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Anticuerpos no-HLA e Inmunofenotipificación de subpoblaciones de células B en el pretrasplante pulmonar

Non-HLA antibodies and Immunophenotyping of B cell subsets in lung pre-transplantation

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Resumen

El rechazo mediado por anticuerpos (RMA) es una de las principales barreras del trasplante pulmonar. Son característicos los anticuerpos anti-HLA donante específicos (ADEs), sin embargo, en algunos pacientes, al diagnóstico de RMA se realiza sin la detección de ADEs circulantes en suero. El objetivo de este estudio es intentar encontrar la causa del rechazo en estos pacientes a través del papel potencial de los anticuerpos no-HLA pretrasplante y asociarlos con el balance pretrasplante de subpoblaciones de células B. Este estudio retrospectivo de casos y controles encontró que la mediana de anticuerpos anti-HLA pretrasplante fue significativamente mayor en aquellos con RMA sin ADEs: 2 [0-16] vs 0 [0-1], p<0.01. Además, los pacientes con >7 anticuerpos anti-HLA pretrasplante tuvieron más riesgo de RMA sin ADEs (sensibilidad 44,4% y especificidad 95,24%, AUC 71,3%), destacando la utilidad de los estudios de anticuerpos no-HLA para identificar pacientes en riesgo de RMA. Por otra parte, el inmunofenotipaje mostró una tendencia a mayores subconjuntos de células B pretrasplante en el grupo de los casos. Estos resultados demuestran que existe una asociación entre los anticuerpos no-HLA pretrasplante y el RMA sin ADEs e indican que estos pacientes podrían tener una respuesta humoral activada incluso antes del trasplante.

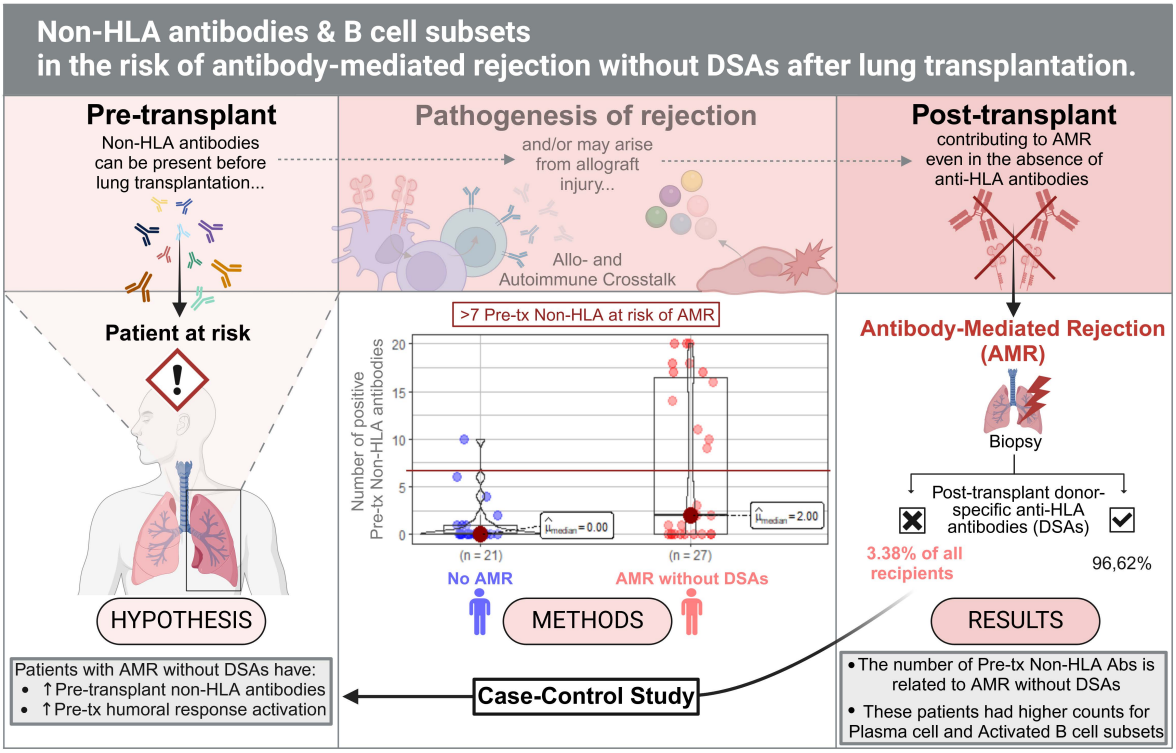
Abstract

Antibody-mediated rejection (AMR) has been for years one of the main barriers in lung transplantation. It is characterised by the presence of donor-specific anti-HLA antibodies (DSAs), however, in some patients, the diagnosis of AMR is not accompanied by the detection of circulating DSAs in serum. The aim of this study is to attempt to find the cause of rejection in these patients through the potential role of pre-transplant non-HLA antibodies and associate them with the balance of pre-transplant B cell subsets. This retrospective case-control study found that the median of pre-transplant non-HLA antibodies was significantly higher in the group with AMR without DSAs than in the control group: 2 [0-16] vs 0 [0-1], p<0.01. Furthermore, patients with >7 pre-transplant non-HLA antibodies had a higher risk of developing AMR without DSAs (sensitivity 44.4% and specificity 95.24%, AUC 71.3%), highlighting the utility of non-HLA antibody studies in identifying patients at risk of AMR. Moreover, immunophenotyping showed a trend towards a higher number of pre-transplant B cell subsets in the case group. These results demonstrate an association of non-HLA antibodies with AMR without DSAs and indicate that these patients might have an activated humoral response even before transplantation.

Keywords

Antibody-mediated rejection, anti-HLA antibody, donor-specific antibody, humoral response, immunophenotyping, lung, non-HLA antibody, transplantation.

58 Graphical Abstract



Introduction

Organ transplantation is a life-saving treatment for advanced lung disease. Despite advances in donor matching, immunosuppression treatments and organ preservation, acute and chronic rejection rates are higher compared to other solid organ transplants. Facing these difficulties requires an in-depth understanding of the immunology of lung rejection, starting with deciphering the mechanisms behind host immune response.

Alloresponse is defined as the reaction of the host immune system against non-self tissues. Firstly, there is the allorecognition, regarding the detection of foreign antigens by immune cells of the lung recipient; and secondly, there is a consecutive destructive effector response[1]. The main target for allorecognition is the human leukocyte antigen (HLA) system[2]. The class-I and -II HLA gene complex is located on the short arm of chromosome 6, being the most polymorphic gene locus in humans with 38,008 alleles identified in January 2024[3]. In lung transplantation, the number of class-I and class-II HLA mismatches are directly related to the development of rejection. HLA-A, -B, and -C, class-I antigens present to CD8+ T cells peptides that have undergone processing within the cytoplasm on nearly every nucleated cell. On the contrary, class-II antigens (HLA-DRα1 and β1, -DQα1 and β1, -DPα1 and β1 loci) present exogenous material to CD4+ T cells and are expressed in antigen-presenting cells (APCs)[4]. However, many other factors are involved in allograft rejection.

Rejection can manifest itself as hyperacute, acute, or chronic rejection. Current strategies largely avoid hyperacute lung rejection by preserving ABO-blood group compatibility and managing recipients without preformed complement-binding donor-specific anti-HLA antibodies (DSAs). These antibodies commonly develop due to pregnancy, blood product transfusion, prior organ transplantation, or human tissue implantation[5]. In acute rejection, the main focus is on complement-binding and non-complement-binding anti-HLA DSAs. But DSAs may also develop *de novo* after transplantation, most frequently directed at complement-binding and non-complement-binding class-II HLA[6], leading to late acute rejection and chronic rejection. Thus, the post-hyperacute period incorporates a large number of sub-optimally controlled cases due to the interplay of a wide range of other players involved.

Contemporary mindset proposes the following sequence of immunological events in alloresponse: ischaemia and tissue injury during organ harvest release damage-associated molecular patterns (DAMPs) activating immune and parenchymal cells within the graft, together with the consequent cytokine storm, leading to an “activated” state of the donor organ that initiates recipient’s innate immune response after anastomosis[7]. This in conjunction with allorecognition-driven response, triggers and amplifies adaptive immune response in the host. As illustrated in Figure 1A, recipient T-cells can recognise donor antigens via three mechanisms[8]: in the direct pathway recipient T cells recognise alloantigens on the surface of donor APCs mainly contributing to acute

rejection. Indirect pathway implies alloantigens processing by APCs for further presentation to alloreactive T cells which is relevant to late rejection. Finally, in semi-direct pathway, recipient APCs express intact donor alloantigens through mechanisms still not completely elucidated such as *cross-dressing*[9]. Subsequently, as detailed in Figure 1B, T- and B-cell interactions could result in the production of antibodies against the HLA system, but also non-HLA antibodies[10] against other polymorphic antigens that differ from donor to recipient including minor histocompatibility antigens (mHAs)[11]. Additionally, autoantibodies can be formed against lung restricted nonpolymorphic self-antigens (SAgs)[12]. Possibly, non-HLA antigens are related to certain damaging circumstances which may lead to an abnormal expression, the exposure of cryptic epitopes, or neoantigen formation[13]. For instance, non-HLA antigens may be expressed on injured endothelial and/or airway epithelial cells leading to the development of antibodies against major histocompatibility complex class I chain-related molecule A (MICA)[14], angiotensin type 1 receptor (AT1R)[15], endothelin A receptor (ETAR)[15] or the C- terminal laminin-like globular domain of perlecan (LG3)[16]. Antibodies may also develop against collagen I (Col I) or collagen V (Col V)[17] in the extracellular matrix, against K-alpha-1-tubulin (Kα1T)[17] in intercellular gap junctions, or even against vimentin[18] or rho GDP dissociation inhibitor beta (ARHGDIB)[19] intracellular molecules. All these may contribute to rejection in a process known as antibody-mediated rejection (AMR), that perpetuates by the expansion of alloreactive T and B cells, *intramolecular epitope spreading* and/or *cross-reactivity* (antigen mimicry)[10].

Aside from AMR, the direct effector role of cells in lung rejection is starting to gain more attention. On the innate side, natural killer (NK), donor-derived plasmacytoid dendritic cells (pDCs), and innate lymphoid cells (ILCs) have been demonstrated to participate, among others[20]. From an adaptive perspective, new candidates have emerged: regulatory B cells (Bregs), regulatory T cells (Tregs), CD8+ T cells (CTLs), Th1, Th2, and Th17 subpopulations of CD4+ T cells, circulating memory T cells and tissue-resident memory T cells (TRM cells)[21]. Also, there is an increasing interest in the role of soluble factors[22], potential biomarkers like thioredoxin levels[23], miRNAs and transcription factors[2], donor-derived exosomes[24,25], circulating donor-derived cell-free DNA[26] and instead of traditional allele-level HLA matching[27] opting for molecular matching, that considers *eplets,* HLA epitopes recognised by antibody complementarity determining regions (CDRs)[28].

Post-transplant complications remain a huge challenge in lung transplantation. According to International Society for Heart and Lung Transplantation (ISHLT) registries focusing on Europe between 2013 and 2016, survival rates are around 70% and 63% at 3- and 5-years post-lung transplantation[29]. Primary graft dysfunction (PGD) is a similar condition to acute respiratory distress syndrome and occurs within 72 hours after transplantation in approximately 30% of recipients[30], being the major cause of early posttransplant morbidity and mortality[31]. The proposed mechanism contributing to PGD is the aforementioned ischaemia-reperfusion injury (IRI)[32]. In addition, post-transplant rejection events have a deleterious effect on graft survival. Regarding lung

acute rejection classification, there are two main categories: acute cellular rejection (ACR) and antibody-mediated rejection (AMR)[33]. Finally, long-term rejection is included in chronic lung allograft dysfunction (CLAD)[34] (see Figure 2A).

ACR is predominantly produced by T cells that recognise alloantigens and initiate a tissue-damaging response. It occurs within the first year following transplantation, when AMR also occurs[34]. AMR is less common, and the most recognised actors are DSAs against donor HLA antigens, which exert graft damage through complement-dependent and -independent mechanisms[35]. CLAD, takes place after the first year and is the main barrier to long-term success, involved in both graft and patient survival. It comprises two differentiated clinical phenotypes: bronchiolitis obliterans syndrome (BOS), which is the most common and recognisable by obstructive physiology, and restrictive allograft syndrome (RAS), defined by restrictive physiology[36]. CLAD is a complex process of fibrosis that results from a combination of alloimmune and non-alloimmune damage, ultimately leading to aberrant remodelling. Major risk factors of CLAD include ACR, AMR (especially those DSAs fixing complement and against HLA-DQ), PGD and infections. However, a wide variety of insults can compromise the graft and in the setting of impaired immune responses, potentially leading to autoimmune phenomena and increasing the risk of CLAD[12].

The diagnosis of AMR in solid organ transplantation is complex and in lung transplantation is accompanied by unclear pathological, serological, and clinical features[35]. Although it was initially related to hyperacute rejection, clinical experience has led to the acceptance of AMR importance beyond the immediate post-transplant setting[37]. Currently, the clinical importance of AMR is widely accepted, but AMR comprehension remains fraught with difficulties.

As described in Figure 2B, the central concept of pulmonary AMR is based on the development of DSAs by allospecific B cells and plasma cells and the focus has been on DSAs against HLA class-I and -II, which have been associated with both acute and chronic allograft rejection, although not all DSA portends the same prognosis[38,2]. These antibodies can initiate complement-dependent mechanisms through the classical complement pathway or trigger complement-independent mechanisms, such as signalling cascades in endothelial and smooth muscle cells. Moreover, they can interact with leukocytes Fc-receptors (FcR) and exert antibody-dependent cellular cytotoxicity (ADCC) and activate the expression of cytokines and chemokines[39]. Emerging evidence suggests that the inflammatory milieu would be conducive to autoimmune phenomena and the development of non-HLA autoantibodies (alloimmunity-induced autoimmunity)[10].

Studies suggest that DSAs lead to increased proinflammatory cytokine production, immune infiltration, and more cellular immune responses to mismatched donor HLA molecules but also to SAgs (Kα1T and Col V). Vice versa, antibodies developed *de novo* against SAgs, will induce a subsequent cytokine response repeating the cycle[40]. The most widely accepted hypothesis

suggests that alloimmune responses precede autoimmune responses[12], but once peripheral tolerance is broken, the presence of antibodies against non-HLA autoantigens, or antibodies against mismatched HLA, may induce the other[40], suggesting a cross-talk between allo- and autoimmunity, both of which may play a role in AMR after lung transplant.

The incidence of AMR has been reported from less than 4% to greater than 50%[41,42,43,44] and there is not yet a standardized approach to treatment nor a definitive diagnostic approach. The current view of lung AMR recognises it as a clinicopathologic spectrum that starts with preformed or *de novo* DSAs. In 2016 ISHLT consensus established the degree of certain AMR diagnosis in “definite” (total absence of ACR and presence of AMR pathological criteria), “probable” or “possible” depending on the number of serologic, pathologic, clinical, and immunologic criteria present[35]. Current criteria according to the 2019 “Banff Lung Report” include: graft dysfunction, histopathology consistent with AMR, capillary complement component 4d (C4d) deposition, circulating DSAs and exclusion of other causes of allograft dysfunction[45]. However, DSAs may not always be detectable, perhaps due to phasic release, absorption into the allograft or limitations of the diagnostic tests in retrospective studies[46]. Similarly, the detection of C4d deposition is variable[47] and histopathologic features are non-specific[48], including neutrophil margination, neutrophil capillaritis and arteritis. Pulmonary AMR it is also divided into clinical, when there is allograft dysfunction as alterations in pulmonary physiology, gas exchange properties, radiologic features or deteriorating functional performance, which can be asymptomatic; or subclinical when allograft function is preserved[35]. Despite these advances in standardisation, confounding factors such as bronchopulmonary infection, the lack of specific diagnostic features and the variable relation between DSAs and graft damage or dysfunction, keep AMR a challenging diagnosis to make.

In the past decade, major progress has been made in understanding the pathogenesis behind alloantibodies due to sensitive and specific solid-phase assays for identification of DSAs[49] and integration of molecular transcripts to characterize the range of graft damage mediated by alloantibodies[50]. Today’s techniques allow us to determine HLA and several non-HLA specificities, levels, and functions. While there is controversy regarding whether these non-HLA antibodies detected solely by highly sensitive techniques are clinically relevant and how to address variability between centres; clearly more studies, especially prospective ones, and sensitive assays are needed for non-HLA antibodies to fully diagnose all cases of AMR, especially, those cases with positive histologic-AMR but without circulating anti-HLA DSAs, which will be the subject of discussion here.

This retrospective study aims to characterise in patients with AMR rejection but without serological AMR criteria, without circulating DSAs, the levels of pre-existing non-HLA antibodies and the balance of different B cell subsets, to shed some more light on the intricate humoral response in lung rejection.

Results

Demographic characteristics of the studied cohort

Two groups of lung transplant recipients were studied, one with AMR without DSAs and a control group transplanted at the same period. The demographics for the studied patients are summarized in Table S1. There was no significant difference in the mean of age at transplantation [58.42 (55.46-62.03) vs 53.63 (40.53-61.93) years] and approximately one-half of the patients were males (77.78% for AMR group and 66.67% for non-AMR group). Furthermore, there was no significant difference between both groups regarding underlying lung diseases, type of transplant, time of ischemia, induction therapies, and PGD diagnosis. In terms of rejection-free time, as expected it was significantly shorter in the AMR group without DSAs, with a median time free of AMR of 12 months. Regarding the incidence of pre-transplant DSAs, no significant differences were obtained, actually, very few patients were positive. In terms of *epitope* HLA mismatch (molecular mismatch) between donor and lung recipients, the number of verified HLA class-I and -II *epitope* was comparable between the two groups. There were also no significant differences in the detection of DQ risk epitope mismatch (REM), whose presence was associated with poorer outcomes.

There were significant differences in the follow-up time (50.71 vs 73.32, $p = 0.039$). Besides, 62.96% percent of patients with AMR without DSAs ended up developing CLAD in the long-term period, while only 28.57% of control patients eventually progressed to CLAD ($p = 0.001$). Cases of *exitus* were higher in the AMR group, doubling the number of the control group (66.66% vs 33.33%, $p = 0.022$).

Frequency of positive non-HLA antibody reactions

Retrospectively, a study of 39 non-HLA antibodies in the pre-transplant serum of each of the 48 patients enrolled in the study was carried out. As a first approximation to analyse the results, the frequencies of positive reactions for each non-HLA antibody were calculated. Figure 3 depicts the relative magnitude of non-HLA positive reactions in each group. From the tested panel containing 39 non-HLA antibodies, 12 antibodies recognised as lung-related and those most frequently detected were chosen.

In the group of patients with AMR without DSAs, the presence of non-HLA antibodies was more frequently detected. Seventy percent of the patients with AMR had at least one positive result for some non-HLA specificity. In contrast, in the control group, 52.38% of the patients were positive for at least one non-HLA. In many cases, the frequency of positive reactions was double in the AMR group compared to the control, with the most notable differences observed in 4 cases, where the difference between groups was quadrupled. The antibodies that almost reached statistical significance were TUBA1B, Col V, LG3 and GSTT1, with the first three being previously associated with poorer lung outcomes in numerous studies.

It is noteworthy that in 2 cases, the frequencies were reversed, with more positive reactions detected in the control group. This is the case for PRKCZ and IFNG, the first is still under investigation and the latter has been more associated with renal and pancreatic transplant rejection. This observation is not a conclusive finding, as the aim of the graph is rather to provide an overview suggesting a strong involvement of non-HLA antibodies in AMR.

Non-HLA antibodies associated with AMR without DSAs

The association of each non-HLA antibody with AMR is summarised in Table S2. To filter out background noise and attempt to focus on those antibodies that appear to have greater significance in the group of patients with rejection, only those antibodies with a 75th percentile (P75) above 1,000 MFIs were selected. For a list of 14 antibodies, nine were significantly associated with AMR without DSAs. Anti-LMNA and anti-GSTT1 antibodies were the best predictors of AMR, but only identified 44.44% ($p = 0.0004$) and 40.74% ($p = 0.0061$) of AMR-positive cases, respectively.

Impact of pre-transplant non-HLA antibodies on AMR without DSAs

A quantitative analysis was conducted to better highlight the differences in non-HLA positive reactions between the study groups. A slightly narrower profile than in the original analysis, 20 non-HLA antibodies with high MFI thresholds and/or less extensively studied in recent years were selected this time. The median of the pre-transplant non-HLA positive antibodies in the group with AMR without DSA was significantly higher than in the control group: 2 [0-16] vs 0 [0-1], $p < 0.01$, $CI_{99\%}$ [-0.71, -0.02] (see Figure 4). It is remarkable that positive reactions tend to cluster for the controls, with only one outlier exceeding the threshold of 7 non-HLA positive reactions, which could be a false positive. However, the AMR group shows the contrary image, where a notable proportion of patients have more than 7 positive reactions. Therefore, it may be considered that having more than 7 positive reactions significantly increases the risk of AMR without DSAs.

The previous statement is further confirmed by a ROC analysis. As shown in Figure 5, results reaffirmed that the number of positive profile-20 non-HLA antibodies in pre-transplant serum predicted AMR (AUC = 0.713 [0.580-0.847]), with a sensitivity of 44.40% and specificity of 95.24%. From the retrospective study of non-HLA antibodies in the pre-transplant serum, it is concluded that patients with >7 pre-transplant non-HLA antibodies were more likely to develop AMR without DSAs.

B cell subsets immunophenotyping

Beyond the study of pre-transplant antibodies, the aim was to deepen in the knowledge of the aetiology underlying AMR by characterising those cell subsets that have more relevance in triggering and perpetuating AMR. Pre-transplant flow cytometry studies were retrieved from 8 patients out of the initial 48 (4 cases and 4 controls). The results for each cell subset studied are shown in Table 1. Although no statistically significant results were obtained, the balance of B lymphocyte subsets was slightly increased in the AMR group without DSAs compared to the control group.

The AMR group without DSAs had a higher mean frequency for B1 (CD5+CD19+), B2 (CD5-CD19+) and unswitched (IgD+CD27+) B cells and there was a revealing trend in the subset of activated B cells (CD268+) and plasma cells/plasmablasts (CD38+CD138+). Regarding the latter, it is remarkable that its *p-value* (highlighted in bold in Table 1) was much closer than the rest to statistical significance despite the very small sample size.

Discussion

In lung transplantation it is widely accepted that AMR is usually triggered by anti-HLA DSAs, however, in some cases, these anti-HLA antibodies are unable to be identified. In order to develop a more comprehensive profile of patients diagnosed with AMR who lack detectable circulating DSAs, the following hypothesis could be formulated: the progression to AMR in these particular patients may be primarily driven by pre-existing non-HLA antibodies. The presence of these non-HLA antibodies could explain why the outcome of these patients is AMR even though HLA-mismatched alloresponse cannot be detected in their sera. Consequently, non-HLA antibodies may act as course modifiers or at least could be potential predictors of poorer outcomes following lung transplantation.

In a cohort of 48 lung transplant recipients, patients diagnosed with AMR but without detectable DSAs exhibit a higher incidence of pre-transplant non-HLA antibodies than the control group (Figure 3). The number of these antibodies prior to transplantation was related to AMR without DSAs (Figure 4).

Nine non-HLA antibodies were significantly associated with AMR but due to the small sample size studies in larger cohorts are needed to verify these results. Moreover, each non-HLA antibody had poor sensitivity in predicting AMR individually with anti-LMNA and anti-GSTT1 antibodies being the best but only identified in 44.44% and 40.74% of AMR-positive cases, respectively (Table S2). This result aligns with a heterogeneous view of AMR aetiology. According to this idea, the risk of AMR would rather increase per incremental non-HLA antibody. Similarly, the increase of pre-transplant non-HLA antibodies has recently been shown to increase the risk of CLAD[51]. Finally, ROC analysis established its potential performance as an AMR-risk test where patients with a higher number of pre-transplant non-HLA antibodies could be at higher risk of developing AMR without DSAs in peripheral blood (number >7 of profile-20), see Figure 5.

On the other hand, no significant results were obtained for any particular B cell subset analysed in the pre-transplant flow cytometry analyses, but it is worth noting that, despite the small sample size used, interesting trends are observed in the plasma cell/plasmablast (CD38+ CD138+) population and in the activated B cell (CD268+) subset. Cell counts for these two subsets were higher in the AMR group without DSAs (see Table 1 and Figure S2). This may indicate the presence of non-HLA antibodies producing plasma cells before transplantation, secondary to a stimulus not yet characterized. However, non-HLA antibody positivity was independent of underlying diseases and induction therapy (Table S1). It is assumed that these statements could not be explained by differences in the baseline characteristics of the patients between the two groups, as the study of the cohort characteristics removed many of these confounding factors.

Despite its potential transferability, there are limitations in this study: it is crucial to conduct this study on a larger cohort to be able to answer these questions with adequate statistical power. Additionally, other confounding factors such as infections, causes of death or type of immunosuppression were not considered.

Other limitations in the study of non-HLA antibodies: only a single pre-transplant serum was studied, results could not be examined together with post-transplant follow-up data of both anti-HLA and non-HLA antibodies, and the dynamics of non-HLA and DSAs remain unclear. The specific situation of the patients according to their clinical situations was also not assessed. Furthermore, the control group did not have the same size as the case group although efforts were made to match case-control pairs. The non-HLA positivity thresholds were determined according to the 75th percentile values obtained for each of the 39 non-HLA antibodies with the raw MFI data of the 48 subjects (Table S2). These cutoffs may represent the non-HLA antibody backgrounds; thus, they were considered adequate to determine positive/negative reactions. Currently, establishing thresholds of positivity for anti-HLA and non-HLA antibodies is extremely challenging because there are very few studies in healthy and diseased populations.

Moreover, there are issues surrounding *Luminex* technique (Figure S1B left), such as inter- and intra-assay variability, its propensity to both false negatives and false positives, the presence of denatured HLA molecules on the beads which reveal cryptic epitopes, and the ongoing debate to establish the clinical impact of antibodies detected solely by highly sensitive SAB assays[52].

Concerning the immunophenotyping study (Figure S1B right), the use of a larger cohort is also indispensable. The results may indicate a trend but should be studied in comparison to other post-transplant analyses and characterise more precisely each cell subset, where it would be interesting to focus on the interaction of B cell subpopulations with germinal centre follicular helper T cells[53] and consequently, being able to interpret in detail how B cells differentiate into plasmablasts and produce class-switched allo- or autoantibodies. As a post-transplant study, sera could be evaluated for circulating HLA-specific memory B cells that have been found to exist in the absence of detectable serum DSAs of the same specificity in kidney transplants, suggesting that this may also explain some cases of AMR without DSAs here[54].

Altogether, our findings highlight the possible utility of non-HLA antibody studies and B cell immunophenotype assays in identifying patients at risk of developing AMR and enhance our understanding of non-HLA antibodies and AMR.

As discussed earlier, the aetiology, pathogenesis, clinical presentation, and treatment in lung transplant AMR is still unclear, and this is essentially the reason why, despite the progress made, it remains a major cause of impaired long-term outcomes in lung transplantation. In response to this challenge, the importance of DSAs targeting both HLA and non-HLA antigens has begun to be taken into consideration.

The association between DSAs to mismatched HLA and antibodies against SAgs has been widely recognised. Studies find that patients who develop antibodies to both HLA antigens and SAgs show poorer post-transplant outcomes[51]. In most cases, it is observed that the development of anti-HLA DSAs preceded the development of antibodies to SAgs, and while there are cases where the detection of DSA is transient, antibodies to SAgs usually persist[55]. However, rejection does also occur in the absence of detectable circulating DSAs, and these cases cannot be distinguished by either histological or clinical criteria from those with measurable levels[56]. In this study, based on a cohort of 800 lung transplant recipients, 3.38% developed AMR without DSAs. In other organ transplants, 3 non-mutually exclusive possibilities have been reported to explain AMR in the absence of anti-HLA DSAs[57,58]:

(1) Anti-HLA DSAs may be the main mediators of rejection, but we are simply not able to detect them. This could be due to limitations in current detection methods or to the “DSA-masking phenomenon”, as it has been reported that after certain therapies such as immunoglobulin M depletion DSAs that were previously not detected circulating on standard testing can be unmasked[59]. The “Sponge effect” has also been observed, where DSAs are adsorbed by the high capillary surface of the lung graft and only after organ removal will they become detectable in serum[58]. In some patients, it has been shown that in the absence of serum DSAs, tissue-bound graft DSAs (gDSAs)[60] are present, which appears to be a biomarker to identify pathogenic DSAs in patients with higher risk for graft loss[61]. The existence of these gDSAs could be attributed to a strong intragraft-binding or to a local alloantibody production within the graft. In chronically rejected allografts, B cells form in-graft tertiary lymphoid organs[62], which are believed to promote a local alloimmune response. Thus, graft-damaging DSAs could be generated in the graft's own microenvironment, abundant with alloantigens and autoantigens[56].

(2) Perhaps a direct antibody-independent innate response is taking part. In this context, NK cells are increasingly recognised as important mediators of both lung allograft tolerance and injury. They can promote tolerance through the depletion of donor APCs and, alternatively, they can drive rejection by complement-independent mechanisms through the activation of their FcR with graft-specific antibodies; or due to cytotoxic effects via killer cell immunoglobulin-like receptor (KIR) or NKG2D receptor ligation, denominated as “missing self” or “stressed” recognition, respectively[63]. Despite their contradictory effects, NK cells may play an important role in diagnosing allograft dysfunction.

(3) Lastly, non-HLA antibodies may be involved in the pathogenesis. This is the alternative that we believe lies behind the results of this study. Preliminary studies about non-HLA antibodies focused on those against non-HLA endothelial antigens and rapidly began to be associated with allograft rejection. From its beginnings to the present, the question of whether these antibodies cause the injury or are simply biomarkers of prior damage has remained unanswered[64]. Research to resolve this cause-effect paradox has first aimed to

characterise more targets of these antibodies. Target antigens can be polymorphic, which implies the development of alloantibodies; or non-polymorphic, which means that autoantibodies would form against them (Figure 1B).

In lung transplantation, the autoantigens Col V[17], Kα1T [17], AT1R[15], and ETAR[15] have received the most attention as triggers of an autoimmune response that promotes allograft rejection. In this study, only the first two were evaluated and indeed the frequency of detection of both was markedly higher in the AMR group than in the control group (40.74% versus 4.76% for both antibodies, see Figure 3). However, the thresholds estimated with P75 for these two antibodies were <1,000 MFIs (573 and 999, respectively, *p-value*=0.328) and are therefore not shown in Table S2. In the lung, collagen V co-assembles with collagen I into heterotypic fibrils and remains sequestered until allograft injury increases the activity of matrix metalloproteinases and leads to its exhibition[65]. Equivalently, Kα1T is a normally sequestered gap junction protein but is exposed with inflammation and tissue repair[66]. The binding of specific antibodies results in increased secretion of fibrogenic growth factors, cell cycle signalling activation and fibroproliferation, suggesting a direct pathogenic effect in BOS[66].

Numerous studies have identified an important role for autoimmune responses in heart[67], kidney[68], and lung[69] allograft rejection. The most supported theory holds that sequestered self-antigens are exposed due to transplant-associated injury and tissue repair (Figure 2B). However, reversing the timeline, this study found a high incidence of pre-transplant non-HLA antibodies, which was markedly higher in the group of patients with rejection (Figures 3 and 4). The development of these pre-existing non-HLA antibodies could be due to a wide range of factors where the impact of underlying lung disease has gained interest, demonstrating that around 30% of patients with pulmonary fibrosis and CF had pre-transplant autoantibodies compared with only about 18% of patients with COPD[17]. Following this idea, it would be interesting to repeat this study stratifying according to the underlying pathology of each patient. Regardless of how they are formed, pre-existing autoantibodies appear to have an impact on post-transplant outcomes. Importantly, a study of murine lung transplantation showed that pre-existing autoantibodies (anti-Col V, anti-Kα1T) increase the risk of PGD and AMR[70], and another study in human lung recipients associated them with *de novo* development of DSA and higher risk of BOS[71].

It has been proposed that pre-existing autoantibodies may mediate graft injury through the following mechanisms[72]: promoting pro-inflammatory responses for example by complement activation or Fc gamma receptor, being able to fine tune the activation or suppression of the immune response. In addition, these autoantibodies could recognise non-polymorphic antigens or even SAgs that are exposed in the graft. Consequently, the recipient's immune system would be primed to attack the graft prior to transplantation. After reperfusion, autoantibodies would mediate damage in the graft releasing other SAgs and ultimately promoting the generation of more autoantibodies by B cells. Thus,

pre-existing autoantibodies would then trigger *epitope spreading* and *de novo* antibody production in the post-transplantation period.

Despite having pre-existing non-HLA antibodies before transplantation or generating them afterwards, many patients do not experience rejection or graft dysfunction, indicating that non-HLA antibody pathogenicity is conditional upon other factors such as the allograft microenvironment or the ligand expression, the latter of which represents a challenge in establishing the clinical significance of each non-HLA allo- and autoantigen, as they vary according to their location and inflammatory milieu[10]. As early as 2010 it was reported that BOS grades could be differentiated by a profile of autoantibodies including down-regulation as well as up-regulation of specific reactivities and some of them may reflect pathological allograft processes[73]. On the other hand, the fact that in several studies most DSA-negative AMR patients were allo-sensitised[74] supports the idea that all AMRs, regardless of whether they are negative or positive for DSAs, reflect an adaptive alloimmune response increased by previous sensitisation.

In summary, this retrospective study shows the association of non-HLA antibodies with AMR without DSAs and highlights the utility of non-HLA antibody studies in identifying patients with end-stage lung diseases at risk of AMR without DSAs. The contributions of this study, in line with existing literature, indicate that pre-transplant non-HLA antibodies and activated humoral response may play a significant role in mediating allograft rejection, but prospective and larger studies should be addressed to confirm these results.

New research directions seem to suggest that the focus should rather be on understanding in depth the antibody and antigen characteristics that may regulate the pathogenic mechanisms of non-HLA antibodies. It will be crucial to narrow down the range of different non-HLA antibodies to those specificities that are truly clinically important.

Methods

Patient cohort

As depicted in Figure S1A, a case-control study was designed with a retrospective cohort of non-consecutive lung transplant recipients (n = 48) from Marqués de Valdecilla University Hospital (HUMV in Spanish) in Cantabria, Spain, transplanted from 2012 to 2022. From an available cohort of 800 lung transplants performed at HUMV between 2012 and 2023, 773 patients who did not meet the inclusion criteria for the case group were excluded. The following lung transplant recipients (LTRs) were excluded: patients who had circulating detectable DSAs and/or who did not meet all diagnostic criteria for AMR according to the anatomic pathology laboratory (APL), i.e., patients whose biopsies were free of histologic patterns evocative of AMR (according to 2019 "Banff Lung Report" criteria) and/or negative for C4d deposition were not included. Twenty-seven patients formed the case group as they met inclusion criteria: they were diagnosed with AMR (or had strong clinical suspicion) and did not have circulating anti-HLA antibodies identified post-transplantation.

It was decided to follow the strategy of matched pair case-control studies, where each case was assigned a control patient who had undergone transplantation on a similar date, as it is an important confounding factor. This was extremely relevant to avoid bias in the subsequent analysis. It allowed the conditions under which the transplantation was performed to be extremely similar between each case-control pair, so the induction therapy and detection techniques used in each pair were virtually the same. Due to non-HLA kit supply problems, only 21 control patients could be included in the study. They were categorized as stable as they were free from any kind of lung acute rejection (neither AMR nor ACR) evidence in the same follow-up period.

All included patients had available demographic characteristics, clinical data, and available stored serum from before and after transplantation. In addition, it was ensured that each patient met the following characteristics: uni- or bilateral primary deceased-donor lung transplant, availability of pre- and post-transplant stored serums, follow-up time greater than 1 year after transplantation, anti-HLA class-I and -II monitoring studies determined by enzyme-linked immunosorbent assay (ELISA) or *Luminex* techniques and determination of the antibody-verified status of *eplets* listed in the HLA Epitope Registry. Availability of biopsies at least once post-transplant and study of C4d deposition. Information of ischaemia time, *exitus*, use of induction therapy and determination of high-risk epitope mismatch (REM), which is found in DQA1∗05 + DQB1∗02/DQB1∗03:01 and is associated with de novo donor-specific antibodies after lung transplantation. It has been reported that DQ REM may identify patients at risk of poor outcomes[75], but there are discrepancies in its association with CLAD and graft survival. Other available data was also considered, such as the diagnosis of PGD and/or CLAD (BOS, RAS, mixed or undefined), cytomegalovirus (CMV) infection and underlying diseases including cystic fibrosis (CF), interstitial lung disease (ILD), chronic obstructive pulmonary

disease (COPD) and others. For AMR patients in the case group, the onset of rejection history was made always after post-transplantation serum samples were collected and the stage of acute pulmonary rejection was made according to the ISHLT Working Formulation criteria from grade A0B0 to A4B1 using from the version available in 2013 to the latest one.

Anti-HLA and non-HLA antibody detection

Anti-HLA antibodies were identified prospectively with *Luminex* screening or single antigen bead (SAB) assay (LABScreen class-I and -II, Thermofisher, One Lambda) as part of routine clinical care towards six HLA targets: HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ and HLA-DP. Pre-transplant positive DSAs were generally considered above the 1,500 MFI threshold. For this study, the results of anti-HLA tests performed before each transplantation were retrieved.

Alternatively, pre-transplant non-HLA antibodies were tested retrospectively for both cases and controls. This test was not part of routine patient care and was performed according to vendor-provided protocol, but slightly optimised with larger centrifugation time to work with deteriorated samples after freeze-defrost cycles. Tests were conducted with banked serum using *Luminex SAB assay* with non-HLA antibody panels (LABScreen Autoantibody Assay, groups 1, 2, and 3, Lot #004, Thermofisher, One Lambda) in the LABScan3D™ system. Figure S1B (left) summarises the basic principles of Luminex technology.

Multiplexing of 39 non-HLA targets: Alpha-enolase (ENO1), fibronectin leucine rich transmembrane protein 2 (FLRT2), vimentin (VIM), tubulin alpha 1b (TUBA1B), cluster of differentiation 36 (CD36), interferon induced with helicase C domain 1 (IFIH1), myosin (MYO), angiotensinogen (AGT), protein tyrosine phosphatase receptor type N (PTPRN), aurora kinase A (AURKA), peptidylprolyl isomerase A (PPIA), eukaryotic translation initiation factor 2A (EIF2A), glutathione S-transferase theta 1 (GSTT1), lamin A/C (LMNA), lamin B1 (LMNB), protein kinase C zeta (PRKCZ), peroxisomal trans-2-enoyl-CoA reductase (PECR), protein kinase C eta (PRKCH), CXC motif chemokine ligand-9 (CXCL9), -10 (CXCL10) and -11 (CXCL11), agrin, rho GDP dissociation inhibitor beta (ARHGDIB), heterogeneous nuclear ribonucleoprotein K (HNRNPK), interferon gamma (IFNG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), chromatin assembly factor 1 subunit B (CHAF1B), phospholipase A2 receptor 1 (PLA2R), nucleolin (NLC), tumor necrosis factor-alpha (TNFA), regenerating family member 3 alpha (REG3A), glial cell line-derived neurotrophic factor (GDNF), perlecan LG3 C-terminal fragment (LG3), fibronectin 1 (FN1), collagen-I (Col I), -II (Col II), -III (Col III), -IV (Col IV) and -V (Col V). Among these 39 antibodies, the supplier highlights 7 lung-coverage (ENO1, CD36, MYO, HNRNPK, TUBA1B, LG3, Col V) and 14 currently under investigation (LRT2, IFIH1, AURKA, PPIA, EIF2A, PRKCZ, PRKCH, LMNB, CXCL10, ARHGDIB, GDNF, GAPDH, TNFA and Col II).

Positive thresholds for non-HLA antibodies were determined using an approach similar to that applied by Luminex kits vendor with One Lambda™ HLA Fusion

Research Software. The 75th, 85th and 95th percentiles for each non-HLA antibody were calculated. Those MFI results that exceeded the calculated percentile value for each particular antibody were considered as non-HLA positive reactions. Also, MFIs were corrected for background with un-conjugated beads and two negative, and two positive controls were used. It was decided to choose the 75th percentile (P75) value as the threshold for non-HLA positivity. Thus, the non-HLA positivities considered for further analysis were assigned on the basis of the P75 threshold, which converted the MFIs of non-HLA antibodies into binary positive/negative reactions.

Immunophenotyping of B cell subsets

Immunophenotyping of B cell subsets was performed by retrieving the results of pre-transplant flow cytometric studies for 8 participants, 4 cases and 4 controls. For each of the 8 subjects, results were obtained for two panels (B1 and B2) to characterise a larger number of B cell subsets.

Given the age of some studies and the poor homogeneity of the conditions under which each study was performed, homogeneous flow cytometry analyses could only be selected for these 8 patients. Depending on the date of the study, a different flow cytometer was used and the information for each one is not available.

With the retrieved files a conventional flow cytometry analysis was performed using *Kaluza* software analysis (*Beckman Coulter*). Fluorochrome compensation was corrected, a new gating strategy was developed and statistics for certain populations were obtained. Figure S1B (right) summarises the gating strategy and details the combination of cluster of differentiation (CDs) and fluorochromes included in each of the two panels and the cell subsets defined in each case.

Statistical analysis

Regarding the analysis of the demographic characteristics of the studied cohort, qualitative variables were reported as absolute numbers (*n*) and percentages (%) and compared using Chi-square or Fisher exact tests as appropriate. Quantitative variables were expressed as median using 25%-75% interquartile range (IQR) or mean ± standard deviation (SD). Normally distributed data was compared with Student's *t*-test and non-normally distributed data with the nonparametric Mann-Whitney test. The Shapiro-Wilk test for small sample sizes determined whether normality existed in the distributions.

For a qualitative assessment of positive non-HLA antibody reactions, a balloon plot of a contingency table of 12 non-HLA antibodies, previously reported to be relevant in lung transplantation, compares the pre-transplant non-HLA frequency for each group. Non-HLA antibodies significantly associated with AMR were determined with Chi-square or Fisher exact tests as appropriate, for 14 antibodies with MFIs > 1,000. In order to provide a quantitative assessment,

the nonparametric Mann-Whitney U test was carried out with a profile of 20 lung-associated antibodies, to compare the central tendency (median) of the pre-transplant non-HLA positive antibodies between the group with AMR without DSAs and the control group. From the profile of 20 antibodies, receiver operating curve (ROC) analysis was used to determine the optimal threshold for the number of positive non-HLA antibodies in predicting AMR without DSAs. For all cases, the 75th percentile calculated was used as the threshold for non-HLA positivity.

Profile of 12 non-HLA antibodies (frequency analysis):
IFNG, PRKCZ, MYO, GAPDH, NLC, IFIH1, VIM, ENO1, LG3, GSTT1, Col V and TUBA1B.

Profile of 14 non-HLA antibodies (non-HLA antibodies associated with AMR):
ENO1, LG3, GSTT1, AURKA, LMNA, PECR, CHAF1B, TNFA and GDNF, plus: Col I, Col II, Col III, CXCL9 and PRKCZ.

Profile of 20 non-HLA antibodies (Mann-Whitney U test and ROC analysis):
VIM, ENO1, LG3, GSTT1, Col V, TUBA1B, FLRT2, CD36, AGT, AURKA, EIF2A, LMNA, LMNB, PECR, CXCL10, CXCL11, AGRIN, CHAF1B, TNFA and GDNF.

Demographic and immunophenotyping studies of the cohort were performed with *IBM SPSS version 28*, and the remaining statistical analyses and their respective figures were generated with *R Studio version 2022.12.0+353.* Schematics and figures on cellular mechanisms were designed with *BioRender*. R scripts and datasets can be found at: https://github.com/ppa978/R-Scripts-figures-3-5-TFG-Paula-Padron-Anceaume.git

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Figure Legends

Figure 1. Allorecognition and Antibody Responses in allograft rejection.

(A) Pathways of T cell Allorecognition.
In the direct pathway, alloantigens are presented on human leukocyte antigen (HLA) class-I and class-II molecules on the surface of donor antigen-presenting cells (APCs) to CD8 and CD4 T cells respectively, which is relevant in acute rejection. The indirect pathway involves the processing of donor antigens (typically HLA antigens) by recipient APCs (commonly dendritic cells) for the presentation of peptide fragments to recipient CD4 T cells, primarily contributing to late rejection. Finally, it has been suggested that in semi-direct pathway recipient APCs express intact donor antigens through mechanisms such as "*cross-dressing*" on the basis of donor-derived extracellular vesicles (EV), which can be categorized into exosomes, microparticles or apoptotic bodies and could carry surface HLA molecules, bound peptides and even lung-self antigens. It has been proposed that donor-derived EV could activate T cells, after being internalized and recycled to the surface of recipient APCs without peptide–processing or through capture and fusion to the APC surface.

(B) Targets of Antibody Responses following solid organ transplantation.
Antibody responses can be directed towards self or non-self antigens. Most research has focused on mismatches between highly polymorphic major histocompatibility (MHC) antigens expressed on the allograft. Human leukocyte antigens (HLA) are genes in MHC and the measurement of anti-HLA antibodies in circulation is used to determine the risk of rejection. Most IgG subclasses can activate complement and Fc-receptors, where IgG3 shows the strongest enhancement whereas IgG4 antibodies exert pathogenic effects by blocking enzymatic activity or disrupting signal transduction pathways, but not by activating complement. Complement binding by anti-HLA antibodies correlates with poorer graft outcomes, activating a downstream cascade that mediates tissue damage. Nowadays, two assays are available for the detection of C1q- or C3d-binding. On the other hand, antibodies against non-HLA antigens may be clinically relevant, especially in B cell alloimmunity. Minor histocompatibility antigens (mHA) represent a target group within non-HLA antibody responses, however scarce studies report conflicting results. mHA are derived from allelic variants (regularly T cell epitopes) of polymorphic proteins that differ between recipient and donor and could trigger allogeneic immune responses. There have been reported three major types of mHA: autosomal single nucleotide polymorphism (SNP) variant (e.g., amino acid substitution), H-Y antigen (sequence identity between proteins encoded on the Y chromosome compared to X chromosome) and loss-of-function variant (homozygous recipient for gene deletion and donor expresses at least one functional allele). The ABO blood group antigens are the most immunogenic of all the blood group antigens and ABO histo-incompatibility is still a barrier in solid organ transplant due to the presence of "natural" preformed ABO antibodies, predominantly IgM subclasses, against those blood group A or B antigens that are not expressed on the individual's red blood cells. Besides, numerous studies have reported

antibodies to self-antigens in the context of solid organ transplant, but the exact mechanism underlying this autoreactivity phenomenon is still unknown. Potentially relevant non-HLA antibodies in lung transplantation include angiotensin type 1 receptor (AT1R) antibodies, endothelin-1 type A receptor (ETAR) antibodies, major histocompatibility complex class I chain-related molecule A (MICA) antibodies, K-alpha-1-tubulin (Kα1T) antibodies, C- terminal laminin-like globular domain of perlecan (LG3) antibodies, vimentin antibodies, collagen I (Col I) and V (Col V) antibodies and rho GDP dissociation inhibitor beta (ARHGDIB) antibodies.

Figure 2. Classification and Main Actors of rejection after lung transplantation.

(A) Rejection Classification and its Features within the allograft.
The International Society for Heart and Lung Transplantation (ISHLT) consensus of rejection after lung transplantation encompasses: primary graft dysfunction (PGD) within 72 hours after transplantation probably as a result of ischaemia-reperfusion injury. Lung acute rejection, which occurs within the first year following transplantation and is divided into acute cellular rejection (ACR), predominantly produced by alloreactive T cells, and antibody-mediated rejection (AMR), primarily triggered by preformed or de novo donor-specific antibodies (DSAs) against donor human leukocyte antigens (HLA), which exert graft-damage through complement-dependent and -independent mechanisms. Finally, chronic lung allograft dysfunction (CLAD), which takes place after the first year post-transplantation and is a complex process of fibrosis that results from a combination of alloimmune and non-alloimmune damage. Additionally, emerging data illustrate that non-HLA antibodies may induce AMR and play a role in CLAD after lung transplantation. Overall, lung transplants have a higher chance of being successful when ABO and HLA compatibility between donor and recipient are carefully assessed and organ damage during retrieval and reperfusion processes is minimised. Lung injury post-transplant leads to neutrophil recruitment and degranulation, macrophage and antigen-presenting cell (APC) recruitment and activation. Donor-derived passenger APCs migrate to the graft-draining lymphoid tissues where they prime donor T cells directly or alternatively deliver antigens to resident APC populations. Exosome shedding (and likely other extracellular vesicles, EVs) from allograft cells could also be involved in alloresponse-bearing donor HLA and lung-self-antigens to recipient APCs. Circulating non-HLA, anti-HLA and ABO antibodies could be measured in patients' serum as indicators of rejection. Damage from acute and later chronic rejection manifests within the allograft through damage-associated features: insults to the bronchial epithelium and a pro-inflammatory environment which, in turn, will continue to foster the destructive immune response. In addition, there is progressive endothelial damage, GAP junctions formation and platelet adhesion and aggregation begin to be affected. Ultimately, the persistence of an uncontrolled alloresponse becomes histologically evident: the edematous interstitium widens, pulmonary fibrosis develops and eventually, organ function and graft survival decline considerably.

(B) Pulmonary Antibody-mediated Rejection and Alloimmunity-induced Autoimmunity.

While acute pulmonary cellular rejection (ACR) is associated with T-cell-initiated alloreactive responses, antibody-mediated rejection (AMR) focuses on the development of donor-specific anti-HLA antibodies (DSAs). Following a germinal centre reaction, allospecific B cells, plasma cells (PC) and long-lived plasma cells (LLPC) allow long-term upkeep of the donor-specific humoral immune response. The presence of preformed circulating DSAs occurs due to sensitisation by pregnancy, blood product transfusion, prior organ transplantation, or previous human tissue implantation. On the other side, *de novo* DSAs are formed after transplantation. DSAs exert damage to the graft by complement-dependent but also through complement-independent (not shown) mechanisms. These antibodies can initiate classical complement pathway leading to membrane attack complex (MAC) formation, C4d deposition, C3b fragment-induced opsonisation and anaphylatoxins secretion (not shown). Moreover, DSAs can interact with leukocytes Fc-receptors (FcR) and exert chemotaxis. Leukocyte recruitment and activation with the consequent secretion of chemokines and cytokines generates a proinflammatory environment. Emerging evidence suggests that the inflammatory milieu together with damage circumstances such as injury generated by natural antibodies (not illustrated), DSAs and extraction and reperfusion processes (ischaemia-reperfusion injury), perhaps in addition to underlying diseases or infections; may lead to an abnormal antigen expression, the exposure of cryptic epitopes or neoantigen formation, that can further initiate autoimmune responses against the graft. Antibodies against non-HLA antigens of the graft would start to be generated and since the majority of them are not polymorphic between donor and recipient, self-antigens (SAgs) would also be attacked, such as collagen V (Col V) and K-alpha-1-tubulin (Kα1T). Furthermore, the damage response could lead directly to the exposure of recipient autoantigens and thus to a “direct” production of autoantibodies against them. The development of these non-HLA autoantibodies would be triggered by the loss of peripheral B cell self-tolerance and could result in the generation of autoreactive PCs, LLPCs and memory B cells that perpetuate the autoimmune response. Another consideration for the development of responses against lung autoantigens is that the stressed organ releases donor extracellular vesicles (EV), for example, exosomes, which carry donor HLA and lung-self antigens. In summary, DSAs lead to increased proinflammatory cytokine production, immune infiltration, and augmentation of cellular immune responses to mismatched donor HLA molecules but also to SAgs (Kα1T and Col V). Vice versa, antibodies developed *de novo* against SAgs, will induce a subsequent cytokine response repeating the cycle. This suggests a cross-talk between allo- and autoimmunity.

Figure 3. Frequency of positive pre-transplant non-HLA antibody reactions.
A balloonplot from a contingency table of 12 pre-selected non-HLA antibodies was created (profile-12 in methods). Several studies and the supplier of the detection kits associate some of these antibodies with lung transplantation and other ones had MFI values highly suggestive of positivity (>1,000 MFI). The frequencies (freq) of positive reactions for each of the 12 antibodies in each of the two groups of lung transplant recipients are shown. On the right, results are shown for the group of patients with antibody-mediated rejection (AMR) without of donor-specific anti-HLA antibodies (DSAs), and on the left side values for the control group. The size of each bubble reflects the relative magnitude of positive reactions for that particular non-HLA antibody.

Figure 4. Comparison of pre-transplant non-HLA antibodies between both groups of lung transplant recipients.
Mann–Whitney *U* nonparametric test was used to compare the medians of pre-transplant non-HLA positive reactions between case and control groups. Twenty non-HLA antibodies with high mean fluorescence intensity (MFI) thresholds and/or more widely studied in recent years were included in this analysis (profile-20 in methods). Statistically significant differences ($p < 0.01$) were found between the medians of the antibody-mediated rejection (AMR) group and the control group of lung transplant recipients.

Figure 5. ROC analysis for the number of pre-transplant non-HLA antibodies in predicting AMR without DSAs.
ROC (receiver operating curve) for the number of pre-transplant non-HLA antibodies in predicting antibody-mediated rejection (AMR). The dataset used contains the mean fluorescence intensity (MFI) values for 20 pre-selected non-HLA antibodies (profile-20 in methods). The area under the curve (AUC) is 0.713 (0.580 - 0.847), the sensitivity was 44.40%, the specificity 95.24% and Youden's index was 7.5%.

Table 1. Descriptive Statistics of B cells Immunophenotyping.
Results of the pre-transplant flow cytometry studies of 4 cases and 4 controls from the initial cohort. The percentage of each B cell subset was obtained using total B cells as the parent population in all cases. The subset with the largest differences between case and control groups is highlighted in bold and corresponds to the plasma cell/plasmablast population (CD38+ CD138+). Student's *t*-test or Mann-Whitney test were used for normally distributed and non-normally distributed data, respectively.

Figure Legends of Supplementary Information

Figure S1. Study flow diagram and Methods basic principles.

(A) Flowchart of cases and controls selection.
The starting point was a database of 800 transplants performed between 2012 and 2023 at Marqués de Valdecilla University Hospital (HUMV in Spanish). Lung transplant recipients (LTRs) who had detectable donor-specific anti-HLA antibodies (DSAs) and/or incomplete criteria for antibody-mediated rejection (AMR) according to the anatomic pathology laboratory (APL) were excluded. Thus, patients negative for complement component 4d (C4d) deposition and/or with a negative AMR-histological pattern were not included in the study. In this way, 27 patients were included in the case group. At that point, patients without evidence of AMR who were transplanted on similar dates as the cases were retrospectively searched, creating case-control pairs. Of the 27 matched controls, only 21 could finally be included in the study due to problems with the detection kit supply. This case-control study would consist of 48 participants transplanted between 2012 and 2022. For each participant, pre-transplant sera were retrieved for the determination of non-HLA antibodies by *Luminex* technology and pre-transplant routine clinical care data about anti-HLA antibodies positivities was rescued (A in the figure). Besides antibody serological detection, immunophenotyping of lymphocyte subsets by flow cytometry (B in the figure) was carried out for certain participants in both groups.

(B) Serum antibody detection (left) and Immunophenotyping gating strategy (right)
Luminex-based assays (left portion of figure) are a type of immunoassays that precisely measure multiple analytes in one sample with a microbead platform. *Luminex* LABScreen kits use up to 100 colour-coded polystyrene beads dyed with different proportions of red and infrared fluorophores that correspond to a unique spectral signature and are coated with antigens of interest. In the case of LABScreen tests for pre-transplant screening of anti-HLA antibodies, beads are coated with a purified pool of HLA class-I and -II antigens for detecting HLA class-I and -II antibodies in patient sera. In particular, six HLA targets are detected: HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQ and HLA-DP. On the contrary, in the LABScreen single antigen bead (SAB) assay, each bead is coated with a purified single recombinant HLA allele, providing a more accurate assignment of patient HLA antibody specificities. Moreover, LABScreen Autoantibody assays also employ this SAB technology to characterise and monitor a broad range of autoantibody targets in serum, simultaneously detecting 39 targets. All these kits use a similar analysis algorithm and protocol: patient sera are incubated in 96-well plates with the beads, and if anti-HLA or non-HLA antibodies are present, they bind to the corresponding antigens in the bead's surface. Bound detection is achieved using fluorescently labelled secondary antibodies, phycoerythrin-conjugated goat anti-human IgG (anti-human IgG-PE). During the protocol unbound excess serum is removed by washing the beads five times and plates are incubated on a rotating platform in

the dark. Afterwards, test samples, for this study 48 samples for non-HLA antibodies determination, were analysed on the LABScan3D™ system, including both in-house as well as manufacturer's positive and negative controls. LABScan3D™ system is a multiplex flow analyser where beads are excited by two lasers. One laser (638 nm) identifies bead specificity by its unique colour signature, determining the bead region and corresponding assigned analyte. The other laser (532 nm) quantifies binding events by detecting the magnitude of the PE-derived signal because it is proportional to the amount of analyte bound. The measurement of mean fluorescence intensity (MFI) correlates with the amount of bound antibodies and is used as a surrogate marker for the level of antibody titres. MFI values allow quantitative analysis of anti-HLA and non-HLA antibodies, but currently there is a lack of consensus regarding the optimum MFI cut-offs for classifying antibodies as positive or those that are significant.

The right side of the figure describes how conventional flow cytometry analysis using *Kaluza* software was performed. Two panels with different combinations of clusters of differentiation (CDs) and fluorochromes were used to define various B cell subsets. In both panels cells were gated in a forward versus side scatter (FSC vs SSC) dot plot to select lymphocytes. This gate identifies cells of interest based on size and granularity (complexity) using only the optics of the flow cytometer. All cell subsets of interest were then identified according to the principle of fluorochrome fluorescence spectra. Total B cells served as the parent population in all cases, but each panel used its own gating strategy: In panel B1, subsets of B1, B2 and transitional B cells were obtained from the total B cell population. In panel 2, switched, unswitched and naive B cells subsets were differentiated. IgM and IgD were used to characterise the maturational stage. Plasma cells (PCs) and plasmablasts (PBs) were characterised by their constitutive marker CD38. Finally, activated B cells were identified by their BAFF receptor (CD268).

Figure S2. Differences in the number of Plasma cells/Plasmablasts between two recipients of each studied group.

The figure provides a visual comparison between the pre-transplant flow cytometry histograms of a control patient (left) and a patient with AMR without DSAs (right). Notable differences in the percentage gated of plasma cells/plasmablasts between both subjects are noted. In this case, prior to transplantation the control patient had 2.66% plasma cells/plasmablasts, in contrast, the AMR patient had 7.15%. The subsets presented in the figure are plasma cells/plasmablasts with CD19+CD27highCD38high phenotype (with CD138 expression, not shown). Indeed, this is the B cell subset with the largest differences between case and control groups (see Table 1).

Table S1. Study cohort demographics.

Demographic characteristics of the studied cohort of 48 lung transplant recipients. Significant *p-values (< 0.05)* are highlighted in bold. Qualitative variables were reported as absolute numbers (*n*) and percentages (%) and

compared using Chi-square or Fisher exact tests as appropriate, while quantitative variables were expressed as median using 25%-75% interquartile range (IQR) or mean ± standard deviation (SD) and were compared with the nonparametric Mann-Whitney test or the Student's *t*-test, respectively.

Table S2. Nine non-HLA antibodies in pre-transplant serum were significantly associated with AMR.

Association of each non-HLA antibody with AMR without DSAs when their positivity was determined with 75th percentile thresholds (MFI values of positive cutoff). The number of positive reactions for each non-HLA antibody in both groups and their corresponding frequency in percentage are shown. Significant *p-values (< 0.05)* are highlighted in bold and were calculated with the Chi-square or Fisher exact tests as appropriate. From the initial 39 non-HLA antibodies tested in the *Luminex* panel, only 14 antibodies that obtained MFI levels above 1,000 and have been associated with lung rejection are shown in an attempt to avoid including false positives (profile-14 in methods).

Figure 1. Allorecognition and Antibody Responses in allograft rejection.

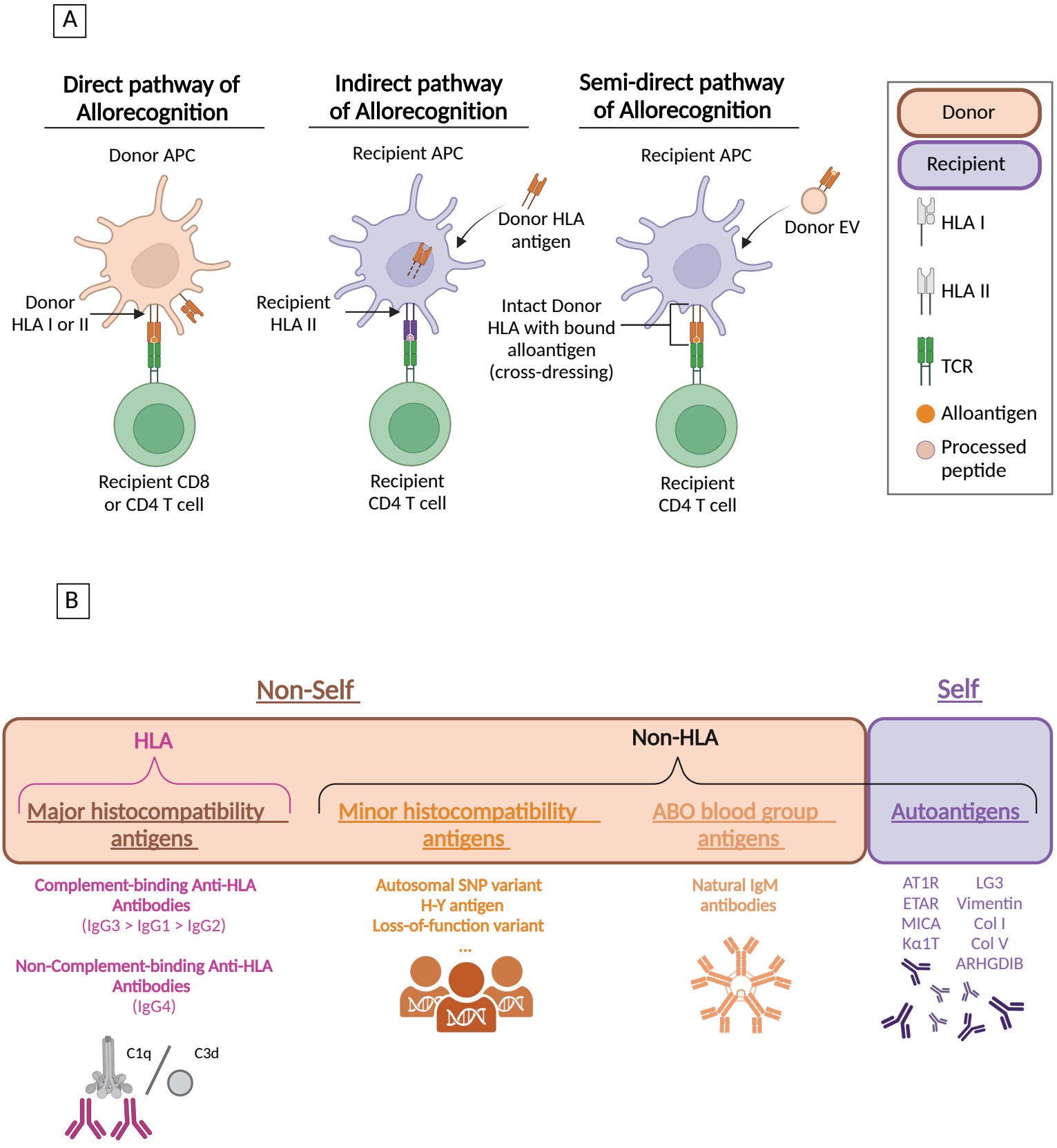

Figure 2. Classification and Main Actors of rejection after lung transplantation.

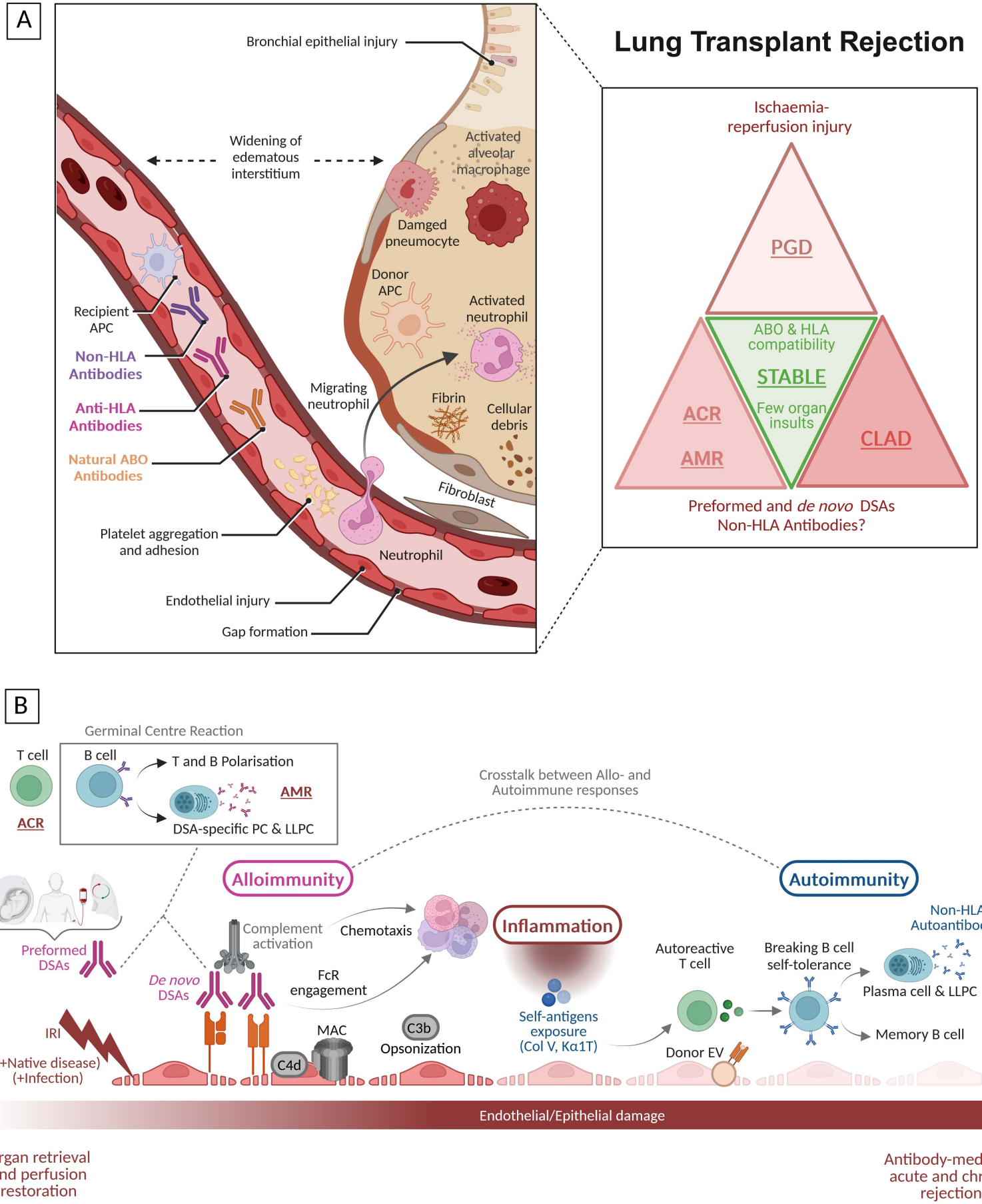

Figure 3. Frequency of positive pre-transplant non-HLA antibody reactions.

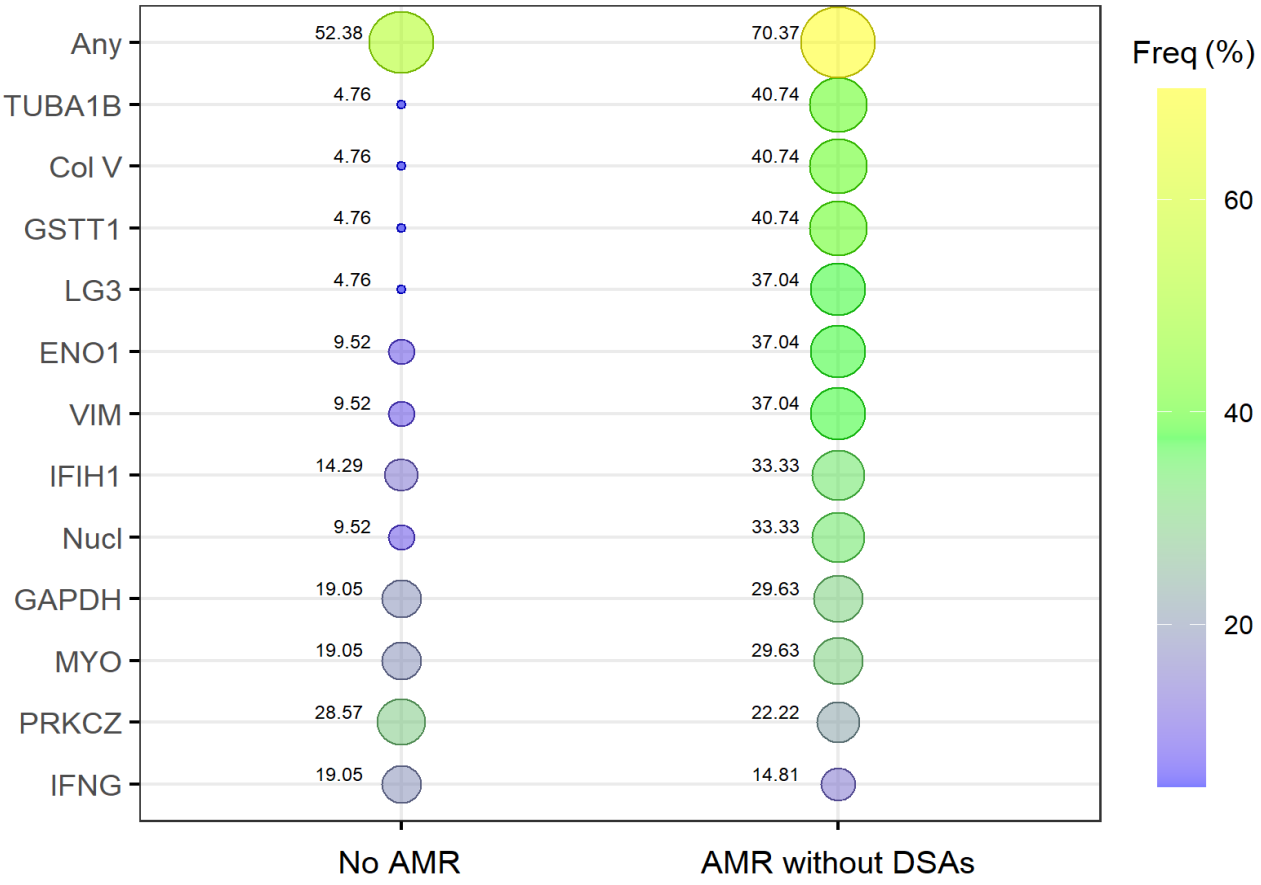

Figure 4. Comparison of pre-transplant non-HLA antibodies between both groups of lung transplant recipients.

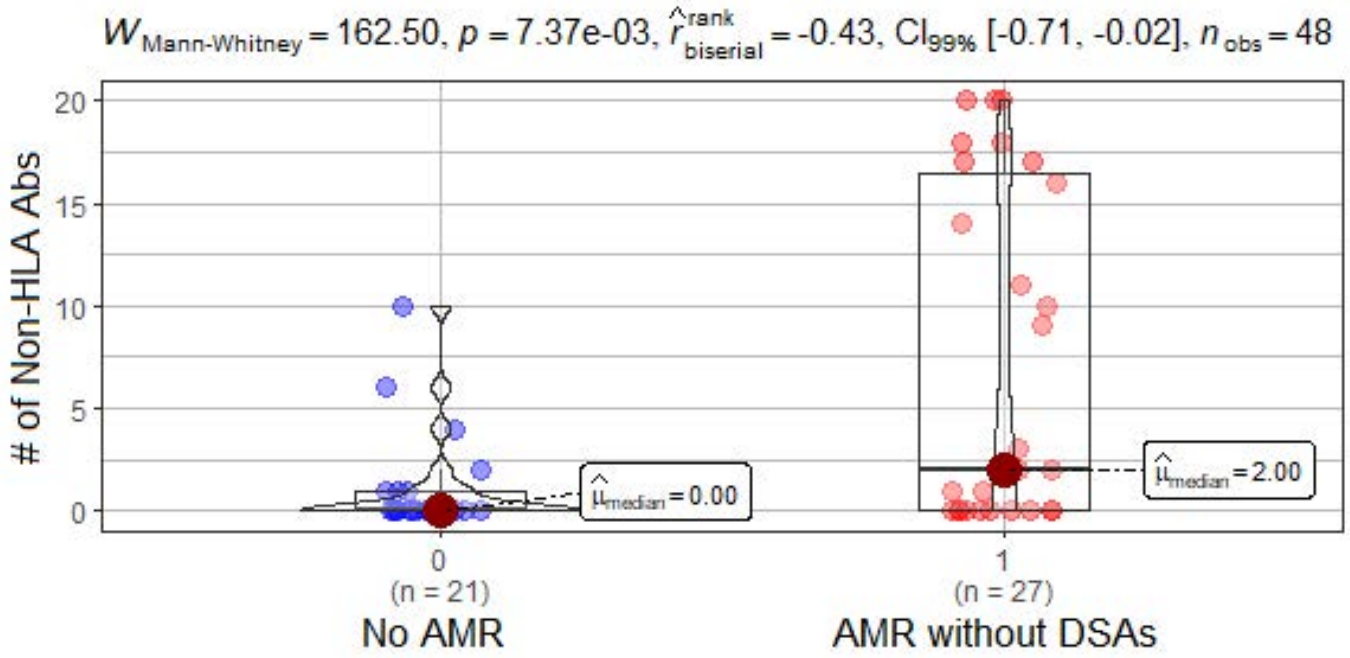

Figure 5. ROC analysis for the number of pre-transplant non-HLA antibodies in predicting AMR**.**

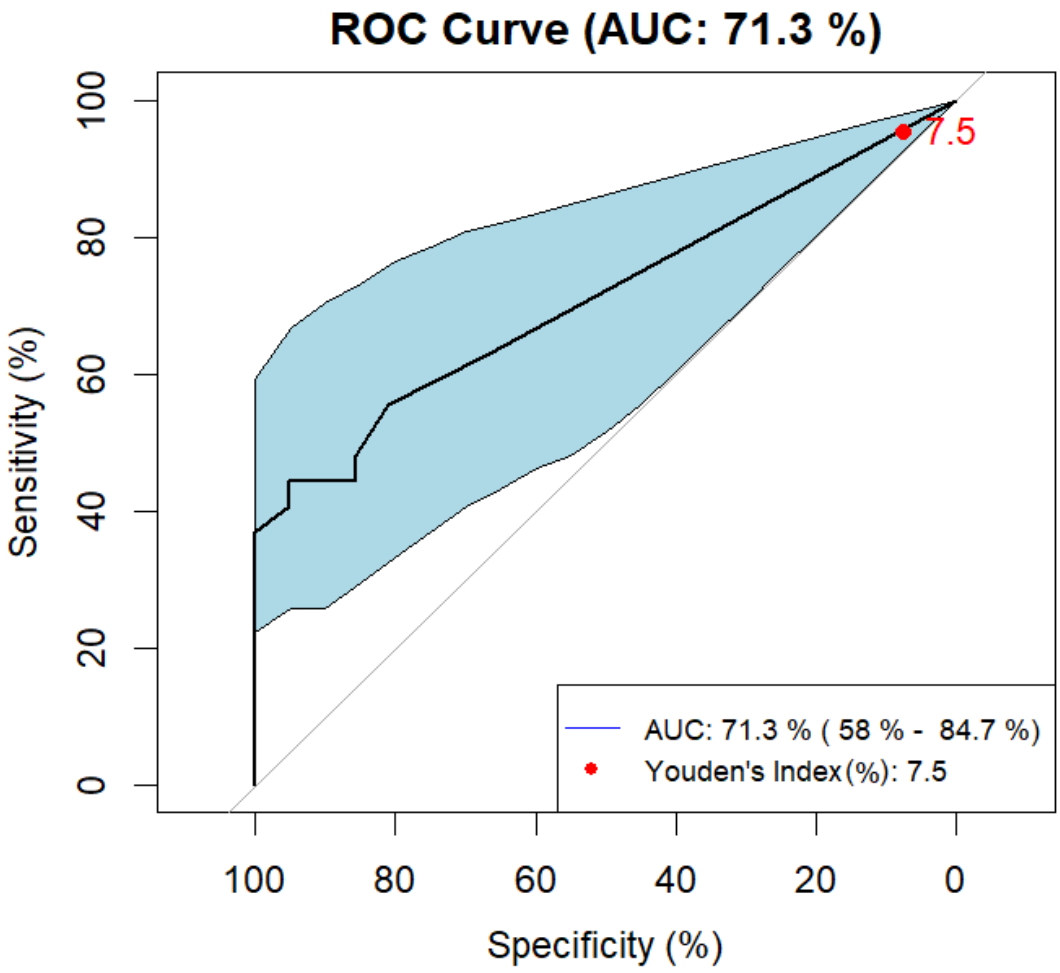

Table 1. Descriptive Statistics of B cells Immunophenotyping.

Variables	AMR without DSAs (n = 27)	No AMR (n = 21)	*p-value*
B cells, mean ± SD	2.49 ± 0.85	3.97 ± 2.70	0.336[a]
CD5+ CD19+, median (IQR)	7.90 (6.49-8.47)	5.76 (3.51-16.32)	0.386[b]
CD5- CD19+, median (IQR)	91.46 (86.05-93.32)	83.30 (66.84-94.14)	0.773[b]
CD38+ CD21+, mean ± SD	61.95 ± 9.81	60.24 ± 17.12	0.868[a]
CD38+ CD21-, median (IQR)	4.12 (2.83-6.16)	9.78 (5.09-16.89)	0.149[b]
CD38- CD21+, mean ± SD	23.32 ± 11.77	11.53 ± 7.46	0.142[a]
CD38- CD21-, mean ± SD	10.23 ± 5.67	17.25 ± 11.73	0.338[a]
CD38+ CD24+, median (IQR)	1.76 (1.05-2.55)	1.21 (0.44-6.31)	0.564[b]
CD38+ CD138+, mean ± SD	2.22 ± 1.11	0.70 ± 0.44	**0.064**[a]
CD268+, mean ± SD	91.16 ± 4.68	88.54 ± 10.32	0.666[a]
IgM+ IgD+, mean ± SD	14.96 ± 11.31	4.51 ± 5.06	0.143[a]
IgM+ IgD-, mean ± SD	3.78 ± 2.77	1.47 ± 1.67	0.203[a]
IgM- IgD+, mean ± SD	56.47 ± 38.97	47.36 ± 22.40	0.699[a]
IgM- IgD-, mean ± SD	30.54 ± 9.96	23.46 ± 13.27	0.426[a]
Naïve, mean ± SD	52.15 ± 15.46	60.65 ± 13.84	0.444[a]
Switched, mean ± SD	16.27 ± 7.99	16.17 ± 3.36	0.983[a]
Unswitched, median (IQR)	10.04 (3.35-20.24)	6.24 (5.38-6.84)	1.000[b]

Suggestive *p-value* is highlighted in bold.

[a]Parametric Student's *t*-test.

[b]Nonparametric Mann-Whitney test.

Figure S1. Study flow diagram and Methods basic principles.

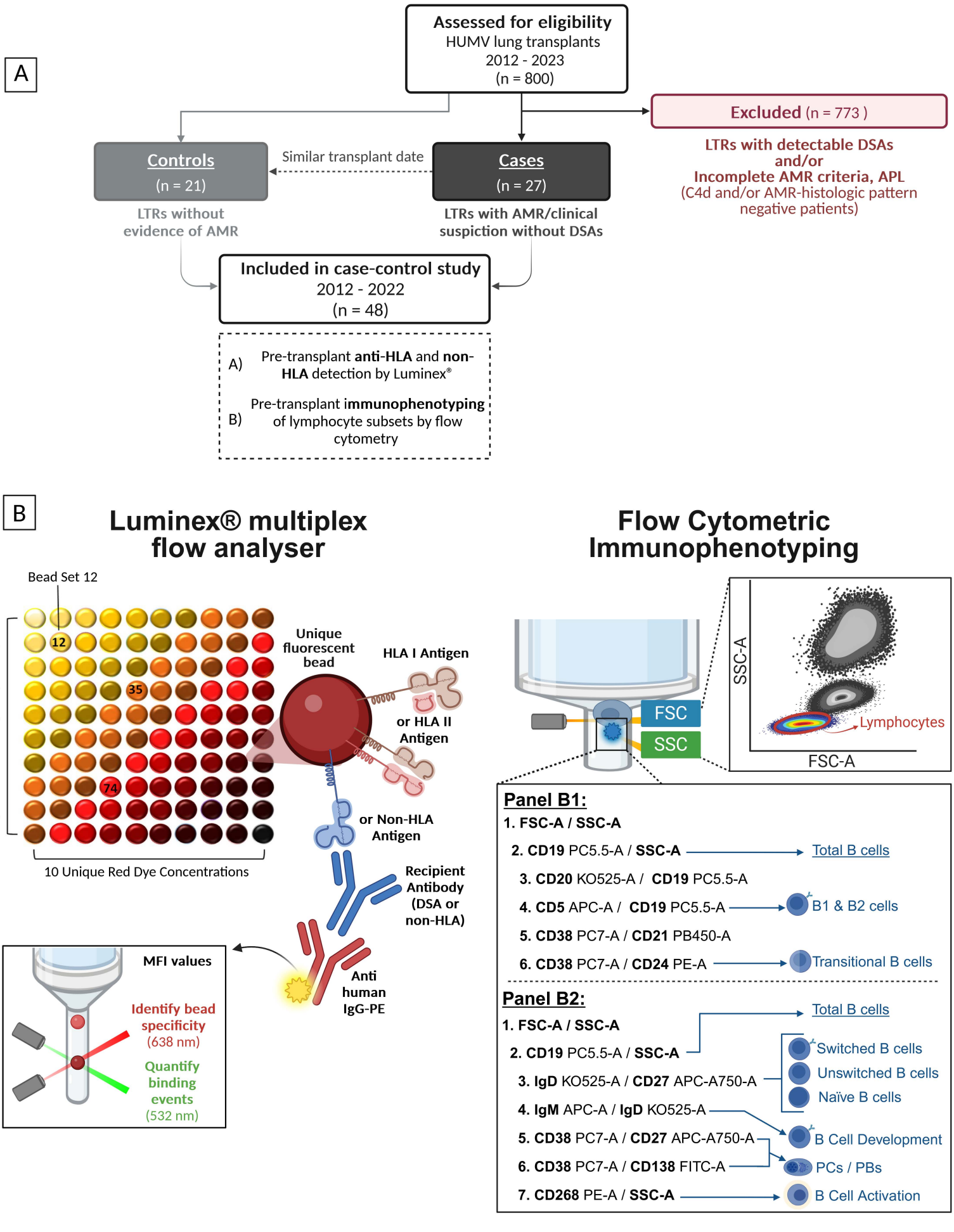

Figure S2. Differences in the number of Plasma cells/Plasmablasts between two recipients of each studied group**.**

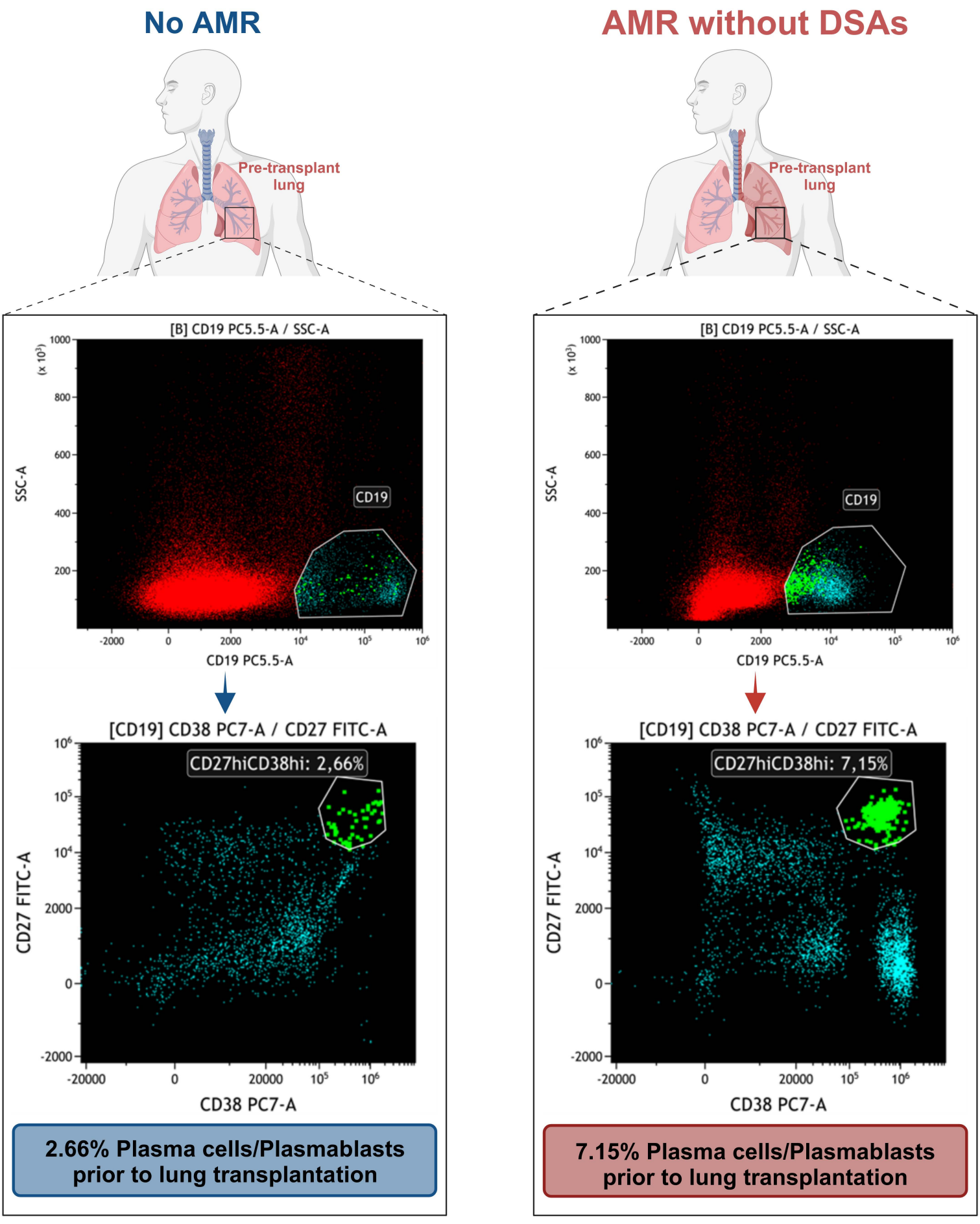

Table S1. Study cohort demographics.

Variables	AMR without DSAs (n = 27)	No AMR (n = 21)	*p-value*
Age, years, median (IQR)	58.42 (55.46-62.03)	53.63 (40.53-61.93)	ns[a]
Male sex, *n* (%)	21 (77.78%)	14 (66.67%)	ns[b]
Follow-up, months, mean ± SD	50.71 ± 39.99	73.32 ± 31.75	**0.039**[c]
Time free of rejection, days, median (IQR)	27 (21.50-814.50)	299 (26-1,466)	ns[a]
Time free of AMR, months, median (IQR)	12.83 (1.80-27.10)	-	NA
Underlying disease (n, %)			ns[b]
A: CF	2 (7.40%)	4 (19.05%)	ns
B: ILD	16 (59.26%)	10 (47.62%)	ns
C: COPD	7 (25.93%)	5 (23.81%)	ns
D: Others	2 (7.41%)	2 (9.52%)	ns
Type of transplant			ns[b]
A: Bipulmonar	21 (77.78%)	17 (80.95%)	ns
B: Unipulmonar	6 (22.22%)	4 (19.05%)	ns
Time of ischemia, hours, mean ± SD	4.45 ± 1.42	4.15 ± 0.99	ns[c]
Induction, *n* (%)			ns[b]
A: No induction	7 (25.93%)	9 (42.86%)	ns
B: Basiliximab	20 (74.07%)	12 (57.14%)	ns
PGD	9 (33.33%)	8 (38.09%)	ns[b]
CLAD	17 (62.96%)	6 (28.57%)	**0.001**[b]
A: BOS	8 (29.63%)	4 (19.05%)	ns
B: RAS	5 (18.52%)	1 (4.76%)	ns
C: Mixed	3 (11.11%)	1 (4.76%)	ns
D: Undefined	1 (3.70%)	0	ns
Exitus, *n* (%)	18 (66.66%)	7 (33.33%)	**0.022**[b]
Pre-transplant DSAs, *n* (%)			
Negative	26 (96.3%)	18 (85,72%)	ns[b]
Positive	1 (3.70%)	3 (14.28%)	ns[b]
Ab-verf Ep class I, mean ± SD	11.87 ± 6.17	10.00 ± 2.45	ns[c]
Ab-verf Ep class II, mean ± SD	10.07 ± 4.38	8.58 ± 2.47	ns[c]
REM, *n* (%)	5 (18.52%)	2 (9.52%)	ns[b]

Antibody-mediated rejection (AMR), donor-specific antibodies (DSAs), cystic fibrosis (CF), interstitial lung disease (ILD), chronic obstructive pulmonary disease (COPD), primary graft dysfunction (PGD), chronic lung allograft dysfunction (CLAD), bronchiolitis obliterans syndrome (BOS), restrictive allograft syndrome (RAS), antibody-verified status of eplets (Ab-verf Ep), high-risk epitope mismatch (REM).

Significant *p-values* < 0.05 are highlighted in bold.
[a]Nonparametric Mann-Whitney test.
[b]Chi-square test or Fisher exact tests as appropriate.
[c]Parametric Student's *t*-test.

Table S2. Nine non-HLA antibodies in pre-transplant serum were significantly associated with AMR.

Non-HLA antibody	Positive cutoff (MFI)	AMR without DSAs (n = 27)	No AMR (n = 21)	*p-value*
LMNA	1,198	12 (44.44%)	0 (0%)	**0.0004** [a]
GSTT1	1,489	11 (40.74%)	1 (4.76%)	**0.0061** [a]
ENO1	2,036	10 (37.04%)	2 (9.52%)	**0.0439** [a]
AURKA	1,783	10 (37.04%)	2 (9.52%)	**0.0439** [a]
PECR	2,865	10 (37.04%)	2 (9.52%)	**0.0439** [a]
CHAF1B	1,729	10 (37.04%)	2 (9.52%)	**0.0439** [a]
TNFA	1,145	10 (37.04%)	2 (9.52%)	**0.0439** [a]
GDNF	1,438	10 (37.04%)	2 (9.52%)	**0.0439** [a]
LG3	3,969	10 (37.04%)	2 (9.52%)	**0.0439** [a]
CXCL9	1,938	8 (29.63%)	4 (19.05%)	0.5101 [a]
Collagen II	4,972	8 (29.63%)	4 (19.05%)	0.5101 [a]
PRKCZ	7,639	6 (22.22%)	6 (28.57%)	0.6143 [b]
Collagen III	3,442	7 (25.93%)	5 (23.81%)	0.8666 [b]
Collagen I	1,993	7 (25.93%)	5 (23.81%)	0.8666 [b]

Human leukocyte antigen (HLA), mean fluorescence intensity (MFI), antibody-mediated rejection (AMR), donor-specific antibodies (DSAs), lamin A/C (LMNA), glutathione S-transferase theta 1 (GSTT1), alpha-enolase (ENO1), aurora kinase A (AURKA), peroxisomal trans-2-enoyl-CoA reductase (PECR), chromatin assembly factor 1 subunit B (CHAF1B), tumor necrosis factor-alpha (TNFA), glial cell line-derived neurotrophic factor (GDNF), perlecan LG3 C-terminal fragment (LG3), protein kinase C zeta (PRKCZ), CXC motif chemokine ligand-9 (CXCL9).

From the initial 39 non-HLA antibodies tested in the Luminex panel, only 14 antibodies that obtained MFI levels >1,000 are shown.

Significant *p-values* < 0.05 are highlighted in bold.

[a] *p-values* calculated with Fisher exact test.

[b] *p-values* calculated with Chi-square test.