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POST-TRANSPLANT NIVOLUMAB PLUS UNSELECTED AUTOLOGOUS LYMPHOCYTES IN REFRACTORY HODGKIN LYMPHOMA PATIENTS: A SAFE AND EFFECTIVE THERAPY ASSOCIATED WITH EXPANSION AND MATURATION OF NK CELLS

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Abstract

Hodgkin Lymphoma (HL) is a B-Cell neoplasia with a favourable outcome in responding patients. However, despite the efficacy of first line therapy about 30% of patients eventually relapse or are refractory (R/R). Recently, the immune checkpoint inhibitor (CI) nivolumab demonstrated good activity in R/R HL patients although the complete response (CR) rate was less than 20%. The efficacy of nivolumab is strictly related to the host degree of immune competence, which is greatly impaired in heavily pre-treated HL patients after autologous stem cell transplantation (ASCT). To enhance the activity of CI, we explored the feasibility of the infusion of autologous lymphocytes (ALI) in combination with the pre-emptive administration of nivolumab, early post-ASCT, in patients affected by R/R HL. Eight patients (median age 29, range 18-56) with active R/R disease, who had already failed at least two chemotherapy lines and Brentuximab, were eligible for the trial. HL patients underwent early lymphocyte apheresis, with a target cell dose of $5x10^7$ CD3+/kg. All patients then received ASCT with FEAM conditioning followed by ALI at a median time of 14 days after infusion, starting with $1x10^4$ CD3+ cells/kg in the first infusion to a maximum of $1x10^7$cells/kg in the fourth and last infusion. Each ALI was followed after 48 hours by the administration of nivolumab 240 mg flat dose. As a control cohort, two patients, in CR after second line chemotherapy, were given ALI only, without nivolumab.

No grade 3 or 4 adverse events were recorded. All treated patients achieved negative PET scan after immunotherapy and are alive and disease-free after a median follow-up of 20 months. Two patients did receive allogeneic stem cell transplantation while in CR. Notably, compared to control patients, a faster expansion/reconstitution of highly differentiated NK cells was observed as well as a quicker T-cell recovery. These data suggest the potential role of PD-1 receptor in the direct or indirect control of NK cell maturation/development and, probably, NK

anti-tumor activity. Thus, the combination of adoptive cell therapy with CI may represent a novel approach for chemorefractory HL patients.

Introduction

Hodgkin lymphoma (HL) is a lymphoid malignancy of B-cell origin with a high cure rate.[1] However, despite the efficacy of frontline therapy, about 30% of patient are refractory or relapse.[2] In this subset, standard salvage treatment includes high-dose chemotherapy followed by autologous stem cell transplantation (ASCT).[3] However, in order to be effective, ASCT should be performed in chemoresponsive patients.[3-5] Unfortunately, 30-50% of patients treated with salvage chemotherapy fail to achieve at least PR and Brentuximab-Vedotin (BV, an anti-CD30 immuno-conjugated antibody) is able to induce a response in only 30-50% of cases.[6,7] Indeed, HL patients not achieving at least a partial response after second or third line chemotherapy have a poor prognosis.[8]

Recently, the clinical application of immune checkpoint inhibitors (CI), in particular the PD-1 targeting antibody nivolumab, has dramatically improved the prognosis of patients with advanced phase solid tumors.[9] Nivolumab has also shown promising results in HL patients relapsing after ASCT and is currently approved for this setting. Unfortunately, the expected CR rate is only about 20% and median PFS is about 18 months, with no evidence of plateau.[7,8,10,11] Earlier administration of PD-1 targeting antibodies as post-ASCT consolidation may be more effective in selected patients, as observed with post-ASCT BV therapy.[12] Moreover, several reports have highlighted the importance of patient immune-competence to achieve durable response with anti-PD-1 immunotherapy.[13-15] In this view, ASCT, especially in heavily pre-treated patients, leads to a prolonged and deep immunosuppression.[13]

In solid oncology, re-infusion of previously criopreserved unselected lymphocytes has been reported to boost T-cell count during radio and chemotherapy.[16] To the best of our knowledge, such strategy has not been described for patients with hematological malignancies undergoing ASCT.

Several lymphocyte populations have been shown to play a role in the complex

immunological mechanism of checkpoint inhibitors therapy.[17]

Notably Reed Sternberg cells display a low/absent expression of HLA I-II complex thus excluding a major contribute of T lymphocyte subpopulations in response to PD-1/PDL-1 blockade in HL setting.[18] In cancer immunotherapy, Natural killer (NK) lymphocytes represent important innate effector cells able to kill tumor cell targets not expressing HLA class I molecules.[19]

The aim of this small study was to assess the feasibility of early post ASCT CI therapy supported by unselected autologous lymphocytes infusions (ALI) and to evaluate post-ASCT immunoreconstitution with specific reference to NK cells. Our results show a very good clinical response rate associated with the fast expansion of highly differentiated NK cells after ASCT, suggesting that PD-1 receptor may have a role in accelerating NK cell maturation and, probably, in their anti-tumor activity. The combination of adoptive cell therapy with pre-emptive CI may therefore represent a promising novel approach for R/R HL patients.

Methods

Study endpoints

Primary endpoint of this study was to investigate the feasibility and toxicity of post-ASCT nivolumab immunotherapy with the support of autologous lymphocyte reinfusions (ALI) in R/R HL patients. Secondary clinical endpoint was the assessment of the efficacy of the strategy.

A supplementary biological endpoint was the immunophenotypic evaluation of peripheral blood lymphocyte subpopulations, performed pre- and post-adoptive immunotherapy and CI administration, with particular attention to NK cell characterization.

Selection of patients, study population and treatment

High risk HL patients (i.e. patients resistant to first line ABVD or showing early relapse) underwent steady-state unselected lymphocyte apheresis, with a target cell dose of $5x10^8$ CD3+/kg. They subsequently received salvage therapies including BV if necessary. Patients failing to achieve CR were enrolled in the treatment group whereas responding patients were included in the control group. All patients underwent ASCT conditioned with FEAM chemotherapy (see supplementary materials). The protocol outline is shown in Figure 1.

Eight R/R HL patients potentially candidate to ASCT failing to achieve at least a PR after three lines of therapy including BV were included in the treatment cohort and received ALI plus nivolumab 240 mg flat dose, delivered 48 hours after each ALI. Two patients achieving CR before ASCT with either IGeV salvage chemotherapy or third line BV, did receive ALI only, without nivolumab, serving as a control group. In both groups the same peripheral blood analysis was performed at the same time points (see below for details).

The number of planned ALI for each patient was four. After ASCT, the first ALI was performed at a median of 14 days after stem cell reinfusion (range 12-16), with at least 3 days wash out from the last G-CSF administration. Second ALI was delivered after 14 days and the other two infusions took place every three weeks.

For safety reasons CD3+ dosing was incremental, with an increase of 1 log for each of the 4 planned infusion (i.e $1x\ 10^4$/Kg up to $1X10^7$/Kg). Furthermore, the starting dose of cycle 1 was planned to increase by one log every four patients if no grade >2 adverse events were observed. Thus, the first four patients received $1x10^4$ CD3+/kg in the first infusion and then we escalated the dose to $1x10^5$ CD3+/kg. The schedule of ALI reinfusion and CI treatment is detailed in Figure 2.

Assessment of disease response was performed in all patients after the first two cycles of nivolumab and 21 days after completion of the fourth ALI + nivolumab. Patients achieving CR were offered allogeneic stem cell transplant (HSCT), if feasible.

Lymphocyte subpopulations analysis

To assess immune-reconstitution, lymphocyte subpopulation analysis has been performed on peripheral blood samples by flow cytometry (BD-FACSCanto II cytometer). Lymphocyte subpopulations have been assessed at the following timepoints: 2 hours before and 48 hours after each of the four scheduled ALI and, in treatment group only, 48 hours after each nivolumab administration.

Single platform absolute counts of major lymphocyte subsets are performed by standard peripheral blood immunophenotyping. Fresh EDTA-anticoagulated peripheral blood (50 ul) are stained with the following conjugated monoclonal antibody combination in 8 color multiparametric flow cytometry (FITC, fluorescein isothiocyanate/PE, phycoerythrin/ PerCP-Cy™5.5, peridinin-chlorophyll proteins-cychrome 5.5 /PE-Cy™7, PE-cyanine dye 7/APC, allophycocyanin/ BD™ APC-H7, allophycocyanin-H7/ V450, BD Horizon™ V450 /V500, BD Horizon™V500: CD3/(CD16 and CD56)/CD4/CD19/CD38/CD8/CD20/CD45 in TruCount tubes. After 10 minutes incubation at room temperature (RT), blood is lysed with 2 ml lysing solution (BD Pharmlyse 1X) for 5 minutes and 250000 lymphocytes are acquired in a BD FACSCanto II cytometer. T lymphocytes (CD3+), T cell subsets (CD3+, CD4+ CD8neg and CD3+, CD8+, CD4neg), B lymphocytes (CD19+, CD20+), NK cells (CD3-, CD16+, CD56+ and CD3-, CD16+, CD56-)[27] are evaluated by using FACS DIVA software. Based on CD45 expression in a CD45 vs side scatter (SSC) a leukocyte gate is drawn to include granulocytes (CD45+/high SSC), monocytes (CD45/medium SSC), and lymphocytes (CD45+/low SSC). Results are expressed as leukocytes and lymphocytes counts, % of lymphocytes and % of monocytes of the total

leukocytes. Lymphocyte populations are expressed as % of total leukocytes % lymphocytes and absolute counts (N/mmc).

To study antigenic expression describing maturation, activation, disfunction, senescence of the T cell compartment and maturation of NK cells, blood (2ml) is bulk lysed with 15 ml lysing solution (BD Pharmlyse 1X) for 10 min, centrifuged at 1500 rpm, washed once with 5 ml PBS-1%FCS, 0,1% NaN3 (PBS) and the pellet resuspended in PBS at 20 x 10^6/ml. Cells (50ul) are incubated with the following conjugated monoclonal antibody combinations: 1) CD45RA/CD62L/CD3/CD27/CD28/CD8/CD4/PD1, 2) CD45RA/CD62L/ HLADR/CD25/CD38/CD8/CD4/PD1, 3)CD57/CD45RO/CD27/CD28/CD8/ - /CD45, 4) CD57/CD16/CD3/CD56/-/CD14/-/CD45.

All antibodies are purchased from Becton Dickinson (San José, CA).

Differential expression of CD45RA, CD62L, CD27 and CD28 is used to identify maturation subsets of CD4 and CD8 T cell populations (CD3+, CD4+ or CD8+) as shown in Table I-S (Supplementary Materials).

The activation markers CD25, HLADR, CD38 are studied on naïve (CD45RA+, CD62L+) versus non naïve CD8 and CD4 T cells. Dysfunction (previously called “exhaustion”) is studied by the expression of inhibitory receptor PD-1 by gating on the various differentiation subsets based on CD45RA and CD62L expression levels and on activation markers (Legat et al, 2013).

NK cells (CD56+CD3-/CD56-CD16+CD3-) are further characterized in four subsets: $CD56^{bright}CD16^{neg/dim}$ (immature), $CD56^{dim}CD16^{bright}$ / $CD56^{neg}CD16^{bright}$ (mature), $CD56^{dim}CD16^{dim}$ (unconventional NK cells). For a fine analysis of NK cells Kaluza software (v.2.1, Beckman Coulter) has been used.

Statistical Methods

Dichotomous variables were compared using the Chi-Square test or by Fisher's exact test when necessary. Continuous variables were compared using Student's T test, or if normal distribution could not be confirmed, by Wilcoxon's rank test.[20]

Overall Survival (OS) was calculated from the time of transplantation until death by any cause, or last follow-up.

Survival curves were built using the Kaplan Meier method, and univariate survival analysis was performed using the Log-rank test.[20]

All statistical analyses, except competing risk analysis, were performed using IBMSPSS v22© running on a Debian (Linux) operating system.

All two-tailed p values <0.05 were considered statistically significant.

Results

Patients

Eight R/R HL patients have been treated so far with ALI + nivolumab in this feasibility trial. All patients had failed to achieve CR with first and second line chemotherapy and progressed during BV therapy. Median age was 28.5 years (range 18-56). PET scan before ASCT showed progressing disease in all patients, with multiple-extra nodal involvement in 6/8. Two additional patients who achieve CR with salvage chemotherapy were enrolled in the control group and received ALI alone. Patient's features are provided in Table I. All patients underwent ASCT and achieved complete neutrophil and platelet engraftment after a median of 10 days.[8-10]

Toxicities

During ASCT, patients experienced the following adverse events correlated to ASCT: fever of unknown origin in 4/10 patients, grade 2-3 mucositis in 6/10 patients, sepsis in two/ten patients.

No grade 3 or 4 adverse events related to ALI or nivolumab were recorded in the first four patients, so that the other patients received the first ALI at the increased dose of $1x10^5$ CD3+/kg, without any complication. In the whole study, no infectious complications or other adverse events were observed during ALI +/- nivolumab therapy, in particular, no patient experienced CMV reactivation/infection.

Biological results

Our preliminary results show that ALI allowed to achieve significantly quicker immune-recovery in all patients, in terms of absolute CD3+ count, if compared to historical HL patients receiving the same conditioning without ALI ($p < 0.05$), especially in the T-lymphocyte effector-memory compartment ($p<0.03$).

Mature/cytotoxic NK cells (Figure 3) and naïve CD8+ cells (data not shown) showed a significant increase after ALI and CI ($p < 0.03$ and $p<0.05$, respectively). Nivolumab administration was followed by a modest and transient increase in T-effector-memory population (data not shown).

We analyzed the development of NK cells in all 10 enrolled patients (Figure 4). Our data suggest that after ASCT, as expected, NK cells are the first lymphoid population detectable in peripheral blood. Interestingly, a faster expansion/reconstitution of NK cells displaying a highly differentiated surface phenotype was observed in the treatment cohort. In detail,

different patterns of NK-cell development could be identified. Indeed, starting from one month after transplantation, in the group of patients undergoing ALI plus post-ASCT nivolumab, a relevant fraction of NK cells displayed a mature phenotype characterized by $CD56^{dim}CD16^{bright}$ expression (Figure 4). On the contrary, in patients undergoing ALI without early post-ASCT nivolumab (control group), NK cells were characterized by a more immature phenotype (high frequencies of $CD56^{bright}CD16^{neg}$ NK cells) even at late time points (three months) after transplantation (Figure 4). In addition, in patients undergoing ALI + nivolumab, an aberrant and hyporesponsive subset of mature NK cells (namely $CD56^{neg}CD16^{+}$), reminiscent of that described in patients with viremic CMV/HIV, was detected. Of note, this subset was absent in the control group (Figure 4). CMV DNA was assessed two times a week during all study period in all patients, performed by standard RT-PCR diagnostic test, and was always found to be undetectable in all patients. Finally, peripheral blood from patients undergoing ALI + nivolumab was enriched in unconventional $CD56^{dim}CD16^{dim}$ NK cells (Figure 4a). A phenotypically similar subset of NK cell, endowed with multifunctional activity (including potent killer and IFN-gamma producing capacity), was found in the bone marrow both of healthy children and of pediatric leukemic patients.

Clinical Results

Early response assessment performed after cycle 2 showed a negative PET in all patients and a complete CT response in 5/8. End of treatment evaluation showed complete PET and CT response in all patients. Three patients were bridged to HSCT and are alive, in ongoing CR and free of graft- versus- host disease (GVHD) at the time of analysis. Three of the remaining patients refused HSCT, one patient did not find a donor while one patient had recently completed the procedure. All of them are alive and disease free after a median follow-up of 20

months (95% IC 16.58-24.56 months).

Detailed therapeutic outcome is provided in Table II.

Discussion

The results of this feasibility study suggest that early post-ASCT administration of nivolumab supported by ALI is safe and highly effective in R/R HL patients. The combination of ALI and nivolumab may significantly improve the results of either single anti-PD-1 agent therapy or ASCT performed in patients with active disease.[10,11,20] Armand et al. reported that pembrolizumab administration as post-ASCT consolidation in HL patients achieving at least PR before transplant, but considered at high risk of relapse resulted in a significant improvement of PFS.[11] In our study, we explored the clinical benefit of nivolumab in the worse setting of truly refractory HL patients that are usually not considered candidate for ASCT. The high risk of early progression and the expected severe post-transplant immune suppression in these heavily pretreated patients, prompted us to plan pre-emptive nivolumab supported by ALI.[21] Post-ASCT ALI was very well tolerated and allowed a quick recovery of selected lymphocyte subsets.[21] The impressive CR rate may be explained by the synergistic effect of the deep and rapid cytoreduction following ASCT conditioning and the immune response triggered by early CI therapy supported with ALI. It has been widely reported that HSCT may provide a substantial contribute to cure advanced stage refractory HL patients if performed in CR.[22] The high activity and the good tolerability of our strategy may therefore allow more refractory patients to enter CR and benefit from HSCT. Indeed, three patients in our cohort were able to receive allo transplant in a CR status. Nevertheless, regardless of subsequent HSCT, all enrolled patients maintain CR after discontinuation of immunotherapy, that was limited to 4 doses of nivolumab instead of standard until progression schedule.[10] With the limitation of the small

number of patients and the relatively short follow up period, a shorter duration of CI therapy did not result in a worse survival.[10] In this view a lower cumulative dose of nivolumab may contribute in lessening the risk of GVHD, which is very high in patients receiving allo-SCT after CI therapy.[23]

The observation of a significant expansion of the mature/cytotoxic NK compartment during ALI+nivolumab treatment suggests that NK cells may play a significant role in the anti-lymphoma response in this setting and is consistent with the observed trend for NK cell expansion in Armand et al study.[11] Differently from what is observed in solid tumors Reed Sternberg (RS) cells, albeit expressing PD-1 ligand, show an HLA class I down-regulation, hampering the possibility of a cytotoxic $CD8^+$- mediated killing.[10,11,16,17] In this view, it has been recently reported that not only T-lymphocytes, but also NK cells express the PD-1 receptor.[9,24] Taken together, these observations suggest a key role for NK cells in response to PD-1 blockade in HL, as these innate cells kill tumor cell targets not expressing HLA class I molecules and, once activated, release high amount of pro-inflammatory cytokines that can shape other immune cell responses.[24-26]

Moreover, we documented a quicker NK cell maturation in patients receiving both ALI and nivolumab, compared to the control cohort. In physiological conditions, human NK cells include different cell subsets corresponding to different stages of NK cell differentiation.[27] $CD56^{bright}$ $CD16^{neg/dim}$ NK cells (the major subset of NK cells in secondary lymphoid tissues) are considered as precursors of $CD56^{dim}$ $CD16^{pos}$ NK cells and have been usually considered as "regulatory NK cells". On the other end, $CD56^{dim}$ NK cell population is the most represented in peripheral blood and is considered as the "cytotoxic population". The terminally differentiated phenotype of $CD56^{dim}$ cells is marked by the expression of the CD57 molecule and high levels of CD16.[26,27] After HCMV infection/reactivation, an increased proportion of terminally

differentiated $CD57^+$ NK cells, characterized by high expression of NKG2C, is induced (the so-called "adaptive" NK cells). Interestingly, unknown cofactors associated with HCMV infection may induce the generation of an additional type of fully mature NK cells characterized by the expression of the inhibitory receptor PD-1 (not necessarily co-expressed with NKG2C). These cells, called $PD\text{-}1^+$ NK cells, are marked by compromised effector functions against tumor cells expressing PD-1 ligands. Notably, this impaired antitumor NK cell activity can be partially restored by antibody-mediated disruption of PD-1/PD-L interaction.[8,25,27]

A remarkable acceleration of NK cell maturation was described also in leukemic patients receiving different types of allograft (i.e. receiving UCBT or $CD34^+$ haplo-HSCT or $\alpha\beta$T/B cell-depleted HLA-haploidentical HSCT containing variable numbers of donor-derived NK cells and γ/δ+ T cells). In all these recipients, CMV infection/reactivation favored the preferential expansion of highly differentiated NK cells and their persistence overtime.[28] However, our patients resulted always negative for CMV, by PCR assessment, thus this NK cell expansion is not related to CMV infection/reactivation. These observations suggest a possible critical role for anti-PD-1 agents in controlling NK cell maturation/development.

In this view, our data support the idea that lymphocyte repletion during early post-ASCT anti-PD-1 consolidation may lead to a rapid accumulation of mature/cytotoxic $CD56^{dim}CD16^{bright}$ NK cells, with anti-neoplastic and anti-infective activity.

If a pivotal role of NK cells in CI clinical response in HL will be confirmed, this therapeutic strategy may pave the way to further innovative approaches, such as supporting CI therapy with positively selected autologous NK cells. Alternatively, highly purified NK cells from selected haploidentical donors may be used, considering that NK cells, differently from T-cells, do not induce GvHD.[28-30]

Table I: Patients characteristics

	Age at diagnosis	Sex	Stage at diagnosis	Previous Therapies and Response	Disease status at Enrollment	Stage at Enrollment	Extranodal disease at Enrollment
ALL PATIENTS (n=8)	**28.5 (median)**	**Female: 5/8 Male: 3/8**	**II: 4/8 III: 1/8 IV: 3/8**	-	**8/8 PD**	**IV: 8/8**	**Multiple in 7/8 Single in 1/8**
Patient #1	26	F	IIB	2xABVD-> PD 2xIGeV-> PD 4xBV-> PD	PD	IVB	Lung, liver
Patient #2	19	M	IIA	2xABVD-> PR 2xIGeV-> NR 4xBV-> PD	PD	IVA	Lung, pericardium
Patient #3	56	M	IIA	6xABVD-> PD 2xIGeV-> NR 4xBV-> PD	PD	IVA	Lung
Patient #4	32	F	IVA	6xABVD-> PD 4xIGeV-> NR 4xBV-> PD	PD	IVB	Lung, liver
Patient #5	22	F	IVB	6xABVD-> PD 3xBeGeV-> PD 4x BV-> PD	PD	IVB	Lung, liver, bone
Patient #6	37	F	IIB	2xABVD-> PR 4xIGeV-> PD 4xBV-> PD	PD	IVB	Lung, bone
Patient #7	31	F	IIIB	2xABVD->PD 4xBeGeV->RP 4xBV-> PD	PD	IVB	Lung, pancreatic
Patient #8	20	M	IVB	2xABVD->PR 4xBeGeV-> NR 4xBV-> PD	PD	IVB	Lung, bone, bone marrow
Control #1	52	F	IIB	2xABVD-> PR 4xIGeV-> CR	CR	-	-
Control #2	34	F	IIIA	6xABVD-> PD 4xIGeV-> CR	CR	-	-

Table II: Therapy Outcome after ALI / nivolumab

	CT scan after cycle 2	PET scan after cycle 2	CT scan at EOT	PET scan at EOT	Allogeneic Stem-cell Transplant	Disease Status at Allogeneic SCT	Donor and Source of Allogeneic SCT	Disease and survival status at last FUP
ALL PATIENTS (n=8)	**PR: 2/8 PR CR: 6/8 CR**	**CR: 8/8**	**CR: 8/8**	**CR: 8/8**	**NO: 5/8 YES: 3/8**	**CR: 3/3**	**Haploidentical BM: 2/3 HLA identical sibling BM: 1/3**	**Alive and CR: 8/8**
Patient #1	CR	CR	CR	CR	YES	CR	Haploidentical, BM	Alive and CR
Patient #2	CR	CR	CR	CR	NO	-	-	Alive and CR
Patient #3	CR	CR	CR	CR	NO	-	-	Alive and CR
Patient #4	CR	CR	CR	CR	NO	-	-	Alive and CR
Patient #5	PR	CR	CR	CR	YES	CR	HLA-identical sibling, BM	Alive and CR
Patient #6	PR	CR	CR	CR	NO	-	-	Alive and CR
Patient #7	PR	CR	CR	CR	YES	CR	Haploidentical, BM	Alive and CR
Patient #8	CR	CR	CR	CR	NO	-	-	Alive and CR

BM= Bone Marrow

Figure 1: Study flowchart

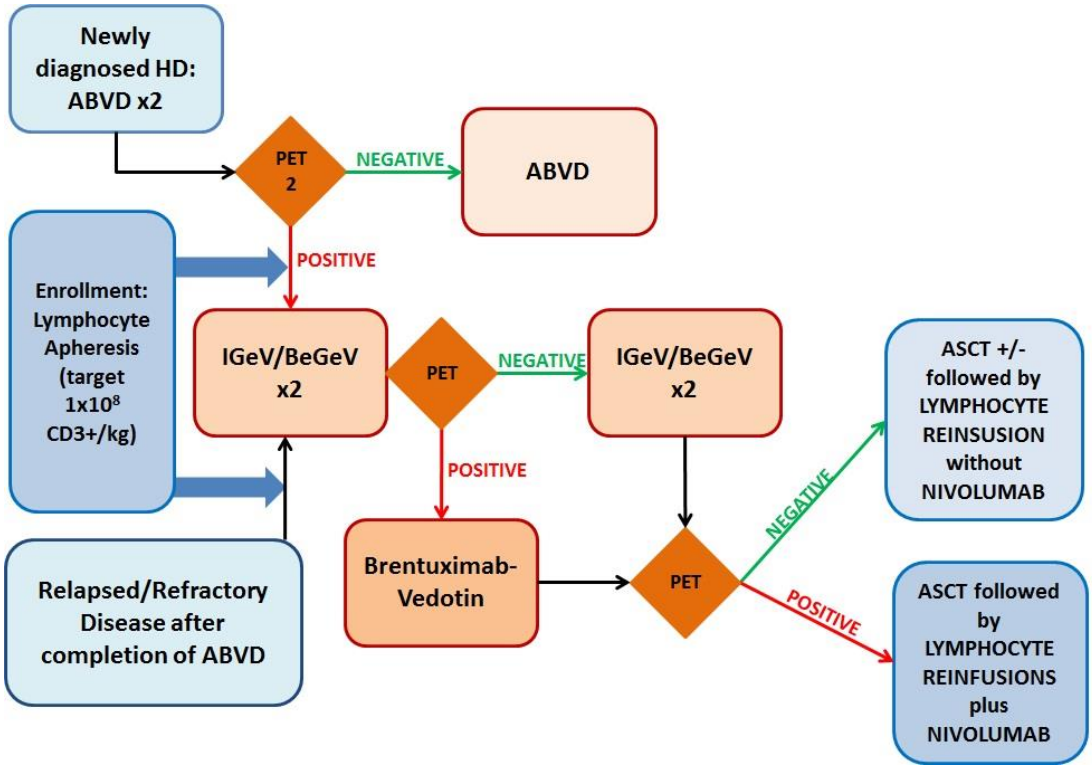


Figure 2: Study Treatment

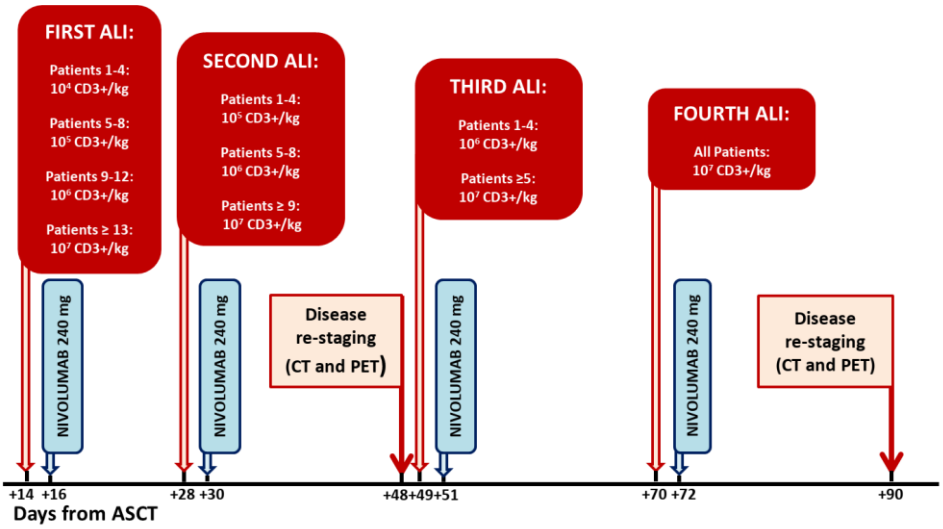


Figure 3: Cytotoxic NK cell/ul before and after ALI.

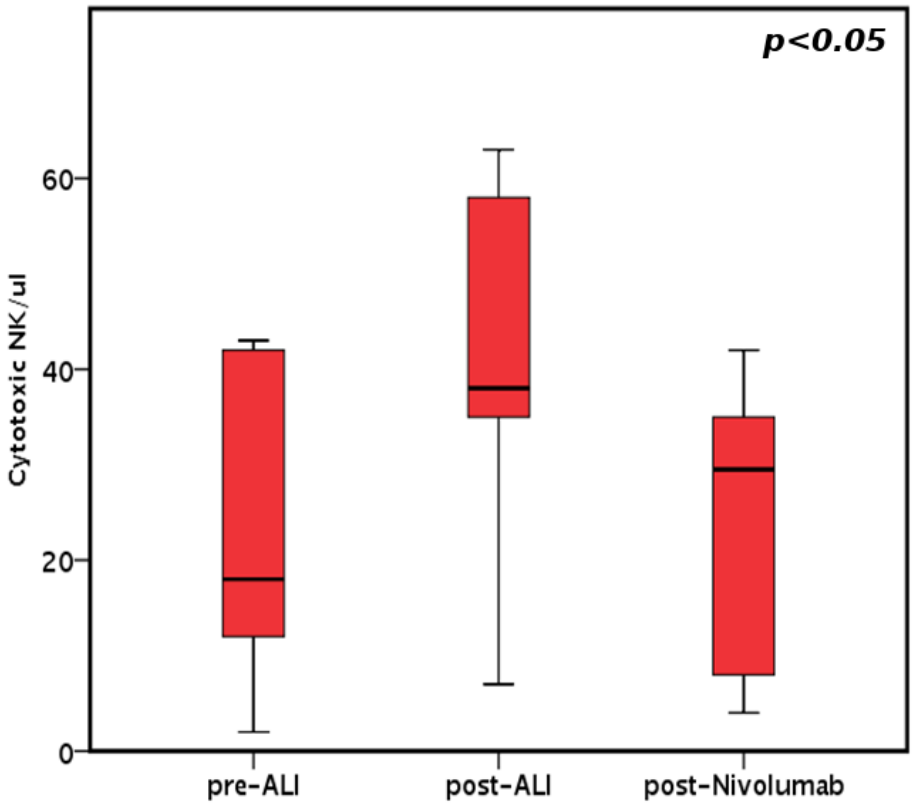
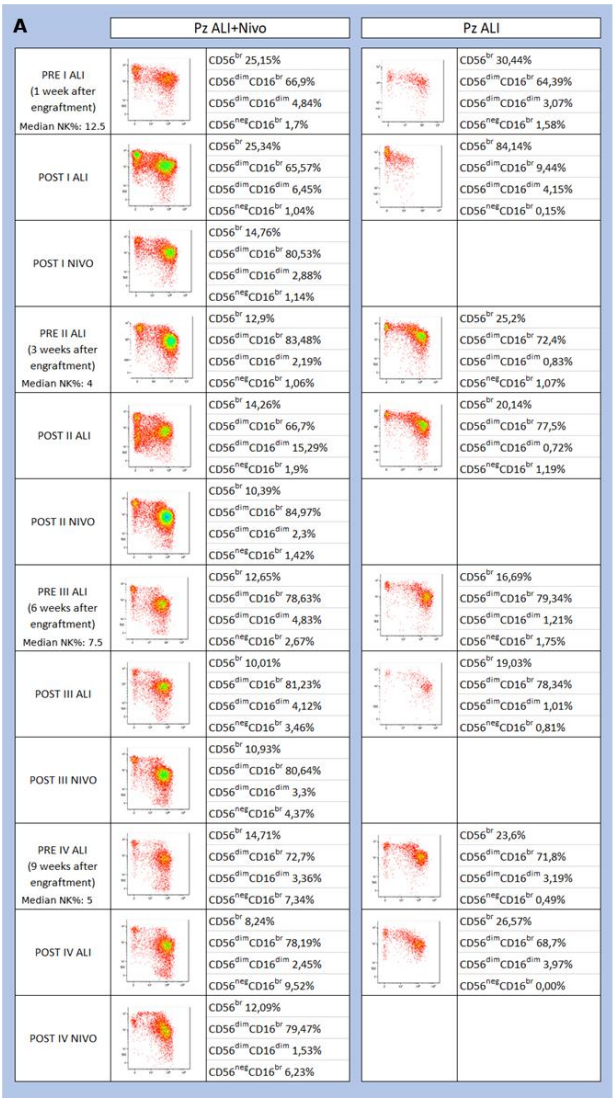
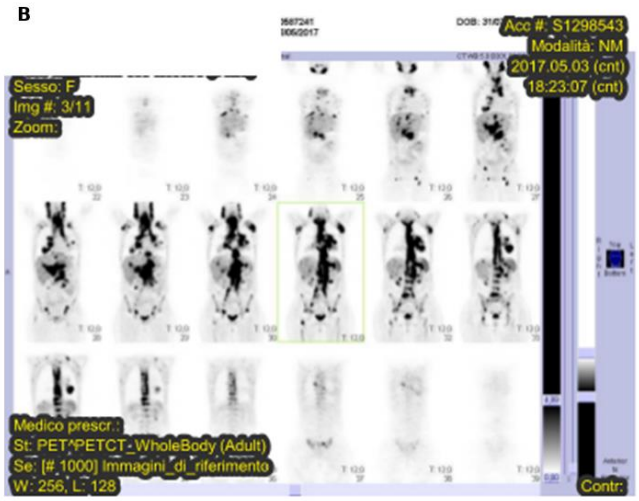


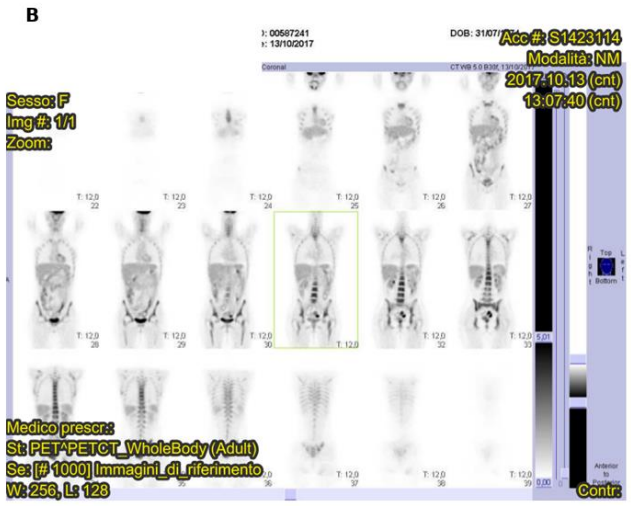
Figure4:

A: Development of NK cells in patients affected by R/R HL undergoing ASCT and receiving ALI + nivolumab (left panel A) or ALI alone (right panel A).

B: PET scan from a patient receiving ALI + nivolumab at enrollement (upper panel B) and at EOT (lower panel B)







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SUPPLEMENTARY MATERIALS

LYMPHOCYTE APHERESIS, CRYOPRESERVATION AND THAWING

The unstimulated lymphocyte apheresis procedure has been performed in steady state (e.g. patient must not have received any chemotherapy or steroids in the last 14 days) by processing a blood volume of about 5 liters, with a single-needle discontinuous flux machine.

The lymphocyte aphaeresis arrived to the Cell Factory from the Collection Center with attached complete blood count (CBC) of the product and one accompanying test tube. The test tube has been assessed for the CD3+ count at flow cytometry.

1 ml of heparin sodium was added to the product and, if necessary, the cell suspension has been dilute with physiological solution and 5% final of Human Albumin up to the final freezing volume. Volume has been calculated by maintaining a cell concentration equal to $50x10^6$/ml.

The cryoprotectant used is dimethyl sulfoxide(DMSO) 10% ; the product has been immediately transferred into 4-ml cryopreservation tubes and frozen at controlled-rate freezer (Ice cube SyLab).

After freezing procedures, samples have been stored in a liquid nitrogen cryo-container until the reinfusion, under temperature control with H24 remote alarms.

Blood cultures (BCs)for aerobic, anaerobic and fungi germs have been carried out on the final product.

The target aphaeresis quantity of CD3+ cell has been minimum of $5x10^8$/kg, if target dose has not been reached, a second unstimulated apheresis has been performed.

The thawing of the required dose of lymphocytes took place at the cryopreservation laboratory using a thermo stated bath at 37° C. In order to limit the DMSO toxicity, the thawed volume has been diluted 1: 1 with 0.9% Sodium chloride in a sterile environment. The reinfusion has always been performed within and no more than 15 minutes after thawing.

CONDITIONING CHEMOTHERAPY

ASCT conditioning has been performed according to conventional FEAM high dose chemotherapy (Fotemustine 150 mg/sqm days -7 and -6, Etoposide 200 mg/sqm + Cytarabine 400 mg/sqm days -5 to -2, Melphalan 140 mg/sqm day -1). A minimum of 3 x 10^6/kg CD34+ autologous stem cells have been re-infused on day 0 in all patients. Granulocyte stimulating factor (G-CSF) has been administered from day 3 until the complete granulocyte recovery (i.e. ANC >2000/mmc). All patients achieved complete hematological engraftment after a median of 10 days (8-12).

Table I-S: T-Lymphocytes subpopulation definitions

		% CD8 cells in PB of healthy individuals	% leukocytes in healthy individuals CD8% 10-20% lymph
Naïve (NA)	CD45RA+, CD62L+, CD27+, CD28+	16%-66%	1,6-7
Central Memory (CM)	CD45RAneg, CD62L+, CD27+, CD28+	1%-8%	0,1-0,8
EffectorMemory-CD27+ (EM-CD27+)	CD45Raneg, CD62L neg, CD27+, CD28+ (EM1) or CD28neg (EM2)	EM1 7-37% EM2 0-4%	0,7-4 0,1-0,4
Effector Memory-CD27neg	CD45RAneg, CD62Lneg, CD27neg, CD28+ (EM4) or CD28neg (EM3)	EM3 0-7% EM4 1-7%	0,1-0,7 0,1-0,7
EM RAINT	CD45RA+/-, CD62Lneg, CD27neg o basso, CD28neg	2-18%	0,2-1,8
EM RA+	CD45RA+, CD62Lneg, CD27+, CD28+ or CD28neg	2-18%	0,2-1,8
Effectors (E)	CD45RA++, CD62Lneg, CD27neg, CD28neg	2-53%	0,2-5