Mature cytotoxic CD56\textsuperscript{bright}/CD16\textsuperscript{+} Natural Killer cells can infiltrate lymph nodes adjacent to metastatic melanoma

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Abstract

Melanomas are characterized by high metastatic potential, with regional lymph node (LN) representing the most frequent site of early dissemination in this disease. These regional LN also represent the primary site for differentiation of natural killer cells (NK cells). While blood-derived NK cells can efficiently melanoma cells isolated from metastatic LN (M-LN), there has been no study of the properties of the most disease-relevant NK cells isolated from M-LN in melanoma patients. Here we report that M-LN contain 0.5-11% of CD56bright NK cells among CD45+ hematopoietic cells present and that this cell population surrounded tumor cell clusters in M-LN. This NK cell population was characterized by expression of CD62L, chemokine receptors and high levels of natural cytotoxicity receptors (NCRs), NKG2D and DNAM-1. Expression of NCR-NKp30 and NKG2D correlated negatively with percentages of tumor cells in M-LN. Interestingly, M-LN contained a unique subset of mature CD56brightCD16+ NK cells displaying co-regulated expression of NCR and NKG2D activating receptors. Ex vivo analyses suggested that M-LN-derived NK cells were inactive but could be activated by appropriate cytokine signals (IL-2 or IL-15), could lyse metastatic melanoma cells in a highly efficient manner compared to blood-derived NK cells. Taken together, the results offer evidence that adjuvant immunotherapy that targets NK cells in M-LN for activation may improve treatment of sentinel LN-positive melanoma patients.
Introduction

Melanomas are highly metastatic tumors for which the treatment of advanced stages is still unsatisfactory. Stage I and II melanoma patients display a localized disease. The main way of dissemination is the lymph and the invasion of sentinel LN, the initial tumor draining LN, is correlated to prognosis. Stage III patients are characterized by LN metastases in the draining basin. These patients are treated by LN dissection and pathological examination determines both the total number of excised LN and the number of metastatic LN, the latter being of prognostic significance. Thus, stage III patients constitute a heterogeneous subset (stage III A to C) of patients that present one or several metastatic LN either microscopically or macroscopically involved. Some characteristics of the primary melanoma are also taken into account for staging in the three subgroups (A to C) such as Breslow thickness and ulceration. These stage III patients have no distant metastases.

Metastatic dissemination is a multistage process that is highly inefficient, as less than 0.1% of tumor cells that penetrate the circulation, either in lymphatic or blood vessels, end up forming metastatic colonies (1). Thus, before being able to penetrate and expand in the lymph nodes and distant organs, circulating tumor cells likely undergo apoptosis, are destroyed by immune cells or enter a state of dormancy for an indefinite time. This period represents a good time window for boosting immune response towards low tumor invasion.

As potent cytotoxic antitumor effectors and strong inducers of adaptive immune response, Natural Killer (NK) cells (CD3-CD56+ lymphocytes) are important targets in the immunotherapy of melanoma. They can be efficiently expanded in vitro, used in autologous as well as in allogeneic settings for adoptive cellular therapy (2-4). NK cells kill tumor cells without prior sensitization, detecting harmful changes in cellular self. They are also pivotal catalysts of adaptive T cell responses as they are considered the major source of IFN\(\gamma\) in vivo and NK-derived IFN\(\gamma\) is crucial in priming T helper 1 responses (5, 6). They differentiate in the bone marrow and in peripheral LN and they represent 5 to 20% of the blood lymphocytes. Two major NK sub-populations are described in humans: CD56\textit{dim}CD16+ and CD56\textit{bright}CD16- cells. The CD56\textit{dim} population predominates in blood (90% of NK cells) and at site of inflammation, exhibits a high cytotoxic potential and broadly expresses MHC-I specific inhibitory receptors. In contrast, the CD56\textit{bright} subset predominates in lymph nodes (95% of
NK cells), produces cytokines upon activation, displays a low cytotoxic potential and is considered to be a precursor of the terminally differentiated CD56dim NK cells (7, 8).

NK cell activation depends on an intricate balance between activating and inhibitory signals that determines whether the target will be susceptible to NK-mediated lysis. Three main natural cytotoxicity receptors (NCR) involved in NK cell activation were identified: NKp46 and NKp30, expressed by resting NK cells, and NKp44, induced after stimulation by cytokines (9, 10). A specific ligand for NKp30, B7H6, was recently identified (11). In addition, the use of blocking anti-NCR mAbs in functional assays confirms the implication of NCR in the lysis of various tumor cells (12). Activation of NK cells is triggered by additional receptors. NK group 2 member D (NKG2D) receptor, expressed by a majority of peripheral NK cells, binds MHC-related antigens MIC-A/B molecules, and UL16-binding proteins 1-4 (ULBP1-4), induced on membrane of stressed cells (13, 14). DNAX accessory molecule 1 (DNAM-1), an adhesion molecule belonging to the immunoglobulin superfamily, promotes the elimination of tumor cells that are comparatively resistant to NK cell-mediated cytotoxicity caused by the paucity of other NK cell-activating ligands (15). Simultaneous engagement of NKp46 and DNAM-1 induces cytotoxicity and cytokine secretion by resting NK cells (16).

NK cell activation is controlled by HLA-I-specific inhibitory NK receptors: Killer Ig-like receptor (KIR) present on NK CD56dim subset and the ubiquitous C-type lectin CD94/NKG2A receptor that binds to HLA-E molecules (17). KIR receptors, CD158a/b and e, recognize HLA-Cw4/Cw3 and HLA-Bw4 molecules respectively (10, 18).

Reports in the literature indicate that melanoma cells express different molecules that trigger NK cells (19). The frequent alterations of HLA-I molecule expression by melanoma cells may also contribute to NK cell activation. There is an inverse relationship between NK cell activity and magnitude of lymphocyte infiltration as well as partial regression of the primary tumor in melanoma patients in some cases (20), suggesting that NK cell activity may be an additional prognostic factor in melanoma patients.

Here we have studied the phenotype and functions of NK cells infiltrating LN from organ donors and stage IIIB and C melanoma patients. Interestingly, our observations suggest that M-LNs from patients are infiltrated by a unique subset of mature CD56brightCD16+ NK cells endowed with high lytic potential towards metastatic melanoma cells following activation by cytokines.
Materials and Methods

Samples, patients, melanoma cell lines

Twenty two stage III melanoma patients with clinically palpable LN, who had to be treated by radical LN dissection, were included after obtaining their written informed consent. The study protocol was approved by an ethic committee “Ile de France” (CPP: 2834) and the Declaration of Helsinki protocols were followed. Fragments from M-LN selected by the pathologist (to allow the histological analysis for the validation of the resection quality) were obtained from the operating room. For 6 patients, fragments from one macroscopically non-invaded and one tumor-invaded, were obtained and analyzed separately. Freshly excised nodes were trimmed of fat, minced, and gently teased through a 100 µm cell strainer. Cells were washed twice with complete medium (RPMI 1640 with 10% FCS (GIBCO Invitrogen). Viability always exceeded 90% by trypan blue exclusion. Lymphoid cell yields varied from 10 to 90%. A blood sample (25 ml on EDTA) was obtained from each patient and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) density gradient centrifugation. In addition, the regional Organ Procurement Organization procured human mediastinal LN from 6 brain-dead donors (D-LN) dissected during lung exportation, obtained after informed consent from appropriate individuals.

The MelC primary melanoma cell line was previously derived in the lab from a regional M-LN from a stage III melanoma patient (21). MelC and K562 cells (0.3 to 1x10^6/ml) were maintained in complete medium. K562 and MelC were recently authenticated byDNA profiling (DSMZ).

Immunohistochemistry of donor and patient LN

Fragments from 6 D-LN and 10 M-LN were paraffin-embedded, sectioned and further stained by routine hematoxylin and eosin method. Immuno-histochemistry was performed to detect NK cells: sections were stained with anti-NKp46 mAb (R&D systems) as previously described (22). Tissue sections were scanned using NanoZoomer (Hamamatsu Photonics). NKp46-positive NK cells were then counted using NDP View software in 10 fields of 1 mm^2 in the center and in the pericapsular areas of the M-LN.

Phenotypic analyses
PBMC and LN cells were suspended in PBS1x (GIBCO Invitrogen) supplemented with 5% human serum AB (Biowest) and incubated 30 min in ice to block nonspecific FcR binding before staining. Cells were then washed and stained 30 min at 4°C with the mAbs diluted in PBS1X/FCS2%/EDTA2mM at predetermined optimal concentrations. NK cells, defined as CD3\(^-\) CD56\(^+\) cells (BD Pharmingen) within CD45\(^+\) (BD Pharmingen) lymphocyte FSC/SSC subset, were analyzed for the expression of NCR (NKp46, NKp30, NKp44), NKG2D, DNAM-1, NKG2C (BD Pharmingen), CD16, CD158a, CD158b, CD158e, NKG2A receptors (Beckman Coulter), CD69, CXCR3, CCR7 and CD62L (Miltenyi Biotech) after dead-cell and doublet exclusion. The percentages of positive cells were determined on more than 3000 NK-gated events. CD4 and intracellular staining with Foxp3 were performed to quantify T regs. The percentages of CD45\(^-\) cells infiltrating M-LN were determined and labeled with anti-MICA/B, anti-ULBP2, anti-MCAM, anti-CD56 and control isotype (BD Pharmingen). These cells produced black pigments in the supernatant. Quantification by RT-PCR of Tyrosinase (Tyro U1 and U2), MCAM and house-keeping gene (TBP) mRNA transcripts was determined in 3 M-LN cell suspensions.

**CD107a degranulation, IFN\(_\gamma\) secretion and perforin assays**

The functional capacities of LN-NK were evaluated *ex vivo* by flow cytometer analyses on gated CD3\(^-\) CD56\(^+\) NK cells. From LN cell suspensions, 10\(^6\) cells were stimulated with 10\(^5\) K562 targets (10/1 effector/target ratio) in V-bottom plates in presence or absence of PMA/Iono and monensin (BD Biosciences) for 4h. Cells were then labeled for 30 min at 4°C with anti-CD16-PeCy7 (Beckman Coulter), anti-CD56-HorizonV450, anti-CD3-APC-H7, anti-CD107a-FITC (BD Biosciences), washed, fixed and permeabilized (kit) (BD Biosciences) for 30 min at 4°C before intracellular staining with anti-IFN\(_\gamma\)-APC (Miltenyi Biotech) or anti-perforin-PE (eBioscience) for 30 min at 4°C. Cells were collected on a FACSCanto II flow cytometer. Results are expressed as the percentages of CD107a, IFN\(_\gamma\) or perforin positive NK-gated cells. Baseline NK cell cytokine secretion and degranulation were determined in absence of targets or in presence of isotype control for the perforin staining.

In addition, for some LNs and blood samples, we have proceeded to the negative immunoselection of NK cells using the NK selection kit (Miltenyi Biotech). Purified NK cells (0.5 to 1x10\(^5\)/ml) were cultured for 6 days in presence of IL-2 or IL-15 (10 ng/mL; R&D System).
Cell-mediated lysis assay using xCELLigence system

Tumor cell lysis by immunoselected NK cells was assessed using the xCELLigence System (Roche). The system dynamically measures electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture E-Plates. The impedance values, expressed as Cell index (CI) provide quantitative information of cell adhesion, number and morphology. For cell-mediated lysis assay, 15000 MelC cells/well were seeded into 96X E-Plates and their adhesion monitored for 3 h. IL-2-activated LN-NK or blood NK cells were added at different concentrations in a volume of 50µl/well. Co-cultures were assessed by the system with a measure every 15 min for up to 300 min. Results are expressed as % of quadruplicate of lysis determined from Cellular Index (CI) normalized with RTCA Software (nCI): % of lysis = (nCI (no effector) – nCI (effector))/ nCI (no effector) x100. The mean CI value at 510 min of MelC targets incubated with medium was 1.5 (range: 1.2-1.8).

Statistics

Statistical tests and graphics were generated by Prism version 5 (GraphPad Software Inc). Non parametric Mann-Whitney (M-W) test was used to compare the medians of % of CD45+, NK, Tregs cells between D-LN and M-LN. The same test was used to compare the medians of NK and CD45+ cells proportions, the % of NK receptors, CD107a and IFNγ production by NK cells between two groups (“D-LN vs M-LN” and “stage IIIB vs IIIC”). Wilcoxon matched paired test was used to compare the expression of NK receptors between CD56brightCD16+ and CD56brightCD16− cells (p values noted as * ≤0.05, ** p<0.01 and *** p<0.0001). Correlations between the different parameters analyzed were assessed by Pearson test.

Results

NK cells infiltrate M-LN from melanoma patients

The distribution of NK cells was assessed by immunohistochemistry in 10 M-LN and 6 mediastinal LN from organ donors (D-LN). The staining with anti-NKp46 mAb revealed large granular NK cells scattered in the T cell zone of the D-LN. In M-LN, the LN architecture was altered by invading tumor cells: NK cells were distributed around the lymphoid structures, squeezed under the LN capsule and rarely detected in the tumor cell clusters (Fig. 1A). Phenotypic analyses by multiparametric flow cytometry were performed in 22 M-LN obtained from 16 stage III melanoma patients treated by LN
dissection (Table 1) and in 4 of 6 D-LN. In LN suspensions, the percentages of NK (CD3−CD56+) cells were determined among CD45+ cells and analyzed following the gating strategy depicted (Supplementary Fig. S1). In M-LN, NK cells represented 0.5-11% of CD45+ cells, percentages in the range of those present in LN from donors (Fig. 1B). The frequency of Tregs (CD4+Foxp3+) determined in 4 LN suspensions represented 12 to 16% of CD4+ T cells and was not related to the percentages of CD45+CD56+ invading cells (Supplementary Fig. S2).

Most M-LN contained large subsets of CD45−CD56− cells constituting 0.3 to 95% of living cells of the LN suspensions (Fig. 1C), while low % of CD45−CD56− cells were detected in D-LN (data not shown). These CD45−CD56− cells expressed NKG2D ligands (MICA/B, ULBP2) and MCAM (CD146) (Fig. 1C), further indicating that they likely corresponded to metastatic melanoma cells. In addition, these cells express transcripts for the melanoma antigen, tyrosinase and MCAM (Fig. 1D).

**Activated phenotype of NK cells infiltrating M-LN from melanoma patients**

NK cells infiltrating M-LN displayed an activated phenotype. NKp30 was highly expressed, and the expression of NKp46 and NKG2D were elevated and homogeneous on M-LN NK cells (Fig. 2A). The activating receptor NKp44 was remarkably expressed (mean 16.75%; range 2.2-57.3) by NK cells from 13/16 M-LN and DNAM-1 was significantly higher in NK cells from M-LN compared to D-LN (Fig. 2A, p=0.0415). The expression levels (MFI ratios) of activating NK receptors were comparable in D- and M-LN NK cells: LN NK cells were characterized by a high expression of NKp46 (Fig. 2B).

NK cells from M-LN were characterized by a high expression of CD62L, found on 30% of M-LN NK cells versus less than 10% of D-LN NK cells (Fig. 2A, p=0.0022). Moreover, we observed a trend for a higher expression of CXCR3 and CCR7 on patient LN-NK cells (Fig. 2A). Activation marker CD69 was expressed by 60% (range 20-88%) of M-LN NK cells. HLA-DR was present on 20% (range 4-75%) of M-LN NK cells (Fig. 2A).

The large numbers of CD45−CD56− melanoma cells in LNs may interfere with NK cell activation. Interestingly, we found negative relations between the expression of NKp30 (%) and NKG2D (%) and MFI ratio) and the proportions of CD45− melanoma cells (Fig. 2C), while NKp46, NKp44, CD16, DNAM-1 and CD62L expressions were not related to LN invasion by tumor cells (data not shown).

When the phenotypic profiles of M-LN NK cells were assessed according to the disease stage (III B or C), we found a trend for lower NKp30 and NKG2D expression and a significantly higher NKG2C
expression by LN NK cells from stage IIIC patients (Supplementary Fig. S3). Stage IIIC LN contained high percentages of CD45^CD56^cells.

**M-LN contained a unique subset of CD56^brightCD16^ NK cells**

Remarkably, we found that 40 to 60% of CD56^bright M-LN NK cells contained an important subset co-expressing CD16 (Fig. 3A). This novel CD56^bright/CD16^ NK subset was further characterized and compared to the CD56^bright/CD16^- subset. The percentages of Nkp46 and NKG2D were significantly higher on CD16^- than on CD16^ NK cells (Fig. 3B). Remarkably, CD16^- NK cells also displayed higher expression of KIR receptors (CD158a, b and e) compared to CD16^ NK cells suggesting a more differentiated CD16^- subset. NKG2A and NKG2C expression levels were comparable in the two subsets of NK cells (Fig. 3C).

The statistical analyses of the paired NK subsets revealed that the expression of Nkp30, NKG2D and Nkp46 were strongly correlated on CD56^bright/CD16^- cells. On the contrary, in the CD16^- subset, there was a weak correlation between Nkp30 and NKG2D while Nkp46 was not correlated with Nkp30 or NKG2D. In addition, NKG2A was correlated with Nkp30 in CD16^ but not in the CD16^- subset and with Nkp46 in both subsets (Supplementary Fig. S4).

It is of note that in mediastinal D-LN, CD56^bright/CD16^- and CD16^- NK cell subsets expressed similar phenotype (data not shown), suggesting that the CD16^- NK cell subset in M-LN is activated in response to tumor.

We have also collected blood samples from stage IIIB/C patients. Blood NK cells were mainly CD56^dim/CD16^- (>90%) and CD56^bright/CD16^- NK cells were not detected. Blood NK cells showed low expression levels (median % values) of NCR (Nkp30 and Nkp44) and NKG2D compared to M-LN NK cells (Fig. 2 and Supplementary Fig. 5A). Thus, in M-LN, CD56^bright/CD16^- NK cells are more mature and activated than the CD56^bright/CD16^- NK cells, also differ from blood CD56^dim/CD16^- NK cells and may exert antitumor activities.

**Functional status of M-LN NK cells from melanoma patients**

The functional capacities of M-LN NK cells were assessed ex vivo in response to K562 stimulation. CD16^- and CD16^- subsets from LN NK cells displayed comparable degranulation potential (Fig. 4A) and did not produce IFN\(\gamma\) (data not shown). In 5 M-LN suspensions, CD16^- cells displayed higher
basal perforin content than in CD16\(^+\) (31.6\% versus 17\%, \(p=0.018\)) (data not shown) and increased perforin degranulation in response to K562 stimulation (Fig. 4B). Addition of PMA/Iono increased the degranulation and induced IFN\(\gamma\) production towards K562 in certain samples indicating that LN-NK cells display the capacity to produce cytokines (Fig. 4C). In presence of PMA/Iono, the % of CD107a\(^+\) but not the % IFN\(\gamma\) producing NK cells inversely correlated with the invasion of the LN by CD45\(^+\) tumor cells (Fig. 4D). The CD107a % were not related to those of IFN\(\gamma\) production (Fig. 4E). Finally, we showed that CD16 expressed by LN-NK cells is functional since CD16 engagement by murine P815 cells coated with anti-CD16 mAb triggered degranulation of LN-NK cells (Fig. 5).

**High anti-tumor capacities of cytokine-activated M-LN NK cells**

From 6 M-LNs and 1 D-LN, NK cells were immunoselected and stimulated for 6 days with IL-2 or IL-15. NK cell-mediated lysis of melanoma cells was assessed using the xCELLigence system that allow the dynamic measure of adherent target cell index (CI), that is correlated to cell viability (23). Rapid and E/T ratio dependent decreases of MelC CI were observed after addition of LN-NK cells (Supplementary Fig. S6). Cytokine-activated LN-NK cells efficiently killed melanoma MelC cells independently of the % of tumor cells invading the LN (Fig. 6A). The NK-mediated lysis curves from the 6 patients were above the curves obtained for the D-LN NK cells, indicating that M-LN NK cells stimulated by IL-2 or IL-15 acquired high lytic potential (Fig. 6A).

In addition, we showed that IL-2-activated M-LN NK cells induced rapid and high percentages of MelC lysis compared to autologous blood NK cells stimulated in the same conditions in 4 out of 5 experiments (Fig. 6B). Resting LN-NK as well as blood-derived NK cells exhibited low lytic potential at least at the used 1/1 E/T ratio (data not shown).

**Discussion**

Our series represents the first large ex vivo study of NK cells infiltrating LN from stage III melanoma patients treated by radical LN dissection for macroscopic regional metastases. We described a unique NK cell population CD56\(^{bright}\)/CD16\(^-\)/NCR\(^-\)/NKG2D\(^+\) infiltrating metastatic LN and demonstrated their high anti-melanoma function upon cytokine activation.

LN are known sites of human NK cell differentiation (24, 25). In the few previous studies on donor LN-NK cells, these cells were described as CD56\(^{bright}\)/CD16\(^-\), NCR\(^-\) cells endowed with a primarily
immunoregulatory function (26-28). They respond to IL-2 and efficiently secrete IFNγ (29). However, a recent immunohistological study reported high level of NKp46 on CD56+ NK cells from mesenteric LN (30). In mediastinal LN from donors and in patients M-LN, we found that NK cells expressed high levels of NCR (NKp46, NKp30 and NKp44) and NKG2D. In addition, we found CD56brightCD16- and CD16+ subsets. The origin of the LNs (peripheral versus mediastinal, resting versus inflamed LN) may account for these phenotypic differences in donors. A likely chronic stimulation of mediastinal LNs would explain the presence of the CD16+ subset. The group of Ferlazzo previously showed that LN can be the site of NK cell final maturation during the course of an immune reaction and resident CD56bright NK cells from paracortical/follicular hyperplasic LN may acquire CD16 and KIR and recirculate in the blood (31). It is interesting to note that our data indicate different regulation of NK receptors in CD16- versus CD16+ M-LN NK cells and in blood NK cells from patients. In particular, NKp30 and NKG2D, as well as NKp30 and NKp46 were co-regulated in CD16+ M-LN and in blood NK cells from patients. In contrast, we previously showed that in donor blood NK cells, NKp30 and NKG2D were not correlated but that NKp46 was correlated with NKG2D (21, 23, 32). Expression of CD16 by blood CD56bright was reported: these cells are CD56brightCD94high and may represent functional intermediary between CD56bright and CD56dim subset (33).

It is assumed that physiologically, CD56brightCD16dim NK cells enter the LN via afferent lymphatic vessels, migrate to the T-cell zone, and eventually in cooperation with dendritic cells, mature into CD56dimCD16bright NK cells that leave LN via efferent lymphatics. However, it was shown that during tumor-induced sentinel node lymphangiogenesis, an increased lymph flow occurs before tumor cell invasion (34) and that DC activation may precede the invasion by tumor cells. Thus, M-LN NK cells may derive from locally resident CD56brightCD16dim NK cells that mature and become activated in response to tumor cell invasion. The absence of CD56dim NK cells in M-LN whereas we found a remarkable subset of mature CD56brightCD16+NCR+ NK cells would favor this hypothesis. Alternatively, some blood CD45+CD56+CD62L+ NK cells may migrate to M-LN and upregulated CD56, as we found substantial % of CD56brightCD62L+ or CCR7+ infiltrating M-LN but not in D-LN. CD62L was previously found at higher density on CD56bright NK cells and increased in response to IL-12, IL-10, or IFNα (8) and required for NK cell recruitment in tumor draining LN in mice (35). There were also lower % of CD117+CD56bright CD16+ M-LN NK cell subset (n=3, data not shown) suggesting they came from blood. Our data indicate an impact of tumor burden on the phenotype and function of NK cells.
infiltrating M-LN. The co-expression of activating receptors (NKp30, NKG2D) by M-LN NK cells and their ex vivo degranulation were inversely correlated with the % of tumor cells, indicating a local suppression of NK activation by metastatic tumor cells.

As immunohistological staining of M-LN showed poor NK cell infiltration of the tumor mass, contact-dependent inhibitory effects are unlikely. However, melanoma cells may produce PgE2 and IDO, that inhibited the expression of NKp30, NKG2D and Nkp44, prevented recognition and killing by IL-2 activated NK cells and decreased cytotoxic molecules, granzymes and perforin (36).

Immunosuppression associated to melanoma invasion with increased IL10 and IDO was found in sentinel LN (37) and may be even more relevant in heavily metastatic LNs. In addition, the tumor may affect other immune cells in the environment that may in turn alter NK cell function. While we did not observe large numbers of FoxP3+ Treg cells in metastatic LN, we found a significant increase of CD14+ cells in correlation with the % of tumor cells in the LN (data not shown). Moreover, as described for primary melanoma, fibroblasts may participate to the functional anergy of LN NK cells producing PgE2 and downregulating activating NK receptors (38).

Contrasting with the low ex vivo lytic potential of LN-NK cells, activation by IL-2 or IL-15 greatly potentiated the lytic capacities and the expansion of M-LN NK cells in vitro. Cytokine activated M-LN NK cells lysed melanoma cells more rapidly and efficiently than donor-derived NK cells, or paired blood NK cells. Cytokine activation likely reverses the ex vivo functional anergy of NK cells infiltrating M-LN from melanoma patients that are mature NK cells endowed with high lytic potential (natural cytotoxicity and Ab dependent-cell-cytotoxicity) towards melanoma cells, known to express NK ligands (39).

A previous work reported in vivo IL-2-induced lymphokine-activated killer (LAK) cells in cervical LN of patients with head and neck tumors. Interestingly, these LAK NK cells were CD16+ and efficiently killed K562 and Daudi (40). Our data bring additional arguments for the presence of CD56brightCD16+ NK cells in M-LN expressing high levels of activating receptors and perforin, thereby acquiring high antitumor activity in response to IL-2 activation.

Immunotherapy may be considered as a complementary approach that can be integrated in therapeutic strategies at different stages of the disease. In metastatic melanoma patients, blocking immune checkpoint of T cell activation with specific mAbs (anti-CTLA4, anti-PD1) is a promising issue to restore efficient antitumor immune response. Ongoing randomized trial with Ipilimumab (anti-CTLA4
mAb) in stage III resected melanoma patients (ECOG E1609) will determine if recurrence free and overall survival is ameliorated in patients. While anti-CTLA4 mAb primarily affect T cells, there is evidence in mice that during MCMV infection, CTLA4 is highly induced on activated memory NK cells (Karo J, J. Immunol, 2012; Meeting Abstract Suppl, 168.3). Presumably, Ipilimumab treatment may result in increased IL-2, IFNγ production by T cells and could lead to activated NK cells in LN.

NK-based therapies would be probably more effective for the treatment of minimal residual disease when associated to therapy inducing NK ligands on tumor cells and enhancing the NK recruitment (32). Our data suggest that, if activated NK cells also infiltrate positive sentinel LN, novel adjuvant therapies aimed at potentiating in situ NK cell activation (cytokine administration, bispecific mAbs, triggering NKR antibodies) might be interesting to consider for a future clinical trial for stage IIIA patients. In vivo continuous infusions of low doses of IL-2 would favor enhanced NK-cell differentiation from bone marrow progenitors, rather than proliferation of mature NK cells in the periphery and it would also delay NK-cell death in vivo (41). This treatment could favor the activation of tumor draining LN NK cells while avoiding Treg expansion. The presence of CD56brightCD16+ in M-LN NK cells offers the possibility to activate ADCC by direct triggering of CD16 NK cell activation, increasing NK cell cytolytic activity and cytokine production against tumor targets (42, 43) using bispecific mAbs (44).

Finally, the treatment with MAP kinase inhibitors (such as anti-B-RAF) induces high percentages of responses in melanoma patients with B-RAF mutated tumors although most patients develop resistance and relapse. Beside a direct effect on cancer cells, these inhibitors could sensitize cancer cells to immune attack (45). Combining immunotherapy with B-RAF inhibitors would be worthwhile to explore in melanoma patients, as emerging experiences show a favorable microenvironment, spared function of immune cells in B-RAF inhibitors treated patients (46-49).

Author Contributions
Conception and design: MM, GF, EFN, MFA, AC
Analysis and interpretation: MM, GF, MFA, LZ, IC, SR, AC
Writing of manuscript: MM, GF, MFA, IC, LZ, AC
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References


### Table 1: Patients characteristics

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<th>Gender</th>
<th>Stage AJCC</th>
<th>Stage TNM</th>
<th>Site of Primary tumor</th>
<th>LN (Nb)</th>
<th>Lymphocytes /mm³</th>
<th>LDH</th>
<th>Other Cancer</th>
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LN: lymph nodes
**Figures Legends**

**Figure 1:** Characterization of NK cells infiltrating M-LN. (A). Representative sections of paraffin-embedded D-LN and M-LN stained with anti-NKp46 mAb: original magnifications x10, x20 and x40 (insert) followed by computer magnification. (B) Percentages of CD3−/CD56+ NK cells from 22 dissociated M-LN. (C) Representative SSC/FSC and SSC/CD45 FACS plots to discriminate CD45+ and CD45− cells in M-LN suspension (left panels). Percentages of CD45−/CD56+ invading cells from 22 dissociated M-LN (right). Below, analysis of CD45− cells from a representative M-LN for expression of CD56, MCAM and NKG2D ligands (MICA/B and ULBP2). (D) Quantification by RT-PCR of Tyrosinase (Tyro U1 and U2), MCAM and house-keeping gene (TBP) mRNA transcripts in 3 M-LN cell suspensions.

**Figure 2:** Multiparameter flow cytometry analyses of D- and M-LN NK cells. (A) Percentages of expression of activating and chemokine receptors. (B) Expression levels (MFI ratio) of NK receptors. Values of D and M-LN NK cells were compared using the non parametric M-W test. (C) Correlations between the % and MFI ratio of NKp30 and NKG2D and the % of CD45− invading cells. Correlations were analyzed with Pearson test. Regression lines ($r^2$) and p-values are reported.

**Figure 3:** CD56−/CD16+ infiltrating M-LN NK cells. (A) Percentages of CD16+ NK cells in D and M-LN suspensions (left) and representative dot plots of CD16+ and CD16− NK subsets from D and M-LN. (B) Percentages of activating NK receptors in paired M-LN CD56−/CD16− (white triangles) and CD16+ (black triangles). On the right, FACS plots of NKp46 and NKG2D expression in M-LN NK cells from a representative M-LN. (C) Expression of HLA-I specific receptors by CD16+ and CD16− M-LN NK subsets. Values of CD16+ and CD16− NK subsets were compared using the non parametric M-W test.

**Figure 4:** Functional activity of LN NK cells. (A) Degranulation (CD107a%) and (B) perforin secretion (%) of CD16+ and CD16− NK subsets in response to K562: a representative patient (left panels) and paired analyses on CD16− and CD16+ NK cell subsets from M-LN (right panels). (C) PMA-ionomycin/target induced degranulation and IFNγ production by D- and M-LN NK cells compared to basal condition. (D) Correlations between function (CD107a% and IFNγ) and M-LN invasion by CD45−
cells %. (E) Correlations of degranulation and IFNγ production by NK cells. Correlations were analyzed with Pearson test: regression lines ($r^2$) and p-values are reported.

Figure 5: Functional activity of CD56bright CD16+ M-LN NK cells. Lysis of murine FcR+ P815 target by M-LN NK cells ex vivo (top) and IL-15-activated-NK (bottom) determined by reverse-ADCC assay in presence of growing concentrations of anti-CD16 mAb.

Figure 6: Dynamic lysis of metastatic melanoma cells (MelC) by IL-2 activated immunoselected NK cells. (A) Percentages of MelC lysis by IL-2 activated LN-NK cells from 6 patients (black symbols) and 1 donor (white symbols). (B) MelC lysis % by NK cells from paired blood (white symbols) and M-LN (black symbols) from 5 patients. The % of tumor cells invading the LN are indicated in brackets.
Figure 2

A

B

C

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Figure 5

CD56

CD107a

Ex-vivo
M-LN NK

IL15-activated
M-LN NK

α CD16 (0.1 μg/ml)
5,1

α CD16 (1 μg/ml)
9,3

α CD16 (10 μg/ml)
13,4

27

31,9

35,3

42,2

5,1

9,3

10,1

13,4

27

31,9

35,3

42,2
Figure 6

(A) Graph showing lysis over time for different samples:
- D-LN NK
- Pt1 LN-NK (20%)
- Pt2 LN-NK (45%)
- Pt3 LN-NK (15%)
- Pt4 LN-NK (60%)
- Pt5 LN-NK (36%)
- Pt6 LN-NK (45%)

(B) Graphs for individual samples:
- Pt1 (20%)
- Pt2 (45%)
- Pt3 (15%)
- Pt4 (60%)
- Pt5 LN-NK (36%)
- Pt6 (45%)

Time (Min) vs. % of lysis
Mature cytotoxic CD56bright/CD16+ natural killer cells can infiltrate lymph nodes adjacent to metatatic melanoma

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