Natural killer cells kill human melanoma cells with characteristics of cancer stem cells

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Abstract

Experimental and clinical data suggest that tumours harbour a cell population retaining stem cell characteristics that can drive tumorigenesis. CD133 is considered an important cancer stem cells (CSC)-associated marker. In a large variety of human malignancies, including melanoma, CD133⁺ cells have been reported to comprise CSC. In this study, we show that melanoma cell lines are highly heterogeneous for the expression of several stem cell-associated markers including CD133, c-kit/CD117 and p75 neurotrophin receptor/CD271. Since no information is available on the ability of NK cells to recognize and lyse melanoma stem cells, we assessed whether melanoma cell lines, characterized by stem cell-like features, were susceptible to lysis by IL-2-activated NK cells. We show that activated NK cells efficiently kill malignant melanoma cell lines that were enriched in putative CSC by the use of different selection methods (i.e. CD133 expression, radioresistance or the ability to form melanospheres in stem cell-supportive medium). NK cell-mediated recognition and lysis of melanoma cells involved different combinations of activating NK receptors. Since CSC have been reported to be both drug resistant and radioresistant, our present data suggest that NK-based adoptive immunotherapy could represent a novel therapeutic approach to possibly eradicate metastatic melanoma.

Introduction

The incidence of malignant melanoma (MM) increased in western populations over the past few decades. Although surgical resection of primary melanoma lesions is associated with high cure rates, metastatic MM is difficult to treat and largely refractory to current adjuvant therapies including chemotherapy, radiation therapy and/or immunotherapy (1). In view of their resistance to current therapies, melanomas remain a significant cause of mortality in Caucasians. Melanomas are believed to arise from the transformation of neural crest-derived melanocytic stem or immature progenitor cells (2). Until a few years ago, all neoplastic cells within a tumour were thought to be capable of tumorigenic growth capacity. Recent evidence, however, hints to the concept that malignant tumours are composed of (i) cancer stem cells (CSC), which have self-renewal capability, tumorigenic capacity and the ability to form metastases and (ii) more differentiated cancer cells, characterized by a limited proliferative potential (3, 4). The existence of CSC has been clearly proved in the context of human acute myeloid leukaemia, where only a small subset of undifferentiated leukaemic cells isolated from patients was able to recapitulate the same leukaemia in severe immunocompromised mice (5). Subsequently, CSC have been identified and characterized for their phenotypic profile also in solid tumours, including breast, brain, colon and pancreatic cancers (6–12). Since CSC are hypothesized to be both drug resistant and radioresistant (due to high expression levels of ATP-binding cassette (ABC) transporters, active DNA repair capacity and resistance to apoptosis) (13–15), these cells should represent an optimal therapeutic target to achieve complete eradication of the tumour. Several evidences support the presence of melanoma stem-like cells that fit the criteria of CSC (16, 17). Melanoma stem-like cells have been obtained and expanded in vitro from both cultured melanoma cells and fresh clinical specimens as non-adherent spheres (called melanospheres) in stem cell-supportive media. These melanoma stem-like cells could self-renew, differentiate into various mesenchymal lineages and, upon xenotransplantation in...
a non-obese diabetic/SCID mouse model, can initiate tumours even when infused in small numbers (16). Furthermore, their gene expression profile resembles the stem cell signature. Thus, melanoma cells tend to express development genes and stem cell markers including Notch receptors, WNTs proteins, CD133, c-KIT/CD117 and nestin antigens (18–22). Recently, different types of ABC transporters have been described as potential markers of human malignant melanoma stem cells (hMMSC) (17, 23, 24). Interestingly, the ABCB5 protein has been found to be expressed preferentially by a distinct subset of chemoresistant CD133+ melanoma cells (20).

Cytolytic effector cells, including CD8+ T cells and natural killer (NK) cells, are thought to play a role in defences against tumours (25, 26). Regarding NK cells, their activation, and function are regulated by triggering and inhibitory surface receptors (27–29). Inhibitory receptors include HLA class I-specific killer Ig-like receptors (KIRs) and CD94/NKG2A. These receptors allow NK cells to discriminate between normal MHC class I+ cells and cells that have lost surface MHC class I molecules (as it frequently occurs in tumour cells, including melanomas). In the absence of inhibitory signals (as in MHC class I-negative tumour cells), activating NK receptors [including NKp46, NKp30, NKp44, NKGD2 and DNAX accessory molecule-1 (DNAM-1)] upon interaction with specific ligands mediate NK cell triggering and target cell lysis. Some of these ligands have been identified. The best characterized are represented by the NKGD2 ligands [i.e. the stress-inducible molecules MHC class I-related chain A/B (MICA/B) and UL16-binding proteins (ULBPs)] (30, 31) and the DNAM-1 ligands (Nectin-2 and the poliovirus receptor (PVR)] (32). In most instances, these molecules are not (or only marginally) expressed by normal resting cells, while they may become highly expressed in tumour cells belonging to different histotypes, as well as in virus-infected cells or, in general, in ‘stressed’ cells.

Targeting CSC may represent a novel and, possibly, a more effective clinical approach for cancer therapy (33). In this context, the susceptibility of CSC to immune effector cell-mediated lysis has been poorly explored so far. In particular, no information is available on the ability of NK cells to recognize and lyse hMMSC. Recent studies suggest that CD133 may represent a putative marker of CSC in tumours of different histotypes, including melanomas (17, 20, 23). In the present study, we evaluated the expression of CD133 in various melanoma cell lines. Secondly, we assessed the susceptibility of CD133+ melanoma cells to radiation-induced apoptosis as compared with the CD133− cell subset. Finally, the susceptibility to NK cell-mediated lysis of CD133+ or CD133− melanoma cells was analysed. Remarkably, CD133+ cells were highly susceptible to NK cell-mediated killing thus suggesting that NK cell-based immunotherapy might be considered a possible approach in the cure of patients with metastatic melanoma.

Methods

mAbs and flow cytometric analysis

The following mAbs were used in this study: 289 (IgG2a, anti-CD3), c218 (IgG1, anti-CD56), A6136 (IgM, anti-HLA class I), F22 and F5 (IgG1 and IgM, anti-DNAM-1, respectively), BAB281 and KL247 (IgG1 and IgM, anti-NKp46, respectively), c127 (IgG1, anti-CD16), A220 and F252 (IgG1 and IgM, anti-NKp30, respectively), BAT221 (IgG1, anti-NKG2D), L95 (IgG1, anti-PVR), L14 (IgG2a, anti-Nectin-2), BAM195 (IgG1, anti-MICA), 14D12D2 (IgG1, anti-ICAM-1/CD54), DF305 [IgM, anti-leukocyte function-associated antigen (LFA)-3], 10F18 (IgG2a, anti-Mel-CAM/CD146), EB6 (IgG1, anti-KI2DL1/S1), GL183 (IgG1, anti-KIR2DL2/L3/S2), Z77 (IgG1, anti-KI3DL1/S1), DEC66 (IgM, anti-KIR3DL2), Z770 (IgG1, anti-NKG2A) and F278 (IgG1, anti-LIR-1); all were produced in our laboratory.

Anti-human disialoganglioside GD2 (14.G2a, IgG2a) and anti-vascular cell adhesion molecule (VCAM)-1/CD106 mAbs were purchased from BD-Pharmingen (BD Biosciences Pharmingen, San Diego, CA, USA). Anti-ICAM-3/CD50 and anti-iCAM-2/CD102 mAbs were purchased from Dako (Carpinteria, CA, USA). Anti-CD133/1 (AC133) pure, allopheycocyanin (APC)-conjugated anti-CD133/1 (AC133-APC), PE-conjugated anti-i-Ki/CD117 (AC126-PE), anti-p75 neurotrophin receptor (NTK)/CD271-PE mAbs and their isotype-matched controls were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Anti-nestin (IgG1) and anti-MelanA/MART-1 (IgG2b) mAbs were purchased from Abcam plc (Cambridge, UK). PE-conjugated anti-isotype goat anti-mouse mAbs were purchased from Southern Biotechnology Associated (Birmingham, AL, USA). For one-colour cytometric analysis, cells were stained with the appropriate mAb followed by PE-conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associated). For intracytoplasmic staining, cells were fixed (4% formaldehyde), permeabilized (0.1% saponin) before staining with anti-nestin mAb. All samples were analysed on an FACSscalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Melanoma cell cultures

Metastatic melanoma biopsies (six) were obtained in accordance with consent procedures approved by the Internal Ethics Board of the National Cancer Institute (IST, Genova, Italy). They were obtained within 1–2 h after surgical removal. Tumours were finely minced with scissors and then subjected to enzymatic dissociation in 500 U ml−1 collagenase type IA and 300 U ml−1 hyaluronidase (Sigma–Aldrich, Saint Louis, MO, USA) in serum-free RPMI 1640 (Lonza, Basel, Switzerland) for 2 h at 37°C temperature. The tumour cell suspensions were washed in RPMI 1640 + 10% foetal bovine serum (FBS) (Euroclone, Celsio S.p.a., Milan, Italy) and passed through a 70-μm pore size filter (Celsio S.p.a., Milan, Italy) to remove undigested tumour tissue macroaggregates. Contaminating red blood cells were removed by osmotic lysis. Single cells were washed with PBS (Celsio S.p.a.), re-suspended in culture medium and plated onto non-coated flasks. Primary cultures were obtained from five subcutaneous and one lymph node melanoma metastases. For growing both primary culture of melanoma cells (MeBO, MeCoP, MeMO, MeOV, MePA and MeTA) and established β2microglobulin-deficient
melanoma cell lines (FO-1 and Me1386), the culture medium used was RPMI 1640 supplemented with 10% FBS, 1% antibiotic mixture (5 mg ml\(^{-1}\) penicillin and 5 mg ml\(^{-1}\) streptomycin stock solution). For spheroids formation (melanospheres), FO-1 melanoma cells were plated at clonal density of 500 cells cm\(^{-2}\) in serum-free medium (SFM) optimized for growth of stem cells containing DMEM/F12 (GIBCO; Invitrogen, Carlsbad, CA, USA), B27 supplement (1:50; GIBCO; Invitrogen), recombinant human fibroblast growth factor-2 (FGF-2; 20 ng ml\(^{-1}\); Sigma–Aldrich) and recombinant human epidermal growth factor (EGF; 20 ng ml\(^{-1}\); Sigma–Aldrich). Fresh FGF-2 and EGF were added twice each week. For subculture, melanospheres were mechanically dissociated through a sterile Pasteur pipette and replated every 5–7 days into fresh SFM. Single CD133\(^+\) and CD133\(^-\) FO-1 melanoma cells were isolated using CD133 Cell Isolation Kit (Miltenyi Biotec GmbH). Cells were magnetically labelled with CD133 microbeads and separated on a suitable MACS Separator. Both the magnetically labelled CD133\(^+\) cells and the unlabelled CD133\(^-\) cells were collected and evaluated for purity by the use of anti-CD133/2 (293C3)-PE mAb (Miltenyi Biotec GmbH) followed by cyt fluorimetric analysis. Purities ranged from 85 to 95% for CD133\(^+\) cells and from 80 to 90% for CD133\(^-\) cells.

**Radiation treatment**

Cells derived from FO-1 monolayer cultures were dissociated with trypsin–EDTA (Euroclone, Celbio S.p.a.) and immediately irradiated at indicated doses with an IBL437C irradiator composed of \(^{137}\)Cs \(\gamma\) ray source (CIS Bio International, France). Corresponding controls were sham irradiated. In some experiments, FO-1 cultures were treated with sequential administration of ionizing radiation (IR) (2.5 and 5 Gy) (three cycles) followed by recovery periods during which cell populations were allowed to regrow.

**Active caspase-3 staining**

FO-1 melanoma cell cultures were either untreated or treated with IR (10, 20 and 30 Gy). Twenty-four hours after *in vitro* IR treatment, cells were collected and double stained with an anti-CD133-APC mAb and the carboxyfluorescein Fluorocent Labelled Inhibitor of Caspases reagent (Vybrant FAM caspases-3 and -7 assay kit, Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Samples were then analysed on a FACScalibur flow cytometer (BD Biosciences).

**Isolation and culture of NK cell populations**

Enriched NK cells were isolated using the Human NK Cell Enrichment Cocktail-RosetteSep (StemCell Technologies Inc., Vancouver, British Columbia, Canada) and then cultured on irradiated feeder cells in the presence of IL-2 (100 U ml\(^{-1}\); Proleukin, Chiron Corp., Emeryville, CA, USA) and PHA (1.5 ng ml\(^{-1}\); GIBCO, Paisley, GB, UK) to obtain polyclonal NK cell populations.

**Cytolytic assays**

NK cell populations derived from two different donors were tested for cytolytic activity in a 4-h \(^{51}\)Cr-release assay as previously described (34). The concentrations of the various mAb added for masking experiments were 10 \(\mu\)g ml\(^{-1}\). The E:T ratios are indicated in the figures.

**Results**

**Melanoma cell lines are heterogeneous for the expression of 'putative' CSC markers**

CSC have been identified in leukaemias (5, 35) as well as in solid malignancies, including MM (16, 17, 23, 24). We therefore analysed a panel of melanomas, derived either from metastatic primary cultures (i.e. MeBO, MeCoP, MeMO, MeOV, MePA and MeTA) or from established cell lines (i.e. FO-1 and Me1386) for the expression of two informative markers previously described to be preferentially expressed by cells exhibiting stem cell characteristics (i.e. CD133 and c-Kit/CD117) (Fig. 1). CD133 displayed a variable pattern of expression. Thus, certain tumours were homogenously negative (5/8) while two were positive (i.e. MeTA and Me1386). Interestingly, FO-1 melanoma cell line displayed a bimodal distribution of CD133 surface expression. c-Kit (CD117, the receptor for stem cell factor) was expressed on MeBO cell line only. In addition, since neural crest cells give rise to both melanocytes and peripheral neurons as well as other cells of the nervous system, melanoma cell lines were also analysed for the expression of p75NTR/CD271 and nestin, two additional stem/progenitor cell-associated markers. p75NTR/CD271 is present on cells of the central and peripheral nervous system as well as on neural crest precursor cells. On the other hand, nestin, an intracytoplasmic intermediate filament protein, is expressed in neuroepithelial stem cells, glioblastoma multiforme and metastatic melanoma. Phenotypic analysis revealed that p75NTR/CD271 was absent in four out of eight cell lines, whereas variable patterns of expression were detected in the other four cell lines. In particular, two cell lines were p75NTR/CD271\(^+\), whereas the other two displayed a bimodal surface expression (Fig. 1). Finally, all cell lines analysed expressed intracytoplasmic nestin protein although at different levels (Fig. 1).

Taken together, these data indicate that stem cell-associated surface markers (i.e. CD133, c-Kit/CD117 and p75NTR/CD271) are expressed heterogeneously both in primary cultures derived from metastatic melanomas and in established melanoma cell lines.

**Radioresistance of CD133\(^+\) melanoma cells**

In glioblastoma, the CD133\(^+\) cell fraction was found to have reduced sensitivity to radiation-induced apoptosis (15). We investigated whether CD133\(^+\) FO-1 cell fraction was more radioresistant than the CD133\(^-\) one. As shown in Fig. 2(A), treatment of FO-1 cells with IR (2.5 and 10 Gy) resulted in enrichment of the CD133\(^+\) cell fraction (\(\sim \)2-fold as compared with untreated cultures). Notably, maximal effect was observed even at the lowest radiation intensity used. In control experiments, cell irradiation did not result in the expression of CD133 antigen in CD133\(^-\) cells (data not shown). In addition, serial cycles of IR treatment (2.5 and 5 Gy) of FO-1 melanoma cells resulted in further enrichment in CD133\(^+\) cells (Fig. 2B). We next analysed whether increased proportion of CD133\(^+\) cells in irradiated cultures was due to
a reduced sensitivity to apoptosis. Figure 2(C) shows that, upon high-dose IR treatment (10, 20 and 30 Gy), CD133+ FO-1 cells displayed, after 24 h, less caspase-3 activation than the CD133−/C0 cells. Taken together, our results suggest that CD133+ melanoma cells preferentially survived radiations as compared with CD133− cells.

**Both CD133+ and CD133− melanoma cell lines express ligands recognized by different activating NK receptors and are susceptible to NK cell-mediated lysis**

We investigated whether melanoma cell lines characterized by a different CD133 expression would express ligands for activating NK receptors, as well as adhesion molecules known to play a relevant role during NK/tumour cell interactions, thus representing suitable targets for NK-mediated killing. To this end, three representative melanoma cell lines (MePA, MeTA and MeMO), displaying a different CD133 surface phenotype, were analysed. The surface expression pattern of NKG2D ligands (i.e. MICA and ULBPs), DNAM-1 ligands (i.e. PVR and Nectin-2) and adhesion molecules was assessed by the use of specific mAbs. In Fig. 3 (panels A and B), data regarding the selected melanoma cell lines are shown. Cytofluorimetric analysis revealed that MICA was expressed by both the CD133+ MeTA and the CD133− MeMO cell lines. In contrast, the CD133− MePA cell line did not express this molecule (Fig. 3A). ULBPs were not expressed with the exception of ULBP2 in MePA cell line and ULBP3 in MeTA cell line (Fig. 3A). Finally, both PVR and Nectin-2 were expressed in all three melanoma cell lines (Fig. 3A). Thus, both CD133+ and CD133− cells express ligands that represent major molecular targets for tumour detection by NK cells (32, 34, 36). Regarding the expression of the adhesion molecules, all cell lines analysed were found to express ICAM-1, while other major adhesion molecules such as LFA-3, ICAM-2, ICAM-3 and VCAM-1 were absent (Fig. 3B).

Next, we asked whether and to what extent melanoma cells expressing the putative stem cell marker CD133 were susceptible to NK cell-mediated lysis. In these functional experiments, we used, as effectors cells, IL-2-cultured polyclonal NK cell populations derived from two different healthy donors. As shown in Fig. 4(A), the three melanoma cell lines were susceptible to NK cell-mediated lysis regardless of their CD133 surface phenotype. Differences in susceptibility to lysis by allogeneic NK cells could reflect both differences in the HLA class I surface density (on target cells) and in the presence or absence of mismatch between KIRs expressed on NK cells and MHC class I molecules on target cells. Although not shown, the CD133− MeMO melanoma expressed high surface levels of HLA class I molecules and its lysis could be increased upon mAb-mediated masking of HLA class I molecules with a specific mAb of IgM isotype.

We further analysed the involvement of different activating NK receptors in the lysis of each melanoma cell lines. To this end, cytolytic assays were performed either in the absence or in the presence of mAbs specific for the main activating NK receptors (used either singularly or in combination). In agreement with the expression of one or another NKG2D ligand, mAb-mediated masking of NKG2D resulted in inhibition of NK-mediated lysis. mAb-mediated disruption of the interaction between DNAM-1 and its ligands (PVR and Nectin-2) resulted in variable inhibition of lysis (Fig. 4B). Remarkably, also blocking of NKp46 and NKp30 inhibited cytotoxicity thus suggesting that these cells do express the still unknown cellular ligands recognized by these activating receptors. The combined use of mAbs specific for different activating receptors resulted, in some instances, in abrogation of cytotoxicity thus indicating that these receptors play a major role in inducing NK-mediated lysis of these particular melanomas (Fig. 4B).

Taken together, these data show that both CD133+ and CD133− melanoma cell lines express ligands for triggering NK receptors and are susceptible to NK cell-mediated lysis.

**CD133+ and CD133− cells derived from the same melanoma cell line (FO-1) are equally susceptible to NK cell-mediated lysis**

FO-1 melanoma cell line shows several characteristics attributed to CSC, including expression of CD133, radiation resistance and, more importantly, capacity of forming tumours on
transplantation with small cell numbers (data not shown). Since FO-1 cell line is characterized by a bimodal expression of CD133 antigen, we analysed the susceptibility of CD133\(^+\) and CD133\(^-/\) populations or clones to NK cell-mediated killing. In particular, we analysed as target cells (i) CD133\(^+\) or CD133\(^-/\) clones obtained by limiting dilution and (ii) CD133\(^+\)- or CD133\(^-/\)-sorted cell fractions obtained by magnetic beads separation. As shown in Fig. 5 (panels A and B), NK cells lysed all different FO-1 cell populations or clones.

We further applied other established criteria of in vitro culture aimed at a specific enrichment of melanoma stem cells. To this end, we used suitable SFM (see Methods), in which melanoma cells were shown to form typical floating outgrowths termed melanospheres. Interestingly, FO-1 spheroids could be serially subcultured under identical cell culture conditions for long time periods and many cell generations, suggesting that FO-1 melanospheres may have unlimited self-renewal capacity. FO-1-derived melanospheres were analysed for their susceptibility to NK cell-mediated lysis in comparison with their adherent counterpart. Notably, both melanospheres and adherent cells expressed surface ligands recognized by triggering NK receptors (data not shown). Accordingly, they were both efficiently killed by NK cells (Fig. 5C).

Collectively, our data provide evidence that different populations of the FO-1 melanoma, enriched in CSC according to different isolation criteria, were susceptible to NK cell-mediated lysis.

Discussion

In this study, we analysed a panel of melanoma cell lines for the presence of the stem cell-associated marker CD133 and found a high degree of heterogeneity for its surface expression. Importantly, our results clearly indicate that both CD133\(^+\) and CD133\(^-/\) melanoma cells are highly susceptible to NK-mediated lysis.

CD133 is a five-transmembrane domain glycoprotein that is normally expressed on haematopoietic stem cells, endothelial precursors cells and neural stem cells. While its function remains unknown, CD133 has been shown to mark malignant stem cells from various tumours. Accordingly, it has been proposed as a putative stem cell-related marker shared by tumours cells belonging to different histotypes (7–10). Our present study shows that adherent MM cell lines, established in vitro under standard conditions, can express several stem cell-related antigens including CD133, p75NTR/CD271 and nestin. Interestingly, CD133 displayed a heterogeneous pattern of expression in different melanoma cell lines. In particular, cytofluorimetric analysis revealed the expression of CD133 in 3/8 specimens. Although stem cell factor (c-kit ligand) has been shown to play a role in melanocyte
Fig. 3. Cytofluorimetric analysis of MeTA (CD133⁺), MePA and MeMO (CD133⁻) melanoma cell lines. (A and B) Melanoma cell lines were analysed by one-colour fluorescence and cytofluorimetric analysis for the expression of ligands for activating NK receptors (A) and adhesion molecules (B). PE-conjugated goat anti-mouse isotype-specific antibody was used as second reagent. White profiles represent cells incubated with the second reagent only.

Fig. 4. Susceptibility of MeTA (CD133⁺), MePA and MeMO (CD133⁻) melanoma cell lines to NK cell-mediated lysis. (A) IL-2-activated NK cells from Donor 1 were tested for cytolytic activity against melanoma cell lines. (B) Analysis of the receptor/ligand interactions involved in NK cell-mediated killing of melanoma cell lines. NK cells from Donor 1 were analysed for cytolytic activity against melanoma cell lines either in the absence or in the presence of mAbs specific for different activating NK receptors. mAbs were used either alone (light grey bars) or in combination (black bars).
migration and proliferation, seven out of eight metastatic melanoma cultures analysed did not express the corresponding receptor (i.e. c-Kit/CD117). This data are consistent with previous studies suggesting that the metastatic potential of human MM cell lines may be associated with loss of both c-Kit/CD117 and membrane-bound stem cell factor expression (37, 38).

In agreement with a previous study on CD133$^+$ cells from glioblastomas (15), we show that also CD133$^+$ cells of melanomas are less susceptible to radiation-induced apoptosis. These data support the concept that CD133$^+$ tumour cells are more radioresistant than their CD133$^{-}$ counterpart.

Phenotypic analysis of melanoma cell lines characterized by a different expression of CD133 revealed that, regardless of their CD133 phenotype, they expressed the melanocytic differentiation antigen MART-1 as well as the melanoma-associated antigen Mel-CAM/CD146, thought to facilitate invasion and metastasis (39, 40), but not GD2 (data not shown). More important for the potential susceptibility to NK-mediated lysis, both CD133$^+$ and CD133$^{-}$ cell lines expressed ligands recognized by the activating NK receptor DNAM-1 (i.e. PVR and Nectin-2). On the other hand, MICA and ULBPs (i.e. the NKG2D ligands) displayed a heterogeneous pattern of expression, which did not appear to be related to the CD133 surface phenotype. Thus, MICA was detected in two out of three melanoma cell lines, while ULBPs were absent with the exception of ULBP2 in the CD133$^-$ MePA and of ULBP3 in the CD133$^+$ MeTA cell line. Remarkably, all cell lines expressed the adhesion molecule ICAM-1, known to play a crucial role in mediating cell adhesion during NK-mediated killing. FO-1 melanoma cell line displayed a bimodal distribution of CD133. Also in this cell line, no substantial differences existed in ligand expression in CD133$^+$ and CD133$^{-}$ cell fractions as well as in CD133$^+$ and CD133$^{-}$ clones (data not shown). Taken together, these data suggested that CD133$^+$ melanoma cells may display susceptibility to NK cell-mediated lysis similar to that of their CD133$^-$ counterpart. This was confirmed by experiments in which both CD133$^+$ and CD133$^{-}$ melanoma cell lines (or cell fractions isolated from FO-1) were efficiently killed by IL-2-activated NK cells. NK-mediated recognition and cytolytic activity were found to involve different combinations of

![Fig. 5. Cytolytic activity of IL-2-activated NK cells against different melanoma cell suspensions derived from FO-1 cell line. IL-2-activated NK cells from Donor 1 were tested for cytolytic activity against: (A) CD133$^+$ or CD133$^{-}$ FO-1 clones, (B) CD133$^+$ or CD133$^{-}$ FO-1-sorted cell fractions and (C) FO-1 cell line propagated either as adherent monolayer cultures or as floating spheroid cell clusters (melanospheres).](#)
activating NK receptors that recognized the corresponding ligands on both CD133\(^+\) and CD133\(^-\) tumour cells.

Also non-adherent spheroid tumour cells were shown to be enriched in CSC as compared with their adherent counterpart. Sphere formation was initially observed in cultured neural stem cells. Cells within neural spheres displayed stem cell properties (e.g. self-renewal and multi-lineage differentiation potential). Recently, tumour cells cultured in SFM were found to form floating spheroid cell clusters with a stem cell-like phenotype. Such spheroids were obtained from malignant tissue as well as from established cell lines of malignant glioma, mammary carcinoma and melanoma (16, 41, 42). As shown in this study, after 5–7 days of culture in SFM, FO-1 melanoma formed non-adherent spheroids (melanospheres).

In order to further explore the susceptibility of putative melanoma CSC to NK-mediated lysis, we analysed whether cells isolated from melanospheres were recognized by NK cells. Also in this case, cells were efficiently killed by NK cells. To our knowledge, this is the first study that directly analyses the susceptibility of melanospheres to NK cell-mediated killing.

Taken together, our data support the notion that NK cells can kill melanoma cell fractions enriched in CSC according to both phenotypic and functional criteria. While none of these criteria has been unequivocally shown to precisely identify CSC, our data show that NK cells were equally efficacious in killing different melanoma cell subsets. Indeed, they could efficiently lyse melanoma cells resistant to radiotherapy or that form melanospheres in vitro or that express the stem cell-associated marker CD133. Although, in the melanoma setting, a clear correlation between CD133 expression and self-renewal and multi-lineage differentiation potential and tumorigenicity have not been clearly determined, a recent report by Schatton et al. (23) described a melanoma-initiating cell population characterized by the co-expression of the ABCB5 drug resistance-related molecule and CD133.

The observation that melanomas harbour a population of cells retaining stem cell characteristics that can drive tumorigenesis should provide us a useful information for designing new and more effective therapies. In this context, also strategies of immunotherapy should be improved, mostly focusing on the capability of specifically eliminating CSC. Since long-term survival of patients with cancer requires effective removal of CSC, our present data may provide a clue for applying novel NK-based immunotherapeutic strategies. Indeed, NK cells appear as a suitable candidate for efficiently targeting the putative CSC.

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Abbreviations

- ABC: ATP-binding cassette
- APC: allogeneic拼音cytocyanin
- CSC: cancer stem cell
- DNAM-1: DNA-Accessory molecule-1
- EGF: epidermal growth factor
- FBS: foetal bovine serum
- FGF-2: fibroblast growth factor-2
- hMMSC: human malignant melanoma stem cell
- IR: ionizing radiation
- KIR: killer Ig-like receptor
- LFA: leukocyte function-associated antigen
- MICA: MHC class I-related chain A
- MM: malignant melanoma
- NK: natural killer
- PVR: poliovirus receptor
- p75NTR: p75 neurotrophin receptor
- SFM: serum-free medium
- ULBP: UL16-binding protein
- VCM: vascular cell adhesion molecule

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