of doxorubicin and melphalan chemotherapeutic drugs activate the DNA Damage Response on myeloma cells.
(A) SKO-007(J3) cells were starved for 24h in culture medium with 2% fetal calf serum, and then left untreated or stimulated with doxorubicin (0.05μM) or melphalan (22μM), for the indicated times at 37°C. Cell lysates were immunoblotted with anti-phospho-ATM, or with anti-p85, used as loading control. Data shown are representative of 1 out of 4 independent experiments. **(B)** Phosphorylation of H2AX upon 24h drug treatment was evaluated on SKO-007(J3) cells by immunofluorescence and FACS analysis by staining with anti-γH2AX or anti-cIgG. **(C)** SKO-007(J3) cells were treated as previously described for 10h. Lysates were probed with antibodies to different phosphorylation sites of Chk1 and Chk2 or β-actin, used as loading control. Numbers represent densitometric analysis of Chk1/2 normalized to β-actin relative to the control untreated cells. Data shown are representative of 1 out of 2 independent experiments. **(D)** SKO-007(J3) cell line was preincubated 1h with caffeine (1mM), and then treated with doxorubicin or melphalan for the indicated times. Lysates were probed with antibodies to phospho-p53-Ser15 or total p53. β-actin was used as loading control. Numbers represent densitometric analysis of phospho-p53 normalized to β-actin relative to the control untreated cells. Data shown are representative of 1 out of 3 independent experiments.

Collectively, our findings clearly demonstrate that low doses of doxorubicin and melphalan are able to trigger the activation of DDR.

Thus, we focused our attention on the role of drug-induced DDR signaling on MICA, MICB and PVR enhanced expression on MM cells and we wondered whether up-regulation of MICA, MICB, and PVR was dependent on the activity of ATM and ATR, and on their downstream molecules. Thus, we tested whether caffeine, a widely

used inhibitor capable of blocking both ATM and ATR catalytic activity **(Sarkaria 1999)** or KU-55933, a specific inhibitor of ATM **(Hickson 2004)** could interfere with the induction of MICA, MICB, and PVR expression on doxorubicin or melphalan-treated SKO-007(J3) cells. Cells were pretreated with doses of caffeine (1mM) or KU-55933 (10μM) that do not affect cell viability, and then incubated with doxorubicin or melphalan for 48h. We found that MICA, MICB, as well as PVR up-regulation was completely inhibited by caffeine and partially reduced by KU-55933 treatment **(Fig. 15)**, whereas both inhibitors did not impair constitutive ligand expression thus suggesting that the two sensor of DNA damage response ATM/ATR are involved in the regulation of both NKG2D **(Gasser 2005)** and DNAM-1 ligand expression.

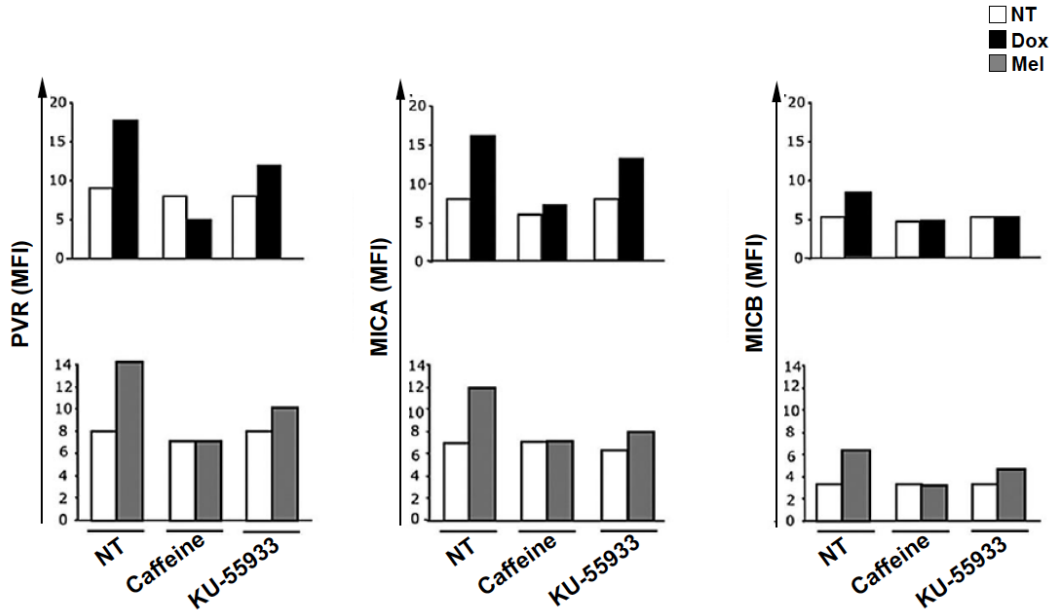


Figure 15. Up-regulation of DNAM-1 (PVR) and NKG2D (MICA and MICB) ligands by doxorubicin or melphalan treatment depends on DNA damage sensors ATM and ATR.
The SKO-007(J3) cell line was preincubated for 2h with caffeine (1mM) or KU55933 (10μM), then treated with doxorubicin (0.05μM) or melphalan (22μM) for a further 48h. The expression of PVR, MICA, and MICB was analyzed by flow cytometry. On the y-axis is shown the median fluorescence intensity (MFI) values of ligand expression obtained by subtracting the MFI of the isotype control antibody. Results are representative of 1 of 4 independent experiments.

Moreover, SKO-007(J3) cells were treated with SB218078, UCN-01 and Pifithrin-α, pharmacological inhibitors of Chk1, Chk1/2 and p53 respectively, before doxorubicin and melphalan treatment, and ligand expression was assessed by

immunofluorescence and FACS analysis. A role for the Chk1/2 kinases was established by the finding that the drug-induced MICA, MICB, and PVR ligand up-regulation was almost completely inhibited by both the Chk1/2 inhibitors **(Fig. 16)**. In an attempt to explore whether UCN-01-induced inhibition of ligand expression could be also related to its ability to block conventional PKC subfamily, we analyzed the effect of Gö 6983, a widespread PKC inhibitor, on drug-induced NKG2D and DNAM-1 ligand expression. Pretreatment of MM cells with this inhibitor did not significantly affect ligand induction, probably excluding a role of PKC kinases (data not shown).

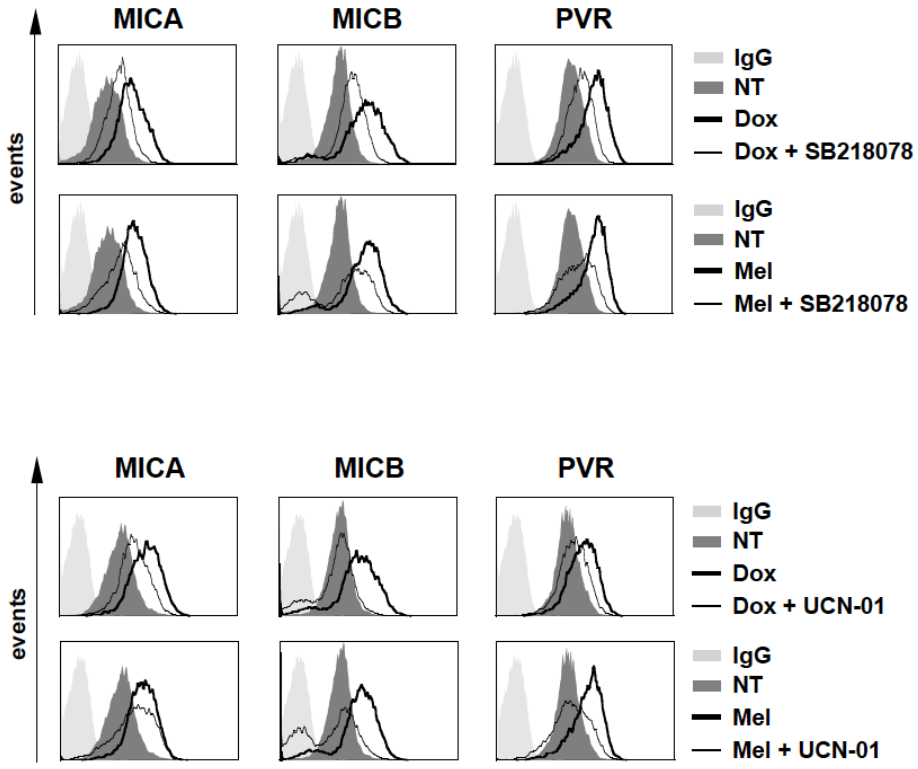


Figure 16. Involvement of Chk1/2 activity in NKG2D (MICA and MICB) and DNAM-1 (PVR) ligand up-regulation triggered by low doses of doxorubicin or melphalan treatment. MICA, MICB and PVR surface expression was analyzed by immunofluorescence and flow cytometry on SKO-007(J3) cell preincubated 1h with SB218078 (1μM) or UCN-01 (20nM), before 72h treatment with doxorubicin (0.05μM) or melphalan (22μM). Data shown are representative of 1 out of 3 independent experiments.

By contrast, the p53 inhibitor Pifithrin-α at the experimental conditions that blocked drug-increased p21 expression, did not affect ligand up-regulation **(Fig.17A)**. To confirm that p53 was not involved in MICA, MICB and PVR induction by

doxorubicin and melphalan, p53 expression was silenced in SKO-007(J3) cells using RNA interference. MM cells were infected with a retrovirus encoding shRNA specific for p53 (shRNA p53) or with a control retrovirus (shRNA GFP). After drug selection, we obtained resistant cells where p53 was specifically inhibited, as confirmed by Western Blot. As shown, up-regulation of MICA, MICB and PVR expression persists in shRNA p53 SKO-007(J3) cells **(Fig. 17B)**.

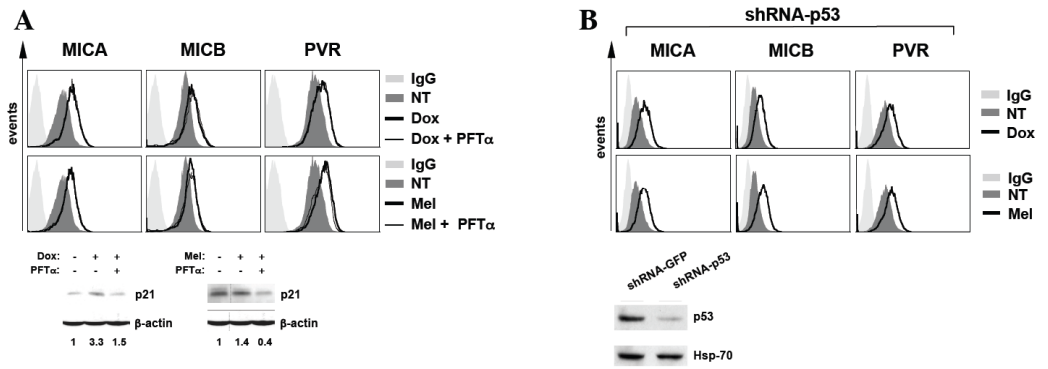


Figure 17. Low doses of doxorubicin and melphalan up-regulate NKG2D and DNAM-1 ligand in a p53 independent manner.
(A) SKO-007(J3) cells were pre-incubated 1h with Pifithrin-α (30 μM) before 72h treatment with doxorubicin (0.05μM) or melphalan (22μM). MICA, MICB and PVR surface expression was analyzed by immunofluorescence and flow cytometry. Western blot of total cell lysates immunoblotted with anti-p21 Ab, or with anti-βactin, used as loading control is shown on the bottom panel. **(B)** SKO-007(J3) were infected with a retrovirus encoding a shRNA specific for p53 (shRNA p53) or with a control retrovirus (shRNA GFP). The expression of MICA, MICB and PVR was analyzed upon doxorubicin or melphalan treatment by immunofluorescence and flow cytometry. Western blot was performed using whole-cell extracts from shRNA p53 or shRNA GFP retrovirus-infected SKO-007(J3) cells. The proteins transferred to nitrocellulose membranes were immunoblotted with anti-p53 or anti-Hsp70 to verify that similar amounts of protein had been loaded in each lane.

4.5. DNAM-1 and NKG2D ligands are up-regulated on doxorubicin- or melphalan-induced senescent MM cells

Senescent cells display an increase of cell size, senescence associated expression of β-galactosidase activity, and an altered pattern of gene expression **(Roninson 2003, Collado 2007)** and in response to drug-induced stress they are arrested in the G_2M cell-cycle phase **(Chang 1999, Chang 2002)**. The ATM/ATR signaling pathway has been found constitutively active in drug-induced senescent tumor cells, and senescence

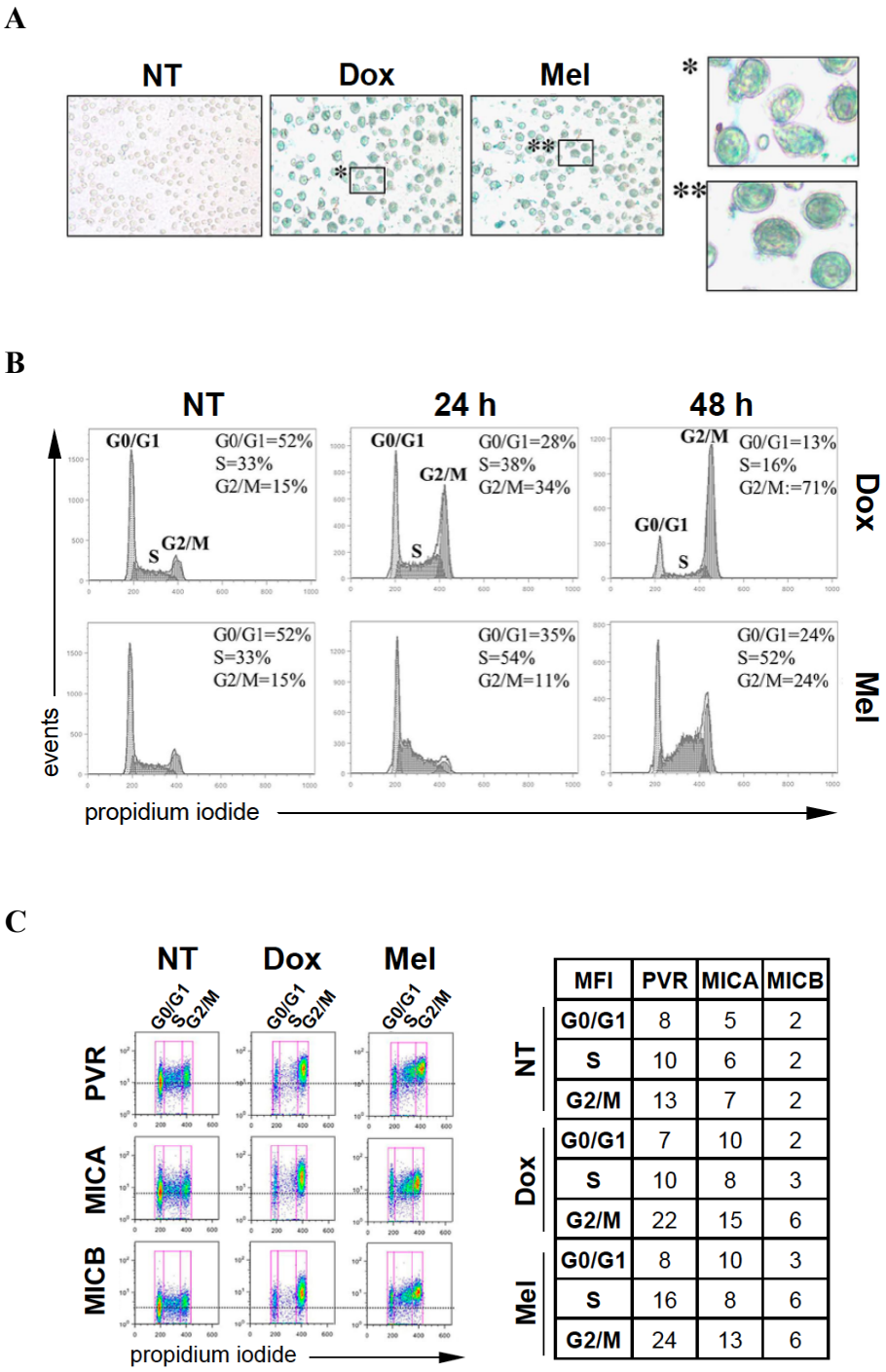
induced by sublethal concentrations of anticancer drugs can be regarded as a form of permanently maintained DDR **(Schmitt 2007)**. In addition, some evidences demonstrate the ability of doxorubicin to induce a senescent phenotype on both normal and tumor cells **(Elmore 2002, Maejima 2008)**.

Our goal was to determine whether drug-induced up-regulation of NKG2D and DNAM-1 ligands on MM cells was associated with a senescent phenotype. To this end, we first analyzed whether SKO-007(J3) cells undergo senescence after exposure to the low doses of doxorubicin or melphalan that up-regulate NKG2D and DNAM-1 ligands and do not induce apoptosis, by evaluating SA-βGalactosidase activity. β-Galactosidase activity visualized by microscopy, through the appearance of a blue color in the cells, was already present in the SKO-007(J3) MM cells after 48h of incubation with the chemotherapeutic agents (data not shown), but the staining became more intense and was present in virtually every cell when they were left for further 24h in the absence of the drug **(Fig. 18A)**.

We then tested cell-cycle progression of doxorubicin- or melphalan-treated MM cells, and the NKG2D and DNAM-1 ligand expression on cells at different cell-cycle phases. We observed that treatment of SKO-007(J3) with low doses of doxorubicin or melphalan induced a G_2M cell-cycle arrest that was already present at 24h, increased at 48h, and was almost complete after 72h of treatment with doxorubicin **(Fig. 18B and Fig. 21)**. Similarly, melphalan-treated cells underwent a G_2M cell cycle arrest, but with a slower kinetics.

Analysis of cell cycle phase-associated ligand expression revealed that in response to drug treatment, MICA was up-regulated in all cell-cycle phases, whereas PVR and MICB up-regulation was more prominent on cells arrested in the G_2M phase **(Fig. 18C)**. As a control, we evaluated CD138 expression at different cell cycle phases and this was not affected by drug treatment (data not shown).

Collectively, these results indicate that the NKG2D and DNAM-1 ligands are up-regulated on drug-induced senescent MM cells.



MFI	PVR	MICA	MICB
NT G0/G1	8	5	2
NT S	10	6	2
NT G2/M	13	7	2
Dox G0/G1	7	10	2
Dox S	10	8	3
Dox G2/M	22	15	6
Mel G0/G1	8	10	3
Mel S	16	8	6
Mel G2/M	24	13	6

Figure 18. DNAM-1 (PVR) and NKG2D (MICA and MICB) ligands are up-regulated on doxorubicin- or melphalan-induced senescent MM cells.
(A) SKO-007(J3) cells were incubated with doxorubicin (0.05μM) or melphalan (22μM) for 48h and left for an additional 24h in the absence of the drug. MM cells were then fixed, and incubated overnight at 37°C without CO_2 with SA-β-Gal staining solution ('Senescence associated-β-galactosidase staining'). Senescent cells were identified as blue-stained cells by microscopy. Results are representative of 1 of 3 independent experiments. **(B)** SKO-007(J3) cells were treated for 24h and 48h with doxorubicin or melphalan, then fixed and stained with PI to analyze cell distribution among the different cell-cycle phases**. (C)** To correlate PVR, MICA, and MICB up-regulation with a specific cell-cycle phase, the untreated and treated SKO-007(J3) cells were incubated with PI and stained for these ligands. The analysis was performed by flow cytometry. The corresponding MFI values of the ligand expression obtained by subtracting the MFI of the isotype control antibody are reported in the table. Results are representative of 1 of 5 independent experiments.

4.6. Genotoxic agents trigger ROS-dependent DDR activation and up-regulation of MICA, MICB and PVR expression on myeloma cells

Based on the evidence that low doses of doxorubicin and melphalan increase superoxide and peroxide intracellular production (data not shown), and ATM and components of the ATM-dependent signaling cascade are regulated by ROS **(Shackelford 2004, Kurz 2004b, Guo 2010)**, we wondered whether changes of redox state could have a role in drug-induced activating ligand up-regulation.

Thus, we evaluated ATM-Ser1981, Chk1-Ser345 and Chk2-Thr68 phosphorylation upon treatment with the anti-oxidant agent NAC. Cells were pretreated with NAC (10mM), and then incubated with doxorubicin or melphalan. Exposure of SKO-007(J3) cells to free radical scavenger resulted in a complete inhibition of ATM, Chk1 and Chk2 enhanced phosphorylation **(Fig. 19A-B)**.

Thus, we tested whether NAC treatment could also interfere with the induction of MICA, MICB and PVR expression on doxorubicin- or melphalan-treated MM cells. Cells were pre-treated with NAC, and further incubated with doxorubicin or melphalan for a maximum of 72h. We found that ligand up-regulation was completely inhibited at both protein and mRNA levels by this antioxidant agent **(Fig. 19C-D)**, that however did not impair constitutive ligand expression **(Fig. 19D** and data not shown**)**.

Our results demonstrate that changes in the redox state are involved on drug-induced regulation of both NKG2D and DNAM-1 ligand expression.

4.7. Doxorubicin and melphalan-dependent changes on redox state control ligand up-regulation on MM senescent cells

Changes in the redox state have been reported to be critical for induction of cellular senescence **(Balaban 2005)** and we have demonstrated that drug-induced up-regulation of NKG2D and DNAM-1 ligands on MM cells is associated with a senescent-dependent G_2M cell cycle arrest **(Fig. 18)**.

Thus, we decided to analyze whether changes in the redox state could interfere with the induction of ligands on MM cells displaying a senescent phenotype. We observed that NAC pretreatment of myeloma cells caused a complete block of doxorubicin and melphalan-dependent G_2M cell cycle arrest, being the percent of cells

in G_2M phase comparable to that of untreated cells **(Fig. 20A)**.

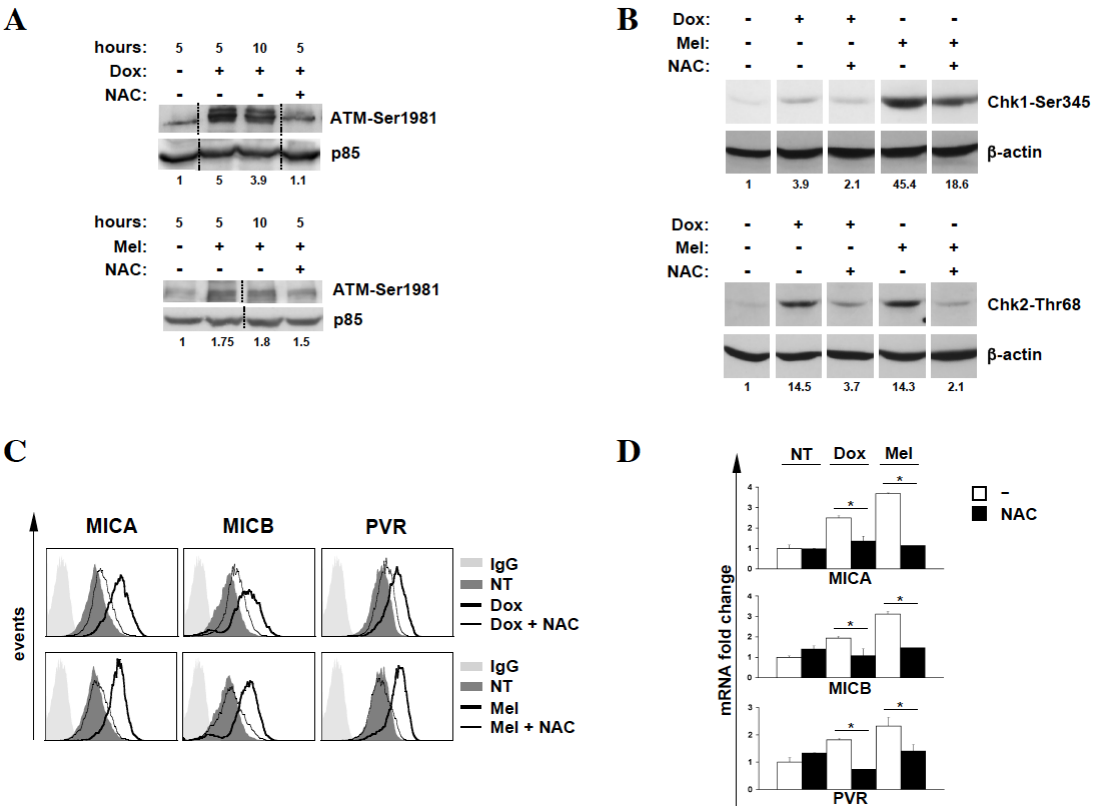


Figure 19. Doxorubicin- and melphalan-induced changes in the redox state trigger DNA Damage Response and up-regulation of MICA, MICB, and PVR expression on myeloma cells.

(A) SKO-007(J3) cells were starved for 24h in medium containing 2% fetal calf serum, pre-incubated 1h with NAC (10mM), and then treated with doxorubicin (0.05μM) or melphalan (22μM) for further 5h or 10h. Cell lysates were immunoblotted with anti-phospho-ATM or with anti-p85, used as loading control. Data shown are representative of 1 out of 3 independent experiments. Numbers represent densitometric analysis of ATM normalized to p85 relative to the control untreated cells. **(B)** SKO-007(J3) cells were pre-incubated 1h with NAC (10mM), and then treated as previously described for 12h. Lysates were probed with antibodies anti-phospho-Chk1-Ser345, anti-phospho-Chk2-Thr68, or anti-β-actin. Numbers represent densitometric analysis of Chk1/Chk2 normalized to β-actin relative to the control untreated cells. Data shown are representative of 1 out of 2 independent experiments. **(C)** MICA, MICB and PVR surface expression was analyzed by immunofluorescence and flow cytometry on SKO-007(J3) cells pretreated 1h with NAC (10mM) and then treated 72h with doxorubicin or melphalan. Data shown are representative of 1 out of 4 independent experiments. **(D)** The corresponding increase in mRNA levels has been tested after 24h by Real Time PCR. The level of ligand expression was measured using the Threshold Cycle value (Ct). The ΔCt was obtained by subtracting the Ct value of the gene of interest (MICA, MICB or PVR) from the housekeeping gene (β-actin) Ct value. We used ΔCt of NT sample as the calibrator. The fold change was calculated according to the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt was the difference between ΔCt of the sample and that of the calibrator (according to the formula, the value of the calibrator in each run is 1).

In addition, as we previously described, in response to drug treatment MICA

expression was up-regulated in all cell cycle phases, whereas PVR and MICB up-regulation was more prominent on senescent cells **(Fig. 18C)**. NAC treatment completely abrogated drug-induced ligand expression on all cell populations independently of their cell cycle phases **(Fig. 20B)**.

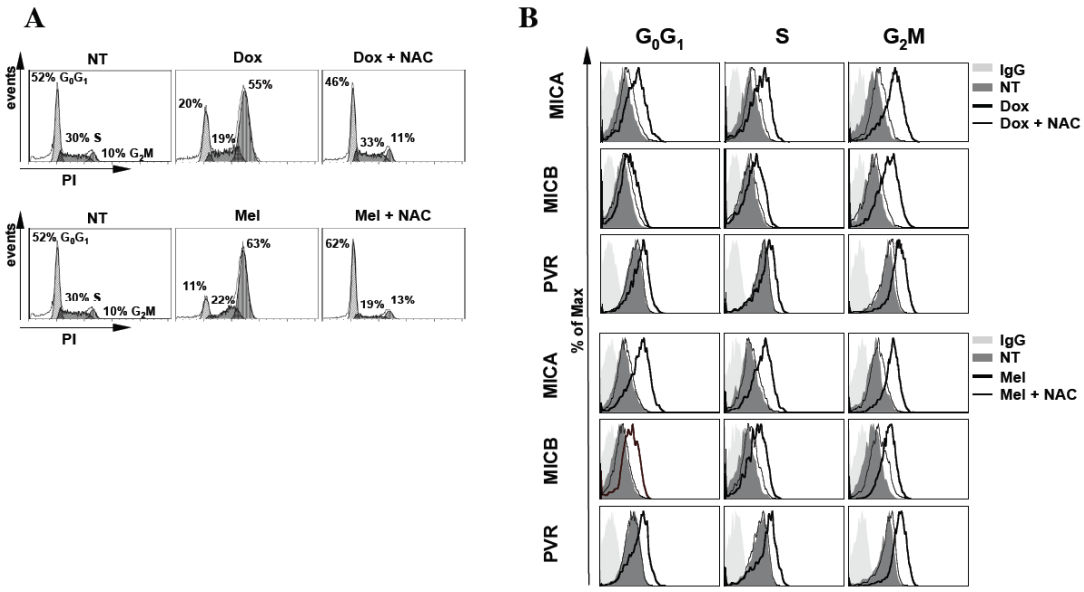


Figure 20. Doxorubicin and Melphalan-dependent ROS production controls senescent-associated G_2M cell cycle phase arrest on SKO-007(J3) myeloma cells.
(A) SKO-007(J3) cells were pre-incubated 1h with NAC (10mM), treated with doxorubicin (0.05μM) or melphalan (20μM) for further 72h, then fixed and stained with PI to analyze cell distribution among the different cell cycle phases. The analysis was performed by flow cytometry. Results are representative of 1 out of 3 independent experiments. **(B)** In order to correlate the effect of NAC to the MICA, MICB and PVR up-regulation in the different cell cycle phase, SKO-007(J3) cells were treated 72h with doxorubicin or melphalan after 1h-NAC pre-treatment, and then stained with antibody specific for these ligands before fixing and incubating with PI. Results are representative of 1 out of 3 independent experiments.

The expression of NKG2D and DNAM-1 ligands on senescent cells was also evaluated with a recently described method that gives a more quantitative evaluation of SA-βGal activity by using the fluorescent lipophilic βgalactosidase substrate C_{12}FDG, and flow cytometry **(Kurz 2000)**. First of all, we analyzed by flow cytometry both cell cycle analysis and expression of SA-βGal activity, the involvement of DDR and redox signaling on the capacity of low doses of doxorubicin and melphalan to induce a senescent phenotype by employing different pharmacological inhibitors. Cells were pretreated with caffeine (1mM), UCN-01 (20nM), or NAC (10mM) and then incubated

with therapeutics drugs for further 72h. The percentage of cells arrested on the G_2M cell cycle phase paralleled that of cells showing an increased $C_{12}FDG$ staining (βGal^{high}). In addition, we found that the percentage of senescent cells was reduced by all the inhibitors tested, suggesting a role for DDR and redox signaling cascade in the establishment of drug-induced senescence **(Fig. 21)**.

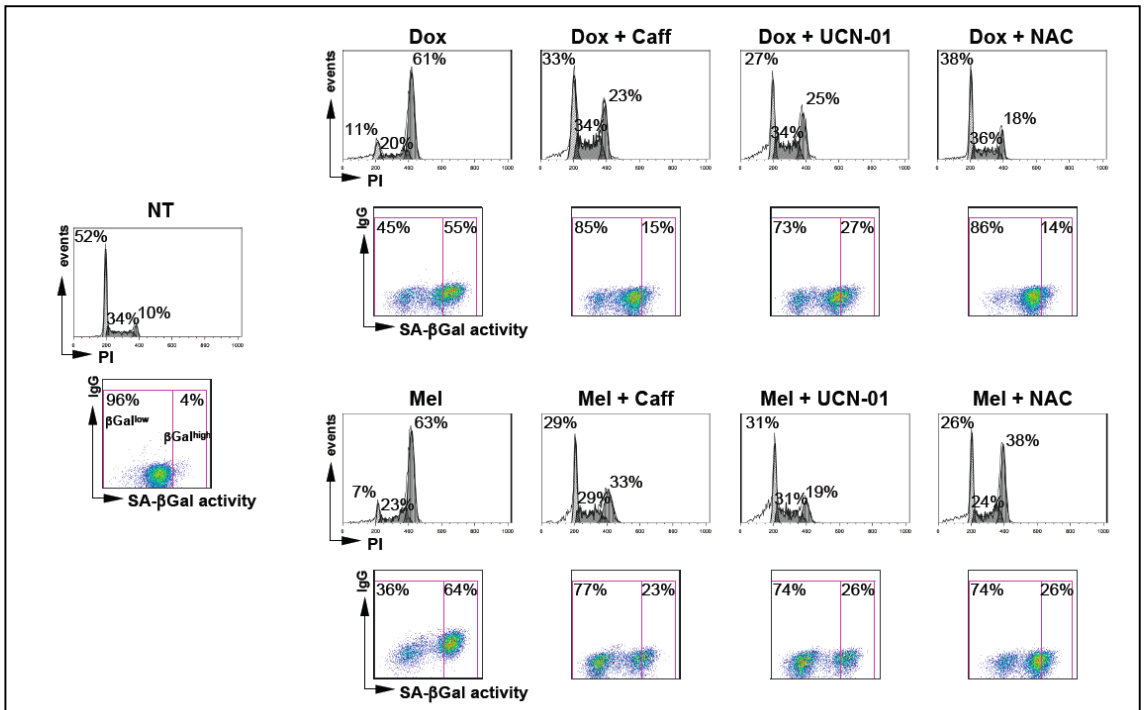


Figure 21. Doxorubicin and Melphalan-induced G_2M cell cycle arrested-cells are comparable to βGal^{high} activity-cells analyzed by flow cytometry.
SKO-007(J3) cells, upon 72h-treatment of chemoterapeutic drugs, were pretreated 1h with 100nM bafilomycin A1 to induce lysosomal alkalinization, followed by 1h incubation with $C_{12}FDG$ (33μM). In the panel are shown the flow cytometric detection of SA-βGal activity compared to the cell distribution among the different cell cycle phases of SKO-007(J3) cells preincubated 1h with caffeine (1mM), NAC (10mM) or UCN-01 (20nM), before 72h treatment with doxorubicin (0.05μM) or melphalan (22μM).

The analysis of NKG2D and DNAM-1 ligand expression on $C_{12}FDG$-stained MM cells revealed that in response to doxorubicin treatment MICA, PVR and MICB up-regulation was more prominent on βGal^{high} cells **(Fig. 22A)**.

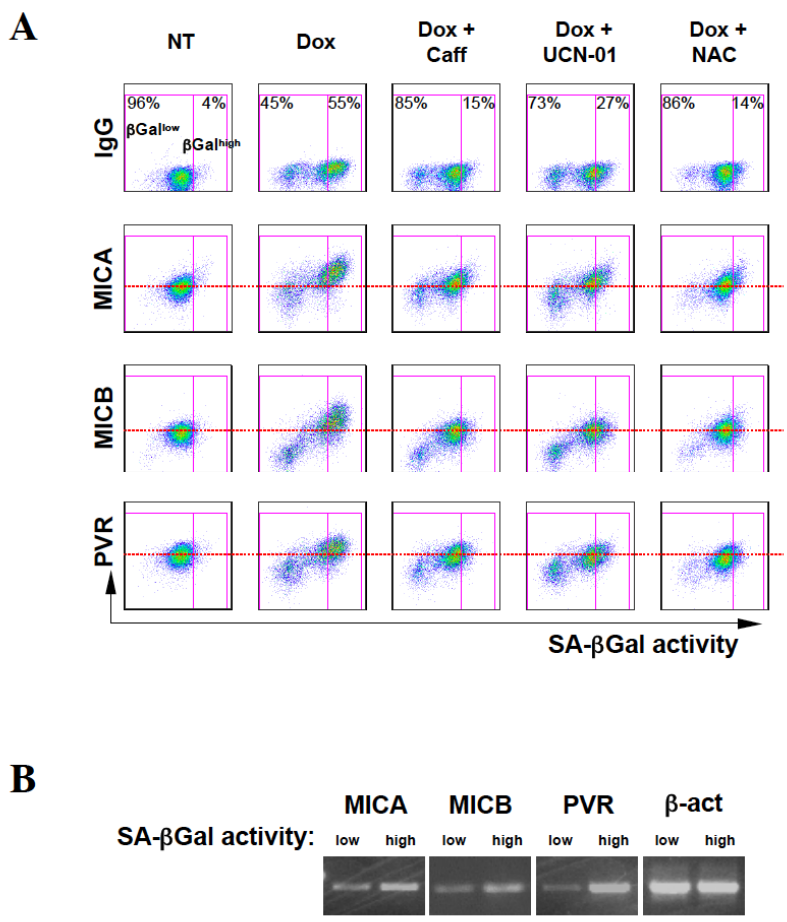


Figure 22. Redox signalling and DNA damage response activation control ligand up-regulation on doxorubicin-induced βGal^{high} senescent cells.
(A) SKO-007(J3) cells, upon 72h-treatment of chemoterapeutic drugs, were pretreated 1h with 100 nM bafilomycin A1 to induce lysosomal alkalinization, followed by 1h incubation with C_{12}FDG (33μM) and the expression of MICA, MICB, and PVR on drug-induced βGal^{high} senescent cells was analyzed by flow cytometry. On the *y*-axis is shown ligand expression while on *x*-axis the SA-βGal activity. Numbers represent the percentage of βGal^{low} and βGal^{high} cells. Results are representative of 1 out of 3 independent experiments. **(B)** Seventytwo hour doxorubicin-treated SKO-007(J3) cells were incubated with C_{12}FDG as previously described, and selected by a FACSAria cell sorter (BD Biosciences) through FL-1 green fluorescence emission. mRNA levels of MICA, MICB, and PVR has been tested by RT-PCR. The housekeeping gene β-actin was used as loading control. Results are representative of 1 out of 2 independent experiments.

Moreover, we sorted MM cells for the levels of SA-βGal activity and performed a RT-PCR assay to establish MICA, MICB and PVR mRNA expression in βGal^{low} and βGal^{high} cell populations. Accordingly with our previous findings, ligand expression was more pronounced in βGal^{high} cells confirming that also at mRNA levels NKG2D and DNAM-1 activating ligands are preferentially increased on senescent cells **(Fig. 22B)**. Inhibition of ATM/ATR and Chk1/2 activation by caffeine and UCN-01 treatment,

respectively resulted in a decreased percentage of βGal^{high} doxorubicin-treated MM cells that returned to almost basal levels, associated with reduced MICA, MICB and PVR expression. In the presence of NAC, doxorubicin treatment failed to trigger the cellular senescence program and the concomitant NK receptor activating ligand expression **(Fig. 22A)**. We obtained similar results with melphalan treatment (data not shown).

Overall, our findings suggest that ROS-dependent activation of DDR is involved in the regulation of both NKG2D and DNAM-1 ligand expression on MM senescent cells.

4.8. Chk1/2 activation and ROS generation are involved in DNAM-1 and NKG2D ligand up-regulation on MM patient-derived PCs

Analysis of patient-derived myeloma cells obtained from bone marrow samples gave us the possibility to extend our observations on primary malignant plasma cells as well as to study the regulation of NKG2D ligands belonging to the ULBP family and the DNAM-1 ligand Nec-2, that are not present on the surface of MM cell lines and are up-regulated on patient-derived cells. We analyzed 8 patients affected by multiple myeloma at different stages according to Durie & Salmon's staging system **(Table 1**, Pt. 11-18). CD138$^+$ malignant PCs were purified from bone marrow aspirates and treated with doxorubicin or melphalan. As previously shown, up-regulation of surface NKG2D and DNAM-1 ligands on drug-treated *ex vivo* PCs was variable among different patients **(Fig. 11)** and for sake of simplicity we decided to only report the effect of inhibitors on the expression of the NKG2D and DNAM-1 ligands undergoing up-regulation **(Fig. 23)**.

In order to analyze the role of Chk1/2 in drug-mediated up-regulation of NK activating receptor ligand in patient-derived malignant PCs (Pt. 11-13), CD138$^+$ cells were preincubated with UCN-01 (10nM) before doxorubicin and melphalan treatment. We confirmed, such as with MM cell lines, that Chk1/2 has an important role also in *ex vivo* ligand up-regulation on malignant PCs induced by low doses of chemotherapeutic drugs **(Fig. 23A)**.

We also investigated the role of p53, that has been recently shown to be involved in ULBP1/2 up-regulation in cancer cell line carrying inducible wtp53 or mutp53 **(Textor 2011)**. We could still observe no changes in the up-regulation of NKG2D and DNAM-1 ligands on *ex vivo* CD138$^+$ PCs incubated with Pifithrin-α before melphalan

treatment suggesting that also the ULBPs are up-regulated upon drug treatment in a p53 independent manner **(Fig. 23C)**.

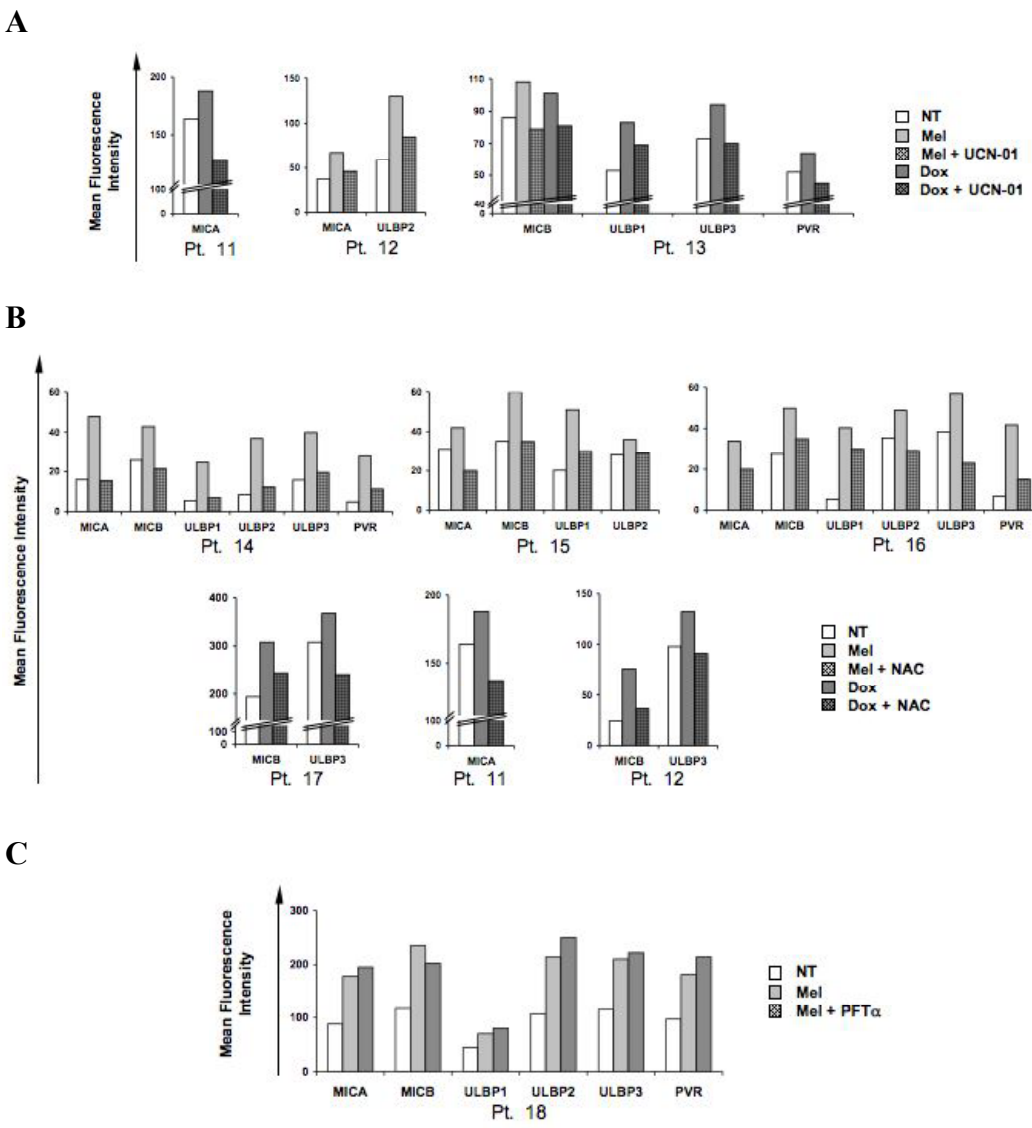


Figure 23. Low doses of therapeutic agents upregulate DNAM-1 and NKG2D ligands on patient-derived PCs in a Chk1/2 and ROS-dependent manner.
Purified CD138$^+$ cells were pre-incubated 1h with UCN-01 (10nM) **(A)**, NAC (2mM) **(B)**, or Pifithrin-α (30μM) **(C)**, and then cultured with doxorubicin (0.05μM) or melphalan (20μM) for further 48h in complete medium supplemented with 20ng/ml of IL-3 and 2ng/ml of IL-6. Upon treatment, the expression of MICA, MICB, ULBP1, ULBP2, ULBP3, PVR and Nec-2 was analyzed by immunofluorescence and flow cytometry on the indicated patients. On the *y*-axis is shown the MFI values obtained by subtracting the MFI of the isotype control antibody. Pt=patient.

We then tested the role of redox signaling, by preincubating purified CD138$^+$ PCs with NAC before drug treatment. Depending on the amount of PCs obtained from

the different patients, samples were treated with melphalan (Pt. 14-16) or doxorubicin (Pt. 17, 11-12) drugs. Consistent with the data obtained with MM cell lines, NAC pretreatment of drug-treated *ex vivo* malignant cells was able to completely block NKG2D and DNAM-1 ligand surface up-regulation **(Fig. 23B)**. In addition, our findings indicate that NAC does not have a strong effect on ligand basal level (data not shown). The observation obtained on isolated $CD138^+$ PCs indicates that ligand up-regulation is triggered by ROS generated by PCs and not by other BM-derived cells.

All together these data further support the findings obtained on MM cell lines and strongly indicate that redox signaling dependent DDR activation is responsible of the drug-triggered NKG2D and DNAM-1 ligand up-regulation on patient derived malignant PCs.

5. DISCUSSION

In the present study aimed at identifying new NK cell-based chemo-immunotherapeutic approaches against MM, we investigated NKG2D and DNAM-1 ligand expression induced by genotoxic agents on cancer cells, as well as the mechanisms underlying their regulation. We demonstrated that commonly used chemotherapeutic drugs, when used at low doses, induce the up-regulation of NKG2D and DNAM-1 ligand expression on MM cells, and, consequently, enhance NK cell degranulation toward drug-treated tumor cells. Moreover, ligand up-regulation on drug-treated MM cells occurs in conjuction with the establishment of a chemotherapy-induced senescent phenotype. These effects depend on the activation of a ROS-dependent DNA damage response, and, in particular, drug-induced NKG2D and DNAM-1 ligand up-regulation occurs in a ATM/ATR and Chk1/2-dependent but p53-independent manner.

We showed that doxorubicin or melphalan, when used at doses that do not affect cell viability, induce the up-regulation of MICA, MICB and PVR expression on MM cell lines both as extracellular protein and mRNA levels. This observation was also confirmed on primary malignant PCs treated with melphalan, bortezomib or doxorubicin. Thanks to the possibility of performing experiments with patient-derived myeloma PCs, we observed drug-induced up-regulation of ULBP and Nec-2 molecules and, unlike MM cell lines, the up-regulation occurred also for ligands not costitutivelly expressed.

Interestingly, we demonstrated that the presence of doxorubicin or melphalan-treated MM cells leads to enhanced NK cell degranulation, being the NKG2D and DNAM-1 receptors the major triggering molecules. Moreover, we could rule out the involvement of NK cell inhibitory receptors because we did not observe HLA class I down-regulation on treated MM cells (data not shown).

Our results confirm that NK cells have a major role in MM tumor suppression. The efficacy of several therapeutic agents that prolong survival of MM patients has been widely attributed to their ability of enhancing NK cell numbers and cytotoxic functions **(Davies 2001, Tai 2005)**. Previous evidence demonstrated that bone marrow myeloma cells have higher levels of MICA expression as compared to pleural effusion-derived myeloma cells **(Carbone 2005)**. In addition PCs from MM patients have higher

MICA expression as compared to healthy donor plasma cells but lower level as compared to plasma cells from MGUS patients, suggesting an association between MICA, its shedding from cell surface, and the progression of multiple myeloma **(Jinushi 2008)**. Moreover, a role for DNAM-1 and its ligands has been demonstrated in NK cell-mediated killing of myeloma cells **(El-Sherbiny 2007)**. Most of these reports addressed the role of NK cells against MM disease focusing mainly on MGUS or active MM; in our study, malignant PCs were also from patients with smoldering MM, an intermediate stage that can often progress to fully developed malignancy. Of note, for a long time, PVR and Nec-2 were mainly considered only adhesion molecules, and there are few evidences still now demonstrating their role in NK cell activation. Our results strongly support the notion of the NK cell activating ability of DNAM-1 ligands.

Our studies provide new evidences on the molecular mechanisms regulating NKG2D and DNAM-1 ligand expression. In the last years, a major role for the DDR in NKG2D ligand expression has been demonstrated while the mechanisms underlying DNAM-1 ligand regulation remain still obscure. The DDR pathway is initiated by ATR and ATM kinases: in particular, ATM is primarly responsible for detecting double-strand DNA breaks, whereas ATR is mainly responsible for the detection of stalled DNA replication. The major ATR substrate is the Chk1 kinase while Chk2 is for ATM, even though it is now widely demonstrated that Chk1 and Chk2 pathways are not parallel branches of the DDR but show a high degree of cross-talk and connection **(Sancar 2004)**. Gasser *et al.* demonstrated that NKG2D ligand expression was induced after exposure to DNA replication inhibitors or agents that induce chromatin remodeling through the activation of the ATM/ATR/Chk1/Chk2 pathway **(Gasser 2005)**. Moreover, our group demonstrated a role for ATM/ATR in the regulation of MICA and PVR expression on antigen-activated T lymphocytes **(Cerboni 2007b, Ardolino 2011)**.

Our findings report for the first time the involvement of ATM/ATR and Chk1/2 in the drug-induced increased expression of not only NKG2D ligands, but also of DNAM-1 ligands. In particular, we demonstrated that no toxic doses of doxorubicin or melphalan on MM cell lines are sufficient to lead ATM autophosphorylation at Ser1981, activation of Chk1 and Chk2 cell cycle kinases, phosphorylation of p53 at Ser15, and up-regulation of the DNA damage marker γH2AX. Chk1 phosphorylation upon doxorubicin or melphalan treatment occurs mostly on Ser345 residue, usually phosphorylated by ATR in response to UV, IR, and hydroxyurea **(Liu 2000)**. Of note, drug-induced phosphorylation of Chk2 occurs mostly on Thr68, an ATM- and ATR-

dependent phosphorylation site, while Ser33/35 residues that are phosphorylated only in the presence of high amounts of DNA damage in an ATM-dependent but ATR-independent manner **(Buscemi 2004, Buscemi 2006)** are not modified, confirming that our chemotherapeutic treatments trigger a mild DDR not leading to apoptosis. Our findings are in line with previous observation demonstrating that in response to radiation, Chk2 undergoes phosphorylation only on Thr68 in cells arrested to G_2M phase while cells in G_1 and S phases display Chk2 also phosphorylated on Ser33/35 **(Buscemi 2006)**.

A key role for ATM/ATR and Chk1/2 in drug-induced NKG2D and DNAM-1 ligand up-regulation was indicated by the findings that ligand up-regulation was complety abrogated in the presence of specific pharmacologic inhibitors. By contrast, we tend to rule out the involvement of the drug-induced activation of p53 in ligand up-regulation, because its inhibitor, Pifithrin-α, did not impair NKG2D and DNAM-1 ligand up-regulation on drug-treated MM cells. Moreover, the up-regulation of MICA, MICB and PVR expression still persisted in p53-silenced SKO-007(J3) cells. This data was also confirmed on patient's malignant PCs demonstrating that also ULBP molecules are regulated in a p53-independent manner upon treatment with melphalan. These results are in accordance with previous observation by Gasser *et al.* **(Gasser 2005)** that demonstrating increased NKG2D ligand expression in $p53^{-/-}$ ovarian epithelial cells in response to ionizing radiation and inhibitors of DNA replication. On the other hand, they are in disagreement with recent data indicating the involvement of p53 in ULBP1/2 up-regulation on carcinoma and sarcoma cell lines **(Textor 2011, Li 2011)**. This may be partially explained by the fact that we used a different tumor model and by the recent observations suggesting that NKG2D ligand expression is regulated by p53-dependent and p53-independent pathways in response to different stimuli. Hence, our findings strongly suggest that DDR is a common strategy involved in drug-induced regulation of the ligands for NK cell activating receptors.

We also demonstrated that our chemotherapeutic treatment, not only induces the up-regulation of NK cell activating ligands, but also activates a premature senescence program on MM cells. Our observations are in line with previous data demonstrating that sublethal doses of genotoxic agents trigger a weak persistent activation of the DDR pathway that lead to premature senescence and not to apoptosis **(Schmitt 2007)**. In accordance with the evidences that drug-induced senescent cells preferentially arrest in the G_2M cell cycle phase **(Chang 1999, Bozko 2005)**, our findings describe for the first

time a correlation between the senescence phenotype and the NK cell activating ligand expression, showing that drug-induced NKG2D (MICA, MICB) and DNAM-1 (PVR) ligands are preferentially expressed on the G_2M-arrested MM cells, with PVR and MICB expression almost completely confined to senescent cells. We strengthened these data also analyzing MICA, MICB and PVR mRNA levels on isolated β-Gal^{high} senescent cells. We suggested a model in which the senescence tumor cells alert the immune system that in turn recognizes and eliminates them through NK cell mediated killing. This hypothesis is supported by findings indicating that p53-mediated activation of a senescent program in murine liver carcinoma cells triggers macrophage- and NK cell-mediated immune responses leading to enhanced tumor clearance **(Xue 2007)**. In addition, NK cells where found to facilitate the resolution of liver fibrosis by eliminating senescent-activated stellate cells **(Krizhanovsky 2008)**. Our hypothesis is also supported by evidences demonstrating that NKG2D preferentially recognizes pre-malignant lesions or early stage tumors **(Guerra 2008, Unni 2008)** that are associated with an oncogene-induced senescent phenotype **(Collado 2007, Schimitt 2007)**. Overall, there are several evidences suggesting that NK cells represent an immunosurveillance mechanism against stress-induced premature senescent cells triggered by drugs or oncogenic signals.

It is widely demonstrated that the persistence of unrepaired double-strand DNA breaks and the chronic mild activation of the DNA damage response pathway are common features of several forms of senescence, but the precise nature of these DNA breaks remains unclear. Several evidences suggest a role of elevated ROS levels in the accumulation of DNA damage **(Kurz EU 2004b, Shackelford 2001)** and in the induction of a senescent phenotype **(Balaban 2005)**. In addition, it has been also demonstrated a positive feedback through which senescent cells produce high amount of ROS to sustain the senescent phenotype and ensure a stable cell cycle arrest **(Takahashi 2006)**. Despite the difficulty of analyzing the intracellular presence and the biological role of the reactive oxygen species and other reactive species, it is largely accepted that ROS are not only able to damage biomolecules but can also act as intracellular second messangers. Hence, the ROS-induced DDR activation and ROS-induced senescence may reflect the cellular damage and/or the physiologic signaling response **(Irani 1997, Rai 2009)**. Of note, it is widely demonstrated that the mechanisms of action of doxorubicin, melphalan and of several DNA damaging chemotherapeutic drugs depends on oxidative stress production **(Jung 2001, Donepudi 2001, Mukhopadhyay 2009)**.

Moreover, there are some evidences suggesting a regulation of NKG2D and DNAM-1 ligand expression by oxidative stress. Borchers *et al.* demonstrated that hydrogen peroxide induces MIC and ULBP molecule expression on airway epithelial cells **(Borchers 2006**. In addition, ROS-regulated transcription factors NF-kB and AP-1 are involved in the regulation of MICA gene expression **(Cerboni 2007b, Molinero 2004)**, and a putative site for the oxidative stress-induced transcription factor Nrf1 has been identified in the promoter region of PVR **(Solecki 2000)**.

We showed that low dose-treatments of doxorubicin or melphalan in MM cells do not trigger a massive production of reactive species, instead lead to a mild perturbation of the cellular oxidative status, not easily detectable by specific probes (data not shown) but preventable by using the wide ROS scavenger NAC. We demonstrated that redox perturbation is the major event triggering the activation of the DDR pathway upon drug treatment because NAC abolished ATM and Chk1/2 phosphorylation in MM cells. Moreover, we showed that ROS-induced DDR is responsible of NKG2D and DNAM-1 ligand up-regulation on MM cell lines and patient's malignant PCs both as extracellular protein and mRNA levels. This result confirms previous data demonstrating that NKG2D ligand expression is affected by cellular oxidative status while it is one of the first evidences describing a role for ROS in the regulation of DNAM-1 ligand expression. Our group has also demonstrated that ROS produced by monocytes induce PVR up-regulation on activated T cells **(Ardolino 2011)**. Given the low levels of ROS production induced in drug-treated MM cells, it is conceivable to hypothesize that DDR is not activated directly by reactive species but rather by oxidized cellular macromolecules. Moreover, we suggest that redox-dependent DDR activation upon chemotherapeutic treatment is critical for MM cell entry in premature senescence as it is shown by the impaired G_2M cell cycle arrest and SA-βGal positivity in the presence of DDR inhibitors or NAC.

Our results support recent evidences suggesting that ROS, usually considered oncogenic and pro-ageing, have also a good face because they can be used to kill cancer cells **(Wang 2008)**. In this regard a recent paper demonstrated that dexamethasone-induced oxidative stress sensitizes MM cells to radiotherapy while spare normal hematopoietic progenitor cells **(Bera 2010)**. It has been also demonstrated that chronic treatments with sub-apoptotic doses of chemotherapeutic drugs, such as resveratrol, induce senescence in tumor cells through ROS increase **(Heiss 2007)**, and numerous evidence clearly demonstrated that the expression of NK cell activating ligands is

regulated by redox signaling **(Borchers 2006, Cerboni 2007b, Molinero 2004, Solecki 2000)**, but the correlation between these events has been observed so far.

Overall, we demonstrated that low dose-treatments of genotoxic agents on MM cells trigger an alteration of the cellular physiologic redox status and the subsequent activation of the DDR. The redox-activated DDR leads MM cells to enter in premature senescence and to up-regulate NKG2D and DNAM-1 ligands on cell surface leading to an increment of NK cell degranulation. This is in line with a large body of previous experimental and clinical evidences demonstrating that exposure to low doses of chemotherapeutic drugs is able to increase immune responses, including NK cell activity, whereas high doses of the same agents are immunosuppressive **(Ehrke 2003)**. In this regard, in older patients affected by MM, administration of intermediate doses of melphalan increases response rate and improves remission duration and survival. We can envisage that its action might also be attributable to induction of innate immune responses **(Palumbo 2006)**. Induction of cellular senescence by chemotherapeutic agents has also emerged as a primary mechanism of tumor regression through its anti-proliferative power. Our findings indicate that in addition to this action, the success of senescence-based anticancer therapies may also be related to their ability to trigger anti-tumor immune responses both rendering senescent tumor cells more visible to NK cell action, and inducing the production by senescent tumor cells of a specific secretory phenotype that might activate and attract the immune cells **(Rodier 2011)**.

Future efforts will focus in defining the possible cellular targets of ROS action, in characterizing the transcriptional factor(s) responsible of NKG2D and DNAM-1 ligand synthesis, and in analyzing the specific cytokines and chemokines secreted by senescent tumor cells. Moreover, it is now largely accepted that expression of NKG2D ligands on tumor cells is regulated at different levels, including epigenetic, transcriptional, and post-transcriptional mechanisms, and there is not reason to dismiss that it can also occurs for DNAM-1 ligands. Thus, a better knowledge of the mechanisms of regulation could help us in achieving the highest expression of NK cell activating ligands by treatment with a combination of compounds targeting the different levels of regulation.

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