Mature Cytotoxic CD56<sup>bright</sup>/CD16<sup>+</sup> 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 representing the most frequent site of early dissemination in this disease. These regional lymph nodes also represent the primary site for differentiation of natural killer (NK) cells. Although blood-derived NK cells can efficiently lyse melanoma cells isolated from metastatic lymph node (M-LN), there has been no study of the properties of the most disease-reactive NK cells isolated from M-LN in patients with melanoma. Here, we report that M-LN contains 0.5% to 11% of CD56<sup>bright</sup> NK cells among CD45<sup>+</sup> hematopoietic cells present and that this cell population surrounds 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 (NCR), NK group 2 D (NK2D), and DNAX accessory molecule 1 (DNAX-1). Expression of NCR-NKp30 and NK2D correlated negatively with percentages of tumor cells in M-LN. Interestingly, M-LN contained a unique subset of mature CD56<sup>bright</sup>/CD16<sup>+</sup> NK cells displaying coregulated expression of NCR and NK2D activating receptors. Ex vivo analyses suggested that M-LN-derived NK cells were inactive but could be activated by appropriate cytokine signals [interleukin (IL)-2 or IL-15], and could lyse metastatic melanoma cells in a highly efficient manner compared with blood-derived NK cells. Taken together, the results offer evidence that adjuvant immuno-therapy that targets NK cells in M-LN for activation may improve treatment of patients with sentinel lymph node–positive melanoma. Cancer Res; 74(1); 81–92. ©2013 AACR.

Introduction

Melanomas are highly metastatic tumors for which the treatment of advanced stages is still unsatisfactory. Stage I and II patients with melanoma display a localized disease. The main way of dissemination is the lymph and the invasion of sentinel lymph node, the initial tumor-draining lymph node (LN), is correlated with prognosis. Stage III patients are characterized by lymph node metastases in the draining basin. These patients are treated by lymph node dissection and pathologic examination determines both the total number of excised lymph node and the number of metastatic lymph node (M-LN), the latter being of prognostic significance. Thus, stage III patients constitute a heterogeneous subset (stage III A–C) of patients that present one or several M-LN either microscopically or macroscopically involved. Some characteristics of the primary melanoma are also taken into account for staging in the three subgroups (A–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 node 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 toward low tumor invasion.

As potent cytotoxic antitumor effectors and strong inducers of adaptive immune response, natural killer (NK) cells (CD3<sup>−</sup>CD56<sup>+</sup> 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 the tumor cell surface, such as loss of MHC class I, leading to NK cell activation.
cellular self. They are also pivotal catalysts of adaptive T cell responses as they are considered the major source of IFN-γ in vivo and NK-derived IFN-γ is crucial in priming Th1 responses (5, 6). They differentiate in the bone marrow and in peripheral lymph node and they represent 5% to 20% of the blood lymphocytes. Two major NK subpopulations are described in humans: CD56dimCD16+ and CD56brightCD16− cells. The CD56dim 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 CD56bright 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 monoclonal antibodies (mAb) 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 MHC class I polypeptide-related sequence (MIC)-A/B molecules, and UL16-binding proteins 1–4 (ULBPs-1–4), 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 presence 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 patients with melanoma in some cases (20), suggesting that NK cell activity may be an additional prognostic factor in patients with melanoma.

Here, we have studied the phenotype and functions of NK cells infiltrating lymph node from organ donors and stage IIIB and C patients with melanoma. 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 toward metastatic melanoma cells following activation by cytokines.

Materials and Methods

Samples, patients, and melanoma cell lines

Twenty-two patients with stage III melanoma with clinically palpable lymph node, who had to be treated by radical lymph node 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 histologic analysis for the validation of the resection quality) were obtained from the operating room. For 6 patients, fragments from one macroscopically noninvaded 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% fetal calf serum (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 lymph node 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 patient with melanoma (21). MelC and K562 cells (0.3–1 × 106/mL) were maintained in complete medium. K562 and MelC were recently authenticated by DNA profiling (DSMZ).

Immunohistochemistry of donor and patient lymph node

Fragments from six D-LN and 10 M-LN were paraffin-embedded, sectioned, and further stained by routine hematoxylin and eosin method. Immunohistochemistry (IHC) 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 (Hamasatsu Photonics). NKP46-positive NK cells were then counted using NDP View software in 10 fields of 1 mm² in the center and in the pericapular areas of the M-LN.

Phenotypic analyses

PBMC and lymph node cells were suspended in PBSIX (GIBCO, Invitrogen) supplemented with 5% human serum AB (Biowest) and incubated 30 minutes in ice to block nonspecific Fc receptor (FcR) binding before staining. Cells were then washed and stained 30 minutes at 4°C with the mAbs diluted in PBSIX/FCS 2%/EDTA 2 mmol/L at predetermined optimal concentrations. NK cells, defined as CD3−CD56+ cells (BD Biosciences) within CD45− (BD Biosciences) lymphocyte forward scatter/side scatter (FSC/SSC) subset, were analyzed for the expression of NCR (NKP46, NKP30, and NKP44), NKG2D, DNAM-1, and NKG2C (BD Biosciences), CD16, CD158a, CD158b, CD158c, and NKG2A receptors (Beckman Coulter), CD69, CXC5R3, CCR7, and CD62L (Miltenyi Biotech) after dead
cell and doublet exclusion. The percentages of positive cells were determined on more than 3,000 NK-gated events. CD4 and intracellular staining with Foxp3 were performed to quantify regulatory T cells (Treg). 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 Biosciences). These cells produced black pigments in the supernatant. Quantification by real-time (RT) PCR of tyrosinase (Tyro U1 and U2), MCAM, and house-keeping gene (TBP) mRNA transcripts was determined in three M-LN cell suspensions.

**CD107a degranulation, IFN-γ secretion, and perforin assays**

The functional capacities of lymph node NK cells were evaluated *ex vivo* by flow cytometer analyses on gated CD3−CD56− NK cells. From lymph node cell suspensions, 10⁶ cells were stimulated with 10⁷ K562 targets (10/1 effector/target ratio) in V-bottom plates in the presence or absence of PMA/ionomycin (PMA/I) and monensin (BD Biosciences) for 4 hours. Cells were then labeled for 30 minutes at 4°C with anti-CD16-PeCy7 (Beckman Coulter), anti-CD56-horizonV450, anti-CD3-allophycocyanin (APC)-H7, and anti-CD107a-FITC (BD Biosciences), washed, fixed, and permeabilized (kit; BD Biosciences) for 30 minutes at 4°C before intracellular staining with anti-IFN-γ-APC (Miltenyi Biotech) or anti-perforin-phycocerythrin (eBioscience) for 30 minutes at 4°C. Cells were collected on a FACSCanto II flow cytometer. Results are expressed as the percentages of CD107a+, IFN-γ+, or perforin-positive NK-gated cells. Baseline NK cell cytokine secretion and degranulation were determined in the absence of targets or in the presence of isotype control for the perforin staining.

In addition, for some lymph nodes and blood samples, we have proceeded to the negative immunoselection of NK cells using the NK selection Kit (Miltenyi Biotech). Purified NK cells (0.5−1 × 10⁶/mL) were cultured for 6 days in the presence of interleukin (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 microelectrodes 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, 15,000 MeC cells per well were seeded into 96X E-plates and their adhesion monitored for 3 hours. IL-2−activated lymph node NK or blood NK cells were added at different concentrations in a volume of 50 μL/well. Cocultures were assessed by the system with a measure every 15 minutes for up to 300 minutes. Results are expressed as percentage of quadruplicate of lysis determined from cellular index (CI) normalized with RTCA Software (nCI): percentage of lysis = [nCI (no effector) − nCI (effector)]/nCI (no effector) × 100. The mean CI value at 510 minutes of MeC targets incubated with medium was 1.5 (range, 1.2−1.8).

**Statistical analysis**

Statistical tests and graphics were generated by Prism version 5 (GraphPad Software Inc). Nonparametric Mann-Whitney test was used to compare the medians of percentage of CD45−, NK, and Tregs cells between D-LN and M-LN. The same test was used to compare the medians of NK and CD45− cells proportions, the percentage of NK receptors, CD107a and IFN-γ 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: *, P ≤ 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 patients with melanoma**

The distribution of NK cells was assessed by IHC in 10 M-LN and six mediastinal lymph node 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 lymph node architecture was altered by invading tumor cells: NK cells were distributed around the lymphoid structures, squeezed under the lymph node 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 patients with melanoma treated by lymph node dissection (Table 1) and in four of six D-LN. In lymph node 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% to 11% of CD45− cells, percentages in the range of those present in lymph node from donors (Fig. 1B). The frequency of Tregs (CD4+Foxp3+) determined in four lymph node 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 lymph node suspensions (Fig. 1C), whereas low percentage 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 patients with melanoma**

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 of 16 M-LNs and DNAM-1 was significantly higher in NK cells from M-LN compared with D-LN (Fig. 2A; P = 0.0415). The expression levels [mean fluorescence intensity (MFI) ratios] of
activating NK receptors were comparable in D- and M-LN NK cells: lymph node 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 lymph node 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 lymph nodes may interfere with NK cell activation. Interestingly, we found negative relations between the expression of NKp30 (%) and NKG2D (percentage and MFI ratio) and the proportions of CD45⁻ melanoma cells (Fig. 2C), whereas NKp46, NKp44, CD16, DNAM-1, and CD62L expressions were not related to lymph node 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 lymph node NK cells from stage IIIC patients (Supplementary Fig. S3). Stage IIIC lymph node contained high percentages of CD45⁻CD56⁺ cells.

**M-LN contained a unique subset of CD56brightCD16⁻ NK cells**

Remarkably, we found that 40% to 60% of CD56bright M-LN NK cells contained an important subset coexpressing CD16 (Fig. 3A). This novel CD56brightCD16⁻ NK subset was further characterized and compared with the CD56brightCD16⁻ 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 with 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, whereas 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 percentage values) of NCR (NKp30 and NKp44) and NKG2D compared with M-LN NK cells (Fig. 2 and Supplementary Fig. S5A). 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 patients with melanoma**

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

**Table 1. Patients characteristics**

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<tr>
<th>Gender</th>
<th>Stage (American Joint Committee on Cancer)</th>
<th>Site of primary tumor</th>
<th>Lymphocytes/mm(^3)</th>
<th>Lactate dehydrogenase</th>
<th>Other cancer</th>
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<td>1N(^-)/15</td>
<td>Lung neuroendocrine tumor</td>
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Abbreviation: M, metastasis.
High antitumor capacities of cytokine-activated M-LN NK cells

From six M-LNs and one 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 allows the dynamic measure of adherent target CI, which is correlated with cell viability (23). Rapid and E/T ratio-dependent decreases of MelC CI were observed after addition of lymph node NK cells (Supplementary Fig. S6). Cytokine-activated lymph node NK cells
efficiently killed melanoma MelC cells independently of the percentage of tumor cells invading the lymph node (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 with autologous blood NK cells stimulated in the same conditions in four of five experiments (Fig. 6B). Resting lymph node 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 lymph node from stage III patients with melanoma treated by radical lymph node dissection for macroscopic

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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). 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 nonparametric Mann–Whitney test. (P values noted as *, P < 0.05; **, P < 0.01; and ***, P < 0.0001).
regional metastases. We described a unique NK cell population CD56<sup>bright</sup>/CD16<sup>−</sup>/NCR<sup>+</sup>/NKG2D<sup>+</sup> infiltrating M-LN and demonstrated their high antimalanoma function upon cytokine activation.

LNs are known sites of human NK cell differentiation (24, 25). In the few previous studies on donor lymph node NK cells, these cells were described as CD56<sup>bright</sup>/CD16<sup>−</sup> cells endowed with a primarily immunoregulatory function (26–28). They respond to IL-2 and efficiently secrete IFN-γ (29). However, a recent immunohistologic study reported high level of NKp46 on CD56<sup>+</sup>NK cells from mesenteric lymph node (30). In mediastinal lymph node 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 CD56<sup>bright</sup>/CD16<sup>−</sup> and CD16<sup>+</sup> subsets. The origin of the lymph nodes (peripheral vs. mediastinal, resting vs. inflamed lymph node) may account for these phenotypic differences in donors.

A likely chronic stimulation of mediastinal lymph nodes would explain the presence of the CD16<sup>+</sup> subset. Romagnani and colleagues previously showed that lymph node could be the site of NK cell final maturation during the course of an immune reaction and resident CD56<sup>bright</sup> NK cells from paracortical/follicular hyperplastic lymph node 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<sup>−</sup> versus CD16<sup>+</sup> M-LN NK cells and in blood NK cells from patients. In particular, NKp30 and NKG2D, as well as NKp30 and NKp46 were coregulated in CD16<sup>−</sup> 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.

Figure 4. Functional activity of lymph node NK cells. A and B, degranulation (CD107a%) and perforin secretion (%) of CD16<sup>+</sup> and CD16<sup>−</sup> NK subsets in response to K562; a representative patient (left) and paired analyses on CD16<sup>−</sup> and CD16<sup>+</sup> NK cell subsets from M-LN (right). C, PMA–target induced degranulation and IFN-γ production by D- and M-LN NK cells compared with basal condition. D, correlations between function (CD107a% and IFN-γ) and M-LN invasion by CD45<sup>+</sup> cells%. E, correlations of degranulation and IFN-γ production by NK cells. Correlations were analyzed with Pearson test; regression lines (r<sup>2</sup>) and P values are reported.
but that NKp46 was correlated with NKG2D (21, 23, 32). Expression of CD16 by blood CD56\textsuperscript{bright} was reported: these cells are CD56\textsuperscript{bright}CD94\textsuperscript{high} and may represent functional intermediary between CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subset (33).

It is assumed that physiologically, CD56\textsuperscript{bright}CD16\textsuperscript{dim} NK cells enter the lymph node via afferent lymphatic vessels, migrate to the T-cell zone, and eventually in cooperation with dendritic cells, mature into CD56\textsuperscript{dim}CD16\textsuperscript{bright} NK cells that leave lymph node 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 dendritic cell activation may precede the invasion by tumor cells. Thus, M-LN NK cells may derive from locally resident CD56\textsuperscript{bright}CD16\textsuperscript{dim} NK cells that mature and become activated in response to tumor cell invasion. The absence of CD56\textsuperscript{dim} NK cells in M-LN, whereas we found a remarkable subset of mature CD56\textsuperscript{bright}CD16\textsuperscript{+} NCR\textsuperscript{+} NK cells would favor this hypothesis. Alternatively, some blood CD45\textsuperscript{-}CD56\textsuperscript{-}CD62L\textsuperscript{-} NK cells may migrate to M-LN and upregulated CD56, as we found substantial percentage of CD56\textsuperscript{bright}CD62L\textsuperscript{-} or CCR7\textsuperscript{-} infiltrating M-LN but not in D-LN. CD62L was previously found at higher density on CD56\textsuperscript{bright} NK cells and increased in response to IL-12, IL-10, or IFN-\(\gamma\) (8) and required for NK cell recruitment in tumor D-LN in mice (35). There were also lower percentages of the CD117\textsuperscript{+}CD56\textsuperscript{bright}CD16\textsuperscript{+} 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 coexpression of activating receptors (NKp30 and NKG2D) by M-LN NK cells and their \textit{ex vivo} degranulation were inversely correlated with the percentage of tumor cells, indicating a local suppression of NK activation by metastatic tumor cells.

As immunohistologic 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 indoleamine 2,3-dioxygenase (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 with melanoma invasion with increased IL-10 and IDO was found in sentinel lymph node (37) and may be even more relevant in heavily M-LNs. In addition, the tumor may affect other immune cells in the environment that may in turn alter NK cell function. Although we did not observe large numbers of FoxP3\textsuperscript{+} Treg cells in M-LN, we found a significant increase of CD14\textsuperscript{+} cells in correlation with the percentage of tumor cells in the lymph node (data not shown). Moreover, as described for primary melanoma, fibroblasts may participate to the functional anergy of lymph node NK cells producing PgE2 and downregulating activating NK receptors (38). Contrasting with the low \textit{ex vivo} lytic potential of lymph node NK cells, activation by IL-2 or IL-15 greatly potentiated the lytic capacities and the expansion of M-LN NK cells \textit{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 \textit{ex vivo} functional anergy of NK cells infiltrating M-LN from patients with melanoma that are mature NK cells endowed with high lytic potential (natural cytotoxicity and antibody-dependent cell cytotoxicity) toward melanoma cells, known to express NK ligands (39).

A previous work reported \textit{in vivo} IL-2–induced lymphokine-activated killer (LAK) cells in cervical lymph node of patients with head and neck tumors. Interestingly, these LAK–NK cells were CD16\textsuperscript{-} and efficiently killed K562 and Daudi (40). Our data bring additional arguments for the presence of CD56\textsuperscript{bright} NK cells.
CD16+ 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 patients with metastatic melanoma, blocking immune checkpoint of T-cell activation with specific mAbs (anti-CTLA4 and anti-PD1) is a promising issue to restore efficient antitumor immune response. Ongoing randomized trial with ipilimumab (anti-CTLA4 mAb) in stage III patients with resected melanoma (ECOG E1609) will determine whether recurrence-free and overall survival is ameliorated in patients. Although anti-CTLA4 mAb primarily affect T cells, there is evidence in mice that during mouse cytomegalovirus infection, CTLA4 is highly induced on activated memory NK cells (41). Presumably, ipilimumab treatment may result in increased IL-2, IFN-γ production by T cells and could lead to activated NK cells in lymph node.

NK-based therapies would be probably more effective for the treatment of minimal residual disease when associated with 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 lymph node, novel adjuvant therapies aimed at potentiating in situ NK cell activation (cytokine administration, bispecific mAbs, and 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 (42). This treatment could favor the activation of tumor D-LN NK cells, whereas avoiding Treg expansion. The
presence of CD56\(^{\text{high}}\)/CD16\(^{-}\) in IM-LN NK cells offers the possibility to activate antibody-dependent cell-mediated cytotoxicity (ADCC) by direct triggering of CD16 NK cell activation, increasing NK cell cytolytic activity, and cytokine production against tumor targets (43, 44) using bispecific mAbs (45).

Finally, the treatment with mitogen-activated protein kinase inhibitors (such as anti-B-RAF) induces high percentages of responses in patients with melanoma 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 (46). Combining immunotherapy with B-RAF inhibitors would be worthwhile to explore in patients with melanoma, as emerging experiences show a favorable microenvironment, spared function of immune cells in B-RAF inhibitors treated patients (47–50).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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