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Effect of Immunosuppression on Target Blood Immune Cells Within 1 Year After Lung Transplantation: Influence of Age on T Lymphocytes

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Data Interpretation D
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Background: Lymphocytes are targeted by immunosuppressive therapy in solid organ transplantation and they influence allograft outcome.

Material/Methods: Peripheral blood lymphocyte subsets (PBLs) determined by flow cytometry during the first year post-transplant from patients who underwent a first lung transplantation in a French University Hospital between December 2011 and July 2013 were retrospectively analyzed according to recipient characteristics and allograft outcome.

Results: Fifty-seven recipients were enrolled and 890 PBLs were collected. T lymphocytes and NK cells were rapidly decreased, below normal range, from the first postoperative days. B cells decreased more gradually, remaining within normal range, with the lowest level reached after day 100. In multivariate analysis, greater T lymphopenia was found in older recipients (-414 [-709 to -119] cells/ μ L, $p=0.007$). According to the outcome, multivariate analysis evidenced lower levels of lymphocytes when bacterial and viral infection occurred (-177 [-310 to -44] cells/ μ L, $p=0.009$ and -601 [-984 to -218] cells/ μ L, $p=0.002$, respectively), higher CD8+ T lymphocytes with BOS ($+324$ [$+94$ to $+553$] cells/ μ L, $p=0.006$), and higher leukocytes with restrictive allograft syndrome ($+3770$ [$+418$ to $+7122$] cells/ μ L, $p=0.028$).

Conclusions: Aging is associated in our cohort with more severe T lymphopenia after induction therapy for lung transplantation. The analysis of leukocytes and PBLs is associated with specific profile according to the allograft outcome.

MeSH Keywords: Aging • Graft Rejection • Lung Transplantation • Lymphocyte Subsets • Opportunistic Infections

Full-text PDF: <https://www.annalsoftransplantation.com/abstract/index/idArt/906372>



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Background

Despite recent advances in management of immunosuppressive regimens after solid organ transplantation, with a specific focus on T lymphocytes as a target cell of immunosuppressive therapy [1], there is little data on analysis of peripheral blood lymphocyte subsets (PBLs) in such context and particularly in lung transplantation (LTx). PBLs are easily assessed at the bedside to evaluate the immune status. The balance between over- and under-immunosuppression, with the dual risk of the occurrence of opportunistic infection on one side, and that of allograft rejection on the other, is sometimes difficult to achieve, with inter-variability from one patient to another. Thus, PBLs might be a valuable biomarker to appreciate the individual level of induced immunodeficiency, but to date there are no guidelines for such monitoring. In our practice, PBLs analysis generally looks at the distribution of the T lymphocytes (CD4+ and CD8+), B lymphocytes, and NK cells. In solid organ transplantation, the focus has been on CD4+ T cell count as a marker of the immunosuppression level, permitting the detection of recipients at risk of subsequent opportunistic infections (OI) [2,3]. A strong association has recently been demonstrated between nadir CD4+ T cell count <200 cells/ μ L and subsequent cumulative incidence of viral infections in a series of LTx [4]. Other blood lymphocyte subsets have been neglected in the monitoring of transplanted solid organs. Regarding NK cells, some studies suggest the association between high levels of NK cells and/or NK cell activation and acute rejection (AR) and/or subsequent chronic lung allograft dysfunction (CLAD) [5,6]. In parallel, other authors have shown that NK cell depletion was associated with cytomegalovirus (CMV) infection in a lung transplant cohort [7]. However, this data is supported by little case evidence. Regarding B cells, there is no relevant data about the quantitative kinetics in LTx, but interest is growing, especially through the aspect of donor-specific antibodies production [8–10].

Finally, patient characteristics (e.g., age, sex, and underlying disease) known to impact survival after LTx are also very important factors affecting immune responses, susceptibility to infections, and response to vaccination [11,12]. Thus, the objectives of our study were to evaluate the influence of immunosuppressive regimen on the kinetics of all blood lymphocyte subsets measured in our center within the first year after lung transplantation according to recipient characteristics, and to assess the link between PBLs count and the allograft outcome (OI and AR/CLAD).

Material and Methods

Study population

All consecutive adult patients who underwent a first LTx between December 2011 and July 2013 at the Lung Transplant

Center of Marseille (Aix-Marseille University, France) with at least 1-year follow-up in our center were enrolled in this retrospective study. Patients who underwent redo LTx or who died before 1 year were excluded from the analysis. Written informed consent was obtained from all patients prior to LTx with authorization to use all the medical data recorded through their conventional LTx monitoring.

Study design

All PBLs samples monitoring, including PBLs performed before LTx (up to 3 months before LTx) and those throughout the follow-up until 1 year post-LTx were retrospectively collected. In our clinical practice, PBLs are routinely assessed in monitoring, once a week during the first stay of hospitalization and at each outpatient visit (at least monthly in the first year). For each PBLs monitoring, data on concomitant OI (bacterial, viral, fungal, CMV reactivation) and immunological events (presence of donor-specific antibodies (DSA) class I or II, AR and CLAD) were recorded. All data were extracted from our digitized institutional database.

Immune status assessment and lymphocyte subsets

Leukocyte and lymphocyte counts were obtained from hematological laboratory reports (ADVIA hematology analyzer, Bayer, Leverkusen, Germany). Lymphocyte subpopulation counts were characterized by flow cytometry as follow: in the total lymphocytes gate (CD45^{intermediate}, side scatter^{low}), we subsequently separated into T cells (CD3⁺CD19⁻), B cells (CD3⁺CD19⁺), and NK cells (CD16/CD56⁺), and in the T cells gate, helper T cells (CD4⁺CD8⁻) and cytotoxic T cells (CD4⁻CD8⁺) (Supplementary Figure 1). Phenotyping was performed from whole blood (50 μ L). Tubes were prepared with the COULTER® TQ-Prep system (Beckman Coulter, Marseille, France), which included a red blood lyse. Whole blood was stained with 10 μ L of each antibody (Beckman Coulter) for 15 min, including anti-CD45 FITC (fluorescein), clone B3821F4A; anti-CD3-PC5 (phycoerythrin-cyanin 5), clone UCHT1; anti-CD4-RD1 (rhodamine), clone T4; anti-CD8-ECD (phycoerythrin-Texas Red), clone T8; anti-CD19-ECD, clone J4-119; anti-CD16-PE (phycoerythrin), clone 3G8 and anti-CD56-RD1, clone N901. Determination of PBLs was performed with a Navios flow cytometer (Beckman Coulter), and data were analyzed directly on the acquisition software.

The normal reference values (lower threshold) of cell counts used in the study arises from anterior published data of healthy adult cohorts: leukocyte=4000 cell/ μ L, lymphocytes=1350 cell/ μ L, T cells=814 cell/ μ L, CD4+ T cells=388 cell/ μ L, CD8+ T cells=171 cell/ μ L, CD4+/CD8+ ratio=1.4, B cells=72 cell/ μ L, NK cells=60 cell/ μ L [13,14].

Immunosuppressive and anti-infectious prophylaxis regimens

In the study period, all recipients received a standardized immunosuppressive regimen based on published data [15–17] and in accordance with our institutional protocol. Induction therapy consisted of intravenous administration of 1.5 mg/kg/day rabbit anti-thymocyte globulins (ATG) (Institut Pasteur Mérieux, Lyon, France) given for the first 3 postoperative days, associated with high-dose methylprednisolone (6 mg/kg/d on day 1, 2 mg/kg/d on day 2 and day 3, and 1 mg/kg/d thereafter); intravenous cyclosporine was administered immediately after LTx (to obtain a steady-state serum concentration between 300 and 400 ng/ml) and was switched by oral tacrolimus as soon as possible (to maintain trough blood levels between 12 and 15 ng/ml during the first 3 months and around 10–12 ng/ml thereafter). Standard triple maintenance immunosuppressive regimen consisted of tacrolimus, mycophenolate mofetil, and steroids (prednisone) tapered to 0.25 mg/kg/day over the first 3 months and stopped, when possible, at around 12 months after surgery.

Postoperatively, transplant recipients received a prophylactic antibiotic treatment according to their preoperative and/or concomitant infectious status for at least 14 days. Seropositive CMV recipients received prophylactic IV ganciclovir or oral valganciclovir when available for the first 2 postoperative weeks. Higher-risk CMV-mismatched recipients (D+/R-) were treated for the first 3 months.

Allograft complications

Opportunistic infections were defined by the combination of a systemic inflammatory response syndrome, presence of clinical symptoms of infection, and requirement of anti-infectious treatment. Specifically, bacterial infections were defined as any pulmonary, extra-pulmonary, or blood clinically suspected infections (with or without microbiological identification). Viral infections were defined by the presence of clinical respiratory symptoms and viral DNA in sputum, bronchial aspiration, and/or broncho-alveolar lavage samples, determined using quantitative real-time polymerase chain reaction (PCR) for myxovirus influenzae, respiratory syncytial virus, metapneumovirus, or rhinovirus. CMV reactivation was defined by a positive blood PCR assay (>500 copies/mL) associated with pp65 antigenemia (at least 1 cell per 200 000). All recipients were screened weekly for CMV reactivation for the first 3 months, monthly thereafter, and when clinically suspected. Fungal infections were defined as localized or invasive bronchopulmonary filamentous fungi infections.

Acute rejection (AR) was characterized by histological abnormalities on trans-bronchial biopsies, according to the international standardized criteria [18]. Episodes of AR were treated

with intravenous methylprednisolone (5 mg/kg/d for 3 consecutive days) and then rapidly reduced.

CLAD were defined according to the standardized diagnosis criteria and the new classification system, separating the 2 phenotypes [19,20]: 1. *Bronchiolitis obliterans syndrome* (BOS) was supported by a sustained decline (2 measurements at least 3 weeks apart) in FEV1 of $\geq 20\%$ from the recipient's best post-transplant values, associated with obstructive syndrome (based on FEV1/FVC ratio < 0.7). As recommended, recipients who experienced the diagnosis of BOS were treated earlier with daily 250 mg azithromycin [19–21]. 2. *Restrictive allograft syndrome* (RAS) was defined as persistent decline (2 measurements at least 3 weeks apart) in FVC and total lung capacity accompanied by a decline in FEV1 of $> 20\%$, while the FEV1/FVC ratio remains normal or increases above the normal range.

Statistical analysis

Primary lung disease, sex, and age groups (2 groups separated by the median for age) were first evaluated as potential factors influencing PBLs kinetics. Then, associations between PBLs counts and allograft outcome at each time point were assessed to detect specific immune profile according to the outcome. In this first part, the analysis was performed with the linear mixed model. A linear mixed model is a statistical model containing both fixed effects and random effects. The recipient individuals were considered as random effects to assumed that repeated measures from the same subject are not independent of one another. The model assumes that intercepts and slopes of different subjects may vary randomly depending on the subject. A difference (beta) between groups with confidence interval is thus obtained, which represents a global estimation of the difference between groups over the study period (the first year in our study) integrating the dependency of the repeated measures in the same individual. We used an unstructured covariance as the default strategy for analyzing these longitudinal data, which assumes a nonspecific correlation among measurements. Analysis was performed only on post-transplant PBLs. Multivariate analysis was performed for variables with univariate p-values < 0.2 .

The linear dependence between 2 continuous variables was assessed using the Pearson's correlation test. The ROC curves analysis of repeated measures was performed using R software (v.3.1.2) with the repeatedMeasuresROC function designed by Taylor Andrew; area under the curve (AUC) bootstrap confidence interval based on 100 bootstrap replicates were calculated with the package «boot». Figures were created using R software with the package «ggplot2» (Bioconductor software suite). Due to personalized monitoring, each PBL count was performed at random times. Thus, kinetics of absolute PBLs counts were graphically represented by the loess

Table 1. Patient characteristics. Results are expressed as median [interquartile] or number, n (%). For DSA, allograft dysfunction, and opportunistic infections, results represent the number of recipients with at least 1 event within the first year.

| Characteristics | Median [IQR] or n (%) |
|--------------------------------|-----------------------|
| Lung transplant recipients (n) | 57 (100) |
| Age at transplantation (year) | 48 [38–56] |
| Men/Women | 31 (54.4)/26 (45.6) |
| Disease | |
| Cystic fibrosis | 14 (24.6) |
| Emphysema | 20 (35.1) |
| COPD | 15 (26.3) |
| A1AD | 5 (8.8) |
| Pulmonary fibrosis | 23 (40.3) |
| Idiopathic | 15 (26.3) |
| Scleroderma | 3 (5.3) |
| GvH | 1 (1.7) |
| LCDD | 1 (1.7) |
| Sarcoidosis | 1 (1.7) |
| Sjogren | 1 (1.7) |
| RP | 1 (1.7) |
| CMV status | |
| D–/R– | 20 (35.1) |
| D–/R+ | 13 (22.8) |
| D+/R+ | 18 (31.6) |
| D+/R– | 6 (10.5) |

method (local polynomial regression fitting) to obtain curves which represent the smoothed mean with confidence interval over time. The other statistical analyses were undertaken using IBM SPSS Statistics version 17 (IBM SPSS Inc., Chicago, Illinois, USA). For all tests, the statistical significance was defined as p value <0.05.

Results

Patient characteristics

Sixty-nine consecutive LTx procedures were performed during the study period in our center. Twelve recipients were excluded from analysis. A total of 57 LTx were enrolled in the study: 14 cases of cystic fibroses, 23 cases of pulmonary fibroses, and

| Characteristics | Median [IQR] or n (%) |
|--------------------------|-----------------------|
| Type of LTx | |
| Single | 6 (10.5) |
| Double | 51 (89.5) |
| PGD | 16 (28.1) |
| Stage | 2 [2–3] |
| DSA class I | 14 (24.6) |
| DSA class II | 18 (31.6) |
| Allograft dysfunction | |
| AR | 17 (29.8) |
| RAS | 2 (3.5) |
| BOS | 5 (8.8) |
| Opportunistic infections | |
| Bacterial | 50 (87.7) |
| Viral | 8 (14.0) |
| Fungal | 8 (14.0) |
| CMV reactivation | 26 (45.6) |

COPD – chronic obstructive pulmonary disease; A1AD – alpha-1 antitrypsin disease; GvH – graft versus host disease after bone graft; LCDD – light-chain deposition disease; RP – rheumatoid polyarthritis; D – donor; R – recipients; LTx – lung transplantation; CMV – cytomegalovirus; PGD – primary graft dysfunction; PBLS – peripheral blood lymphocyte subsets; DSA – donor-specific antibodies; AR – acute rejection; RAS – restrictive allograft syndrome; BOS – bronchiolitis obliterans syndrome.

20 cases of emphysema. The median age was 48 [38–56] years (Table 1). In this cohort, a total of 890 PBLS monitorings were performed before transplant and within the first year post-transplantation, with a median of 19 [14–23] PBLS per patient.

Kinetics of leukocytes and lymphocyte subsets

Kinetics of total leukocytes and lymphocyte subsets of the whole cohort are graphically represented in Figure 1. After an initial increase in the immediate postoperative phase, leukocytes kinetics demonstrated a slow and constant decrease until day 365. Compared to baseline values, we observed a decrease below normal range in total lymphocytes, T lymphocytes (CD4+ and CD8+), and NK cells from day 3 to day 30. After this initial decline, T lymphocytes and NK cells increased steadily to reach a plateau at around 100 days, returning to above

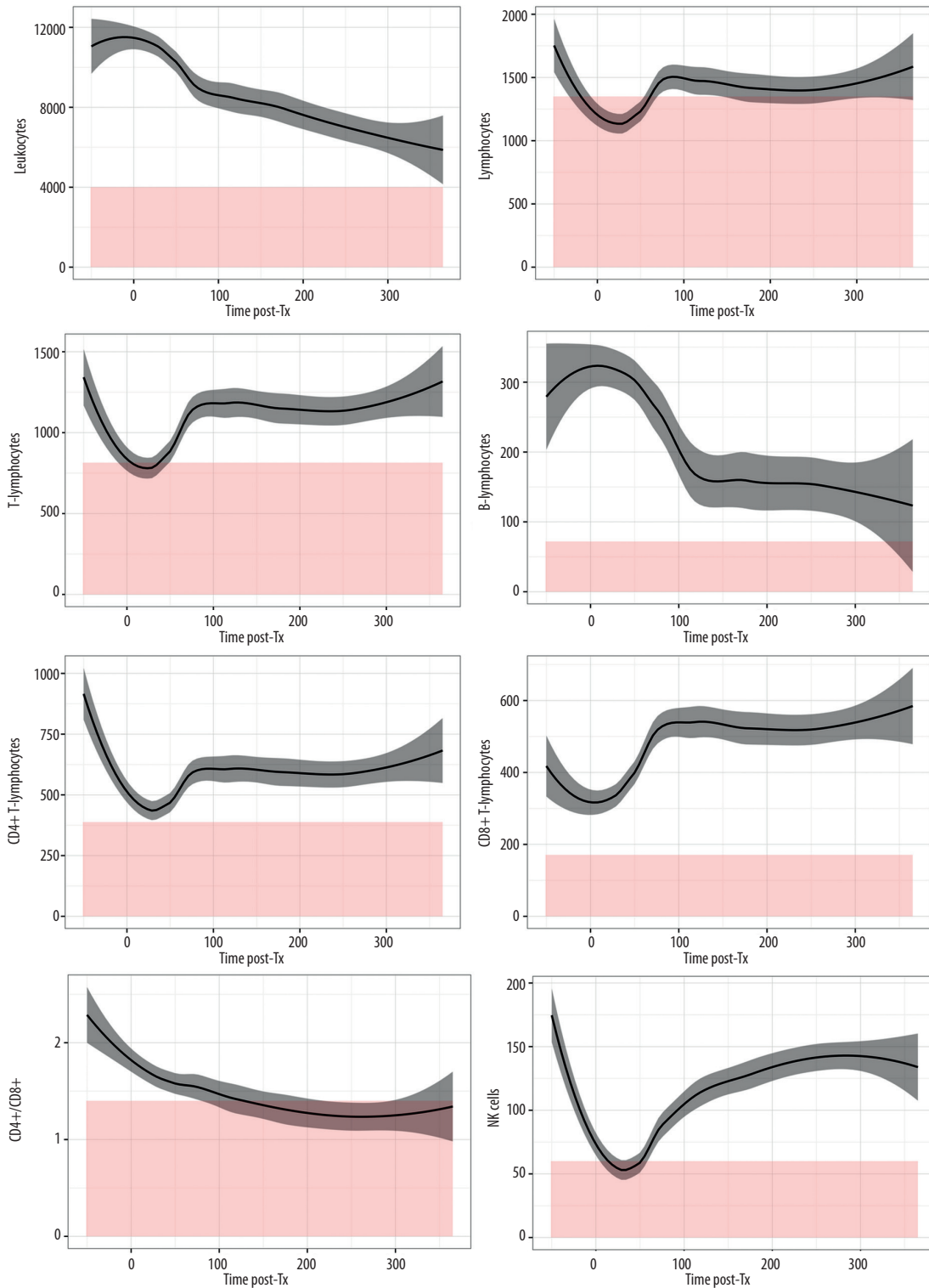


Figure 1. Kinetics of peripheral blood lymphocyte subsets (PBLs) over the first year post-transplantation. Y-axis=cell counts in cells/ μ L and X-axis=time post-transplant in days. Curves represent the smoothed mean with confidence interval. Values before zero represent the pooled counts realized within 3 months before LTx. Red color represents area below inferior normal thresholds from healthy controls.

standard value. CD4+ T lymphocytes and NK cells did not reach their baseline values, whereas CD8+ T lymphocytes exceeded baseline by more than 100 cell/ μ L. B cells decreased more gradually, with the lowest level reached after 100 days, constantly above normal range. CD4/CD8 ratio decreased gradually to almost reach equality between populations from the 200th day and then stabilized.

Kinetics according to recipient characteristics

The analysis of patient characteristics as factors influencing PBLs kinetics revealed in the univariate analysis a more severe T cell depletion: (1) in patients affected with previous emphysema (-359 [-713 to -5] cell/ μ L, $p=0.047$) and pulmonary fibrosis (-404 [-745 to -62] cell/ μ L, $p=0.021$) as compared to those with cystic fibrosis; (2) in males (-305 [-572 to -37] cell/ μ L, $p=0.03$) as compared to females; and (3) in recipients older than median age (-348 [-533 to -163] cell/ μ L, $p<0.001$) compared to younger recipients. Multivariate analysis found a significantly more severe T lymphopenia in older recipients (-414 [-709 to -119] cell/ μ L, $p=0.01$) as compared to younger recipients and in pulmonary fibrosis compared to cystic fibrosis (-354 [-700 to -8] cell/ μ L, $p=0.045$) (Table 2).

Kinetics according to the age

Pearson's test revealed a negative correlation between age and T lymphocytes in the overall PBLs set (Pearson's coefficient $r=-0.40$ [-0.46 to -0.34], $p<0.001$). The analysis by decades revealed that this effect worsened with increasing age, and only recipients older than 40 years crossed the lower normal range (Figure 2).

Analysis of CD4+ T lymphocytes kinetics according to recipient age revealed that older patients (who have lower CD4+ estimated at -194 [-373 to -14] cell/ μ L, $p=0.03$ within first year post-LTx) crossed the line of the lower normal value (388 cell/ μ L) in the early postoperative phase around day 30, whereas younger recipients remained in normal range (Figure 3). Despite significantly lower CD8+ T lymphocytes (-211 [-356 to -65] cell/ μ L, $p=0.005$), older recipients remained in normal range for this subpopulation. Finally, total lymphocytes, probably through the influence of T cells, were lower in older recipients (-436 [-773 to -99] cell/ μ L, $p=0.01$) and constantly below normal range (1350 cell/ μ L) until day 300. There was no relevant difference by age for the other subpopulations (Table 2, Figure 3).

Allograft outcome

Over the 839 PBLs samples obtained during the first year post-transplantation, 228 (27.2%) were performed in a context of bacterial, 15 (1.8%) in viral, 13 (1.5%) in fungal infections, 82

(9.8%) in CMV blood reactivation, 42 (5.0%) during episodes of AR, 18 (2.1%) in occurrence of BOS, and 19 (2.3%) in RAS. For some patients, there were repeated measures of PBLs for the same complication but this effect was integrated in the analysis by the linear mixed model. There were 176 (21.0%) PBLs with missing data on CMV reactivations, not systematically realized at each PBLs monitoring.

The description of bacterial infections reported in Supplementary Table 1 revealed that the lungs are the most frequent site of infection (81%) and that most bacterial infections occurred within 90 days post-transplant. Resistant bacteria are mostly implicated in the early postoperative period (within 60 days) and correspond to hospital-acquired infections, while other bacteria are community-acquired infection or tardive colonization such as *Pseudomonas aeruginosa*.

Univariate and multivariate analyses showed a strong association between lymphopenia and concomitant bacterial (-177 [-310 to -44] cell/ μ L, $p=0.009$) and viral (-601 [-984 to -218] cell/ μ L, $p=0.002$) infections. Except for NK cells, this association was observed in all lymphocyte subpopulations, T cells (CD4+ and CD8+), and B cells. Regarding fungal infections, we demonstrated higher CD4+ T lymphocytes ($+412$ [$+235$ to $+590$] cell/ μ L, $p<0.001$). BOS was associated with higher levels of CD8+ T lymphocytes ($+324$ [$+94$ to $+553$] cell/ μ L, $p=0.006$) and RAS with higher total leukocytes ($+3770$ [$+418$ to $+7122$] cell/ μ L, $p=0.028$). RAS was also associated with lower level of NK cells (-60 [-115 to -5] cell/ μ L, $p=0.034$). CMV reactivations, acute rejections, and presence of class I or class II donor-specific antibodies were not correlated with specific PBLs profile (Table 3).

Based on the association between lymphopenia and infection (bacterial and viral), we evaluated the diagnostic performance of PBLs (T and B lymphocytes) by ROC curves as markers of global infection (bacterial and viral). The analysis revealed the best performance with CD4+ T cells: AUC=0.77 [0.70 to 0.84], Se=75.2%, Sp=68.8%, PPV=47.1%, and NPV=88.2% for a best threshold at 432 cell/ μ L (Figure 4) (data not shown for the other subpopulations).

Discussion

The present study provides detailed and complementary information on the kinetics of lymphocyte subsets in peripheral blood after LTx and their associations with patient characteristics and allograft outcome.

Kinetics of PBLs after LTx distinguished 2 consecutive phases in relationship with the immunosuppressive therapy. In the very early postoperative phase, there was not only a strong

Table 2. PBLs counts according to recipient characteristics. The results (expressed in cells/ μ L) represent the estimated difference [95% CI] in PBLs counts within the first year according to primary lung disease, sex, and age group. The statistical analysis was performed with the linear mixed model as described in Methods.

| Disease | CF (Reference) | Univariate analysis | | | Multivariate analysis | | |
|--------------|---------------------------|----------------------|----------------------|--------------------------|-----------------------|-------------------------|----------------------|
| | | COPD | PF | p Value | COPD | PF | p Value |
| | | Estimated difference | Estimated difference | | Estimated difference | Estimated difference | |
| | | [95% CI] | [95% CI] | | [95% CI] | [95% CI] | |
| Leukocytes | 0 | 1045 [-1262; 3351] | 93 [-2108; 2295] | 0.56 | | | |
| Lymphocytes | 0 | -424 [-756; -92] | -454 [-773; -136] | 0.01 | -276 [-679; 126] | -410 [-805; -15] | 0.12 |
| T-Cells | 0 | -359 [-713; -5] | -404 [-745; -62] | 0.05 | -137 [-489; 216] | -354 [-700; -8] | 0.12 |
| CD4+ T-Cells | 0 | -100 [-311; 111] | -194 [-397; 10] | 0.17 | 20 [-195; 236] | -116 [-327; 95] | 0.37 |
| CD8 T-Cells | 0 | -237 [-410; -63] | -175 [-342; -8] | 0.03 | -128 [-302; 46] | -201 [-372; -31] | 0.07 |
| B-Cells | 0 | -19 [-183; 144] | -36 [-130; 130] | 0.99 | | | |
| NK-Cells | 0 | 0 [-29; 28] | 4 [-23; 31] | 0.93 | | | |
| Sex | Women (Reference) | Men | | p Value | Men | p Value | |
| | | Estimated difference | Estimated difference | | | | Estimated difference |
| | | [95% CI] | [95% CI] | | | | [95% CI] |
| | | | | | | | |
| Leukocytes | 0 | -420 [-1670; 1670] | 0.82 | | | | |
| Lymphocytes | 0 | -313 [-617; 10] | 0.04 | -267 [-589; 56] | 0.10 | | |
| T-Cells | 0 | -305 [-572; -37] | 0.03 | -190 [-472; 92] | 0.18 | | |
| CD4+ T-Cells | 0 | -216 [-369; -63] | 0.006 | -124 [-296; -48] | 0.15 | | |
| CD8 T-Cells | 0 | -81 [-22; 57] | 0.24 | | | | |
| B-Cells | 0 | 44 [-79; 167] | 0.47 | | | | |
| NK-Cells | 0 | -10 [-31; 11] | 0.35 | | | | |
| Age | \leq Median (Reference) | $>$ Median | | p Value | $>$ Median | p Value | |
| | | Estimated difference | Estimated difference | | | | Estimated difference |
| | | [95% CI] | [95% CI] | | | | [95% CI] |
| | | | | | | | |
| Leukocytes | 0 | -1417 [-2581; -253] | 0.02 | 952 [-1337; 3240] | 0.41 | | |
| Lymphocytes | 0 | -385 [-601; -170] | <0.001 | -436 [-773; -99] | 0.01 | | |
| T-Cells | 0 | -348 [-533; -163] | <0.001 | -414 [-709; -119] | 0.007 | | |
| CD4+ T-Cells | 0 | -136 [-253; -19] | 0.02 | -194 [-373; -14] | 0.03 | | |
| CD8 T-Cells | 0 | -179 [-268; -90] | <0.001 | -211 [-356; -65] | 0.005 | | |
| B-Cells | 0 | 22 [-62; 107] | 0.60 | | | | |
| NK-Cells | 0 | 4 [-16; 24] | 0.70 | | | | |

CF – cystic fibrosis; COPD – chronic obstructive pulmonary disease; PF – pulmonary fibrosis; CI – confidence interval.

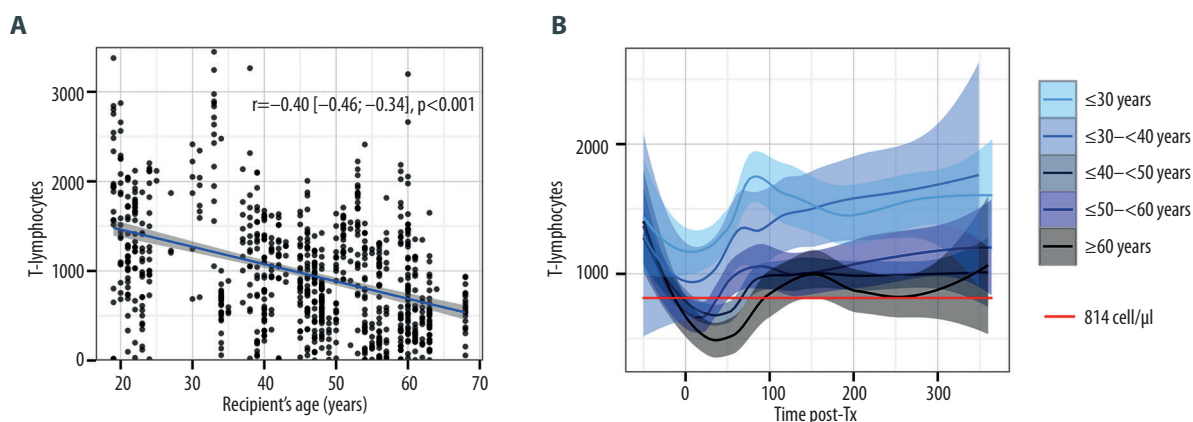


Figure 2. T lymphocytes according to the age. **(A)** Scatterplot and fitted linear regression (blue line) with confidence interval (grey fill). Pearson correlation test, r =coefficient of correlation. **(B)** Kinetics of T lymphocytes over the first year post-transplantation according to age in decades. Y-axis= cell counts in cell/ μ L and X-axis=time post-transplant in days. Curves represent the smoothed mean with confidence interval. Values before zero represent the pooled counts realized within 3 months before LTx. Red line represents the inferior normal threshold from healthy controls.

T cell depletion, as expected with the induction therapy with ATG [22,23], but also a decrease in NK cell counts. The impact of ATG on NK cells has already been reported in kidney transplantation [24,25]. However, this effect could also be attributed to corticosteroids, which are known to inhibit functions and proliferation of NK cells [26–29]. In this early post-operative phase, both T lymphocyte and NK cell counts are the lowest in follow-up and below normal range compared to healthy controls, confirming the high risk of OI during this period. The effect of induction therapy disappears by around day 30. The induction therapy does not influence B cells that are depressed later.

The consecutive phase depends on maintenance immunosuppressive therapy (calcineurin inhibitor and antimetabolites) that depresses all PBLs by their anti-lymphoproliferative effect, which seems to be fully effective by around day 100, and thereafter have a quite stable effect over time. It is noteworthy, however, that NK cells and CD4+ T lymphocytes never return in this phase to baseline pre-transplant values, which reflects a constant immunosuppression over time.

Kinetics of PBLs was then evaluated according to recipient characteristics, and in multivariate analysis revealed significantly lower T lymphocyte counts among the oldest patients within the first year. This effect is demonstrated mainly in the early postoperative phase after lung transplant and then seems to disappear, which more strongly suggests induction therapy than standard maintenance regimen. We also noted that only older recipients (especially more than 40 years old) cross the line of the lower normal value (814 cell/ μ L) in the postoperative phase at around day 30, conferring to them a potential higher risk of OI. This observation in T lymphocytes may be

related to immune senescence. The concept of immune senescence in transplantation has recently been strongly suspected by the observation of higher infectious and cancer risks, with lower incidence of acute rejection in older transplanted patients [30,31]. More generally, the aging of the immune system is well described and results in altered inflammation and response to vaccination [32]. It is also known that the senescence especially alters T cell functions [33], which may explain why in our study the influence of age was only observed in this population. The influence of induction therapy on immune senescence has recently been described in kidney transplantation upon observing lower levels of hematopoietic progenitor cells, lower T cell-relative telomere length, and telomerase activity at 1 year post-transplant in ATG-treated patients as compared with those receiving anti-CD25 monoclonal antibodies [34]. Indeed, hematopoietic progenitor cells defect could explain a slower T cell reconstitution in older recipients after target induction therapy. Thus, it would be interesting to evaluate the effectiveness and impact of adapting immunosuppression according to age, as recently proposed by Krenzien et al. [30].

Finally, we evaluated the associations between PBLs and consequent graft outcomes (OI and rejection); 25% of PBLs performed within the first year were realized in the context of OI versus 5% in the context of AR, which reflects the strong immunosuppression during this period.

Regarding OI, as expected, T and B lymphopenia was associated both with bacterial and viral infections. These findings are well known in infectious diseases and have also been described many times in the kidney transplant literature [2,3]. In our study, CD4+ T cells were the best diagnostic marker of

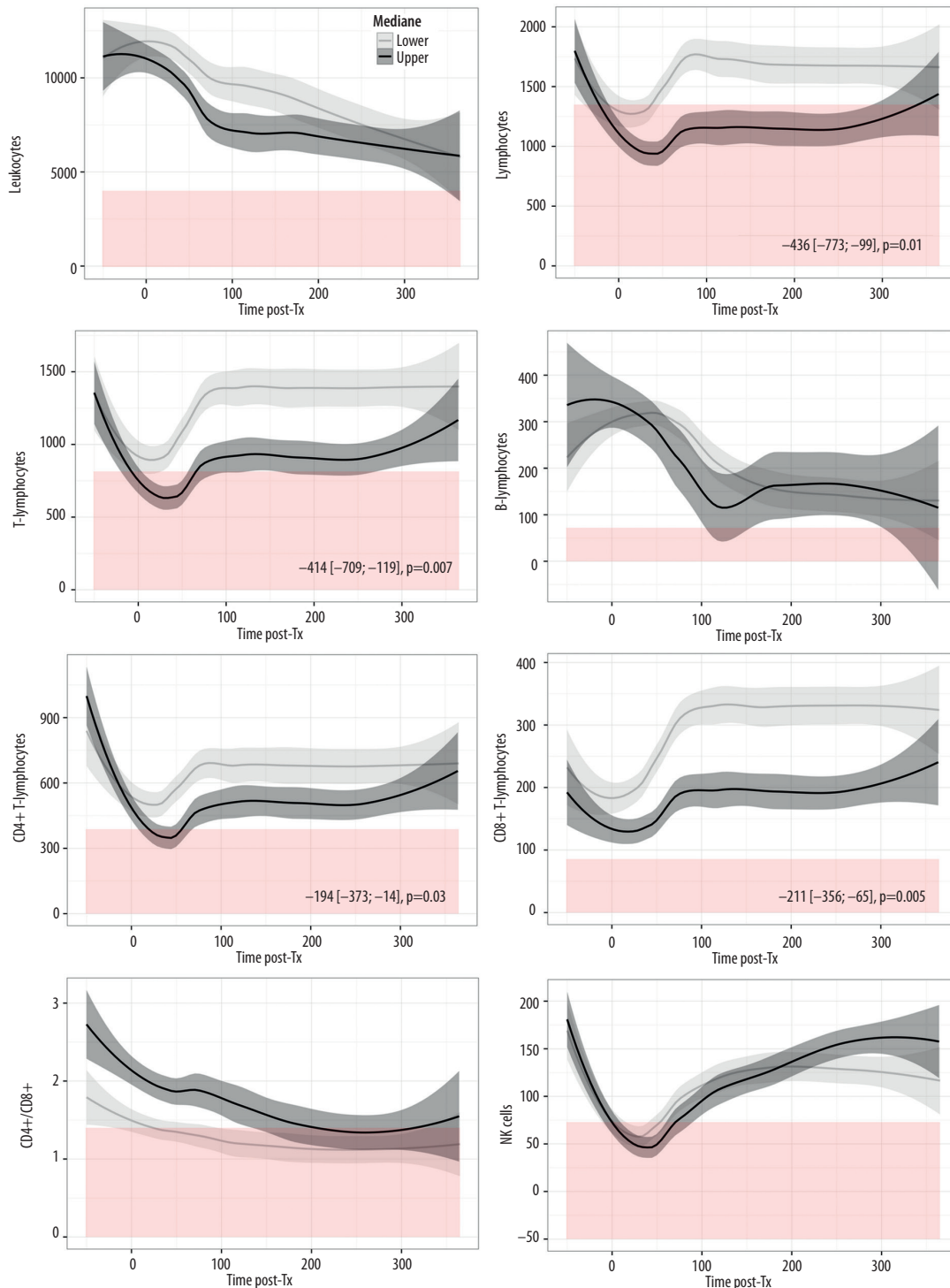


Figure 3. Kinetics of peripheral blood lymphocyte subsets (PBLs) over the first year post-transplantation according to age groups (median). Y-axis=cell counts in cells/ μ L and X-axis=time post-transplant in days. Grey curves=recipients \leq median age (48 years old). Black curves= recipients $>$ median age. Curves represent the smoothed mean with confidence interval. Values before zero represent the pooled counts performed within 3 months before LTx. Red color represents the area below inferior normal thresholds from healthy controls. Analysis by linear mixed model: beta estimation of cell counts differences with confidence interval and p value.

Table 3. PBLs counts according to outcome. The results (expressed in cells/ μ L) represent the estimated difference [95% CI] in PBLs counts performed within the first year according to the concomitant presence of an infectious and/or immunological complication. The statistical analysis was performed with the linear mixed model as described in Methods.

| | Univariate analysis | | Multivariate analysis | | Univariate analysis | | Multivariate analysis | | Univariate analysis | | Multivariate analysis | |
|--------------|----------------------------|---------|-----------------------|--------------|------------------------|---------|-----------------------|--------------|-------------------------|---------|-----------------------|------------------|
| | Estimated difference | p Value | Estimated difference | p Value | Estimated difference | p Value | Estimated difference | p Value | Estimated difference | p Value | Estimated difference | p Value |
| | [95% CI] | | [95% CI] | | [95% CI] | | [95% CI] | | [95% CI] | | [95% CI] | |
| | Bacterial infection | | | | Viral infection | | | | Fungal infection | | | |
| Leukocytes | 461 | 0.19 | -206 | 0.61 | 887 | 0.46 | | | 1675 | 0.19 | -316 | 0.79 |
| | [-234; 1156] | | [-1004; 593] | | [-1478; 3251] | | | | [-814; 4164] | | [-2650; 2017] | |
| Lymphocytes | -190 | <0.001 | -177 | 0.009 | -324 | 0.048 | -601 | 0.002 | 216 | 0.23 | | |
| | [-284; -97] | | [-310; -44] | | [-646; -2] | | [-984; -218] | | [-139; 571] | | | |
| T-Cells | -176 | <0.001 | -137 | 0.014 | -264 | 0.055 | -471 | 0.003 | 205 | 0.17 | 604 | <0.001 |
| | [-255; -98] | | [-246; -28] | | [-533; 6] | | [-784; -159] | | [-92; 503] | | [282; 926] | |
| CD4+ T-Cells | -107 | <0.001 | -80 | 0.01 | -140 | 0.11 | -237 | 0.007 | 162 | 0.09 | 412 | <0.001 |
| | [-157; -58] | | [-140; -19] | | [-309; 30] | | [-410; -65] | | [-26; 349] | | [235; 590] | |
| CD8 T-Cells | -62 | <0.001 | -55 | 0.044 | -129 | 0.03 | -214 | 0.007 | 57 | 0.38 | | |
| | [-96; -28] | | [-109; -1] | | [-246; -12] | | [-368; -60] | | [-71; -186] | | | |
| B-Cells | -34 | 0.008 | -46 | 0.017 | -83 | 0.06 | -144 | 0.009 | -14 | 0.77 | | |
| | [-60; -9] | | [-85; -8] | | [-171; 4] | | [-251; -37] | | [-110; 81] | | | |
| NK-Cells | -107 | 0.08 | -9 | 0.27 | 1 | 0.95 | | | 21 | 0.37 | | |
| | [-23; 1] | | [-26; 7] | | [-40; 42] | | | | [-25; 6] | | | |
| | CMV replication | | | | DSA class I | | | | DSA class II | | | |
| Leukocytes | -923 | 0.08 | -218 | 0.65 | 55 | 0.94 | | | -174 | 0.79 | | |
| | [-1946; 102] | | [-1178; 742] | | [-1424; 1534] | | | | [-1475; 1128] | | | |
| Lymphocytes | -104 | 0.10 | -92 | 0.25 | -111 | 0.28 | | | -114 | 0.20 | -25 | 0.79 |
| | [-228; 21] | | [-250; 66] | | [-310; -89] | | | | [-287; 60] | | [-214; 163] | |
| T-Cells | -84 | 0.11 | -81 | 0.22 | 126 | 0.26 | | | -75 | 0.31 | | |
| | [-188; 20] | | [-210; -49] | | [-95; 347] | | | | [-220; 70] | | | |
| CD4+ T-Cells | -57 | 0.06 | -61 | 0.10 | -62 | 0.24 | | | -47 | 0.31 | | |
| | [-117; 2] | | [-132; 11] | | [-167; 42] | | | | [-138; 44] | | | |
| CD8 T-Cells | -30 | 0.23 | | | -237 | 0.26 | | | -38 | 0.24 | | |
| | [-78; 19] | | | | [-410; -63] | | | | [-100; -25] | | | |
| B-Cells | 27 | 0.16 | 19 | 0.39 | -38 | 0.16 | 12 | 0.76 | -41 | 0.08 | -16 | 0.53 |
| | [-11; 65] | | [-25; 64] | | [-92; 15] | | [-64; 88] | | [-87; 5] | | [-68; 35] | |
| NK-Cells | -4 | 0.61 | | | 12 | 0.37 | | | 4 | 0.73 | | |
| | [-19; 11] | | | | [-14; 38] | | | | [-19; 27] | | | |
| | Acute rejection | | | | BOS | | | | RAS | | | |
| Leukocytes | 900 | 0.20 | -379 | 0.65 | -1053 | 0.51 | | | 3712 | 0.03 | 3770 | 0.028 |
| | [-476; 2276] | | [-2027; 1268] | | [-4171; 2064] | | | | [-304; 7120] | | [418; 7122] | |
| Lymphocytes | -113 | 0.24 | | | 149 | 0.54 | | | -254 | 0.37 | | |
| | [-302; 76] | | | | [-328; 625] | | | | [-809; 301] | | | |
| T-Cells | -117 | 0.15 | -185 | 0.11 | 128 | 0.53 | | | -175 | 0.46 | | |
| | [-276; 41] | | [-412; 42] | | [-272; 528] | | | | [-642; 291] | | | |
| CD4+ T-Cells | -74 | 0.14 | -107 | 0.09 | -12 | 0.92 | | | -90 | 0.54 | | |
| | [-174; 25] | | [-233; 18] | | [-261; 237] | | | | [-379; 198] | | | |
| CD8 T-Cells | -46 | 0.19 | -67 | 0.24 | 146 | 0.11 | 324 | 0.006 | -75 | 0.49 | | |
| | [-115; 23] | | [-179; 45] | | [-35; 327] | | [94; 553] | | [-290; 141] | | | |
| B-Cells | -11 | 0.67 | | | 179 | 0.013 | 92 | 0.34 | -49 | 0.58 | | |
| | [-62; 40] | | | | [-37; 322] | | [-97; 281] | | [-224; 126] | | | |
| NK-Cells | 1 | 0.94 | | | 23 | 0.38 | | | -40 | 0.17 | -60 | 0.034 |
| | [-25; 23] | | | | [-29; 76] | | | | [-98; 17] | | [-115; -5] | |

CMV – cytomegalovirus; DSA – donor-specific antibodies; AR – acute rejection; RAS – restrictive allograft syndrome; BOS – bronchiolitis obliterans syndrome; CI – confidence interval.

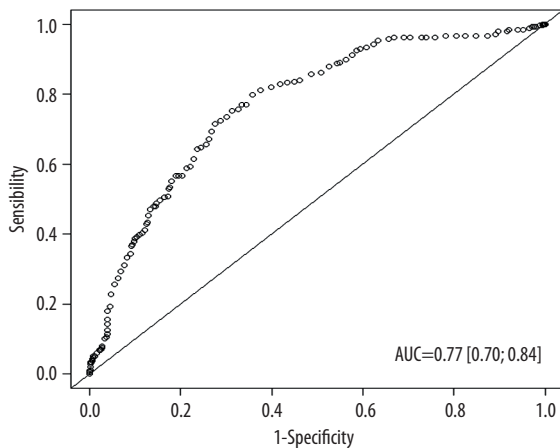


Figure 4. ROC curves of CD4+ T cell counts realized within the first year for diagnosis of infection (bacterial and viral). AUC: area under the curve.

global infection, with a best threshold evaluated at 432 cell/ μ L. Thus, CD4+ T cells, as used in HIV for the prevention of OI [35], could be an interesting biomarker of infection. More recently, immune function monitoring using adenosine triphosphate lymphocytes production was also revealed as being an interesting blood marker of infection after lung transplantation [36,37].

We did not demonstrate any association between PBLs and CMV reactivation, whereas Calarota et al. previously demonstrated a strong association with CD4+ T lymphopenia [4]. Missing data for CMV (19.8% of PBLs) probably underpowered this analysis.

Class I or class II donor-specific antibodies and acute rejection were not associated with specific PBLs profile in our study. This suggests that standard lymphocytes subsets (T, B, and NK cells) are not specific enough to show an association with these risks. Some authors demonstrated a link between activated T lymphocytes that were marked for HLA-DR (type II MHC) and acute rejection in kidney transplantation, suggesting that a more specific marker of immune response must be found [38–41].

Regarding CLAD, the expected low incidence within the first postoperative year (2% of PBLs, 2 RAS and 5 BOS over the 57 recipients included) does not allow a strong conclusion. Nonetheless, multivariate analysis revealed high levels of CD8+ T lymphocytes in BOS and high levels of leukocytes in RAS. These results are consistent with the recent lung transplantation literature. Indeed, there is now strong evidence that adaptive T cell responses play a key role in chronic lung allograft rejection, especially in obliterative bronchiolitis [42,43].

However, the correlation between high CD8+ T lymphocytes and BOS is almost exclusively described in animals, and particularly in murine heterotopic allogeneic airway transplant models that showed the implication of CD8+ T cells in the role of airway allograft rejection [44–49]. More specifically, PD-1 and histone deacetylase 2 involved in immune tolerance of CD8+ T cells was recently demonstrated as being link to lung graft survival [50,51].

The association between RAS and high leukocyte count is also interesting. Leukocytes kinetics are probably indirectly related to neutrophil blood cell population (not evaluated in our study), since neutrophils represent more than half of the total numeration. The mechanisms involved are not clear, but increased neutrophils in broncho-alveolar lavage fluid from recipients with RAS was recently described by Verleden et al. [52], who demonstrated that neutrophils might be involved in the pathophysiology of this CLAD.

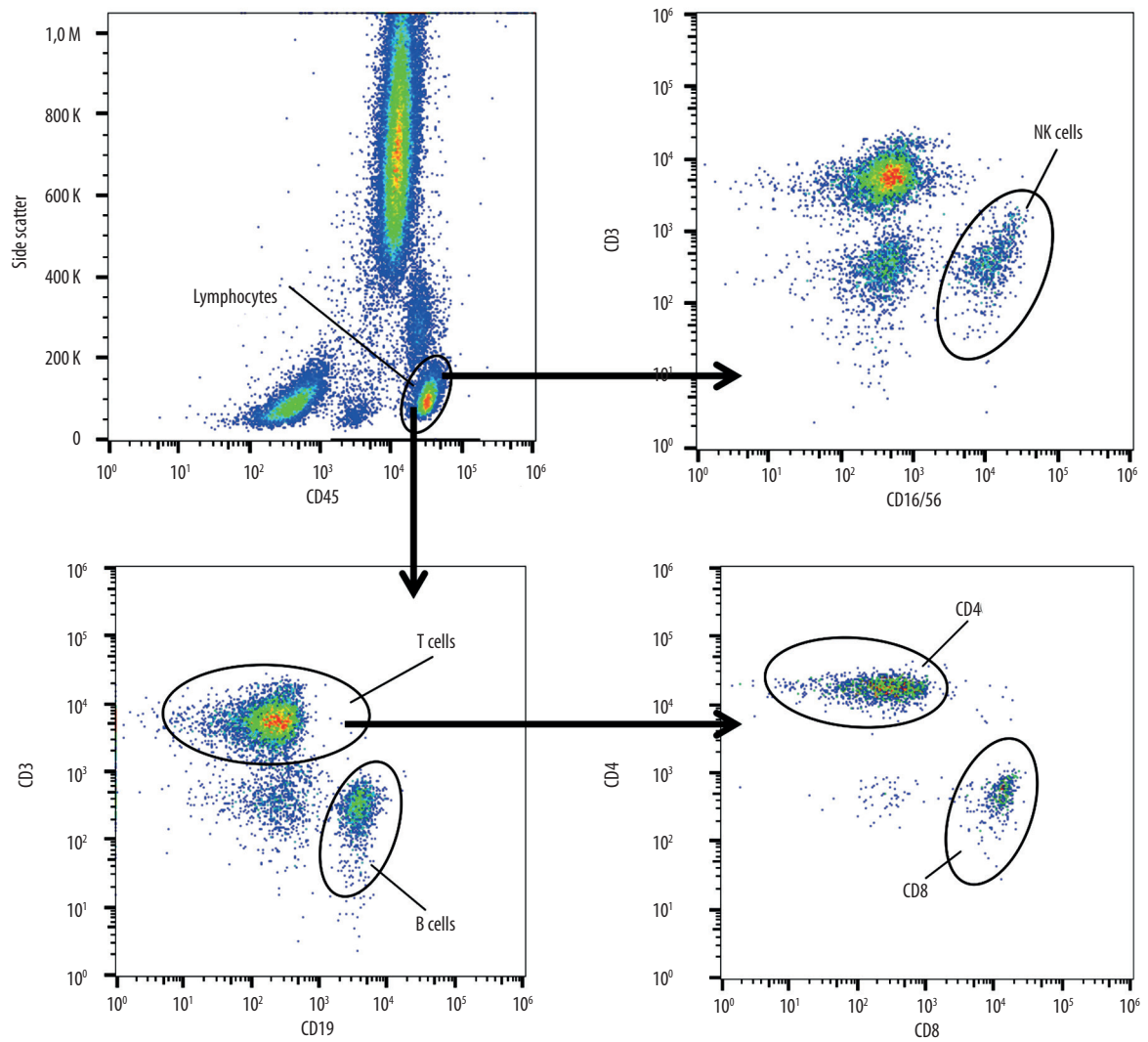
Our study has some limitations. Its monocentric nature makes generalizations difficult but allowed us to have a homogeneous group. All patients received the same immunosuppressive treatment, which is standard in lung transplantation. These results should be confirmed prospectively and assessed in a larger, multicenter cohort.

Conclusions

Our study demonstrated that aging is associated with more severe T lymphopenia after induction therapy for lung transplantation. This effect could be related to immune senescence, which was until now was only indirectly suspected based on the fact that older patients developed more opportunistic infections and cancer, and had less rejection in solid organ transplantation [30]. It would be thus interesting to evaluate the effect of a strategy of age-adapted immunosuppression on the allograft outcome in a proper prospective randomized trial. In the new era of individualized therapy in modern medicine, immunosuppression protocols should probably be more individualized according to the individual recipient characteristics and immune status. Lee et al. recently proposed to guiding the introduction of immunosuppression in liver transplantation based on the preoperative levels of CD8+ T lymphocytes [53].

Furthermore, we found an interesting association between PBLs and allograft outcomes such as CD8+ T cells with BOS and leukocytes with RAS, which is consistent with the recent literature on lung transplantation. These results reinforce the idea that PBLs monitoring is an interesting tool to use in evaluating the risk-benefit balance of immunosuppression and could be a useful biomarker of specific allograft outcome.

Supplementary Files



Supplementary Figure 1. Peripheral blood lymphocyte subsets (PBLs) count by flow cytometry. Cells in the lymphocyte gate were subsequently separated into T cells ($CD3^+$), B cells ($CD19^+$), and NK cells ($CD16CD56^+$). Cells in the T cell gate were separated into helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$).

Supplementary Table 1. Description of pathogens and clinical sites implicated in reported infections (bacteria and viruses) isolated in recipients. Description concerns only PBLs realized after lung transplant (n=839/890).

| | Causal agent | PBLs, n (%) | Time | Site | PBLs, n (%) | Time |
|----------------------|----------------------------------|-------------|---------------|---------------|-------------|--------------|
| Total | | 839 | | | 839 | |
| | | 228 (100) | | | 228 (100) | |
| Bacterial Infections | <i>Staphylococcus aureus</i> | 43 (19) | 38 [25–103] | Lung | 183 (81) | 75 [30–185] |
| | <i>Streptococcus pneumoniae</i> | 7 (3) | 185 [70–220] | Urinary tract | 13 (6) | 64 [42–245] |
| | Enterococcus (faecalis, faecium) | 4 (2) | 49 [45–55] | Bacteriemia | 12 (5) | 57 [44–85] |
| | <i>Neisseria flavescens</i> | 1 (0.5) | 26 [26–26] | Intestin | 2 (1) | 89 [76–103] |
| | GPB (Bacillus, Corynebacterium) | 5 (2) | 121 [42–284] | Others | 16 (7) | 122 [15–230] |
| | Enterobacteria, group I | 7 (3) | 121 [24–291] | | | |
| | Enterobacteria, group II | 21 (9) | 72 [37–111] | | | |
| | Enterobacteria, group III | 26 (11) | 58 [29–152] | | | |
| | Other GNB-S | 5 (2) | 124 [120–129] | | | |
| | Other GNB-R | 21 (9) | 55 [22–72] | | | |
| | <i>Pseudomonas aeruginosa</i> | 69 (30) | 121 [38–206] | | | |
| | Not found | 19 (8) | 176 [98–261] | | | |
| | | | 15 (100) | | | |
| Viral infections | <i>Myxovirus influenzae</i> | 3 (20) | 125 [87–128] | | | |
| | Respiratory syncytial virus | 6 (40) | 280 [185–289] | | | |
| | Rhinovirus | 3 (20) | 61 [59–69] | | | |
| | Human simplex virus | 3 (20) | 67 [63–70] | | | |

GPB – gram-positive bacilli; GNB-S – gram-negative bacilli sensitive to third-generation cephalosporin; GNB-R – gram-negative bacilli resistant to third-generation cephalosporin.

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