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Title: LUNG INTRAGRAFT DONOR-SPECIFIC ANTIBODIES AS A RISK FACTOR FOR GRAFT LOSS

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Abstract

Background: In lung transplantation, the impact of donor-specific anti-HLA antibodies (DSA) on graft survival is recognized, but not all serum DSA appear to be harmful. We wondered whether *in situ* DSA detection from graft biopsies could help in identifying lung transplant recipients (LTRs) at higher risk for graft loss.

Methods: For 53 LTRs, class I and II HLA antibody single antigen flow bead assays were performed to identify IgG DSA in biopsy eluates and in sera, and to evaluate C1q binding ability of DSA in sera. Intragraft antibodies (gDSA) were correlated with serum DSA (sDSA), clinical and histological data, and graft survival.

Results: Twenty-eight (52.8%) LTRs had sDSA, 12 (42.9%) had C1q+ sDSA and 11 (20.8%) had gDSA. Fifty sDSA were found, among which 15 (30%) were C1q+ and 14 (28%) were found in biopsy eluates. One DSA was detected in the biopsy only. Both serum MFI and biopsy fragment size were higher for sDSA detected in biopsies (p=0.003 and 0.02, respectively). LTRs with gDSA displayed a lower one-year post-biopsy graft survival (log-rank test p=0.008). Presence of gDSA at the time of biopsy constituted a risk factor for graft loss in univariate (OR 6.67 [1.51-29.47], p=0.008 and HR 3.44 [1.47-8.01], p=0.005) and multivariate (OR 5.85 [1.23-27.68], p=0.03 and HR 4.51 [1.83-11.13], p=0001) analyses using logistic regression and a Cox proportional hazard model, respectively.

Conclusions: In lung transplantation, intragraft DSA appears as a valuable biomarker to identify pathogenic DSA and recipients with a higher risk for graft loss.

Introduction

Lung transplantation (LT) has benefited from advances in surgical techniques and immunosuppression strategies, but median survival remains limited in comparison with other solid organ transplantations.[1] The main cause of graft loss is bronchiolitis obliterans syndrome (BOS),[1, 2] which is associated with several risk factors: acute cellular rejection (ACR), lymphocytic bronchitis, human leukocyte antigen (HLA) mismatches, community-acquired respiratory viral infections, primary graft dysfunction and gastroesophageal reflux.[3-6]

Previous studies showed that development of HLA antibodies was also associated with BOS and even preceded its development.[7-10] This was further demonstrated using single antigen flow beads (SAFB) assays which greatly improved the resolution and the sensitivity of donor-specific antibodies (DSA) detection.[11-16] However, detection of serum DSA with SAFB has technical limitations, false-negative and false-positive results being respectively caused by complement interference[17-19] and by anti-HLA antibodies of ill-defined pathogenic role recognizing denatured class I HLA molecules.[20-25]

The presence of DSA is not synonymous with lung allograft injury.[26] The lack of direct association may be due to the inability of the DSA to bind to the graft because of the absence of expression of cognate HLA molecules, or because of insufficient binding strength between a low affinity DSA and its target. It was also suggested in kidney[27, 28] and liver[29] transplantation that circulating DSA may not be detectable within the blood during episodes of antibody mediated rejection (AMR) if the DSA are trapped within the allograft. Another difficulty is that the histological features of AMR in LT have not yet been accurately defined. Therefore, the diagnosis of AMR relies on the presence of allograft dysfunction, circulating DSA and pathologic findings, without clear evidence of a pathogenic interaction between the DSA and the transplant.[30]

The SAFB assay has been adapted to the detection of C1q binding HLA antibodies,[31] with promising results in kidney transplantation[32, 33] but, to our knowledge, the use and interest of this new assay have not been studied in lung transplantation.

In a previous study of kidney transplantation, we previously reported that *in situ* detection of DSA was a severity marker for antibody-mediated pathogenic processes.[34] The aims of this study were therefore to evaluate whether intragraft DSA (gDSA) can be detected in lung transplant biopsies, to identify the factors associated with their presence and whether they could represent a risk factor for graft loss.

Methods

Patients and biopsies

In this retrospective single centre study, among the 252 LT recipients (LTRs) transplanted locally from January 1999 to July 2014, we included the 53 LTRs for whom a frozen, non-fixed biopsy and a serum sample obtained close to the day of biopsy were available. Biopsies were performed either for deterioration in clinical status or for routine surveillance. Formalin-fixed, paraffin-embedded biopsy specimens were stained with hematoxylin-eosin for histologic review and classification according to the ISHLT 2007 and ISHLT 2013 reports.[35, 36] Immunohistochemical C4d analysis was performed on deparaffinized sections. Continuous, linear, endothelial/sub-endothelial C4d staining in capillaries was considered positive. We collected available data on HLA sensitization status, graft conditions, immunosuppressive regimen and graft functional status at 12 months post-biopsy and until August 2015. Recipient's death, replacement on the waiting list or re-transplantation defined a graft loss. The study was approved by the institutional review board and did not interfere with standard patient clinical management. Except for cystic fibrosis patients, LTRs received an induction therapy with anti-thymocyte globulins. Maintenance therapy associated cyclosporine, mycophenolate and steroids. Tacrolimus replaced cyclosporine in case of BOS or refractory acute rejection. Everolimus was associated to the triple therapy in two cases: kidney dysfunction, in order to decrease the dose of calcineurin inhibitor, and insufficient improvement of respiratory function after calcineurin inhibitor switch, for patients with BOS. Azithromycin was introduced in case of BOS with airway neutrophilia without infectious manifestation.

Biopsy elution and anti-HLA antibody testing

We used frozen biopsy fragments initially devoted to histopathological analysis. No sampling was specifically performed for this study. Graft biopsies were processed according to the previously described protocol[34, 37] using the Acid Elution kit (Elukit II; Gamma Biologicals, Inc., Houston, TX). Their length, width and thickness were measured. Anti-HLA Class I and II antibodies were identified in serum and biopsy eluates with the LabScreen LS1A04 and LS2A01 SAFB assays (One Lambda, Inc., Canoga Park, CA) on a Luminex 100® analyzer (Luminex, Austin, TX), according to the manufacturer's recommendations. Sera were systematically treated with ethylenediamine tetraacetic acid in order to circumvent the complement interference phenomenon.[17-19] The C1q Screen® assay (One lambda) was performed when a serum DSA (sDSA) was detected. For sDSA, the positivity threshold was set at a normalized mean fluorescence intensity (MFI) of 500 for IgG detection and 300 for C1q binding, using the baseline calculation mode (HLA Fusion software, One Lambda). For biopsy eluates, the positivity threshold was set at a normalized MFI higher than the mean plus 5 standard deviations of the MFI obtained in the biopsy eluate for the alleles not expressed by the donor that displayed a MFI below 500 in serum, i.e. against which the patient was considered not sensitized. When necessary, additional donor HLA typing (Class I HLA-A, -B, -C and Class II HLA-DR,-DR51/52/53, -DQ and -DP) was performed with high-resolution reverse SSO-PCR (LabType, One Lambda) on frozen aliquots of cells collected at the time of transplantation.

Statistical analysis

Categorical and continuous variables were summarized as percentage and median values with range or interquartile range (IQR). The independent-samples Mann–Whitney U-test and the Fisher's exact test were used for group comparisons of continuous and categorical variables, respectively. Optimal threshold for MFI value was determined with receiver operating characteristics (ROC) analysis. Kaplan–Meier analysis was used to construct graft survival curves. Comparisons used the log-rank test. The variables potentially associated with one-year post-biopsy survival were subjected

to univariate and multivariate analysis, using a logistic regression model. A Cox proportional hazards analysis was used for univariate and multivariate analyses for overall graft survival. Multivariate analyses included variables that showed trends in univariate analysis, i.e. $p \leq 0.2$. Variables not independently predictive of graft survival were dropped using the backward elimination procedure. Results were reported as odds ratios (ORs) or hazard risk (HR) with a 95% confidence interval (CI) and corresponding p-value. P-value ≤ 0.05 was considered statistically significant. Analyses were performed with MedCalc software (Mariakerke, Belgium).

Results

Patient characteristics at transplantation and at biopsy

The study included 53 LTRs for whom serum and biopsy eluates were tested with both class I and II SAFB. At the time of biopsy, serum analysis showed that 4 LTRs were not sensitized while 49 LTRs (92.5%) exhibited anti-HLA antibodies, with 6 (11.3%) only exhibiting anti-class I, 9 (17.0%) only anti-class II and 34 (64.2%) both anti-class I and anti-class II. At least one sDSA was present in 28 LTRs (52.8%), with 6 (21.4%) displaying only class I, 18 (64.3%) only class II and 4 (14.3%) both class I and class II DSA. Median sDSA number was 2 (range 1-4) per patient. Twelve (42.9%) among the 28 sDSA+ LTRs displayed at least one C1q+ sDSA.

Among the 53 LTRs, 11 (20.8%) displayed one or more gDSA, 3 (27.3%) of them displaying only class I gDSA and 8 (72.7%) only class II gDSA, with a median of 1 gDSA (range 1-3) per patient. One LTR displayed a DSA in biopsy eluate not found in the serum. Seven (63.6%) among the 11 sDSA+/gDSA+ LTRs displayed at least one C1q+ sDSA.

At the time of transplantation, there was no difference in terms of demographic or clinical characteristics between LTRs who will become gDSA+ or gDSA- (for these latter, sDSA+/gDSA- and sDSA-/gDSA- LTRs being pooled) at the time of biopsy (Table 1). The only difference at the time of biopsy was that gDSA+ patients more frequently displayed acute decline of graft function (Table 2). Median time between serum and biopsy sampling was similar between gDSA- (0 day, IQR 0-42) and gDSA+ (7 days, IQR 0-83) LTRs (p=0.54). Histological findings at time of biopsy evidenced a higher proportion of normal biopsies for gDSA- LTRs (Table 2). Allograft function at time of biopsy was similar between gDSA+ and gDSA- LTRs (Table 3).

Characteristics of sDSA and gDSA

A total of 50 sDSA were found, among which were 14 (28%) class I (6 HLA-A, 2 HLA-B, 6 HLA-C) with a median MFI of 1095 (IQR 652-3366) and 36 (72%) class II (8 HLA-DR, 18 HLA-DQB1, 6 HLA-DQA1, 4 HLA-DP) with a median MFI of 3662 (IQR 1590-9113) (class I vs class II: p=0.004, Figure 1A). Among the sDSA, 15 (30%) were C1q+, all in class II (1 HLA-DR, 10 HLA-DQB1, 4 HLA-DQA1), with a median IgG SAFB MFI of 8819 (IQR 4528–19237) which was significantly higher than for the C1q- sDSA (median MFI 1456, IQR 834–3393) (C1q+ vs C1q-: p<0.0001).

Fourteen (28%) of the 50 sDSA were found in biopsies eluates (s+/g+ DSA) and one DSA was in biopsy only (s-/g+ DSA). These 15 gDSA constituted the gDSA group, composed of 4 (26.7%) class I (2 HLA-A, 1 HLA-B and 1 HLA-C) and 11 (73.3%) class II (1 HLA-DR, 7 HLA-DQB1, 3 HLA-DQA1). The median MFI of sDSA was 1804 (IQR 940–3720) for those not found in biopsies, and 10383 (IQR 3707–19237) for those found in biopsies, which was significantly different (gDSA+ vs gDSA-: p=0.003, Figure 1B). We used a ROC analysis to define the optimal sDSA MFI threshold allowing the gDSA+ status to be predicted. The area under curve was 0.765 (p=0.007) and the optimal threshold was 6515 (IC 3249–9407), offering 66.7% sensitivity and 91.7% specificity, 8 and 0.36 positive and negative likelihood ratios, respectively. Size of the eluted fragment was higher for the gDSA+ (median 8.5 mm^3, IQR 4.9–11.3) than for the gDSA- (median 5.8 mm^3, IQR 1.7–6.4) biopsies (p = 0.02). Relations between sDSA MFI, biopsy fragment size and gDSA status are presented in Figure 1C. Of note, C1q+ sDSA and gDSA constituted overlapping but not identical groups as 7/15 C1q+ sDSA were not found in biopsy eluates and 7/15 gDSA did not bind C1q in serum.

DSA status and post-biopsy outcomes

At one year post-biopsy, 20 (37.7%) LTRs had lost their graft, with a median of 92 days post-biopsy (IQR 20-231). Graft survival was comparable between the sDSA- LTRs and the sDSA+ LTRs, whichever their gDSA status was (Figure 2A). In contrast, graft survival was lower for the gDSA+

LTRs than for the gDSA- and the sDSA- LTRs (Figure 2B and 2C). Identical findings were observed after censoring LTRs who survived with their graft less than 3 months post-biopsy (Figure 2, D to F). At the time of biopsy, presence of gDSA and infection were the only risk factors for graft loss in univariate and multivariate analyses (Table 4). During the first year post-biopsy, the only difference between gDSA- and gDSA+ LTRs in terms of immunosuppression or infection was that gDSA+ LTRs were more frequently treated for humoral rejection than sDSA+/gDSA- LTRs (Table 5). The median of total post-biopsy follow-up was 391 days (IQR 214-434) and 25 (47.2%) recipients lost their graft at a median of 212 days post-biopsy (IQR 44-358). We analyzed several parameters at time of biopsy with a Cox proportional hazards model. Only presence of gDSA and infection at the time of biopsy were associated with an increased risk for graft loss (Table 4). There was no difference in cause of graft loss (Table 6) or allograft dysfunction at the end of follow-up between gDSA+ and gDSA- LTRs (data not shown).

Discussion

We report for the first time, to our knowledge, that DSA can be identified in eluates from lung transplant biopsies. Anti-DQ DSA were the most frequently identified as sDSA and also as gDSA. Their role has been less studied than that of anti-class I and anti-DR DSA, because of the difficulties in identifying anti-DQ DSA prior to the use of SAFB. Moreover, expression of HLA-DQ by the graft has been scarcely studied. It is noteworthy that the assignment of the DQ antigenic specificities has traditionally relied on the beta chain only, ignoring the possible contribution of the alpha chain and potentially underestimating its importance. Nowadays, thanks to a more accurate evaluation, the highly pathogenic role of HLA-DQ DSA is increasingly reported for lung as well as for other solid organ transplantations.[38-40] Our results strongly support these findings by showing that they can be found in biopsy eluates, i.e. are able to interact with donor HLA-DQ molecules expressed by the lung allograft.

The prevalence of gDSA was lower than that of s+/g- DSA, which could reflect an insufficient sensitivity of the biopsy elution assay. Indeed, s+/g+ DSA had higher serum MFI values than s+/g- DSA, and were preferentially, but not systematically, detected in eluates from larger biopsy fragments. This indicated that we might have missed sDSA bound to the donor tissue when the amount and/or strength of the sDSA were too low and/or when the biopsy fragment was too small.[42] Nevertheless, we could detect several sDSA of very low MFI in small biopsies whereas others with higher MFI were not retrieved *in situ*, despite tissue fragments of substantially bigger size. In line with this, the serum MFI threshold calculated with ROC analysis to predict the presence of gDSA offered quite weak sensitivity and positive likelihood ratio. Moreover, for one LTR we were able to detect one gDSA which was not retrieved in serum, which suggested that the DSA were completely trapped within the allograft.[27, 28] Therefore the search for gDSA would greatly benefit from disposing of larger tissue fragments in order to ascertain that a negative result is a proof of absence of DSA *in situ*.

In our cohort, gDSA+ LTRs displayed a lower post-biopsy survival when compared with all other LTRs, i.e. those with sDSA only, without DSA or without anti-HLA antibodies. No difference

was observed between sDSA+ and sDSA- LTRs. These findings were confirmed in a multivariate analysis showing that the presence of gDSA but not of sDSA was an independent risk factor for graft loss. Then, besides the technical considerations discussed above, the clinical management of LTRs could also benefit from gDSA detection. Indeed, although almost all the gDSA were also sDSA, detection of a gDSA confirms that the sDSA is able to interact with the transplant and to exert pathogenic effects. On the contrary, sDSA not detectable *in situ* could have an affinity/avidity and/or be produced at a concentration that are not adequate, or could target antigens not expressed high enough locally. Interestingly, more than half of gDSA+ recipients were diagnosed as having ACR, infectious disease or normal biopsy, but not acute or chronic AMR. In these cases, prospectively identifying gDSA could help refining the diagnosis and more precisely guiding recipient management.

We observed that the C1q+ sDSA and gDSA populations were overlapping but not identical, and that the presence of C1q+ sDSA was not associated with a higher risk for graft loss. However, complement activation is only one of the possible effector mechanisms for a DSA and we recently showed that non complement binding DSA are indeed harmful.[42] The ability of the DSA to bind to the transplant could therefore provide more valuable information than its ability to bind C1q on SAFB.

Another approach for evaluating AMR in lung transplantation could be the search for DSA in bronchoalveolar lavage fluids. However, it could be less sensitive, and it might not bring the same information as biopsy elution through detection of DSA unable to strongly interact with the graft.

Our study has several limitations. It is a single-center, retrospective and nonrandomized study on a rather small LTRs cohort, and the comparison of LTRs characteristics at time of transplantation or biopsy could have been underpowered, not precluding that some risk factors for the presence of gDSA might exist.[38] Lung graft function deterioration was often at an advanced stage at the time of the biopsy, as nearly 40% of recipients already had severe BOS. Therefore, our findings cannot be used for estimating the role of gDSA in the early phase of AMR. Moreover, we did not observe a decreased post-biopsy graft survival for sDSA+ LTRs in comparison with LTRs without sDSA or anti-HLA antibodies. This was in disagreement with reports from the recent literature[11-13, 15, 16] and could be

explained by a short follow-up after biopsy which did not allow graft loss caused by chronic AMR to be observed.

In conclusion, by showing that the presence of gDSA was associated with graft loss, we highlight the interest of searching DSA in biopsy eluates. This work paves the way for further prospective investigations on larger cohorts to analyze whether gDSA could represent a valuable biomarker of AMR in a field where other approaches, such as histopathology, are frequently unhelpful.

Disclosure statement

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References

1. Yusen RD, Christie JD, Edwards LB, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Adult Lung and Heart-Lung Transplant Report--2013; focus theme: age. J Heart Lung Transplant 2013;32:965-78.

2. Finlen Copeland CA, Snyder LD, Zaas DW, Turbyfill WJ, Davis WA, Palmer SM. Survival after bronchiolitis obliterans syndrome among bilateral lung transplant recipients. Am J Respir Crit Care Med 2010;182:784-9.

3. Estenne M, Maurer JR, Boehler A, et al. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002;21:297-310.

4. Khalifah AP, Hachem RR, Chakinala MM, et al. Respiratory viral infections are a distinct risk for bronchiolitis obliterans syndrome and death. Am J Respir Crit Care Med 2004;170:181-7.

5. Daud SA, Yusen RD, Meyers BF, et al. Impact of immediate primary lung allograft dysfunction on bronchiolitis obliterans syndrome. Am J Respir Crit Care Med 2007;175:507-13.

6. D'Ovidio F, Mura M, Tsang M, et al. Bile acid aspiration and the development of bronchiolitis obliterans after lung transplantation. J Thorac Cardiovasc Surg 2005;129:1144-52.

7. Jaramillo A, Smith MA, Phelan D, et al. Development of ELISA-detected anti-HLA antibodies precedes the development of bronchiolitis obliterans syndrome and correlates with progressive decline in pulmonary function after lung transplantation. Transplantation 1999;67:1155-61.

8. Sundaresan S, Mohanakumar T, Smith MA, et al. HLA-A locus mismatches and development of antibodies to HLA after lung transplantation correlate with the development of bronchiolitis obliterans syndrome. Transplantation 1998;65:648-53.

9. Girnita AL, McCurry KR, Iacono AT, et al. HLA-specific antibodies are associated with high-grade and persistent-recurrent lung allograft acute rejection. J Heart Lung Transplant 2004;23:1135-41.

10. Palmer SM, Davis RD, Hadjiliadis D, et al. Development of an antibody specific to major histocompatibility antigens detectable by flow cytometry after lung transplant is associated with bronchiolitis obliterans syndrome. Transplantation 2002;74:799-804.

11. Morrell MR, Pilewski JM, Gries CJ, et al. De novo donor-specific HLA antibodies are associated with early and high-grade bronchiolitis obliterans syndrome and death after lung transplantation. J Heart Lung Transplant 2014;33:1288-94.

12. Safavi S, Robinson DR, Soresi S, Carby M, Smith JD. De novo donor HLA-specific antibodies predict development of bronchiolitis obliterans syndrome after lung transplantation. J Heart Lung Transplant 2014;33:1273-81.

13. Ius F, Sommer W, Tudorache I, et al. Early donor-specific antibodies in lung transplantation: risk factors and impact on survival. J Heart Lung Transplant 2014;33:1255-63.

14. Smith JD, Ibrahim MW, Newell H, et al. Pre-transplant donor HLA-specific antibodies: characteristics causing detrimental effects on survival after lung transplantation. J Heart Lung Transplant 2014;33:1074-82.

15. Witt CA, Gaut JP, Yusen RD, et al. Acute antibody-mediated rejection after lung transplantation. J Heart Lung Transplant 2013;32:1034-40.

16. Kim M, Townsend KR, Wood IG, et al. Impact of pretransplant anti-HLA antibodies on outcomes in lung transplant candidates. Am J Respir Crit Care Med 2014;189:1234-9.

17. Schnaidt M, Weinstock C, Jurisic M, Schmid-Horch B, Ender A, Wernet D. HLA antibody specification using single-antigen beads--a technical solution for the prozone effect. Transplantation 2011;92:510-5.

18. Guidicelli G, Anies G, Bachelet T, et al. The complement interference phenomenon as a cause for sharp fluctuations of serum anti-HLA antibody strength in kidney transplant patients. Transpl Immunol 2013;29:17-21.

19. Visentin J, Vigata M, Daburon S, et al. Deciphering complement interference in anti-human leukocyte antigen antibody detection with flow beads assays. Transplantation 2014;98:625-31.

20. Morales-Buenrostro LE, Terasaki PI, Marino-Vazquez LA, Lee JH, El-Awar N, Alberu J. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. Transplantation 2008;86:1111-5.

21. El-Awar N, Terasaki PI, Nguyen A, et al. Epitopes of human leukocyte antigen class I antibodies found in sera of normal healthy males and cord blood. Hum Immunol 2009;70:844-53.

22. Otten HG, Verhaar MC, Borst HP, et al. The significance of pretransplant donor-specific antibodies reactive with intact or denatured human leucocyte antigen in kidney transplantation. Clin Exp Immunol 2013;173:536-43.

23. Visentin J, Guidicelli G, Bachelet T, et al. Denatured class I human leukocyte antigen antibodies in sensitized kidney recipients: prevalence, relevance, and impact on organ allocation. Transplantation 2014;98:738-44.

24. Visentin J, Marroc M, Guidicelli G, et al. Clinical impact of preformed donor-specific denatured class I HLA antibodies after kidney transplantation. Clin Transplant 2015;29:393-402.

25. Visentin J, Guidicelli G, Moreau JF, Lee JH, Taupin JL. Deciphering allogeneic antibody response against native and denatured HLA epitopes in organ transplantation. Eur J Immunol 2015;45:2111-21.

26. Glanville AR. Antibody-mediated rejection in lung transplantation: myth or reality? J Heart Lung Transplant 2010;29:395-400.

27. Del Bello A, Congy N, Sallusto F, et al. Anti-human leukocyte antigen immunization after early allograft nephrectomy. Transplantation 2012;93:936-41.

28. Del Bello A, Congy-Jolivet N, Sallusto F, et al. Donor-specific antibodies after ceasing immunosuppressive therapy, with or without an allograft nephrectomy. Clin J Am Soc Nephrol 2012;7:1310-9.

29. Neau-Cransac M, Le Bail B, Guidicelli G, et al. Evolution of serum and intra-graft donor-specific anti-HLA antibodies in a patient with two consecutive liver transplantations. Transpl Immunol 2015;33:58-62.

30. Westall GP, Snell GI. Antibody-mediated rejection in lung transplantation: fable, spin, or fact? Transplantation 2014;98:927-30.

31. Chen G, Sequeira F, Tyan DB. Novel C1q assay reveals a clinically relevant subset of human leukocyte antigen antibodies independent of immunoglobulin G strength on single antigen beads. Hum Immunol 2011;72:849-58.

32. Loupy A, Lefaucheur C, Vernerey D, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. N Engl J Med 2013;369:1215-26.

33. Calp-Inal S, Ajaimy M, Melamed ML, et al. The prevalence and clinical significance of C1q-binding donor-specific anti-HLA antibodies early and late after kidney transplantation. Kidney Int 2015. doi: 10.1038/ki.2015.275.

34. Bachelet T, Couzi L, Lepreux S, et al. Kidney intragraft donor-specific antibodies as determinant of antibody-mediated lesions and poor graft outcome. Am J Transplant 2013;13:2855-64.

35. Stewart S, Fishbein MC, Snell GI, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. J Heart Lung Transplant 2007;26:1229-42.

36. Berry GJ, Burke MM, Andersen C, et al. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. J Heart Lung Transplant 2013;32:1147-62.

37. Martin L, Guignier F, Mousson C, Rageot D, Justrabo E, Rifle G. Detection of donor-specific anti-HLA antibodies with flow cytometry in eluates and sera from renal transplant recipients with chronic allograft nephropathy. Transplantation 2003;76:395-400.

38. Lobo LJ, Aris RM, Schmitz J, Neuringer IP. Donor-specific antibodies are associated with antibody-mediated rejection, acute cellular rejection, bronchiolitis obliterans syndrome, and cystic fibrosis after lung transplantation. J Heart Lung Transplant 2013;32:70-7.

39. Smith JD, Banner NR, Hamour IM, et al. De novo donor HLA-specific antibodies after heart transplantation are an independent predictor of poor patient survival. Am J Transplant 2011;11:312-9.

40. Willicombe M, Brookes P, Sergeant R, et al. De novo DQ donor-specific antibodies are associated with a significant risk of antibody-mediated rejection and transplant glomerulopathy. Transplantation 2012;94:172-7.

41. Martin L, Guignier F, Bocrie O, et al. Detection of anti-HLA antibodies with flow cytometry in needle core biopsies of renal transplants recipients with chronic allograft nephropathy. Transplantation 2005;79:1459-61.

42. Guidicelli G, Guerville F, Lepreux S, et al. Non-Complement-Binding De Novo Donor-Specific Anti-HLA Antibodies and Kidney Allograft Survival. J Am Soc Nephrol 2015;27:615-25.

Figures legends

Figure 1: Characteristics of DSA detected in serum and in biopsy eluates with SAFB. (A) Serum MFI of class I and II DSA and (B) serum MFI of DSA detected in biopsy eluates or not. The vertical line encompasses the range, the "outliers" being represented as dots. MFI were compared using the Mann-Whitney U-test. (C) Link between serum MFI, biopsy fragment size and gDSA positivity. DSA: donor specific antibodies, gDSA: intragraft DSA, MFI: mean fluorescence intensity, SAFB: single antigen flow beads.

Figure 2: One-year post-biopsy graft survival according to DSA status. (A) Comparison of graft survival between recipients having DSA or not, with those having gDSA or sDSA not detected in biopsy eluates being pooled. (B) Comparison of graft survival between recipients having gDSA or not, with those having sDSA not detected in biopsy eluates and those with no DSA being pooled. (C) Comparison of graft survival between recipients having gDSA, sDSA not detected in biopsy eluates and no DSA. Panels (D), (E) and (F) represent the same analysis as in (A), (B) and (C), respectively, after exclusion of recipients that survived less than 3 months post-biopsy. Graft survival was compared using the log-rank test. DSA: donor specific antibodies, gDSA: intragraft DSA.

Table 1. Patients' characteristics at the time of transplantation.

	Total	gDSA-	gDSA+	p-value
Number of patients (%)	53 (100)	42 (79.2)	11 (20.8)	
Recipient gender				0.18
Female (%)	27 (51.0)	19 (45.2)	8 (72.7)	
Male (%)	26 (49.0)	23 (54.8)	3 (27.3)	
Recipient age	48 (27 – 56)	47 (26 – 56)	45 (32 – 55)	0.78
Diagnosis				
Emphysema (%)	19 (35.8)	16 (38.1)	3 (27.3)	0.73
CF (%)	18 (34.0)	15 (35.7)	3 (27.3)	0.73
Fibrosis (%)	9 (16.0)	6 (14.3)	3 (27.3)	0.37
PAH (%)	3 (5.7)	2 (4.8)	1 (9.1)	0.51
Other (%)	4 (7.5)	3 (7.1)	1 (9.1)	1.00
Type of transplant				
Double lung (%)	47 (88.7)	39 (92.9)	8 (72.7)	0.10
Single lung (%)	3 (5.7)	2 (4.8)	1 (9.1)	0.51
Heart-Lung (%)	3 (5.7)	1 (2.4)	2 (18.2)	0.11
Donor age	41 (32 – 55)	42 (32 – 53)	41 (33 -60)	0.78
Extended criteria donor (%)	34 (64.2)	28 (66.7)	6 (54.5)	0.50
Ischemic time (min)	377 (301 – 427)	366 (295 – 420)	380 (360 – 459)	0.21
Induction therapy* (%)	29 (54.7)	23 (54.8)	6 (54.5)	1.00
Early acute cellular rejection (≤3 months) (%)	23 (43.4)	20 (47.6)	3 (27.3)	0.31
HLA-A/B MM	3 (3 – 4)	4 (3 – 4)	3 (2 – 3)	0.07
HLA-DR/DQ MM	3 (2 – 4)	3 (2 – 4)	3 (2 – 4)	0.67
HLA A/B/DR/DQ MM	6 (5 – 7)	6 (5 – 7)	5 (5 – 7)	0.11
Pre-transplant HLA sensitization				
None (%)	31 (58.5)	27 (64.3)	4 (36.4)	0.17
Anti-HLA (%)	11 (20.8)	8 (19.0)	3 (24.3)	0.68
DSA (%)	11 (20.8)	7 (16.7)	4 (36.4)	0.21
Positive CDCXM (%)	0	0	0	NA
Positive FCXM (%)	2 (3.8)	0	2 (18.2)	NA

CMV status				
D+/R- (%)	20 (37.7)	17 (40.5)	3 (27.3)	0.50
D+ or D-/R+ (%)	19 (35.8)	15 (35.7)	4 (36.4)	1.00
D-/R- (%)	14 (26.4)	10 (23.8)	4 (36.4)	0.45

Values are expressed as median (interquartiles) or as number (%) when specified. CDCXM: complement dependent cytotoxicity crossmatch; CF: cystic fibrosis; CMV: cytomegalovirus; D: donor; DSA: donor-specific antibodies; FCXM: flow cytometry crossmatch; gDSA: intr agraft DSA; HLA: human leukocyte antigens; MM: mismatch; NA: not applicable; PAH: pulmonary arterial hypertension; R: recipient. Statistical tests: categorical variables were analyzed with Fisher's exact test and continuous variables with Mann-Whitney U test. *: anti-thymocyte globulins

Table 2. Patients' characteristics and histology at the time of biopsy.

	Total	gDSA-	gDSA+	p-value
Number of patients (%)	53 (100)	42 (79.2)	11 (20.8)	
Recipient age	50 (31 – 58)	49 (29 – 58)	52 (34 -58)	0.83
Time from transplantation (days)	507 (75 – 1128)	281 (80 – 1102)	554 (135 – 1119)	0.90
Early biopsies (≤ 3months from transplantation) (%)	16 (30.2)	13 (31.0)	3 (27.3)	1.00
Acute decline of graft function (%)*	24 (64.9)	16 (55.2)	8 (100)	0.03
Type of biopsy				0.30
Surgical (%)	20 (37.7)	14 (33.3)	6 (54.5)	
Transbronchial (%)	33 (62.3)	28 (66.7)	5 (45.5)	
Biopsy indication				
Respiratory function decline (%)	27 (50.9)	21 (50.0)	6 (54.5)	1.00
ARDS (%)	10 (18.9)	7 (16.7)	3 (27.3)	0.42
Dyspnea (%)	7 (13.2)	7 (16.7)	0	NA
Protocol biopsy (%)	8 (15.1)	6 (14.3)	2 (18.2)	0.67
Radiologic (%)	1 (1.9)	1 (2.4)	0	1.00
Serum sample to biopsy (days)**	3 (2 – 13)	6 (1 – 13)	3 (2 – 19)	0.72
Treatment***				
Cyclosporine (%)	36 (67.9)	28 (66.7)	8 (72.7)	1.00
Tacrolimus (%)	17 (32.1)	14 (33.3)	3 (27.3)	1.00
Everolimus (%)	7 (13.2)	6 (14.3)	1 (9.1)	1.00
Azithromycin (%)	29 (54.7)	23 (54.8)	6 (54.5)	1.00
Histologic findings				
Diffuse alveolar damages (%)	5 (9.4)	4 (9.5)	1 (9.1)	1.00
ACR (%)	10 (19.9)	7 (16.7)	3 (27.3)	0.42
High grade ACR (≥A3) (%)	1 (1.9)	1 (2.4)	0	1.00
Recurrent ACR (any A grade) (%)	4 (7.5)	4 (9.5)	0	0.57
Neutrophilic capillaritis (%)	0	0	0	NA
Obliterative bronchiolitis (%)	10 (18.9)	6 (14.3)	4 (36.4)	0.19

C4d deposition (%)****	3 (6.4)	2 (5.3)	1 (11.1)	0.48
High grade lymphocytic bronchiolitis (B2R) (%)	7 (13.2)	5 (11.9)	2 (18.2)	0.63
Infectious Disease (%)	12 (22.6)	9 (21.4)	3 (27.3)	0.70
Normal biopsy (%)	20 (37.7)	19 (45.2)	1 (9.1)	0.04
Thrombosis (%)	1 (1.9)	1 (2.4)	0	1.00

Values are expressed as median (interquartiles) or as number (%) when specified. ACR: acute cellular rejection; ARDS: acute respiratory distress syndrome; NA: not applicable. Statistical tests: categorical variables were analyzed with Fisher's exact test and continuous variables with Mann-Whitney U test. *: among recipients who experienced their biopsy more than 3 months post-transplantation, **: absolute value, ***: treatment associated cyclosporine or tacrolimus plus mycophenolate acid and corticosteroids +/- everolimus and/or azithromycin, ****: C4d staining was performed for 47/53 biopsies from 38 gDSA- and 9 gDSA+ recipients.

Table 3. Allograft function at the day of biopsy.

	Total	gDSA-	gDSA+	p-value*
Number of patients (%)	53 (100)	42 (79.2)	11 (20.8)	
Allograft function at biopsy				
BOS 0 (%)	8 (15.1)	7 (13.2)	1 (9.1)	1.00
BOS 0p (%)	6 (11.3)	4 (7.5)	2 (18.2)	0.59
BOS 1 (%)	6 (11.3)	6 (11.3)	0	NA
BOS 2 (%)	11 (20.8)	6 (11.3)	5 (45.5)	0.04
BOS 3 (%)	9 (17.0)	9 (17.0)	0	NA
BOS (%)	26 (49.1)	21 (50)	5 (45.5)	1.00
High-grade BOS (≥2) (%)	20 (37.7)	15 (35.7)	5 (45.5)	0.73
ND (%)	13 (24.5)	10 (18.9)	3 (27.3)	1.00

* Fisher's exact test. BOS: bronchiolitis obliterans syndrome; NA: not applicable; ND: not determinable because of recipient intubation.

Table 4. Univariate and multivariate analysis of risk factors at time of biopsy associated with graft loss at one-year post-biopsy (logistic regression) and overall graft loss (cox proportional hazards model).

	Graft loss at one-year post-biopsy						Overall graft loss					
	Univariate			Multivariate			Univariate			Multivariate		
	OR	95% CI	p-value	OR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
ARDS	1.04	0.29-3.78	0.95				1.39	0.58-3.33	0.46			
Acute rejection	1.87	0.47-7.49	0.38				1.35	0.58-3.29	0.50			
Infection	3.02	0.80-11.32	0.10	5.65	1.13-28.21	0.03	2.62	1.12-6.18	0.03	3.67	1.45-9.27	0.006
BOS	1.06	0.35-3.34	0.91				0.88	0.39-1.98	0.76			
Severe BOS	2.30	0.73-7.25	0.15	3.39	0.85-13.54	0.08	1.43	0.64-3.20	0.39			
DSA	2.23	0.71-7.01	0.16*				1.65	0.73-3.74	0.22			
C1q+ DSA	1.93	0.52 – 7.10	0.32				1.65	0.68 – 3.97	0.29			
gDSA	6.67	1.51-29.47	0.008	5.85	1.23-27.68	0.03	3.44	1.47-8.01	0.005	4.51	1.83-11.13	0.001

ARDS: acute respiratory distress syndrome; BOS: bronchiolitis obliterans syndrome; CI: confidence interval; DSA: donor specific antibodies; gDSA: intragraft DSA; HR: hazard risk, OR: odds ratio.*: No OR value in multivariate analysis because of an exclusion from the model during the backward elimination procedure.

Table 5. Treatment modifications and infections during the first year post-biopsy

	Total	gDSA-	gDSA+	p-value
Number of patients	53 (100)	42 (79.2)	11 (20.8)	
Corticosteroid bolus	20 (37.7)	15 (35.7)	5 (45.5)	0.73
Thymoglobulins	7 (13.2)	5 (11.9)	2 (18.2)	0.63
Tacrolimus switch*	18 (34.0)	13 (31.0)	5 (45.5)	0.48
Everolimus introduction	15 (28.3)	11 (26.2)	4 (36.4)	0.71
Azithomycin introduction	11 (20.8)	9 (21.4)	2 (18.2)	1.00
IVIg + RTX +/- PE**	14 (50.0)	5 (29.4)	9 (81.8)	0.018
Bacterial infection after biopsy	38 (71.7)	29 (69.0)	9 (81.8)	0.48

Values are expressed as number (%) analyzed with Fisher's exact test. *: substitution of ciclosporin by tacrolimus, **: Intravenous immunoglobulin + rituximab +/- plasma exchange, comparison of recipients with sDSA only.

Table 6. Cause of graft loss for patients with gDSA or no gDSA

	Total	gDSA-	gDSA+	p-value*
Number of patients (%)	25 (100)	16 (64.0)	9 (36.0)	
Cause of graft loss				
BOS (%)	14 (56.0)	9 (56.3)	5 (55.6)	1.00
ACR (%)	1 (4.0)	1 (6.3)	0	1.00
Infection (%)	7 (28.0)	4 (25.0)	3 (33.3)	0.67
Other (%)	3 (12.0)	2 (12.5)	1 (11.1)	1.00

* Fisher's exact test. ACR: acute cellular rejection, BOS: bronchiolitis obliterans syndrome; NA: not applicable.

FIGURE 1

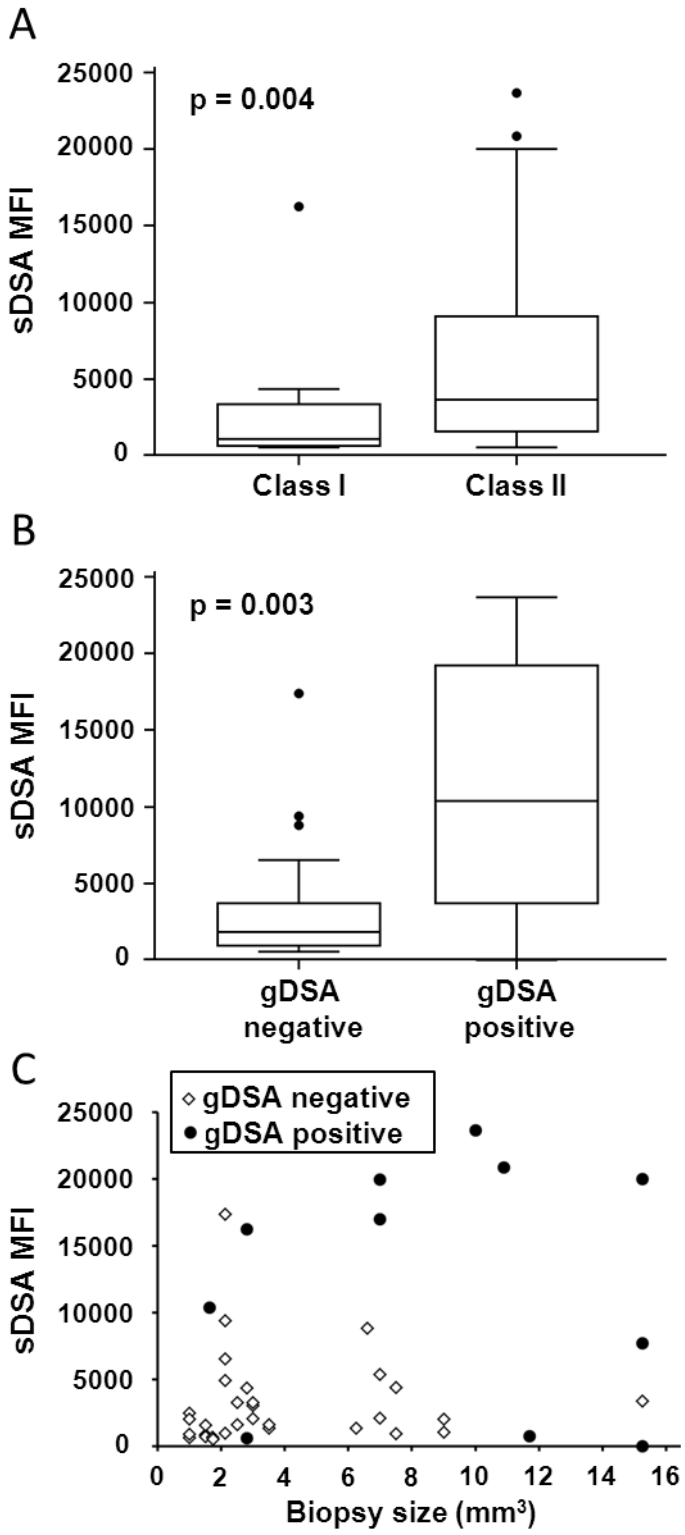
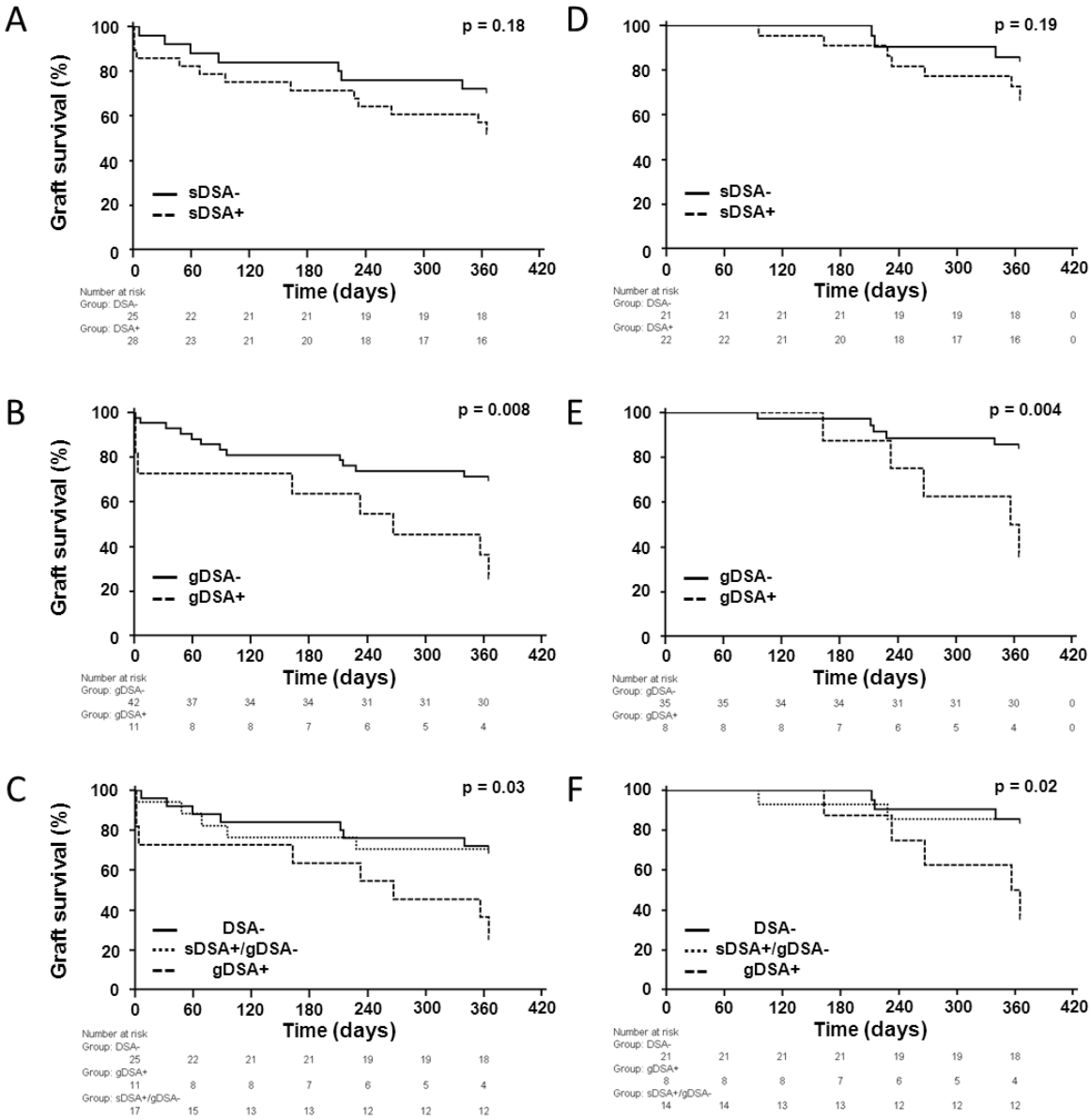

A
p = 0.004
sDSA MFI
25000
20000
15000
10000
5000
0
Class I
Class II
B
p = 0.003
sDSA MFI
25000
20000
15000
10000
5000
0
gDSA negative
gDSA positive
C
gDSA negative
gDSA positive
sDSA MFI
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
12
14
16
Biopsy size (mm3)

FIGURE 2


A
p = 0.18
Graft survival (%)
sDSA-
sDSA+
Time (days)
Number at risk
Group: DSA-
25 22 21 21 19 19 18
Group: DSA+
28 23 21 20 18 17 16
D
p = 0.19
sDSA-
sDSA+
Time (days)
Number at risk
Group: DSA-
21 21 21 21 19 19 18 0
Group: DSA+
22 22 21 20 18 17 16 0
B
p = 0.008
Graft survival (%)
gDSA-
gDSA+
Time (days)
Number at risk
Group: gDSA-
42 37 34 34 31 31 30
Group: gDSA+
11 8 8 7 6 5 4
E
p = 0.004
gDSA-
gDSA+
Time (days)
Number at risk
Group: gDSA-
35 35 34 34 31 31 30 0
Group: gDSA+
8 8 8 7 6 5 4 0
C
p = 0.03
Graft survival (%)
DSA-
sDSA+/gDSA-
gDSA+
Time (days)
Number at risk
Group: DSA-
25 22 21 21 19 19 18
Group: gDSA+
11 8 8 7 6 5 4
Group: sDSA+/gDSA-
17 15 13 13 12 12 12
F
p = 0.02
DSA-
sDSA+/gDSA-
gDSA+
Time (days)
Number at risk
Group: DSA-
21 21 21 21 19 19 18
Group: gDSA+
8 8 8 7 6 5 4
Group: sDSA+/gDSA-
14 14 13 13 12 12 12