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Absence of Donor-Specific Anti-HLA Antibodies After ABO-Incompatible Heart Transplantation in Infancy: Altered Immunity or Age?

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Specific B-cell tolerance toward donor blood group antigens develops in infants after ABO-incompatible heart transplantation, whereas their immune response toward protein antigens such as HLA has not been investigated. We assessed *de novo* HLA-antibodies in 122 patients after pediatric thoracic transplantation (28 ABO-incompatible) and 36 controls. Median age at transplantation was 1.7 years (1 day to 17.8 year) and samples were collected at median 3.48 years after transplantation. Antibodies were detected against HLA-class I in 21 patients (17.2%), class II in 18 (14.8%) and against both classes in 10 (8.2%). Using single-antigen beads, donor-specific antibodies (DSAs) were identified in six patients (all class II, one additional class I). Patients with DSAs were significantly older at time of transplantation. In patients who had undergone pretransplant cardiac surgeries, class II antibodies were more frequent, although use of homografts or mechanical heart support had no influence. DSAs were absent in ABO-incompatible recipients and class II antibodies were significantly less frequent than in children with ABO-compatible transplants. This difference was present also when comparing only children transplanted below 2 years of age. Therefore, tolerance toward the donor blood group appears to be associated with an altered response to HLA beyond age-related effects.

Key words: ABO incompatibility, B-cell tolerance, HLA-antibodies, immune response, immunomodulatory effects, pediatric heart transplantation, population studies and HLA

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Introduction

Infants differ fundamentally in their capacity to mount immune responses to polysaccharide antigens (i.e. blood group antigens, ABO) *versus* protein antigens (i.e. HLA). Because infants do not normally manifest responses to foreign blood groups, ABO-incompatible heart transplantation was found to be a safe clinical approach to enhance donor availability (1). After ABO-incompatible transplantation, a majority of recipients develop spontaneous B-cell tolerance to the donor blood group antigens, showing a pattern of persistent absence of antibodies toward the donor blood group, whereas antibodies toward other polysaccharide antigens, such as the nondonor blood group, develop normally (2). An analysis of the underlying mechanisms of tolerance in this setting demonstrated absence of active B cells specific to the donor blood group (3). However, immune responses also vary with age. Young animals respond poorly if at all to challenge by certain antigens. Moreover, Medawar and colleagues demonstrated in the 1950s that exposure of newborn animals to allogeneic cells induced a state of specific immune tolerance to subsequent antigen exposure in skin grafts (4). Whether and to what extent acceptance of grafts by young recipients reflects immaturity and hence absence of responses, and to what extent it reflects tolerance, is poorly understood. We now explore how tolerance or immunologic immaturity may affect responses to other donor antigens, such as HLA, proteins to which human infants are normally capable of mounting an immune response.

Anti-HLA antibodies are associated with hyperacute and acute antibody-mediated rejection if present at the time of transplantation (5). *De novo* antibodies that develop after transplantation have been shown to accelerate graft

vasculopathy and lead to decreased graft survival in adults after heart (6) and other solid organ transplants (7). In children requiring heart transplantation, smaller studies have described the same consequences, especially if the antibodies were directed toward specific HLA present on the donor organ (8,9). However, most of the available pediatric data concern patients who were presensitized or whose antibody status was unknown prior to transplantation.

The purpose of this study was to determine if children after ABO-incompatible transplantation show differences when compared to ABO-compatible recipients regarding the presence and specificity of *de novo* anti-HLA antibodies. We further assessed the influence of age at transplantation and previously described risk factors such as pretransplant cardiac surgery, use of surgically implanted 'homografts' (10,11) and mechanical cardiac support (12) on the posttransplant development of HLA-antibodies. With the inclusion of a control group without obvious sensitizing events such as prior surgery or transfusion, we also investigated the development of 'natural' HLA-antibodies in children, which have been found in more than 60% of healthy male adults (13).

Methods

The patient cohort was recruited in three pediatric thoracic transplant centers, Edmonton and Toronto, Canada, and Munich, Germany. In Canada, patient plasma was collected within a study on cardiac transplant in infancy after approval from the institutional ethical review boards and written informed consent of each patient's parents or guardians. The patients from Munich underwent antibody screening as part of routine clinical follow-up. Clinical data were collected within a follow-up database for thoracic transplant patients with written consent of the patient's parents or guardians and approved by local ethical review board. The demographic, clinical and test data were blinded for analysis.

Study subjects included patients after pediatric heart or heart–lung–transplantation and a control group of otherwise healthy children with minor cardiac disorders (atrial septal defect, patent arterial duct) who underwent cardiac catheter examination without having had prior surgery or blood transfusion and consented within the Canadian study protocol. The controls were matched to the patient cohort by age, gender and blood group. Patients with HLA-sensitization detected pretransplant with either cytotoxic assays, ELISA or flow cytometry were excluded, as well as patients with congenital immunodeficiency, chromosomal abnormalities or prior use of B-cell–depleting antibodies (rituximab).

The plasma samples for antibody screening were frozen at −80°C directly after drawing and thawed in batches for immediate analysis. Antibody screening was performed by flow beads (FlowPRA®, One Lambda, Canoga Park, CA) and enzyme-linked immunosorbent assay (ELISA; AbScreen® HLA class I and II Biotest, Dreieich, Germany; licensed and approved for clinical use). Following manufacturers' recommendations ELISA screen greater than twice the mean of the negative controls was defined as positive.

A positive FlowPRA® screen was defined as ≥5% or clear pattern of reactivity with screening beads. Positive screens were further analyzed with FlowPRA® Single Antigen I and II beads (One Lambda). Manufacturer's instructions for staining and acquiring were followed. Beads were analyzed on a BD FACSCalibur™ cytometer (Becton Dickinson Biosciences, Mississauga, Ontario, Canada). Single-antigen beads were used to test for antibodies against HLA-A, HLA-B, DRB1, DRB3, DRB4 and DRB5 and DQB1. As the patients included in the study were transplanted since 1989, methods of pretransplant HLA typing improved over time and breadth of tested HLA antigens increased. We therefore limited the analysis for donor specificity to the generally available loci HLA-A, HLA-B and DR.

Statistical analysis was performed using Sigma Plot®/Sigma Stat® and SPSS®. Not all variables followed a normal distribution. Therefore, quantitative values were analyzed using Mann–Whitney rank sum test for comparison of two groups and Kruskal–Wallis test for comparison of more than two groups. Qualitative parameters were compared using z-test, χ^2 or Fisher's exact test; correlations were determined using linear regression and Spearman correlation. To exclude confounding effects of the young age at transplantation, a separate analysis was performed comparing ABO-incompatible recipients to an age-matched group of ABO-compatible recipients.

Results

Patient characteristics

The demographic and clinical history of the patient cohort is shown in Table 1. A total of 122 patients were included, of whom about half were transplanted in the first 2 years of life. This relatively young group appears representative for the demographic structure in many pediatric heart transplant centers (14,15). Table 2 compares clinical data of children who received ABO-incompatible *versus* compatible transplantation younger than 2 years of age. There were no significant differences regarding immunosuppressive medication or target trough levels at time of sample collection, or presence of any type of induction treatment. There was no significant difference in immunosuppressive treatment comparing patients with *versus* without class I, class II or donor-specific HLA-antibodies. The standard maintenance treatment in all participating centers was tacrolimus combined with mycophenolate mofetil, with

Table 1: Demographic and clinical characteristics of the patients

Type of tranplantation	
Heart ABO compatible	86 (70%)
Heart ABO incompatible	28 (23%)
Heart–lung	8 (7%)
Age at transplantation (median and range, years)	1.7 (0.01–17.8)
Interval between transplantation and sample collection (median and range, years)	3.48 (0.6–17.1)
Age of control group at sample collection (median and range, years)	3.14 (0.04–16.7)
Cause for transplantation	
Cardiomyopathy	54 (44%)
Congenital heart disease	53 (43%)
Myocarditis	5 (4%)
Primary pulmonary hypertension	4 (3%)
Kawasaki disease	1 (1%)
Other/unknown	5 (4%)

Table 2: Comparison of clinical and technical aspects comparing all patients versus patients transplanted in the first 2 years ABO incompatible or compatible

		All	ABOi	ABOc <2 years
No. of patients		122	28	37
HLA mismatch (mean ± SD)	Class I (A/B) mismatch	3.1 ± 0.83	2.9 ± 0.89	3.2 ± 0.81
	Class II (DR) mismatch	1.5 ± 0.65	1.6 ± 0.53	1.5 ± 0.52
	No mismatch A/B	0	0	0
	No mismatch DR	1.60%	0	0
Immunosuppressive medication				
Current maintenance (% of patients)	Tacrolimus	85.2	89.3	82.4
	Cyclosporine A	9.8	10.7	14.7
	Mycophenolate	69.7	59.3	75.0
	Azathioprine	7.4	11.1	9.4
	Sirolimus	4.1	3.6	0
	Everolimus	1.6	0	2.9
	Low-dose steroids	14.8	21.4	7.7
Induction (% of patients)	Any induction	73.8	100	75.7
	ATG	56.6*	100*	64.9*
	IL-2 receptor-antibody	17.2	0	10.8
	None	25.4*	0*	21.6*
Method of antibody screening	Flow-PRA	54.1	85.7	67.6
	ELISA	40.2	10.7	29.7
	Both	5.7	3.6	2.7

Similar regimens for immunosuppressive therapy except for significantly more frequent use of ATG-induction in children with ABOi transplantation *($p < 0.05$). No significant differences in HLA-mismatch and used antibody-screening method.

steroids over the first 6–12 months in weaning doses. Individual patients received different medications for particular clinical reasons. All ABOi-transplanted children received induction with commercial or generic polyclonal antithymocyte globulin (ATG), which was standard treatment in the Canadian centers for all patients with only single patients excepted. In Munich, induction with polyclonal antibodies was not standard for ABO-compatible transplantation but rather anti-CD25 antibodies (daclizumab, basiliximab) or no specific induction, whereas all ABOi-transplanted children received ATG following the Toronto protocol. Therefore, ABOi-transplanted children were significantly more likely to have received ATG for induction. The use of ATG, however, was not associated with a lower or higher presence of HLA-antibodies of either class in the cohort.

HLA-mismatch is only shown for HLA-A, HLA-B and DR antigens, as typing for other loci (e.g. Cw and DQ) was only performed in the more recently transplanted children; there were no differences between the groups. The trend to use FlowPRA more frequently for testing of younger and ABOi-transplanted children was not significant.

Frequency of de novo *HLA-antibodies among thoracic transplant recipients and healthy children*

In samples from 60 patients (48.8%) and all 36 controls, HLA-antibodies were analyzed by 'flow beads', whereas ELISA assays were used for samples from 55 patients (45.6%), and both methods for seven patients (5.7%).

Anti-HLA antibodies were detected in 29 of 122 patients (23.8%). Of these, 21 patients (17.2%) had antibodies against HLA class I and 18 (14.8%) had antibodies against HLA class II. The presence of antibodies against class I antigens significantly correlated with presence of antibodies against class II ($p < 0.01$): 11 patients had antibodies against class I only, 8 against class II only and 10 against both classes. Donor-specific *de novo* antibodies (DSAs) were found in six patients (4.9%) all against class II, and in one patient additionally against class I. Patients with DSAs were significantly older at transplantation than subjects lacking those antibodies ($p < 0.05$). The distribution of age at transplant comparing patients with HLA-antibodies *versus* without is shown in Figure 1. No patient transplanted in the first 30 months of age had detectable DSAs, whereas antibodies against nondonor antigens were present in the same proportion as in patients transplanted later in life. As shown in Figure 2, these differences in immune responses were not owed to differences in the interval between transplantation and sample collection.

During the period of time in which these patients were followed, graft survival was not significantly influenced by the presence of *de novo* HLA-antibodies (Figure 3). Patients with antibodies specific for HLA class II do exhibit what appears to be lower survival after 10 years, however the number of patients followed for this period of time is too few to exclude chance as the basis for the difference. The 10-year survival was above 90% in all patients.

In the control group of 36 healthy children, seven (19.4%) had detectable anti-HLA antibodies. Of these, four (11%) had antibodies specific only for HLA class I, two (6%) only for HLA class II and one (3%) for both. Age distribution of positive and negative findings is shown in Figure 4. Although class I-directed antibodies were found throughout

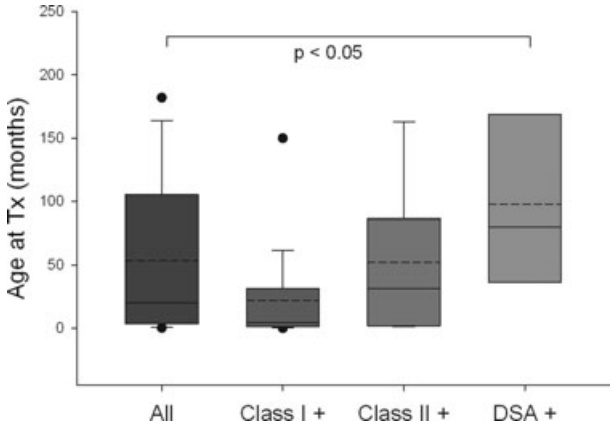

Figure 1: Age at transplantation *versus* presence of antibodies: Distribution of age at transplantation of all patients and patients with class I, class II and donor-specific antibodies. The box represents 25th, 50th and 75th percentile, the whiskers indicate 10th and 90th percentile, the circles show the 5th and 95th percentile, the dotted line represents the mean. Patients with donor-specific antibodies were significantly older at transplantation than the whole cohort ($p < 0.05$).

childhood beginning as early as 26 days of life, the youngest patient with class II antibodies was 5.7 years old.

Influence of risk factors for HLA sensitization in thoracic transplant recipients

The groups with class I, class II and donor-specific antibodies (DSAs) were analyzed for the presence of previously described risk factors for HLA sensitization. We found no significant influence of 'homograft' implantation and of ventricular assist devices (VADs) or extracorporeal membrane oxygenation (ECMO) (Figure 5). Pretransplant cardiac surgery was more common in the group with class II antibodies (Figure 5) but this observation did not reach statistical significance ($p = 0.075$). Comparing patients with pretransplant cardiac surgery toward those without, we found a highly significant difference ($p < 0.001$): class II-specific antibodies were present in nine of 40 patients (22.5%) with surgery but only in seven of 79 (9.1%) without. Occurrence of class I antibodies was not significantly different regarding presence *versus* absence of pretransplant surgery.

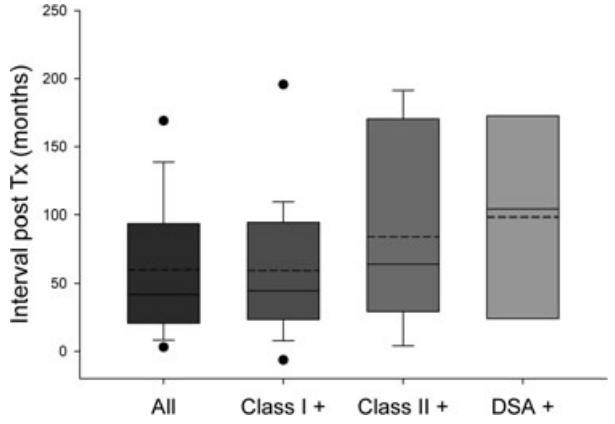

Figure 2: Interval post transplantation *versus* presence of antibodies: Interval between transplantation and sample collection comparing all patients and patients with class I, class II and donor-specific antibodies. The box represents 25th, 50th and 75th percentile, the whiskers indicate 10th and 90th percentile, the circles show the 5th and 95th percentile, the dotted line represents the mean. No significant difference between the groups.

Influence of blood group compatibility on production of anti-HLA antibodies

To determine whether and how ABO-incompatibility between donor and recipient might influence anti-HLA responses, the patient cohort was analyzed for presence or absence of anti-HLA antibodies by ABO-compatibility status. Antibodies to HLA class II were present significantly less frequently ($p < 0.05$) in patients after ABO-incompatible transplantation than after ABO-compatible transplantation (Figure 6). Class I-specific antibodies were present in about the same proportion in both groups. DSAs were completely absent in all 28 ABO-incompatible recipients ($p < 0.01$ compared to ABO-compatible). As all ABO-incompatible transplants were performed within the first 24 months of life, we compared them to the identical age group of ABO-compatible recipients. Although class II antibodies were significantly rarer in the ABO-incompatible group ($p < 0.05$), DSAs were also absent in all ABO-compatible recipients transplanted in the first 2 years of life.

Discussion

This is the first study investigating the development of *de novo* HLA-antibodies in children after ABO-incompatible heart transplantation, and addressing the potential differences in the development of anti-HLA antibodies following thoracic transplantation in early *versus* later childhood. Whereas previous studies in children did not focus on presence of *de novo* HLA-antibodies, we have analyzed a unique patient group by excluding children who were sensitized prior to transplantation. We have found that patients developing DSAs were significantly older at transplantation than those who did not. The DSAs in all our patients were directed against class II antigens, with additional class I-specific DSAs in only one patient. This may be of some importance regarding the immunologic function of class II HLA molecules as they are a crucial part of the indirect pathway of antigen presentation. Whereas HLA class I molecules are present on nearly all cells and can be directly recognized by antigen-specific CD8+ T cells (16), class II molecules are required for specific antigen presentation. Thus, immaturity in indirect antigen presentation may contribute to this difference in immune

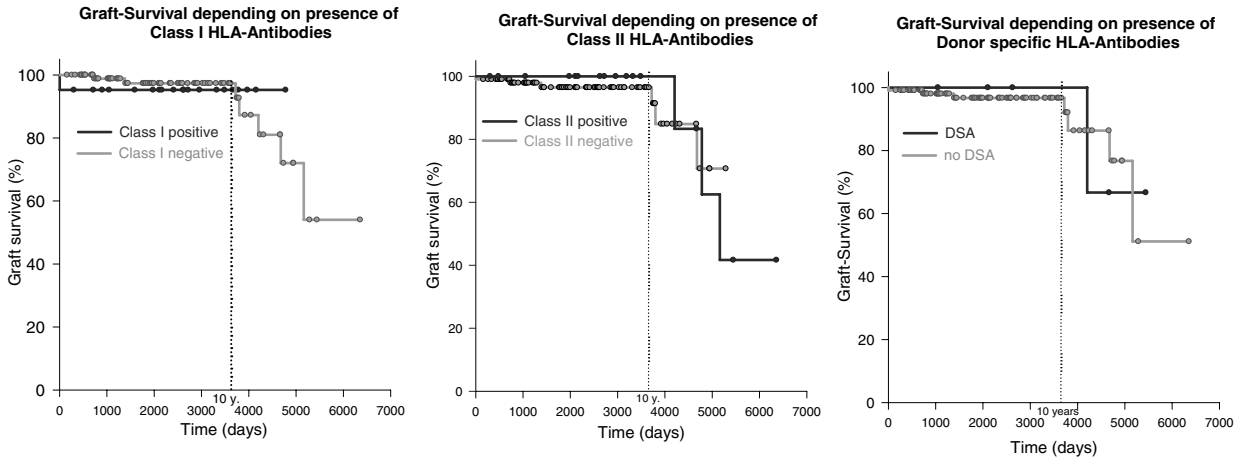

Figure 3: Graft survival: Kaplan–Meier survival curves comparing graft survival in patients with *versus* without antibodies against HLA-class I, HLA-class II and donor-specific HLA. No significant difference in survival (Gehan–Breslowtest $p > 0.05$).

responses of early childhood. In the heart, HLA class II expression is found predominantly on vascular endothelium (17) whereas cardiomyocytes show only weak expression of both HLA classes (18). In addition to vascular endothelium, expression of HLA class II molecules is generally limited to highly specialized cells of the immune system such as macrophages, dendritic cells and B cells (19). In this context, the role of B cells as specific antigen-presenting cells, in addition to their function as a main component for initiation of antibody-mediated rejection (20), may be crucial. The interaction of B and T cells has been found to be less efficient in early childhood than later, for example as in reduced expression of CD40 and CD40 ligand (CD154) (21). Studies on infection and vaccination have further demonstrated that early childhood immunity is skewed toward a less persistent cell-mediated and more prominent Th2 immune response. Despite these elements of immunologic immaturity, human infants are known to be sufficiently competent to mount a vigorous and broad HLA-antibody response to implanted vascular allograft tissue patches when not pharmacologically immunosuppressed (10,11). Thus, our results suggest that the immune response to a transplanted organ in infancy, under systemic immunosuppression, may be less likely to lead to specific antibody production than when transplantation is performed later in life. This impairment of specific antibody production could, in part, explain the observation of the far better long-term survival of children transplanted at an early age when compared to patients transplanted at any later age (15).

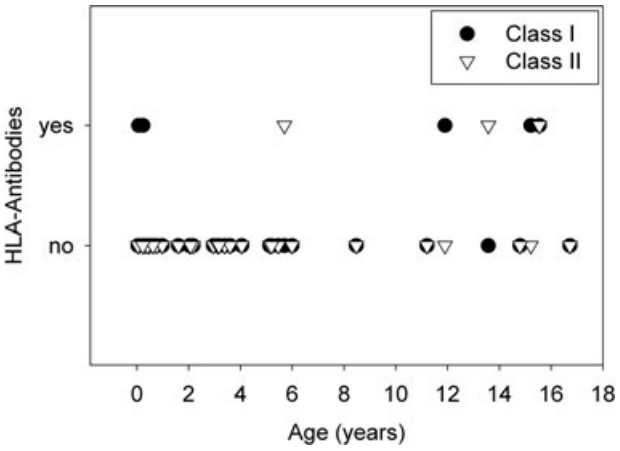

Figure 4: Presence of HLA-antibodies and age distribution of in the control group: Presence of HLA-specific antibodies in the group of 36 matched controls *versus* age at sample collection. HLA-class II antibodies (white triangles) were present only in patients more than 5 years old, while class I were found as early as the first months of life.

The influence of young age on the absence of especially class II antibodies was further confirmed by our control group: while prevalence of HLA-antibodies overall was lower than reported in adults, class II antibodies were found exclusively in children older than five years. 'Natural' HLA-antibodies are speculated to develop following intraintestinal exposure to mimicry antigens (13). The lower incidence of these antibodies in our pediatric control cohort than described for adults might therefore either be due to lack of exposure to the particular organisms or be due to differences in the immune response in early childhood.

In unique contradistinction to these generalizable aspects of T-dependent B-cell immunity related to transplantation in infant recipients, the most striking finding in our patient cohort is the significantly lower presence of HLA class II antibodies and absence of DSAs following ABO-incompatible heart transplantation compared to ABO-compatible. The new clinical approach to infant transplantation afforded by use of ABO-incompatible donors is an extrapolation of the knowledge of general B-cell immaturity in early childhood,

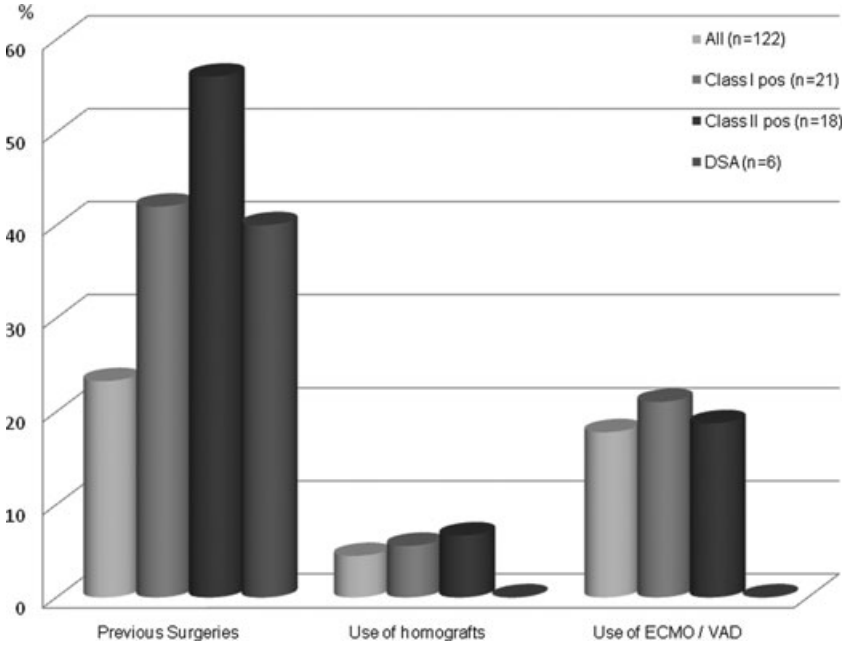

Figure 5: Presence of risk factors in patients with and without HLA-antibodies: Presence of pre-described risk factors comparing all patients and patients with class I, class II and donor-specific antibodies (% of patients). High prevalence of pretransplant cardiac surgery in patients with class II antibodies statistically not significant ($p = 0.075$).

and is largely limited to children in the first year or two of life. This age group is known to have an impaired immune response toward pure polysaccharide antigens, leading to high susceptibility to infections with encapsulated bacteria and particularly severe courses of these infections (22). Beyond the lack of hyperacute and acute antibody-mediated rejection such as described in ABO-incompatible transplantation in older individuals, recipients of ABO-incompatible heart grafts at this immature stage of immune development show long-term absence of antibodies toward the donor blood group (2,23). This was shown to be a form of tolerance, represented by absence of antigen-specific active B cells in patients' peripheral blood (3) and confirmed by equivalent long-term clinical outcomes when compared to ABO-compatible transplantation of the same demographics. The timing and environment of B-cell exposure to polysaccharide antigens seem to play a crucial role in the balance of the immune system toward immunity *versus* tolerance to a specific antigen (24), as well as the interplay of B cells with the innate immune system, especially complement components (25). The lack of response to polysaccharide vaccines in early childhood has been overcome by the creation of conjugate vaccines that connect the polysaccharide to a protein component (26). It is not well understood why the activation of T cells against the protein stimulates the B-cell response to the connected polysaccharide but it was proven to be highly efficient (27), even when the sensitized B-cell later encounters the polysaccharide antigen without the protein. A possible explanation for our findings could be that the observed B-cell tolerance or anergy toward the donor blood group in ABO-incompatible recipients may in reverse manner modulate the response to donor HLA, in particular toward class II antigens. This hypothesis needs to be explored in experimental assays and may be unique to the setting of suppressed immune function posttransplant. There were no significant differences in the immunosuppressive treatment of the ABOi-transplanted children regarding maintenance immunosuppression as determined

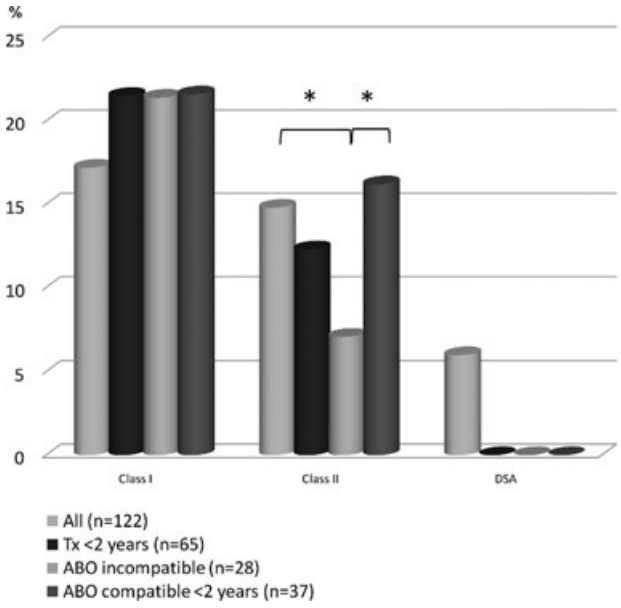

Figure 6: Influence of ABO compatibility on presence of HLA-antibodies: Presence of HLA antibodies comparing ABO-incompatible heart transplant recipients with ABO-compatible recipients of all ages, and ABO-compatible recipients transplanted within the first 24 months of life (% of patients). Difference significant for HLA-class II-specific antibodies in both of these groups ($p < 0.05$). DSA were absent in all patients transplanted in the first 24 months of life regardless of ABO-compatibility.

at the time of sample collection and use of any induction treatment. ATG induction was more common in the ABOi-transplanted group and could therefore be a confounding event. However, this is not supported by the fact that ATG induction was not associated with a difference in the occurrence of *de novo* HLA-antibodies in the overall patient cohort.

Surprisingly, neither the use of homografts nor of VAD or ECMO was found to be a relevant risk factor for development of *de novo* antibodies in our cohort. This is most likely due to the exclusion of presensitized patients in whom these risk factors are often present, as these patients typically develop antibodies early after the intervention and therefore prior to transplantation (10–12). Although some of the earlier patients in our cohort may have been presensitized, with HLA-antibodies that might have been detected with the more sensitive methods which were not available at the time of pretransplant screening, the majority of patients were transplanted after 2003, when solid phase assays were standard for pretransplant screening in the participating centers. The higher prevalence of class II antibodies after pretransplant surgery may indicate a subclinical sensitization that becomes manifest later on. This hypothesis is supported by the fact that these antibodies were predominantly not donor specific. The mechanism of sensitization following cardiac surgery is not perfectly determined and most likely a combination of different factors: activation of the immune system by the artificial surfaces of the heart–lung machine (12), need for blood-transfusion, mandatory because the small blood volume of young children does not allow pump priming with patient blood, and finally activation of the immune system by the massive intervention and subsequent healing process.

In our cohort, we did not find a significant detrimental effect of *de novo* HLA-antibodies on graft survival. Although a high incidence of graft vasculopathy has been observed in patients with DSAs, this aspect was not systematically analyzed in all patients in our cohort and therefore cannot be judged. In adults, several studies have demonstrated a negative impact of class II and DSAs on graft survival, however in children there are very few published data (28). A study by Stastny et al. (9) addressed this question and did not show a significant negative impact of *de novo* antibodies in children, in contrast to the presence of antibodies prior to transplant. However, their cohort was markedly older than ours and had an unusually low overall survival. A second study of a pediatric cohort also found no significant negative effect when separately analyzing the presence of *de novo* antibodies (8). A recently presented analysis of data from the UNOS database showed significant influence of the antibody status (29), however this was not confirmed by other reports (30). All these investigations are hampered by the same limitations as our study: the low prevalence of *de novo* antibodies in patients after pediatric thoracic transplantation and the small number of patients in the long-term follow-up groups. Whether graft accommodation has occurred in these patients is unclear, particularly in relation to possible protective effects mediated by persistent exposure to low levels of antidonor antibodies. A further limitation of our study is the use of different antibody assays at different centers, as the flow-based assays are more sensitive than older cell-based assays (31,32) and require further evaluation of their clinical impact. The control group was only analyzed using the more sensitive FlowPRA®, so the presence of natural antibodies may be overestimated in comparison to the patient cohort. The characteristics of the patient groups analyzed with the different assays were not significantly different. However, as samples from most of the younger patients and all but two ABO-incompatible transplanted patients were analyzed by FlowPRA®, any potential bias would have pointed in the opposite direction than our results. Further, due to the evolution of methods and practices for HLA-typing in the donors and recipients, many of the donors did not have typing at Cw and DQ, and DP typing is not currently standard practice. Testing for donor specificity therefore did not include HLA Cw and HLA DP. Consequently, some DSAs may remain undetected, as well as antibodies against non-HLA epitopes.

In summary, this study has demonstrated that generation of *de novo* anti-HLA antibodies following pediatric thoracic transplantation is clearly age dependent. Whether the absence of antibodies in patients transplanted at a younger age is due to combined effects of antigen exposure during immunologic immaturity and concomitant systemic immunosuppression, or due to other factors, remains unclear. Beyond this age-related phenomenon, infant recipients of ABO-incompatible heart transplants, in particular, not only develop B-cell tolerance to donor ABO antigens as previously described, but were found in this study also to show reduced development of antibodies specific for class II HLA, including DSAs. Understanding the underlying mechanisms behind these results could give access to new strategies to exploit inherent immaturities of the infant immune system to improve transplant outcomes. Moreover, mechanistic understanding of these physiologic events may allow approaches to be developed to avoid or to treat the major threat of long-term survival following organ transplantation: antibody-mediated acute and chronic rejection. Although our findings provide another piece of evidence that ABO-incompatible heart transplantation in early childhood is noninferior, one could even postulate a benefit of this approach in terms of long-term antibody development. However, the limited number of patients and follow-up time prohibit such extensive conclusions until our findings are confirmed by further studies including larger patient numbers.

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Conflict of Interest Statement

There is no funding or financial affiliation of any of the above named authors influencing the content of the paper or leading to a conflict of interest. This study was supported in part by a grant from the National Institutes of Health (USA) HL79067.

References

1. West LJ, Pollock-Barziv SM, Dipchand AI et al. ABO-incompatible heart transplantation in infants. N Engl J Med 2001; 344: 793–800.
2. West LJ. B-cell tolerance following ABO-incompatible infant heart transplantation. Transplantation 2006; 81: 301–307.
3. Fan XH, Ang A, BarZiv SMP et al. Donor-specific B-cell tolerance after ABO-incompatible infant heart transplantation. Nat Med 2004; 10: 1227–1233.
4. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature 1953; 172: 603–606.
5. Nwakanma LU, Williams JA, Weiss ES, Russell SD, Baumgartner WA, Conte JV. Influence of pretransplant panel-reactive antibody on outcomes in 8160 heart transplant recipients in recent era. Ann Thorac Surg 2007; 84: 1556–1562; discussion 1562–1553.
6. Tambur AR, Pamboukian SV, Costanzo MR et al. The presence of HLA-directed antibodies after heart transplantation is associated with poor allograft outcome. Transplantation 2005; 80: 1019–1025.
7. Terasaki P, Lachmann N, Cai J. Summary of the effect of de novo HLA antibodies on chronic kidney graft failure. Clin Transplant 2006; 455–462.
8. Di Filippo S, Girnita A, Webber SA et al. Impact of ELISA-detected anti-HLA antibodies on pediatric cardiac allograft outcome. Hum Immunol 2005; 66: 513–518.
9. Stastny P, Lavingia B, Fixler DE, Yancy CW, Ring WS. Antibodies against donor human leukocyte antigens and the outcome of cardiac allografts in adults and children. Transplantation 2007; 84: 738–745.
10. Meyer SR, Campbell PM, Rutledge JM et al. Use of an allograft patch in repair of hypoplastic left heart syndrome may complicate future transplantation. Eur J Cardiothorac Surg 2005; 27: 554–560.
11. Hawkins JA, Breinholt JP, Lambert LM et al. Class I and class II anti-HLA antibodies after implantation of cryopreserved allograft material in pediatric patients. J Thorac Cardiovasc Surg 2000; 119: 324–330.
12. Yang J, Schall C, Smith D et al. HLA sensitization in pediatric pre-transplant cardiac patients supported by mechanical assist devices: The utility of Luminex. J Heart Lung Transplant 2009; 28: 123–129.
13. 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: 1111–1115.
14. Dionigi B, Razzouk AJ, Hasaniya NW, Chinnock RE, Bailey LL. Late outcomes of pediatric heart transplantation are independent of pre-transplant diagnosis and prior cardiac surgical intervention. J Heart Lung Transplant 2008; 27: 1090–1095.
15. Kirk R, Edwards LB, Aurora P et al. Registry of the International Society for Heart and Lung Transplantation: Eleventh official pediatric heart transplantation report–2008. J Heart Lung Transplant 2008; 27: 970–977.
16. Moretta A, Biassoni R, Bottino C et al. Major histocompatibility complex class I-specific receptors on human natural killer and T lymphocytes. Immunol Rev 1997; 155: 105–117.
17. McDouall RM, Batten P, McCormack A, Yacoub MH, Rose ML. MHC class II expression on human heart microvascular endothelial cells: Exquisite sensitivity to interferon-gamma and natural killer cells. Transplantation 1997; 64: 1175–1180.
18. Sundstrom JB, Jollow KC, Braud V et al. Magnitude of alloresponses to MHC class I/II expressing human cardiac myocytes is limited by their intrinsic ability to process and present antigenic peptides. Clin Dev Immunol 2003; 10: 213–226.
19. Holling TM, Schooten E, Van Den Elsen PJ. Function and regulation of MHC class II molecules in T-lymphocytes: Of mice and men. Hum Immunol 2004; 65: 282–290.
20. Zarkhin V, Li L, Sarwal M. 'To B or not to B?' B-cells and graft rejection. Transplantation 2008; 85: 1705–1714.
21. Durandy A, De Saint Basile G, Lisowska-Grospierre B et al. Undetectable CD40 ligand expression on T cells and low B cell responses to CD40 binding agonists in human newborns. J Immunol 1995; 154: 1560–1568.
22. Rijkers GT, Sanders EAM, Breukels MA, Zegers BJM. Infant B-cell response to polysaccharide determinants. Vaccine 1998; 16: 1396–1400.
23. Roche SBM, O'Sullivan J, Wallis J et al. Multicenter experience of ABO-incompatible pediatric cardiac transplantation. Am J Transplant 2008; 8: 208–215.
24. Rickert RC. Regulation of B lymphocyte activation by complement C3 and the B cell coreceptor complex. Curr Opin Immunol 2005; 17: 237–243.
25. Lee Y, Haas KM, Gor DO et al. Complement component C3d-antigen complexes can either augment or inhibit B lymphocyte activation and humoral immunity in mice depending on the degree of CD21/CD19 complex engagement. J Immunol 2005; 175: 8011–8023.
26. Granoff DM, Holmes SJ, Belshe RB, Osterholm MT, McHugh JE, Anderson EL. Effect of carrier protein priming on antibody responses to Haemophilus influenzae type b conjugate vaccines in infants. JAMA 1994; 272: 1116–1121.
27. Toltzis P, Jacobs MR. The epidemiology of childhood pneumococcal disease in the United States in the era of conjugate vaccine use. Infect Dis Clin North Am 2005; 19: 629–645.
28. Feingold B, Bowman P, Zeevi A et al. Survival in allosensitized children after listing for cardiac transplantation. J Heart Lung Transplant 2007; 26: 565–571.
29. Rossano JW, Morales DL, Denfield SW et al. Impact of panel-reactive antibodies on long-term outcome in pediatric heart transplant patients: An analysis of the United Network of Organ Sharing Database. J Heart Lung Transplant 2009; 28: S232.
30. Irving C, Hasan A, Carter V, Gennery A, Parry G, Kirk R. Association between Donor-specific HLA antibodies and adverse outcome following paediatric cardiac transplantation. J Heart Lung Transplant 2009; 28: S232.
31. Gebel HM, Bray RA. Sensitization and sensitivity: Defining the unsensitized patient. Transplantation 2000; 69: 1370–1374.
32. Tait BD, Hudson F, Cantwell L et al. Review article: Luminex technology for HLA antibody detection in organ transplantation. Nephrol 2008; 14: 247–254.