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TITLE : Chronic lung allograft dysfunction is associated with an early increase of circulating cytotoxic CD4+CD57+ILT2+ T cells, selectively inhibited by the immune check-point HLA-G.

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ABSTRACT

Background: Survival after lung transplantation (LTx) still remains limited by chronic lung allograft dysfunction (CLAD), thought to represent a form of chronic rejection.

We investigated whether the immune checkpoint HLA-G/ILT2 expressed by peripheral T-cell subpopulations could predict CLAD.

Methods: We used data for 150 LTx recipients from COLT (Cohort-For-Lung-Transplantation) cohort with ≥ 1 available blood sample at 1, 6, or 12 months post-Tx. Analysis of T cells by flow cytometry focused on the ILT2 receptor of HLA-G and other markers (CD57, CD25, CD127). T-cell subset analyses compared stable patients and those with CLAD at 3 years post-LTx.

Results: With data for 78 stable and 72 CLAD patients, among 21 T-cell subsets expressing ILT2, only CD4+CD57+ILT2+ T cells were associated with outcome. At 1 month post-Tx, low proportion of CD4+CD57+ILT2+ T cells was associated with reduced 3-year incidence of CLAD (CD4+CD57+ILT2+ T cells \leq first IQR[25%] vs $>$ first IQR, log-rank test, $p=0.028$). Furthermore, the incidence of CLAD was higher with >2.6 - versus ≤ 2.6 -fold increased proportion of CD4+CD57+ILT2+ T cells over the first year post-LTx (3-year freedom frequencies: 27% [95%CI: 8-50] vs 64% [95%CI: 48-77] (log-rank test, $p=0.014$).

On multivariable analysis, increased proportion of CD4+CD57+ILT2+ T cells over the first year predicted CLAD (hazard ratio 1.25; 95%CI: 1.09-1.44; $p=0.001$). Focusing on CD4+CD57+ILT2+ T cells, we demonstrated *ex vivo* that they are cytotoxic CD4+ T cells, selectively inhibited by HLA-G.

Conclusion: Our data suggest that an early increase of CD4+CD57+ILT2+ T cells after LTx may be associated with CLAD onset.

INTRODUCTION

Long-term survival after lung transplantation (LTx) still remains limited because of chronic lung allograft dysfunction (CLAD), thought to represent a form of chronic rejection (1). The most prevalent morphological feature in CLAD is bronchiolitis obliterans syndrome, but in the last decade, a restrictive allograft syndrome (RAS) phenotype has been described (2, 3). The current immunosuppressive therapies cannot prevent the occurrence of CLAD in many LTx patients (4), whereas other recipients, under the same standard immunosuppression therapy, remain free of CLAD for a prolonged time (4), as reported in other solid organ Tx (SOT) (5). Hence, we need a better understanding of immune checkpoints leading to a tolerogenic state in some patients and conversely, the identification of predictive markers of graft acceptance breakdown in the medium- and long-term. In the last decade, attention has been paid to the immune checkpoint human leukocyte antigen G (HLA-G) molecule, well known for its tolerogenic role in materno-foetal tolerance, but that is also commonly neo-expressed in some pathological situations, including allograft in SOT and cancer. HLA-G expression in the graft has been associated with acceptance of various allografts, as reflected by fewer episodes of acute rejection (AR) and reduced incidence of chronic rejection (6-10). Similarly, in the context of LTx, HLA-G expression in bronchial epithelial cells, the primary targets of rejection, is associated with allograft acceptance (9, 11). HLA-G exerts its immune modulatory functions via two main inhibitory receptors : leukocyte immunoglobulin (Ig)-like receptor B1 (LILRB1) and LILRB2 (also known as Ig-like transcript 2 [ILT2]/CD85j and ILT4/CD85d), which are differentially expressed by immune cells. ILT2 is

expressed by some T and natural killer (NK) cells, B cells and antigen-presenting cells (APCs), whereas ILT4 is myeloid-specific (12).

Through these receptors, HLA-G inhibits the cytolytic function of NK and T cells, the alloproliferative response of CD4⁺ T cells, the antibody production by B cells, and the function of dendritic cells (13). Hence, expression of HLA-G by graft cells seems to induce an inhibition of the recipient's anti-graft effector cells, effectively protecting the graft cells against immune-induced CLAD lesions (9, 11). For HLA-G to exert its tolerogenic properties, the immune effectors expressing ILT2 or ILT4 receptors should be present in the graft vicinity and possibly observable in the peripheral blood. Given that the CLAD process is mainly mediated by immune recognition of the graft, we hypothesized that involvement of the HLA-G/ILT2 immune checkpoint via graft HLA-G expression could be tuned by variations in ILT2 expression on peripheral T lymphocytes. ILT4 receptor, which is not expressed on T lymphocytes, was not investigated.

According to this hypothesis, peripheral T-cell sub-populations could represent a prognostic non-invasive biomarker of CLAD development and also give new insights into the pathways of CLAD onset. This hypothesis is supported by our previous observation in bladder cancer: a significant increase in proportion of the peripheral cytotoxic CD8⁺ILT2⁺T-cell sub-population was a strong predictor of cancer recurrence (14). Accordingly, because ILT2 is normally expressed only by a minority of CD4⁺ T cells (0–4%) and CD8⁺ T cells (5–20%), variations in its expression might affect the ability of HLA-G to inhibit immune cell effectors in the context of cancer (14) or LTx.

In the current study, we investigated whether the proportion of circulating immune cells expressing ILT2 might be associated with CLAD occurrence. For this purpose, we used flow cytometry to analyse peripheral-blood T cells from the French multicentre Cohort for Lung Transplantation (COLT) for cell-surface marker expression at predefined post-transplant times. Analysis was focused on the ILT2 receptor of HLA-G, first when associated with CD57 marker, which has been previously shown to be linked to other pathological conditions including rejection in solid-organ transplant recipients (15)(26, 27, 28), and second on other markers known or suspected to be linked to graft stability and/or CLAD development, including CD57, PD1, CD25, and CD127^{low} T-cell markers (23).

MATERIALS AND METHODS

Patients and study design

Data for LTx patients from the COLT cohort were analysed. The COLT cohort was from 11 French LTx centres (Programme transplantation 2008, PRTP-13, ClinicalTrials.gov NCT00980967) (see supplemental data). Inclusion criteria were at least 1 available blood sample at 1 and/or 6 months and/or 12 months post-Tx, a date of CLAD diagnosis after 12 months post-Tx in patients showing CLAD within 3 years post-Tx, and a stable lung function at 3 years post-LTx in the stable group. This study enrolled 150 patients from the COLT cohort, selected according to the availability of blood samples (Figure 1, Flow chart, and supplemental data). All CLAD patients from the COLT cohort with these criteria were enrolled (n=72), and concomitantly, n=78 successive stable patients of the COLT study were selected according to the availability of blood samples, among all stable patients of COLT cohort (see detailed in supplemental data). Monitoring protocols of patients, immunosuppressive therapies, HLA typing, and detection of alloimmunization in LTx candidates were left to the

physician's discretion in each local LTx centre (see supplemental data). Pulmonary function tests, including spirometry and plethysmography when available, clinical data, and blood samples were collected at 1 month post-LTx and every 6 months within the 3 years post-LTx. CLAD was diagnosed according to International Society for Heart and Lung Transplantation criteria (3) in the presence of bronchiolitis obliterans syndrome (3, 16) or RAS onset (2, 17, 18) (see supplemental data). Criteria for diagnosis of primary graft dysfunction (PGD) (19), acute rejection (AR) episodes, and CLAD are detailed in supplemental data (2, 3, 20). Stable graft function (STA) or CLAD status at 3 years post-LTx were confirmed by repeated sessions of an ad-hoc adjudication multidisciplinary committee (3) (see supplemental data).

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) obtained from COLT patients were analyzed by flow cytometry. PBMCs were isolated by Ficoll gradient centrifugation (Ficoll-Paque, LifeSciences), frozen and stored at -80°C (see details in supplemental data). All phenotyping experiments were systematically set up with five samples from patients and one sample from a healthy blood donor. For analyses, cells were labeled with different antibodies targeting cell-surface markers from CD4⁺ and CD8⁺ T cells, and analysis was first focused on T-cells expressing both CD57 and ILT2 markers, and CD4⁺CD25⁺CD127⁺ regulatory T-cells, based on their suspected associations with CLAD development (21) or known cytotoxicities (15, 22, 23). Investigations of other combinations of cell-surface markers were also performed, which allowed us to characterize a total of 37 different T-cell sub-populations (see below and supplemental data). In parallel, control PBMC from healthy individuals were analyzed (no.13/EFS/101, supplemental data). The following monoclonal

antibodies (mAb) were used for cell-surface staining of PBMC: From Miltenyi Biotec: CD19-APC, CD3 PerCP-Vio700, CD4 Vio-Bright FITC, CD8 APC-Vio770, CD57 PE-Vio770, CD127 PE-Vio770. From eBioscience: ILT2 PE, ILT2-APC, and CD25 eFluor450. From Biolegend: PD1 BV421 (see supplemental data). To control for background staining, an isotypic control panel was run for each sample, that included CD19, CD3, CD4, CD8, and CD57 antibodies, and isotypic control antibodies for non-lineage markers such as ILT2.

Results of flow cytometry were analysed at each time point (1, 6, and 12 months) and also according to kinetics of each T-cell subset within the first year post-LTx, because variations in peripheral T-cell subpopulations have been associated with CLAD onset (24). For these kinetics analyses, we measured the variation in T-cell subsets proportions over a 6-month period during the first year post-Tx. By comparing data obtained at 1 and 6 months post-Tx or at 6 and 12 months post-Tx for all patients with available samples at these timepoints, we investigated a single variable defined as the 6-month T-cell ratio within the first-year post-Tx.

Functionnal assays

Cell lines

Functional analysis was performed to assess the cytolytic properties of T-cell subpopulations of interest identified in the LTx cohort. The monocytic cell line THP1 (ATCC) transduced or not to express membrane-bound HLA-G1 (*i.e.*, THP1-HLA-G and THP1, respectively) was used as target cells for T cells (See details un supplemental data) (22).

Cytotoxicity assay: peripheral T-cell degranulation

Cytotoxicity assays were CD107a degranulation assays. CD107a is a lysosome-associated membrane glycoprotein (LAMPs) that is expressed at the surface of immune cells upon cytolytic degranulation. The percentage of CD107a-expressing cells at the end of the assay was used as a marker of cytolytic degranulation, and thus of cytotoxic killing of the target cells (see details in supplemental data) (25). Briefly, THP1 or THP1-HLA-G cells were placed in a 96-well culture plates in supplemented RPMI culture medium (Sigma) and cultured with PMA for 72 hr to obtain a confluent macrophage layer (mTHP1/mTHP1-HLA-G). Then, cells were coated for 15 min with CD3 mAb (clone OKT3, Orthoclone). In blocking experiments, PBMCs from LTx recipients were incubated for 20 min at 37°C with 20 µg/mL of a blocking ILT2 antibody (clone GHI/75, BioLegend) or a control antibody (BioLegend). PBMCs were then added to the OKT3-coated mTHP1/mTHP1-HLA-G target cells in culture medium supplemented with monensin and brefeldin A (Protein Transport Inhibitor Cocktail, eBioscience) in the presence of a PE-conjugated CD107a antibody (clone H4A3, BioLegend) or isotype control. After 4 hr of co-incubation at 37°C, cells were washed and stained for flow cytometry analysis.

Statistics

Analysis of continuous and categorical variables are detailed in supplemental data. Freedom from CLAD and graft survival were estimated by the Kaplan-Meier estimator and the log-rank test was used to compare the event-free survival curves between groups. Univariate and multivariable Cox models were built to assess the association between the proportion of cell subpopulations at 1 month post-TxP or the

6-month T-cell ratio and freedom from CLAD survival and graft survival, with adjustment for potential confounding factors. Factors associated with freedom from CLAD and graft survival on univariate analyses (at a significance of $p < 0.2$) were selected for multivariable analyses. A backward stepwise selection was used to perform the final multivariable model (model 1). Among cell populations, CD4+CD25+CD127^{low}, CD8+ CD25+, and CD4+CD57+ILT2+ T cells assessed at 1-month post-LTx and the 6-month CD4+CD57+ILT2+ T-cell ratio were included in the Cox model as a time-independent covariate (see supplemental data). Additionally, due to the major potential impact of induction therapy (Thymoglobulin or IL2 receptor antagonist) on T-cell subsets, multivariate Cox models were also built that includes only induction immunosuppression and the T-cell subset variables selected by univariate analyses (model 2).

The last follow-up date for all survival models was 3 years post-LTx. All analyses were two-tailed and $p < 0.05$ was considered statistically significant. Analyses involved the use of Stata v12 for Windows (StataCorp LP, College Station, TX).

This study was approved by an institution ethics committee and conducted in accordance with recommendations concerning human research of the Declaration of Helsinki. All patients gave their informed consent.

RESULTS

Baseline characteristics of patients

Among the 150 patients, 78 remained with stable condition throughout the study (STA group), and in 72, CLAD developed within 3 years post-LTx (Figure 1, Flow chart). Characteristics of patients are in Table 1. Mean (SD) recipient age was 43 (15) and 44 (15) years in the STA and CLAD groups, respectively ($p=0.66$). Most of the patients received bilateral LTx ($n=64$ and $n=58$ in STA and CLAD groups, respectively) with no difference in lung-Tx indication between the 2 groups ($p=0.80$). The proportion of induction therapy was lower in the CLAD than STA group, including the use of thymoglobulin or IL-2 antagonists (38 [53%] vs 55 [70%] in the STA group, $p=0.01$). Other factors, including initial disease, graft ischemic time, proportion of cytomegalovirus mismatches and PGD grade 3 (19), HLA mismatches at class I (HLA-A, HLA-B) and class II (HLA-DR, HLA-DQ) loci and preformed donor-specific antibodies did not differ between the 2 groups (Table 1).

Comparison of peripheral blood lymphocytes between STA and CLAD patients at 1, 6 and 12 months post-LTx

At 1 month post-LTx, STA and CLAD patients significantly differed in some peripheral blood lymphocyte subpopulations (Table 2). For CD4⁺ T cells, the proportion of CD4⁺CD25⁺CD127^{low} T cells was higher in CLAD than STA patients (1.8 ± 2.3 vs 0.9 ± 1.2 , $p=0.02$). The proportion of CD4⁺CD57⁺ T cells was higher in STA than CLAD patients (3.4 ± 6.5 vs 1.5 ± 2.5 , $p=0.04$). No significant differences were found for all other cell populations studied at 6 and 12 months post-LTx.

Increase in CD4⁺CD57⁺ILT2⁺ T-cell subset over a 6-month period during the first year post-LTx is higher in CLAD than in STABLE patients.

We measured the variation in T-cell subsets over a 6-month period during the first year post-LTx. For this, we compared data obtained at 1 and 6 months or at 6 and 12 months post-transplantation. We included 59 patients (n=31 STA and n=28 CLAD) with available samples at 1 and 6 months or at 6 and 12 months in this analysis.

Increased CD4+CD57+ILT2+ T-cell subset over a 6-month period during the first year post-LTx was associated with CLAD development. Indeed, this subpopulation was reduced by 20% in STA patients but increased 2.6-fold in CLAD patients ($p=0.01$) (Table 2). Representative flow cytometry results obtained for CD4+CD57+ILT2+ cell populations can be seen in Figure S1. Figure S1A shows data obtained for one phenotyping experiment (5 patients + 1 healthy control) in which two patients and the healthy donor had no CD4+CD57+ILT2+ circulating T cells whereas 2 patients had significant CD4+CD57+ILT2+ cell populations. Figure S1B shows the CD4+CD57+ILT2+ populations observed at 1 and 6 months for one stable patient with no change and two CLAD patients with increase in CD4+CD57+ILT2+ population proportions. We found no association between other T-cell subset variations within the first year and CLAD occurrence, and in particular, no significant difference between STA and CLAD groups in variation in CD4+CD57+ T cells not expressing ILT2 (CD4+CD57+ILT2^{neg} T cells) over the same period (6month CD4+CD57+ILT2- T-cell variation: 1 [1.2] in STA patients vs 1.2 [1.5] in CLAD patients, $p=0.62$).

Freedom from CLAD by increase in CD4+CD57+ILT2+ T cells over a 6-month period in the first year post-LTx.

CLAD developed in 26/57 (46%) patients with available data for 6-month T-cell subset variation during the first year post-LTx. Freedom from CLAD at 3 years was lower with a 6-month CD4+CD57+ILT2+ T-cell increase > 2.64-fold (n=15, 26% of patients) than \leq 2.64-fold (n=42): 3-year freedom frequencies were 27% (95% confidence interval [CI]: 8-50) versus 64% (95%CI: 48-77) (log-rank, p=0.014) (Figure 2A).

Graft survival by increase in CD4+CD57+ILT2+ T cells over a 6-month period in the first year post-LTx

Graft survival frequency did not significantly differ with an increase of CD4+CD57+ILT2+ T-cell > 2.64-fold versus \leq 2.64-fold over a 6-month period during the first year (log-rank=0.19): 3-year graft survival frequencies were 80% (95%CI: 50–93) versus 93% (95%CI: 79–98) (Figure 2B).

Freedom from CLAD and graft survival by proportion of CD4+CD57+ILT2+ T cells at 1, 6, and 12 months post-LTx

Besides results of kinetics of CD4+CD57+ILT2+ T cells within the first year post-LTx, we also looked at the incidence of CLAD and graft survival by proportion of CD4+CD57+ILT2+ T cells at different times. At 1 month post-LTx, freedom from CLAD at 3 years post-Tx was higher with proportion CD4+CD57+ILT2+ T cells \leq first quartile (25%) versus > first quartile (Figure 3A, log-rank test, p=0.028), with no difference in CLAD incidence according to this T-cell subset at 6 and 12 months post-

LTx. Graft survival did not differ by proportion of CD4+CD57+ILT2+ T cells at 1 month (Figure 3B, log-rank, $p=0.78$), 6 months, or 12 months post-LTx.

Determinants of CLAD onset and graft failure

The association of clinical, functional, histologic, and immunologic factors with CLAD onset and graft loss were assessed in univariate analysis and backward-elimination multivariable Cox regression analyses in 107 patients with available data for T-cell subsets at 1 month post-LTx. Several variables were associated with CLAD onset on univariate analysis, including no induction therapy ($p=0.04$), proportion of CD4+CD25+CD127^{low} lymphocytes at 1 month ($p=0.02$), and increased proportion of CD4+CD57+ILT2+ T cells over a 6-month period during the first year ($p=0.001$) (Table 3).

On multivariable analysis (model 1), only increased proportion of CD4+CD57+ILT2+ T cells over a 6-month period during the first year (per 0.1 unit increase) was an independent predictor of CLAD onset (hazard ratio = 1.25; 95% CI: 1.09-1.44; $p=0.001$, model 1; and hazard ratio = 1.3; 95% CI: 1.1-1.5; $p<0.001$, model 2, respectively) (Table 3). Similarly, a 6-month CD4+CD57+ILT2+ T-cell increase > 2.64-fold was also an independent predictor of CLAD onset (HR:3.04; 95% CI: 1.32-6.99; $p=0.009$, model 3)

On univariate analysis, only increased proportion of CD4+CD25+CD127^{low} T cells at 1 month was associated with graft failure ($p=0.005$) and remained associated on multivariable analysis (model 1) ($p=0.01$) (Table 3). Same observations were observed with model 2 of multivariable analysis taking into account only induction immunosuppression and T-cell subsets variables, showing that : 1) an increased proportion of CD4+CD57+ILT2+ T cells over a 6-month period during the first year

was associated with CLAD (HR:1.3 ; CI95%[1.1-1.5]; p<0.001), and that 2) an increase % of CD4+CD25+CD127low T cells at 1 month was associated with graft failure (HR: 1.2; CI95%[1.0-1.3]; p=0.02). Additionally, we also observed in model 2 of multivariable analysis that an increase % of CD4+CD25+CD127low T cells at 1 month was also associated with CLAD (HR: 1.2; CI95%[1.1-1.4]; p=0.003) (Table 3).

FUNCTIONAL EXPERIMENTS

CD4+CD57+ILT2+ T cells are cytotoxic and selectively inhibited by target-expressed HLA-G

After characterizing the CD4+CD57+ILT2+ T-cell phenotype, we investigated whether these cells display cytotoxic functions, and could act as proper CD4+ CTLs. For this purpose, we used a method published for CD8+ T cells that we adapted for CD4+ T cells and that allows for the selective analysis of cytotoxic degranulation by ILT2+ and ILT2- T cells in one tube (25). We used the monocyte cell line THP1 as target cells for COLT patient PBMCs and a selective analysis of cytotoxic degranulation with CD3+CD4+CD57+ILT2- or CD3+CD4+CD57+ILT2+ T cells from 1 healthy individual and 2 LTx recipients (patients 7044V2 and 6007V2; Figure 4A). Indeed, after engagement by anti-CD3-coated THP-1 cells, CD4+CD57+ILT2+ T cells showed cell-surface expression of CD107a, indicative of cytotoxic degranulation. This was not the case for their CD4+CD57+ILT2- counterparts.

Furthermore, expression of HLA-G1 by target cells (THP1-HLA-G1) significantly reduced the degranulation of CD3+CD4+CD57+ILT2+ T cells by 30% (Figure 4B).

HLA-G expressed by the target cells was directly responsible for the cytotoxic degranulation inhibition by CD4+CD57+ILT2+ T cells because the use of an anti-ILT2

antibody blocking the HLA-G1:ILT2 interaction totally reversed this inhibition (Figure 4B).

DISCUSSION

In this study, we aimed to investigate the potential role of the immune checkpoint HLA-G/ILT2 expressed by different peripheral T-cell subsets in predicting CLAD onset in LTx-recipients from the COLT cohort. Increased proportion of CD4⁺CD57⁺ILT2⁺ T cells in a 6-month period within the first year post-LTx (>2.6-fold increase) was associated with CLAD onset at 3 years. This subset increase was confirmed on multivariable analysis as the only independent predictor of CLAD among all other analyzed T-cell subsets. This increase at an early time, 1 month after LTx, was also associated with CLAD occurrence. Furthermore, functional experiments showed unexpected cytolytic properties of this newly identified CD4⁺CD57⁺ILT2⁺ T-cell subpopulation, which was selectively inhibited by target-expressed HLA-G. Also, an early increase at 1 month post-LTx of regulatory CD4⁺CD25⁺CD127^{low} T cells was associated with graft failure, which confirmed previous published findings (21).

Non-invasive tools for predicting graft dysfunction remain to be identified in LTx, in an attempt to intervene earlier to halt the progression of CLAD (3, 16). Our results showed the potential of the newly identified CD4⁺CD57⁺ILT2⁺ T-cell subset as a kinetics biomarker to stratify LTx recipients with increased risk of CLAD. The marker CD57 is a carbohydrate epitope present on replicative senescent cells (26, 27). It has been well described in the context of CD8 T cells (28, 29) and recently identified in kidney Tx recipients in whom CD4⁺CD57⁺PD1⁻ T cell populations were associated

with belatacept-resistant rejection after Tx (30). Phenotyping of CD4+CD57+ T cells showed that these cells were not senescent by traditional indices, with increased density in rejecting allograft tissue and high cytolytic properties (30). Our identification of the ILT2 receptor of HLA-G on CD4+CD57+ T cells allowed for predicting CLAD by using their increased proportion within the first year post-LTx, which was not the case for CD4+CD57+ T cells as a whole or other analyzed T-cell subsets. The identification of circulating CD4+CD57+ILT2+ T cells with a potential role for predicting CLAD suggests new pathophysiological pathways of CLAD. ILT2 is the only HLA-G receptor expressed by peripheral blood lymphocyte effectors and thus the key player of the immune checkpoint HLA-G for these immune cells. In the context of cancer, HLA-G expression by solid tumors has been associated with worse prognosis in numerous cancers including kidney, ovary, breast, lung, and colon (13). In bladder cancer, increased proportion of peripheral cytotoxic CD3+CD8+ILT2+ T cells was a strong predictor of cancer recurrence, which confirms that the HLA-G pathway may allow the immune evasion of tumoral cells through its *in situ* expression by carcinologic tissue (14, 22). In LTx, recipients without neo-expression of HLA-G in the graft may be more prone to such cytotoxic CD4+CD57+ILT2+ T cells insidiously insulting the lung graft, as other senescent T cells associated with BOS, such as CD28null lymphocytes recently identified (31). Because ILT2 is normally expressed by only a minority of CD4+ T cells (0–4%) and CD8+ T cells (5–20%), variations in its expression might affect the HLA-G capability to inhibit alloimmune effectors (14). HLA-G has a low polymorphism and its normal expression is highly tissue-restricted: besides being expressed in fetal tissues, such as trophoblast cells, HLA-G constitutive expression is found only in adult thymic medulla, cornea, pancreatic islets and erythroid and endothelial cell precursors (32).

Neo-expression of HLA-G in allograft cells, especially target cells in chronic rejection such as bronchial cells, have been found associated with graft stability and reduced risk of rejection in LTx and SOT recipient cohorts (6-10).

We showed that CD4⁺CD57⁺ILT2⁺ T-cells from healthy donors and LTx patients are cytolytic, as shown by their increased surface expression of the marker of cytolytic degranulation CD107a upon stimulation by target cells. Importantly, HLA-G expression by target cells significantly reduced the degranulation of CD3⁺CD4⁺CD57⁺ILT2⁺ cells while having no effect on their CD3⁺CD4⁺CD57⁻ILT2⁻ counterparts. This inhibition was reversed by anti-ILT2 antibody treatment, which demonstrates that it involved the HLA-G:ILT2 checkpoint. Taken together, our results suggest a dysregulated role of HLA-G in LTx patients with rejection, owing to an increased proportion of cytotoxic CD4⁺CD57⁺ILT2⁺ T cells, capable of damaging the lung graft without sizeable *in situ* HLA-G graft expression.

In addition to our focus on the HLA-G/ILT2 pathway, we also looked at circulating CD4⁺CD25⁺CD127^{low} regulatory cells, whose early percentage increase at 1 month was associated with increased CLAD onset at 3 years. These Tregs have been explored after SOT and LTx (21, 24, 33), with conflicting results. First, increased levels of CD4⁺CD25^{high}CD127^{low} and/or CD4⁺CD25^{high}FoxP3⁺ T cells were protective for CLAD in LTx cohorts (24, 33). In contrast, LTx recipients in whom CLAD developed within 3 years showed an early increased proportion of CD4⁺CD25^{high}FoxP3⁺ T cells at 1 month post-LTx (21). Our findings are in agreement with this last study (21), and we hypothesize that these Tregs could be negative immunoregulators attempting (without success) to counteract cellular insults directed toward the graft.

Our study has some limitations. Although the analysis involved a sizeable number of patients, the availability of T-cell sampling in the COLT study did not allow for a high number of cases for kinetics analysis of T cells during the first year post-LTx. Another potential limitation of our study is the possible bias due to significant differences in the proportion of CLAD and stable patients that received induction immunosuppression, because induction therapy (thymoglobulin or IL2 receptor antagonist) may have significant impact on T-cell subsets. To investigate this issue, multivariate Cox models were also built (model 2) including only induction immunosuppression and T-cell subset variables selected by univariate analyses. Same findings were observed, with increased proportion of CD4+CD57+ILT2+ T cells in a 6-month period associated with CLAD, and increase % of CD4+CD25+CD127low T cells at 1 month associated with poorer outcome (increased risk of graft failure and CLAD onset at 3 years). Validation of these preliminary results in larger cohorts of patients is needed to assess the precise role in clinical practice. Also, classification of the patient's medical condition is a crucial issue for identifying predictive biomarkers, and we did our best to classify patients with stable graft function (STA) or CLAD at 3 years post-LTx by using repeated sessions of an ad-hoc adjudication multidisciplinary committee. Biologics targeting rationally selected disease-relevant pathways have become the standard of care for many immunological diseases. A similar development has not yet occurred in the field of transplantation. Identification of new specific T-cell subsets may offer the opportunity to investigate targeted therapies, such as counteracting insults of CD4+CD57+ILT2+ with HLA-G-inducing treatment or an HLA-G-vehicle component.

In conclusion, increased proportion of CD4+CD57+ILT2+ T cells during the first year post-LTx allowed for discriminating subsequent CLAD and STA patients at 3 years post-Tx in our cohort. This potential novel biomarker may represent a non-invasive predictor for stratifying the risk of CLAD onset. We could demonstrate the cytotoxic properties of these CD4+CD57+ILT2+ T cells, which may be involved in cellular injuries observed in CLAD patients. Additional studies are needed in larger cohorts to validate this new HLA-G–linked biomarker, in parallel with deciphering the precise role of the HLA-G:ILT2 immune checkpoint after LTx.

Discloser statement

The authors have no conflict of interest to disclose

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Authors' contributions

O.B., N.R-F., J.L., and E.C. developed concept and designed experiments; O.B. wrote the manuscript, and N.R-F. and J.L. performed critical review of the manuscript and revised accordingly. D.M., J.L., and I.K. processed

sampled and performed experiments; S.H., B.R-P., M.R-G., A.N., V.B., J.M., X.D., J.M., J.L., G.D., C.S-R., L.F., A.M., C.P., A.R., collected the samples. O.B., N.R-F., J.L., AV and J.T analyzed data.

LEGENDS OF FIGURES

Figure 1. Flow Chart of patients

Figure 2. (A) Comparison of freedom from chronic lung allograft dysfunction (CLAD) between patients with a 6-month CD4+ILT2+CD57+ T cells (% of CD4+ T cells) fold increase >2.64 and those with a variation ≤ 2.64 fold increase. **(B)** Comparison of freedom from chronic lung allograft dysfunction (CLAD) between patients with a 6-month CD4+ILT2+CD57+ T cells (% of CD4+ T cells) fold increase > 2.64 and those with a variation ≤ 2.64 fold increase.

Figure 3. (A) Comparison of graft survival between patients with a % of CD4+CD57+ILT2+ T cells (% of CD4+ T cells) \leq first IQR (25%) at 1 month post-LTx versus those with % CD4+ILT2+CD57+ T cells (% of CD4+ T cells) $>$ first IQR (25%). **(B)** Comparison of freedom from chronic lung allograft dysfunction (CLAD) between patients with a % of CD4+CD57+ILT2+ T cells \leq first IQR (25%) at 1 month post-LTx versus those with % CD4+ILT2+CD57+ T cells $>$ first IQR (25%).

Figure 4. Example of (A) cytotoxic function of CD3+CD4+CD57+ILT2+ T cells from healthy donor, or two patients TxP 7044V2 and TxP 6007V2; and (B) inhibition of this cytotoxic function by HLA-G. (A) The monocytic cell line THP1 (ATCC) was used as target cells facing either CD4+CD57-ILT2- or CD4+ CD57+ ILT2+ effector cells from healthy donor or from two patients TxP 7044V2 and TxP 6007V2. (B) THP1 Target cells were transduced or not to express membrane-bound HLA-G1 (THP1 and THP1-HLA-G cells). THP1 and THP1-HLA-G cells were used as target cells facing either CD4+CD57-ILT2- or CD4+ CD57+ ILT2+ effector cells. ILT2 was blocked by the use of 20 μ g/mL of a blocking anti-ILT2 antibody (clone GHI/75, BioLegend) or a control antibody. The Effector:Target ratio used here was PBMC:Target of 2:1 in each well. The cytolytic degranulation by CD4+CD57+ILT2+ or CD4+CD57-ILT2- T cell subsets were evaluated using the percentage of CD107a-positive cells.

Figure S1. Representative flow cytometry results obtained for the CD4+CD57+ILT2+ cell population. Analyses shown were gated on CD19⁻ CD3⁺CD8⁻ CD4⁺ lymphocytes (CD4+ T cells). Percentages indicate the proportion of CD57+ILT2+ cells within the CD4+ T cell gate. **(A)** Results obtained for one phenotyping experiment, which always contained samples from five patients and one healthy blood donor. CD57 and ILT2 expression by CD4+ T cells is shown. **(B)** Evolution of CD4+CD57+ILT2+ T cell populations for one stable patient (STABLE) and two CLAD patients (CLAD) between the first month (M1) and the sixth month (M6) after transplantation is shown.

SUPPLEMENTAL DATA

Prediction of chronic lung allograft dysfunction by measurement of circulating cytotoxic CD4+CD57+ILT2+ T cells, selectively inhibited by the immune check-point HLA-G

MATERIALS AND METHODS

Patients and study design

LTx patients from the multicentric longitudinal cohort COLT (Cohort in Lung Transplantation, NCT00980967) were analyzed in the present study. The cohort COLT included 11 French lung transplantation centers (Programme transplantation 2008, PRTP-13, ClinicalTrials.gov Identifier: NCT00980967). The University Hospital Ethical Committee of Nantes and the Committee for the Protection of Patients from Biologic Risks approved this study (Comité de Protection des Personnes Ouest 1-Tours, 2009-A00036-51). All patients who participated in this study gave informed consent. Inclusion criteria of patients in this study were as follows : (i) at least 1 available blood samples at 1 and/or 6, and/or 12 months post-Tx, and (ii) date of CLAD diagnosis after 12 months post-Tx in patients who developed CLAD within the 3 years post-Tx, so that all the patients in the following analysis display normal graft function at all time-points of cells analysis, prior to CLAD onset in the CLAD group. One hundred fifty patients from the COLT cohort were enrolled. We planned to compare relatively similar numbers of patients in each group (STABLE group versus CLAD group). These successive patients of the COLT study were selected according to the availability of blood samples. We started first with the screening of all successive CLAD patients previously included in COLT study who met our predefined criteria for enrollment in our study, that is “ (i) at least 1 available blood samples at 1 and/or 6, and/or 12 months post-Tx, and (ii) date of CLAD diagnosis after 12 months post-Tx, and within the first 3 years post-LTx. All CLAD patients from COLT cohort with these criteria were enrolled (n=72 CLAD patients). Concomitantly, we screened a relatively similar number of patients who remained stable at 3 years post-TxP, to allow statistical comparison between STABLE group versus CLAD group. Hence, n=78

successive stable patients of the COLT study were selected according to the availability of blood samples, among all stable patients of COLT cohort.

Among the n=150 patients, n=91 had only 1 available sample, including n=60 at M1, n=25 at M6, and n=6 at M12. Additionally, n= 59 had at least 2 samples. Among these n=59 patients, n= 47 had 1 and 6 months, and n=12 had 6 and 12 months sampling and were studied for their Δ 6 month ratio.

Monitoring protocols of patients.

Diagnosis of primary graft failure (PGD)

Diagnosis of primary graft failure (PGD). was diagnosed and graded according to ISHLT criteria (1).

Diagnosis of acute rejection (AR) episodes

Surveillance of cellular acute rejection (AR) episodes was left at the physician's discretion of each local LTx centers. Diagnosis of cellular acute rejection (AR) episode and results of transbronchial biopsies (TBBx) were recorded when performed during a protocol visit at month 1, 6, 12, 18, 24, 30, 36 post-LTx. An AR rejection episode was defined as AR grade ≥ 1 according to the International Society for Heart and Lung transplantation (ISHLT).

Diagnosis of chronic allograft dysfunction (CLAD)

Pulmonary function tests (PFTs), including spirometry and plethysmography when available, clinical data, and blood samples were collected at 1-month post-LTx and every six months post-transplantation within the 3 years post-LTX. Clinical data collection allowed for diagnosis of chronic allograft dysfunction (CLAD) during follow-up at 3 years post-LTx, according to International Society for Heart and Lung Transplantation (ISHLT) (2). Diagnosis of CLAD was performed in case either of diagnosis of bronchiolitis obliterans syndrome (BOS) (2, 3), or restrictive allograft syndrome (RAS). BOS was defined by the classical definition of ISHLT (2, 3), and RAS defined by recent classification of ISHLT (4), or by previously used spirometric criteria of RAS previously (5). Stable graft function (STA) or CLAD status at 3 years-post-LTx were confirmed by repeated sessions of an ad-hoc

adjudication multidisciplinary committee using pulmonary function tests, CT-scan, and after elimination of confounding factors according to the ISHLT practice guidelines (3). All included patients were followed-up at least 3 years post-LTx and classified according to their 3-years post-Tx functional status as stable patients (STA group) or patients with CLAD (CLAD group). Characteristics of recipient and donor, and immunologic data were recorded, including the followings: recipient age, initial disease, type of LTx, cytomegalovirus (CMV) mismatch (yes or no), ischemic time (in minutes), primary graft dysfunction grade 3 at 72 hours post-LTx (yes or no) (1), perioperative ECMO (yes or no), post-LTx diabetes melitus (yes or no), AR episode, when occurring at the date of follow-up visit of COLT study, number of HLA-A, or -B, or -DR, or -DQ mismatches, detection of pre-formed donor-specific antibodies (DSA) (yes or no), and the use of induction therapy (including thymoglobulin or IL-2 antagonist).

Immunosuppression

The post-LTx immunosuppressive treatment was left at the physician's discretion of each local LTx centers. Data of each patient included in COLT cohort were recorded at each time point of COLT protocol (day-0, month 1, 6, 12, 18, 24, 30, 36 post-LTx), including: 1) the use or not of induction therapy with antithymocyte globulin or IL-2 inhibitors (Daclizumab or basiliximab; 2) Type of maintenance immunosuppressive therapy with calcineurin inhibitors (tacrolimus or ciclosporine).

HLA typing

HLA typing of recipients was performed according to protocol of each local LTx center. For the 4 centers located in Ile-de France area, HLA typing was performed by molecular biology. For all LTx donors, HLA-A/B/DR/DQ tissue typing involved the microlymphocytotoxicity technique with tissue-typing trays (One Lambda Inc.), and was controlled by molecular biology (Innolipa HLA typing kit; Innogenetics, Gent, Belgium) (6).

Detection of allo-immunization in LTx candidates

Detection of allo-immunized candidates for LTx was performed by Lumionex assay, according to a technique let at the discretion of each center. For the 4 LTx center of Ile-de-France area, detection of anti-HLA abs directed against HLA Class I and Class II antigens by Luminex assay (LSA, One Lambda, Canoga Park, CA) was performed in routine in the Immunology laboratory of Saint-Louis hospital. Patients were screened for anti-HLA antibodies by Luminex assay, and Luminex-detected abs directed against HLA Class I and Class II antigens were also identified by SAFB Luminex assay (One Lambda, Canoga Park, CA). Identification of anti-HLA-A, -B, -Cw, -DR, -DQ, and -DP antibodies by Luminex used the references LS1A04 and LS2A01 from One Lambda for classes I and II respectively. Antibodies were detected by the use of goat anti-human IgG coupled with phycoerythrin. The fluorescence of each bead was detected by use of a reader (LABscan; Luminex, Austin, TX) and recorded as mean fluorescence intensity (MFI). The antibody positivity threshold was set at a normalized MFI of 500 according to the baseline formula of the Fusion software (One Lambda).

Flow cytometry analysis

Peripheral blood Mononuclear Cells (PBMCs) obtained from COLT patients at each visit were here analyzed. PBMCs were isolated by Ficoll gradient centrifugation (Ficoll-Paque, LifeSciences), frozen and stored at -80°C until investigation. After thawing, cells were washed and incubated for 1h at 37°C. In order to avoid any non-specific binding of the labeling antibodies, Fc receptors were blocked with polyclonal human Ig, prior to labelling. To ensure that all stainings were comparable, automated staining was used: antibody mixes were prepared beforehand for several experiments, and the robotic arm of the cytometer performed all pipetting and incubations times prior to automatically acquire the samples.

Cells were then labeled using automated pipetting by the flow cytometer prior to being automatically acquired. This ensured that all incubation times and pipetting were perfectly identical among all samples of the study. To control for inter-experiment variability, each staining experiment consisted of 6 PBMC samples, from 5 COLT patients and one healthy blood donor. Control peripheral blood mononuclear cells (PBMCs) were obtained from voluntary blood donors of the French Blood Establishment: this collection was authorized by the Etablissement francais du sang- Commissariat a l'energie atomique convention (no. 13/EFS/101).

The following monoclonal antibodies (mAb) were used for cell-surface staining of PBMC:

From Miltenyi Biotec: CD19-VioGreen, CD3 PerCP-Vio700, CD4 Vio-Bright FITC, CD8 APC-Vio770, CD57 PE-Vio770, CD127 PE-Vio770. From eBioscience: ILT2 PE, ILT2-APC, and CD25 eFluor450. From Biolegend: PD1 BV421. To control for background staining, an isotypic control panel was run for each sample, that included CD19, CD3, CD4, CD8, and CD57 antibodies, and isotypic control antibodies for non-lineage markers such as ILT2.

The following T cells subsets, combining different cell-surface were analyzed, as % of CD4+ or CD8+ T cells: CD4+CD57+ T cells, CD4+ILT2+ T cells, CD4+PD1+ T cells, CD4+ILT2+PD1+ T cells, CD4+ILT2negCD57+ T cells, CD4+ILT2+CD57+ T cells, CD8+CD57+ T cells, CD8+ILT2+ T cells, CD8+PD1+ T cells, CD8+ILT2+CD57+ T cells, CD8+ILT2+PD1+ T cells, CD8hiCD57+ T cells, CD8hilILT2+ T cells, CD8hiPD1+ T cells, CD8hilILT2+CD57+ T cells, CD8hilILT2+PD1+ T cells, CD8loCD57+ T cells, CD8loILT2+ T cells, CD8loPD1+ T cells, CD8loILT2+CD57+ T cells, CD8loILT2+PD1+ T cells, CD4+CD25+ T cells, CD4+CD127+ T cells, CD4+ILT2+CD25+ T cells, CD4+ILT2+127+ T cells, CD8+CD25+ T cells, CD8+CD127+ T cells, CD8+ILT2+CD25+ T cells, CD8+ILT2+CD127+ T cells, CD8hiCD25+ T cells, CD8hiCD127+ T cells, CD8hilILT2+CD25+ T cells, CD8hilILT2+CD127+ T cells, CD8loCD25+ T cells, CD8loCD127+ T cells, CD8loILT2+CD25+ T cells, CD8loILT2+CD127+ T cells. Acquisition was made on a MACSQuant 10 flow cytometer (Miltenyi Biotec); analysis was performed using the MACSQuantify software (Miltenyi Biotec) and FlowJo software using appropriate isotypic controls to account for

nonspecific background staining of the cell subsets of interest. All available blood sample at time points 1 month (mo), 6 mo, and 12 mo were analyzed by cytometry. Analysis was performed according results of flow cytometry at each time point (1, 6, and 12 months), and also according to kinetics of each T-cell subset within the 1st year post-LTx, because variations of peripheral T-cell subpopulations have been associated to CLAD onset (7). For kinetics analysis, we measured the variation of T-cell subsets over a 6-month period during the 1st year post-Tx. For this, we compared data obtained at 1 and 6 months post-Tx, or at 6 and 12 months post-Tx in all LTx recipients with at least 2 available samples at these time-points. Percentage of variation of 1-6 months or 6-12 months was calculated for all cell subsets, and expressed as 6 months T cell subpopulations variation within the 1st year (6 month T-cell subsets ratio).

Functionnal Assays

Cell lines

Functional analysis was performed to assess the cytolytic properties of T cell subpopulations of interest identified in the LTx cohort. For functional studies, the monocytic cell line THP1 (ATCC) transduced or not to express membrane-bound HLA-G1 (*i.e.*, THP1-HLA-G1 and THP1, respectively) was used as target cells. Lentiviral vector and transduction protocol were previously described (8). Cell-surface expression of HLA-G on THP1-HLA-G1 cells was confirmed using a PE-conjugated HLA-G mAb (clone MEM-G9, Exbio), and lack of ILT2 cell-surface expression was confirmed, using a PE-conjugated ILT2 mAb (clone HP-F1, eBioscience) by flow cytometry as previously described (8). THP1/THP1-HLA-G1 cells were cultured in RPMI (Sigma) supplemented with 10% FCS and antibiotics.

Peripheral blood T cell degranulation

Cytotoxicity assays were CD107a degranulation assays. CD107a is a lysosome-associated membrane glycoprotein (LAMPs) that is expressed at the surface of immune cells upon cytolytic degranulation. The percentage of CD107a-expressing-cells at the end of the assay

was used as a marker of cytolytic-degranulation, and thus of cytotoxic-killing of the target cells, as previously described (9). Briefly, THP1 or THP1-HLA-G cells were first incubated for 72h in flat-bottom 96-well culture plates in culture medium containing 50ng/ml PMA (Sigma) in order to obtain a confluent macrophage layer (mTHP1/mTHP1-HLA-G). Prior to use in functional experiments, macrophage-differentiated mTHP1/mTHP1-HLA-G1 cells were coated for 15 min with 50ng/ml anti-CD3 mAb (clone OKT3, Orthoclone). In blocking experiments, PBMCs from LTx recipients were incubated for 20 min at 37°C with 20 µg/mL of a blocking ILT2 antibody (clone GHI/75, BioLegend) or a control antibody (BioLegend). PBMCs were then added to the OKT3-coated mTHP1/mTHP1-HLA-G target cells in culture medium supplemented with monensin and brefeldin A (Protein Transport Inhibitor Cocktail, eBioscience) in the presence of a PE-conjugated CD107a antibody (clone H4A3, BioLegend) or isotype control. After 4 hr of co-incubation at 37°C, cells were washed and stained for flow cytometry analysis. Acquisition was performed on a MACS Quant10 flow cytometer (Miltenyi Biotec) and data analysis on FlowJo10 Software. The cytolytic degranulation by CD3+ T cell subsets was evaluated using the percentage of CD107a-positive cells. Advantages of the CD107a assay have been recently highlighted when investigating ILT2+ immune cell subsets (9). Briefly, CD107a is a lysosome-associated membrane glycoprotein (LAMPs) that is expressed at the surface of immune cells upon cytolytic degranulation. Consequently, the percentage of CD107a-expressing-cells at the end of the assay is used as a marker of cytolytic-degranulation, and thus of cytotoxic-killing of the target cells. This flow cytometry-based assay allowed us to discriminate between ILT2⁺ and ILT2⁻ T cells, thereby confirming that HLA-G inhibits CD4⁺T cell-mediated cytotoxicity through ILT2 interaction.

Statistics

Continuous variables are described with mean (SD), or median (range), and were compared by Student *t* test or Mann–Whitney U-test. Categorical variables are described number (%) and were compared by chi-square test or Fisher' exact tests. Proportions of cell subsets at

different time points post-TxP or the 6-month T-cell ratio were compared between stable and CLAD patients groups, using a Bonferroni correction for the n=4 T-cells subsets expressing both CD57+marker and ILT2 receptor (15)(26, 27, 28), and the n=32 T-cells subsets tested at random, whereas no correction was applied for the CD4+CD25+CD127+ T-cells (23).

Freedom from CLAD and graft survival were estimated by the Kaplan-Meier estimator and the log-rank test was used to compare the event-free survival curves between groups..

Univariate and multivariable Cox models were built to assess the association between % of cells subpopulations at time-point 1-month post-TxP or 1-12 months T-cells ratio and freedom from CLAD survival and graft survival, with adjustment for potential confounding factors. Factors associated with freedom from CLAD and graft survival by univariate analyses (at a significance of $p < 0.2$) were selected for multivariate analyses. A backward stepwise selection was used to perform the final multivariate model (model 1), with an alpha-to-stay level of 0.05. Among cells populations, CD4+CD25+CD127low, CD8+ CD25+, and CD4+CD57+ILT2+ T cells assessed at Time-point 1-month post-LTx and the 6-month CD4+CD57+ILT2+cells ratio were included in the Cox model as a time independent covariate. For univariate and multivariable Cox models built to assess the association between 6-month T cells ratio and freedom from CLAD survival and graft survival, analysis was reduced to the n=59 patients with at least 2 available different time-point samplings during follow-up. By comparing data obtained at 1 and 6 months post-Tx or at 6 and 12 months post-Tx for all patients with available samples at these timepoints, we investigated a single variable defined as the 6-month T-cell ratio within the first-year post-Tx. Graft survival, freedom from CLAD and follow-up were computed from the 12-month post-LTx according to the 6-month CD4+CD57+ILT2+ T cells variation calculation, because all events as CLAD onset or death occurred after 12-month post-LTx in all analyzed patients. Hence, for all variables collected after time-0 post-LTx, that is, 1-month T-cell variable and 6-month T-cell ratio calculated at 6-month or 12-month post-LTx, the landmark time was 12-month post-LTx.

This allowed to observe all event as CLAD onset or death, because predefined inclusion criteria of patients included a date of CLAD diagnosis after 12 months post-Tx in patients showing CLAD within 3 years post-Tx, and a stable lung function at 3 years post-LTx in the stable group.

Additionally, due to the major potential impact of induction therapy (Thymoglobulin or IL2 receptor antagonist) on T-cell subsets, multivariate Cox models were also built that includes only induction immunosuppression and the T-cell subset variables selected by univariate analyses (model 2). Also, Mean % of CD4+CD57+ILT2+ T-cell and CD4+CD25+CD127+ T-cell subsets at month1 (M1), M6, M12 were compared between patients according to their induction therapy (no induction therapy, no induction group; Thymoglobulin group; and IL2 receptor antagonist [IL2 R group]).

The last follow-up date for all survival models was 3 years post-LTx. All analyses were two-tailed and $p < 0.05$ was considered statistically significant. Analyses involved the use of Stata v12 for Windows (StataCorp LP, College Station, TX).

Because induction therapy (thymoglobulin or IL2 receptor antagonist) may have significant impact on T-cell subsets, we also compared mean % of CD4+CD57+ILT2+ T-cell and CD4+CD25+CD127+ T-cell subsets at month1 (M1), M6, M12 between patients according to their induction therapy (no induction therapy, no induction group; Thymoglobulin group; and IL2 receptor antagonist [IL2 R group]). We checked the assumptions underlying our Cox models, and had confirmation of the proportional hazards assumption (model 1 and 2). Additionally, we assessed the linearity assumption for continuous covariates, and also built a third Cox model (model 3) including the CD4+CD57+ILT2+ T-cell ratio as a categorical variable.

This study was approved by an institution ethics committee and conducted in accordance with good clinical practices and the recommendations concerning human research contained

in the Declaration of Helsinki. All patients gave their informed consent to be included in the study.

RESULTS

Comparison of the mean % of CD4+CD25+CD127+ T-cell subset at M1 showed a significant difference between the 3 groups with a mean of 1.25% (1.38%) in the group without induction, versus 1.98% (2.62%) in the Thymoglobulin group, and 0.76% (1.20%) in the IL2-RA group ($p=0.04$), suggesting that induction therapy could alter the % of these cells subset, with the higher % with thymoglobulin induction, and the lower % with IL2RA at M1.

Nevertheless, when performing multivariate analysis taking into account %

CD4+CD25+CD127low T-cell subset at 1 month and only induction therapy as co-variate (model 2), the increased % CD4+CD25+CD127low T-cell subset at 1 month still remained independently associated with both CLAD and graft failure which argues for an independent role of increased % of this T-cell subset at M1 as a predictor of CLAD.

Comparison of the mean % of CD4+CD57+ILT2+ T-cell subset at M1 showed only a trend without significant difference between the 3 groups, with a mean of 1.07% (2.40%) in the no induction group, versus 0.61% (1.19%) in the Thymoglobulin group, and 1.74% (0.46%) in the IL2RA group ($p=0.06$). No significant differences were observed between the 3 groups at M6 (no induction group: 2.36% (5.52%); Thymoglobulin group: 0.46% (1.07%); IL2-inh group 0.76% (0.96%), $p=0.11$), or at M12 (no induction group: 1.37% (2.12%); Thymoglobulin group: 0.87% (0.94%); IL2-inh group 0.80% (0.74%), $p=0.47$). This could also suggest a direct role of induction therapy on the association between increase CD4+CD57+ILT2+ T-cell subsets ratio within the 1st year and CLAD onset that we observed. Nevertheless, multivariate analysis taking into account CD4+CD57+ILT2+ T-cell subsets ratio and only induction therapy as co-variate in the model (model 2), the increased CD4+CD57+ILT2+ T-cell subsets ratio still remained independently associated with CLAD.

We also compared the proportion of CD4+CD57+IL2+ T cells at M1, M6, and M12 in CLAD patients between those that did versus did not receive induction. No differences were observed at M1, M6, and M12 between the 3 groups of patients: no induction (1.02% [1.04%] at M1; 2.36% [4.86%] at M6; 0.82% [1.58%] at M12), versus patients who received thymoglobulins (0.26% [0.29%] at M1; 0.26% [0.18%] at M6; 0.91% [1.08%] at M12), versus those with IL2RA (1.00% [1.74%] at M1; 0.38% [0.33%] at M6; 0.17% [0.24%] at M12) (difference between the 3 groups: p=0.31 at M1, p=0.33 at M6; p=0.77 at M3). We also compared CD4+CD25+CD127^{low} T cells at M1, M6, and M12 in CLAD patients between those that did versus did not receive induction. No differences were at M1, M6, and M12 between the 3 groups of patients: no induction (0.82% [1.36%] at M1; 1.96% [4.08%] at M6; 1.40% [2.28%] at M12), versus patients who received thymoglobulins (0.44% [0.63%] at M1; 0.30% [0.19%] at M6; 0.91% [1.08%] at M12), versus those with IL2RA (0.64% [1.23%] at M1; 0.93% [1.12%] at M6; 0.16% [0.17%]), (p=0.49 at M1, p=0.31 at M6, and p=0.54 at M12).

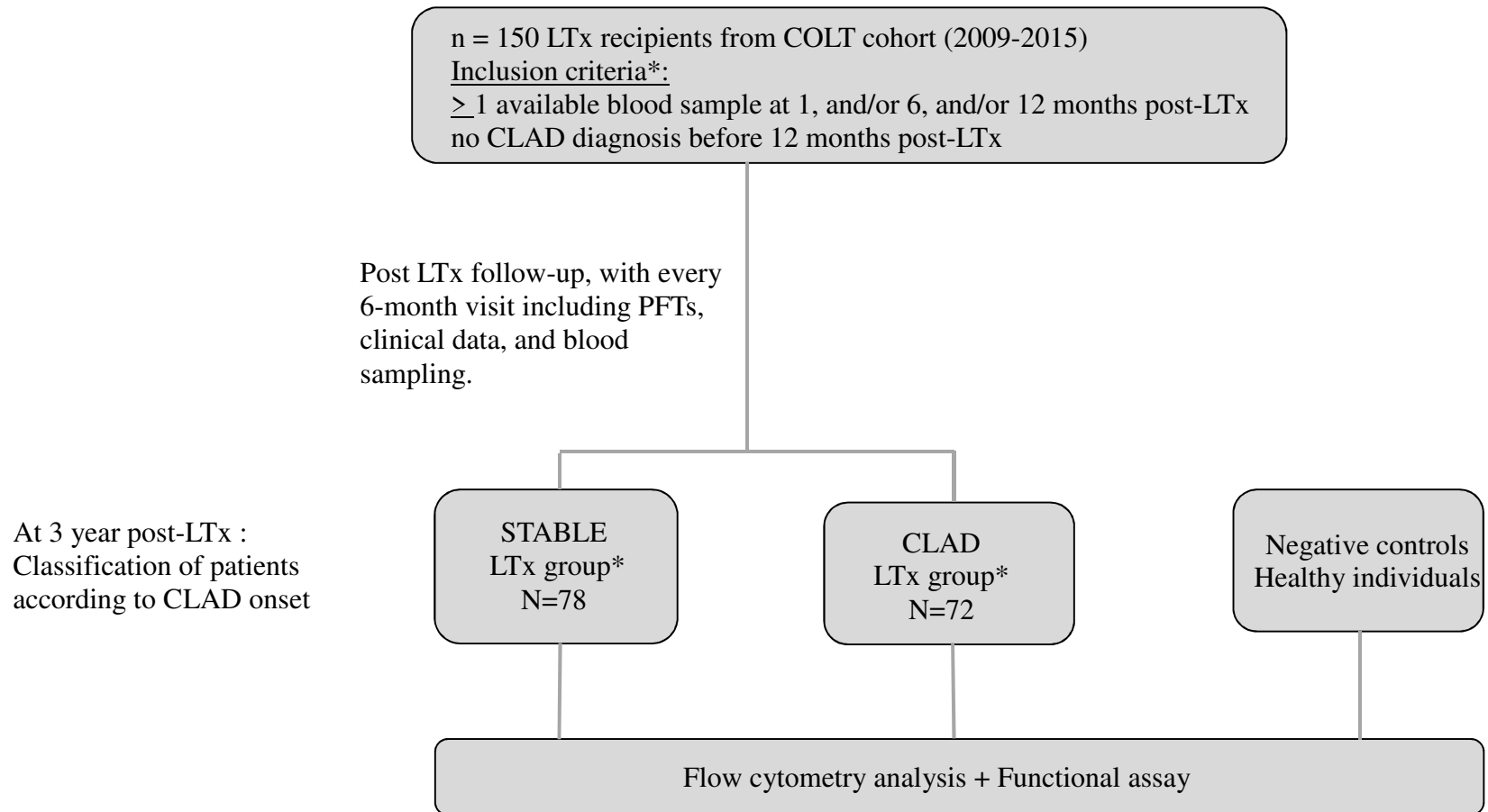
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Figure 1. Study Flow Chart



LTx, lung transplantation; COLT, cohort for Lung Transplantation; PFTs, pulmonary function tests; CLAD, chronic lung allograft dysfunction.* All CLAD patients from the COLT cohort with these criteria were enrolled (n=72), and concomitantly, n=78 successive stable patients of the COLT study were selected according to the availability of blood samples, among all stable patients of COLT cohort

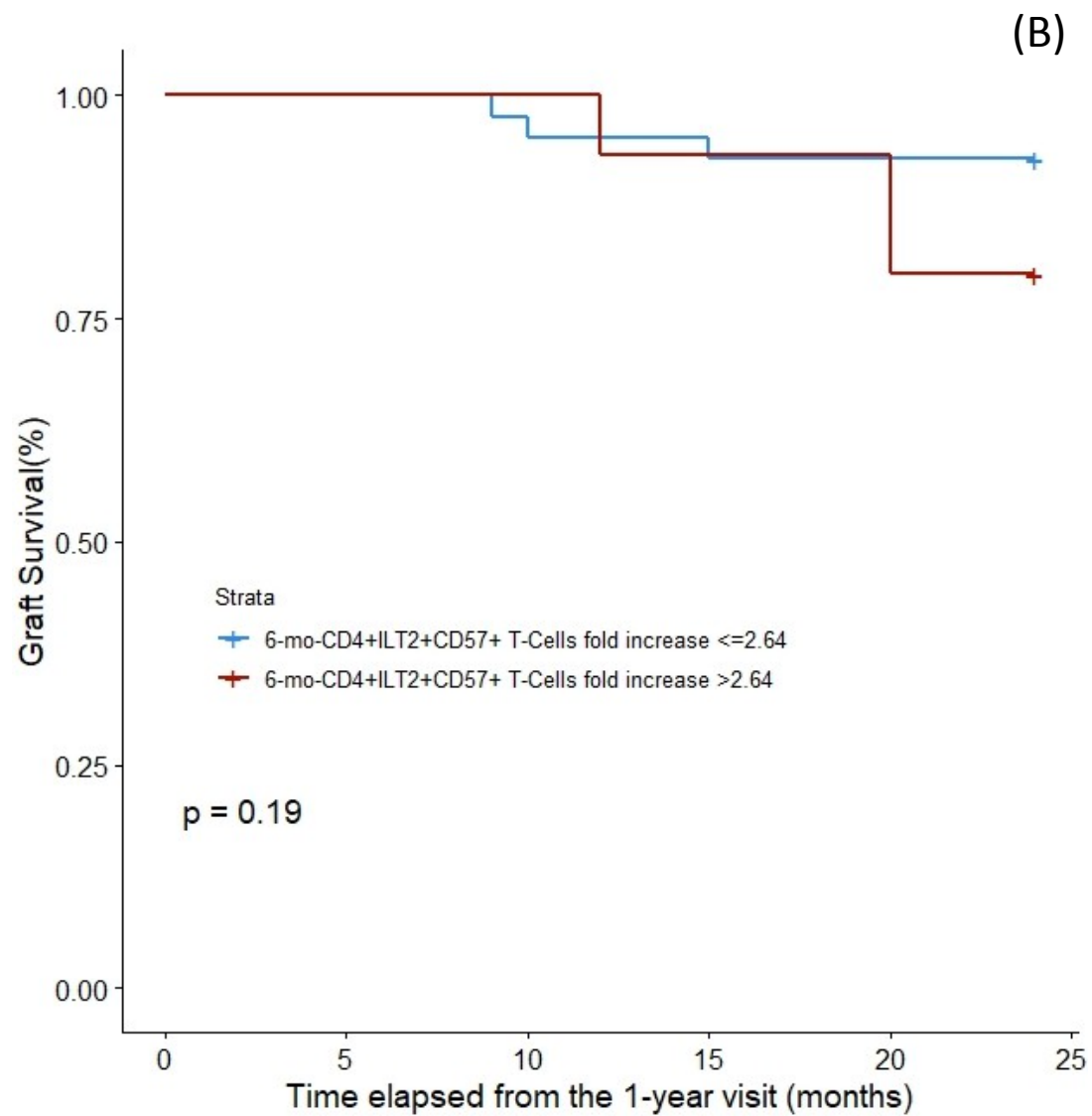
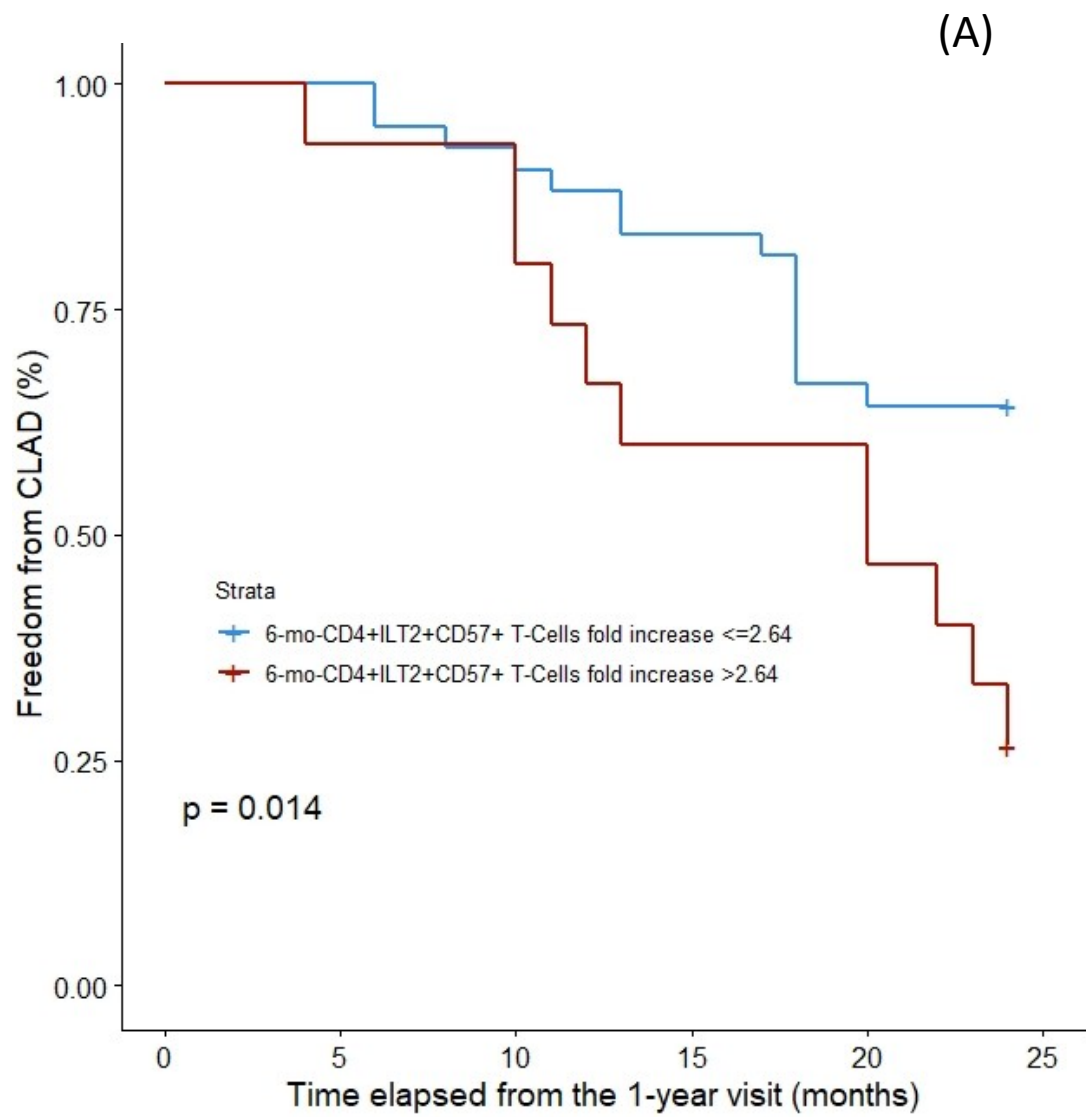


Figure 2.

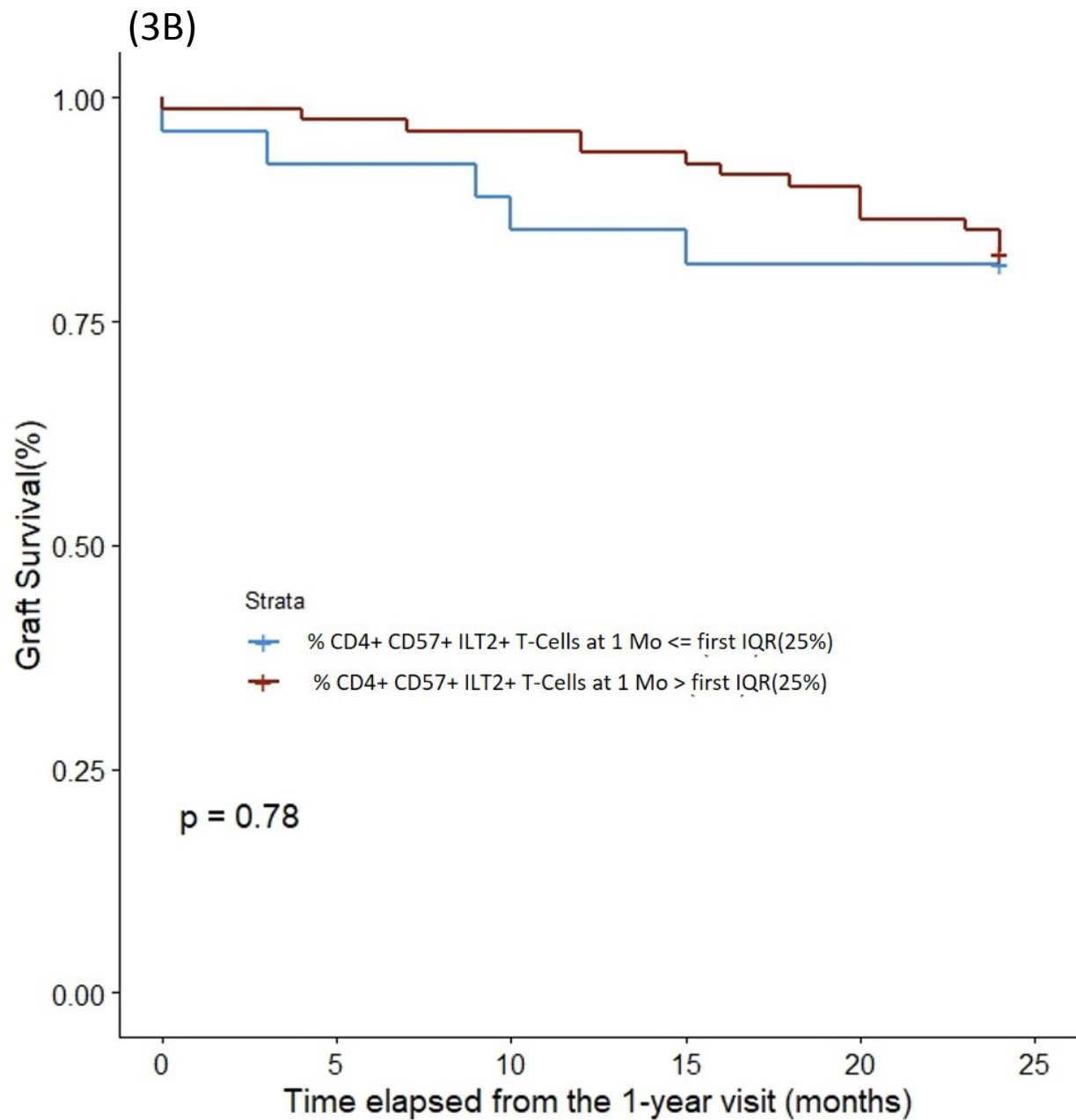
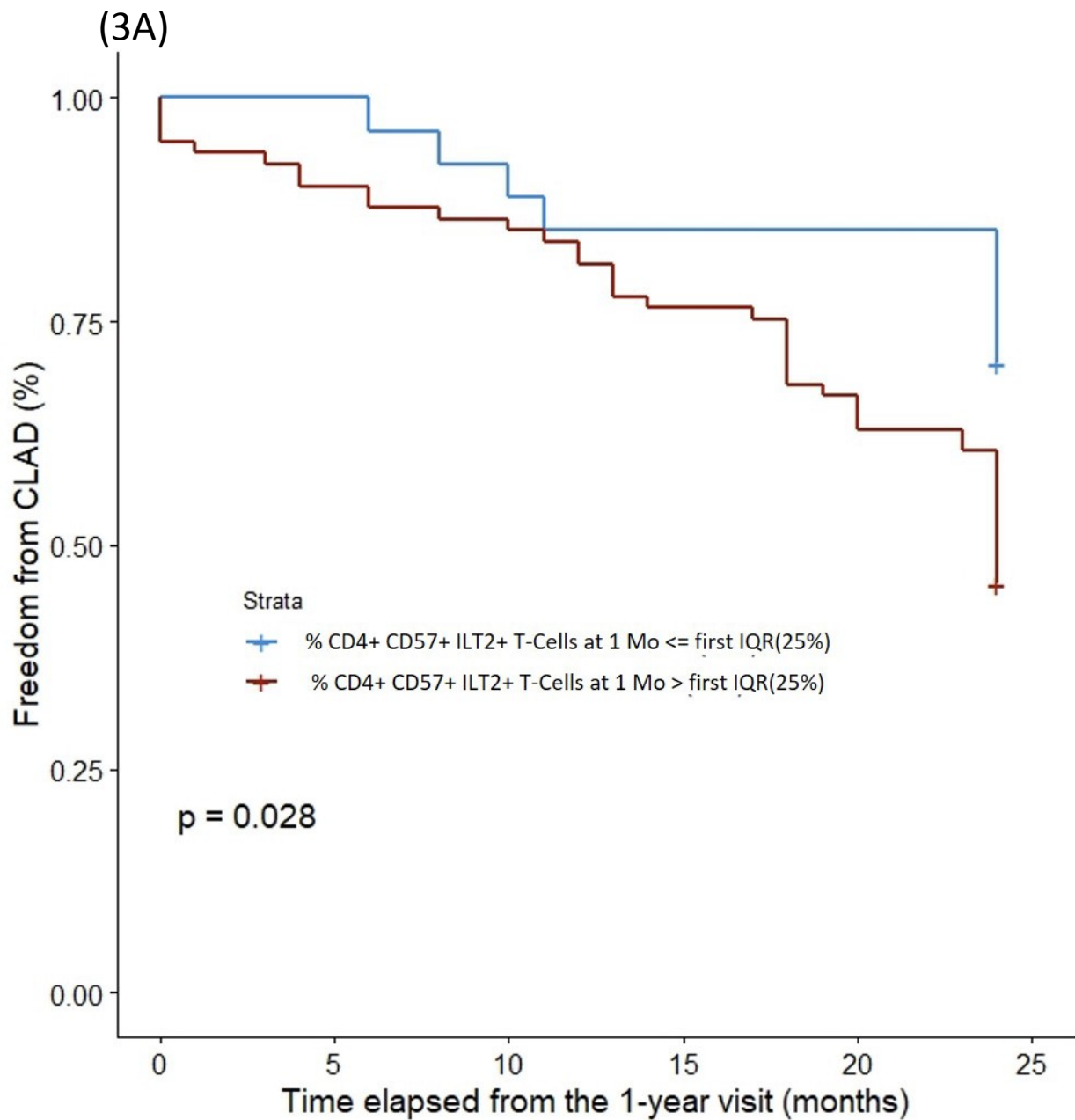
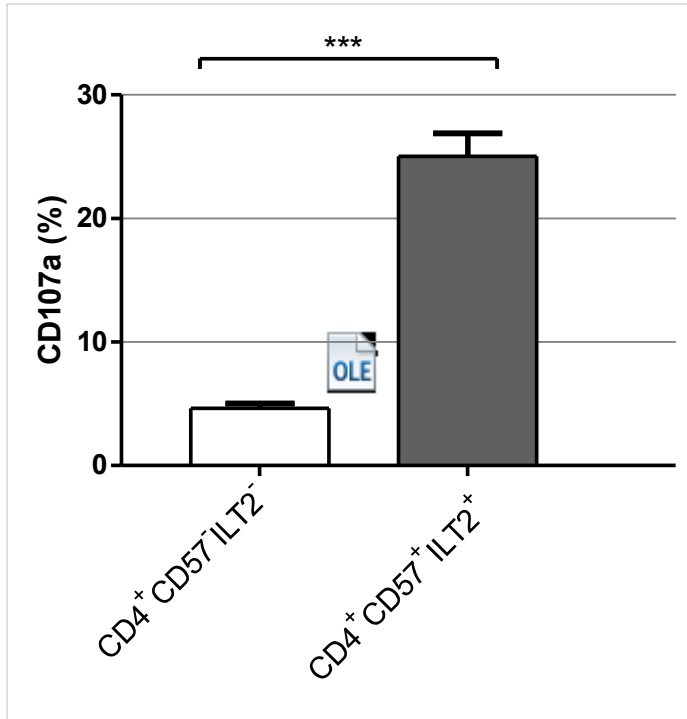


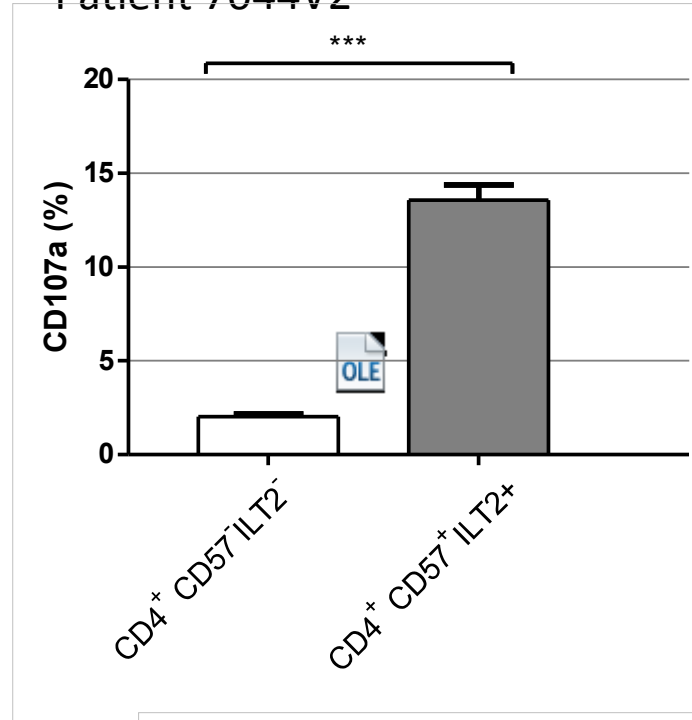
Figure 3

A. Cytotoxic function of CD4+CD57+ILT2+ T cells

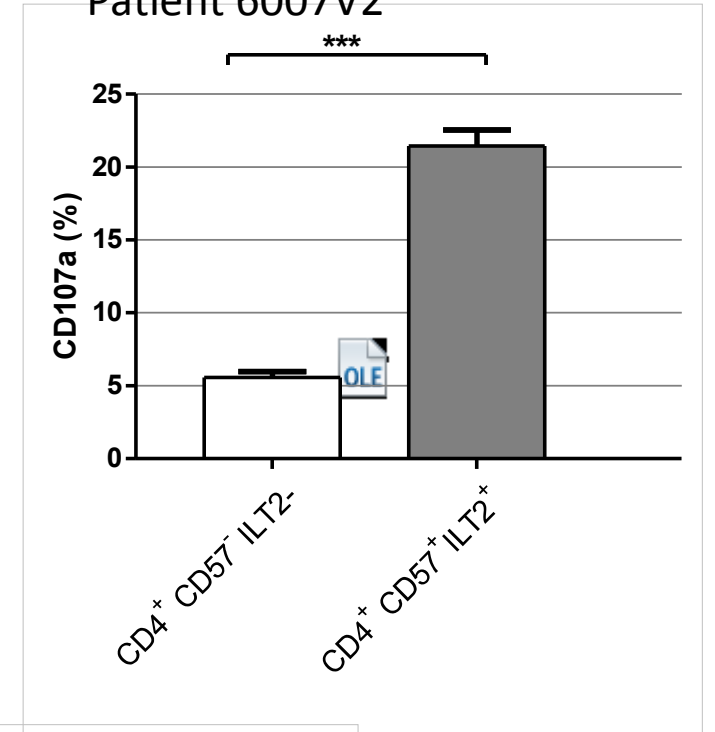
Healthy donor



Patient 7044V2



Patient 6007V2



B. Inhibition of cytotoxic function by HLA-G

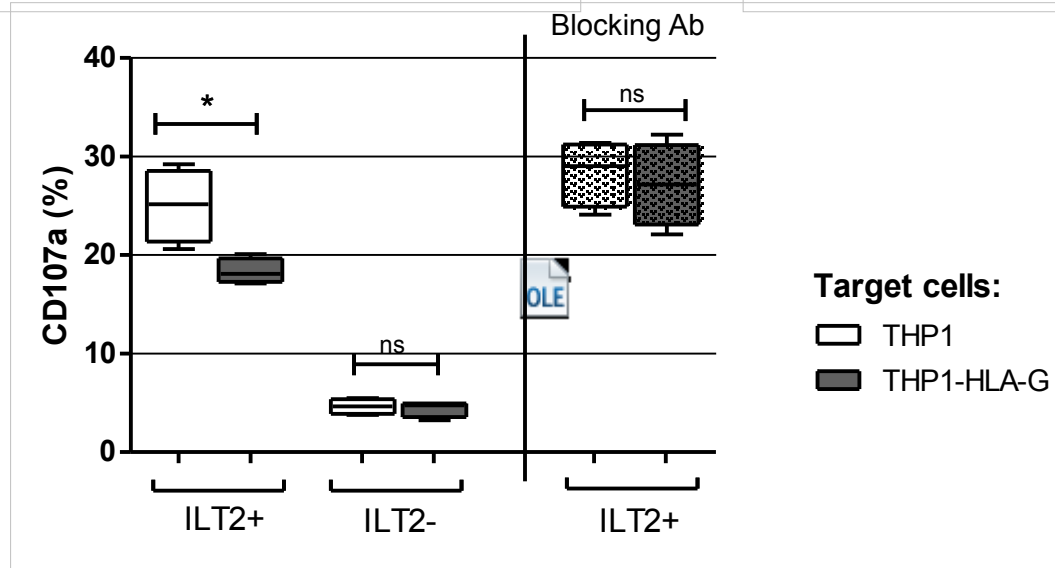


Table 1. Characteristics of patients

Recipients	All patients	STABLE Patients N=78	CLAD Patients N=72	P value
Age, mean [SD]	44 (15)	43 (15)	44 (15)	0.66
Lung Tx indication, n				0.80
DLTx	122 (81)	64 (82)	58	
SLTx	25 (17)	12 (15)	13	
CPTx	3	2	1	
Initial disease, n.				0.70
1Emphysema/COPD	52 (35)	27 (34)	25 (34)	
2Cystic fibrosis	53 (35)	31 (39)	22	
3Fibrosis	30 (20)	14 (17)	16	
4PAH	7 (5)	3 (3)	4	
5others	8 (5)	3 (3)	5	
CMV mismatch (D+/R-) N (%)	57 (38)	27 (37)	30 (45)	0.30
ischemia time (min), mean [SD]	312 (101)	304 (82)	319 (117)	0.37
Primary Graft Dysfunction grade 3				0.30
Yes	54 (38)	31	23	
no	89	43	46	
Per-operative ECMO, n (%)				0.30
Yes	54 (38)	31	23	
no	89	43	46	
Diabetes post-LTx, n (%)	67 (51)	36	31	0.99
No of HLA-A or -B, (class 1) mismatches (MM)				0.45
4 MM	60 (48)	43	17	
0,1,2, or 3 MM	65	28	37	
No of HLA-DR or DQ (class 2) MM				0.67
4 MM	30 (24)	16	14	
0,1,2, or 3 MM	95	55	40	
Preformed DSA (Yes vs no)				0.18
Yes	40 (32)	17	23	
No	85	48	37	
Induction therapy				0.01
No induction	56 (37)	23	33	
Thymoglobulin	60 (40)	31	29	
IL-2 antagonist	33 (23)	24	9	

DLTx, double-lung transplantation; SLTx, single-LTx; CPTx, cardio-pulmonary Tx; ECMO, extra-corporeal membrane oxygenation; COPD, chronic obstructive pulmonary disease; PAH, Pulmonary arterial hypertension; CMV, cytomegalovirus; ECMO, extra-corporeal membrane oxygenation; HLA, human leukocyte antigen; DSA, donor-specific antibody;

Table 2. Comparison of peripheral T-cells subsets between Stable and CLAD patients at time points 1-month (M1), and M6 post-LTx, and according 6 months T-cells subsets variation within the 1st year.

	STABLE patients N=78	CLAD patients N=72	p
At 1 month post-LTx*			
CD4+CD25+CD127low T cells (% of CD4+ T cells)	0.9% (1.2)	1.8% (2.3)	0.02*
6 months T-cell subset ratio			
CD4+CD57+ILT2+ T cells (% of CD4+ T cells) ratio	0.8 (1.3)	2.6 (3.4)	0.01*

LTx, lung transplantation; * all T-cell subsets at time point 1 and 6 months post-LTx are mean % of CD4+ or CD8+ T cells; 6 months T-cells subsets ratio, percentage of variation of T-cells subset over 6 months within the 1st year which was calculated for all T-cell subsets. *p < 0.05.

Table 3. Clinical, functional, and immunologic factors associated with chronic lung allograft dysfunction (CLAD) onset and graft survival after lung transplantation (Univariate and multivariate Analysis).

Variable	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Univariate Analysis	for CLAD onset		for graft failure	
Recipient age (by 1 unit increase)	1.0 (1.0-1.0)	0.62	1.0 (1.0-1.0)	0.91
Lung Tx indication		0.77		0.99
SLTx	1.0		1	
DLTx	0.9 (0.5-1.6)		1.1 (0.4-3.2)	
Cardio-pulmonary Tx	0.5 (0.0-3.7)		-	
Initial disease		0.65		0.51
1Emphysema/COPD	1.0		1.0	
2Cystic fibrosis	0.8 (0.4-1.4)		1.2 (0.4-3.2)	
3Fibrosis	1.1 (0.6-2.0)		1.6 (0.5-4.7)	
4PAH	1.3 (0.5-3.9)		1.1 (0.1-8.8)	
5others	1.3 (0.5-3.5)		3.2 (0.8-12.6)	
CMV mismatch (yes versus no)	0.7 (0.4-1.2)	0.2	0.6 (0.2-1.3)	0.18
ischemia time, per 60 units increase.	1.0 (1.0-1.0)	0.16	1.0 (1.0-1.0)	0.28
Primary Graft Dysfunction grade 3 (Yes vs No)	2.1 (0.9-4.8)	0.08	1.3 (0.4-4.4)	0.65
Peroperative ECMO or cardiopulmonary bypass (yes vs no)	1.2 (0.8-2.1)	0.37	1.0 (0.5-2.4)	0.9
Diabetes (Post-LTx, Yes vs no)	1 (0.6-1.6)	1	1.3 (0.5-3.2)	0.54
No of HLA-A or -B, (class 1) mismatches (MM) 4 MM vs 0,1,2, or 3 MM	0.8 (0.4-1.4)	0.48	1.2 (0.7-2.2)	0.45
No of HLA-DR or DQ (class 2) MM 4 MM vs 0,1,2, or 3 MM	1.0 (0.5-1.8)	0.95	0.7 (0.5-1.1)	0.18
Preformed DSA (Yes vs no)	0.7 (0.4-1.3)	0.28		
Induction therapy		0.04		0.57
No induction	1.0		1.0	
Thymoglobulin	0.8 (0.4-1.3)		1.3 (0.5-3.1)	
IL-2 antagonist	0.3 (0.1-0.7)		0.7 (0.2-2.3)	
1-12 mo CD4+ILT2+CD57+ T cells (% of CD4 T cells) ratio, per 0.1 unit increase**	1.25 (1.09-1.44)	0.001	1.1 (0.9-1.3)	0.17
% CD4+CD25+CD127low T cells (% of CD4+ T cells) at 1mo post-Tx	1.1 (1.0-1.2)	0.02	1.2 (1.0-1.3)	0.005
% CD4+CD57+ILT2+ T cells (% of CD4+ T cells) at 1mo post-Tx	0.8 (0.7-1)	0.13	0.6 (0.3-1.1)	0.13
% CD8+CD25+ T cells (% of CD4+ T cells) at 1mo post-Tx	1.0 (1.0-1.08)	0.07	1.0 (0.9-1.1)	0.23

Variable	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Multivariate Analysis (models 1)				
1-12 mo CD4+ILT2+CD57+ T cells (% of CD4 T cells) ratio, per 0.1 unit increase**	1.25 (1.09-1.44)	0.001	1.1 (0.9-1.4)	0.18
% CD4+CD25+CD127low T cells (% of CD4+ T cells) at 1mo post-Tx, per 0.1 unit increase.	1.1 (0.9-1.4)	0.22	1.1 (1.0-1.3)	0.01
Multivariate Analysis (models 2)				
1-12 mo CD4+ILT2+CD57+ T cells (% of CD4 T cells) ratio, per 0.1 unit increase**	1.3 (1.1-1.5)	<0.001	1.1 (0.9-1.3)	0.14
Induction therapy				
No induction	1	0.06	1	0.37
Thymoglobulin	1 (0.4-2.25)		4.7 (0.5-40.4)	
IL2 receptor antagonist	0.2 (0.0-0.8)		0	
% CD4+CD25+CD127low T cells (% of CD4+ T cells) at 1mo post-Tx, per 0.1 unit increase.	1.2 (1.1-1.4)	0.003	1.2 (1.0-1.3)	0.02
Induction therapy				
No induction	1	0.26	1	0.42
Thymoglobulin	1 (0.5-1.8)		2.1 (0.6-6.5)	
IL2 receptor antagonist	0.5 (0.2-1.2)		1.2 (0.3-5.5)	

* Data are hazard ratios (HRs) and 95% confidence intervals (CIs); ECMO, extra-corporeal membrane oxygenation; Transplantation (Tx); COPD, chronic obstructive pulmonary disease; PAH, Pulmonary arterial hypertension; CMV, cytomegalovirus; ECMO, extra-corporeal membrane oxygenation; HLA, human leukocyte antigen; MM, mismatch; DSA, donor-specific antibodies;

**Univariable analysis are performed in the cohort of 107 patients with available 1-mo Time-point for all analyzed parameters, except for the 1-12mo CD4+CD57+ILT2+ T cells ratio, for which only n=57 patients had available 2 available time-point sampling within the 12 months post-LTx to calculate a cell ratio.