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Decreased NKp46 and NKG2D and elevated PD-1 are associated with altered NK-cell function in pediatric transplant patients with PTLD

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Abstract

Post-transplantation lymphoproliferative disorders (PTLD) are life-threatening complications of organ transplantation caused by EBV infection and the use of chronic immunosuppression. While T cell impairment is known to play a critical role in the immunopathogenesis of EBV complications post-transplantation, the role of NK cells is still under investigation. Here we have characterized NK cell phenotype and function in peripheral blood from asymptomatic pediatric thoracic transplant patients, patients with PTLD, and healthy controls. Overall, asymptomatic pediatric Tx patients presented significant expansion of the $CD56^{bright}CD16^{\pm}$ subset and displayed effective NK cell function, while PTLD patients accumulated $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$ NK cell subsets. In addition, NK cells from PTLD patients down-regulated NKp46 and NKG2D, and significantly up-regulated PD-1. These phenotypic changes were associated with NK functional impairment, resembling cellular exhaustion. Disrupting PD-1 inhibitory pathway improved IFN-γ release, but didn't enhance cytotoxicity in PTLD patients, suggesting that these defects were partially PD-1 independent. Our results indicate the important role of NK cells during EBV surveillance post-transplantation, with implications for the immunopathogenesis of EBV complications, and suggest that monitoring NK cells in transplant patients may hold clinical value.

Keywords

pediatric; thoracic organ transplantation; EBV; PTLD; NK cells

Introduction

Epstein-Barr virus (EBV) is a gamma herpes virus that latently infects > 90% of the world adult population [1]. While EBV has significant growth transforming potential of B lymphocytes and epithelial cells, effective anti-viral T cells maintain EBV infection latent in immunocompetent individuals [2]. However, immunocompromised patients, such as solid

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organ transplant (Tx) recipients, often develop EBV-associated post-transplant lymphoproliferative disorders (PTLD), since chronic administration of immunosuppressive (IS) drugs to prevent graft rejection impairs anti-viral T cell immune-surveillance [1, 3]. Clinical monitoring of EBV load in peripheral blood of pediatric Tx patients who EBV sero-converted after transplantation has identified three groups of clinically asymptomatic children: approximately 30% that exhibited undetectable (<100 copies/ml) EBV loads (UVL), resembling normal EBV latency; 50% that displayed persistent low (100-16,000 copies/ml) EBV loads (LVL); and 20% that showed persistent, high (>16,000 copies/ml) EBV loads (HVL) in peripheral blood for months to years after primary post-Tx EBV infection [4]. These findings are indicative of an EBV latency switch to chronic productive infection in these two latter cohorts of pediatric Tx patients. We have further shown that chronic HVL carrier state is an independent and strong (45%) predictor of '*de novo*' or '*recurrent*' late onset PTLD, frequently with aggressive histology [5].

As a part of innate immunity, natural killer (NK) cells are critical in protecting hosts during the early response to viral infections or tumor growth [6, 7]. NK cells have been defined based on the level of CD56 and CD16 expression in the absence of CD3, and constitute approximately 5–15% of peripheral blood mononuclear cells [8]. In healthy individuals, two subsets of circulating NK cells have been identified: approximately 90% NK cells express $CD56^{dim}CD16^{+}$, and display cytolytic activity against susceptible targets, while 10% of NK cells express $CD56^{bright}CD16^{\pm}$, that have immunoregulatory properties, as they readily produce large amounts of cytokines, including IFN-γ [8-10]. In secondary lymphoid organs, the distribution of these two major NK subsets was found to be reversed, reflecting the distinct functional requirements of these subsets at different sites of infection [11, 12]. The complexity of NK cell function is modulated by a myriad of activating and inhibitory receptors expressed on cell surfaces [13, 14]. The major classes of triggering NK cell receptors include natural cytotoxicity receptors (NCR) and the c-type lectin receptor NKG2D.

While the importance of NK cells in the control of primary EBV infection during early immune responses in healthy individuals has been documented [15, 16], the role of NK cell surveillance during EBV latency or during chronic EBV infection after organ Tx and under IS still remains to be elucidated. Here we aimed to characterize the phenotype and function of NK cells from asymptomatic pediatric thoracic Tx patients that carry an EBV load (LVL and HVL carriers) or symptomatic patients diagnosed with PTLD, and to compare these to NK cells from healthy controls (HC) or asymptomatic pediatric Tx patients with undetectable loads (UVL carriers) that carry EBV infection in its latent form. Our results demonstrate that while UVL and LVL asymptomatic Tx patients exhibit NK cell phenotype and function comparable to HC, patients with PTLD display critical changes in NK cell phenotype paralleled by impaired function and accumulation of unusual NK cell subsets. In addition, NK cells from asymptomatic HVL patients who are at higher risk of EBV complications, demonstrated similar phenotypic trends as PTLD patients in addition to a selective decrease in cytotoxicity.

Results

NK cell subsets distribution is altered in asymptomatic pediatric Tx patients and in patients with PTLD

NK cell subset characterization was performed on peripheral blood $CD3^{-}CD19^{-}$ cells, out of the lymphocyte gate, as shown in Figure 1A. NK cells were defined based on CD56 and CD16 expression, and four subsets were further identified as follows: $CD56^{bright}CD16^{\pm}$, $CD56^{dim}CD16^{+}$, $CD56^{dim}CD16^{-}$, and $CD56^{-}CD16^{+}$ populations (Figure 1A). While the overall frequencies (%) of all NK cells were not different among groups (data not shown),

the analysis of NK cell subsets revealed that pediatric thoracic Tx patients (including patients with PTLD) displayed significantly lower levels of the $CD56^{dim}CD16^{+}$ NK subset (mean± SD: UVL: 52±20%; LVL: 55±14%; HVL: 55±15%; PTLD: 34±26%), a subset previously described to be the most abundant NK cell subset in peripheral blood of HC (77±4%) (Figure 1B). In addition, asymptomatic pediatric thoracic Tx patients displayed a trend of higher percentages of circulating $CD56^{bright}CD16^{\pm}$ NK cells (UVL: 25±20%; LVL: 22±13%) as compared with HC (6±3%) (Figure 1C). Conversely, PTLD patients displayed increase in peripheral blood $CD56^{dim}CD16^{-}$ subset (PTLD: 43±7% versus HC: 10±6%) and $CD56^{-}CD16^{+}$ NK subset (PTLD: 19±20%; HC: 7±2%) (Figure 1D, E).

NK cells from patients with PTLD down-modulate NKp46 and NKG2D

We next investigated the levels of triggering receptor expression on NK cells. Previous reports have documented that the activating receptors are expressed at highest levels on $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{+}$ NK subsets in healthy subjects [8]. Our results show significant down-modulation of NKp46 expression on total NK cells from PTLD patients (mean±SD= 42±23%) as compared with those from asymptomatic pediatric Tx patients (UVL: 70±24%; LVL: 84±13%) or HC (85±5%) (Figure 2A). Similar decrease of NKp46 expression was detected on all four NK cell subsets, including the $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{+}$ (Figure 2B, C). Similar to NKp46, the NKG2D expression was also significantly decreased on all NK cells from PTLD patients (4±4%) as compared with NK cells from asymptomatic Tx patients (LVL: 21±12%) or HC (22±5%) (Figure 2D). Similar findings were also observed on $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{+}$ NK cell subsets (Figure 2E, F) as well as on the unusual $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$ subsets (data not shown). Interestingly, chronic HVL carriers displayed trends of diminished triggering receptor (NKp46: 60±27% and NKG2D: 15±4%) expression on all circulating NK cells and NK subsets, similar to PTLD patients (Figure 2A-F).

NK cells from patients with PTLD up-regulate PD-1

PD-1 has been implicated in the negative regulation of T lymphocyte function during chronic viral infections [17]. Therefore, we next analyzed whether PD-1 expression was detectable on NK cells from Tx patients. Our results demonstrate a significant up-regulation of PD-1 expression on all NK cells from patients with PTLD (36±24%), as compared with those from asymptomatic pediatric Tx patients (UVL: 16±3%; LVL: 15±5%) or HC (14±6%) resembling “exhausted” T cell phenotypes (Figure 3A). PD-1 up-regulation was also detected on $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{+}$ NK cell subsets from PTLD patients (Figure 3B, C) as well as on the unusual $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$ subsets (data not shown). In addition, a trend of PD-1 up-regulation on NK cells was noted in chronic HVL carriers (22±13%) (Figure 3A, B).

NK cells from asymptomatic Tx patients display memory-like responses to EBV-antigens, while NK cells from PTLD patients exhibit functional exhaustion

We next analyzed the ability of $CD56^{bright}CD16^{\pm}$ NK cells to respond by IFN-γ production and of $CD56^{dim}CD16^{+}$ NK cells to up-regulate CD107a (as a measurement of active granule (perforin) exocytosis and NK cytotoxic potential) [18] to non-specific stimulation (pro-inflammatory Type-1 promoting cytokines), or to EBV-antigen specific stimulation with autologous lymphoblastoid cell lines (LCL). In particular, hrIL-12p70+hrIL-18 stimulation triggered strong IFN-γ responses from $CD56^{bright}CD16^{\pm}$ NK cells from asymptomatic Tx patients (UVL: 30±14%, LVL: 33±16%; HVL: 25±15%) and HC (32±10%) (Figure 4A). EBV-antigen specific stimulation with LCL triggered lower levels of IFN-γ release as compared with the non-specific stimulation, but still most effective with $CD56^{bright}CD16^{\pm}$ NK cells from HC (6±4%) and LVL (6±3%) patients (Figure 4B). Surprisingly, although NK cells from UVL patients showed IFN-γ responses to hrIL-12p70+hrIL-18 stimulation

comparable to those from HC or to asymptomatic patients that carry an EBV load (LVL and HVL) (Figure 4A), they displayed lower IFN-γ (UVL: 3±3%) responses following EBV-antigen specific LCL (Figure 4B). In contrast, PTLD patients showed impaired IFN-γ production by $CD56^{bright}CD16^{\pm}$ cells to non-specific (13±12%) as compared with UVL, LVL, and HC or to EBV-specific stimulation (2±3%) as compared with LVL and HC (Figure 4A,B) suggesting their profound functional alteration.

Furthermore, while the CD107a response was not significantly modulated by hrIL-12p70+hrIL-18 cytokine treatment (Figure 4C), it was significantly boosted by EBV-LCL stimulation resulting in $CD107a^{+}$ $CD56^{dim}CD16^{+}$ NK cells from HC (4±2%) and LVL (3±3%) patients (Figure 4D). Similar to the IFN-γ response, the CD107a response to EBV-LCL stimulation was decreased in UVL patients (1±2%) as compared with that of HC and LVL carriers (Figure 4D). Conversely, both PTLD (1±1%) and HVL (1±1%) patients presented with significantly decreased $CD107a^{+}$ $CD56^{dim}CD16^{+}$ NK cells in response to LCL trigger (Figure 4D). In addition, CD107a release seems to be affected before the IFN-γ secretion in recipients with chronic high EBV challenge (HVL and PTLD patients) (Figure 4A-D).

Disrupting PD-1 pathway partially restores NK cell function in PTLD patients

Since phenotypic analysis of NK cells (including $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{+}$ NK cell subsets) from PTLD patients has identified PD-1 up-regulation (Figure 3), we next investigated whether disrupting PD-1 receptor binding during NK cell stimulation may result in NK cell function restoration in this cohort. To test the mechanism of PD-1 regulation, we incubated NK cells with autologous LCL in the presence or absence of PD-1 blocking mAb (or isotype control). This treatment restored the IFN-γ response by $CD56^{bright}CD16^{\pm}$ (Figure 5A) NK cells, while CD107a release by $CD56^{dim}CD16^{+}$ (Figure 5B) was only partially increased in PTLD patients. Interestingly, similar experiments performed on NK cells from LVL patients, who displayed low levels of PD-1 expression but maintained high NKp46 and NKG2D expression, have showed that blocking PD-1 resulted in increased IFN-γ and CD107a expression (Figure 5A, B).

Discussion

NK cells, as part of innate immunity, play an important role in the initial immunologic defense against viral infections [6, 7]. However, the role of NK cell surveillance during EBV latency, or chronic EBV infection with increased viral loads after Tx, or during PTLD remains elusive. Overall our results show that NK cell phenotype and function are profoundly impaired in pediatric Tx PTLD patients (with a similar trend for chronic HVL carriers), indicating a possible NK cell contribution to the immunopathogenesis of EBV complications in the Tx setting.

Here, we have identified for the first time significant differences in NK cell subset distribution between EBV seropositive HC and pediatric Tx patients carrying, or not, an EBV load. On one hand the $CD56^{bright}CD16^{\pm}$ subset was increased in asymptomatic Tx patients, suggesting possible differences in the NK functional (IFN-γ) requirements in pediatric Tx recipients versus HC. In contrast, PTLD patients showed decreased $CD56^{bright}CD16^{\pm}$ and $CD56^{dim}CD16^{+}$ subset levels with an accumulation of $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$ NK subsets. These changes in the NK cell subset levels may be a consequence of high EBV challenge of NK cells seen with PTLD patients, leading to the possible CD56 receptor down-modulation on the conventional “functional” NK cell subsets. Interestingly, recent studies have also described unusual accumulation of circulating dysfunctional $CD56^{dim}CD16^{-}$ and $CD56^{-}$ $CD16^{+}$ NK cell subsets in patients with

complications of chronic HIV and HCV infections, indicating a direct correlation between NK cell subset defective function and chronic viral uncontrolled challenge [19-21].

Early protection against EBV replication and against proliferation of EBV-infected targets was shown to rely on NK cell ability to release IFN-γ and to mediate cytotoxicity in response to cytokine milieu instructions and to triggering receptor ligation by molecules on EBV-infected target cells [15, 16]. Here we have identified that NK cells from HC and from UVL pediatric Tx patients carrying latent EBV infection, or from patients with moderate EBV reactivation (LVL) mount effective IFN-γ by $CD56^{bright}CD16^{\pm}$ and CD107a responses by $CD56^{dim}CD16^{+}$, respectively. Interestingly, our data further revealed that functional responses to non-specific pro-inflammatory cytokine stimulation were comparable among HC and asymptomatic Tx patients. Conversely, EBV-specific stimulation resulted in different levels of IFN-γ and CD107a responses in these same cohorts, indicating a role of recall EBV-antigen stimulation in shaping anti-viral NK cell function independently of Type-1 promoting cytokine stimulation. Indeed, recent reports have demonstrated that although still acknowledged as members of innate immunity, NK cells also possess nearly all the features of adaptive T cell immunity [22, 23]. Using a MCMV model of viral infection, long-lived MCMV-specific memory NK cells displayed enhanced capacity to produce IFN-γ and degranulate upon re-encounter with murine CMV, as compared with resting NK cells from naïve mice [22, 23]. The Ly49H receptor was responsible for this NK cell MCMV-cognate recognition, and appeared not to recognize other viral antigens [22]. Future work is therefore needed to elucidate whether viral (including EBV) recognition by human NK cells is mediated by a single common receptor or by multiple viral antigen-specific receptors. Our results have further identified significant and broad (IFN-γ and CD107a) functional impairment of NK cells from PTLD patients both in response to non-specific and to EBV antigen–specific stimulation. NK cells from asymptomatic HVL carriers displayed similar trends, suggesting a progressive loss of NK cell functions (exhaustion) in these patients that parallels the increased EBV-antigenic load, and with cytotyoxicity being affected early. These NK cell functional data resemble the functional features of exhausted viral-specific $CD8^{+}$ T cells identified during chronic high viral load infections, with IFN-γ being the last function maintained by Ag-specific T cells [24].

Furthermore, our results identified the decreased expression of NKp46 and NKG2D and concomitant up-regulation of PD-1 on NK cells from EBV viremic PTLD patients as potential regulatory mechanisms responsible for the NK cell functional abnormalities. The decreased expression of activating NCRs was previously described in chronic viremic (HIV- and HCV-) patients, and was shown to lead to significant NK cell functional impairment of cytolytic activity and IFN-γ release [25, 26]. In another study, down-regulation of NKG2D activation pathways provided Kaposi's sarcoma-associated herpesvirus with a mechanism for evasion of NK cell efficient viral clearance [27]. The mechanisms leading to decreased NK cell triggering receptors on NK cells from viremic patients are not entirely clear. Anti-inflammatory cytokines associated with chronic viral infection/tumors such as TGF-β or IL-10 were incriminated for triggering receptor down-modulation on NK cells in AIDS patients [28, 29]. Alternatively, up-regulation of ligands for triggering receptors on virally-transformed targets, as well as chronic antigenic pressure, may play a role in rendering altered NK phenotypes. While the ligands for NKp46 are not well defined, two structurally distinct families of molecules, MICA/B and ULBP (UL16-binding proteins) have been identified as ligands for NKG2D and shown to play a role in NKG2D down-modulation [30, 31].

Prior reports have demonstrated a critical role for PD-1 expression in rendering $CD8^{+}$ T cells exhausted during chronic viral infections, such as HCV, HBV and HIV [32-34]. The

role of PD-1 expression on NK cells from HCV-viremic patients has been recently identified, but no mechanistic studies were performed to clarify the specific PD-1 functional significance in this model of viral infection [35]. Our results from *in vitro* PD-1 blocking experiments during EBV-antigen stimulation with LCL have demonstrated only partial (IFN-γ) NK cell functional restoration in PTLD patients. Disrupting PD-1 recognition on NK cells from PTLD patients which have concomitantly decreased NKp46 and NKG2D expression indicate a potential complex regulatory mechanism of cross-talk between PD-1 and NCR in this setting. Indeed, we have found that LCL (which are the *in vitro* correspondent of the *in vivo* EBV-transformed B cells) co-express PD-L1/PD-L2 (as the ligands for PD-1), and MICA/B and ULBP1 (as NKG2D ligands) (Supporting Information Figure 1). Alternatively, restoration of IFN-γ release, but not of CD107a, by NK cells from PTLD patients suggests that cytotoxicity and IFN-γ may be differently regulated in this setting, and future studies are required to dissect these regulatory mechanisms. Moreover, blocking PD-1 experiments during EBV-antigen stimulation of NK cells from LVL patients revealed a significant up-regulation of IFN-γ secretion and CD107a release, and suggests that the presence of preserved NKp46 and NKG2D receptor expression is essential for NK activation.

In summary, our results indicate that while HC and asymptomatic pediatric Tx patients that control well EBV infection (UVL and LVL carriers) mount effective non-specific and memory-like EBV-specific NK cell responses, patients with PTLD display functionally exhausted EBV-specific NK cell responses, regulated by a complex cross-talk between triggering receptors and the inhibitory PD-1 receptor, with possible implications for EBV disease immunopathogenesis. Future prospective multicenter studies focusing on PTLD patients before and after disease onset are needed for additional insights into the pathogenesis of PTLD in solid organ transplant recipients, and to allow in depth potential correlations between these parameters and the development of PTLD. Of note, asymptomatic HVL carriers have NK phenotype and functional characteristics that more closely resemble PTLD patients. This observation carries clinical importance given the high rate of progression of HVL state to late PTLD development [5], and suggests a potential role for NK cell immune monitoring after Tx.

Materials and Methods

Human subjects and PBMC isolation

Thirty-seven clinically asymptomatic pediatric thoracic Tx recipients with no signs of allograft rejection or EBV infectious complications at the time of blood donation (Table 1) and 6 patients with biopsy confirmed PTLD (Table 2) were consented to this cross-sectional study under IRB-approved protocols at Children's Hospital of Pittsburgh of UPMC. In addition 14 healthy controls were also recruited to the study (Table 1). Blood samples were collected between January 2008 and April 2009. Asymptomatic pediatric Tx patients were divided into three groups according to their peripheral blood EBV loads as: UVL carriers (n=12), LVL patients (n=10) and HVL patients (n=9) (see definition of EBV load below). PTLD (n=6) patients displayed HVL in their peripheral blood at that time of analysis with one exception of a patient who displayed LVL. Immunosuppressive regimens of asymptomatic pediatric thoracic Tx recipients or of patients with PTLD at the time of diagnosis consisted of a calcineurin inhibitor (tacrolimus or microemulsion cyclosporine), variable usage of anti-proliferative agents (mycophenolate mofetil or sirolimus) with or without corticosteroids (Table 1, 2). In addition, 12 asymptomatic and 4 symptomatic (PTLD) patients received induction therapy with polyclonal anti-thymocyte immunoglobulins (Thymoglobulin® or ATGAM®) 0.5 or more years prior study sampling. For PTLD patients, decreased/discontinued immunosuppression and PTLD treatments were initiated only after the biopsy confirmed diagnosis and after blood sampling (Table 2). All

patients and healthy subjects were EBV-positive at the time of the study (Table 1, 2), as determined by serology (Clinical Immunopathology, Central Laboratory Services Inc., UPMC). Heparinized whole blood was collected from each subject, according to their age and body mass, as stipulated by the IRB guidelines. The sample was used to isolate peripheral blood mononuclear cells (PBMC) by Ficoll-Hypaque density gradient centrifugation, as previously described [36]. Aliquots of whole blood were used for flow cytometric analysis in Figure 1, while purified PBMC were frozen and banked for subsequent phenotypic and analyses.

Definition of EBV load

EBV load was determined as previously described [37]. UVL pediatric thoracic Tx patients had no EBV load detected by PCR (<100 EBV genomic copies/ml whole blood) in more than 80% of determinations including the time of analysis; LVL carriers had EBV loads ranging between 100-16,000 EBV genomic copies/ml whole blood, detected in more than 20% of measurements, including the time of analysis; and HVL carriers had EBV loads above 16,000 EBV genomic copies/ml whole blood, on at least 50% of determinations, and over a period of at least 6 months prior to the current immunologic analysis.

Media, reagents and cell lines

RPMI-1640 (Cellgro, Manassas, VA) supplemented with 2mM L-glutamine, 10mM HEPES, 100IU/ml penicillin/streptomycin and 10% heat inactivated fetal calf serum (FCS) (all from Gibco BRL, Grand Island, NJ) was used as complete media. For *in vitro* NK cell co-cultures, FCS was replaced by 5% normal human AB serum (NHS) (Nabi, Boca Raton, FL). Recombinant human (rh)IL-12p70 and rhIL-18 were purchased from R&D Systems Inc. (Minneapolis, MN) and from *Bender MedSystems* Inc. (Burlingame, CA), respectively. Ficoll-Paque™ was obtained from Amersham Biosciences AB (Uppsala, Sweden). Monensin and Brefeldin A were purchased from eBioscience (San Diego, CA) and Sigma (St.Louis, MO), respectively. Autologous LCL were generated in our laboratory as previously described and were used as EBV^+ stimulators in functional assays [38].

Phenotypic analyses by flow cytometry

NK cell phenotype was determined by seven color flow cytometric analysis as previously described [8]. Briefly, 100μl whole blood or 0.1×10^6 PBMC aliquots were incubated for 30 min at romm temperature or 4°C, respectively, in the dark with different combinations of fluorochrome-conjugated mAbs such as anti-CD3, anti-CD19, anti-CD56, anti-CD16, anti-NKG2D and anti-PD-1 (all from e-Bioscience), anti-NKp46 (Miltenyi Biotech GmbH, Auburn CA). Stained aliquots from whole blood were further incubated for 10 min at room temperature with 2ml/tube of lysing buffer (BD Bioscience) to allow red blood cell lysis. All tubes were then washed twice with FACS buffer (PBS supplemented with 1% FCS, and 0.05% NaN_3) and fixed with 2% paraformaldehyde-containing FACS buffer (Sigma). Appropriate isotype negative controls were always used to define background staining. Data acquisition was performed using a LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland OR).

NK cell co-cultures and functional assays

Thawed PBMC (1×10^6 cells/ml) were plated in 48 well plates (Costar Corning Inc, Corning NY) in the presence of i) hrIL-12p70 (10 ng/ml) + hrIL-18 (20 ng/ml) or ii) autologous LCL (at 5:1 NK:LCL ratio) for 18 hr at 37° C, 5% CO_2. PBMCs cultured in media alone were used as negative controls. In selected experiments, neutralizing antibodies against PD-1 (R&D Systems) were added at 20μg/ml at co-culture initiation. NK cell degranulation upon activation, as a direct measurement of cytotoxicity, was assessed by CD107a staining, as

previously described [39]. Briefly, anti-CD107a mAb (eBioscience) was added at co-culture initiation. During the last 4 hr of co-culture, monensin (2μM) and brefeldin A (15μg/ml) were added to each condition according to manufacturer instructions. Cells were then harvested, washed, surface stained and fixed as described above. NK cell intracellular cytokine staining was detected simultaneously by further cell permeabilization with 2% saponin (Sigma) and intracellular staining with anti-IFN-γ mAb (eBioscience). Appropriate isotype negative controls were always used to define background staining.

Statistical analysis

The two-tailed Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison post-test were applied to determine overall differences between groups using GraphPad Prism software (La Jolla CA). Results were considered statistically significant if p-value was < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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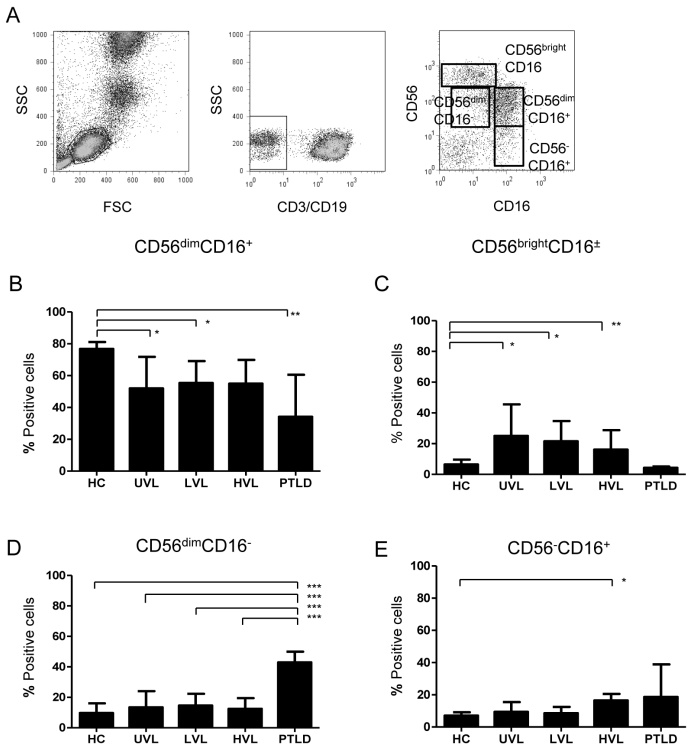

Figure 1. Identification of NK cells and NK-cell subset distribution in peripheral blood

(A) Detection of NK cells was done by flow cytometric analysis on lymphocyte gate in whole blood. NK cells were identified among $CD3^{-}/CD19^{-}$ lymphocytes and were defined by CD56 and CD16 expression. Four NK cell subsets were identified. Data are shown for one representative experiment performed on a sample obtained from a LVL Tx patient. The frequency (%) of (B) $CD56^{dim}CD16^{+}$, (C) $CD56^{bright}CD16^{\pm}$, (D) $CD56^{dim}CD16^{-}$ and (E) $CD56^{-}CD16^{+}$ NK-cell subsets was shown as mean ± SD for each group. Results represent experiments obtained from 6 UVL, 8 LVL, 7 HVL asymptomatic Tx patients, 2 patients with PTLD and 8 HC. *p<0.05, ** p<0.01, ***p<0.001, as determined by ANOVA with Tukey's multiple comparison post-test in (B, D, and E) and by two tailed Student's *t*-test in (C).

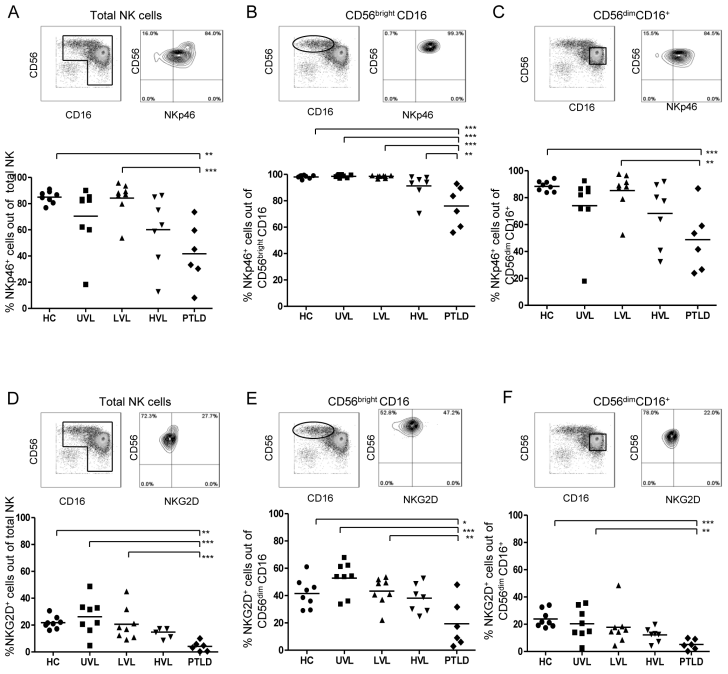

Figure 2. Expression of triggering NKp46 and NKG2D receptors of NK cells in peripheral blood

The gating strategy and representative (A-C, top) NKp46 and (D-F, top) NKG2D staining from one LVL patient are displayed in flow cytometry plots. Results indicating the % of NKp46 and of NKG2D expression on (A, D) total NK cells, (B, E) $CD56^{bright}CD16^{\pm}$, and (C, F) $CD56^{dim}CD16^{+}$ NK cells were obtained from 8 UVL, 8 LVL, 7 HVL asymptomatic Tx patients, 6 patients with PTLD and 8 HC. Each symbol in every column represents a subject, while the horizontal lines stand for the mean values. *$p<0.05$, ** $p<0.01$, ***$p<0.001$, as determined by ANOVA with Tukey's multiple comparison post-test.

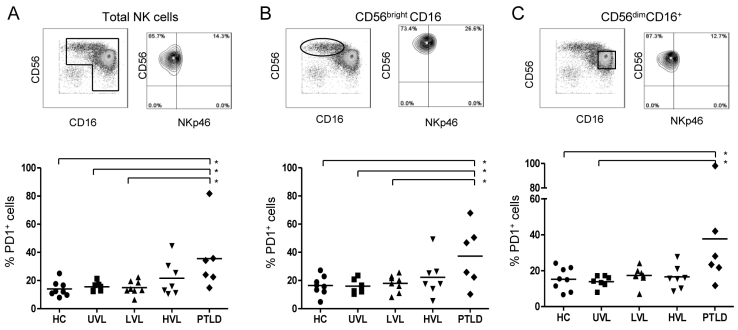

Figure 3. Expression of PD-1 of NK cells in peripheral blood

(A-C, top) The gating strategy and a representative PD-1 staining from one LVL patient are displayed. Quantification of PD-1 expression (%) on (A) total NK cells, (B) $CD56^{bright}CD16^{\pm}$ and (C) $CD56^{dim}CD16^{+}$ NK cells was performed from asymptomatic UVL (n=7), LVL (n=8), HVL (n=7) Tx patients, patients with PTLD (n=6) and from HC (n=8). Each symbol in every column represents a single subject, while the horizontal lines stand for the mean values. *$p<0.05$ as determined by ANOVA with Tukey's multiple comparison post-test.

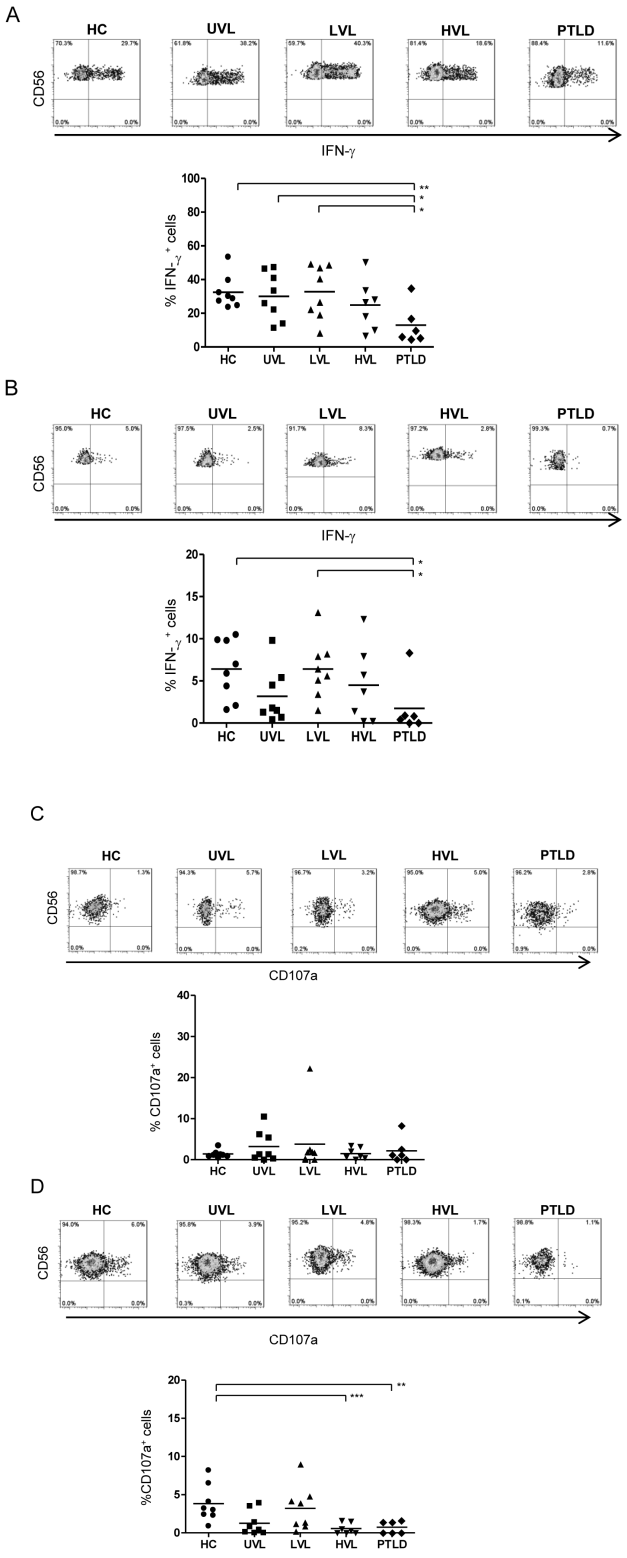

Figure 4. NK-cell function in response to non-specific (hrIL12+hrIL18) and EBV-specific (LCL) stimulation

NK cells were subjected to in vitro (A, C) hrIL-12 + hrIL-18 stimulation, or to (B, D) EBV-specific (LCL) stimulation. Data shown have had background values (cells stimulated by media alone) subtracted. (A-D, top) Dot plots from one representative patient in each cohort and one HC are shown. Results were generated from 8 UVL, 8 LVL, 7 HVL asymptomatic Tx patients, 6 patients with PTLD and 8 HC for (A, B) intracellular IFN-γ production by $CD56^{bright}CD16^{\pm}$ NK cells, and (C, D) CD107a release by $CD56^{dim}CD16^{+}$ NK cells. Each symbol in every column represents a single subject, while the horizontal lines in each graph indicate mean values. *$p<0.05$, ** $p<0.01$, ***$p<0.001$ as determined by two tailed Student's *t*-test in (A, B) and by ANOVA with Tukey's multiple comparison post-test in (D).

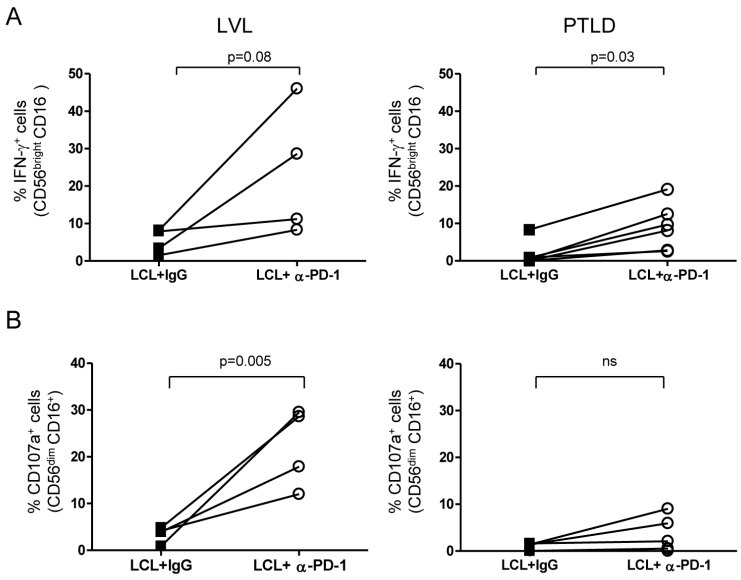

Figure 5. The impact of PD-1 blockade on NK-cell function

Flow cytometric staining for (A) intracellular IFN-γ production by $CD56^{bright}CD16^{\pm}$ NK cells, and (B) CD107a release by $CD56^{dim}CD16^{+}$ NK cells in response to in vitro EBV-specific (LCL) stimulation in the presence of IgG control (LCL+IgG; black squares) or of anti-PD-1 (LCL+α-PD-1; open circles) are shown for LVL (n=4) Tx recipients and for PTLD (n=5) patients. Statistical significance was determined by the Student's *t*-test.

Table. 1

Demographics of asymptomatic pediatric Tx patients

Subjects	Type of Tx	Gender	Induction Therapy	Mean Yrs ± SD at Tx	Mean Yrs ± SD at blood draw	EBV Status pre-Tx	EBV load range post-Tx
UVL (n=8)	7H 1 H/L	2F/6M	0%	5.5 ± 6.0	10.3 ± 5.2	negative=5 positive=3	ND
LVL (n=8)	8H 1H/L	1F/7M	37.50%	6.7 ± 5.5	11.9 ± 4.0	negative=6 positive=2	100-6,700
HVL (n=8)	4H 3L 1H/L	4F/4M	62.50%	5.3 ± 4.4	10.1 ± 5.8	negative=7 positive=1	21,000-110,000
Healthy Controls (N=10)	N/A	8F/2M	N/A	N/A	37 ± 10	N/A	ND

UVL = undetectable EBV load (< 100 EBV genomic copies/ml whole blood); LVL = low EBV load (100-16,000 EBV genomic copies/ml whole blood); HVL = high EBV load (>16,000 EBV genomic copies/ml whole blood); N/A = non applicable , ND= non detectable H=heart ; L=lung

Table 2

Demographics of symptomatic pediatric Tx patients with PTLD

Patients*	Type of Tx	Gender	Age at Tx (years)	Age at PTLD (years)	EBV-status pre-Tx	EBV-status post-Tx	Site and type of PTLD	EBV load at PTLD
CHP-0162	Heart	M	0.8	7.2	Negative	HVL	Intestine - Polymorphous	250,000
CHP-1021	Heart (2x)	F	5	21.4	Negative	HVL	Intestine - Polymorphous	140,000
CHP-0243	Heart	M	1.7	2.2	Positive	LVL	Stomach - Polymorphous	35,000
CHP-1043**	Lung	F	15.5	17.2	Negative	NA	Lung - Polymorphous	1,300
CHP-0085	Heart	F	0.3	10.4	Negative	HVL	Lung - Polymorphous	160,000
CHP-1039	Lung	M	6.7	10	Negative	HVL	Lung - Polymorphous	33,000

* Patients were on CNI-based IS with variable use of anti-proliferative agents and/or steroids prior to diagnosis with the exception of patient CHP0-0243 who was off immunosuppression at time of sampling. All samples were taking prior any treatment of PTLD.

** Patient received a kidney Tx 4 months after lung Tx.