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Expansion of CMV-Mediated NKG2C+ NK Cells Associated with the Development of Specific De Novo Malignancies in Liver-Transplanted Patients

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Solid cancers are a major adverse outcome of orthotopic liver transplantation (OLT). Although the use of chronic immunosuppression is known to play a role in T cell impairment, recent insights into the specificities of NK cells led us to reassess the potential modulation of this innate immune cell compartment after transplantation. Our extensive phenotypic and functional study reveals that the development of specific de novo noncutaneous tumors post-OLT is linked to unusual NK cell subsets with maturation defects and to uncommon cytokine production associated with the development of specific cancers. Remarkably, in CMV+ patients, the development de novo head/neck or colorectal tumors is linked to an aberrant expansion of NK cells expressing NKG2C and a high level of intracellular TNF-α, which impact on their polyfunctional capacities. In contrast, NK cells from patients diagnosed with genitourinary tumors possessed a standard immature signature, including high expression of NKG2A and a robust production of IFN-γ. Taken together, our results suggest that under an immunosuppressive environment, the interpaly between the modulation of NK repertoire and CMV status may greatly hamper the spectrum of immune surveillance and thus favor outgrowth and the development of specific de novo tumors after OLT. The Journal of Immunology, 2014, 192: 000–000.

Orthotopic liver transplantation (OLT) provides life-saving therapy for patients with end-stage liver diseases. The categories of fatal liver diseases that may be treated by liver grafting include primary cancers such as hepatocarcinoma (HCC) and severe nonmalignant destructive liver cell diseases, including cirrhosis, fulminant hepatic failure, and chronic hepatitis infection, which remains a leading cause of liver-related mortality despite the development of effective antiviral agents in the past decade (1, 2). Although transplant outcomes have improved, de novo malignancies are the second major adverse outcome, after cardiovascular complications, with an incidence that ranges from 3 to 16% after OLT. Cancers cause 25% of the deaths following OLT, a rate significantly higher than in the general population (3–5). This increased risk was originally attributed, at least in part, to prolonged immunosuppressive treatments following transplantation (6).

As a part of innate immunity, NK cells are critical in protecting hosts from viral infections and tumor growth (6). An 11-γ general population follow-up study showed that high NK cytotoxic activity is associated with reduced risk of cancer (7). In addition, NK cell infiltration into tumor tissues is associated with better disease prognosis in non–small-cell lung carcinoma (8), renal cell carcinoma (9), and colorectal carcinoma (10). NK cells are heterogeneous and differ in their proliferative potential, homing characteristics, functional capacities, and responses to different cytokines. They are divided into two major subsets: CD56bright and CD56dim cells, accounting, respectively, for 10 and 90% of the NK cells in the peripheral blood. On activation, CD56bright NK cells proliferate and produce cytokines; mostly IFN-γ and/or TNF-α, but their cytotoxic activity is minimal. CD56dim NK cells, in contrast, proliferate minimally, produce relatively low amounts of cytokines, but are highly cytotoxic (11). It is not yet clear which NK cell subpopulation controls tumor growth most effectively. Recent experimental evidence demonstrates that NK development proceeds from a CD56bright to CD56dim phenotype (12, 13). NK cell function is regulated by a vast network of inhibitory and activating signals. Under normal immune surveillance, NK cells have inhibitory receptors that recognize MHC class I molecules as their cognate ligands; these receptors include killer Ig-like receptors (KIR)-L, LIR-1/ILT-2, LAIR-1, and the CD94/NKG2A heterodimer. Cytotoxicity occurs when stimulatory signals outweigh inhibitory ones by a critical threshold. Several of these activating receptors have been characterized, including NKG2C, NKG2D, and the natural cytotoxicity receptors (NKp30, NKp44, and NKp46) (14–17).
Recent investigations have revealed that persistent viral infections influence NK cell phenotypes and functions. These include, in particular, the highly prevalent herpes viruses, such as CMV and EBV, which infect at least 50 and 80%, respectively, of the adult white population (18, 19). During latency, these viruses appear to be silent and cause no clinical symptoms as long as they are kept in balance with the host immune system. Rapid and vigorous proliferation of activated and differentiated CD56dimNKG2C+ NK cells in CMV-positive individuals was recently described upon additional encounters with viruses, including HIV-1, hantavirus, hepatitis B virus (HBV), and hepatitis C virus (HCV) (20–22). Whereas the frequency of NK cells expressing NKG2C is low or undetectable in CMV-seronegative individuals (23), NKG2C recognizes HLA-E, although with a lower affinity than NKG2A does (24). In immunocompromised individuals, the reactivation of latent virus or the development of an acute CMV infection is associated with the promotion of a lasting increase in differentiated CD56dimNKG2C+ NK cells, as observed in a SCID child infected with CMV, but also after allogeneic transplantation or in acute CMV infection after organ transplantation (25–28).

Although the importance of NK cells in the control of CMV infection has been well documented, the implication of NK cell during CMV latency or reactivation after organ transplantation remains to be elucidated. The aim of this study was to determine the role of NK cells from liver-transplanted patients diagnosed with de novo solid malignancies. Our results suggest that CMV could drive the expansion of NKG2C+ NK cells associated with the development of specific tumors after OLT.

Materials and Methods

Patients and healthy donors

The characteristics of OLT patients are summarized in Table I. Samples were provided of patients at the time of diagnosis for their cutaneous tumors (CT; n = 18), or noncutaneous tumors (NCT; n = 24). OLT patients without de novo tumors and matched for sex and age were enrolled as controls (CTL) during the same period (n = 35). All patients came from either Paul Brouss (Villejuif, France) or Pitié-Salpêtrière (Paris, France) hospitals and provided signed written informed consent. The appropriate institutional review board approved the study. Blood samples from 35 anonymous healthy donors (HD), provided by the hospital blood center (Etablissement Français du Sang), served as CTL.

Cell lines

K562 (ATCC CCL–243) and P815 (ATCC TIB-64) were obtained from and was observed significantly more often in patients with CT

Flow cytometric analysis

NK cells were analyzed after staining with an appropriate Ab mixture, as previously described (13): anti-CD54 (Chrome Orange; #F133), anti-CD3 (ECD; #UCHT1), anti-CD56 (PC7; #N901), anti-CD159a/NKG2A (APC; #13299), anti-CD336/Nkp44 (PE; #2231), anti-CD335/Nkp46 (APC; #BAB281), anti-NKG2D (allophycocyanin; #ON72), and anti-CD85j/ILT-2 (PE; #HP-F1), anti-CD69 (allophycocyanin; #FN50), anti–HLA-DR (PE; #Immu357), and anti-CD57 (FITC; #5-HCL-1) from Beckman Coulter; anti-CD161 (FITC; #DX12) and DNAM-1 (FITC; #DX11) from BD Biosciences; anti-CD337/Nkp30 (PE; #AF29–D12) and anti-KIR2DL2/KIR2DL3/ (APC; #DX27) from Miltenyi Biotec; anti-NKG2C (PE; #134591), anti-KIR2DL1 (FITC; #134211), and anti-KIR3DL1 (allophycocyanin; #DX9) from BD and System; and anti-CD11b (AF700; #CBRM1/5), anti-CD11c (APC; #3.9), anti-CD62L (allophycocyanin eFlour780; #DREG-56), anti–integrin-α4 (PE; #PF10), and anti–integrin–β7 (FITC; #FIB04) from eBioscience.

For intracellular staining, PBMCs were fixed and permeabilized with 0.1% saponin in 0.5% BSA/PBS buffer and then stained with perinor PE; #6G9; BD Biosciences), granzyme K (PE; #GM6C3; Santa Cruz Biotechnology), or granulocyte (AF647; #DH2; BD Biosciences), as described (15).

Populations of interest within the CD45+ lymphocyte population were identified by using the software’s “Boolean gate” algorithm. Pestle software was used to remove the background, and pie charts, generated with Speck software (NIAI freeware) (32), present the frequency of NK cells positive for zero, one, two, or three functions was defined with the software’s polyfunctional assay. Statistical analysis

Statistical analyses were performed with Prism 5 software (GraphPad). Tests for comparisons were assessed with the Fisher exact test for categorical variables, and the nonparametric Kruskal–Wallis test with Dunn posttest was used to define the significance of results from three independent groups of subjects, when compared 2 by 2. The nonparametric Mann–Whitney U test was used to compare two unrelated samples. Nonparametric correlations were assessed by determination of Spearman rank correlation coefficient. Statistical significance was defined by p < 0.05 with a two-tailed test: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Phenotypic signature of NK receptors in OLT patients developing de novo tumor

A total of 77 OLT patients (53 men and 24 women) were enrolled at the time of diagnosis, then categorized in function of the development of either de novo nonmelanoma CT or NCT, and compared with patients without tumors (CTL group). Table I summarizes their characteristics in detail; they received similar immunosuppressive treatments except for tacrolimus, which is reported to inhibit NK cell function, at least after renal transplantation (33) and was observed significantly more often in patients with CT (p < 0.0005). They did not differ significantly for the other variables considered, including sex, age, liver disease, age at transplantation, or follow-up post-OLT (Table I). Of 24 NCT patients who had de novo noncutaneous malignancies, five developed posttransplant lymphoproliferative disorders (PTLD); the other NCT included various genitourinary cancers (n = 7), colorectal cancers (n = 3), head/neck tumors (n = 3), various gynecological tumors (n = 3), lung cancer (n = 1), and hepatocarcinoma (n = 1) (Table II), as reported (33).

Flow cytometry was used to measure CD3+ T cells and CD3−CD56+ NK cells in OLT and HD. The absolute count and fre-
quency of lymphocytes subsets showed that OLT patients had significantly lower T cells than HD (Fig. 1A), whereas the distribution of NK cells was higher in OLT, in frequency and absolute count, irrespective of the presence of de novo tumors, as compared with HD (Fig. 1A). The analysis of NK cell subsets revealed that both CT and NCT displayed significantly higher levels of the CD56bright NK subset (mean ± SD; CT 16.5 ± 6.1%; NCT 14.1 ± 5.5%; p, 0.0001 for both) than OLT CTL without tumors (10.5 ± 5.7%) or HD (7.8 ± 4.1%) (Fig. 1B).

We further compared the phenotypic properties of the NK cells in OLTs and show that NK cells from patients without de novo tumors were indistinguishable from those of HD, in terms of cell-surface expression of the major NK receptors studied (Fig. 1C, Supplemental Fig. 2). Importantly, the proportion of several markers was significantly decreased in OLT patients with de novo tumors (CT and NCT), notably the expression of CD57, CD11b, and integrin-α4β7.

In addition, as compared with the CT, the frequency of cells expressing NKG2D (p = 0.0018) and NKp30 (p = 0.0006), two activating receptors, was very significantly decreased in NCT, whereas CD62L increased (p = 0.042) (Mann–Whitney tests) (Fig. 1C). Overall, these results demonstrated that NK cells from OLT patients who developed de novo tumors display specific phenotypic NK cell features distinct from those of OLT CTL and HD.

### Dichotomization of NCT patients by a hierarchical clustering of NK cell markers

To gain insight into the full characterization of NK cells in OLT patients with de novo tumors, we used a large-scale flow cytometric analysis.
analysis of the frequency of all 15 different phenotypic NK markers used in this study (Fig. 2). This hierarchical clustering analysis revealed specific clusters of patients relatively to the phenotypic expression of NK markers; thus, OLT patients who developed CT were similar to those in HD. In contrast, the NK cell repertoire of patients with NCT was substantially different and can be divided into two particular groups called 2C and 2C+. Thus, 2C- are mainly NKG2A^highCD62L^highILT2^lowCD57^lowNKG2C^2, whereas the 2C+ was characterized by NKG2A^lowCD62L^lowILT2^highCD57^highNKG2C^+ NK cells. Notably, OLT CTL patients without tumor represent an intermediate cluster between the HD/NCT and the CT, with three individuals (CTL-91, 211, and 226) with an NK profile similar to 2C- (Fig. 2).

To confirm these data, we further compared the phenotypic properties of some specific NK markers, both in CD56^{bright} and CD56^{dim} NK subsets, highlighted by the hierarchical analysis in the NCT patients of 2C- and 2C+. Thus, 2C- are mainly NKG2A^highCD62L^highILT2^lowCD57^lowNKG2C^-, whereas the 2C+ was characterized by NKG2A^lowCD62L^lowILT2^highCD57^highNKG2C^+ NK cells. Notably, OLT CTL patients without tumor represent an intermediate cluster between the HD/NCT and the CT, with three individuals (CTL-91, 211, and 226) with an NK profile similar to 2C- (Fig. 2).

**NKG2C expression skewed NK cell function in NCT patients**

We next assessed the overall functional ability of NK cells. One of their main functions is the synthesis and release of cytokines, especially IFN-γ and TNF-α (Supplemental Fig. 1A), which participate in triggering the adaptive immune response (35, 36). Fig. 4A shows that the level of intracellular IFN-γ and TNF-α production was significantly different in 2C- and 2C+. Thus, IFN-γ production was almost undetectable in 2C+, but remained significantly higher (p = 0.012) in 2C- than in CTL. In contrast, intracellular production of TNF-α was significantly upregulated in samples from 2C+ (p = 0.03), compared with 2C- and CTL (Fig. 4A). Importantly, the percentage of NK cells expressing NKG2C was inversely correlated with their intracellular production of IFN-γ (Spearman coefficient = -0.76; p < 0.0001) and positively correlated with their TNF-α expression (Spearman coefficient 0.76; p = 0.0002) (Fig. 4B).

We further assessed the overall cytolytic activity of NK cells. Fig. 4C shows that granzyme B, perforin, and granulyzin were expressed at equivalent levels in all samples, whereas granzyme K, a marker of immaturity, increased only in NK cells from 2C-.
To obtain further insight into the function of NK cells, polyfunctional assays were performed in the presence of various target cells to determine simultaneously the levels of degranulation and cytokine production. As expected, in the absence of target cells, the level of CD107a was close to the baseline level in all study groups, but NK cells from 2C+ produced only TNF-α, whereas those from 2C produced mainly IFN-γ (Fig. 5). In the presence of K562 or 721.221, two target cell lines that do not express MHC class I molecules, the proportion of polyfunctional cells is very low in 2C+ NCT, in which most TNF-α-positive cells are not cytotoxic. In contrast, the NK cells in the other groups had a greater tendency toward polyfunctionality with a high proportion of CD107+ cells producing cytokines (Fig. 5). In the presence of target cells expressing HLA-E (221.AEH), the ligand of the activating NKG2C receptor, and its inhibitory counterpart NKG2A, the degranulation activity is strongly repressed in all groups, whereas their capacity to produce cytokines is preserved at the levels previously detected in the absence of targets (Fig. 5). Of note, a redirected killing assay against FcγR+ P815 target cells in the presence of anti-NKG2C mAb showed the continuing functionality of the activating NKG2C receptor expressed on NK cells of 2C+ NCT (Fig. 5, Supplemental 1A). Altogether, these data confirm that the inhibitory signaling mediated by NKG2A expression prevailed in all groups in the presence of HLA-E+ target cells, despite the expression of NKG2C in 2C+ NCT (Fig. 5), and suggest that NK cells from both NCT groups are mainly distinguished by their polyfunctional capacities.

Effect of CMV on NK cells in the development of de novo–specific malignancies in NCT patients

Finally, we sought to determine the contribution of these unique phenotypic and functional modulations of the NK repertoire in the
development of specific malignancies. Table II revealed that $2C^-$ and $2C^+$ are different in regards to their primary disease; with a higher proportion of cirrhosis in $2C^-$ than in $2C^+$ (Fisher exact test, $p = 0.03$).

Intriguingly, all $2C^+$ patients (100%) are seropositive for CMV, compared with 26.6% (4 out of 15) of those in $2C^-$ (Fisher exact test, $p < 0.0005$), suggesting a striking association between $2C^+$ and CMV. Of note, these two groups did not differ significantly for any of the other routinely tested viruses, including EBV, HBV, or HCV (Table II). Moreover, quantitative PCR also detected CMV in 6 out of 9 $2C^+$, compared with 0 out of 15 in $2C^-$ (Table II) and 1 out of 35 in control OLT patients without cancer (Supplemental Table I). Although it is possible that the immunosuppressive protocols used in OLT recipients could impair specific antiviral immunity, NK cell function is apparently only sometimes affected by these therapeutic regimens. Thus, pre- and postinduction treatment did not differ significantly for any of the other routinely tested viruses, including EBV, HBV, or HCV (Table II).

More importantly, Table II shows significant differences in regards to the diagnosis of de novo malignancies. $2C^-$ and $2C^+$ are different in regards to their primary disease; with a higher proportion of cirrhosis in $2C^-$ than in $2C^+$ (Fisher exact test, $p = 0.03$).

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More importantly, Table II shows significant differences in regards to the diagnosis of de novo malignancies in the two NCT groups: colorectal (Fisher exact test, $p = 0.0415$) and head/neck (Fisher exact test, $p = 0.0415$) tumors appeared exclusively in $2C^-$, whereas gynecological (breast, uterine/ovary, or urothelial) tumors and PTLD were diagnosed in both NCT groups (Table II). These data suggest that phenotypic and functional features in NK cells from NCT, possibly associated with CMV, could drive the development of specific de novo malignancies.

**Discussion**

In this study of a cohort of 77 liver-transplanted recipients, we investigated the phenotypic and functional features of NK cells and their relation to the development of de novo cancers. Our results clearly show that NK cells from OLT with de novo tumors display an unusual phenotype and impaired functioning. These changes are particularly evident in patients with NCT, and they may have the potential to affect the disease outcome. This highlights the possibility that unique NK cell subsets, closely associated with maturation defects and uncommon patterns of cytokine production, are expanded in NCT and may lead to the development of de novo tumors. Flow cytometric characterization of NK cells enabled us to distinguish two groups among the NCT patients: $2C^-$ is mainly composed of $\text{NKG2A}^{\text{high}}\text{CD62L}^{\text{high}}\text{CD57}^{\text{low}}\text{NKG2C}^{\text{low}}\text{TNF-}\alpha^{\text{low}}\text{IFN-}\gamma^{\text{high}}$, with an immature signature, whereas $2C^+$ is characterized by nonconventional differentiated $\text{NKG2A}^{\text{low}}\text{CD62L}^{\text{low}}\text{CD57}^{\text{high}}\text{NKG2C}^{\text{+}}\text{TNF-}\alpha^{\text{high}}\text{IFN-}\gamma^{\text{low}}$ NK cells.
Our in-depth analysis reveals a striking link between the expansion of highly differentiated NK cells in 2C+ and CMV infection with all patients seropositive for CMV and a high proportion of CMV reactivation. Note that 60–90% of transplant recipients develop CMV infection due either to reactivation of a latent virus or to a new infection after long-term immunosuppressive treatments (37). CMV infection of tumor cells likely impairs NK cell function because CMV has evolved a unique strategy to circumvent its own virally mediated downregulation of MHC class I Ags to subvert NK cell recognition of infected cells (38). After intense debate and despite the difficulty of proving this experimentally, it is well accepted today that ∼20% of the global cancer burden is linked to infectious agents, including viruses, bacteria, and parasites (39). Although CMV has not yet been clearly implicated in human cancers, it has been detected in some malignant tumors, including colon cancer (40) and malignant glioblastoma with noncancer CMV-negative cells in close proximity to them (41). Recently, Baryawno et al. (42) showed significantly longer survival rates in medulloblastoma patients without CMV infection in the tumor than in those with high-grade infections. Interestingly, Di Cocco et al. (43) recently described a case of de novo gastric cancer after renal transplantation with a concomitant diagnosis of CMV disease.

Accumulating experimental evidence indicates that inflammatory cytokines, such as TNF-α, IFN-α, and GM-CSF, could be important in CMV-mediated tumors (44). In particular, TNF-α has been linked to increased replication of CMV (45). In vitro, TNF-α directly stimulates the CMV immediate-early promoter and can dramatically increase malignant transformations by triggering the NF-κB transcriptional activator, thus contributing to the immune failure seen in various malignancies (46). Our experimental evidence supports this possibility: intracellular cytokine production in NK cells differed quite notably between both OLT groups with de novo tumors, with NK cells of 2C+ expressing mainly TNF-α and those of 2C+ produced more IFN-γ. Popivanova et al. (47) showed that blocking TNF-α reduces carcinogenesis of this solid colorectal tumor, whereas IFN-γ levels, as mediated by NK cells, predict long-term survival in patients with gastrointestinal stromal tumors after treatment with imatinib mesylate (48).

Our study underlines the complexity of NK cell responses and their clinical impact after liver transplantation. Our data confirm in CMV-negative patients the persistence of immature NK cells. In contrast, in CMV+ patients, a specific expansion of differentiated NK cells specific to the virus, as previously observed (21–23, 27, 28), partially biases the NK repertoire by a restriction of its capacities of the immune surveillance, as shown by a decrease of their polyfunctional capacities. This could favor the development of the specific malignancies, including colorectal and head/neck tumors, observed in 2C+. In contrast, the presence of immature NK cells in 2C− leads to the development of de novo genitourinary tumors. Further investigations with larger numbers of patients are required to confirm these hypotheses; nonetheless, the findings that

![FIGURE 4. Intracellular cytokine production and expression of cytolytic markers in NK cells from 2C+ and 2C− NCT patients. (A) Box plot plots summarize intracellular production of IFN-γ and TNF-α among CD3−CD56+ NK cells from 2C− (n = 8) and 2C+ (n = 5) NCT compared with HD (n = 10) and OLT patients without tumors (CTL; n = 10). Intergroup comparisons were assessed with Kruskal–Wallis test and Dunn posttest. (B) Linear regression between NKG2C expression and intracellular production of IFN-γ or TNF-α. Open circles represent 2C− and closed circles 2C+ NCT. (C) Expression of cytolytic markers among CD3−CD56dim NK cells from 2C− (n = 10) and 2C+ (n = 6) NCT compared with HD (n = 12) and OLT patients without tumors (CTL; n = 12). Intergroup comparisons were assessed with Kruskal–Wallis test and Dunn posttest. *p < 0.05, **p < 0.001.](http://www.jimmunol.org/)

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FIGURE 5. Polyfunctionality of NK cells from NCT patients. Polyfunctionality assays of CD3⁺ CD56⁺ NK cells from 2C⁻ (median; n = 5), and 2C⁺ (median; n = 5) NCT compared with HD (median, n = 12). Cell-surface expression of CD107a and intracellular production of IFN-γ and TNF-α were assessed in absence of target cells (None) or in the presence of K562, 721.221 that did (221.AEH) or did not (721.221) express HLA-E, and P815 ± anti-NKG2C mAb (αNKG2C). FlowJo software and its Boolean gate function were used to determine whether cells expressed zero, one, two, or three functions simultaneously. Data represent the mean of frequency of five independent samples and are presented as pie charts representing the number of different responses and colored arcs the frequency of cells producing each cytokine, created with Pestle and Spice software.

several specific cancer forms are linked to a possible association between the NK cell maturation status and/or the patient’s CMV status could open up new possibilities for neutralizing development of certain NCT after OLT.

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Disclosures

The authors have no financial conflicts of interest.

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