University of Genoa

Center of Excellence for Biomedical Research (CEBR)

PhD in Clinical and Experimental Immunology

Myeloma cells induce the accumulation of activated CD94^{low} NK cells by cell-to-cell contacts involving CD56 molecules

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CONTENTS

Abstract

Natural Killer (NK) cells represent innate effector cells potentially able to play a role during the immune response against Multiple Myeloma (MM).

To better define the distribution and the specific properties of NK cell subsets during MM disease, we analyzed their features in the bone marrow and peripheral blood of newly diagnosed MM patients. Our findings revealed that, in both compartments, NK cells were more abundant than in healthy donors. Among total MM-NK cells, a significant increase of $CD94^{low}CD56^{dim}$ NK cell subset was observed, which already appears in clinical precursor conditions leading to MM, namely monoclonal gammopathy of undetermined significance and smoldering MM, and eventually accumulates with disease progression. Moreover, a consistent fraction of $CD94^{low}CD56^{dim}$ NK cells was in a proliferation phase. When analyzed for their killing abilities, they represented the main cytotoxic NK cell subset against autologous MM cells.

In vitro, MM cells could rapidly induce the expansion of the $CD94^{low}CD56^{dim}$ NK cell subset, thus reminiscent of that observed in MM patients. Mechanistically, this accumulation relied on cell to cell contacts between MM and NK cells and required both activation via DNAM-1 and homophilic interaction with CD56 expressed on MM cells.

Considering the growing variety of combination treatments aimed at enhancing NK cell-mediated cytotoxicity against MM, these results may also be informative for optimizing current immunotherapeutic approaches.

Key words: NK cells, Human Multiple Myeloma, NCAM-1/CD56, CD94/NKG2A, Immunobiology, Immunotherapy.

Introduction

Multiple Myeloma

Multiple Myeloma (MM) is a hematologic malignancy characterized by a monoclonal expansion of malignant plasma cells (PCs) within the bone marrow (BM), often accompanied by osteolytic bone lesions and modifications of normal immune responses.[1] MM represents about 1% of total neoplastic diseases, and 13% of hematologic cancers, currently the treatments rely on autologous stem cell transplantation and subministration of several classes of drugs, such as alkylating agents (melphalan), IMiDs (thalidomide, lenalidomide) or proteasome inhibitors (bortezomib). New treatment protocols have improved the management of MM course, and extended patients survival, though this pathology is still classified as incurable.

The clonal proliferation of malignant PCs in the bone marrow microenvironment is associated with increase in the level of monoclonal (M) protein in blood and serum. Myeloma cells are characterized by high rates of somatic hypermutation of immunoglobulin (Ig) genes and isotype class switching, but differ from healthy PCs with respect to the abundance of certain cell surface molecules, including CD138 and CD38.

MM arises consistently from asymptomatic precursor conditions, specifically monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM (sMM) (**Figure 1**), with a cumulative risk of overall progression of 1% and 10% per year, respectively.[2,3]

MM evolves from these premalignant disorders via progressive molecular events that lead to altered plasma cell surface protein expression, such as loss or decrease of CD45 molecules, aberrant expression of neural cell adhesion molecule (NCAM-1/CD56)[4] and abnormally enhanced proliferation upon interactions with BM microenvironment.[5]

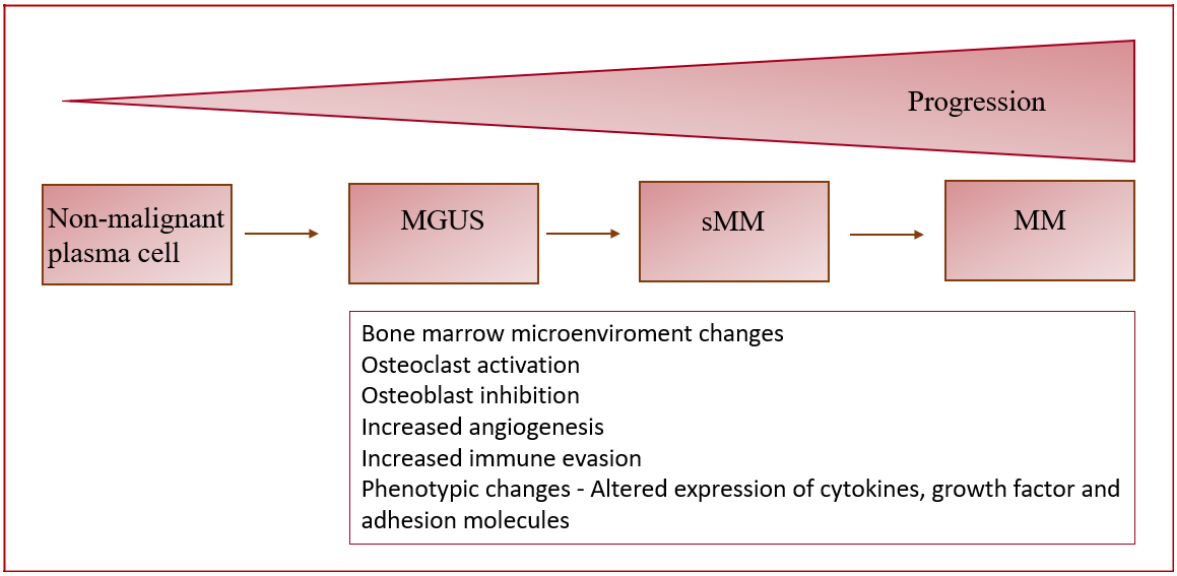


Figure 1. Progression of Multiple Myeloma

Natural Killer cells

Natural Killer (NK) cells are innate lymphocytes involved in the initiation, enhancement, and regulation of anti-tumor immune response and in the control of viral infection spreading.

NK cells express a wide range of activating and inhibitory receptors, as well as adhesion and co-stimulatory molecules, allowing them to recognize and kill infected or transformed cells[6,7], whereas they are prevented from attacking normal tissues by the interaction between major histocompatibility complex (MHC) class I molecules and MHC-class I-specific inhibitory receptors.

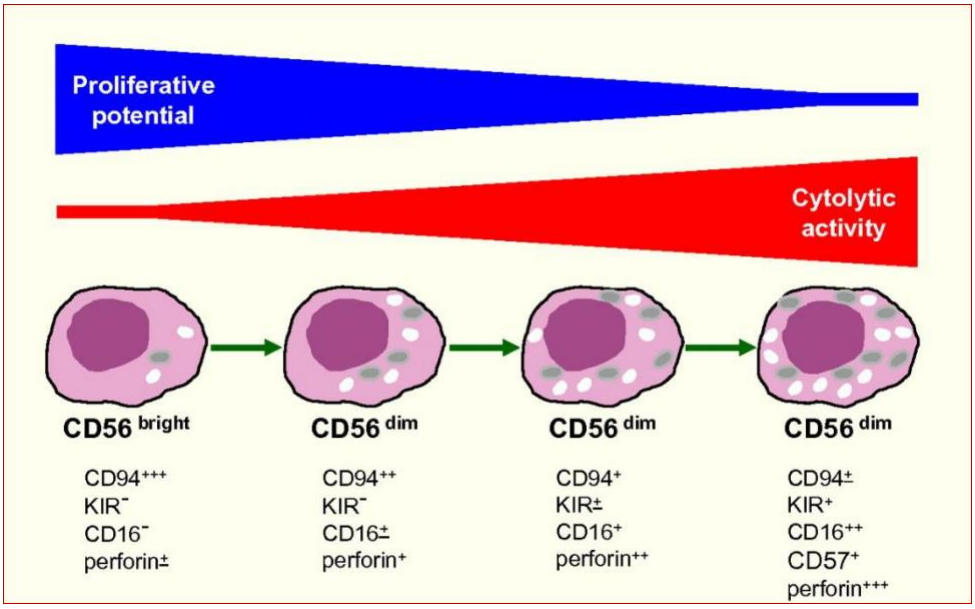
Therefore, in the absence or down-regulation of MHC class I expression, for example, on tumor cells or virus-infected cells, engagement of the activating receptors on NK cells by the corresponding ligands triggers target cell killing.

NK cells represent 5-10% of circulating lymphocytes, but they are widely present in other tissue compartments. In particular, they are abundant in secondary lymphoid tissues (lymph node, spleen, tonsils) and in inflamed tissues.

Among the lymphocyte population, they are defined by the positive expression of CD56 and the lack of expression of the T cell receptor and the CD3 complex. CD56 expression is not restricted to NK cell population: it represents the 140 kDa isoform of the neural cell adhesion molecule (N-CAM), and it is also expressed by a T cell subtype and some cancer cells.[8] Although this molecule is used for human NK cell phenotypic definition and selection, its role on NK cell function is still unknown.

One key function in the development of NK cells is the CD56-driven migratory behavior of NK cells on stromal cells, forming a developmental synapse. NK cells acquire motility with progressive maturation, correlated with the expression of CD56 on developing NK cells.[9] Blocking of CD56 therefore perturbs both NK cell motility and maturation. In general $CD56^+$ immune cells are also able to form strong immune synapses with each other through CD56 binding. For example, $CD56^+$ DCs have been shown to induce the preferential activation and expansion of $CD56^+$ γδ T cells via CD56.[10] In particular, homophilic interaction between CD56 molecules on $CD56^+$ cells can be formed, including immune cells but also, for example, tumor cells. In this way, $CD56^+$ CIK cells are able to kill $CD56^+$ leukemic cells.[11] The expression of another NK cell surface molecule, the low-affinity receptor for the Fc portion of IgG CD16 (FcγRIIIA), is also used to define NK cells; like CD56, its expression is not restricted to these cells. The definition of NK cells by a unique marker is still debated. NK cells are usually distinguished into two main subsets $CD56^{bright}$ and $CD56^{dim}$ NK cells, which represent sequential stages of maturation[12] and show a dichotomy in phenotypic and functional properties.[13] $CD56^{dim}$ NK cells account for ~90% of the population in peripheral blood (PB) and are capable of potent cytotoxicity. $CD56^{bright}$ NK cells, on the other hand, are poor mediators of direct cytotoxicity but are competent for cytokine production and, primarily, reside in secondary lymphoid tissue and other solid tissues.[14]

Despite some common features, several NK cell subsets with specialized functions have been described in human and mice by using different markers.[15] The progression of CD56^{bright} toward the more differentiated CD56^{dim} NK cells can be identified on the basis of the progressive down-regulation of CD94[16], CD62L and the expression of KIRs and CD16, while CD57 expression is acquired at later stages and marks terminally differentiated cells (**Figure 2**).[17,18,19]



L. Moretta Blood, 2010;116:3689-3691

Figure 2. Model of human CD56^{dim} NK-cell differentiation

Among these markers, CD94 particular interest since useful to distinguish functionally distinct subsets in both human and mouse. CD94 is a type II integral membrane protein that is related to the C-type lectin superfamily and can covalently associate with NKG2A/C. In human, the natural ligand for these CD94/ NKG2A-C heterodimers is the non-classic MHC class I molecule HLA-E.

The level of CD94 expression can segregate human NK cells into three distinct subsets and defines phenotypic and functional intermediaries existing between $CD56^{bright}$ and $CD56^{dim}CD57^{+}$ NK cell subsets (**Figure 3**).[16,17]

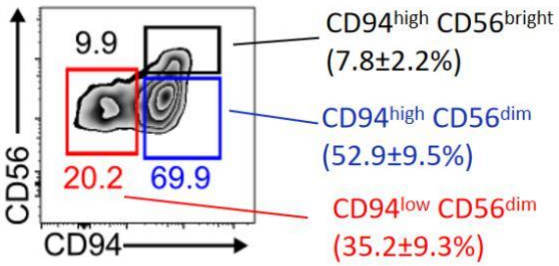


Figure 3. Dissection of PB-NK cells from healthy donor by using the CD94 marker. Flow cytometry analysis of NK cells derived from PB of Healthy Donor, dissected on the basis of CD56 and CD94 surface expression levels. NK cell subsets are identified as $CD94^{low}CD56^{dim}$ (red), $CD94^{high}CD56^{dim}$ (blue) and $CD94^{high}CD56^{bright}$ (black) and numbers indicate the percentage of cells in each gate.

NK cells function

Natural killer cells are able to lyse virus-infected and transformed cells without any priming and are particularly activated towards targets that display low levels of major histocompatibility complex (MHC) class I molecules via two main cell-to-cell contact dependent mechanisms: the Antibody Dependent Cell Cytotoxicity (ADCC) and the Natural Cytotoxicity.

The binding to a target cell is accompanied by the formation of a complex structure at the cell–cell interface, named immunological synapse. Following the recognition of a target cell, NK cytotoxic granules containing perforin (a membrane-disrupting protein) and granzymes (serine proteases) are secreted by a Ca2+-dependent exocytosis in the immunological synapse. This mechanism is also known as degranulation. Perforin polymerizes and forms a transmembrane pore that allows the delivery of granzymes to the cytosol where they cleave and activate different caspases leading to the apoptosis of the target cell.[20] Lining the membrane of the lytic granules is the lysosomal-associated-membrane protein-1 (LAMP-1, or CD107a).

Has been demonstrated that the up-regulation of this molecule on NK cell membrane is a marker of NK cell activation and strongly correlates with both cytokine secretion and NK cell-mediated lysis of target cells.[21]

Target cell recognition can occur through direct recognition of the target cell mediated by activating and inhibitory receptors or through antibody-dependent cellular cytotoxicity (ADCC) mediated via ligation of the CD16 receptor expressed on $CD56^{dim}$ NK cells. ADCC is an important immune effector mechanism by which antigens of tumor or infected cells coated by IgG antibodies are recognized by Fcγ receptors present on effector cells. Thus, antibodies mediate contacts between effector and target cells allowing the subsequent killing of target cells. Natural Killer cells are one of the major effector cells involved in this mechanism due to their expression of CD16, the FcγRIIIA. In particular, $CD56^{dim}$ NK cells, highly expressing this receptor, are more implicated in ADCC than $CD56^{bright}$ NK cells. The interaction between CD16 and its ligand induces NK cell degranulation with the exocytosis of perforin and granzymes at the immune synapse. This mechanism has been well studied and is now exploited in mAb-based cancer immunotherapy. The production of antibodies directed against specific antigens overexpressed by tumor cells is an active field in immunotherapy.

NK cell cytotoxicity can be mediated by another mechanism that involves the direct binding between NK cell molecules belonging to the Tumour Necrosis Factor (TNF) family, like FasL and the soluble TNF-related apoptosis-inducing ligand (TRAIL), and their receptors expressed by target cells (Fas, and TRAIL-R). This second pathway is less implicated in the host defense against pathogens in vivo but is important for the elimination of auto-reactive lymphoid cells and homeostasis. Fas (CD95) is expressed by numerous cells and belongs to the TNF receptor family. This receptor contains a conserved intracytoplasmic “death domain” that, following the interaction with Fas-L, indirectly activates the cleavage of caspases and induces apoptosis.

Natural cytotoxicity refers to the capacity of Natural Killer cells to lyse a target cell without prior sensitization and without antibody-mediated recognition. In 1990, Klas Karre proposed "the missing self" hypothesis to explain how NK cells are capable to lyse target cells lacking or displaying low expression of MHC class I molecules.[22] This theory derived from the numerous experimental observations that tumor cells deficient for MHC class I molecules were efficiently lysed by NKs, conferring them a role in the control of NK cell activation. The missing self hypothesis was proposed before the discovery of the receptors implicated in the recognition of MHC-I molecules (KIRs, CD94/NKG2 and ILT).

NK cell Receptors and Ligands

Through the identification of activating and inhibitory NK cell receptors, the missing self theory has evolved. It is currently accepted that NK cell activation depends on an intricate balance between positive and negative signals derived from membrane receptors (**Figure 4**).

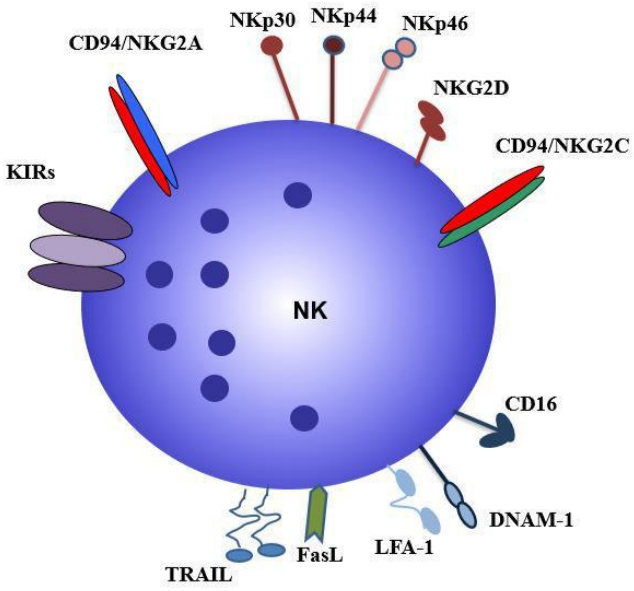


Figure 4. NK cell receptors

Killer-cell immunoglobulin-like receptors

Killer cell immunoglobulin-like receptors (KIR) constitute the main group of inhibitory receptors expressed on human NK cells. Located on chromosome 19, stochastic expression of the KIR genes is epigenetically controlled via the KIR promoter.[23] The KIR nomenclature is based on the length of the cytoplasmic tail, short (S) or long (L), and the number of extracellular Ig-like domains (2 or 3). While the long cytoplasmic tail receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIM), the short tails contain immunoreceptor tyrosine-based activation motifs (ITAM) that aid in binding to the adaptor molecule DAP12.

Phosphorylation of ITIMs on inhibitory receptors results in the recruitment of tyrosine phosphatases which in turn dephosphorylate adaptor molecules associated with activating receptors.[24] This ensures inhibitory receptor signaling dominating over activating receptor signaling. KIR bind to specific allelic variants of human leukocyte antigen (HLA) A, B and C, the human equivalent of MHC class I proteins.[25] Non-classical HLA-F and HLA-G have also been identified as interacting with KIR receptors. As the highly diverse KIR locus is both polygenic and polymorphic, many ligands for this large repertoire of KIR receptors still remain to be discovered.

For simplicity, two KIR haplotypes are used to group KIR genotypes within individuals. Haplotype A contains a restricted number of inhibitory receptors and one activating receptor, KIR2DS4. The less common haplotype B includes a larger repertoire of both inhibitory and activating receptors61. On top of the stochastic expression via epigenetic regulation of the KIR gene promoter, variation in terms of KIR gene copy number furthers adds to the diversity. The three main inhibitory receptors commonly studied include KIR2DL1, KIR2DL3 and KIR3DL1.

Other notable KIR-ligand interactions include KIR3DL2 binding to HLAA3/A11 and HLA-F and the activator receptors KIR2DS1 binding to HLA-C2 and KIR3DS1 binding to HLA-F Activating ligands, in particular, are still largely undiscovered.

CD94/NKG2 heterodimers

CD94 is a type II integral membrane protein that is related to the C-type lectin superfamily and can covalently associate with 5 different members of the NKG2 family (NKG2A, B, C, E, and H), but not with NKG2D. This association is important for the translocation of NKG2 receptors to cell surface and for ligand recognition. NKG2 receptors share a common structure composed of a C-type lectin-like domain, a transmembrane domain and a cytoplasmic segment. According to the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains and to their binding with adaptor molecules, they were classified as inhibitory or activating receptors. NKG2 and CD94 genes are located in a region of chromosome 12 known as “Natural Killer complex”. NKG2A and the alternative spliced form B contain two ITIM motifs, form a heterodimer with CD94 molecule and are thus inhibitory receptors. After interaction with the ligand, the tyrosine residue in each ITIM domain is phosphorylated leading to the recruitment and activation of SHP-1 and SHP-2 tyrosine phosphatases and finally blocking the NK cell activation cascade[26] In contrast, NKG2C, E and H variants lack ITIM domains and are associated with the ITAM-bearing DAP12 adaptor molecule. Thus, CD94/NKG2C molecules form heterodimeric activating receptors. The affinity of NKG2 receptors with ligands was investigated and seemed to be different for each receptor: in particular, CD94/NKG2A displayed a higher affinity for HLA-E molecules compared to CD94/NKG2C. Interestingly, binding affinity was dependent on the peptide sequence and not on HLA-E allelic differences.[27]

Cell surface expression of HLA-E is regulated by the expression of other classical class I molecules, as they are the major source of HLA-E binding peptides in normal cells. In particular, signal sequence peptides from HLA-I molecules (HLA-A, B and C as well as the non-classical HLA-G molecule) bind to HLA-E allowing its surface expression. Consequently, CD94/NKG2 receptors, through the recognition of HLA-E, let NK cells indirectly survey the global expression of class I molecules, frequently altered in transformed or infected cells. The decrease of classical HLA molecules at the surface of tumor cells is a major mechanism involved in tumor immune escape from cytotoxic T effectors while it leads to NK cell activation. Conversely, non-classical HLA-E and HLA-G molecules are frequently overexpressed by cancer cells participating in tumor immune escape through the interaction with inhibitory receptors and the subsequent inhibition of NK and cytotoxic T cell (CTL) activation. The production of soluble HLA molecules (sHLA; classical and non-classical) have been described as another potential mechanism involved in cancer immune escape and in vitro studies demonstrated that sHLA could induce inhibition and apoptosis of NK cells.

Along with NKG2C and NKG2D, a number of other activating receptors exist which play important roles in regulating the cytotoxic capability of NK cells. Notably these include the germline encoded natural cytotoxicity receptor (NCR) family consisting of NKp30, NKp44 and NKp46, CD16 and DNAX accessory molecule-1 (DNAM-1).

Additional molecules appear to be important in the activation of NK cell cytotoxicity, among them NKp80, 2B4 and NTB-A. These are considered as co-receptors since their function is dependent on the concomitant stimulation by true activating receptors.

Natural Cytotoxicity Receptors (NCRs)

Three NK cell triggering surface receptors that display a critical role in the induction of NK cell-mediated non-MHC-restricted cytotoxicity of tumor and transformed cells have been identified. These important activating receptors for NK cell lytic function are named "Natural Cytotoxicity Receptors" (NCR). The cross-linking of NCR with specific mAb strongly increase the cytolytic activity in redirected killing assays, whereas blocking of NCR by mAbs inhibits NK cell cytotoxicity against most target cells. Furthermore, NCR surface density on NK cells correlates with the magnitude of cytotoxicity against NK-susceptible target cells.[28-30] NKp30 (NCR3) and NKp46 (NCR1) are ubiquitously expressed on resting NK cells in peripheral blood, while NKp44 (NCR2) is upregulated on activated NK cells in response to IL 2 stimulation. NKp46, evolutionarily conserved in mammals, contains two extracellular Ig domains, similar to Ig-like receptors, while NKp30 and NKp44 only contain one domain each. All three receptors signal via coupling to adaptor molecules, either FceRIg and CD3z (NKp30, NKp46) or DAP12 (NKp44). B7-H6, the ligand for NKp30, is expressed on tumor cell lines as well as on neutrophils and monocytes after toll-like receptor and pro-inflammatory cytokine stimulation. Similar to CD16, NKp30 also has immune-regulatory functions on top of its important role in immune surveillance. The ligands for NKp44 and NKp46 have been suggested to be viral hemagglutinins.

DNAM-1 (CD226)

DNAM-1, also known as CD226, is a leukocyte adhesion molecule involved in the induction phase of NK cell activation. DNAM-1 gene is encoded on chromosome 18 and is expressed by NK cells, T cells and monocytes.

Its structure is characterized by an extracellular portion with two Ig-like domains and a cytoplasmic tail containing three tyrosine residues. Following DNAM-1 cross-linking, tyrosines are phosphorylated and NK cell cytotoxicity is triggered. Interestingly, DNAM-1 expression is dependent on the surface expression of lymphocyte function associated-antigen 1 (LFA-1).[31] In humans, DNAM-1 expression is coordinated with LFA-1 undergoing conformational changes, as they co-localize at the immune synapse.

Two ligands of DNAM-1 have been identified: the poliovirus receptor (PVR; CD155) and Nectine-2 (CD112). These molecules are strongly expressed by tumor cell lines of epithelial and neuronal origin like carcinomas, melanomas and neuroblastomas.[32] In association with NCR or NKG2D receptors, DNAM-1 is involved in the recognition and lysis of different types of cancer cells: myeloma[33], melanoma[34], ovarian carcinomas.[35] Its role in anti-tumor immunity is prominent when ligands for other activating receptors are poorly expressed.[36]

Aim of the study

The anti-MM potential of NK cells has been of rising interest in recent years. Although it has been reported that NK cell recognition and killing of MM cells mainly involves the activating receptors DNAM-1, NKG2D and/or NKp46, MM cells utilize specific immunoevasive strategies to reduce NK cell recognition and cytotoxicity. The presence of high levels of both classical HLA class I and HLA-E on MM cells can efficiently provide inhibitory signaling, mediated by KIRs and CD94/NKG2A, respectively (**Figure 5**), therefore representing an important stumbling block for NK cell activity against autologous MM cells.[37,38]

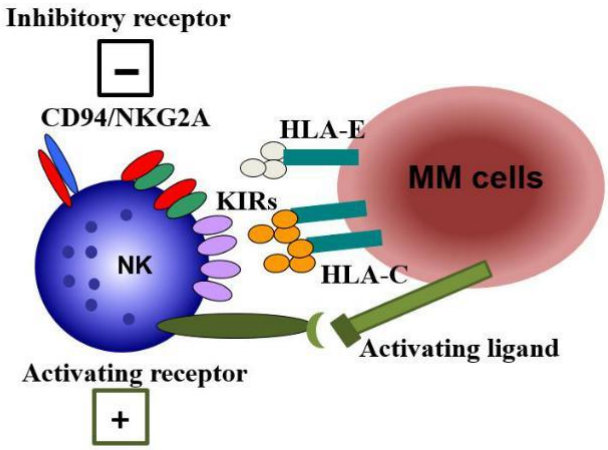


Figure 5. Mechanisms of resistance for NK cell lysis of MM cells

The aim of this study was to better define the distribution and the specific properties of NK cell subsets during MM disease. Here we analyzed NK cells features in the bone marrow and peripheral blood of newly diagnosed, untreated, MM patients and during MM progression. Moreover, we analyzed different NK cell subsets on the basis of their receptor expression, including CD94/NKG2A molecules, representing one of the dominant NK cell inhibitory signal involved in MM resistance to NK cell killing.

Materials and Methods

Patients' recruitment

Peripheral blood (PB) and bone marrow (BM) samples were obtained from a cohort of 23 newly diagnosed, untreated, MM pts (Table 1). Additional PB and BM samples were also collected from pts with MGUS (8 PB and 4 BM samples) and sMM (5 PB and 3 BM samples). All the pts were admitted at the Hematology Unit of University Hospital Policlinico *G. Martino*, Messina. The study was approved by the institutional ethics committees and all participants gave written informed consent according to the Declaration of Helsinki.

PTs no.	Age (years) /Gender	Clinical Stage (ISS)	Monoclonal Ig	% PCs in BM	CD56 expression on MM cells
1	60/M	III	IgG κ	50	++
2	65/M	I	IgG κ	28	++
3	59/M	III	IgG κ	60	++
4	60/F	I	IgG κ	60	+
5	65/F	III	IgA κ	50	+
6	67/F	III	IgG κ	40	++
7	62/F	II	IgG κ	70	+
8	60/M	III	IgG κ	70	++
9	54/F	I	IgG κ	35	++
10	65/M	I	IgG κ	50	++
11	60/F	II	IgG κ	30	++
12	62/M	II	IgA κ	35	++
13	58/F	I	IgG λ	60	+
14	65/M	II	IgG κ	50	++
15	70/M	I	IgG κ	40	++
16	62/F	III	IgA κ	50	++
17	65/F	III	IgG λ	40	++
18	63/F	II	IgA κ	45	++
19	64/M	III	IgG λ	38	++
20	60/F	III	IgG λ	80	+
21	61/F	III	IgG λ	38	++
22	65/M	III	IgG κ	30	+
23	63/F	II	IgG λ	35	+

Table 1. Patient's baseline characteristics and CD56 expression on BM-MM cells.
++: CD56 positive MM cells (70-100%); +: CD56 positive MM cells (30-69%)

Cell Purification and Isolation

Peripheral blood mononuclear cells (PBMCs), as well as bone marrow mononuclear cells (BMMCs), were isolated by Ficoll Hypaque density gradient centrifugation from heparinized PB and BM samples (30 min, 25 °C, 400 × g). To isolate MM cells (which reside in BM and hardly circulate in PB) BMMCs from MM pts were stained with anti-CD38 PerCp-Cy5.5 (clone HIT2) and anti-CD138 PE (clone M115) mAbs and then FACS sorted by gating on $CD38^{high}CD138^{+}$cells.

To isolate NK cells, PBMCs from MM pts and/or HDs were stained with anti-CD56 APC-Vio770 (clone REA196) and anti-CD3 Vioblue (clone BW264/56) and then FACS sorted by gating on $CD56^{+}$ $CD3^{-}$ cells. In selected experiments, NKp46 has been employed as additional marker to identify, count and/or sort NK cells. The gating strategy to identify and/or sort both NK cells and plasma cells in the BM is shown in **Figure 6**. Alternatively, human NK cells were previously isolated by negative magnetic separation (NK Cell Isolation Kit/ Miltenyi). Cell populations sorted either by negative magnetic separation or by flow cytometry displayed purity above 95%.

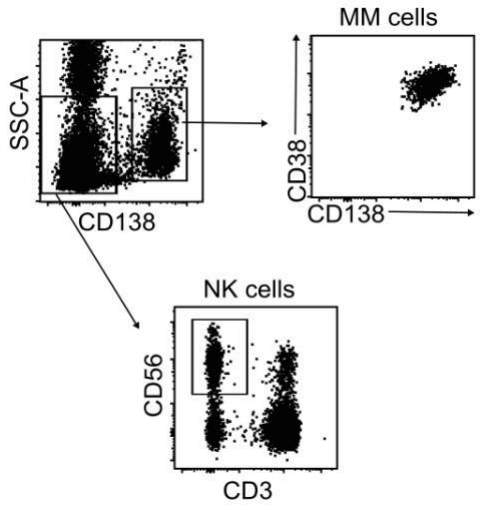


Figure 6. Gating strategy used to identify and/or sort both NK cells and MM cells in BM. Representative flow cytometry dot plots of gating strategy used to identify and /or sort NK cells and MM cells in BM. MM cells were isolated by gating on $CD38^{high}CD138^{+}$cells. NK cells were isolated as $CD56^{+}CD3^{-}$ cells after gating on the $CD138^{-}$ cell fraction.

Multicolor Flow Cytometry

To investigate the phenotype of NK cells in MM pts, PBMCs or BMMCs were stained with the following mAbs: anti-CD94 FITC (clone HP3D9), anti-CD57 PE (clone NKI), anti-CD16 PeCy5.5 (clone 3G8), anti-CD19 APC-R700 (clone HIB19), anti-CD62L PE (clone SK11), anti-CD69 PeCF594 (clone FN50), anti-HLA-DR APC-H7 (clone L243), anti-NKp46 APC (clone 9E2), anti-NKp44 Alexa Fluor 647 (clone p44-8), anti-CD38 PerCp Cy5.5 (clone HIT2) and anti-CD138 PE (clone M115), purchased from BD Biosciences; anti-CD56 APC-Vio770 (clone REA196), anti-CD3 Vioblue (clone BW264/56), anti-CD45 Viogreen (clone 5B1), anti-NKp30 PE (clone AF29-4D12), anti-NKG2D PE-Vio770 (clone BAT221), anti-NKG2A APC (clone REA110), anti-CD158a/h PE (KIR2DL1/DS1) (clone 11PB6), anti-CD158b PE (KIR2DL2/DL3/DS1) (clone DX27) from Miltenyi; anti-CD56 PC7 (clone N901, NKH-1) and anti-CD3 ECD (clone UCHT1) from Beckman Coulter. For surface markers, cells were stained for 30 min at 4°C. For indirect labeling, the following mAbs were used: anti-HLA class I (clone A6-136, and clone W632), anti-CD56 (clone A6-220), anti-DNAM-1 (clone F5), anti-NKp46 (clone KL247), anti-NKp30 (clone F252), anti-NKG2D (clone BAT221), anti-LIR/LT2 (clone F278). All the antibodies used for indirect labeling were produced in our laboratory or kindly provided by Prof. Alessandro Moretta (University of Genoa, Italy). For indirect labeling, cells were first incubated with the primary antibody for 30’ at 4 °C. After incubation, cells were extensively washed and incubated with isotype-specific fluorochrome-conjugated secondary antibody for 30’ in the dark. NK cell proliferation was assessed by the expression of the intranuclear marker Ki67, performing fixation/permeabilization procedures according to manufacturer’s protocol (eBioscience) followed by staining with an anti-Ki67-BV421 mAb (clone B56) purchased from BD Biosciences. Dead cells were excluded by staining with LIVE/DEAD FIXABLE Aqua Dead dye (Invitrogen).

Sample acquisition was performed on FACSCantoII (BD Biosciences) or Gallios (Beckman Coulter) flow cytometers. Data were analyzed by FACSDiva (BD Biosciences) or FlowJoVX (Tree Star Inc) software.

Cell lines

The human MM cell lines U266B1 and MM1S were kindly provided by Prof. F. Cimino (University of Messina), whereas K562 (chronic myelogenous leukemia, CML) and 721.221 (B-lymphoblastoid cell line, LCL) were provided by Prof. M.C. Mingari. All the above cell lines were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml).

In order to assess the relevance of NCAM-1 in the interaction between NK cells and tumor cells, K562 cell line was transfected with the expression construct pCDNA3.1/V5/His-TOPO-NCAM-1 containing the cDNA coding for NCAM-1 (NM_181351), utilizing Lipofectamine 2000 (ThermoFisher) following manufacturer's instructions. After 72 h, NCAM-1 expression was analyzed by flow cytometry using anti-CD56 PC7 mAb. Transfected cells were cultured in the presence of 1.2 mg/ml G418 (Calbiochem) in order to obtain stable transfectants. At the end of the selection period, cells were sorted based on NCAM-1 expression, and subcloned by limiting dilution.

NK/MM co-cultures

To assess accumulation and proliferation of $CD94^{low}$ $CD56^{dim}$ NK cell subset, NK cells isolated from healthy donors (HDs) were plated at $1x10^5$/well in 96-well V-bottom plates, with or without primary MM cells (i.e. freshly isolated from pts BM) or MM cell lines (U266B1, MM1S), 721.221 lymphoblastoid cell line, K562 erythroleukemia cell line, at NK cell to target cell (E:T) ratio of 1 to 1, for 24 hours.

In selected experiments, co-cultures were preceded by incubation of cells (either effector or target cells) with blocking mAbs against NKp46 (IgM, clone KL247), NKp30 (IgM, clone F252), NKG2D (IgG1, clone BAT221), DNAM-1 (IgM, clone F5), CD56 (IgM, clone A6-220) (kindly provided by Prof. A. Moretta), or isotype matched controls.

To assess NK-cell cytolytic potential and IFN-γ production, CD107a degranulation assay and intracellular IFN-γ staining were performed by co-incubating NK cells isolated from both HDs and MM pts, with primary MM cells or U266B1 cell line or K562. In selected experiments, co-cultures were preceded by incubation of target cells with HLA class I (IgM, clone A6-136) blocking mAb. Co-cultures were performed, at an E:T ratio of 1:1, in the presence of anti-CD107a APC (BD Biosciences). After 1 h, Golgi Stop (1:1500 dilution, BD Biosciences) was added, and cultures were incubated for an additional 5 hrs. Upon surface staining, cells were fixed and permeabilized (Cytofix/Cytoperm Kit, BD Biosciences) according to the manufacturer's protocol and incubated with anti-IFN-γ PE-conjugated mAb (BD Biosciences) for 30 min at 4°C. Surface CD107a and intracellular IFN-γ expression were finally assessed by flow cytometry on effector cells. To detect spontaneous degranulation and constitutive expression of cytokine, NK cells incubated without target cells were analyzed as control.

To assess the killing capacity of HDs- and MM-NK cells we performed a flow cytometric and non-radioactive target-based assay. In this assay, NK cells isolated from both HDs and MM pts were co-incubated at an E:T ratio of 1:1 with a target tumor cell lines (K562 or U266B1). The target cells were pre-labeled with a red fluorescent dye PKH-26 (Sigma-Aldrich), according to the manufacturer's instructions, to allow their discrimination from the effector cells (NK cells). After short term in vitro incubation (3h), killed target cells are identified by a 7AAD stain, which specifically permeates dead cells. Data analysis is performed first by gating on PKH-26 positive target cells, followed by the analysis of the 7AAD positive subpopulation.

The following control conditions were included in this assay: Target cells only and Tween mediated killing of target cells. The target cells death was calculated on the bases of the ratio: [(% sample cytotoxicity - % spontaneous cytotoxicity)/(% total cytotoxicity - % spontaneous cytotoxicity)].

Statistical Analyses

A paired Student's t-test was used to evaluate statistical significance. *P*-values <0.05 were considered statistically significant. Statistics were calculated using GraphPad Prism 6 software.

Results

NK cells are increased in both PB and BM of MM patients

In order to analyse NK cells in MM disease, newly diagnosed, not treated pts, were enrolled in the study. We first determined the frequency of NK cells in both PB and BM compartments and compared it with the one in healthy donors (HDs). We found a higher amount of NK cells in both PB (18.2%±1.153% vs 10.37%±0.57%, *P*=0.002) and BM of MM pts (17.57%±0.44% vs 9.54%±0.44%, *P*=0.002) (**Figure 7A**), whereas total leukocyte and lymphocyte counts did not show significant differences between MM pts and HDs. Remarkably, a consistent fraction of NK cells in both PB and BM of MM pts expressed Ki67 (**Figure7B**), therefore indicating that NK cells in these pts are largely in a proliferative phase, which may account for their higher amount in MM pts.

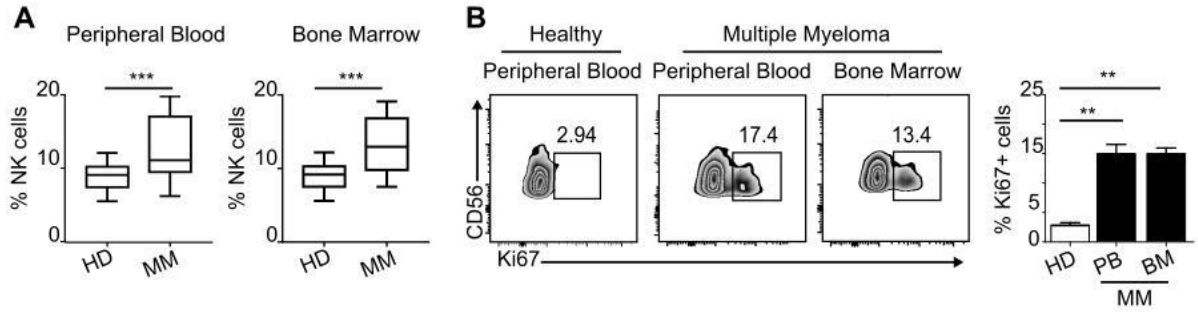


Figure 7. Assessment of NK cell frequency and proliferation in MM patients. (**A**) PBMCs and BMMCs of MM pts or HDs were analysed for the frequency of NK cells (gated on total live $CD56^+$ $CD3^-$ lymphocytes). Box and whiskers plots represent percentage of NK cells in PB (MM=n.23; HD=n.23) and BM (MM=n.23; HD=n.3) samples; (***: p< 0.001), Student's t-test. (**B**) Proliferating NK cells were assessed by Ki67 staining in PBMCs and BMMCs derived from MM pts and PBMCs from HDs as control. Representative dot plots show the percentage of $Ki67^+$ cells among total NK cells. Data derived from 23 MM pts are summarized in the right panel and compared with HDs (n=20). Bars represent mean values ± SEM of $Ki67^+$ NK cells (**: p< 0.01), Student's t-test.

The $CD94^{low}CD56^{dim}$ NK cell subset is enriched and proliferating in MM patients

We then analyzed the distribution of major NK cell receptors and other relevant molecules expressed by NK cells of MM pts. As a whole, MM-NK cells displayed features consistent with a more activated and differentiated phenotype, highlighted by the up-regulation of both activating receptors and

activation markers and including a lower amount of $CD62L^+$ cells and a higher frequency of $CD57^+$ cells (**Figure 8**).[17,19,39] In agreement with these observations, NK cells expressing significant levels of CD94 molecules were drastically decreased in MM pts and this distribution was accompanied by a concomitant lower frequency of $NKG2A^+$ NK cells, while NK cells expressing NKG2C (also associated to CD94) were only slightly increased.

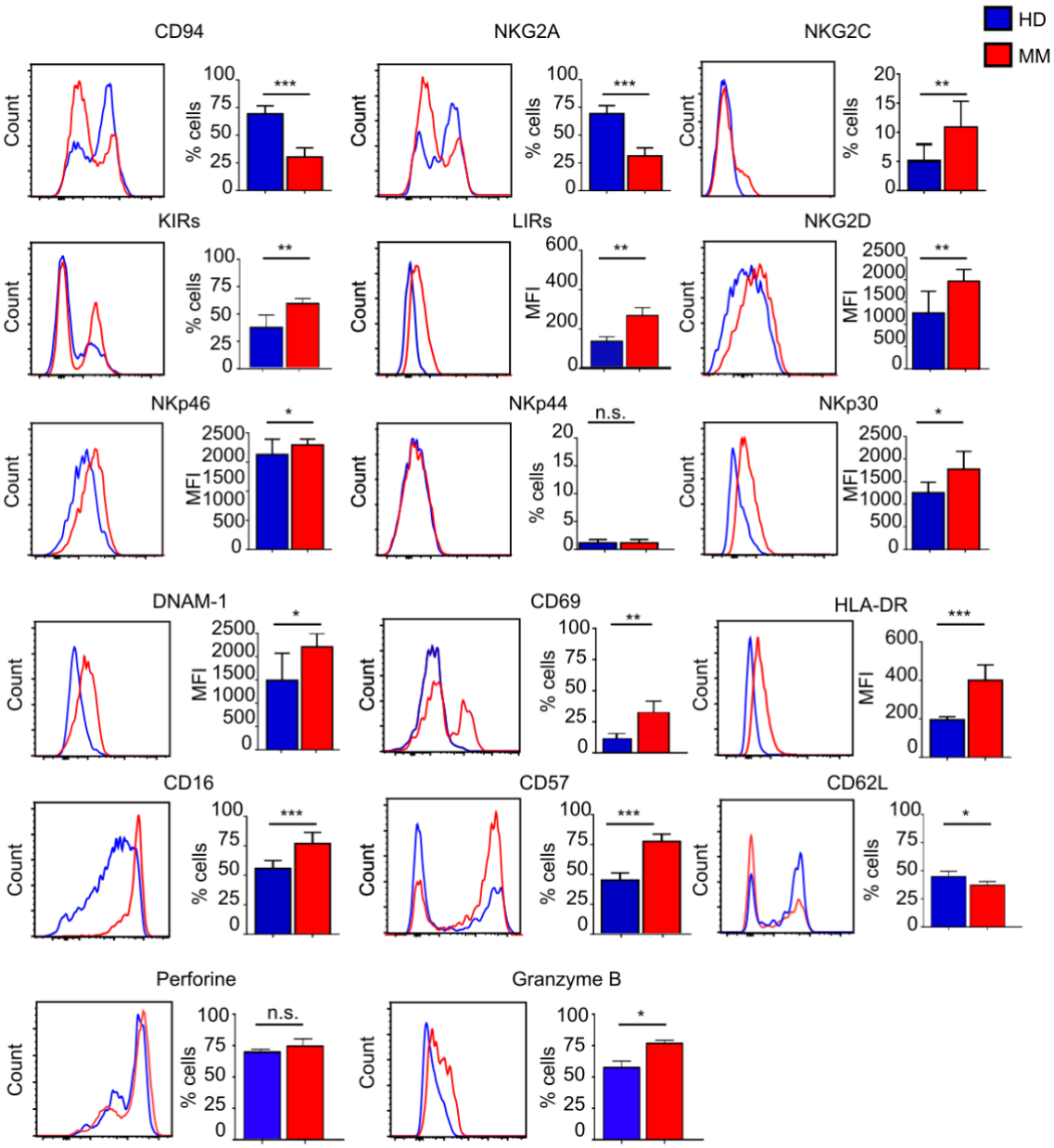


Figure 8. Phenotypic characterization of PB NK cells from MM patients. Flow cytometric analysis of the indicated markers assessed on NK cells freshly isolated from PB of MM pts or HDs. Data from analyses of at least 20 pts are summarized in graphs beside each flow cytometry histogram overlay; bars represent the mean values of the percentage of positive cells or mean fluorescence intensity (MFI) ± SEM for the indicated cell marker (*: $p<0.05$; **: $p< 0.01$; ***: $p< 0.001$). Student's t-test.

Considering that CD94 density identifies three functional distinct NK cell subsets and the relevance of CD94/NKG2A inhibitory molecules in the recognition of MM cells, we further dissected NK cell subsets on the basis of CD94 expression. $CD94^{low}CD56^{dim}$ subset was consistently increased in both PB (63.3%±7.8% vs 35.2%±9.8%, *P*=0.001) and BM (65.9%±7.4% vs 36.2%±9.4%, *P*=0.001) of MM pts, when compared to NK cells of HDs (**Figure 9**). Accordingly, also the amount of $NKG2A^{low/neg}$ NK cells was increased.

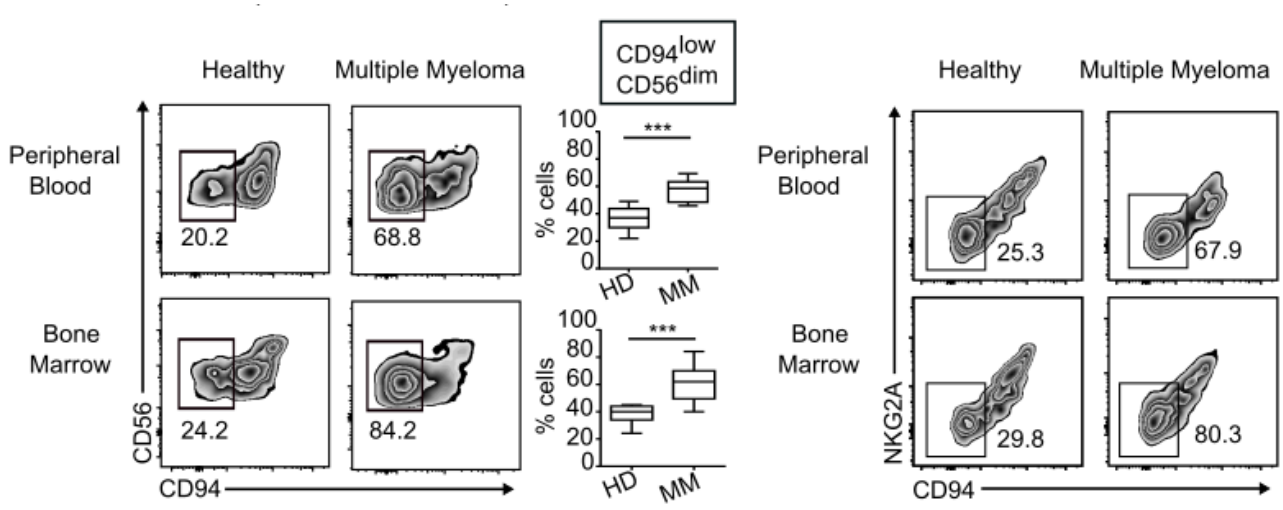


Figure 9. (**A**) Representative dot plots of NK cells from PB and BM of MM pts and HDs showing the percentage of $CD94^{low}CD56^{dim}$ NK cells. Box and whiskers plots represent the frequency of $CD94^{low}CD56^{dim}$ NK cells in PB or BM of 23 MM pts compared to HDs (PB=n.20, BM=n.3), (***: p< 0.001), Student's t-test. In the right panel, representative dot plots of NK cells from PB and BM of MM pts and HDs showing the frequency of $CD94^{low}NKG2A^{-}$ NK cells.

Further phenotypic analysis conducted on NK cell subsets dissected according to CD94 expression confirms the higher activation status of $CD94^{low}CD56^{dim}$ subset compared to the other two $CD94^{high}$ counterparts. Among the different markers, it is noteworthy that, in MM pts, DNAM-1 AND granzyme B were higher in the $CD94^{low}CD56^{dim}$ subset (Figure 10).

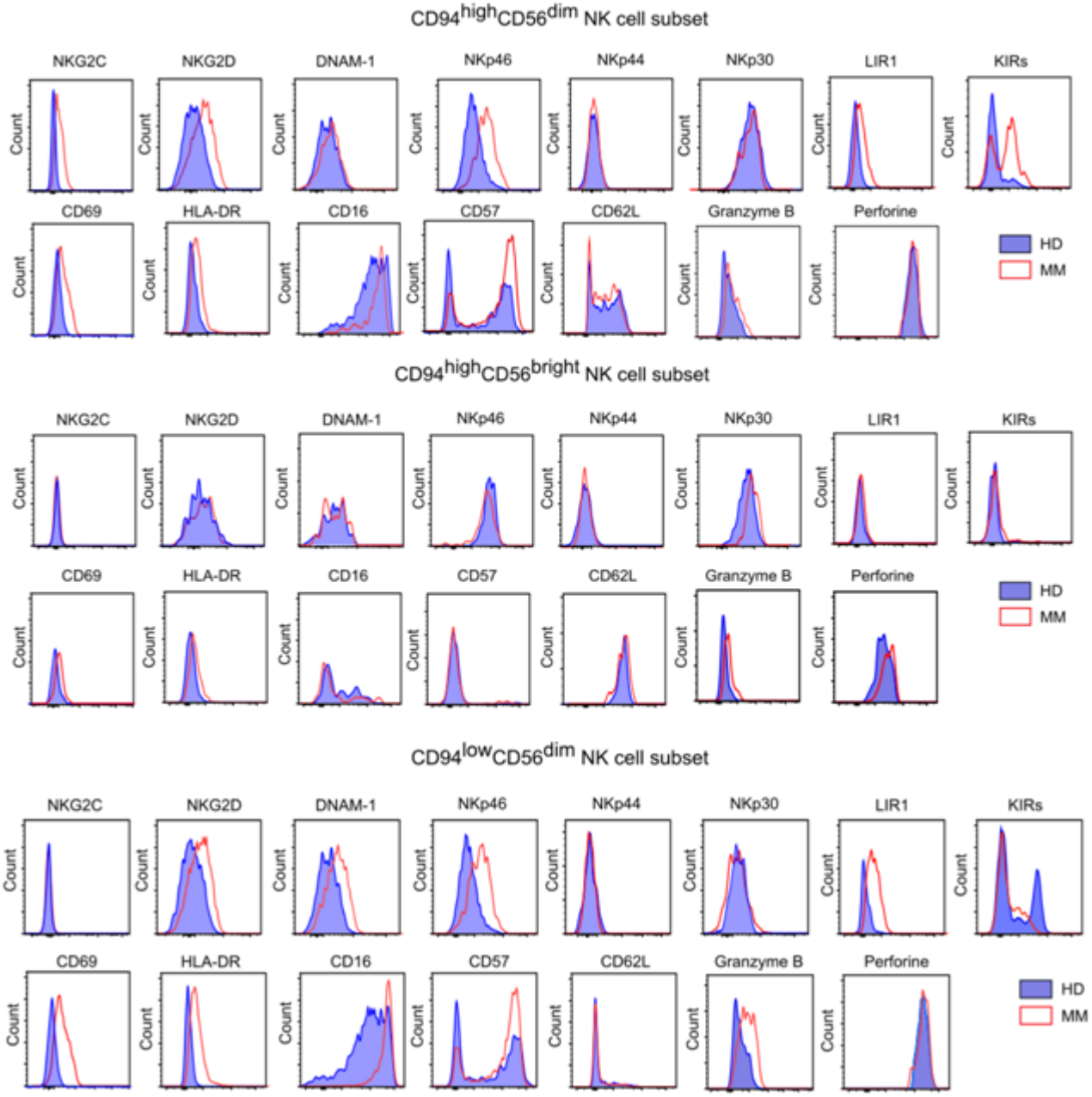


Figure 10. Phenotypic characterization of PB-NK cell subsets from MM patients dissected on the basis of CD94 expression. NK cells were identified as CD94lowCD56dim (upper panel), CD94highCD56dim (middle panel) and CD94highCD56bright (low panel) and flow cytometric analysis of the indicated markers was performed on freshly isolated PB NK cells of MM pts or HDs. Histograms shown are representative of data obtained from the analyses of at least 20 pts/HDs.

Interestingly, the accumulation of $CD94^{low}CD56^{dim}$ NK cells was accompanied by their concomitant expression of Ki67 (**Figure 11**) and a further dissection of $CD94^{low}CD56^{dim}$ NK cells based on the expression of the main KIRs (namely KIR2DL1/DS1 and KIR2DL2/DL3/DS2) revealed that Ki67 expression was confined to $CD94^{low}NKG2A^{-}KIR^{-}$ NK cells (**Figure 11, lower panel**). These results suggest that, in MM pts, $CD94^{low}NKG2A^{-}KIR^{-}$ NK cells, devoid of the main inhibitory signals, might be prone to proliferate upon their activation.

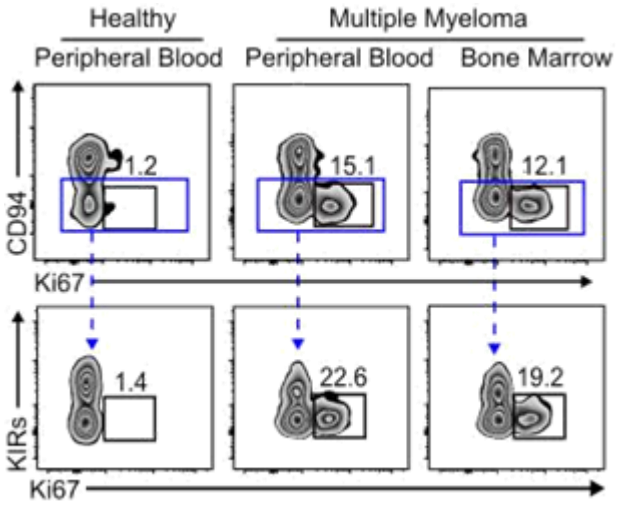


Figure 11. Analysis of proliferative rate of NK cells isolated from PB and BM of MM pts and from PB of HDs based on the expression of main inhibitory receptors (*upper row*). Numbers adjacent to gates indicate the frequency of $CD94^{low}CD56^{dim}$ $Ki67^{+}$ NK cells. *Lower row* shows Ki67 $^{+}$ cells within the $CD94^{low}CD56^{dim}$ NK cell population according to KIR (KIR2DL1/DS1 and KIR2DL2/DL3) expression.

The increase of CD94^{low}CD56^{dim} proliferating NK cells is an early hallmark of MM disease

Because MM disease is often preceded by precursor conditions, namely MGUS and sMM, we analyzed MM-NK cell features during MM progression. Interestingly, the modifications detected in MM pts were already present in MGUS pts and apparently accumulated along disease progression (**Figure 12A**). Total NK cell frequency, CD94^{low} NK cells, and Ki67^{+} NK cells were increased in MGUS and sMM pts, both in PB and BM, reaching however their highest levels in MM pts (Figure 5A). **Figure 12B** shows the modifications of NK cell compartments progressively occurring along disease evolution in a representative patient followed up during progression from MGUS to MM.

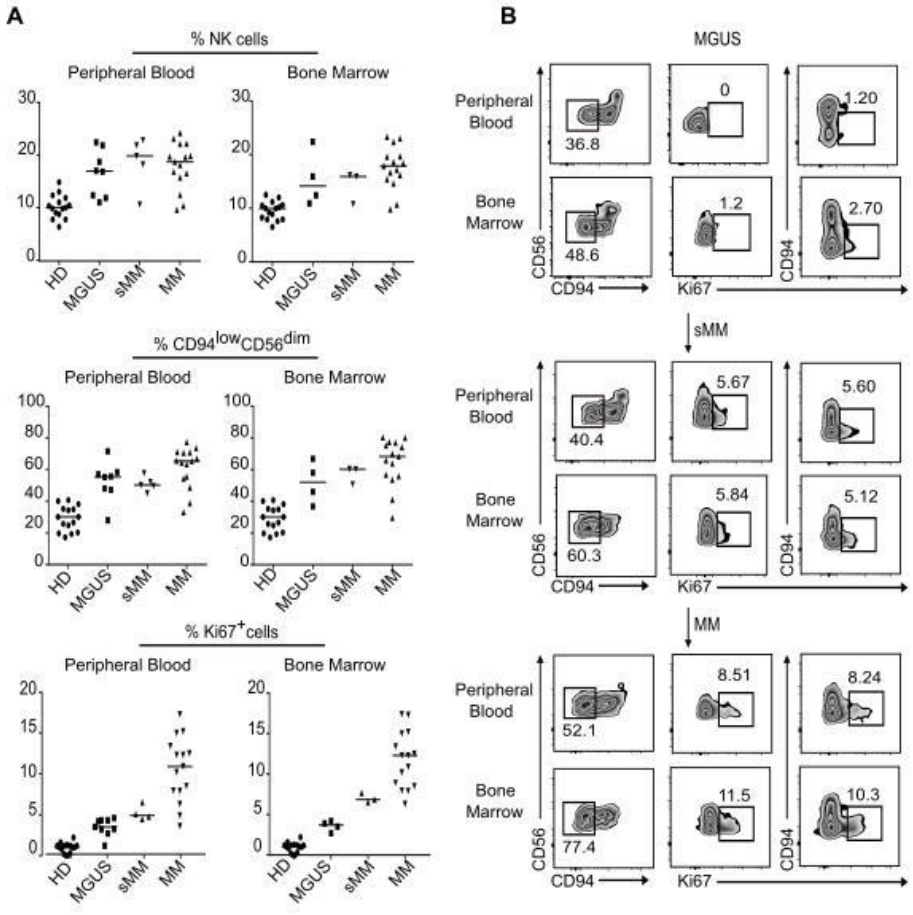


Figure 12. **NK cell features during the early phases of disease: from MGUS to MM. (A)** Comparative analysis of frequency of total NK cells (*upper row*), frequency of CD94^{low}CD56^{dim} NK cells (*middle row*) and frequency of Ki67^{+} NK cells (*lower row*) in PB and BM of MGUS, sMM and MM pts compared to HDs. **(B)** Representative patient evolving from MGUS to sMM and eventually to MM. Dot plots depict frequency of CD94^{low}CD56^{dim} NK cells and Ki67 analyzed on NK cells from both PB and BM of the patient along disease progression.

$CD94^{low}CD56^{dim}$ NK cells represent the main cytotoxic subset against autologous MM cells

We then investigated the functional capability of MM-NK cells against MM cells. To this aim, we used either primary MM cells or U266B1 cell line as cellular target for both HD- and MM-NK cells. MM-NK cells were more competent than HD-NK cells in both cytolytic ability and IFN-γ secreting properties against both allogeneic primary MM cells, U266B1 (**Figure 13A-B**), suggesting that NK cells of MM pts are not functionally impaired.

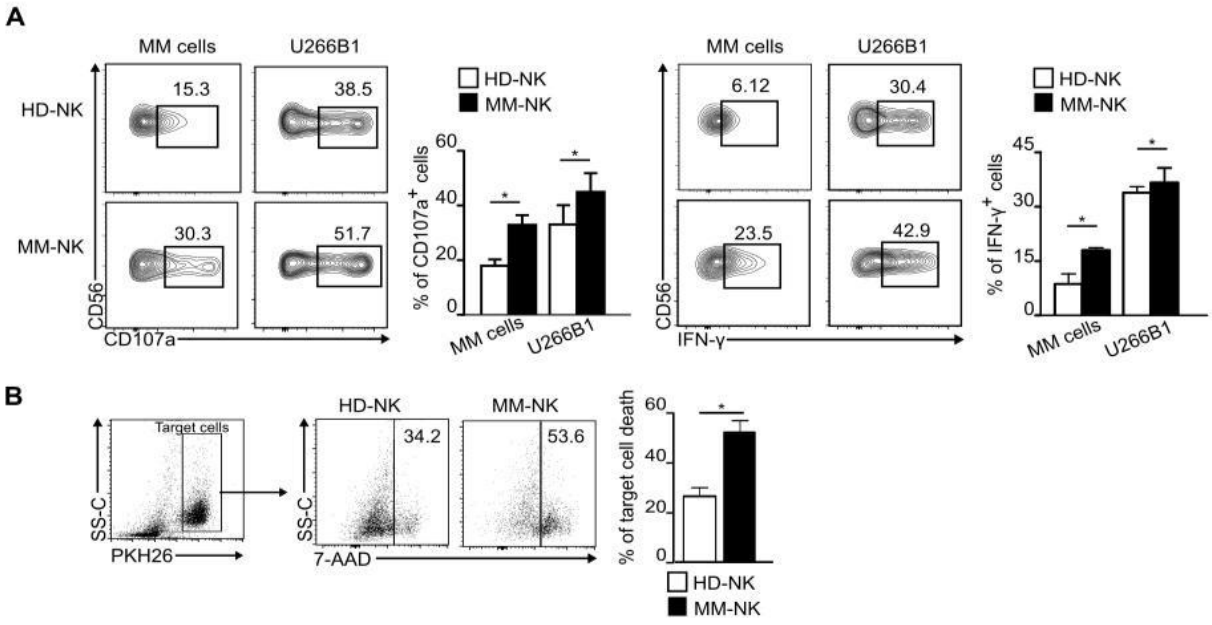


Figure 13. Degranulation ability and IFN-γ production of NK cells from MM patients. (**A)** NK cells isolated from PB of MM pts or HDs were co-cultured with allogeneic primary MM cells or U266B1 cell line at an E/T ratio 1:1 for 6hrs. In the *upper panel*, degranulation activity was assessed by evaluating the percentage of $CD107a^+$ NK cells. One representative experiment out of eight is shown. Summary of data is represented in the bars as mean ± SEM of $CD107a^+$ cells. (*: $p<0.05$), Student's t-test. In the *lower panel*, IFN-γ production by NK cells was analysed by flow cytometry. One representative experiment out of eight is shown. Results of all experiments performed are summarized in the bars as mean ± SEM of IFN-γ^+ cells. (*: $P<0.05$), Student's t-test. (**B**) NK cells isolated from PB of MM pts or HDs were co-cultured with K562 at an E/T ratio 1:1 for 3hrs. Killing capability of NK cells was assessed by evaluating target cell death upon co-culture. Gating strategy for detection of target cells. The initial gate was set in PKH26/SSC-A plot. U266B1 are gated as $PKH26^+$ events. Dead target cells (7AAD) are gated in the subsequent 7AAD/SSC-A plot within the PKH26+ target cell population.

Nevertheless, the cytolytic activity of MM-NK cells against autologous MM cells was significantly defective (**Figure 14**). This flawed cytotoxic capability against autologous MM cells seems to be dependent on the high level of HLA-class I molecules expressed by MM cells, which resulted, as a whole, as high as in normal tonsillar plasma cells (**Figure 14, right panel**).

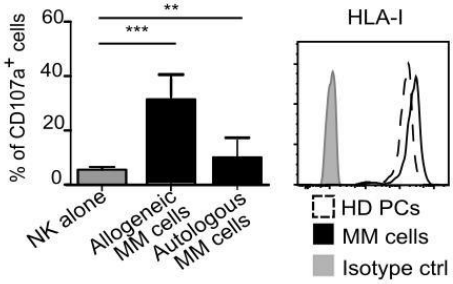


Figure 14. Degranulation ability of pts-derived NK cells against matched autologous or allogeneic MM cells. In the *left panel* data from 10 independent experiments are represented as mean ± SEM of $CD107a^+$ positive cells (*: $P<0.05$), Student's t-test. MM cells used for the degranulation assay were further analyzed for the expression of total HLA class I (clone W632, IgG) by flow cytometry. One representative patient is shown where MM cells were compared with healthy B plasma cells isolated from human tonsil (*right panel*).

Masking of total HLA-class I molecules on MM targets resulted in a relevant recovery of NK cell degranulation (**Figure 15**).

Although, as expected, NK cells were impaired in the autologous setting, it is noteworthy that $CD94^{low}CD56^{dim}$ NK cells mostly account for the residual degranulating activity against MM cells, while $CD94^{high}$ counterpart showed cytolytic ability only upon blocking of total HLA-I molecules (**Figure 15, right panel**).

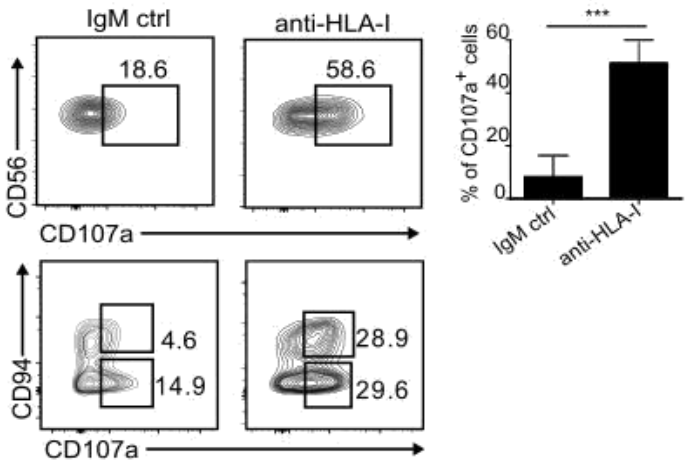


Figure 15. *(Upper panel):* degranulation ability of pts-derived NK cells was analyzed against autologous MM cells, either in the absence (isotype control, IgM) or in the presence of an anti-HLA-I blocking antibody (clone A6-136, IgM). One representative experiment is shown and numbers in each gate represent the percentage of $CD107a^+$ cells. Data from six independent experiments are summarized in the bars and presented as mean ± SEM of $CD107a^+$ NK cells (*: $P<0.05$), Student's t-test. In the *lower panel*, CD107a expression on pts-derived NK cells upon co-culture with autologous MM cells, in the absence or presence of an anti-HLA class I blocking antibody (clone A6-136, IgM) is shown in relation to CD94 marker distribution.

MM cells induce the accumulation of $CD94^{low}CD56^{dim}$ cells

With the aim of identifying the mechanisms leading to the accumulation and proliferation of $CD94^{low}CD56^{dim}$ NK cell subset, we evaluated whether MM cells could directly be responsible for the observed effect.

After 24h co-culture of HD-NK cells with primary MM cells (i.e. freshly isolated from pts BM) or with MM cell lines (namely U266B1 and MM1S), we observed that the percentage of $CD94^{low}CD56^{dim}$ NK cells was increased and this effect was specifically induced by MM cells, since we did not detect any significant increase when NK cells were co-cultured with other highly susceptible target cells such as K562 and LCL721.221 cell lines (**Figure 16A**).

Accordingly, both pts BM-derived primary MM cells and MM cell lines were able to induce the proliferation of $CD94^{low}CD56^{dim}$ NK cell subset in the absence of any exogenous growth factors, while this proliferation response was not detected employing K562 cell line in the co-culture (**Figure 16B**). Additionally, we sorted $CD94^{high}$ and $CD94^{low}$ HDs-NK cell subsets before co-culturing with MM cells and we compared their proliferative ability. Consistently with previous results we observed that the $CD94^{low}$ NK cell subset proliferated than $CD94^{high}$ NK cell subsets (**Figure 16C**). These results indicated that the observed accumulation of $CD94^{low}CD56^{dim}$ NK cells was dependent on their proliferation during co-culture with MM cells.

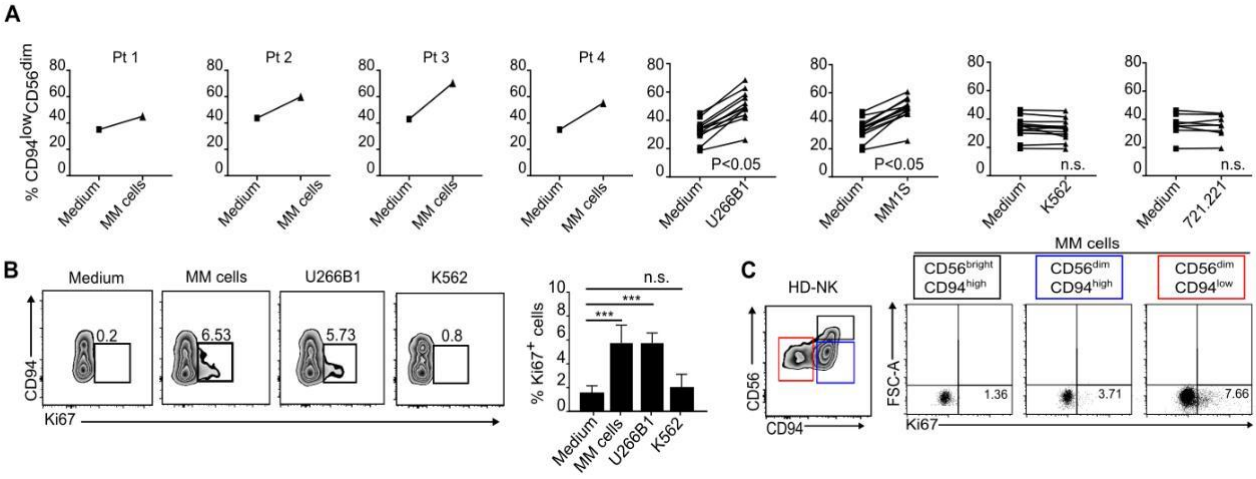


Figure 16. Mechanisms determining the expansion of $CD94^{low}CD56^{dim}$ NK cell subset induced by MM plasma cells (A) Frequency of $CD94^{low}CD56^{dim}$ NK cells among total NK cells from HDs upon 24h co-culture with or without allogeneic MM cells isolated from the BM of four distinct pts (*upper panel*) or with the indicated tumor cell lines (*lower panel*). E/T ratio of 1:1. **(B)** *In vitro* proliferation of NK cells from HDs cultured in the absence or presence of primary MM cells, U266B1 and K562 cell lines. The Ki67 expression on NK cells is shown in relation to CD94 molecule distribution. Summary of data from three independent experiments is shown as mean ±SEM (***: p< 0.001). Student's t-test. (**C**) . $CD94^{high}$ and $CD94^{low}$ HDs-NK cells were sorted and co-cultured for 24h with MM cells. One representative experiment out of three is shown.

To gain more insight into the specific mechanism underlying the accumulation of $CD94^{low}$ subset induced by MM cells, we determined CD94 expression upon 24h culture of NK cells in the presence of MM cell culture supernatant. Conditioned medium was ineffective for the accumulation of $CD94^{low}CD56^{dim}$ subset, suggesting that cell-to-cell contacts between NK cells and MM cells were required.

Thus, we assessed the contribution of the main NK cell activating receptors that might potentially be involved in the interaction with MM cells[33]. Remarkably, blocking DNAM-1 receptor significantly reduced the accumulation of $CD94^{low}CD56^{dim}$ NK cells, while inhibition of NKp46, NKp30, and NKG2D receptors did not significantly affect $CD94^{low}CD56^{dim}$ NK cell subset accumulation (**Figure 17A**). Since the ligands of DNAM-1 are widely expressed in several other tumor cell lines that do not induce $CD94^{low}CD56^{dim}$ NK cell expansion, we wondered whether MM cells may additionally provide some specific signal that might account for this peculiar accumulation in MM pts. NCAM-1, also known as CD56, is a glycoprotein typically expressed on NK cells but also present on neoplastic plasma cells.

CD56 has been implicated as having a role in cell-cell adhesion by homophilic interaction. Recently it has been reported that the expression of CD56 in breast cancer enhance the formation of cytotoxic immunological synapse becoming a factor responsible for the sensitivity to NK cell killing. In light of this, we hypothesized that CD56, known to be commonly expressed also by MM cells, might account for the observed effect on NK cell activation.

To address this issue, we sorted with high purity by flow cytometry either $CD56^{neg}$ or $CD56^{+}$ MM cells from patient's BM (**Figure 17B**) and we observed that only $CD56^{+}$ MM cells were able to induce $CD94^{low}$ accumulation (**Figure 17B**). Accordingly, the addition of CD56/NCAM-1 blocking mAb to MM/NK cell co-cultures prevented the accumulation of $CD94^{low}CD56^{dim}$ NK cells and, remarkably, their proliferation in response to MM cells (**Figure 17C-D**).

To further support the relevance of CD56 in the observed proliferation of NK cells, we stably transfected the cDNA coding for CD56 protein in the highly susceptible NK cell target K562 cell line. Reliable expression of CD56 was confirmed by staining with anti-CD56 mAb (**Figure 17E**). Consistent with our hypothesis, K562 cells expressing CD56 acquired the ability to induce proliferation of NK cells, while parental K562 were unable to mediate the same effect (**Figure 17E**), confirming that CD56-CD56 interaction between tumor and NK cells can play a relevant role in the activation of NK cells.

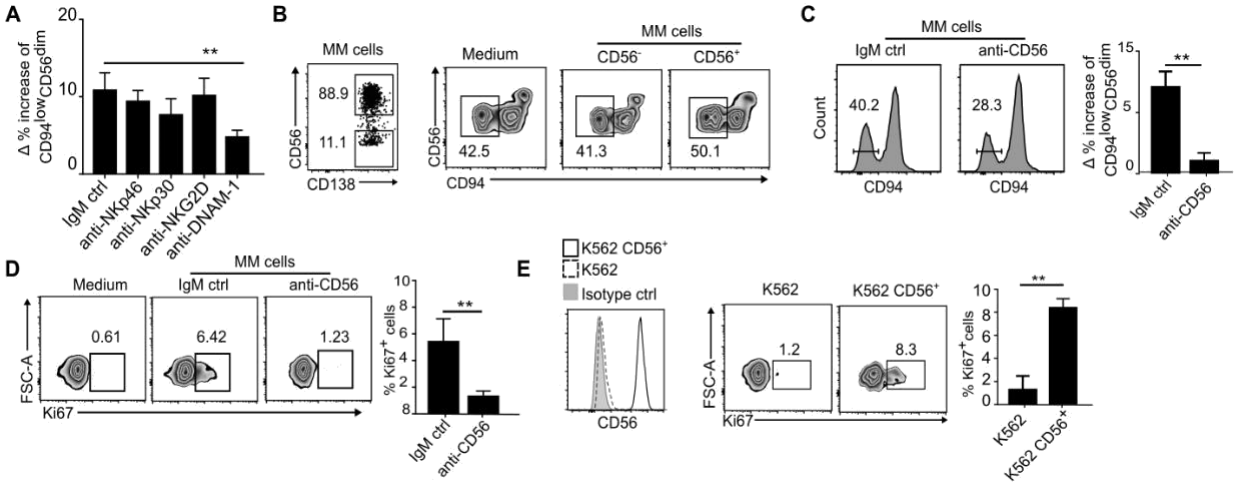


Figure 17. Mechanisms determining the expansion of CD94^{low}CD56^{dim} NK cell subset induced by MM plasma cells. (**A)** NK cells from HDs were cultured with U266B1 cells for 24h in the presence of blocking mAbs for the indicated receptors (anti-NKp46, anti NKp30, anti-NKG2D, anti-DNAM-1) or isotype controls (IgM ctrl). Histograms represent delta (Δ) values ± SEM of CD94^{low}CD56^{dim} NK cells calculated as the percentage of CD94^{low}CD56^{dim} NK cells cultured with U266B1 subtracted the percentage of CD94^{low}CD56^{dim} NK cells cultured without a target, in each condition. **(B)** CD56^{neg} and CD56^{+} MM were sorted from patient's BM (as shown in the left dot plot) and co-cultured for 24h with HD NK cells (*right dot plots*). One representative experiment out of three is shown. **(C)** NK cells from HDs were cultured with MM cells isolated from pts's BM for 24h in the presence or absence of blocking mAb for CD56/NCAM-1 and then analyzed for the expression of CD94 by flow cytometry. Histograms show representative expression of CD94 in cultured NK cells in the presence of blocking mAb for CD56/NCAM-1 (A6-220) or IgM control. Bars represent delta (Δ) values ± SEM of CD94^{low}CD56^{dim} NK cells calculated as the percentage of CD94^{low}CD56^{dim} NK cells cultured with MM cells subtracted the percentage of CD94^{low}CD56^{dim} NK cells cultured without a target. **(D)** *In vitro* proliferation of HDs NK cells cultured with MM cells in the absence or in the presence of an anti-CD56/NCAM-1 blocking antibody. Numbers adjacent to gates indicate the frequency of Ki67^{+} NK cells. Bars represent data from four experiments shown as mean ± SEM of Ki67^{+} cells. Student's t-test. (**: $p < 0.01$). **E)** *In vitro* proliferation of HDs NK cells co-cultured (24h) with either K562 or K562-CD56^{+} cells. A representative experiment and mean of data ± SEM from three independent experiments are shown (**: $p < 0.05$). Student's t-test.

Discussion

Immune effector cells, including NK cells, are known to play a crucial role in the control of tumor growth. The therapeutic potential of NK cells against cancers has stimulated their study and led to the discovery of several NK cell subsets, each of which is endowed with different functions.

The precise identification of tumor-associated NK cell subsets that are specifically activated along the disease may provide information regarding an ongoing immune response against the tumor and might be of help to identify an immuno-editing process occurring in a given patient.[40]

Although several studies have shown that tumor-associated NK cells in advanced disease can have profound functional defects and therefore be inefficacious against tumor, the further level of complexity due to the wide heterogeneity and plasticity of NK cells can allow us to identify specific NK cell subsets endowed with enduring antitumor activity.[41]

Thus, the challenge is to understand which subpopulations mediate the antitumor response and which environmental factors can modulate their activity. In this regard, a large body of evidence reveals enrichment of selective NK cell subsets in both solid and hematological tumors[42], indicating that the outcome of NK cell antitumor effector functions is not always predictable, since largely dependent on the specific tumor microenvironment. Although the mechanisms underlying the enrichment in specific NK cell subsets have not always been fully elucidated, it is conceivable that neoplastic cells or tumor-derived factors could take part in this process. In MM disease, tumor plasma cells appear to be mainly confined to the microenvironment of the BM and are rarely detected in the periphery.

Since several studies indicated that tumor-induced impairment of NK cell functions correlates with alterations of NK cell subset distribution, we analyzed different NK cell subsets on the basis of their receptor expression, including CD94/NKG2A molecules, one of the dominant NK cell inhibitory signal involved in MM resistance to NK cell killing.

Surprisingly, we observed an enrichment of the $CD94^{low}CD56^{dim}$ subset in both PB and BM of MM pts that was more evident in the latter. The $CD94^{low}CD56^{dim}$ NK cell subset showed the highest cytotoxic potential against autologous MM cells and, interestingly, these cells were proliferating in MM pts, thus suggesting they can undergo *in vivo* activation in pts.

Our data show that MM-associated NK cells are fully competent *per se*, at least in the disease stages included in this study, ranging from I to III, but most of them lose their cytotoxic potential in the autologous setting, most likely because of the high expression of HLA class I molecules displayed by MM cells. Analysis of NK cell subset distribution based on CD94 expression reveals that $CD94^{low}CD56^{dim}$ NK cells maintain the capability to degranulate against autologous MM. Although *in vitro* studies have demonstrated a higher killing potential of KIR/ligand mismatched $NKG2A^{neg}$ NK cells against MM cells[38], an accurate prediction of NK cell activity in pts was lacking. Our data not only corroborated previous reports but also provided *in vivo* evidence for a strong anti-myeloma activity of a specific NK cell subpopulation devoid of the main inhibitory receptors.

The accumulation of $CD94^{low}CD56^{dim}$ NK cell subset accumulation also in MGUS and smoldering MM suggests an active role of NK cells not only in MM but also in the early phase of disease development. These findings represent an interesting observation that calls for further investigation considering that these initial phases of the disease are not currently treated and a future NK cell-based strategy might be envisaged for these precursor forms with the aim of blocking or, at least, slowing down the progression of the disease.

Altogether, these findings clearly highlight the relevance of inhibitory receptors, including NKG2A/CD94, in the anti-MM immune response. NKG2A has recently been identified as an immune checkpoint therapeutic target for both T cells and NK cells leading to the ongoing clinical testing of anti-NKG2A blocking antibodies.

In this context, it has been recently shown that the use of novel anti-NKG2A mAbs in combination with anti-PD-L1 mAbs had a synergistic anti-tumor effect, improving the control of tumor growth.[43,44] Another approach to bypass NKG2A-mediated inhibition is the generation of highly functional NK cells lacking NKG2A, achieved by using modern gene-editing methodologies based on meganucleases, TALEN, or CRISPR/Cas9. The $NKG2A^{null}$ cells generated in this way had a superior antitumor capacity compared to that of their $NKG2A^{+}$ counterparts and could induce, in mice, a durable tumor remission.[45]

More in general, novel NK cell-based immunotherapeutic strategies are now oriented to the identification, isolation, expansion, and administration of multifunctional NK cell subsets with high anti-tumor potential. In the case of MM, expansion, and infusion of a high number of $CD94/NKG2A^{neg}$ NK cells could represent a way to optimize NK cell therapy and thus improve its efficacy.

CD56 expression is not restricted to NK cell population since it is also expressed by a T cell subtype and some cancer cells. Our current data highlighted the relevance of CD56 molecule as an activating signal provided by tumor plasma cells to NK cells.

Being an adhesion molecule, CD56 is involved in cell-to-cell interactions through multiple cis and trans homophilic binding.[46,47] $CD56^{+}$ immune cells are able to form strong immune synapses with each other through CD56 binding. For example, $CD56^{+}$ DCs have been shown to induce the preferential activation and expansion of $CD56^{+}$ γδ T cells via CD56. Otherwise, homophilic interaction between CD56 molecules can also occurs between immune cells and cancer cells. For instance, CD56 expressed by cytokine-induced killer cells (CIK) confers them a stronger cytotoxic effect against $CD56^{+}$ leukemia cells. [11] Moreover, exploiting fluorescence microscopy, a recent study clearly showed that CD56 expression in breast cancer is associated with immunological synapse and granzyme B transfer to target cells, thus concluding that CD56 enhances the formation of cytotoxic immunological synapse.

Our observation that CD94^{low}CD56^{dim} NK cells displayed a high proliferative rate in these pts is of particular interest since this NK cell subset has been previously associated with a more differentiated phenotype endowed with low proliferative potential, at least upon cytokine stimulation.[17]

We here showed that CD94^{low}CD56^{dim} NK cells are able to actively proliferate in vitro upon interaction with MM cells and CD56 signal is critical for inducing this effect.

Our data also demonstrate that DNAM-1 activating receptor is required to induce the accumulation and a proper activation of CD94^{low}CD56^{dim} NK cells by MM cells. The immunological synapses forming between NK cells and target cells are extremely relevant to obtain a proper NK cell activation. In this context, the homophilic interaction between CD56 molecules *in trans* seems conferring to MM cells a particular activating potential for NK cells. Therefore, CD56 homophilic binding between NK and MM cells occurs and represents an additional activating signal able to improve myeloma cell recognition by NK cells and, at the same time, a critical factor to prompt their proliferation upon interaction with target cells.

In conclusion, our current results represent an advance in understanding the crucial signals involved in anti-MM response by NK cells, providing further support for the clinical employment of anti-NKG2A mAbs in the treatment of these pts and, potentially, also of those in the earlier stages of the disease. This information might as well be useful for designing novel and more effective NK cell-based immunotherapeutic strategies for MM disease.

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