UNIVERSITAT DE BARCELONA

Tracking preformed serological and T-cell alloimmune memory together with donor/recipient Molecular Human Leukocyte Antigen (HLA) disparity to improve immune-risk stratification in Kidney Transplantation

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Tracking preformed serological and T-cell alloimmune memory together with donor/recipient Molecular Human Leukocyte Antigen (HLA) disparity to improve immune-risk stratification in Kidney Transplantation

Doctoral thesis report submitted by **Maria Antonia Emilia Meneghini**
to obtain a doctoral degree by the University of Barcelona

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CERTIFY

That Maria Antonia Emilia Meneghini, graduated in Medicine and Surgery by the Università degli Studi di Milano, has carried out under our direction the research work to elaborate her Doctoral Thesis untitled **"Tracking preformed serological and T-cell alloimmune memory together with donor/recipient Molecular Human Leukocyte Antigen (HLA) disparity to improve immune-risk stratification in Kidney Transplantation"**, and through this writing they authorize its presentation to achieve the degree of Doctor in medicine.

This is made evident to all effects in Barcelona, the 2nd of August, 2021

Oriol Bestard Matamoros — Thesis Director

Josep Maria Grinyó Boira — Thesis Co-director

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TABLE OF CONTENTS

GLOSSARY

AR: acute rejection
ABMR: antibody-mediated rejection
ADCC: antibody-dependent cell mediated toxicity
AIM: activation-induced cell marker
APC: antigen presenting cell
BCR: B-cell receptor
BL: borderline lesions
BPAR: biopsy-proven acute rejection
CDC-XM: complement-dependent cytotoxicity crossmatch
CNI: calcineurin inhibitors
CREG: Cross reactive group
DSA: donor-specific alloantibodies
eGFR: estimated glomerular filtration rate
ELISPOT: Enzyme-Linked immunoSPOT
FC-XM: flow cytometry crossmatch
HLA: Human Leukocyte Antigens
IL: Interleukin
IFN: Interferon
IFN-γ ELISPOT: Interferon gamma Enzyme-linked ImmunoSpot
Ig: immunoglobulin
mBc: Memory B cells
MHC: Major Histocompatibility Complex
MFI: mean fluorescence intensity
MLR: mixed lymphocyte reaction
MM: mismatches
MMF: mycophenolate mofetil
mTORi: mTOR inhibitors
NK: natural killer cells
PIRCHE-II: predicted indirectly recognizable HLA epitopes
SAB: single antigen beads
SPA: solid phase assay
TCMR: T cell–mediated rejection
TCR: T-cell receptor

List of the articles that comprise the thesis. Thesis in the form of a collection of published articles.

This thesis comprises 4 objectives and 3 articles:

1.

Maria Meneghini, Edoardo Melilli, Jaume Martorell, Ignacio Revuelta, Elisabet Rigol-Monzó, Anna Manonelles, Nuria Montero, David Cucchiari, Fritz Diekmann, Josep M. Cruzado, Salvador Gil-Vernet, Josep M. Grinyó and Oriol Bestard

Combining Sensitive Crossmatch Assays With Donor/Recipient Human Leukocyte Antigen Eplet Matching Predicts Living-Donor Kidney Transplant Outcome

Kidney International Reports (2018) 3, 926–938; doi.org/10.1016/j.ekir.2018.03.015
Impact factor: 3.374 (2019). Quartile 1 Nephrology (2020)

2.

Maria Meneghini, Elena Crespo, Matthias Niemann, Alba Torija, Nuria Lloberas, Vincent Pernin, Pere Fontova, Edoardo Melilli, Alexandre Favà, Nuria Montero, Anna Manonelles, Josep Maria Cruzado, Eduard Palou, Jaume Martorell, Josep Maria Grinyo´ and Oriol Bestard

Donor/Recipient HLA Molecular Mismatch Scores Predict Primary Humoral and Cellular Alloimmunity in Kidney Transplantation

Frontiers in Immunology 11:623276; doi: 10.3389/fimmu.2020.623276
Impact factor: 6.429 (2020). Quartile 1 Immunology (2020)

3.

Oriol Bestard, **Maria Meneghini,** Elena Crespo, Frederike Bemelman, Martina Koch, Hans D. Volk, Ondrej Viklicky, Magali Giral, Bernhard Banas, Juan C. Ruiz, Edoardo Melilli, Liu Hu, Raphael van Duivenvoorden, Björn Nashan, Friedrich Thaiss, Natalie M. Otto, Gantuja Bold, Maik Stein, Anett Sefrin, Nils Lachmann, Petra Hruba, Lucia Stranavova, Sophie Brouard, Cécile Braudeau, Gilles Blancho, Miriam Banas, Juan Irure, Sophia Christakoudi, Alberto Sanchez-Fueyo, Kathryn J. Wood, Petra Reinke and Josep M. Grinyó

Preformed T cell alloimmunity and HLA eplet mismatch to guide immunosuppression minimization with tacrolimus monotherapy in kidney transplantation: Results of the CELLIMIN trial

Am J Transplant. 2021;00:1–13; doi /10.1111/ajt.16563
Impact factor 7.338 (2020). Quartile 1 Immunology and Transplantation (2020)

RESUMEN DE LA TESIS DOCTORAL (Castellano)

Titulo: *Estudio de la memoria aloinmune preformada serológica y celular T, y de la incompatibilidad HLA donante/receptor a nivel molecular para mejorar la estratificación del riesgo inmunológico en el trasplante renal.*

Introducción: La presencia de una respuesta inmunológica donante-especifica impacta negativamente en la evolución del injerto, asociándose a un mayor riesgo de rechazo y de pérdida de injerto. Esta respuesta aloinmune donante-específica puede ser de memoria, y estar preformada antes del trasplante como también, puede activarse *de novo* tras el trasplante.
Consecuentemente, la evaluación del riesgo inmunológico de los receptores de un trasplante renal debe tener en cuenta estos dos aspectos para evitar el desarrollo del rechazo del injerto. Las técnicas inmunológicas usadas en la practica clínica actual presentan algunas limitaciones que no permiten una evaluación completa y precisa de estas 2 respuestas inmunológicas en el momento del trasplante.

Hipótesis: la hipótesis de esta tesis doctoral es que una evaluación más precisa de la memoria inmunológica preformada mediante nuevas herramientas diagnosticas sensibles y específicas que permiten monitorizar la presencia de una respuesta de memoria tanto serológica como celular mediada por linfocitos T donante-específicos, combinado con el estudio de la susceptibilidad de activación de una respuesta aloinmune *de novo* mediante algoritmos bioinformáticos que cuantifican el grado de compatibilidad HLA donante/receptor a nivel molecular, mejorarían la estratificación del riesgo inmunológico individual antes del trasplante y permitirían finalmente guiar el tipo de terapia inmunosupresora de forma personalizada.

Objetivos:
- Comparar la precisión de diferentes tests inmunológicos de detección de anticuerpos circulantes donante-específicos (DSA) pre-trasplante para predecir el riesgo de rechazo tras el trasplante.
- Investigar las cinéticas de activación de la respuesta aloinmune celular T en el post-trasplante renal y estudiar las vías de presentación antigénicas involucradas en su activación.
- Analizar el impacto de la incompatibilidad HLA donante/receptor a nivel molecular sobre el riesgo de desarrollar una respuesta donante-especifica *de novo* tanto celular T como humoral.

- Evaluar la utilidad de combinar la evaluación de la respuesta celular T preformada donante-específica junto al estudio de incompatibilidad HLA molecular donante/receptor para identificar pacientes candidatos a recibir de forma segura una terapia inmunosupresora de mantenimiento basada en monoterapia con tacrolimus .

Métodos: Los estudios de esta tesis doctoral se han fundamentado en dos estudios retrospectivos de cohortes y un ensayo clínico prospectivo multicéntrico guiado por biomarcadores en pacientes trasplantados renales (CELLIMIN). Se ha evaluado la capacidad predictiva de diferentes ensayos inmunológicos de detección de DSA en suero pre-trasplante: cross-match por citometría de flujo, técnicas de fase solida y análisis de la capacidad de los DSA de fijar complemento (C3d) *in vitro*. Además, se ha medido la presencia de células T aloreactivas *in vitro* mediante la técnica de ELISPOT Interferon(IFN)-y tanto antes como después del trasplante.

La incompatibilidad HLA donante/receptor se ha evaluado mediante diferentes algoritmos informáticos que evalúan la composición aminoacídica y las características físicas de las moléculas HLA cuantifican así el grado de incompatibilidad a 3 niveles: de aminoácidos, epletos y péptidos alogénicos presentados por molécula de HLA de clase II por el receptor (incompatibilidad de aminoácidos, HLAMatchmaker y score PIRCHE-II, respectivamente). Se ha estimado el impacto de los resultados de estos algoritmos en la predicción de la aloinmunidad primaria o *de novo* tanto serológica como de células T aloreactivas.

Por ultimo, se ha evaluado la seguridad y eficacia para prevenir el rechazo del injerto renal en un ensayo clínico prospectivo, multicéntrico de no inferioridad, y guiado por biomarcadores de respuesta de memoria pre-trasplante (serológica y celular T donant-específica) para aleatorizar pacientes de bajo riesgo inmunológico pre-trasplante a un régimen inmunosupresor basado en monoterapia con tacrolimus o un tratamiento inmunosupresor convencional basado en triple terapia con tacrolimus, micofenolato mofetil y esteroides..

Resultados principales: Las técnicas más precisas de estudio de la respuesta serológica pre-trasplante que predicen resultados negativos tras el trasplante en forma de mayor rechazo como pérdida del injerto son una combinación de detección de DSA (detectados por técnicas de fase solida) y el cross-match por citometría de flujo. Los DSA con elevado índice de fluorescencia (MFI) y los que fijan complemento *in vitro* predicen elevado riesgo de rechazo post-trasplante con elevada sensibilidad y especificidad.

Por otro lado, todos los algoritmos evaluados de estimación de incompatibilidad HLA a nivel molecular predicen el riesgo de activación de la aloimunidad humoral primaria post-trasplante de forma muy precisa. De forma parecida, una mayor incompatibilidad molecular (sobretodo según el score PIRCHE-II) permite predecir un mayor riesgo de generación de respuesta celular T donante-especifica *de novo* post-trasplante.

Por último, en el ensayo clínico CELLIMIN, los pacientes sin detección de aloreactividad preformada pre-trasplante (ni serológica ni celular T donante-específica) presentaron un riesgo significativamente inferior de rechazo agudo del injerto tanto clínico como subclínico, comparado con pacientes con aloreactividad T donante-específica detectable pre-trasplante bajo el mismo tratamiento inmunosupresor convencional basado en triple terapia. Sin embrago, los pacientes sin aloreactividad celular T ni DSA preformados que recibieron tratamiento con tacrolimus monoterapia presentaron una incidencia significativamente mayor de rechazo agudo del injerto, especialmente aquellos con una elevada incompatibilidad HLA de epletos a nivel de los antígenos DQ.

Conclusiones: Un estudio amplio y preciso de las respuestas inmunológicas de memoria tanto serológica como celular T donante-específica, junto con la evaluación de la incompatibilidad HLA a nivel molecular, podrían permitir una estratificación del riesgo inmunológico de cada receptor frente a su respectivo donante de forma más precisa y en última instancia permitir adaptar el tipo de tratamiento inmunosupresor de una forma personalizada.

RESUM DE LA TESI DOCTORAL (Català)

Títol: Estudi de la memòria al·loinmune preformada serològica i cel·lular T així com de la incompatibilitat HLA donant/receptor a nivell molecular per millorar l'estratificació del risc immunològic en el trasplantament renal

Introducció: La presència d'una resposta immunològica donant-especifica impacta negativament en l'evolució del trasplantament, associant-se a major risc de rebuig i de pèrdua d'empelt. Aquesta resposta immunològica donant-específica pot ser de memòria i estar preformada abans del trasplantament, o bé activar-se *de novo* després del trasplantament. Així, l'avaluació del risc immunològic dels receptors d'un trasplantament renal haurà de tenir present aquests dos mecanismes per monitoritzar el risc de rebuig immunològic de l'empelt. Tanmateix, les tècniques immunològiques emprades en la pràctica clínica actual presenten algunes limitacions que no permeten una avaluació d'aquestes 2 respostes immunològiques d'una forma complerta i precisa en el moment del trasplantament.

Hipòtesis: La hipòtesi d'aquesta tesi doctoral és que una avaluació més acurada de la memòria immunològica preformada mitjançant la utilització de noves eines diagnòstiques sensibles i específiques que permeten monitoritzar la presència d'una resposta de memòria tant serològica com cel·lular mitjançada per limfòcits T donant-específics, combinat amb un estudi acurat de la susceptibilitat d'activació d'una resposta al·loimmune *de novo* mitjançant algoritmes bioinformàtics que quantifiquen el grau de compatibilitat HLA donant/receptor a nivell molecular, millorarien l'estratificació del risc immunològic individual abans del trasplantament i permetrien finalment guiar el tipus de teràpia immunosupressora d'una forma personalitzada.

Objectius:

- Comparar la precisió de diferents tests immunològics de detecció d'anticossos donant-específics (DSA) pre-trasplantament per a predir el risc de rebuig de l'empelt renal

-Investigar les cinètiques d'activació de la resposta al·loinmune cel·lular T post-trasplantament renal i estudiar les vies de presentació antigèniques involucrades en la seva activació.

- Analitzar l'impacte de la incompatibilitat HLA donat/receptor a nivell molecular sobre el risc de desenvolupar una resposta donant-específica *de novo* cel·lular tant T com B.

- Avaluar la utilitat de combinar l'avaluació de la resposta immunològica preformada serològica i cel·lular T donant-específica junt amb l'estudi d'incompatibilitat HLA donant/receptor a nivell molecular per identificar pacients candidats a rebre una teràpia immunosupressora de manteniment basada en monoteràpia amb tacrolimus de forma segura.

Mètodes: S'ha realitzat dos estudis retrospectius de cohorts en pacients trasplantats renals i un estudi prospectiu multicèntric guiat per biomarcadors (CELLIMIN). S'ha avaluat la capacitatpredictiva de diferents tests immunològics de detecció de DSA en sèrum pretrasplantament: cross-match per citometria de fluxe, tècniques de fase sòlida i anàlisi de capacitat dels DSA de fixar complement (C3d) *in vitro*. A més, s'ha avaluat la presència de cèl·lules T al·loreactives in vitro mitjançant la tècnica d'ELISPOT Interferó(IFN)- y tant abans com després del trasplantament. La incompatibilitat HLA donant/receptor s'ha avaluat mitjançant diferents algoritmes informàtics que quantifiquen la composició aminoacídica, i les característiques físiques de les molècules d'HLA mesurant així el grau d'incompatibilitat a 3 nivells: d'aminoàcids, d'eplets i de pèptids al·logènics presentats per molècules d'HLA de classe II pel receptor (incompatibilitat d'aminoàcids, HLA-Matchmaker, PIRCHE-II, respectivament). S'ha estimat l'impacte dels resultats d'aquests algoritmes en la predicció de l'al·loimmunitat primària o *de novo* tant serològica com de cèl·lules T. Per últim, s'ha avaluat la seguretat i eficàcia per a prevenir el rebuig agut de l'empelt renal en un assaig clínic prospectiu, multicèntric de no inferioritat, i guiat per biomarcadors de resposta de memòria pre-trasplantament (serològica i cel·lular T donant-específica) per a aleatoritzar pacients de baix risc immunològic pre-trasplantament a un règim immunosupressor basat en monoteràpia amb tacrolimus o un tractament immunosupressor convencional amb triple teràpia amb tacrolimus, micofenolat mofetil i esteroids.

Resultats principals: Les tècniques més sensibles i específiques d'estudi de la resposta de memòria serològica pre-trasplantament que prediuen resultats negatius post-trasplant en forma de major risc de rebuig com pèrdua d'empelt són una combinació de detecció de DSA (detectats per tècniques de fase sòlida) i el croSs-match per citometria de fluxe. Els DSA amb elevat índex de fluorescència (MFI) i els que fixen complement *in vitro* prediuen un elevat risc de rebuig post-trasplantament amb elevada sensibilitat i especificitat.

Per altra costat, tots els algoritmes avaluats d'estimació del grau d'incompatibilitat HLA a nivell molecular prediuen el risc d'activació d'al·loimmunitat humoral primària o *de novo* de forma molt

precisa. De forma semblant, una major incompatibilitat molecular (sobretot segons l'Score PIRCHE-II) permet predir un major risc de generació de resposta cel·lular T donant-específica *de novo* post-trasplantament. Per últim, a l'assaig clínic CELLIMIN, els pacients sense detecció d'al·loreativitat preformada pretrasplantament (ni serològica ni cel·lular T donant-específica) presentaren un risc significativament inferior de desenvolupar rebuig agut de l'empelt tant clínic com subclínic, en comparació amb pacients amb al·loreactivitat T donant-específica detectable pre-trasplantament sota el mateix tipus de tractament immunosupressor convencional en triple teràpia. No obstant, aquells pacients sense al·loreactivitat cel·lular T ni serològica preformada que reberen tractament amb tacrolimus monoteràpia presentaren una incidència significativament major de rebuig de l'empelt, i especialment aquells amb una elevada incompatibilitat HLA d'eplets a nivell dels antígens DQ.

Conclusions: Un estudi més fi i ampli de les respostes immunològiques de memòria tant serològic com cel·lular T donant-específica, junt amb l'avaluació de la incompatibilitat HLA a nivell molecular, podrien permetre l'estratificació del risc immunològic de cada receptor enfront el respectiu donant de forma més precisa i conseqüentment possibilitar adaptar el tipus de tractament immunosupressor d'una forma personalitzada.

DOCTORAL THESIS SUMMARY (English)

Title: Tracking preformed serological and T-cell alloimmune memory together with donor/recipient Molecular Human Leukocyte Antigen (HLA) disparity to improve immune-risk stratification in Kidney Transplantation"

Introduction: The presence of a donor-specific alloimmune response negatively impacts allograft outcomes, being associated to higher risk of rejection and graft loss. Alloimmunity can be both preformed at time of transplantation (*memory*) or can develop *de novo* after transplant.
Therefore, the stratification of the immunological risk of kidney transplant recipients should take into account both those kinds of responses to avoid the development of allograft rejection.
The immunoassays currently used in clinical practice have several limitations and do not allow a complete and precise evaluation of those two responses at time of transplantation.

Hypothesis: The hypothesis of this doctoral thesis is that at the time of kidney transplantation, an accurate characterization of pretransplant anti-donor alloimmune sensitization using highly sensitive immune assays tracking both serological memory and circulating donor-reactive memory T cells together with the assessment of the individual susceptibility to *de novo* alloimmune activation assessing the degree of donor/recipient HLA matching at the molecular level, would improve current immune-risk stratification and ultimately guide transplant physicians individualizing immunosuppressive therapies.

Objectives:

- To compare the accuracy of different immune assays evaluating the presence of preformed serological immunity (circulating donor(HLA)-specific antibodies), either individually or in combination and their value predicting distinct kidney graft outcomes.
- To investigate the development and kinetics of primary T-cell alloreactivity after kidney transplantation by IFN-γ T cells using an Enzyme-link ImmunoSpot (ELISPOT) assay and evaluate their predominant antigen presenting T-cell priming pathways.
- To analyze the impact of donor/recipient HLA molecular mismatching on the generation of *de novo* donor-specific alloimmunity both at humoral and T-cell level after transplantation using distinct bioinformatic algorithms.

- To evaluate the value of assessing preformed donor-reactive IFN-γ-producing T cells and donor/recipient Molecular HLA mismatching to identify kidney transplant recipients at low risk of developing allograft rejection when receiving reduced immunosuppression based on tacrolimuns monotherapy.

Methods: to support this doctoral thesis we have performed two retrospective clinical studies and one prospective multicenter biomarker-guided clinical studies on kidney transplant recipients (CELLIMIN). The predictive capacity of different assays to detect pretransplant donor-specific antibodies (DSA) has been evaluated: flow cytometry crossmatch, solid phase assays and complement activating (C3d) capacity of DSA in vitro. Furthermore, the presence of alloreactive T cells *in vitro* has been assessed by Interferon-γ ELISPOT both before and after transplantation.
Donor/recipient HLA incompatibility has been evaluated with different informatic algorithms assessing aminoacidic composition and physical characteristics of the HLA molecules at three levels: amino-acids, eplets and peptides indirectly presented by recipient class II HLA molecules (Amino acid mismatch score, HLA-Matchmaker eplet mismatches and PIRCHE-II scores, respectively). It has been assessed the impact of the results of those algorithms on the prediction on primary alloimmunity both at the serological and T-cell level.
Last, in a prospective non-inferiority study guided by biomarkers assessing both pretransplant serological and T-cell alloimmunity we randomized low-risk patients to receive either immunosuppression based on tacrolimus monotherapy or standard of care (steroids, Mycophenolate mofetil and tacrolimus).

Main results: the most accurate serological assays to predict transplant outcomes (rejection and graft loss) were a combination of DSA detected by solid phase assay and flow cytometry crossmatch. DSA with high mean fluorescence intensity (MFI) and those fixing complement *in vitro* predict higher rejection risk with high sensitivity and specificity.
All the informatic HLA molecular mismatch algorithms precisely predicted risk of humoral primary alloimmunity. Similarly, a higher molecular incompatibility (especially by PIRCHE-II score) predicted risk of *de novo* T-cell activation after transplantation. Finally, in the CELLIMIN trial, we observed that patients without preformed alloreactivity (neither serological or T cell-mediated) displayed significantly lower risk of acute rejection both clinical and subclinical, as compared to patients with preformed cellular alloreactivity and receiving the same standard of care immunosuppression.

However, patients without serological/ T cell preformed alloreactivity receiving minimized immunosuppression with tacrolimus monotherapy showed significantly higher incidence of acute rejection especially those patients with high molecular HLA mismatch at the DQ level.

Conclusions: A complete and accurate study of the donor-specific preformed immune responses both at the serological and T-cell level, together with the assessment of the molecular HLA incompatibility, could improve stratification of the alloimmune risk in a more precise way, finally allowing adapted individualization of immunosuppression.

I. **INTRODUCTION**

End stage kidney disease is an increasingly prevalent chronic disease, whose incidence has been growing in the last decades.[1]

Kidney transplantation is the best therapeutic option for all patients with end-stage kidney diseases, as though as compared to any chronic dialysis replacement therapy, it provides significantly higher long-term survival expectancy, better quality of life and is much more cost-effective.[2, 3] . However, transplantation of a solid organ between two non-genetically identical individuals of the same species, is inevitably challenged by the development of an antigen-specific alloimmune response against non-self molecules expressed in the allograft, which will drive the rejection of the graft.[4] To control this *physiologic* immune response, transplant recipients must receive long-lasting chronic immunosuppression, which entails a number of related short and long-term side effects such as increase susceptibility to opportunistic infections, development of malignancies or a direct nephrotoxic damage, thus ultimately challenging the longevity of both patients and transplanted allografts.[5,6]

In the last decades a plethora of diverse immunosuppressive agents have been developed, targeting at distinct molecular levels of the alloimmune response, leading to a significant improvement of both short-term patient and graft survival. However, the type and burden of immunosuppression as well as the different combinations are indicated based on a one-fits-all paradigm rather than based on a personalized manner according to the patient individual immune risk. This suboptimal approach is fundamentally due to a still rather poor understanding of the main determinants and mechanisms of the alloimmune response favouring allograft rejection. Thus, a better evaluation of the main effector mechanisms of rejection as well as its precise monitoring with the implementation of novel and highly sensitive immune technologies are important unmet needs in the field of transplant medicine to ultimately help improving long-term transplant and patient outcomes.

In this thesis, we aimed to address some of these important questions. First, we assessed the value and impact of a number of currently available immune assays, with different readouts, all of them assessing the presence of pretransplant serological alloimmunity, both individually or in combination, in order to establish the most accurate immune test combinations to rule out the risk of a preformed humoral anti-donor immune memory challenging graft rejection and survival. Secondly, current clinical pretransplant immune-risk stratification is exclusively focused on anti-donor serological immunity, thus we aimed to further explore the impact of the other main effector

pathway of adaptive immunity triggering allograft rejection, which is driven by donor-reactive memory/effector T cells. Here, we sought to investigate not only the impact of preformed anti-donor T-cell immunity on kidney graft outcomes but most importantly, we assessed main clinical and molecular determinants tracking main donor/recipient disparities at a molecular level using bioinformatic algorithms, ultimately driving primary or *de novo* anti-donor T-cell immune activation after kidney transplantation. Lastly, and in the context of a prospective, multicenter, randomized biomarker-guided trial we investigated the value of stratifying kidney transplant candidates without anti-donor serological immune memory and no preformed anti-donor T-cell alloimmunity to safely receive a guided minimization immunosuppressive regimen based on tacrolimus monotherapy as compared to current standard-of-care based on a triple drug immunosuppressive regimen.

A. BIOLOGY OF ADAPTIVE ALLOIMMUNITY

1. The human leukocyte antigen (HLA) complex

The main antigens recognized on the endothelium of the allograft by the recipient's immune system are known to be part of the Major Histocompatibility Complex (MHC), known in humans as the human leukocyte antigen (HLA) complex. [7]

The genes encoding for HLA proteins are located in the short arm of chromosome 6. The function of the Major Histocompatibility Complex is to bind pathogen derived peptidic residues and present them on the cell surface so that those peptides can be recognized by T cells leading to a specific adaptive response. [8, 9] Therefore, the necessity of efficiently binding as many as possible different microbial antigens has led to the existence of a high number of possible variants of each HLA gene in the human species. In fact, the MHC genes are known to be the most polymorphic of any mammalian genome and the last 2021-01 IMGT/HLA Database identifies 29417 officially recognized alleles.[10]

HLA proteins are classified according to the type of cells on which they are expressed. Class I molecules are expressed on virtually all human nucleated cells. They are constituted of non-polymorphic protein, β2-microglobulin (whose coding gene is not on chromosome 6 but on chromosome 15) non-covalently linked to a polymorphic α heavy chain of 45-kd. [11] The α chain of the 3 class I proteins A, B and C are encoded by the HLA-A, B or C loci on chromosome 6, respectively. So-called "non classical" HLA class I antigens are HLA-E, F, G and H whose function are less known and involved in negative natural killer (NK) cells regulation.

The function of class I HLA molecules is to present to CD8+ T cells the peptides derived from intracellular infections (mainly viral) after endocytosis and processing, or tumor antigens.[12]

This class of proteins also interact with killer-cell immunoglobulin-like receptors (KIR) expressed on NK cells mediating mainly inhibitory signals. The expression of class I HLA proteins on cell surface is increased by interferon (IFN) α, β and y.[13]

Class I HLA proteins can present peptides of 8-11 aa length that are accommodated in the extracellular peptide-binding cleft constituted by the α1 and α2 segments of the α chain. This portion of the HLA molecules are therefore the polymorphic parts of the molecules, while the rest of the protein is conserved across the species for all class I and provide anchoring to the cell membrane and the site of interaction with CD8 (the α3 immunoglobulin-like domain).

Conversely, class II HLA molecules are not universally expressed, they are only constitutively found on some cell categories with professional antigen presenting features: B lymphocytes, monocytes, macrophages, Langerhans cells, dendritic cells, activated T lymphocytes, endothelial and epithelial cells. Importantly, especially in the case of endothelial cells of renal microvasculature , the expression of class II molecules is increased by IFN-γ.[14,15]

Class II molecules have a heterodimeric structure. They are formed by two polypeptidic chains: α and β. The main isotypes of class II HLA proteins are DR, DQ and DP and the loci encoding for the different chains are named A or B for α and β chain respectively. For the DR molecule, only one non-variable α chain exists that can be associated to different β chains.[16]

Similarly to class I molecules, the most polymorphic part is located in the extracellular peptide-binding groove. Due to their open structure, they can present bigger peptides as compared to class I molecules, usually 12 to 24 amino acids but even longer in some cases.

MHC class II molecules bind peptides derived from extracellular proteins (e.g., bacterial) that are internalized in specialized antigen presenting cells (APC), processed and displayed the peptides to CD4+ T cells.

Importantly, HLA genes are inherited as a haplotype (set of genes expressed on one chromosome) and are covalently expressed on human cells (up to 2 alleles for each locus). Crossing over between the two parental chromosome is infrequent, therefore usually each individual inherits two intact haplotypes, one from each of the two parents. Consequently, due to this low recombination, some combinations of alleles are more frequent than others in a population. *Linkage disequilibrium* is the phenomenon of inheritance of adjacent HLA loci more frequently than it would be expected by chance and it is supposed that it is due to possible effect of positive selection of the haplotype.

At a population level, frequencies of different haplotypes have been studied and collected so that for determined HLA alleles, the probability of other alleles at different loci can be estimated with higher or lower probability. [17]

The nomenclature of HLA molecules is different according to the technology used to perform the HLA determination or typing.[18]

Serologic typing consists in testing cytotoxic effect on the patient's lymphocytes when incubated with sera with antibodies of known HLA specificities, complement and vital dye. [19]

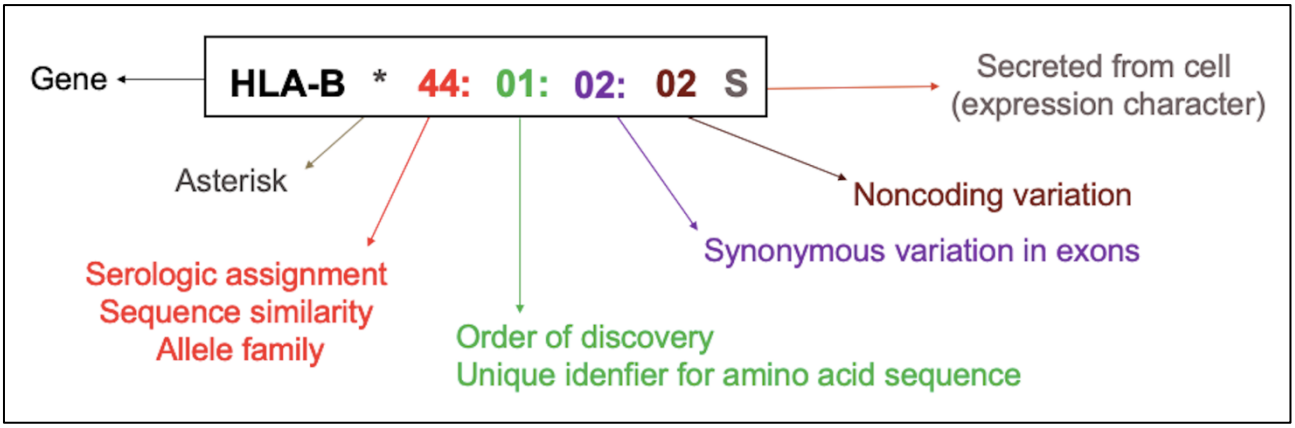
More recently, HLA typing is performed by molecular methods (sequence specific primer PCR: SSP, sequence specific oligonucleotide probes: SSOP, or direct DNA sequencing) that allow a more precise characterization at the amino-acid level. [20] At the allelic level the number of known HLA antigens internal to each serogroup is significantly higher. Both Sanger methodology and direct DNA sequencing permits allelic level typing at high resolution beyond the highly variable recognition site or domain (ARD). [21] Direct DNA sequencing additionally provides information on non-coding gene regions. [22]

HLA types were numbered first according to their serologic group (A1, A2, etc). Subsequent refinement of serologic method led to identification of more antigens, previously thought to represent single allotypes, but which were serologically and genetically unique. Some antigens were therefore "split" into 2 components. Both the components are now considered part of the same cross-reactive group "CREG". CREGs comprehend public epitopes, common to all the members of one CREG, and private epitopes that define the individual serologically defined antigens.

With molecular typing a second nomenclature is added, to distinguish multiple alleles of each serologically defined HLA antigen. After the name of the gene (A, B, DQβ1, …) and an asterisk, the first 2 digits (first field) describe the serologic group or allelic family, and then the next 2 digits (second field) represent a unique allele differing by at least 1 amino acid difference.

By direct DNA sequencing two more fields (4 fields HR typing) can be defined and added to the nomenclature describing finally the complete nucleotide sequence of the HLA allele (coding and noncoding regions). Third field describes synonymous variations in exons while the last 4th field differences in non-coding region of the gene.[23]Last, an expression variant can be added as letters defining expression of the molecule, e.g. N: null (non-expressed), L: low expression and S: secreted.

Figure 1: HLA DNA-based nomenclature system for HLA alleles using HLA-B*44:02:01:02S as an example



Even if the HLA molecules are known to be the most important and most studied antigens in allotransplantation, other non-self structures can be expressed on donor cells in every non genetically identical transplantation and generate an adaptive immune response in the host. All those other proteins are named *minor histocompatibility antigens*. Their relevance is best established in the generation of graft versus host disease in the case of HLA identical stem cell transplantation, however they seem to play a role also in solid organ (kidney) transplantation rejection. Recent advances in genome-wide sequencing technique have amplified the capacity of identifying possible non-syngenic single nucleotide polymorphism (SNPs) coding for non-self molecular structures expressed by donor cells. In addition, it has also been described the sensitizing impact of transplanting an organ with normal expression of a specific allele into a recipient homozygous for a deletion polymorphism and therefore not expressing the allele. [24]

2. Alloantigen presentation pathways

Adaptive response of recipient T cells against donor mismatched HLA antigens, named "alloreactivity", can develop through different pathways of antigen presentation. [25], [26]

In the *direct pathway*, CD4+ or CD8+ T cells recognize intact class I or II antigens expressed on donor antigen presenting cells (APC) transplanted together with the graft that migrate from the allograft to host secondary lymphoid tissue. The actual localization of donor APCs into secondary lymphoid organs of the recipient has been described at least in the case of vascularized allografts such as cardiac transplantation.[27] The existence of this phenomenon has further been proved in basic research studies that showed how in animal models the absence of passenger donor dendritic cells relatively protected from rejection of the graft, and how the infusion of donor's APC leads to prompt rejection supposedly because of direct presentation of donor antigens in secondary lymphoid organs of the recipient. [28]

The existence of T cells directly reacting against entirely non self MHC+peptides is not straightforward, when taking into consideration the physiology of thymic selection of self MHC restricted T cells. However, not only directly primed alloreactive T cells exist but there is a relatively high percentage (1 to 10%) of T cells that can recognize a single MHC alloantigen.

One possible explanation for this phenomenon is that potentially alloreactive T cells are not negatively selected because no foreign MHCs are present in the thymus and if these alloreactive cells show sufficient affinity for both self and allogenic MHC they are not negatively selected. The

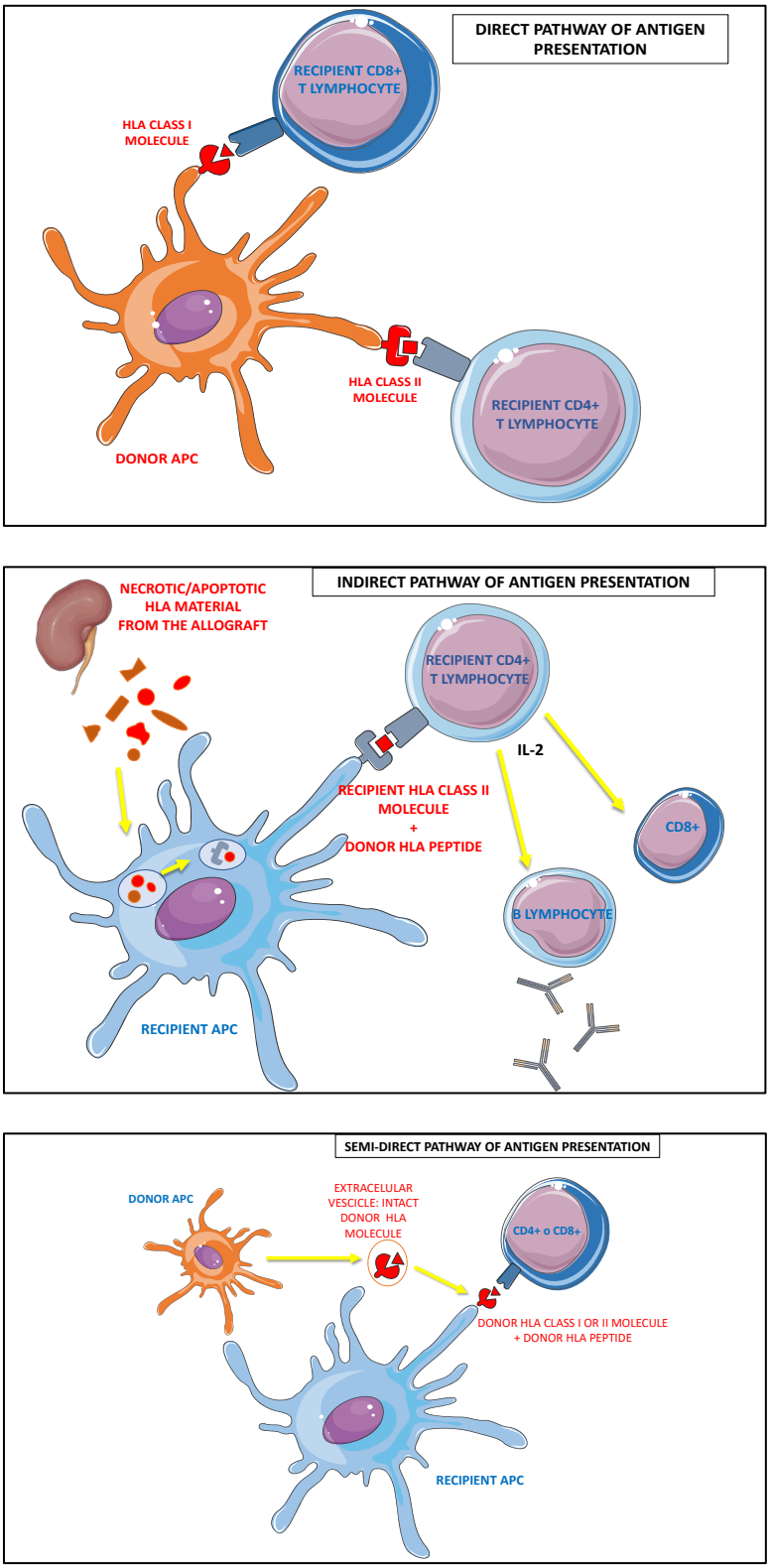
antigen recognized by the recipient T cell can be either the peptide+MHC complex or just the allogenic MHC resulting in a structure similar enough to a self MHC+peptide as to activate T cells. [29]
The first theory proposed to explain the high number of potentially directly primed alloreactive T cells relies on the hypothesis that the same allogenic MHC is the target recognized, being this an antigen present at a high density on donor cells' surface combined with different allogenic peptides. On the contrary, only a lower number of MHC on an APC usually presents peptides of a classic microbial infection. This is known as the *high determinant density model.*[29,30]
The second hypothesis is that different cross-reactive T cells recognize multiple different bound peptides (both allogenic and self), in combination with one allogenic MHC gene product, amplifying the T cells possibly activated by a single MHC (*multiple binary complex model*).
As far as pretransplant responses are concerned, the most likely explanation for the memory T cells reacting against allogenic peptides, is that they have been previously primed and cross-reacting because of similarity between the allogenic MHC and other foreign peptides (especially viral).[31, 32]
This phenomenon, called *heterologous immunity* is due both to molecular mimicry and to the high degree of T-cell receptor cross-reactivity, essential for the proper recognition of a large spectrum of potential peptide epitopes in pathogens.
Classically, the direct pathway of allorecognition has been considered of main importance during the early phases of transplantation when circulating donor APC are still present, and of minor importance thereafter.

The second pathway is the *indirect* pathway and postulates that donor derived peptides shed by the organ can be internalized by recipient APCs, processed, and presented as any other foreign peptide on their class II MHC. This pathway therefore activates recipient CD4+ T cells.
To prove the existence of this pathway, it was first shown that sensitization against donor peptides can lead to rejection of a graft also in absence of passenger donor dendritic cells (absence of direct presentation). [33] Also, classic studies proved that in absence of recipient dendritic cells, survival of a solid allograft is significantly increased. [34]
More recently, attempts to detect *in vitro* indirectly primed alloreactive CD4+T cells have been investigated using donor-derived peptides. The presence of indirectly-primed alloreactive T cells was associated to clinically relevant events such as development of chronic rejection or inferior graft function.[35, 36]

Due to the stable presence of recipient T cells possibly presenting graft derived peptides, the indirect pathway is considered of main importance during all the lifespan of allotransplantation.

More recently, a third possible antigen presenting pathway has been identified and named as *semi-direct*. In this pathway, intact donor MHC are transferred and presented on the surface of recipient APCs together with self MHC (*cross dressing*). Even if earlier studies postulated a role of cell-to-cell contact, the transfer of alloantigen seems to happen mainly though transfer of extracellular vesicles. The capacity of dendritic cells to acquire allogenic MHC has been studied more recently thanks to the availability of new technologies of imaging flow cytometry. It has been demonstrated to be a reproducible phenomenon *in vivo* in animal and in *in vitro* models and with proved capability of activating T cells. [37, 38, 39, 40, 41] The T-cell clones activated by intact donor antigens will be the same that can be activated by direct pathway, with the semi-direct pathway playing a major role in the direct activation also most likely in the long term after transplantation.

Figure 2: T-cell Antigen presenting pathways



Adapted from the book: Hernando Nefrología Clinica, 5th edition, Panamericana

3. Effector T and B-cell immune responses

In physiologic adaptive immunity, T and B-cell responses act complementary to eradicate intracellular and extracellular infections. Both these harms of the adaptive immunity can mediate allograft rejection in the setting of allotransplantation.

3.1. T-cell effector immune responses

T-cell activation is dependent on antigen presentation by APCs. Early after kidney transplantation, activation and maturation of APCs is favoured by the proinflammatory milieu of the immediate post-transplant period when innate immunity is activated by ischemia-reperfusion injury, cell death, release of damage-associated molecular patterns (DAMPs) and complement cascade activation similarly to what happens after other inflammatory events such as graft infections. [42, 43] Activation of APCs results in the expression and upregulation of costimulatory molecules (CD80, CD86) and proinflammatory cytokines production (interleukin (IL)-1, IL-12, tumor necrosis factor-α, chemokine C-C motif ligand 9). Mature APCs (especially dendritic cells) then migrate to the T-cell zone of secondary lymphoid organs where naïve T lymphocytes that had not previously encountered antigens circulate.

When the naïve T cell encounters the APC presenting the peptidic antigen for which it is specific, biochemical signals lead to stop of the T cell and the process of T cell activation starts.

Other types of cells can present antigens to differentiated T cells, such as macrophages or B lymphocytes, but dendritic cells are the main cells responsible for priming of naïve T cells.[44]

The process of T-cell activation requires two more steps after antigen recognition: costimulation and signals mediated by cytokines. Therefore, both these steps have been identified as possible targets of immunosuppressive drugs used in solid-organ transplantation.

The second signal, costimulation, is provided by the interaction of surface receptors on the APC (eg CD80, CD86, CD40, ICOS-L) binding to specific ligands on the T cell (CD28, CD40L, ICOS). It has been observed *in vitro* and *in vivo* that the absence of the costimulatory signal leads to unresponsiveness to the antigen presented by the APC. [45]

CD28 expression is constitutive on naive CD4+ and CD8+ T cells, while the expression of B7 molecules (CD80, CD86) on APCs is increased by inflammatory stimuli mediated by activation of innate immunity, while it is very low in resting APCs limiting spontaneous activation in absence of a

foreign stimuli. Dendritic cells show the higher expression of B7 molecules as well as a high expression of MHC-peptide complexes, being therefore the most efficient APCs for naïve T cells.[46]

After antigen presentation and costimulation, T cells undergo activation and express surface markers involved in the amplification of the cytokinic cascade (third signal) and T-cell mobilization. The best characterized cytokine mediating T-cell activation is IL-2, mainly produced by CD4+T cells after activation. The expression of the complete form of the IL-2 receptor (with α chain CD25) with higher affinity is also increased on activated T cells in a positive loop. IL-2 acts as an autocrine growth factor for survival and proliferation of T cells (clonal expansion). [47] The activation of T cells results in differentiation into an effector or memory phenotype.

By secreting different cytokines, CD4+ T effector cells trigger the activation of other immune cells, mainly macrophages, leukocytes or B lymphocytes. Also, these effector cells participate in the amplification of inflammation (delayed type hypersensitivity).
Different subsets of CD4+ effector cells have been identified, according to the cytokines produced and therefore different functions: type 1 helper (Th1) secreting IFN-y and activating macrophage lineage; type 2 helper (Th2) producing IL-4; IL-5 and IL-13 activating eosinophils and mast cells; and Th17 characterized by secretion of IL-17 and TNF- α targeting neutrophils. Last, follicular T helper cells (TfH) produce IL-21, IFN-y and are involved in the process of antigen-specific B-cell activation.
Effector CD8+ T cells can directly damage and kill target cells (in physiologic responses, infected cells or expressing neoplastic antigens) but also release proinflammatory cytokines and subsequent macrophage activation.
Their lifespan is limited and after mediating their effect, most CD4 and CD8+ effector T cells undergo regulated apoptosis, with a small percentage differentiates into a memory phenotype.

As far as alloreactive T cells are concerned, both the naïve and memory subsets of CD4+ and CD8+ T cells contribute to the responses possibly detected at the time of transplantation.[48]
Once primed and activated, alloreactive effector T cells are attracted to the endothelium of the allograft by chemokines secreted by endothelial, tubular, and interstitial cells. Due to the expression of adhesion molecules on endothelial cells and integrins on activated T cells, CD8+ activated T cells can cross endothelium and tubular basement membrane and penetrate into the intersitium. T cells

primed by a specific antigen recognized at the allograft site particularly express adhesion molecules and can migrate to the extravascular space.

The histological visualization of this process is represented by interstitial infiltration and tubulitis, or even endothelialitis, characteristics of T cell mediated rejection in kidney allograft biopsy.[49]

3.2. T cell alloimmune memory

Memory T cells are generated in conjunction with effector cells after T-cell activation by APCs charged with specific antigens. Usually, memory T cells recirculate in the periphery and in secondary lymphoid organs where a possible encounter with the primary antigen is possible. Those cells can persist for years independently of the presence of the stimulating antigen but with the capacity to react promptly to antigen re-challenge. After priming, the number of memory T cells specific for one antigen is significantly higher than the original naïve pool and these cells can rapidly differentiate into effector cells with lower activation thresholds. Also, these memory T cells are less dependent on costimulatory signals and can be efficiently activated by non-professional APCs [50] and have a wider mobility into peripheral tissues as compared to naïve T cells. Surface markers for the memory T-cell phenotype include IL-7 receptor, CD27 and CD45RO (being CD45RA-, as opposed to naïve T cells).[51]

Beyond those general characteristics, memory T cells can be further classified in various subsets.

Central memory T cells are mainly located in lymph nodes, with increased proliferative capacity (markers: $CD62L^{hi}$ and CCR7+). On the other side, effector memory T cells have direct cytotoxic activity and product inflammatory cytokines such as IFN-y ($CD62L^{Lo}$, CCR7-).

Expression of CCR7 and CD62L of central memory T cells facilitates their homing into secondary lymphoid organs, while T effector memory migrate from blood to peripheral tissues because of an increased expression of integrins and specific chemokine receptors.

Another subtype are tissue resident memory T cells. These cells specialized, non-circulating and non-lymphoid and their main function is antigen surveillance for a rapid response at barrier sites such as skin, lung, intestine, and genitourinary tract. Their surface markers define this characteristic of being non-circulating: drivers of tissue retention such as CD69 and CD103 are highly expressed while those molecules driving return into the circulation such as S1PR1 and CCR7 are reduced. In solid organ transplantation both recipient and donor derived tissue resident T cells exist.[52] Importantly, all those memory T cells residing outside of the circulation (both in lymphoid and non-lymphoid organs)

are less sensitive to depletion by polyclonal antibodies (most used being rabbit anti-thymocyte globulins rATG).[53]

As opposed to all the other subtypes, Terminally differentiated effector memory T cells express $CD45RA^{Hi}$ and $CD45RO^{Lo}$. These cells have been described to have a senescent phenotype: they have low IL-2 requirement, are highly cytotoxic (mainly CD8+) with high IFN-γ production but low proliferative capacity and increased sensitivity to apoptosis. Typically, these cells constitute a large proportion of the T cells during aging and have been associated to a protective effect when predominant pretransplantation[54] but also an inferior graft function and higher risk of neoplasia in the long term. [55] Importantly, CD27 and CD28 are under-expressed in this population, making it especially resistant to costimulatory blockade with Belatacept.

Last, memory follicular T helper cells (CXCR5+) reside in secondary lymphoid organs in B cell follicles and are involved in B cell responses 'generation.

An important body of evidence supports the fact that memory T cells are involved in the development of allograft rejection, being able to migrate fast to the allograft tissues before donor specific priming. On the one hand, CD4+ memory T cells can acquire effector upon reactivation and provide help for CD8 effector T cells activation.[56] On the other, also CD8+ memory T cells have been shown to be directly involved in allograft rejection.[57] These cells can early infiltrate the graft stimulated by the contact with donor endothelium and upregulate ICOS expression and IFN-γ secretion.[58] Importantly, activation of memory CD8+ alloreactive T cells can be triggered by the proinflammatory milieu emerging during ischaemia-reperfusion after transplantation, lowering the threshold for activation of these cells with allogenic specificity.[59]

Memory T-cell immune responses are highly resistant to costimulation blockade but seem to be better controlled by calcineurin-inhibitors, especially tacrolimus.[60]

Also, the use of T-cell depleting agents such as rATG or alemtuzumab do not deplete memory T cells as effectively as naïve T cells, leading to a predominance of the memory subset during homeostatic repopulation.[61]

3.3. B-cell effector immune responses

Effector B cell responses rely on the production of circulating antibodies by plasma cells that differentiate from activated B cells.[62]

Activation of antigen-specific B cells takes place in secondary lymphoid organs where, according to the type of antigen, the cells are activated in T-dependent (protein antigens) or T-independent (microbial antigens with repeated characteristics or polysaccharides) manner.

Since HLA molecules, the main alloantigens in transplantation are of protein nature, their presentation is T-dependent. Protein antigens (as complete HLA molecules) are contemporarily recognized directly through the B cell receptor (BCR) of naïve B cells in the B-cell zone of secondary lymphoid organs and in the form of peptide+class II MHC complex by naïve T cells in the T zone that differentiate into T helper cells.

After BCR recognition, B cells can also act as antigen presenting cells and present the peptides derived from the same antigen to T cells, after internalization and processing.

Stimulated by the expression of CD40 ligand and interaction with CD40 on B cells and cytokines production by activated T Helper cells, B cells undergo activation and migrate to the follicles. There, the germinal center reaction takes place, and through the processes of proliferation and somatic hypermutation, mature B cells finally differentiate into one of the two kinds of cells constituting the B-cell response to an antigen: memory B cells and long-lived plasma cells (and their precursors, plasmablasts).

Short-lived plasma cells are located in secondary lymphoid organs and are important in early responses after B-cell activation by protein antigens. Long lived plasma cells are mainly located in the bone marrow and can secrete antibodies after more than 10 years after first antigen encounter.

Antibodies produced by plasma cells (in bone marrow or secondary lymphoid organs) circulate in the bloodstream, mediating their effect independently from the cells that produced them. Antibodies have several effector functions, distinct according to the immunoglobulin (Ig) subtype.

The main effector mechanisms mediating antibody-mediated graft injury are complement activation and cytotoxicity mediated by natural killer (NK) cells and monocyte-macrophage activation.[63,64]

NK cells express Fc (constant domain) receptor (FCGRIIIA) and are activated by Fc only when the IgG is interacting with target cells (in the case of allograft rejection, endothelial cells expressing specific HLA or non-HLA antigens). NK activation leads to secretion of proinflammatory cytokines such as IFN-y and TNF. Moreover, those cells mediate direct antibody-dependent cell mediated toxicity (ADCC) through degranulation (granulysin, perforin, and granzymes A and B).

Activation of monocyte-macrophage opsonization and phagocytosis is both directly mediated by their interaction with the Fc portion of IgG antibodies and also because of secretion of granulocyte–macrophage colony-stimulating factor (CSF2) by KN cells amplifying monocyte activation and cytotoxicity.

The other important effector mechanism of humoral immunity is the activation of the complement cascade by the classical pathway. Classical complement pathway is initiated by C1 binding to Ch domains of IgG or IgM that have bound the antigens (not in the soluble form) and finally mediates the formation of the membrane attack complex (MAC) and cell lysis of target cells. Apart for direct cell damage, the activation of the complement cascade also produces anaphylatoxins (C5a, C4a, C3a) peptides with proinflammatory effect such as mast cells activation and increased vascular permeability through P-selectin expression on endothelial cells. Additionally, the complement system has been shown to act as positive enhancer of T-cell immunity.[65,66]

3.4. B cell alloimmune memory

Similarly, memory B-cell responses are generated after antigen recognition both out of the germinal center (germinal center independent memory B cells) or after the germinal center process, and are characterized by the capacity of rapidly developing a humoral response in case of antigen rechallenge. [67,68]

Memory B cells (mBc) can belong to different subtypes with different functions. Their common surface marker is CD27.[69]

Both germinal center dependent and independent mBc have high capacity of rapidly differentiating into plasmablasts if stimulated by specific antigen encounter, moreover most of the antibodies produced during the secondary or memory response are IgG with higher affinity as compared with the primary response.

IgM mBc are less prone to differentiate into plasmablasts, and more likely re-enter germinal center reaction. Also, according to the expression of CD80 and PD-L2 markers, cells are distinguished in a doble negative phenotype generating GC reactions or double positive that generate a fast and large isotype-switched secondary antibody response. [70]

Some mBc recirculate in blood and secondary lymphoid organs in a quiescent state until antigen encounter, while others reside in the bone marrow where they have been generated.

B. Impact of anti HLA humoral sensitization in Human kidney transplantation

As commented above, an adaptive response defined by the presence of anti-HLA antibodies, can develop against non-self HLA antigens because of the exposition to those molecules during pregnancy,[71] blood component transfusions [72] and solid-organ transplantation.[73]

When comparing those routes of sensitization, it seems that a previous organ transplantation has the strongest immunizing effect as compared to the other events.[74],[75]

Since anti-HLA circulating antibodies can occasionally be detected also in patients that have never undergone any of those events, it had been proposed that anti-HLA antibodies can be generated because of cross-reactivity with other antigens such as viral infections or vaccination.[76] However, no clear evidence has been generated supporting this theory [77] and one possibility to explain the temporal association between infections and anti HLA appearance is that previously anti-HLA primed B cells are triggered for bystander activation during those events. Conversely, as discussed above, the antigen recognized by the TCR is a combination of self HLA+ exogenous peptides and not the entire protein as it is for the BCR. Therefore, there is stronger evidence of the possibility of cross reactivity with viral or other antigens and human HLA molecules [76, 78,79].

1. Monitoring Serological memory in Human kidney transplantation

In current clinical practice, the presence of humoral donor-specific responses is fundamentally assessed by detection of circulating donor(HLA)-specific antibodies (DSA) in recipient serum that can be produced by either bone marrow long-lived plasma cells or short-lived plasma cells generated after B-cell activation. The presence of DSA in recipients' serum at time of transplantation is detected by tests confronting recipient serum with donor cells or donor HLA antigens (crossmatch tests)[80,81]

A common limitation of the approach used in clinical practice, only assessing serological memory, relies in the dynamic nature of the presence of alloantibodies in the bloodstream. In fact, in absence of detectable serum DSA, it cannot be completely ruled out the presence of antigen specific memory B cells, which may potentially differentiate into antibody-producing plasma cells after re-challenge with the same antigen.[81, 82] While an important body of research is focusing on the functional detection of donor(HLA)-specific memory B cells[82], an indirect approach widely used to partially overcome this limitation is to test not only actual serum but also historic sera (in particular after

every sensitizing events such as graft loss, immunosuppression withdrawal, pregnancies or blood transfusions).

Table 1: serological cross-match tests

	ASSAY	Ref.	Brief description	STRENGHTS	LIMITATIONS
Cell Based	**Complement dependent Cytotoxicity crossmatch (CDC-XM)**	1	45 minutes incubation of recipient's serum with donor's T and B lymphocytes with rabbit complement. Visual assessment of % of donor lymphocytes cytotoxicity (vital stain incorporation).	• Very high positive predictive value for hyper-acute ABMR • Detects anti-HLA and non-HLA antibodies expressed on donor cells	• Low sensitivity for Non-complement binding (low level) antibodies • Possible false positive results because of auto-antibodies • DTT necessary to distinguish IgM/IgG • Subject to interobserver variability • Subject to donor cells 'quality and number
	Flow Cytometry crossmatch (FC-XM)	2, 3	30 min incubation of recipient's serum with donor lymphocytes. Staining with: -fluorochrome-conjugated anti-IgG antibodies -fluorochrome-conjugated anti-CD3 and anti-CD19 antibodies for T and B cells, respectively. Detection of coexpression by flow cytometry.	• Higher sensitivity than CDC-XM for low-level (not-complement binding DSA) • High positive predictive value for acute rejection especially in combination with DSA by SPA • Detects anti-HLA and non-HLA antibodies expressed on donor cells	• Possible false positive results due to treatment with anti-T/B-cell antibody • Possible false positive results because of auto-antibodies • Need for inter-laboratory standardization • Subject to donor cells 'quality and number
Solid-phase assays (SPA)	**ELISA & Bead-based assays on Luminex**	4	3-6 hours incubation of recipient serum with purified HLA antigens presented on a solid-phase platform (microtitre plate or microparticle beads). Addition of fluorescent–conjugated anti-human IgG Analysis on a flow cytometer or Luminex platform.	•Higher sensitivity and specificity than CDC-XM and FC-XM • Possible to perform a virtual XM without need for donor cells	• Interpretation requires expertise • Necessary complete (possibly high-resolution) donor HLA typing • Only detects anti-HLA antibodies • Possible false negative results due to prozone effect or shared antigens • Lack of definition of clinically relevant antibody • Possible variations between laboratories and platforms/kits • Possible false positive results for interaction with denatured beads
	Complement binding assay	5	Incubation of recipient serum with complement factors. Second labelled antibody directed against components of the complement cascade (C1q, C4d or C3d).		

Abbreviations: SPA: Solid-phase assays; CDC-XM: Complement dependent Cytotoxicity crossmatch; FC-XM: Flow Cytometry crossmatch; ABMR: antibody-mediated rejection; DSA: donor-specific antibody; DTT: dithiothreitol

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2. Bray, doi.org/10.1007/978-1-62703-493-7_14
3. Couzi et al, doi.org/10.1097/TP.0b013e31820794bb
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1.1. Complement dependent cytotoxicity crossmatch (CDC-XM)

The first crossmatch assay was described in 1969 by Patel and Terasaki that demonstrated how patients undergoing transplantation with a positive test developed hyperacute rejection in 24/30 patients with 3 more suffering of graft loss at 3 months.[83]

The CDC-XM consists in incubating recipient's serum with donor's T and B lymphocytes (representing both HLA class I and II antigens) together with rabbit complement. The presence of

DSA is detected by the induction of donor lymphocyte' lysis in presence of antibodies activating complement cascade.

The standard technique results positive in presence of IgG and IgM antibodies. IgM can be differentiated from IgG antibodies using dithiothreitol (DTT) reducing the disulfide bonds in the IgM pentamer and consequently turning negative a positive crossmatch due to only IgM antibodies. In fact, in clinical practice it has been observed that IgM antibodies do not confer a significant risk and therefore should not preclude transplantation.[84]

CDC-XM technique in its original form has several limitations causing both false negative and false positive results. First, it detects only complement-fixing antibodies. An implementation of the technique is the augmentation with antihuman globulin where an anti-kappa light chain or the Amos technique removing anti-complement factors, both approaches increasing the sensibility of the test.[85,86] Other technical limitation are the inter-observer variability of the results and the dependency of the sensitivity of every test on the viability of target cells and on the batch of rabbit complement used. Also, autoantibodies in patient's serum can lead to false positive results, possibly verified by the performance of an auto-crossmatch with recipient's own cells.[87] It must be underlined that CDC-XM can detects antibodies reacting both against donor HLA and non-HLA antigens.

As described before, a positive CDC-XM has a high positive predictive value for the risk of hyperacute rejection[88,89], whose incidence has virtually disappeared because of routinary CDC-XM in most transplant centres. When addressing the prediction of longer-term immunological risk beyond hyperacute rejection the CDC-XM lacks sensitivity/negative predictive value (NPV) because it allows the detection of only complement fixing antibodies/high antibody density.

1.2. Flow cytometry crossmatch (FC-XM)

The FC-XM was described in early eighties.[90]

Similarly to CDC-XM, recipient serum is incubated with donor lymphocytes and then stained with both anti-IgG and fluorochrome-conjugated antibodies directed to specific T and B lymphocytes surface markers (CD3 and CD19, respectively) and analysed by flow cytometry.[91]

A semi-quantitative result can be expressed as number of channel shift of mean fluorescence above baseline or against standardized molecules (mean channel shift: MCS) although calibration between different laboratories should be assessed.

The main advantage of FC-XM as compared to CDC-XM is a higher sensibility in detecting weak IgG HLA-specific antibodies and not complement-activating antibodies.[92]

Performing a FC-XM requires a considerable amount of recipient serum and donor cells (1 million viable lymphocytes), needs inter-laboratory standardization and can be affected by previous use of anti-T and anti-B monoclonal/polyclonal antibodies.

A positive FC-XM is not always associated to inferior outcomes. FC-XM has high sensitivity but low specificity in predicting early graft dysfunction caused by antibody-mediated rejection, but it is more sensitive than CDC-XM in detecting long term dysfunction and later rejection episodes. It seems particularly important for risk stratification of anti-HLA-sensitized patients, while it is less clear the importance of an isolated positive FC-XM in non-sensitized recipients.[93,94,95] The predictive value of FC-XM is increased by the combination with solid phase assay that help identifying possible non-specific results, in fact a positive FC-XM in absence of anti-HLA antibodies does not predict rejection.

1.3. Solid-phase assays: ELISA and LUMINEX bead technology

Solid phase assays (SPA) are characterized by the substitution of donor lymphocytes' target with purified HLA molecules that could be fixed on a solid phase (plaque or beads).

The presence of antibodies binding to a specific HLA target molecule is detected by the use of enzyme or fluoresceine conjugated anti-human IgG by colorimetric detection (ELISA) or fluorescent signal (flow cytometry/ Luminex) following excitation by a laser.[96] The fluorescent signal of the anti-human IgG antibody is measured as mean florescence intensity (MFI).

Calculated PRA (cPRA) is the % of antibodies specificities determined on SPA locally and therefore expresses the risk of a transplant candidate to have DSAs against possible donors in the local population.

Solid phase assays allow a sensitive screening of the presence of anti HLA antibodies, using a pool of beads coated with a panel of either class I or II purified antigens. In addition, it is possible to obtain the determination of the precise antigen specificity by the use of single antigen beads (SAB) where every bead carries one unique HLA antigen at higher concentration and therefore further increases the sensitivity.

Using solid phase antibody screening in combination with SAB it is possible to perform a *Virtual Crossmatch* against the HLA typing of a possible donor without the need of donor samples.

Solid phase kits are licensed for qualitative use only, but the semi-quantitative readout (MFI) has been used to indicate antibody load in serum and therefore to stratify immunological risk and monitoring of DSA levels in diagnosis and response to treatment of ABMR. The use of MFI to define antibodies' pathogenicity or strength is debated.[97],[98]

The advantage of these assays is that they do not depend on donor cells' availability, together with the increased sensitivity as compared to the other techniques and the precise identification of antibodies' specificity (anti HLA IgG antibodies only).

Nevertheless, some limitations must be considered.

A lack of consensus exists on the level to define clinically relevant DSA detected by SPA, and both complement binding and not complement binding antibodies are detected. Moreover, possible false-positive results can arise from antibody interaction with denaturized HLA antigens.[99, 100] The addition of mild acetic acid that demonstrates the presence of denaturated antigens or the combined use of a different solid phase and/or cell-based assay have been proposed to overcome this limitation.

False negative results can be due to the missed detection of non-HLA antigens or because of public epitopes that result in low total positivity.[101] The *prozone effect* is another cause of false negative results: the presence of inhibitory factors in serum, causing a false negative or falsely reduced MFI levels. Experimental studied demonstrated that it might be due to C1 complex formation that starts classical complement activation preventing secondary anti IgG antibody binding, especially in presence of high concentration of specific antibodies. It has been demonstrated that pre-treatment of serum with ethylenediaminetetraacetic acid (EDTA) overcomes the prozone effect because it acts dissociating C1 but it must be reminded that EDTA may also remove some antibodies specificities.

Last, it is fundamental to verify if the donor's typing (ideally with high resolution) is fully represented on the used platform before ruling out the presence of DSAs.

The elevated negative predictive value of SPA has led to the proposal of using virtual crossmatch to replace the cytotoxic test with the rationale of reducing cold ischemia time with its known negative impact on graft results.[102],[103]

If using SPA in this context, the limitation on specificity of the assay must be reminded, because the presence of low-titer antibodies or allele specific antibodies could unnecessarily exclude possible donors.

In retrospective studies of cohorts not undergoing any desensitization treatment to remove anti-HLA antibodies, it was observed a higher incidence of acute rejection[104,105] and inferior graft survival [106],[107] in presence of DSA with both negative CDC-XM and FC-XM.

In contrast, other studies including patients undergoing some scheme of desensitization therapy [108],[109] reported an increased risk of graft loss in presence of a positive FC-XM but not in presence of isolated DSA assessed by SPA.

1.4. Complement-binding solid phase assays

An evolution of SPA are the *complement-binding solid phase assays* in which serum is incubated simultaneously with complement factors and the second labelled antibody is directed against components of the complement cascade. Different tests have been developed according to the secondary antibody's target (C1q, C4d or C3d).

By assessing *in vitro* the capacity of circulating antibodies to activate complement, the aim is to identify the antibodies that might activate complement *in vivo* and may produce acute/chronic complement mediated damage of the graft.[66]

However, differently from the positivity of a CDC-XM, complement-binding of solid phase antibodies *in vitro* may only represent a higher title of antibodies and not an intrinsic characteristic of the DSA. A relation between complement-binding capacity and MFI has in fact been described in literature[110],[111] although not universally confirmed.[97,112] Furthermore, the addition of complement can overcome the prozone effect as shown by studies where after dilution or addition of EDTA the relation between MFI and C1q binding capacity was restored.[113]

Only a few studies assessed the impact of pretransplant DSA with complement binding capacity on graft outcomes, producing conflicting results.[114,115, 116, 117,118] In a recent metanalysis of 37 studies, a significant impact on graft outcomes was observed for complement-binding DSA, either pretransplant or *de novo* and independently of the test used.[119]

C. Monitoring T cell alloimmunity in Human kidney transplantation

1. Immune assays tracking donor-reactive T cell responses

While for the humoral immune memory a variety of serological tests are available to clinicians to assess the presence of circulating DSA, no assay is currently implemented in clinical practice to track the presence of anti-donor T cell memory.

Noteworthy, in the research field, several research groups have been addressing this topic and a number of immune-assays have been developed.[120,121]

The assays tracking alloreactive T-cells in vitro have been designed to assess T-cell proliferation, intracellular production of ATP on activated CD4+ T-cells, cytotoxicity and/or cytokine production as well as cell phenotyping after allogeneic stimulation, mainly through the direct pathway of antigen presentation. More recently, molecular approaches using high-throughput T-cell receptor B chain CDR3 region sequencing have been also shown to accurately track donor- alloreactive T-cell proliferating after mixed lymphocyte reaction.[122,123,124]

Main limitations to the development of valuables *in vitro* assays are: i) the timing of assessment that influences the type of donor-antigen presenting pathways to detect, namely direct, indirect or semidirect; ii) the fact that assessing naïve or memory T-cell responses specific with donor antigens or a panel of alloantigens may describe different biological features of the alloimmune response, and, iii) peripheral blood assessment of T-cell alloreactivity may not fully illustrate the global T-cell alloimmune response that originated within secondary lymphoid organs.

Table 2: assays to detect donor specific alloreactive T cells in vitro

ASSAY	Ref.	Brief description	STRENGHTS	LIMITATIONS
Mixed lymphocyte reaction (MLR)	1	6 days coculture of lymphocyte-depleted donor PBMCs with recipient's PBMCs. T cell proliferation evaluated by incorporation of 3H thymidine or BrdU or CFSE dye-dilution assays	• Low cost • Relatively easy technique	• Need of donor cells • Long incubation times • Low reproducibility • Debated clinical relevance
Donor-Specific Interferon- γ T cell ELISPOT	2, 3	20-24 hours incubation of recipient PBMCs with T-cell depleted donor splenocytes or PBMCs in a 96 well plate pre-coated with capture antibody against IFN-γ (or Granzyme B)	• Reproducibility validated in multicentric studies • Standardized technology and automatized readout • Reported association with post-transplant rejection risk	• Need of donor cells • 24 hours delay for results (limited use for deceased donor pre-transplant assessment) • Need of technical skill • Detection limited to circulating T cell pool
Activation induced markers (AIM) assay	4	24-36 hours incubation of recipient PBMCs with T-cell depleted donor splenocytes or PBMCs. Assessment by flow cytometry of AIM. Possible markers: CD40L (CD154), CD69, CD137, CD30	• Detects possibly alloreactive T cells independently of their functionality • Relatively easy technique	• Need of donor cells • Detection limited to circulating T cell pool • Lack of association with clinically relevant outcomes
T-cell receptor sequencing	5	High throughout TCR sequencing at the CDR3 region of the TCR ß chain	• Directly identification of donor-reactive T cells	• Need of donor cells • 3-4 days to obtain results • Need of technical skill and technology • High cost • Lack of association with clinically relevant outcomes

Abbreviations: MLR: Mixed lymphocyte reaction; ELISPOT: Enzyme-linked ImmunoSpot; IFN: interferon; PBMC: peripheral blood mononuclear cells; AIM: Activation induced markers; CFSE: carboxyfluorescein diacetate succinimidyl ester, BrdU: 5- bromo-2'-deoxyuridine; TCR: T-cell receptor;

Brief references:

1. Ghobrial et al. doi.org/ 10.1007/BF00346036
2. Montero et al. doi.org/10.1097/TXD.0000000000000886
3. Heeger et al. J Immunol 163:2267–2275
4. Dan, doi.org/10.4049/jimmunol.1600318
5. DeWolf, doi.org/10.1172/jci.insight.121256

Immune assays can be classified in a) non-antigen-specific characterizing the degree of activation capacity of T-cells after polyclonal stimulation, or b) T-cell immune responses against defined antigens. Non-antigen-specific assays provide information regarding the global immunosuppressive state of the T-cell compartment, assessing the likelihood to develop opportunistic infections or malignancies.[125,126,127] In retrospective studies, antigen specific tests, described below, were associated to the risk of allograft rejection or allograft acceptance (hypo-responsiveness, as defined by absence of anti-donor responses but preserved anti-third-party T-cell immunity).

1.1 Mixed lymphocyte reaction (MLR)

In MLR, T-cells from the recipient are faced for several hours or days to donor APCs (by inactivating donor T lymphocytes) and direct allogeneic reactivity is quantified by different methods.

The original approaches quantified recipient T cells 'proliferation by incorporation of 3H thymidine or other molecules as BrdU (5- bromo-2'-deoxyuridine) as a result of cellular division detected by an ELISA assay, or CFSE dilution, quantified by flow cytometry.

Limitation of the technique are the necessity of donor cells, long incubation times (6 days) and low reproducibility.

The presence of alloreactive T cells as assessed by MLR has been retrospectively associated to risk of allograft rejection.[128,129,130,131]

Due to the limitations listed before, MLR tests have been lately used for specific mechanistic studies more than to be implemented in clinical practice.[48,132]

1.2 Interferon- γ Enzyme-linked ImmunoSpot (ELISPOT) Assay

This assay investigates *in vitro* the frequencies of antigen-specific cytokine-producing T-cells after stimulation. Recipient peripheral blood mononuclear cells (PBMCs) are cultured in the presence

of T-cell depleted donor splenocytes or PBMCs as stimulators in a 96 well plate pre-coated with capture antibody against preferentially IFN-γ or Granzyme B.

With the aim of investigating allogeneic memory/effector T-cell frequencies, cytokine-producing T-cells are quantified after a short time of *in vitro* co-culture (18- 24 h). Hence, each spot represents the cytokine released by a single cell that had previously been primed *in vivo* against the specific antigen. Readout can be processed by computerized readers increasing reproducibility. The main advantage of these assays is the capacity of detecting single-cell responses for particularly low frequencies (less than 1/10000 cells).

The presence of memory T cells detected by IFN- γ ELISPOT has been associated to the risk of posttransplant rejection and inferior graft function by various research groups.[133,134,135,136,137]

The impact of the memory T cells identified at time of transplantation is affected by the use of T-cell depletion[137, 138], possibly because of the specific inhibition of memory T-cells repopulation by the immunosuppressive maintenance treatment.[61]

The methodology of this assay has been validated in multi center consortia, as interlaboratory comparisons and reproducibility are crucial for its potential clinical application[139,140]; no differences were observed between fresh or frozen samples and a high correlation between different laboratories was described (coefficient of variance < 30%)

Those good reproducibility results have allowed its use in the context of non-randomized, multicenter, pilot studies.[141],[142]

Similarly to the cPRA, the degree of T-cell alloreactivity against a panel of target cells expressing distinct HLA molecules can be estimated by the panel of reactive T cells" (PRT) using the IFN- γ ELISPOT assay overcoming the need for donor cells.[143]

Peptides and proteins may also be used to stimulate recipient's cells emulating the indirect pathway of antigen presentation.[35, 36,144,]

1.3 Flow Cytometry analysis of activation-induced markers (AIM)

Another method is the assessment by flow cytometry of activation-induced markers (AIM) after allogeneic stimulation, with the aim of identifying other alloreactive T cells besides those secreting IFN-γ. Candidate markers of early activation are CD40L (CD154), CD69, CD137, CD30 (or soluble CD30 in serum).[145,146,147,148, 149, 150, 151]

1.4 T-cell receptor sequencing

Finally, it has been recently implemented the technology to perform high throughout T-cell receptor (TCR) sequencing at the CDR3 region of the TCR ß chain, allowing the precise definition of the nucleotide sequencing of every T-cell clone proliferating in the MLR and possibly mediating alloreactivity.[122-124]

D. Primary (*de novo*) donor-specific Immune responses in Human Kidney transplantation

While TCMR after kidney transplantation still unpredictably occurs due to the absence of any accurate assay measuring anti-donor T-cell alloimmunity in clinical practice, whether these events are due to preformed or *de novo* T-cell alloimmune priming needs to be investigated. In fact, there is no information regarding the occurrence, kinetics, and potential impact of *de novo* T-cell alloimmunity, besides the direct occurrence of (clinical and subclinical) T-cell mediated rejection.

Conversely, primary alloimmunity can emerge after transplantation against donor antigens in naïve patients.

Similarly to the pretransplant scenario, the appearance of humoral alloresponses in clinical practice has classically been defined by a *de novo* serological presence of circulating DSA (dnDSA). The incidence of dnDSA has been estimated to be around 10-20% up to 10 years after transplantation in the modern era of immunosuppression.[152, 153]

Importantly, several clinical studies have associated the presence of dnDSA to poorer graft outcomes such as ABMR and inferior graft survival. [152, 107, 154]

In the posttransplant setting, it is also debated the relevance of complement binding capacity of the circulating DSAs as predictors of inferior graft outcomes, since absence of complement binding capacity do not rule out pathogenicity of dnDSA.[119, 155]

The analysis of the IgG subclasses of *de novo* DSAs is another appealing way to stratify DSA and ABMR severity according to their potential to activate classic complement pathway or Fc γ receptors of innate immune effectors. IgG1 and IgG3 subtypes were found to be the most represented in patients developing ABMR, while IgG2 and IgG4 are less frequent.[156] It is an expected finding since IgG1 and IgG3 are involved in responses to protein antigens and are the best activators of complement and NK cells mediated toxicity.[157] However, current assays do not meet the criteria for scientifically sound interpretation, and until it is not clearly defined the relevance to the pathologic function *in vivo* of the antibodies detected, all those *in vitro* features cannot be implemented the clinical setting.[158]

As compared to preformed DSA, those appearing *de novo* seem to have a stronger negative impact on graft outcomes.[159] In this line, some evidence suggested that preformed DSA persisting after transplantations are more harmful than those that disappear under chronic immunosuppression: in

the absence of antibody-depleting therapy, pretransplant DSA display stable or decreasing MFI values in the first 2 weeks post-transplant in 75% of patients, they disappear spontaneously in 50% at 3 months and up to 65% at 12 months possibly because of immunosuppression. In contrast, preformed DSA that increase their MFI after transplantation are associated to ABMR risk. Attention has focused on the DSA characteristics predicting persistence, as it might be the class (class II associates to higher risk), the locus (DQ), and higher MFI or a previous kidney transplant as sensitizing event.[160,161,162,163]

1. Monitoring the risk of Primary alloimmune activation

At a clinical level, the main risk factors associated to the advent of primary alloimmune activation possibly leading to immune-mediated graft damage are related with indirect evidence based on the degree of immunosuppression blood exposure together with the immunogenicity of the allograft.

In naïve patients, the decision of minimize immunosuppression by reducing calcineurin inhibitors (CNI) through levels or CNI sparing regimens has been associated to an increased risk of primary alloimmune activation.[164, 165, 166] Therefore, an accurate approach to identify transplant recipients that can safely receive minimized regimens and beneficiate of reduced toxicity is needed.

Similarly, lack of adherence to the prescribed immunosuppressive treatments, when detected, can also augment the risk of *de novo* alloimmunity.[167,168, 169]

1.1. Assessing donor/recipient HLA mismatching at the antigen level

However, while immunosuppressive exposure is key to abrogate the activation of the alloimmune response, the specific immune susceptibility of each individual against the respective donor will be also influenced by the presence and amount of recognizable antigens.

As commented above, the great heterogenicity of the HLA system results in a high possible rate of differences between donor and recipients in non-syngenic allotransplantation and therefore the HLA molecules are the main target of the alloimmune response (consequentially named HLA *antigens*).

The degree of HLA incompatibility between donor and recipient is classically defined by enumeration of the HLA molecules expressed by the donor that are not expressed by the recipient *(*number of *mismatches).* This number varies according to the number of loci that are typed. Until recently, the serologic or allelic typing used for solid organ (kidney) allocation evaluated the loci A, B and DR(β1) and therefore could be in a range from 0 to 6 mismatches.

Large retrospective cohorts have clearly shown that the higher the number of HLA mismatches the worse the graft outcomes, including incidence of allograft rejection and premature graft loss, being the difference especially evident when comparing patients receiving a *full match*ed transplantation, a donor-recipient couple with no HLA mismatches, as compared with all the other donor-recipient combinations.[170,171,172,173]

Between class I and II HLA antigens, it has been shown that class II incompatibility, historically assessed at DR locus, has the strongest impact on graft outcomes.[174] It is not fully understood why class II antigens appear to best correlate with graft immunogenicity and outcomes. One hypothesis is that that one mismatched DR*β*1 molecule possibly corresponds for linkage disequilibrium with more mismatched DRβ3-4-5 chains, and DP and DQ molecules (with 2 polymorphic chains) having a *gene dose effect*. This concept is confirmed and expanded by analyses of the aminoacidic composition of the HLA molecules (discussed below) showing that especially DQ and DRB3-4-5 molecules can be very different across non matched subjects and more easily induce an alloresponse.[175]

Therefore, in most of current allocation systems, donor-recipient HLA compatibility is considered especially at the DR locus, trying to limit the number of HLA-DR mismatched molecules. Moreover, due to the lower number of possible DR antigens as compared to A or B, there are higher possibilities of finding a compatible donor with 0 DR mismatches.

With the availability of more complete HLA typing, similar observations on graft outcomes have been reproduced when assessing HLA compatibility also at the DQ locus.[176,177,178]

As far as DP molecules are concerned, their importance in matching is less clear because of a lower and more variable endothelial expression. It seems important especially in case of preformed donor-specific antibodies at this locus.[179]

Importantly, the degree of HLA mismatch also impacts the level of sensitization after graft failure, significantly limiting regraft opportunities.[180]

1.2. Assessment of donor/recipient HLA mismatching at the molecular level

In the last decades, the concept of HLA incompatibility has evolved, trying to better dissect the importance of the molecular differences between donor and recipient HLAs and not only listing the number of non-matched molecules.[181]

Combining the available biological information on the aminoacidic composition of the HLA molecules with modern informatic technologies, different research groups have developed algorithms evaluating the HLA incompatibility at a molecular level producing easy to use outputs for research and clinical use. The outcomes that have been associated with the degree of donor-recipient HLA molecular incompatibility are mainly related to the risk of development of primary humoral alloimmunity (anti-HLA sensitization after sensitizing events such as pregnancy, kidney transplant failure and transfusions, development of *de novo* DSA, development of antibody-mediated rejection).

The first approach proposed in the Cambridge HLA Immunogenicity algorithm is the quantification of amino acid sequence polymorphisms that differ between the donor and recipient HLA molecules, particularly focusing on their extracellular components. Differences are considered both intralocus and interlocus. The most recent version of this algorithm also takes into account the physical characteristics (hydrophobicity and electrostatic charge) of the two molecules, finally determining likelihood of a paratope-epitope interaction and antibody affinity.[182,183,184,185]
Lately, another algorithm for amino acid mismatch assessment stratified according to solvent-accessibility of the mismatched donor HLA, has been released.[186]
Amino acidic and electrostatic mismatches have been associated in retrospective cohorts to the risk of anti-HLA sensitization after graft loss or pregnancy, as well as risk of dnDSA development after kidney transplantation.[182-185]

Different algorithms have been developed starting from the concept that immunogenicity of a mismatched HLA molecule is due to short aminoacidic sequences expressed in the polymorphic clef of the HLA, that constitute the epitope possibly interacting with Ig complementarity-determining regions (CDRs) constituting the paratope and therefore truly immunogenic.

Additionally, the presence of those specific non-self epitopes can justify the presence of antibodies reacting against different HLA molecules sharing the same epitope, an evolution of the older "CREG" concept.

The first definition of those epitopes was the model of the Terasaki epitopes (TerEps), defined by analyzing the antibody reactivity patterns from serum with known specificities to determine the shared amino acid sequences among the positive single antigens beads.[187]

Afterwards the HLAMatchmaker algorithm has been released. The basic immunogenic units in this case were first named *triplets*, linear sequences of three amino acid residues.[188] More recently the elementary components of the HLAMatchmaker algorithm have been named *eplets,* polymorphic amino acids conformation in a 3-Å radius in antibody-accessible position that are not necessarily continuous in the primary structure of the HLA protein but physically associated in the tertiary one.[189]

Eplets are further classified in "antibody-verified" or not, depending on the performance of an *in vitro* test of antibody reactivity.[190, 191]

In the last years, the research group from Manitoba has proposed a more detailed evaluation of eplet mismatches by calculating the eplets present in each single donor mismatched molecule and not at each locus as a sum. The detailed single molecule DRβ1345 and DQ mismatches are being included by the FDA as a recognized prognostic biomarker (CDER Biomarker Qualification Program) predicting *de novo* DSA generation at the mismatched molecule and other adverse graft outcomes.[192,193]

A higher risk of dnDSA and ABMR was observed in retrospective cohorts in presence of worse eplet (or triplet) matching[194, 195, 185, 196, 197, 193] especially in the context of immunosuppression minimization or non-adherence [165,166, 198,199,200] Moreover, the degree of eplet compatibility has also been associated to allograft survival.[168, 199, 201]

Finally, even though eplet mismatch scores have been developed to estimate the risk of HLA molecule-antibody interaction, some reports show that also the risk of T-cell mediated rejection is higher in presence of a worse molecularly matched donor, especially at class II loci.[193, 199, 202]

Last, the predicted indirectly recognizable HLA epitopes (PIRCHE-II) algorithm calculates the number of donor HLA derived peptides that are likely to be presented by class II HLA molecules of the recipient (indirect pathway of antigen presentation). The peptides are considered as binders to the presenting HLA according to the IC50 values calculated by the NetMHCIIpan algorithm. First versions

of the algorithm only evaluated presentation by DRβ1 molecule, while newer releases also consider the presentation by DQ and DP molecules.[203]

In retrospective studies, PIRCHE-II score predicted risk of sensitization after pregnancy[204], and dnDSA ater kidney transplantation, especially in recipients of a first kidney allograft, possibly reducing the confounder of preformed immune memory.[205,196, 206] Recently, even more impactful outcomes such as allograft survival were shown to be influenced by PIRCHE-II level.[207]

Only a few studies compared between them the different molecular mismatch scores. Even if differences exist between the algorithms, largely overlapping results have been reported between the best-known mismatch scores as predictors of *de novo* DSA [208,209,210,211] and possibly, the combination of algorithms assessing different characteristics of the HLA molecules might increase the sensitivity.[209]

Different publications mentioned earlier proposed and applied different cut-offs of molecular incompatibility to classify donor-recipients' pairs risk, however the fact that even one discrepant amino acid can evocate an immune response and the ongoing continuous update of the algorithms used, still restricts a wide clinical application of any of those cut-offs.[158]

While retrospective evidence is consistent of the association between those algorithms and graft outcomes, only one study by now assessed the effect of HLA molecular mismatches to prospectively allocate organs in a prospective cohort of paediatric kidney transplant recipients.[212] Also, the class I epitope analysis by HLAMatchmaker is included among other parameters in the *Acceptable mismatch program* within the Eurotrasplant organization.[213] In a recent simulation using the Canadian registry, eplet matching especially at class II was demonstrated to be theoretically feasible in waiting lists including a number of 250 patients. Interestingly, good class II eplet matching would be easier to achieve than allele matching, being eplets more uniformly distributed in a population then alleles.[214]

An open issue in this research field is the estimation of the degree of immunogenicity of the different epitopes possibly mismatched between donor and recipients. In fact, while now all those algorithms simply enumerate the mismatched molecular units, probably not all have the same immunogenic potential.[215,216]

II. HYPOTHESIS

The hypothesis of this doctoral thesis is that at the time of kidney transplantation, an accurate characterization of pretransplant anti-donor alloimmune sensitization using highly sensitive immune assays tracking both humoral serological memory and circulating donor-reactive memory T cells together with the assessment of the individual susceptibility to *de novo* alloimmune activation using novel bioinformatic algorithms assessing the degree of donor/recipient HLA matching at the molecular level, would improve current immune-risk stratification and ultimately guide transplant physicians individualizing immunosuppressive therapies.

III. OBJECTIVES

The main objectives of this thesis were:

- To compare the accuracy of different currently available immune assays evaluating the presence of preformed serological immunity by means of circulating donor(HLA)-specific antibodies, either individually or in combination and their value predicting distinct kidney graft outcomes.

- To investigate the development and kinetics of primary T-cell alloreactivity after kidney transplantation by means of *de novo* circulating donor-reactive IFN-γ-producing T cells over a 2-years period of time using a highly sensitive Enzyme-link ImmunoSpot (ELISPOT) assay and evaluate their predominant antigen presenting T-cell priming pathways.

- To analyze the impact of donor/recipient HLA molecular mismatching on the generation of *de novo* donor-specific alloimmunity both at humoral and T-cell level after kidney transplantation using distinct bioinformatic algorithms.

- To evaluate the value of assessing preformed donor-reactive IFN-γ-producing T cells and donor/recipient Molecular HLA mismatching to identify kidney transplant recipients at low risk of developing allograft rejection when receiving reduced immunosuppression based on tacrolimuns monotherapy.

IV. MATERIALS, METHODS AND RESULTS

Article 1.

Combining Sensitive Crossmatch Assays With Donor/Recipient Human Leukocyte Antigen Eplet Matching Predicts Living-Donor Kidney Transplant Outcome

Kidney International Reports (2018) 3, 926–938; doi.org/10.1016/j.ekir.2018.03.015

Objective: *to compare the accuracy of different currently available immune assays evaluating the presence of preformed serological immunity by means of circulating donor(HLA)-specific antibodies, either individually or in combination and their value predicting distinct kidney graft outcomes.*

The aim of this study was to assess, in a cohort of 330 consecutive patients receiving a living-donor kidney transplantation from two distinct high-volume transplant centres, the predictive value on graft outcomes of the currently available immunoassays detecting circulating donor(HLA)-specific antibodies at time of transplantation alone or in combination, together with the assessment of donor/recipient HLA mismatch at the molecular (eplet) level.

In 330 living-donor kidney transplant recipients, we retrospectively analysed the results of 4 immunoassays detecting serum donor(HLA)-specific antibodies (complement-dependent cytotoxicity panel-reactive antibody (CDC-PRA), Flow cytometry crossmatch (FC-XM), solid-phase single antigen beads (SAB) assay and their C3d binding capacity (DSA-C3d), as well as the differences between donor/recipient HLA matching at the allele and eplet level and evaluated their association with main clinical outcomes such as graft function, incidence of acute rejection and death-censored graft loss.

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Combining Sensitive Crossmatch Assays With Donor/Recipient Human Leukocyte Antigen Eplet Matching Predicts Living-Donor Kidney Transplant Outcome

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Introduction: Despite the different assays available for immune-risk stratification before living-donor kidney transplantation (LDKT), the precise type and number of tests to perform remain uncertain.

Methods: In a cohort of 330 consecutive LDKT patients, all of which were complement-dependent cytotoxicity (CDC)–crossmatch negative, we retrospectively analyzed the impact on main clinical outcomes of most sensitive immunoassays (complement-dependent cytotoxicity–panel-reactive antibody [CDC-PRA], flow cytometry crossmatch [FC-XM], donor-specific antibodies [DSAs], and their complement-binding capacity DSA-C3d]), together with donor/recipient HLA eplet matching. Mean follow-up was 67 months (range 24–190 months).

Results: Of 330 patients, 35 (11%) showed a CDC-PRA >20%; 17 (5%) FC-XM+; 30 (9%) DSA+, 18(5%) DSA-C3d+, with low overlapping results (10 patients positive in all donor-specific tests). Unlike HLA allele compatibility, the mean number of HLA class II eplet mismatches was higher in LDKT patients with positive baseline test results. DSA-C3d+ showed higher mean fluorescence intensity (MFI) DSA, with a cut-off MFI of 6192 accurately predicting complement fixation (area under the curve = 0.85, $P = 0.008$). Although all assays were associated with acute rejection (AR), only DSA-C3d+ (odds ratio [OR] = 6.64, $P = 0.038$) or high MFI-DSA (OR = 7.54, $P = 0.038$) independently predicted AR. Likewise, poorly HLA class II eplet–matched patients were at higher risk for AR, particularly patients with negative baseline test results (OR = 1.14, $P = 0.019$). Finally, previous AR and FC-XM+/DSA+, regardless of C3d positivity, independently predicted graft loss.

Conclusion: Combining FC-XM and solid-phase assays with the evaluation of donor/recipient HLA eplet mismatches, are most accurate tools for immune-risk stratification prior LDKT.

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More than 35% of patients on a waiting list for kidney transplantation are sensitized against human leukocye antigen (HLA), having longer waiting times for an HLA-compatible transplant than nonsensitized patients.[1,2] However, the sensitization degree before transplantation and the antidonor specificity may differ according to the immunoassay used,[3–6] thus influencing decision making regarding establishing desensitization strategies or even leading to the withdrawal of the transplantation due to an overreaching rejection risk.

Living-donor kidney transplantation (LDKT) is the best therapeutic option for patients with end-stage renal disease.[7,8] A main advantage over

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deceased-donor transplantation is that a thorough evaluation of the immunological risk is more feasible due to the programmed nature of the transplant: on 1 hand, assessing the presence of preformed anti-HLA DSAs and, on the other, defining the degree of HLA−donor/recipient matching. Indeed, a broad array of assays such as complement-dependent cytotoxicity crossmatch (CDC-XM), FC-XM, and solid-phase assays detecting circulating DSA with or without complement-binding capacity (either C1q- or C3d-binding) may be performed prior to transplantation.[9−12] Although all of these assays are informative about alloimmune status, they may also lead to ambiguous results, not always providing overlapping evidence.[13]

In fact, recent reports assessing cohorts of both deceased-donor and LDKT patients showed an increased risk of acute rejection (AR) and graft loss in the presence of pretransplantation-positive FC-XM but not isolated DSA.[6,14] Conversely, other groups have described lower graft survival only with both high MFI DSA and positive FC-XM but not with low DSA levels regardless of a positive FC-XM.[15] Also, inferior graft outcomes in presence of DSA, independently of CDC-XM and FC-XM have also been reported.[5,16] In addition, the refinement of solid-phase assays by measuring the complement-binding capacity of DSAs, with either C1q or C3d, has revealed a greater likelihood and severity of AR and poorer graft survival in these patients.[12,17−19] Furthermore, although better donor/recipient HLA matching at the allele level still accounts for better graft outcomes,[20] the specific analysis of donor/recipient HLA mismatches at the epitope level has recently emerged as a more accurate tool for identifying the degree of donor/recipient HLA compatibility.[21−23]

However, the clinical value of all of these tests and their combination prior to LDKT has not been evaluated yet in the same cohort of kidney transplant patients to determine the most sensitive and specific assays for stratifying the risk of rejection and graft loss. Hence, we aimed at retrospectively investigating, in a large cohort of consecutive LDKT patients from 2 different transplant programs, the predictive value on clinical outcomes of the currently available immunoassays, alone or in combination, together with donor/recipient HLA mismatch evaluation at the eplet level. Herein we show that pretransplantation DSA, with either high-MFI or C3d-binding capacity, are independent risk factors for AR, and that the combination of preformed DSAs, regardless of their complement-binding capacity, with positive FC-XM discriminates patients with poorer graft survival. Also, a low HLA class II eplet mismatch is key to reducing the likelihood of subsequent immune activation, regardless of pretransplantation sensitization status.

METHODS

Study Population

We retrospectively analyzed 330 consecutive adult LDKT patients from 2 transplant centers in Barcelona, Spain (Hospital de Bellvitge and Hospital Clínic), who underwent transplantation from 2000 until 2013. All patients with negative CDC-XM at the time of transplantation surgery and with available pretransplantation serum samples (within 4 weeks before transplantation) were evaluated with 4 immunoassays: complement-dependent cytotoxicity panel-reactive antibody CDC-PRA, FC-XM, solid-phase single antigen beads (SAB) to assess DSA and those with C3d-binding capacity (DSA-C3d). Minimum follow-up was 2 years, with a mean actuarial follow-up of 67 ± 29 months (range 24−190 months). ABO-incompatible and HLA-identical patients, those receiving other transplant organs, and those with early graft loss for surgically related complications were excluded (Figure 1). CDC-PRA and CDC-XM data were available to the clinicians before transplantation in all patients, and DSA and FC-XM in 134 patients who underwent transplantation after 2011. We retrospectively analyzed, on pretransplantation frozen samples, DSA and FC-XM in the 196 remaining patients who underwent transplantation before 2011 and DSA-C3d tests in all patients with positive DSA. All of the tests were carried out at the immunology laboratory at Hospital Clínic. The study was approved by the institutional review boards from both institutions. Informed consent was obtained from all patients.

Outcome Definitions

Cases of AR were all biopsy proven except for 6 cases due to biopsy contraindication, and were all graded following the last Banff classification.[24] Twelve patients showing borderline changes were considered as AR

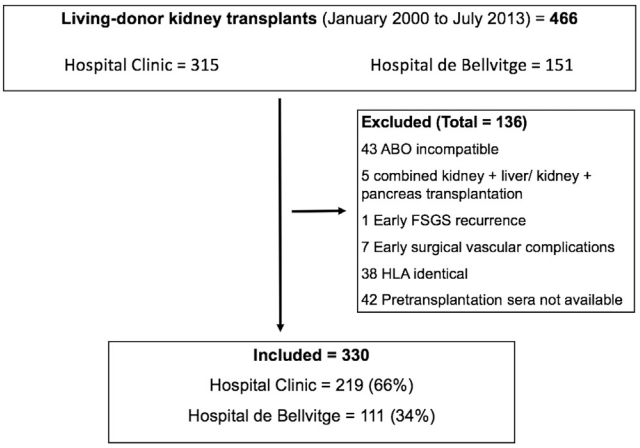

Figure 1. Flowchart of patients in the study cohort. ABO, blood group; FSGS, focal segmental glomerulosclerosis; HLA, human leukocyte antigen.

due to acute graft dysfunction and positive response to antirejection treatment. Graft loss was defined as either return to chronic dialysis or re-transplantation. Renal function was evaluated using the Modification of Diet in Renal Disease (MDRD) equation—estimated glomerular filtration rate (eGFR) at 6, 12, 24, and 60 months after transplantation.

HLA Typing

Recipients' and donors' HLA class I (A, B) and class II (DR, DQ) typing was performed by DNA-based low-resolution typing with sequence-specific primers (SSP) as previously described.[25]

Eplet Mismatch Analysis

The HLAMatchmaker program (Rene Duquesnoy, 2016; University of Pittsburgh Medical Center, Pittsburgh, PA) was used to assess eplet matching (4ABCEpletMatchingVs02protoype.xlsb and DRDQDPEpletMatchingVs02protoype.xlsb from http://www.epitopes.net/downloads.html). Donor and recipient typing (A, B, DR, and DQ) were converted to high resolution using a local frequency table typed by sequence-based typing. Total numbers of incompatible eplets and antibody-proven eplets were calculated.[21] Here, all analyses were performed using the global number of eplet mismatches.

Humoral Alloimmune Risk Characterization

Complement-Dependent Panel-Reactive Antibody

Both CDC-PRA and CDC-XM tests were performed as previously described.[26] CDC-PRA tests were performed using frozen cells obtained from blood bank donors, and CDC-XM was done with fresh donor peripheral blood mononuclear cells (PBMCs). Neither antihuman globulin nor extended incubation was used. To rule out the presence of autoantibodies, all crossmatch tests were carried out with dithiotreitol and without dithiotreitol to discard IgM, evaluating the discrepancy between techniques and by performing an autoXM. The highest CDC-PRA before transplantation was considered for the analysis. CDC-PRA $>$ 20% was defined as positive and $>$80% highly HLA sensitized.

Flow-Cytometry Crossmatch

All donor PBMCs for FC-XM tests were obtained from freshly obtained blood samples. T- and B-cell FC-XM were performed using peripheral blood donor cells.[11] With a scale expressing staining intensity as a linear channel value (0—1024), median channel fluorescence (MCS: median channel fluorescence shift) for anti-human IgG-F(ab)' fluorescein isothiocyanate was quantified on CD3+ T cells and CD19+ B cells. FC-XM was positive when MCS of the sample exceeded the negative control value by 3 SDs. T-cell FC-XM was considered negative when MCS was $\leq$25 and positive when it was $\geq$100. For B-cell FC-XM, an MCS value of $\leq$200 was considered negative and $\geq$300 positive.

Solid-Phase Single-Antigen Assay for DSA Characterization

All patients were tested by SAB assay to detect donor-specific anti-HLA IgG using a single-antigen class I and class II flow beads assay kit (Lifecodes, Division of Immucor, Stanford, CA). All beads showing a normalized MFI of $>$1500 were considered positive if (MFI/MFI lowest bead) was $>$5. The highest MFI value for each DSA was considered for the analysis.

C3d Complement-Binding DSA

In all patients showing DSA+, a C3d test (C3d complement-binding DSA [DSA-C3d]) was performed using a solid-phase assay and following the manufacturer's procedures (Lifecodes; Immucor, Stanford, CA). An MFI threshold of 1500 was considered positive (DSA-C3d+). For a better comparison, DSA-IgG was analyzed using the same lot of beads in both the C3d detection and IgG SAB assay.

Statistical Analysis

All data were expressed as mean $\pm$ SD or as median and interquartile range for continuous variables, and as frequencies for categorical variables.

The number of eplet mismatches was evaluated as a continuous variable. Comparisons between groups were performed using the Pearson χ^2 test for categorical data, and the Fisher test was applied when the number of cases was $<$5. One-way analysis of variance and t tests were used for normally distributed data, and the nonparametric Kruskal–Wallis test and Mann–Whitney U test were used for non—normally distributed data. Receiver operating characteristic (ROC) curve analysis was used to assess the specificity and sensitivity of MFI threshold predicting C3d-binding-capacity of DSA.

Bivariate correlation analyses were performed using the Pearson or Spearman test for nonparametric variables. Both univariate and multivariate logistic regression models were performed to examine the factors associated with AR.

Kaplan–Meier probabilities of graft survival and rejection-free survival were plotted and compared by different immunoassay results using log-rank tests. A Cox regression model was used to estimate hazard ratios for univariate analyses for graft survival and to compare clinical and immunological variables. Analyses of graft loss were censored for patient death. Multicollinearity was assessed using variance inflation factors.

All P values were 2-tailed, and statistical significance was fixed at $P < 0.05$. SPSS version 20.0 software

Table 1. Main demographic, clinical, and immunological characteristics of the studied population

Demographic and clinical variables	n (%)
Recipient age, yr (mean ± SD)	45 ± 14
Donor age, yr (mean ± SD)	52 ± 11
Recipient gender, male	218 (66)
Donor gender, male	112 (34)
Type of donor: child to mother OR husband to wife versus other types of donor	41 (12)
ESRD cause	
Unknown	120 (36)
Glomerulopathy	95 (29)
Polycystic kidney and tubulo-interstitial disease	59(18)
Diabetic and hypertensive nephropathy	32 (10)
Others	24 (7)
Time on dialysis, mo (median, IQR)	8, 0–27
Number of transplants (1/2/≥3)	271 (82) / 38 (12) /21 (6)
Induction treatment: no/rATG /anti-CD25	36 (11) / 114 (34) / 180 (54)
Immunosuppressive therapy	
CNI-based	323 (98)
No CNIs (mTOR i)	7 (2)
Desensitization treatment	23 (7)
Plasmaphreresis + rituximab + i.v. Ig	12 (50)
Plasmapheresis + i.v. Ig	2 (8)
i.v. Ig	8 (34)
Plasmapheresis	1 (4)
Delayed graft function	3 (1)
Acute rejection	65 (19)
TCMR	40 (71)
Banff grades: IA/IB/IIA/IIB/III/BL	7 / 7 / 8 / 5 / 1 / 12
ABMR	19 (29)
Mean eGFR, ml/min (mean ± SD)	
1 yr (n = 308)	71.5 ± 20.2
2 yr (n = 285)	70.3 ± 21.4
5 yr (n = 179)	67 ± 23
1-yr Death-censored graft survival	326 (99)
Actuarial death-censored graft survival	288 (87)
1-yr Patients' survival	324 (98)
Actuarial patients' survival	306 (93)
Immunological variables	
HLA allele mismatches (<3/3/>3)	89 (27) / 99 (30) / 142 (43)
Class I (A, B) (0/1/2/3/4)	5 (2) / 55 (17) / 141 (43) / 71 (22) / 58 (18)
Class II: (DR) (0/1/2)	62 (19) / 175 (53) / 93 (28)
HLA Eplet mismatches (mean ± SD)	
Class I (A, B)	11.4 ± 6
Class II: –DR/–DQ	9.6 ± 9.7 / 8 ± 7.3
CDC-XM+	5 (1)
Peak CDC-PRA >20%	35 (11)
20%–80%	27 (77)
>80%	8 (23)
FC-XM+	17 (5)
FC-XM anti-T+	2 (12)
FC-XM anti-B+	11 (64)
FC-XM+ and T+ and B+	4 (24)
DSA+	30 (9)
Class I+	9 (30)
Class II+	12 (40)
Class I+ and II+	9 (30)
DSA-C3d+	18 (5)
Class I+	2 (11)
Class II+	13 (72)
Class I+ and II+	3 (17)

ABMR, antibody-mediated rejection; BL, borderline changes; CDC-PRA, complement-dependent cytotoxicity panel-reactive antibody; CDC-XM, complement-dependent cytotoxicity crossmatch; CNI, calcineurin inhibitor; DSA, donor-specific antibody (solid phase assay); DSA-C3d, C3d binding donor-specific antibody; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; FC-XM, flow cytometry crossmatch; mTOR i, mammalian target of rapamycin inhibitors; rATG, rabbit anti-thymocyte globulin (Thymoglobulin); TCMR, T-cell–mediated rejection.

(SPSS Inc., Chicago, IL) and GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA) were used for data management and analysis.

RESULTS

Study Population

Main clinical and demographic characteristics of the study cohort are depicted in Table 1. Most patients were Caucasian/white (99%), male (66%), on dialysis therapy (82%), and receiving a first kidney transplant (82%).

Maintenance immunosuppression was based mainly on calcineurin inhibitors (CNIs), mycophenolate mofetil and corticosteroids, and most patients received induction therapy with anti-CD25 monoclonal antibody (basiliximab) or rATG. Desensitization therapies including i.v. Ig (2 g/kg total dose), plasmapheresis (5 sessions), and/or rituximab (475 g/m^2), alone or in combination, were used in a subgroup of patients if there was a positive result in any of the immunoassays. All tests were performed prior to these therapies.

The incidence of AR was 19% (65/330), 29% being antibody-mediated rejection (ABMR) and 71% T-cell–mediated rejection. One-year patient and death-censored graft survival were 98% and 99%. Among 330 LDKT patients, there were 22 graft losses (6.6%): 6 (27%) due to AR (4 = T-cell–mediated rejection, 1 = ABMR, 1 not biopsy proven), 7 (36%) due to chronic ABMR, 5 (23%) due to nonspecific interstitial fibrosis and tubular atrophy, and 2 (9%) due to recurrent glomerulonephritis. In 2 cases, graft biopsy could not be performed to characterize the cause of graft loss. A total of 21 patients (6%) died during the study period. The main causes of death were malignancies in 7 patients (33%), infections in 5 (20%), and cardiovascular events in 3 (12%).

Differences Between Donor/Recipient HLA Allele and Eplet Mismatches

As shown in Table 1, 142 of the 330 LDKT patients (43%) showed >3 total donor/recipient HLA allele mismatches, 99 of 330 (30%) displayed 3, and 89 (27%) showed <3. The mean number of HLA class I (A, B),

DR, and DQ eplet mismatches were 11.4 ± 6, 9.6 ± 9.7, and 8 ± 7.3, respectively. As illustrated in Figure 2, a progressive increase in the number of HLA eplet mismatches was observed as the number of HLA allele mismatches increased ($r = 0.62$, $r = 0.74$, and $r = 0.64$, for A and B, DR, and DQ mismatches, respectively; all $P < 0.001$).

Pretransplantation Immune Assays Identify Different Sensitized LDKT

Five of 330 patients (1%) displayed a positive CDC-XM before desensitization therapy and became negative at the time of transplantation. CDC-PRA was >20% in 35 of 330 patients (11%) and >80% in 8 of 330 patients (2%). A total of 17 patients (5%) showed FC-XM+, 30 of 330 (9%) DSA+, and 18 of 330 (5%) DSA-C3d+. Thirteen patients (4%) showed more than 1 preformed DSA. As shown in Figure 3, 33 of 330 patients (10%) showed a positive result in any of the different donor-specific immunoassays: of these, only 10 of 33 (30%) had a positive result in all tests, 8 of 33 (24%) were FC-XM-/DSA+/DSA-C3d+, 4 of 33 (12%) FC-XM+/DSA+/DSA-C3d−, 8 of 33 (24%) FC-XM-/DSA+/DSA-C3d−, and 3 of 33 (9%) exclusively FC-XM+.

Table 2 shows the main differences between the most relevant clinical and demographic characteristics and the results of the different immunoassays. As depicted, although no disparities were observed regarding donor and recipient age, gender, and cause of end-stage renal disease, patients showing positive results on the different tests more frequently had undergone transplantation previously, were females receiving an allograft from their husband or child, had a longer time on dialysis, and had more frequently received both T-cell−depletion induction and desensitization therapies.

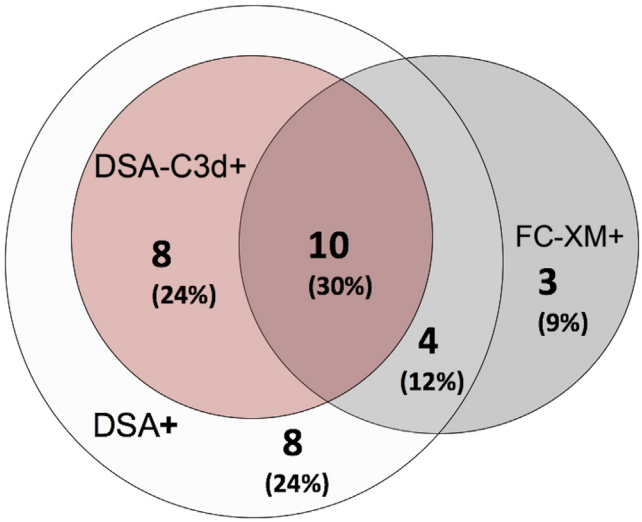

Figure 3. Distribution of patients according to the presence of a positive result of the different immunoassays. In all, 33 of 330 patients (10%) showed a positive result in any of the different donor-specific immunoassays: of these 33, 10 patients (30%) showed a positive result in all tests, 8 (24%) were FC-XM−/DSA+/DSA-C3d+, 4 (12%) were FC-XM+/DSA+/DSA-C3d−, 8 (24%) were FC-XM−/DSA+/DSA-C3d−, and 3 (9%) were exclusively FC-XM+. DSA, donor-specific antibody; DSA-C3d, donor-specific antibody−C3d complement-binding; FC-XM, flow cytometry crossmatch.

Although no differences were observed between the mean number of HLA allele mismatches and results of pretransplantation immunoassays, a significantly higher mean number of HLA class II eplet mismatches was observed among pretransplantation-sensitized LDKT patients with positive test results.

Notably, DSA-C3d+ patients displayed significantly higher mean MFI-DSA than DSA-C3d− patients

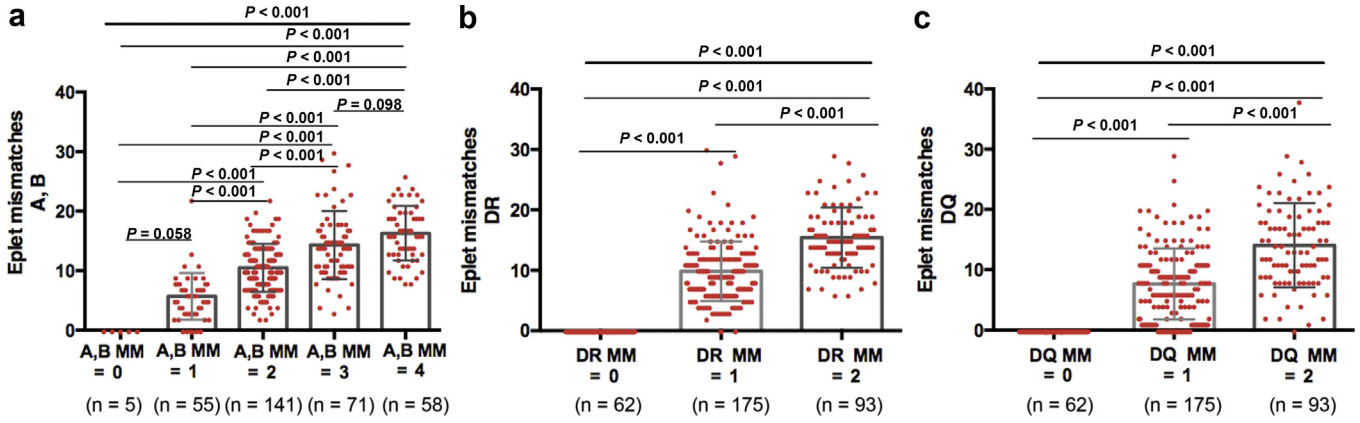

Figure 2. Distribution of donor-recipient human leukocyte antigen (HLA) eplet mismatches (MMs) according to the number of HLA allele MMs. (a) Distribution of donor−recipient HLA eplet MMs according to the number of class I (A, B) HLA allele MMs. (b) Distribution of donor−recipient HLA−eplet MMs according to the number of DR HLA allele MMs. (c) Distribution of donor−recipient HLA eplet MMs according to the number of DQ HLA-allele MMs. Mean ± SD HLA eplet MMs in patients with 0, 1, 2, 3, or 4 HLA allele AB MMs was 0, 5.6 ± 4, 10.5 ± 4, 14.3 ± 6, and 16.3 ± 4.5, respectively. Mean ± SD HLA eplet MMs in patients with 0, 1, or 2 HLA allele DR MMs was 0, 9.8 ± 5, and 15.3 ± 5, respectively. Mean ± SD HLA eplet MMs in patients with 0, 1, or 2 HLA allele DQ MMs was 0, 7.6 ± 6, and 14 ± 7, respectively.

Table 2. Demographical and clinical characteristics according to the results of all immunoassays

Variable	All negative tests (n = 278)	CDC-PRA > 20% (n = 35)	FC-XM+ (n = 17)	DSA+ (n = 30)	DSA-C3d+ (n = 18)	P[a]
Recipient age, yr (mean ± SD)	45 ± 14	44 ± 12	47 ± 15	45 ± 14	48 ± 15	0.45
Donor age (mean ± SD)	52 ± 11	52 ± 12	52 ± 12	52 ± 13	54 ± 14	0.62
Recipient gender: male, n (%)	186 (67)	25 (69)	10 (59)	17 (57)	9 (50)	0.58
Donor gender: male, n (%)	84 (30)[b]	19 (53)[b]	9 (53)	19 (63)[c]	12 (67)[b]	0.006
Donation child to mother/husband to wife versus other types of donors	32 (12)	3 (8)	5 (29)[b]	7 (17)	5 (12)[b]	0.06
ESRD cause, n (%)						0.43
Unknown	108 (39)	8 (23)	2 (12)	7 (23)	5 (28)	
Glomerulopathy	73 (26)	16 (46)	9 (53)	13 (43)	9 (50)	
ADPKD and TID	47 (16)	8 (23)	3(18)	7 (23)	3 (17)	
DN and HTN	29 (10)	2 (6)	1 (6)	1 (3)	0	
Others	21(8)	1 (3)	2 (12)	2 (7)	1 (6)	
Time on dialysis, mo (mean ± SD)	19 ± 43[b]	44 ± 47[b]	36 ± 36	36 ± 35[b]	30 ± 33	0.15
Number of transplants (>1 versus 1), n (%)	38 (14)[c]	22 (61)[c]	10 (59)[c]	19 (63)[c]	13 (89)[c]	<0.001
HLA (allele) mismatches (mean ± SD)						
Class I (A, B)	2.4 ± 1	2.4 ± 0.9	2.3 ± 1	2.4 ± 0.9	2.4 ± 1	0.59
Class II: DR	1.1 ± 0.7	1.2 ± 0.6	1.3 ± 0.6	1.3 ± 0.5	1.3 ± 0.6	0.75
HLA (eplet) mismatches (mean ± SD)						
Class I (A, B)	11 ± 5.9	11 ± 11.4	13 ± 11.2	12 ± 11.3	12 ± 11	0.81
Class II: DR	9 ± 6.6[c]	12 ± 9.2[c]	15 ± 9.2[c]	14 ± 9[c]	14 ± 9.27[b]	0.004
DQ	7 ± 7.2	10 ± 7.8	13 ± 7.76[c]	11 ± 7.7[b]	13 ± 7.75[b]	0.014
Induction treatment: rATG n (%)	82 (29)[c]	22 (61)[c]	15 (88)[c]	24 (80)[c]	16 (89)[c]	<0.001
Desensitization therapy: yes, n (%)	0[c]	15 (42)[c]	13 (76)[c]	17 (57)[c]	12 (67)[c]	<0.001
DGF: yes, n (%)	0[c]	3 (8)[c]	0	0	0	<0.001

ADPKD, autosomal-dominant polycystic disease; CDC-PRA, complement-dependent cytotoxicity panel reactive antibody; DGF, delayed graft function; DN, diabetic nephropathy; DSA, donor-specific antibody (solid phase assay); DSA-C3d, C3d-binding donor-specific antibody; ESRD, end-stage renal disease; FC-XM, flow cytometry crossmatch; HTN, hypertensive nephropathy; rATG, rabbit anti-thymocyte globulin (Thymoglobulin); TID, tubulo-interstitial disease.
[a] P for comparison of the distribution of patients' characteristics according to the results of all baseline immunoassays.
[b] $P < 0.05$ for comparison between patients with positive and negative test results.
[c] $P < 0.001$ for comparison between patients with positive and negative test results.

(Figure 4a), as was the case for both class I and class II DSA (Figure 4b). We subsequently generated a receiver operating characteristic (ROC) curve to identify the most accurate MFI-DSA threshold differentiating DSAC3d-binding capacity. As illustrated in Figure 4d, an MFI cut-off of 6192 best discriminated C3d-binding capacity (area under the curve [AUC] = 0.838, 95% confidence interval [CI] = 0.65−1.00, P = 0.008). Although numerically higher, no statistically significant differences were observed comparing MFI-DSA between FC-XM+ and FC-XM− patients (Figure 4c).

Value of Baseline Immunoassays and HLA-Eplet Mismatching Predicting AR

As shown in Figure 5a to d, patients with any positive assay, either CDC-PRA>20%, FC-XM+, DSA+ or DSA-C3d+, showed significantly higher incidence of AR than those with negative tests. Unlike HLA allele mismatches, mean HLA (DR)-eplet incompatibilities were higher in patients developing AR than those that did not. When analyzing the type of AR, this association was only observed for patients developing ABMR (Supplementary Figure S1).

When assessing the main clinical, demographic, therapeutic, and immunological variables predicting AR in a multivariate logistic regression, although desensitization therapy, high HLA(DR) eplet mismatches, and all positive pretransplantation immunoassays were associated with higher incidence of AR, only DSA-C3d+ independently predicted AR (OR = 6.64, 95% confidence interval [CI] = 1.14−36.56, P = 0.038). Likewise, when MFI-DSA >6190 or the combination of both FC-XM+ and DSA-C3d+ were evaluated in the same model rather than DSA-C3d, they also independently predicted AR (OR = 7.54, 95% CI = 1.11−50.85, P = 0.038, and OR = 4.94, 95% CI = 0.98−24.81, P = 0.05, respectively). The combination of other assays did not reach statistical significance (Table 3).

When breaking down DSA type, both class I and class II DSA (C3d-binding and non−C3d-binding) were associated with increased risk of AR on univariate analysis, and only C3d-binding DSA (predominantly class II) was independently correlated (data not shown).

The predictive values of each assay for AR, as well as either for ABMR or T-cell–mediated rejection, showed high specificity and negative predictive value for those tests providing antidonor reactivity (FC-XM, DSA and DSA-C3d) (Supplementary Table S1).

Interestingly, when excluding from the analysis all patients with any positive immunoassay at baseline,

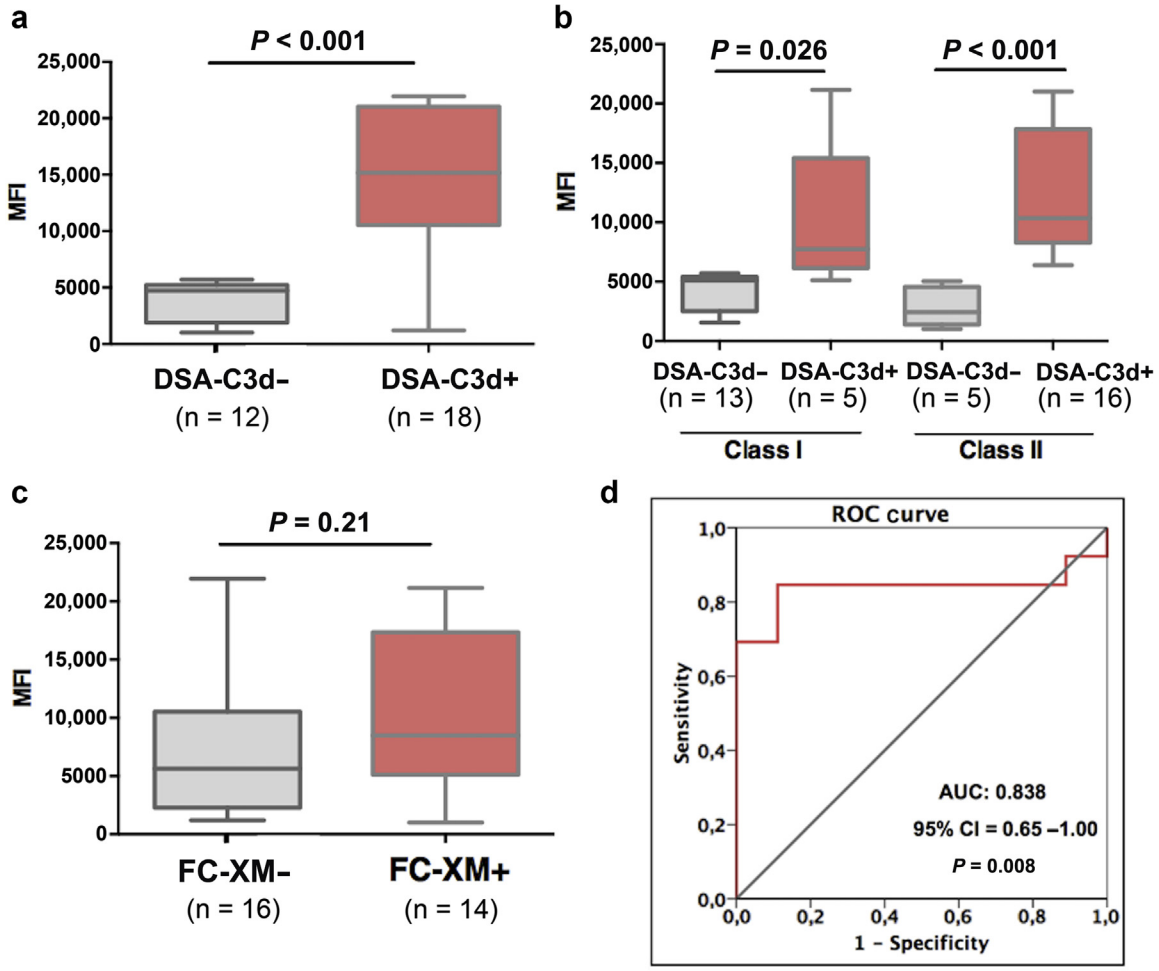

Figure 4. Association between mean fluorescence intensity (MFI)—donor-specific antibody (DSA) and donor-specific antibody—C3d complement-binding (DSA-C3d) and flow cytometry crossmatch (FC-XM) positivity. (a) Comparison between mean MFI and DSA-C3d positivity (both class I and class II). (b) Comparison of MFI between either class I or class II DSA-C3d+ and DSA-C3d—. (c) Comparison between mean MFI-DSA and FC-XM positivity. (d) Receiver operating characteristic (ROC) curve analysis of MFI-DSA predicting DSA-C3d positivity. Mean MFI DSA-C3d– versus DSA-C3d+: 3799 ± 1773 versus 12,414 ± 5884, $P < 0.001$. Mean class I MFI DSA-C3d— versus DSA-C3d+ was 4259 ± 1612 versus 10,608 ± 7569, $P = 0.026$, respectively. Mean class II MFI DSA-C3d— versus DSA-C3d+ was 2801 ± 1629 versus 12,355 ± 5049, $P < 0.001$, respectively. Mean MFI-DSA in FC-XM— and FC-XM+ cases: 7619 ± 6358 versus 10,510 ± 6130, $P = 0.21$. Area under the curve (AUC): 0.838, 95% confidence interval (CI) = 0.65—1.00, $P = 0.008$.

high HLA(DR) eplet mismatches independently predicted higher risk of ABMR (OR = 1.14, 95% CI = 1.02—1.27, $P = 0.02$). During ABMR events in this subgroup, most of the patients (57%) developed anti-DR antibodies *de novo* (Supplementary Table S2).

Pretransplantation Immune Sensitization and Kidney Graft Function Progression

As illustrated in Supplementary Figure S2, patients experiencing AR showed lower 12-month, 24-month, and 5-year eGFR than those who did not. Similarly, DSA+ and DSA-C3d+ patients displayed worse graft function progression at 1, 2, and 5 years than DSA— and DSA-C3d— patients. Conversely, no differences were observed according to CDC-PRA and FC-XM tests.

Pretransplantation Immunoassays and Graft and Patient Survival

Death-censored graft survival was significantly poorer in patients who experienced AR than among those who did not, as well as among patients showing a double-positive test as FC-XM+/DSA+ (Figure 6a and b). On univariate and multivariate Cox-regression analyses (Table 4), whereas young recipient age, previous transplantations, low 6-month eGFR, AR, and a positive result in any immunoassay were associated with graft loss, only AR (HR = 6.68, 95% CI = 2.51—17.78, $P < 0.001$), young age (HR = 0.95, 95% CI = 0.92—0.99, $P = 0.028$), and low 6-month eGFR (HR = 0.96, 95% CI = 0.93—0.99, $P = 0.005$) independently predicted graft loss.

When main clinical, demographic, and immunological variables were evaluated for their influence

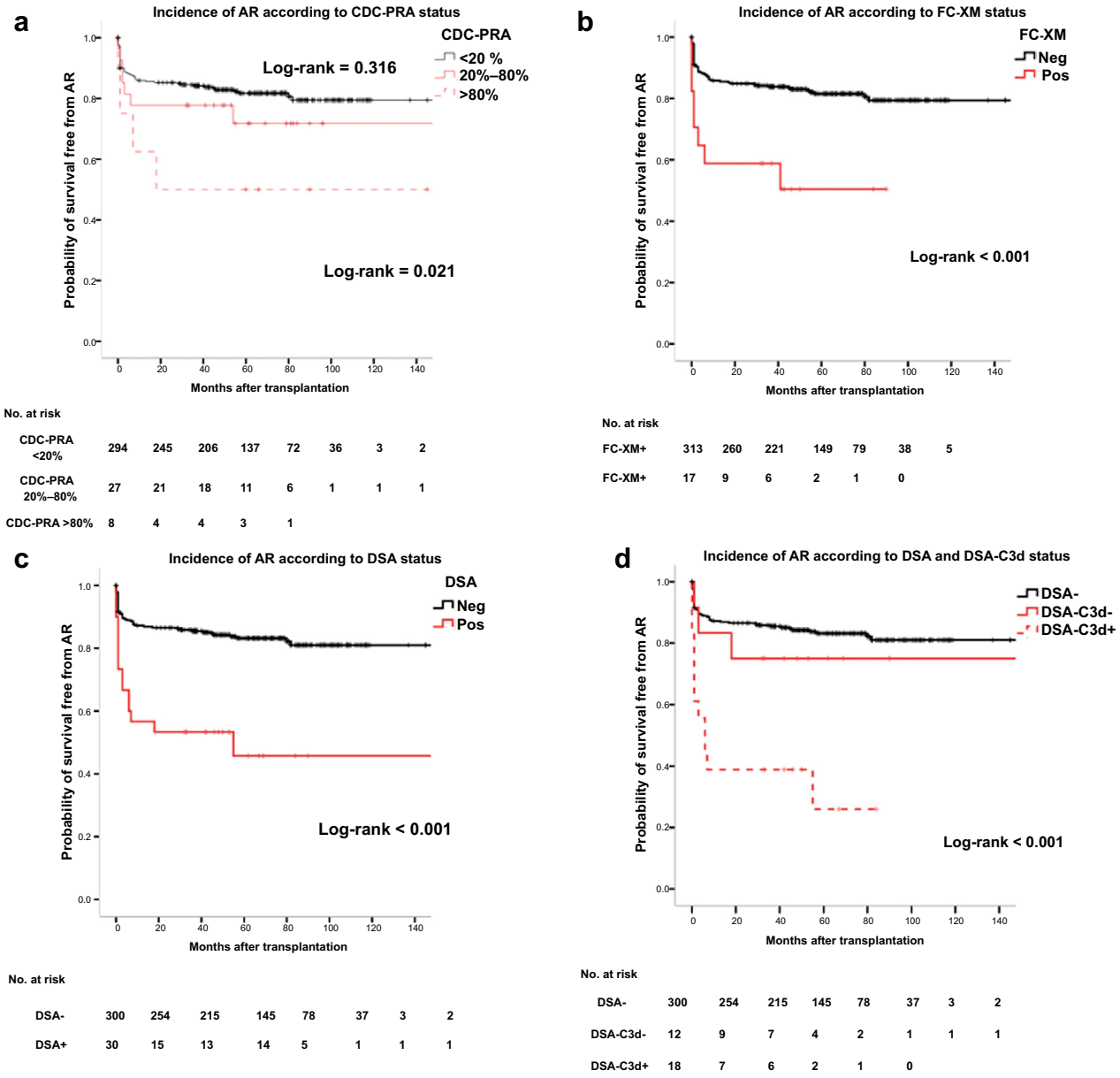

Figure 5. Kaplan—Meier free-survival curves for acute rejection (AR) according to the different immunoassays investigated. (a) Kaplan—Meier survival curves free from AR according to complement-dependent panel-reactive antibody (CDC-PRA) status. (b) Kaplan—Meier survival curves free from AR according to flow cytometry crossmatch (FC-XM) positivity. (c) Kaplan—Meier survival curves free from AR according to donor-specific antibody (DSA) positivity. (d) Kaplan—Meier survival curves free from AR according to donor-specific antibody—C3d complement-binding (DSA-C3d) positivity. Cumulative incidence of AR: CDC-PRA <20%: 53 (18%); CDC-PRA 20—80%: 7 (26%); CDC-PRA >80%: 4 (50%); log rank = 0.043. FC-XM–: 57 (18%); FC-XM+: 8 (47%); log rank = <0.001. DSA—: 50 (17%); DSA+: 15 (50%); log-rank <0.001. Cumulative incidence of AR: DSA—: 50 (17%); DSA-C3d—: 3 (25%); DSA-C3d+: 12 (67%); log-rank <0.001. DSA–, absence of donor-specific antibodies by solid phase assay; DSAC3d–, DSA without C3d binding capacity; DSAC3d+, DSA with C3d binding capacity; Neg, negative; Pos, positive.

predicting patient death, only younger recipient age was significantly associated with lower risk of death (OR = 0.93, 95% CI = 0.90—0.97, $P < 0.001$).

CONCLUSION

Although a number of immunoassays assessing the degree of anti-HLA immune sensitization and a more accurate HLA-matching approach evaluating HLA epitope compatibility have emerged in the last decades, there is no clear consensus regarding the type and number of immune tests that more efficiently may discriminate kidney transplant candidates with poorer graft outcomes. Here, evaluating a large cohort of LDKT recipients from 2 different transplant programs, we first show that although all current immunoassays are capable of identifying transplant candidates with different degrees of humoral sensitization, they display a rather poor overlap among them. Furthermore, although a positive result of any of the tests was

Table 3. Univariate and multivariate binary logistical regression for AR

	Univariate			Multivariate		
Variable	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Recipient age (yr)	0.99	0.97–1.007	0.1			
Donor gender: female	1.31	0.72–2.36	0.37			
Child to mother or husband to wife versus other types of donors	1.19	0.54–2.64	0.66			
Time on dialysis (mo)	1.001	0.99–1.007	0.79			
Transplant number >1 versus 1	1.49	0.79–2.79	0.22			
Induction treatment: rATG	1.32	0.75–2.30	0.33			
CNI-free IS regimen: yes	1.65	0.31–8.71	0.55			
Desensitization therapy: yes	2.88	1.19–6.98	0.019	2.68	0.49–14.85	0.26
CDC-XM + prior to desensitization	2.77	0.45–16.94	0.27			
HLA allele mismatches	1.01	0.84–1.21	0.93			
Class I	1.082	0.82–1.42	0.57			
Class II	1.12	0.75–1.69	0.55			
HLA eplet mismatches						
Class I (A, B)	1.012	0.966–1.060	0.61			
Class II: DR	1.042	1.001–1.084	0.043	1.02	0.98–1–07	0.24
DQ	1.022	0.986–1.060	0.22			
CDC-PRA > 20 %	2.08	0.96–4.51	0.06	1.18	0.35–3.99	0.79
CDC-PRA > 80%	4.35	1.06–17.89	0.042	3.01	0.45–20.3	0.26
FC-XM anti T or B+	3.99	1.48–10.79	0.006	1.53	0.28–8.36	0.62
DSA+	5.00	2.29–10.88	<0.001	1.89	0.41–8.82	0.41
DSA-C3d+	9.77	3.51–27.20	<0.001	6.64	1.14–36.56	0.038
DSA MFI > 6190[a]	10.59	3.54–31.73	<0.001	7.54	1.11–50.85	0.038
FC-XM+/DSA+/[b]	4.45	1.50–13.17	0.007	3.59	0.78–16.51	0.10
FC-XM+/DSA-C3d+[b]	6.64	1.81–24.26	0.004	4.94	0.98–24.81	0.05

CDC-XM, complement-dependent cytotoxicity crossmatch; CDC-PRA, complement-dependent cytotoxicity panel-reactive antibody; CI, confidence interval; CNI, calcineurin inhibitor; DSA, donor-specific antibody (solid phase assay); DSA-C3d, C3d-binding donor-specific antibody; FC-XM, flow cytometry crossmatch; MFI, mean fluorescence intensity; OR, odds ratio; rATG, rabbit anti-thymocyte globulin (Thymoglobulin®).

In the multivariate model for acute rejection evaluating the impact of each test individually (adjusted for desensitization therapy and human leukocyte antigen [HLA] DR-eplet mismatches), DSA+ also appears as an independent variable (OR = 5.1, 95% CI = 1.81–14.41, $P = 0.002$). Both DSA-C3d+ and DSA MFI > 6190 are independently correlated (OR = 12.0, 95% CI = 2.98–48.34, $P < 0.001$; OR = 15.6, 95% CI = 2.97–81.88, $P = 0.001$, respectively).

[a]Analysis adjusted for desensitization therapy, HLA-DR eplet mismatches, CDC-PRA > 20%, CDC-PRA > 80%, FC-XM+, DSA+.

[b]Analysis adjusted for desensitization therapy, HLA-DR eplet mismatches, CDC-PRA > 20%, CDC-PRA > 80%.

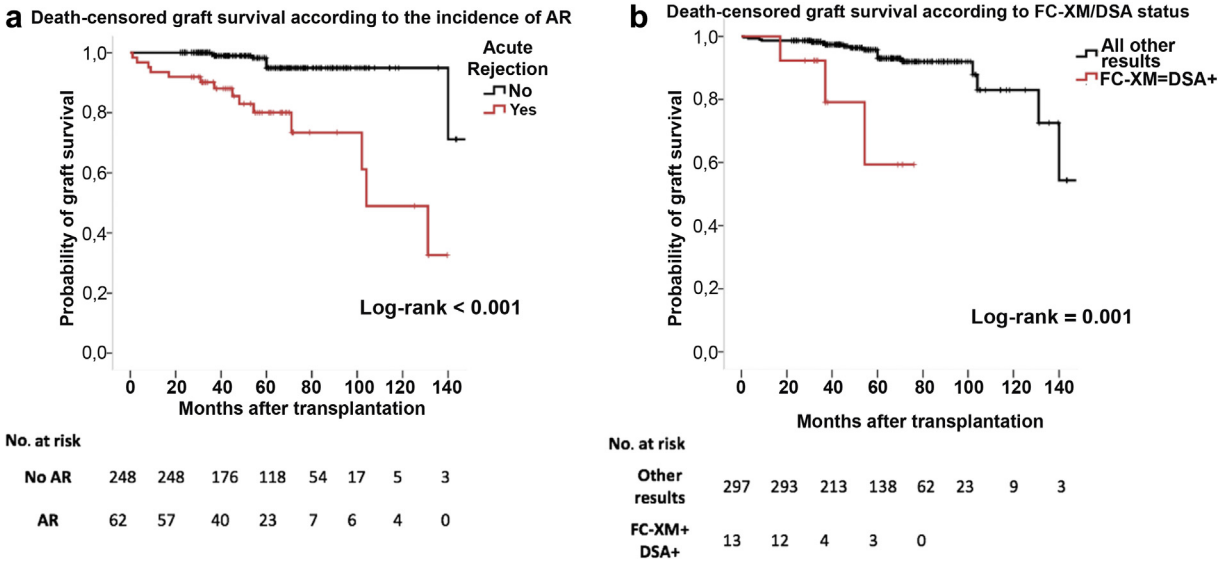

Figure 6. Kaplan—Meier free-survival curves of death-censored graft survival. (a) Kaplan—Meier free-survival curve for death-censored graft survival according to acute rejection (AR). (b) Kaplan—Meier free-survival curve for death-censored graft survival according to flow cytometry crossmatch (FC-XM)/donor-specific antibody (DSA). Cumulative incidence of death-censored graft loss: no AR: 8 (3%); AR: 14 (23%); log-rank <0.001. All other results: 19 (6%); FC-XM/DSA+: 3 (23%); log-rank = 0.001.

Table 4. Univariate and multivariate Cox regression for death-censored graft loss

	Univariate			Multivariate		
Variable	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Recipient age (yr)	0.96	0.93–1.00	0.051	0.95	0.92–0.99	0.028
Donor age (yr)	1.015	0.98–1.053	0.42			
Time on dialysis (mo)	1.006	0.998–1.013	0.13			
Transplant number >1 versus 1	2.23	0.93–5.35	0.071	1.97	0.71–5.44	0.19
Child to mother or husband to wife versus other types of donors	2.23	0.29–16.70	0.43			
eGFR 6 mo (ml/min per 1.73 m^2)	0.97	0.94–0.99	0.009	0.96	0.93–0.99	0.005
HLA allele mismatches	0.93	0.69–1.27	0.67			
Class I	1.03	0.65–1.62	0.89			
Class II	0.79	0.43–1.45	0.44			
HLA eplet mismatches						
Class I (A, B)	1.03	0.95–1.11	0.48			
Class II: DR	1.01	0.927–1.09	0.86			
DQ	1.01	0.93–1.09	0.78			
Induction treatment: rATG	1.62	0.63–4.13	0.31			
Desensitization therapy: yes	3.81	1.09–13.30	0.036	3.57	0.52–24.4	0.19
CDC-XM + prior to desensitization	3.44	0.46–25.92	0.23			
Acute rejection	8.47	3.39–21.12	<0.001	6.68	2.51–17.78	<0.001
CDC-PRA > 20%	3.01	1.19–7.64	0.020	1.59	0.45–5.60	0.48
CDC-PRA >80%	1.028	1.13–8.08	0.97			
FC-XM anti T or B+	5.81	1.66–20.34	0.006	3.43	0.58–20.27	0.17
DSA+	4.42	1.69–11.52	0.002	2.23	0.50–18.38	0.23
DSA-C3d+	4.31	1.24–14.99	0.022	2.61	0.36–18.57	0.34
DSA MFI > 6190[a]	5.07	1.46–17.67	0.011	2.20	0.33–14.81	0.42
FC-XM+/DSA+[b]	6.56	1.88–22.86	0.003	3.99	0.86–18.66	0.07
FC-XM+/DSA-C3d+[b]	5.67	1.29–24.87	0.021	2.89	0.52–15.99	0.22

CDC-PRA, complement-dependent cytotoxicity panel-reactive antibody; CDC-XM, complement-dependent cytotoxicity crossmatch; CI, confidence interval; DSA, donorspecific antibody (solid phase assay); DSA-C3d, C3d-binding donor-specific antibody; eGFR, estimated glomerular filtration rate; FC-XM, flow cytometry crossmatch; HR, hazard ratio; MFI, mean fluorescence intensity; rATG, rabbit anti-thymocyte globulin (Thymoglobulin).

In the multivariate Cox regression model excluding acute rejection and analyzing all tests individually (adjusted for recipient age, transplant number, eGFR 6 mo, and desensitization therapy), only FC-XM+/DSA+ was independently predicting graft loss (HR = 8.01, 95% CI = 1.27–50.48, $P = 0.027$).

[a]Analysis adjusted for recipient age, transplant number, eGFR 6 mo, desensitization therapy, acute rejection, CDC-PRA>20%, FC-XM+, DSA+.

[b]Analysis adjusted for recipient age, transplant number, eGFR 6 mo, desensitization therapy, acute rejection, and CDC-PRA > 20%.

associated with a higher incidence of AR, only DSA-C3d independently predicted high risk of AR, thus highlighting the greater aggressiveness of such preformed DSAs leading to AR. In line with previous works, high MFI-DSAs more likely fixed complement and displayed a stronger AR risk, particularly ABMR. However, the presence of a pretransplantation DSA, regardless of its complement-binding capacity, together with a positive FC-XM, were the strongest correlates of allograft loss, suggesting persistent alloimmune activation over time despite chronic immunosuppression.

An important finding in our study is that despite the fact that no differences were observed regarding HLA allele matching and higher rejection risk, most sensitized individuals were poorly matched at the HLA eplet level. This might be of great importance, particularly among this high-risk population, as poor matching at this molecular level might increase the likelihood of DSA binding to true immunogenic donor epitopes and thus lead to allograft rejection. In this regard, we found that the higher the mean donor/recipient HLA eplet mismatch number, the higher the incidence of AR, particularly ABMR. Remarkably, the importance of optimal matching at this level, was also replicated among nonsensitized LDKT patients, in whom a higher donor/recipient HLA-DR eplet mismatch correlated with a significantly higher incidence of ABMR.

In agreement with previous works[3–6,14–16] although in our cohort all immunoassays were associated with a higher incidence of AR, the respective discrimination capacity significantly varied among them. All of these tests are currently being performed in most transplant programs around the world. Therefore, in our analysis, we assessed in multivariate models the different immunological tests investigated. In fact, although all of them evaluate the degree of anti-HLA humoral sensitization, they all provide different insights regarding the biological mechanisms by which the humoral immune response might be activated, and thus differently predict the immunological risk of transplant patients.

Nevertheless, clearly overlapping immune tests such as DSA/C3d+ and DSA with MFI >6190, or the 2 combinations of FC-XM+/DSA+ and FC-XM+/DSA-C3d+, were analyzed in different models, as they

might provide the same biological information and thus overfit the model.

Indeed, we first observed that patients displaying a positive test result at baseline shared similar clinical backgrounds such as a longer dialysis time, previous transplantations, female sex with previous pregnancies, and receipt of a transplant from donors with whom they had previously been exposed to alloantigens, such as husband or child to wife/mother, respectively.

However, although all tests had similarly high negative predictive value, FC-XM+ and DSA-C3d+ showed the greatest specificity in predicting AR. This observation strongly suggests the greater aggressiveness of such preformed DSAs as compared to those not fixing complement despite similar immunosuppression; thus, guided preventive strategies would be highly recommended.[27] In our study, patients receiving desensitization therapy because of any positive pretransplantation immunoassay result appeared to be at high risk for AR, even if they achieved a negative CDC-XM after such a preventive strategy. However, despite this greater AR risk, desensitization therapy *per se* did not have an impact on graft or patient survival, thus highlighting the relevance of the result of the immunoassay performed after desensitization, which could guide the decision to go further into transplantation or to reconsider alternative approaches such as paired-exchange donation programs.

Although pretransplantation sensitization, regardless of the type of immunoassay used, was associated with poorer graft survival, only low 6-month eGFR, previous AR, and FC-XM+/DSA+ were independent predictors of graft loss. These data suggest the need for considering these 2 tests as main immunoassays for immune risk stratification before transplantation.

Our study has some limitations. As previously reported, not all DSA with high MFI fix complement, and conversely, some low MFI-DSA are capable of binding complement *in vitro,* due to a prozone effect that may lead to falsely low MFI in the presence of a high load of antibodies per bead. A titration or DSA IgG subclass characterization may overcome such a limitation, although this would be costly and labor intensive for daily clinical practice.[28–30] Also, 3 patients displayed a very mild but positive FC-XM without any detectable DSA. Although our main hypothesis is that they were all false-positive test results, 1 patient displayed a single anti-C HLA antibody with a very low MFI (500); thus, in the absence of the HLA C antigen donor type, we cannot exclude the presence of a potential DSA in this patient. The retrospective nature of the study and the fact that the results of some immunoassays were known before transplantation may weaken the impact of our findings. However, the large and consecutive cohort of LDKT patients from 2 different transplant centers, and the multivariate statistical models controlling for main immunologic, clinical, therapeutic, and demographic variables, significantly counterbalance these drawbacks. In addition, although high-resolution HLA typing was not available in this work to enumerate donor/recipient eplet mismatches and was inferred using a local frequency table typed by sequence-based typing, a strong correlation between high- and low-resolution typing predicting the development of *de novo* DSA has been previously shown, thus suggesting that immunogenic epitope mismmatches might also be inferred by using low-resolution HLA typing.[21] Furthermore, as our patient population was highly homogeneous in terms of ethnicity, this significantly reduces the difference in this estimation approach. Finally, the large number of variables analyzed in the multivariate model could have possibly hidden an interaction among them. However, our results were confirmed using separate models assessing each test individually.

In summary, solid-phase antibody identification and flow cytometry crossmatch assays are the 2 main tests that are highly warranted for a compelling stratification of immune risk prior to transplantation. Moreover, special caution should taken in patients displaying high MFI-DSA, as they may be more likely to develop posttransplantation AR despite receiving strong immunosuppression. Finally, a more accurate donor/recipient HLA-matching evaluation at the HLA-DR eplet level is highly recommended to reduce the risk of posttransplantation alloimmune activation.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

MM and EM collected the data, performed data analysis and interpretation and contributed to the writing of the

manuscript. EM, IR, AM, NM, DC, FD, SGV, JMC, JMG, and OB contributed to data collection and clinical follow-up of the included patients. ERM and JM carried out and supervised the immunological tests described in the study. OB designed the study, contributed to the writing of the manuscript, revised the final version and supervised the project. All authors discussed the results and commented on the manuscript.

SUPPLEMENTARY MATERIAL

Table S1. Accuracy of the different humoral alloimmunoassays predicting acute rejection and different types of acute rejection.

Table S2. Univariate and multivariate binary logistical regression for ABMR excluding patients with positive pretransplantation humoral immunoassays (PRA<20%; FC-XM−; DSA−; DSA-C3d−: 278 patients).

Figure S1. Comparison of mean number of eplet mismatches in patients experiencing acute rejection episodes. (A) Acute rejection. (B) T-cell−mediated rejection. (C) Antibody-mediated rejection.

Figure S2. Kidney allograft function progression according to the different immunoassays. (A) Comparison between mean eGFR at 12 months, 24 months, and 5 years according to incidence of acute rejection. (B) Comparison between mean eGFR at 12 months, 24 months, and 5 years according to CDC-PRA (<20% or >20%). (C) Comparison between mean eGFR at 12 months, 24 months, and 5 years according to CDC-PRA (<80% or >80%). (D) Comparison between mean eGFR at 12 months, 24 months, and 5 years according to FC-XM positivity. (E) Comparison between mean eGFR at 12 months, 24 months, and 5 years according to DSA positivity. (F) Comparison between mean eGFR at 12 months, 24 months, and 5 years according to DSA-C3d positivity.

Supplementary material is linked to the online version of the paper at www.kireports.org.

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Supplementary material

Supplemental Table 1: Accuracy of the different humoral alloimmune assays predicting AR and different types of acute rejection

Assay	Accuracy	AR	TCMR	ABMR
CDC-PRA >20%	PPV	31	3	14
	NPV	82	87	95
	Se	17	2	26
	Sp	91	88	90
CDC-PRA >80%	PPV	50	0	37
	NPV	81	88	95
	Se	6	0	16
	Sp	98	97	98
FC-XM +	PPV	47	12	18
	NPV	82	88	95
	Se	12	5	16
	Sp	96	95	96
DSA +	PPV	50	6	33
	NPV	83	88	97
	Se	23	5	53
	Sp	94	90	94
DSAC3d +	PPV	66	5	44
	NPV	83	88	96
	Se	18	2	42
	Sp	98	94	97

CDC-PRA: complement dependent cytotoxicity panel reactive antibodies; FC-XM: Flow cytometry cross match; DSA: donor specific antibody (solid-phase assay); DSAC3d: C3d binding donor specific antibody; AR: acute rejection; TCMR: T cell mediated rejection; ABMR: antibody-mediated rejection; PPV: positive predictive value; NPV: negative predictive value

Supplemental Table 2. Univariate and multivariate binary logistical regression for ABMR excluding patients with positive pre-transplant humoral immune assays (PRA<20%; FC-XM-; DSA-; DSAC3d-: 278 patients)

	UNIVARIATE			MULTIVARIATE		
Variable	OR	95% CI	p	OR	95% CI	p
Recipient age years	0.93	0.95-1.05	0.99			
Donor gender F	0.91	0.17-4.76	0.91			
Time on dialysis (months)	0.99	0.98-1.02	0.92			
Transplant number > 1 vs 1	0.39	0.72-2.05	0.26			
Induction treatment: rATG	0.3	0.66-1.39	0.12			
CNI free IS regimen: YES	8.87	0.89-87.94	0.06	9.82	0.89-108	0.062
HLA allele mismatches	1.24	0.73-2.10	0.42			
HLA eplet mismatches						
- Class I (A,B)	1.07	0.95-1.20	0.26			
- Class II: - DR	**1.14**	1.02-1.27	**0.02**	**1.14**	1.02-1.28	**0.019**
- DQ	1.05	0.96-1.16	0.27			

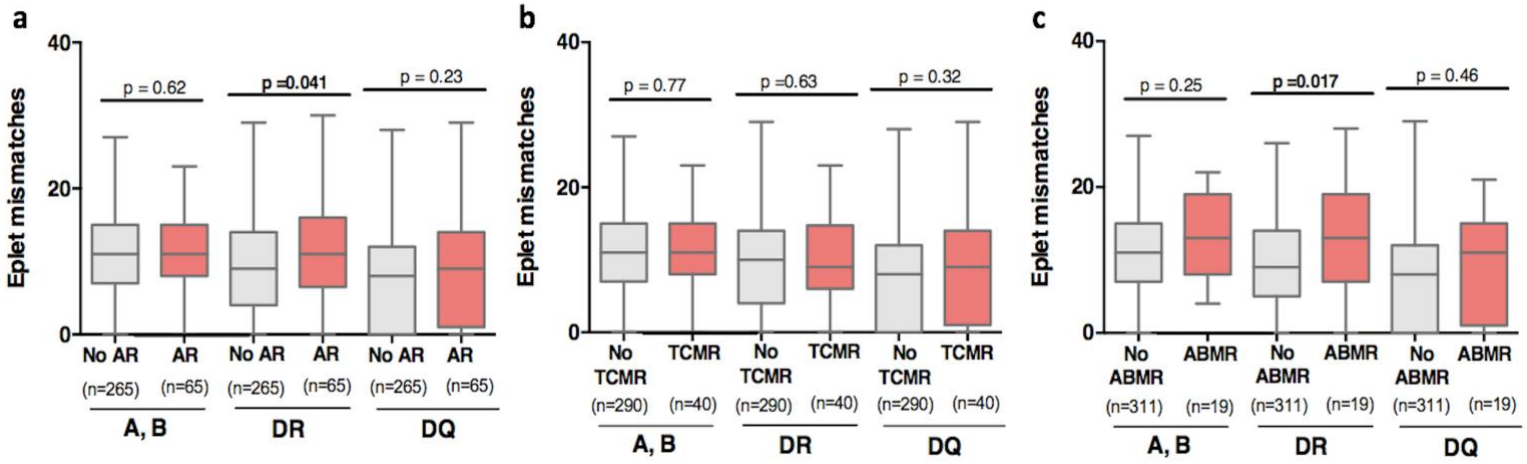
rATG: rabbit anti-thymocyte globulin (Thymoglobulin®); CNI: Calcineurin inhibitor

Supplemental Figure 1. Comparison of mean number of Eplet-mismatches in patients experiencing acute rejection episodes.

S1a. Acute rejection**.**

S1b. T cell mediated rejection.

S1c. Antibody-mediated rejection.



AR: acute rejection; TCMR: T cell mediated rejection; ABMR: antibody-mediated rejection

* Patients not experiencing AR vs AR: AB Eplet-mismatches: 11.3±6 vs 11.8±5, p=0.62; DR Eplet-mismatches: 9.2±7 vs 11.1±7, p=0.04; DQ Eplet-mismatches: 7.8±7 vs 9±8, p=0.23.

Patients not experiencing TCMR vs TCMR: AB Eplet-mismatches: 11.4±6 vs 11.6±5, p=0.77; DR Eplet-mismatches: 9.5±7 vs 10±6, p=0.63; DQ Eplet-mismatches: 7.9±7 vs 9.1±8, p=0.32.

Patients not experiencing ABMR vs ABMR: AB Eplet-mismatches: 11.4±6 vs 12.9±5, p=0.25; DR Eplet-mismatches: 9.3±7 vs 13.1±9, p=0.17; DQ Eplet-mismatches: 7.9±7 vs 9.3±7, p=0.46.

Supplemental Figure 2. Kidney allograft function progression according to the different immune assays.

S2a. Comparison between mean eGFR at 12 months, 24 months and 5 years according to incidence of acute rejection.

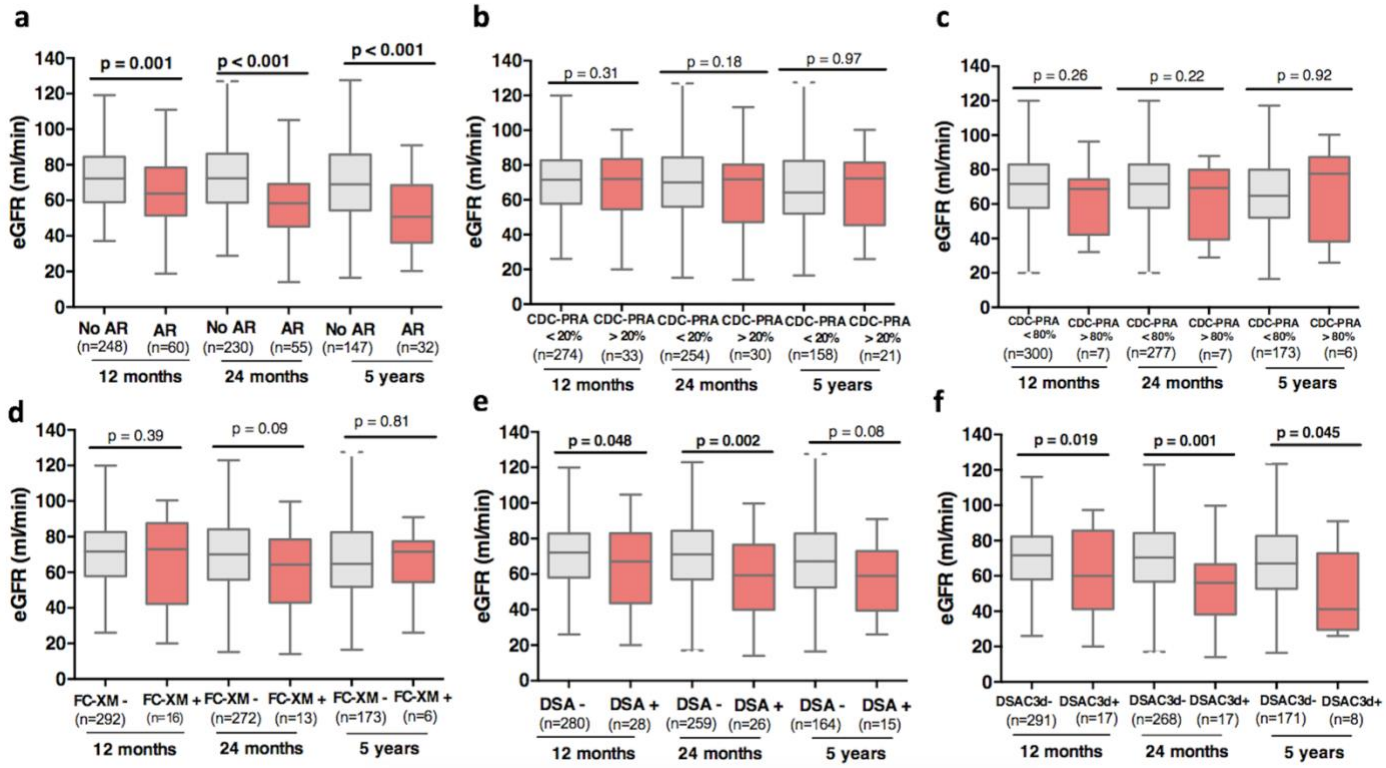
S2b. Comparison between mean eGFR at 12 months, 24 months and 5 years according to CDC-PRA (< or >20%).

S2c. Comparison between mean eGFR at 12 months, 24 months and 5 years according to CDC-PRA. (< or >80%).

S2d. Comparison between mean eGFR at 12 months, 24 months and 5 years according to FC-XM positivity.

S3e. Comparison between mean eGFR at 12 months, 24 months and 5 years according to DSA positivity.

S3f. Comparison between mean eGFR at 12 months, 24 months and 5 years according to DSAC3d positivity.



eGFR: estimated glomerular filtration rate; AR: Acute rejection; CDC-PRA: complement dependent cytotoxicity panel reactive antibodies; FC-XM: flow cytometry crossmatch; DSA: donor specific antibody (solid phase assay); DSAC3d: C3d binding donor specific antibody.

* Patients not experiencing AR vs AR: 12months 73±19 vs 64±23 ml/min, p=0.001; 24months 73±20 vs 59±24ml/min, p<0.001; 5years 70±22 vs 53±24 ml/min, p<0.00.1. CDC-PRA <20% vs CDC-PRA>20%: 12months 72±20 vs 68±24 ml/min, p=0.31; 24months 71±21 vs 65±24 ml/min, p=0.17; 5years 67±23 vs 67±21 ml/min, p=0.94. CDC-PRA <80% vs CDC-PRA>80%: 12months 72±20 vs 63±21 ml/min, p=0.26; 24months 71±21 vs 61±24 ml/min, p=0.22; 5years 68±28 vs 66±23 ml/min, p=0.93. FC-XM- vs FC-XM +: 12months 72±20 vs 67±26 ml/min, p=0.39; 24months 71±21 vs 61±26 ml/min, p=0.09; 5years: 67±23 vs 65±22 ml/min, p=0.81. DSA- vs DSA+: 12months 72±20 vs 64±23 ml/min, p=0.048; 24 months 71±21 vs 58±23 ml/min, p=0.002; 5years 68±23 vs 57±20 ml/min, p=0.08. DSAC3d- vs DSAC3d+: 12months 72±20

vs 60±24 ml/min, p=0.019; 24months 71±21 vs 54±23 ml/min, p=0.001, 5years 68±23 vs 51±24 ml/min, p=0.045.

Article 2.

Donor/Recipient HLA Molecular Mismatch Scores Predict Primary Humoral and Cellular Alloimmunity in Kidney Transplantation

Frontiers in Immunology 11:623276; doi: 10.3389/fimmu.2020.623276

Objectives:

-to investigate the development and kinetics of primary T-cell alloreactivity after kidney transplantation by means of de novo circulating donor-reactive IFN-γ-producing T cells over a 2-years period of time using a highly sensitive Enzyme-link ImmunoSpot (ELISPOT) assay and evaluate their predominant antigen presenting T-cell priming pathways.

- to analyze the impact of donor/recipient HLA molecular mismatching on the generation of de novo donor-specific alloimmunity both at humoral and T-cell level after kidney transplantation using distinct bioinformatic algorithms.

In this study, we aimed at investigating the association of distinct donor/recipient HLA molecular mismatch algorithms with the risk of primary activation of anti-donor alloimmunity both at the T-cell level (DST, evaluated by IFN-γ Enzyme-Linked ImmunoSpot-ELISPOT) and at the humoral level by means of circulating *de novo* DSA detected on a solid phase assay.

In a cohort of 169 non HLA-identical kidney transplant recipients without pretransplant DSA and with available biological samples, we combined the use of IFN-γ T-Cell ELISPOT and Single Antigen test to describe the kinetics of posttransplant donor specific alloimmune responses at different time points during the first 2 years after transplantation. Finally, in a subgroup of patients the role of main T-cell subsets accounting for donor-specific T-cell alloreactivity and the type of alloantigen presenting pathways priming *de novo* DST *in vitro* were further assessed.

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ORIGINAL RESEARCH
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Donor/Recipient HLA Molecular Mismatch Scores Predict Primary Humoral and Cellular Alloimmunity in Kidney Transplantation

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Donor/recipient molecular human leukocyte antigen (HLA) mismatch predicts primary B-cell alloimmune activation, yet the impact on *de novo* donor-specific T-cell alloimmunity (dnDST) remains undetermined. The hypothesis of our study is that donor/recipient HLA mismatches assessed at the molecular level may also influence a higher susceptibility to the development of posttransplant primary T-cell alloimmunity. In this prospective observational study, 169 consecutive kidney transplant recipients without preformed donor-specific antibodies (DSA) and with high resolution donor/recipient HLA typing were evaluated for HLA molecular mismatch scores using different informatic algorithms [amino acid mismatch, eplet MM, and Predicted Indirectly Recognizable HLA Epitopes (PIRCHE-II)]. Primary donor-specific alloimmune activation over the first 2 years posttransplantation was assessed by means of both dnDSA and dnDST using single antigen bead (SAB) and IFN-γ ELISPOT assays, respectively. Also, the predominant alloantigen presenting pathway priming DST alloimmunity and the contribution of main alloreactive T-cell subsets were further characterized *in vitro.* Pretransplantation, 78/169 (46%) were DST+ whereas 91/169 (54%) DST−. At 2 years, 54/169 (32%) patients showed detectable DST responses: 23/54 (42%) dnDST and 31/54 (57%) persistently positive (persistDST+). 24/169 (14%) patients developed dnDSA. A strong correlation was observed between the three distinct molecular mismatch scores and they all accurately predicted dnDSA formation, in particular at the DQ locus. Likewise, HLA molecular incompatibility predicted the advent of dnDST, especially when assessed by PIRCHE-II score (OR 1.014 95% CI 1.001–1.03, p=0.04). While pretransplant DST predicted the development of posttransplant BPAR (OR 5.18, 95% CI=1.64–16.34, p=0.005) and particularly T cell mediated rejection (OR 5.33, 95% CI=1.45–19.66, p=0.012), patients developing dnDST were at significantly higher risk of subsequent dnDSA formation

(HR 2.64, 95% CI=1.08–6.45, p=0.03). *In vitro* experiments showed that unlike preformed DST that is predominantly primed by CD8+ direct pathway T cells, posttransplant DST may also be activated by the indirect pathway of alloantigen presentation, and predominantly driven by CD4+ alloreactive T cells in an important proportion of patients. *De novo* donor-specific cellular alloreactivity seems to precede subsequent humoral alloimmune activation and is influenced by a poor donor/recipient HLA molecular matching.

Keywords: alloreactive, T cell, HLA mismatch, donor-specific antibodies, kidney transplantation

INTRODUCTION

Long-lasting survival of kidney transplantation is greatly challenged by both preformed and primary donor-specific humoral alloimmunity: the former preventing access to transplantation in sensitized patients, and the latter accelerating chronic rejection and premature graft loss (1, 2). Between 5 and 9% of kidney transplant recipients may develop *de novo* donor-specific antibodies (dnDSA) each year mainly against class-II human leukocyte antigens (HLA) (3, 4). This is of significant clinical relevance, being chronic antibody mediated rejection (ABMR) one of the leading causes of death-censored graft loss that may explain to some extent why even with modern immunosuppression, long term graft survival has not improved in recent years (5).

Recent data show that a major determinant of primary humoral alloimmune activation relies on poor donor/recipient HLA matching, especially in case of non-adherence or insufficient immunosuppression exposure (4, 6, 7). Notably, while clinical histocompatibility assessment is still based on alphanumeric class-I/II allele matching, novel computed algorithms have refined its evaluation by assessing the mismatch (MM) degree at a molecular level (8). The definition of the molecular differences between donor and recipient HLA molecules has been an interesting field of research developed in the last decade that led to the creation of informatic algorithms available for research purposes and whose clinical impact on outcomes has been investigated. On the one hand, the calculation of the number of highly polymorphic aminoacids composing the mismatched donor HLA molecules (amino acid MM) has been proposed and showed to predict primary humoral responses (9, 10). Similarly, the HLAMatchmaker algorithm defines the count of specific mismatched polymorphic aminoacidic-residues within 3 Ångstroms radius (eplets) exposed on the HLA molecular surface and constituting the functional epitopes against which anti-HLA antibodies are directed (11). The number of eplets that are mismatched between donor and recipients can be calculated by the HLAMatchmaker software either at each HLA locus, by class (1 or 2) or as a cumulative number or "eplet MM load". Some but not all eplets have been "antibody verified" *in vitro* and since this process is ongoing, newer versions of the calculator are periodically released including the last updates on the eplets' repertoire. An increasing number of eplet MM has been shown to identify kidney transplant recipients at higher risk of developing dnDSA, antibody-mediated rejection (ABMR) and worse allograft survival (12–15). Furthermore, since dnDSA can only be produced by B cells activated by cognate interactions with indirectly primed alloreactive T cells that have previously recognized donor HLA antigens (16), another HLA matching algorithm was developed to predict the number of recognizable donor-HLA-derived peptides that can be processed and presented by recipient's HLA class-II molecules according to the physico-chemical characteristics of donor and recipient HLA molecules (PIRCHE-II). The PIRCHE-II score sums the number of these peptides and defines the risk of primary anti-donor humoral alloimmune activation through indirect pathway of antigen presentation. In clinical studies, this score has also been associated to the risk of dnDSA formation and graft loss (13, 17).

While previous clinical reports suggest that alloreactive T-cell priming precedes humoral activation, (4, 18, 19) there is no evidence yet showing the frequency of *de novo* donor-specific T-cell alloimmune activation (dnDST) after kidney transplantation and its association with donor/recipient HLA molecular matching. Hence, we here investigated the association of distinct donor/recipient HLA molecular mismatch algorithms with the risk of dnDST activation as well as its influence on subsequent dnDSA formation. While there are no readily available tests to monitor the presence of donor-specific T-cell responses in the clinical setting, we used one of the most sensitive immune assays tracking circulating frequencies of donor-reactive memory/effector T cells, the IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT), which has been validated between different research consortiums (20, 21) and has shown important associations between preformed T-cell alloimmune memory and posttransplant rejection risk (22–24). On the other hand, the development of single antigen beads using solid phase assays has revolutionized the field of humoral alloimmune risk-stratification as the most reliable assay tracking anti-HLA antibodies in clinical practice (25). Therefore, to obtain a complete picture of the kinetics of posttransplant donor-specific alloimmune responses, we used these two immune assays to detect dnDST and dnDSA at different time points during the first 2 years after kidney transplantation. Finally, the role of main T-cell subsets accounting DST alloreactivity and the type of alloantigen presenting pathways priming dnDST *in vitro*

were further assessed to characterize the predominant donor-antigen T-cell priming occurring after transplantation.

MATERIAL AND METHODS

Patients of the Study

As illustrated in **Figure 1**, between June 2014 and December 2016 326 adult kidney transplants were performed. Out of them, multiorgan transplant recipients, ABO incompatible, and HLA identical transplant recipients, with preformed DSA, without available donor/recipient PBMCs and/or high-resolution HLA typing and those lost to follow-up were excluded from this study. Clinical data were collected prospectively during clinical follow-up. BPAR was defined according to latest BANFF classification (26). Graft loss was defined either as re-transplantation or return to chronic renal replacement therapy. Minimum patient follow-up was 2 years (mean: 33 ± 16 months, range 24–60). All patients signed informed consent to participate in the study, which had been previously approved by the local Investigator Research Board.

HLA Typing and Donor/Recipient Mismatch Scores

HLA Typing

High-resolution donor and recipient HLA typing was done for both class-I (A, B, C) and class-II (DRB1, DQB1, DPB1) antigens with NGS technology. Exons 2, 3, 4 for class I and exons 2 and 3 for class II were amplified by multiplex PCR. NGS was performed on a MiSeq platform (Illumina, San Diego, California). DRB3/4/and DPA1 could not be assessed in all donor/recipient pairs because of insufficient biological material, thus HLA mismatch scores were performed at A, B, C, DRB1, DQB1 and DPB1 loci. Notably, since all recipients could be typed for DQA1, we evaluated PIRCHE score also taking into account the alloantigen presentation by recipients' DQ(B1/A1)+ DRB1 molecules.

Amino Acid HLA Mismatches

The HLA epitope mismatch algorithm (HLA-EMMA) was used to assess polymorphic amino acids on mismatched donor HLA molecules as previously described (27). Both total amino acid sequences and amino acids in solvent accessible positions were assessed as a global score and at the single HLA locus or molecule. The software package is available at http://www.HLA-EMMA.com.

HLAMatchmaker Algorithm

The HLAMatchmaker program (Rene Duquesnoy, 2016, University of Pittsburgh Medical Center, Pittsburgh, PA HLA-ABCEpletMatchingVersion3.1 and DRDQDPEpletMatching ProgramV3.1 from http://www.epitopes.net/downloads.html) was used to calculate eplet scores as previously described (6). Total number of eplet and antibody verified eplet mismatches were calculated for all HLA molecules (eplet MM load), for each locus and for each donor HLA molecule separately.

Predicted Indirectly Recognizable HLA Epitopes II Algorithm

PIRCHE-II score was calculated as previously described using the latest version3.3 from https://www.pirche.org (28). Briefly, the NetMHCIIpan3.0 algorithm was used to predict the non-americ-binding cores of donor mismatched HLA-derived peptides that can bind to recipient HLA-DRB1. Relevant HLA-DRB1 binders were defined as peptides with an IC50<1,000nM for HLA-DRB1 (15).

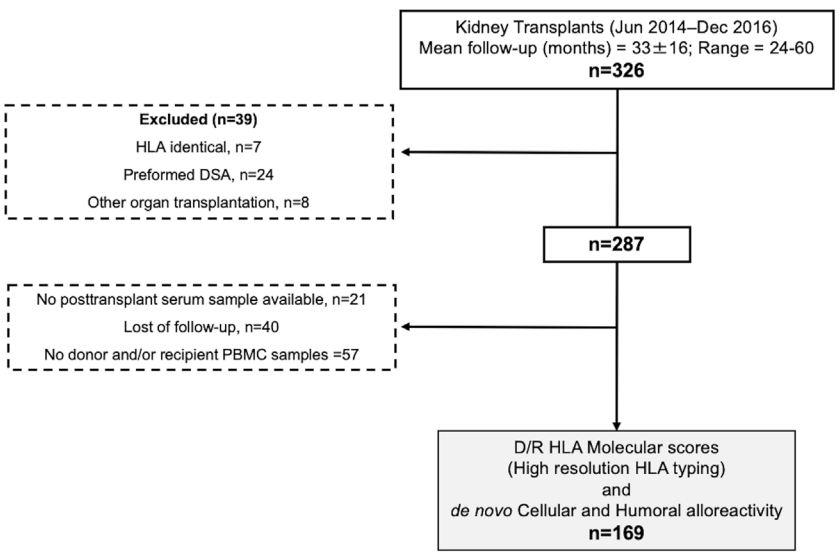

FIGURE 1 | Flow chart of the study. PBMCs, peripheral blood mononucleated cells; DSA, donor specific antibody.

Donor-derived HLA class-II binder peptides that differed at least one amino acid in their non-americ-binding core from recipient's HLA sequence were counted as PIRCHE-II. Donor epitope-HLA complexes that were present multiple times in a donor/recipient couple were counted as a single PIRCHE-II. The analysis of PIRCHE-II global score enumerates all class I/II donor derived peptides, presented by recipient DRB1 molecule. The peptide counts originated from each donor's locus and each donor molecule is also described. The analysis of DRB1 and DQ presentation of donor-derived peptides, and different IC50 cut-offs for peptide binding, were evaluated.

Anti-HLA Antibody Determinations

Patients' sera were tested for the presence of class-I and II anti-HLA IgG antibodies at baseline, 6 and 12 months after transplantation and annually thereafter. A single-antigen class-I and class-II flow beads-assay kit was used (LIFECODES, division of Immucor, Stanford, CA). All beads showing a normalized MFI>500 were considered positive if (MFI/MFI lowest bead)>5.

Donor and Recipient Peripheral Blood Mononuclear Cell Samples Preparation and Evaluation of Circulating Donor-Specific T-Cell Alloreactivity

Donor and Recipient Peripheral Blood Mononuclear Cell Samples

Recipient and donor PBMCs from living donors or splenocytes from deceased donors were harvested and isolated by Ficoll density gradient centrifugation. Donor samples were depleted from T-Cells using either anti-CD3 (Human CD3+Cell Depletion Kit-RosetteSep Kit, STEMCELL, France) or anti-CD2 kits (EasySep1 Human-CD2 Selection Kit, STEMCELL, France), in living or deceased donors, respectively, to avoid any donor T-cell alloimmune response. All samples were frozen in liquid nitrogen at −80°C until their use.

Evaluation of Donor-Specific Alloreactive T-Cell Responses

The assessment of DST in peripheral blood, both prior and posttransplantation, was done using the IFN-γ Enzyme-linked Immunosorbent Spot (ELISpot) assay as previously described (21). Briefly, $3x10^5$ responder PBMC were placed in each Elispot well plate coated with primary IFN-γ antibody wells with $3x10^5$ donor cells, in triplicates. A negative control (complete medium alone: RPMI 1640, GE Healthcare Life Sciences, USA, with 10% inactivated FBS, antibiotics and L-glutamine) and a positive control (Pokeweed, AID, Autoimmun Diagnostika) were also tested in duplicates. Incubation time was 22 h at 37°C, 5% CO2. Results were expressed as frequencies of IFN-γ producing T-cells/$3x10^5$ PBMCs, subtracting responses from negative donor and recipient control wells. As previously reported, a cut off of ≥25 spots/$3x10^5$ PBMCs was considered positive (21, 29).

Analysis of T-Cell Receptor Dependent Activation-Induced T-Cell Markers

To assess the contribution of CD8 and/or CD4 T-cell subsets to the allogenic T-cell response assessed *in vitro*, 22 donor-recipient pairs with remaining available samples (pretransplant DST−, n=5; pretransplant DST+, n=10; dnDST+, n=7) were tested in a T-cell receptor (TCR)-dependent Activation-Induced T-Cell Markers (AIM) assay as previously described (30).

Cells were cultured in 96-wells round bottom plates at $3x10^5$PBMC per well either with 100µl of medium (negative control), $3x10^5$ T-cell depleted donor cells (allo-stimulation) or 50µl of phytohemagluttinin-PHA (positive control). After incubation, cells were stained with the following antibodies: CD4-FITC, CD8-APC-H7, CD134 (OX-40Antigen)-PE, CD69 (very early activation antigen)-PE-Cy7, CD137 (4-1BB)-APC, 7-AAD (BD Biosciences, San Diego, CA). Donor Cells after 22 h incubation with medium were stained with CD4-FITC, CD8-APC-H7 antibodies to test effective T-cell depletion. After 22 h incubation with T-cell depleted donor cells, we assessed by flow cytometry analysis the % of AIM+ cells defined as the % of (CD69+CD137+) cells for CD8+ T cells, and (CD134-OX40+ CD137+) for CD4+. T-cell activation results are presented by subtracting the percentage of AIM+ cells after stimulation with medium (negative control) from % of AIM+ cells after allogenic stimulation.

Flow cytometry was performed on a FACS-Canto flow cytometer and analyzed using the FACS-Diva Software (BD Biosciences, San Diego, CA).

In Vitro Assessment of Alloantigen-Presenting Pathways Priming Donor-Specific T Cells

In order to characterize the predominant alloantigen-presenting pathways of circulating DST *in vitro*, a subset of DST+ patients with available cell samples, either prior and/or after transplantation were functionally re-evaluated (preDST+, n=9; dnDST+, n=9; persistDST+, n=9). For these experiments we modified the functional immune assay by evaluating in the same patient DST responses with the following conditions: 1) using total recipient PBMC as responder cells co-cultured with T-cell depleted donor stimulating cells and, 2) using recipient T-Cells only after being selectively isolated as responder cells co-cultured with donor stimulating cells. In the first assay, both directly and indirectly primed DST frequencies are detected, since recipient PBMCs include T cells (CD3+), B cells (CD19+), monocytes (CD14+) and dendritic cells (HLADR+CD14− CD3− CD19− CD56−) (**Supplementary Figure 1**), whereas in the second experiment only T cells are present as responders thus, DST frequencies primed by the direct pathway (DP) of antigen presentation may be only detected. For these later experiments, a positive selection of recipient CD3+ T-Cells was done (Human T Cell Enrichment Kit-RosetteSep Kit, STEMCELL, France). Importantly, the same number of CD3+ T-Cells present in the all PBMCs sample was seeded in each well when analyzing the DST with enriched responder T-Cells, to avoid any additional response due to higher presence of responder T-Cells. Therefore, to assess the relative role of indirectly primed (IP) DST cells in

the *in vitro* assays, the total number of IFN-γ spots observed in the DP experiment was subtracted from those observed in the same patient when using all PBMCs as responder cells. PBMC subsets were stained with combinations of the following fluorochrome conjugated antibodies: CD3-APC-H7, CD19-FITC, CD14-PECy7, CD56-PE, HLADR-APC (BD Biosciences, San Diego, CA).

Statistical Analysis

All continuous data are presented as mean ± SD or median and interquartile-range. Different groups were compared using X^2 test for categorical variables and student t-test for normally distributed data, and non-parametric Kruskal-Wallis or Mann-Whitney U test for non-normally distributed variables. Bivariate correlation analyses were performed by Pearson or Spearman test (non-parametric variables). Univariate and multivariate logistic regression analyses were used to determine the variables associated with the risk of developing BPAR and dnDST. The time-dependent association of the variables assessed on graft survival and dnDSA development was studied with Cox proportional hazard, Kaplan–Meier plots, and log-rank test. The statistical significance level was defined as 2-tailed $p<0.05$. Statistical analyses were performed with IBM SPSS Statistics, version 26 (Armonk, NY) and GraphPad Prism version6.0 (GraphPad Software, La Jolla, CA).

RESULTS

Patients of the Study and Main Clinical Outcomes

As illustrated in **Figure 1**, 169 consecutives non HLA-identical, single, adult transplant recipients at Bellvitge University Hospital (Barcelona, Spain) without preformed DSA and in whom both donor and recipient HLA typing was characterized using high resolution Next Generation Sequencing (NGS) technology and peripheral blood mononuclear cells (PBMC) to monitor DST were obtained both prior and at different time points after transplantation were evaluated in this study.

As shown in **Table 1**, the patients included in the study were representative of the total kidney transplant patients performed during the study timeline, as there were no differences regarding main demographic, immunological, and clinical outcomes. Most patients of the study were male, Caucasic transplant recipients receiving a deceased donor kidney. Induction immunosuppression was mainly based on basiliximab induction with tacrolimus-based maintenance triple therapy.

Forty-six (27%) patients developed delayed graft function (DGF) and 19 (11%) biopsy-proven acute rejection (BPAR) (79% TCMR, 21% ABMR). 24/169 (14%) patients developed dnDSA: 6 (25%) class-I only (2 against A, 3 anti-B and 1 anti-C), 19 (80%) class-II only (anti-DR n=1, 5%; anti-DQ n=17, 89%, anti-DP n=1, 5%), and 1(5%) patient against both class-I and II. Five (21%) patients developed dnDSA against both donor DQ molecules, thus the majority of dnDSA were directed against DQ antigens (22/30, 73%). Mean time until first dnDSA detection was 24± 20 months (range 6–60). Mean dnDSA mean fluorescence intensity (MFI) was 8,685 (range 1,152–20,338).

TABLE 1 | Main baseline and clinical outcomes of the study population and comparison with patients not included in the study.

Main baseline characteristics	All patients (n=169)	Not studied patients (n=118)	p
Recipient age (years)	52 ± 14	52 ± 14	0.83
Recipient gender (male)	110 (65)	30 (25)	0.09
Race (Caucasic)	158 (94)	113 (96)	0.41
Cause of end stage disease			
Vascular	20 (12)	21 (18)	0.12
Diabetes	8 (5)	14 (12)	
Glomerular	48 (28)	30 (25)	
Polycystic kidney disease	23 (14)	16 (14)	
Interstitial disease	24 (14)	11 (9)	
Others/unknown	46 (27)	26 (22)	
Time on dialysis (months)	25 ± 34	21 ± 25	0.23
Transplant type (deceased)	115 (68)	88 (75)	0.23
Donor age (years)	55 ± 15	54 ± 12	0.86
Transplant number (1)	152 (90)	106 (90)	0.98
Cold ischemia time (hours)	12.8 ± 9.5	11 ± 9	0.18
Pre-transplant anti-HLA (non DSA) antibodies			
Class I	14 (8)	10 (8.5)	0.34
Class II	17 (10)	12 (10.2)	0.30
cPRA (maximum)	2.8 ± 6.6	2.6 ± 5.9	0.88
Main immunosuppression			
- Induction (rATG/basiliximab/none)	32 (19)/126 (74)/11 (6)	30 (25)/85 (72)/3 (2)	0.14
- Maintenance therapy (CNI, tacrolimus)	150 (89)	116 (98)	0.06
- Steroid withdrawal before 6 months (yes)	50 (30)	38 (34)	0.41
Main clinical outcomes			
DGF	46 (27)	36 (31)	0.54
BPAR	19 (11)	17 (14)	0.43
TCMR/ABMR	15/4	16/1	0.21/0.33
Patients developing *de novo* DSA	24 (14)	20	0.49
HLA class I	6	3	
HLA class II	19	18	
HLA class I and II	1	1	
Death-censored graft loss	9 (5)	10 (9)	0.32
Patient death	11 (6)	4 (4)	0.24

Data are mean (standard deviation, SD) or n (%).
cPRA, calculated panel of reactive antibodies; rATG, rabbit anti thymoglobulin; CNI, calcineurin inhibitor; BPAR, biopsy-proven acute rejection; TCMR, T cell mediated rejection; ABMR, antibody-mediated rejection; DSA, donor-specific antibodies.

Death-censored graft loss occurred in 9 (6%) patients, being main causes BPAR (5, 55%), interstitial fibrosis/tubular atrophy (2, 22%), primary glomerulonephritis recurrence (2, 22%). Eleven (6%) patients died with a functioning graft because of malignancies (5, 45%), infections (3, 27%), and cardiovascular events (3, 27%).

A detailed description of the different HLA mismatch (MM) scores of the study population is depicted in **Supplementary Table 1**. Despite the strong positive correlation between the three molecular MM algorithms, a single number of HLA allelic mismatch could correspond to a wide range of molecular MM at the individual patient level (**Supplementary Figure 2**).

No direct association was observed between BPAR and the HLA allelic, amino acid, and eplet MM scores (OR 1.08, 95% CI

0.84–1.38, p=0.54 allelic; OR 1.01, 95% CI 0.99–1.04, p=0.33 global amino acidic and OR 1.02, 95% CI 0.98–1.07, p=0.34 eplet MM), but for global PIRCHE-II score (OR 1.012, 95% CI 1.001–1.023, p=0.038). Patients developing ABMR during follow-up showed a trend towards higher amino acid MM (88±5 *vs.* 63±22, p=0.08); global PIRCHE-II (117±48 *vs.* 80±38, p=0.06) and higher eplet MM load (41.5±7 *vs.* 32±11, p=0.07). There was no effect of molecular MM scores on graft function progression, death-censored graft survival, and patient death (data not shown).

Donor/Recipient HLA Molecular Mismatch Scores Predict Primary Humoral Alloimmunity

As shown in **Figure 2**, significantly higher MM scores of each molecular algorithm against the individual mismatched donor DQ molecule was observed for the respective anti-DQ dnDSA. No association was observed at the allelic MM level (data not shown). A similar association was observed when donor DQ peptides presented by both recipient DRB1 and DQ were assessed (35.19±29 *vs.* 17.70±23, p=0.0002, in dnDSA+ *vs.* dnDSA−, respectively). We did not study the impact of the different molecular algorithms in the two solely anti-DP and anti-DR dnDSA. A positive correlation with anti DQ dnDSA MFI was observed for DQB1 amino acid MM (r=0.57, p=0.02; solvent accessible r=0.60, p=0.013), DQB1 eplet MM (r=0.44, p=0.03), and DQB1 PIRCHE-II score (r=0.36, p=0.08).

Donor/Recipient HLA Molecular Mismatch Scores and Primary T-Cell Alloimmunity

Pretransplant DST Does Not Correlate With Donor/Recipient HLA Molecular MM Scores

Despite the absence of preformed DSA, 78/169 (46%) showed high frequencies of pretransplant DST (preDST+), whereas 91 (54%) did not (preDST−). No association was found between preDST+ and main clinical, demographic characteristics nor with different HLA molecular MM scores (**Supplementary Table 2**). Nonetheless, preDST+ patients showed higher risk of BPAR (OR 5.18, 95% CI=1.64–16.34, p=0.005), mostly TCMR (OR 5.33, 95% CI=1.45–19.66, p=0.012) (**Supplementary Figure 3**), whereas it was not associated with dnDSA nor death-censored graft survival. Multivariate logistic regression analysis showed that while PIRCHE-II and tacrolimus CV (OR 1.02, 95% CI 1–1.04, p=0.047) where associated to BPAR, only preDST+, induction therapy with rATG and DGF were independent correlates of BPAR (preDST+ OR 8.46, 95% CI 1.7–41.8, p=0.009; rATG induction OR 0.13, 95% CI 0.14–1.3, p=0.08; DGF OR 3.9, 95% CI 1.2–13.1, p=0.03).

PIRCHE-II Score Identifies Patients at Risk of Primary Donor-Specific T-Cell Alloreactivity

After transplantation, 54/169 (32%) patients showed DST responses at some timepoint (postDST+), being 23 (42%) dnDST and 31 (57%) persistently positive (persistDST+), whereas 115/169 (68%) were postDST− (68 preDST− and 47 preDST+) (**Figure 3A**). Changes of mean donor-reactive IFN-γ T-cell frequencies between pre and posttransplantation are depicted in **Figures 3B–E**.

While none of the different HLA MM scores associated with postDST+ (persistDST+ and/or dnDST+) (data not shown), a significantly higher global PIRCHE-II score was observed among dnDST+ than within postDST− patients (**Figure 4**). When analyzing the single HLA loci, dnDST patients showed significantly higher solvent-accessible DRB1 amino acid MM, not-Ab-verified (Abv) DRB1 eplet MM, PIRCHE-II DRB1, and PIRCHE-II DQB1 (DRB1 amino acid MM 11.17 ± 6.2 *vs.* 8.18 ± 6.4, p=0.05, not-Abv DRB1 Eplet 6.3±3.05 *vs.* 4.79±3.5, p=0.06 PIRCHE-II DRB1 15.5 ± 11.9 *vs.* 9.44 ± 8.4, p=0.03, PIRCHE-II DQB1 22.65 ± 15.7 *vs.* 16.29 ± 12.5 p=0.05). When assessing the PIRCHE-II score presented by DRB1+DQ molecules, similar results were observed, being the count of DRB1 donor-derived peptides similarly associated to dnDST activation (29.95±24.2 *vs.* 20.15±17.8, p=0.04). However, the difference in global PIRCHE-II score presented by both DRB1+ DQ molecules was not statistically different (199.4±132 in dnDST+ *vs.* 175.1±91 in dnDST−, p=0.4). The relationship between PIRCHE-II and dnDST for different peptide affinity thresholds (IC50: 0–50, 0–125, and 125–1,000), revealed that PIRCHE-II was significantly associated to dnDST especially at less stringent IC50 intervals (**Supplementary Figure 4**). Donor-specific T-cell frequencies did not correlate

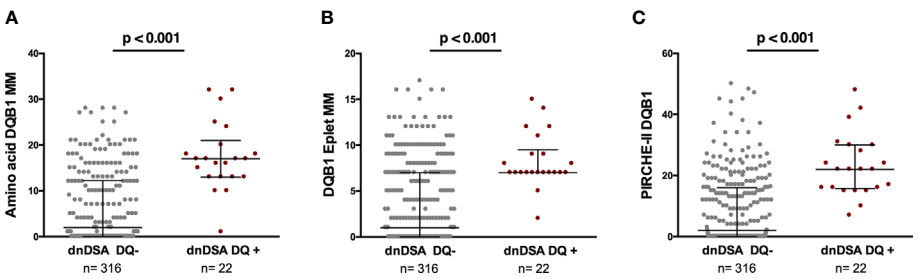

FIGURE 2 | Association between amino acid MM, eplet MM load, and PIRCHE-II score for each donor DQ molecule and the respective dnDSA formation. Each dot illustrates the single molecular MM score of each donor DQ molecule against which the patients developed or not dnDSA. **(A)** Amino acid MM 6.5 ± 7.9 vs 17.6 ± 7.4, p<0.001. Solvent accessible 4.82 ± 6.18 vs 12.87 ± 6.29, p<0.001. **(B)** Eplet MM load 3.56 ± 4.33 vs 8.27 ± 2.95, p<0.001. Antibody-verified 1.31 ± 1.87 vs 2.82 ± 1.26, <0.001. **(C)** PIRCHE-II 8.98 ± 11.54 vs 23.2 ± 10.84, p<0.001. MM, mismatch; dnDSA, *de novo* donor-specific antibody.

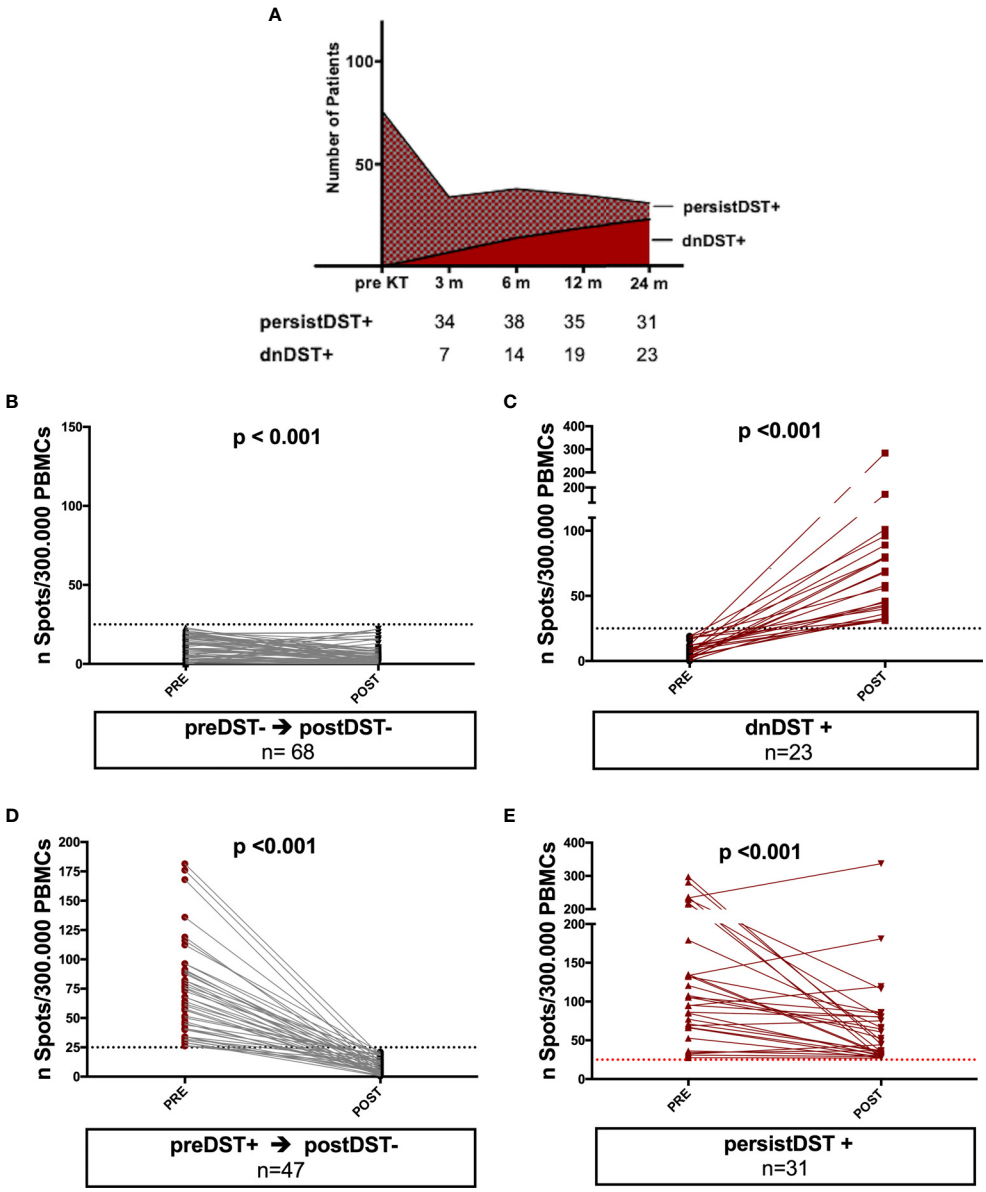

FIGURE 3 | Kinetics of posttransplant *de novo* DST and changes of mean donor-reactive IFN-γ T-cell frequencies between pre and posttransplantation in different groups of patients. **(A)** At month 3, 6, 12, and 24 months 7, 7, 5, and 4 patients developed dnDST, and 34, 38, 35 and 31 were PersistDST+ respectively. **(B–E)** All preDST– remaining DST– and preDST+ becoming postDST– showed significantly lower T-cell frequencies posttransplantation. PersistDST+ although remaining positive, showed weaker responses. Only dnDST+ patients showed a significant increase of spots number. **(B)** preDST– and postDST–: preDST mean 9.76 ± 7.2 IFN-γ spots/300.000 PBMC; postDST mean 5.28± 6.27 IFN-γ spots/300.000 PBMC **(C)** dnDST: postDST mean 70.7±55.9 spots/300.000PBMCs. **(D)** preDST+ and postDST–: preDST+ mean 67.04 ± 35.9 IFN-γ spots/300.000PBMC; postDST: mean 8.76±6.35 IFN-γ spots/300.000 PBMC **(E)** persistDST+: postDST mean 66.56±61.04 IFN-γ spots/300.000 PBMC. DST, donor specific T cell alloreactivity; preDST, pretransplant donor-specific T-cell alloimmune response; postDST, posttransplant donor specific T-cell alloreactivity; dnDST, *de novo* donor specific T-cell alloreactivity; persistDST, persistent donor specific T-cell alloreactivity.

with amino acid MM (r=0.17, p=0.14) nor Eplet MM load (r=0.1, p=0.34), whereas showed a weak but positive linear correlation with the global PIRCHE-II score (r=0.24, p=0.025).

In the univariate and multivariate logistic regression analysis for the prediction of dnDST, high global PIRCHE-II score and delayed graft function were independent correlates (**Table 2**). Conversely, persistDST+ was only predicted by absence of T-cell depletion (OR 0.09, 95% CI 0.01–0.62, p=0.01) and high pre-transplant IFN-γ ELISpot frequencies (OR 1.02, 95% CI 1.009–1.03, p=0.001).

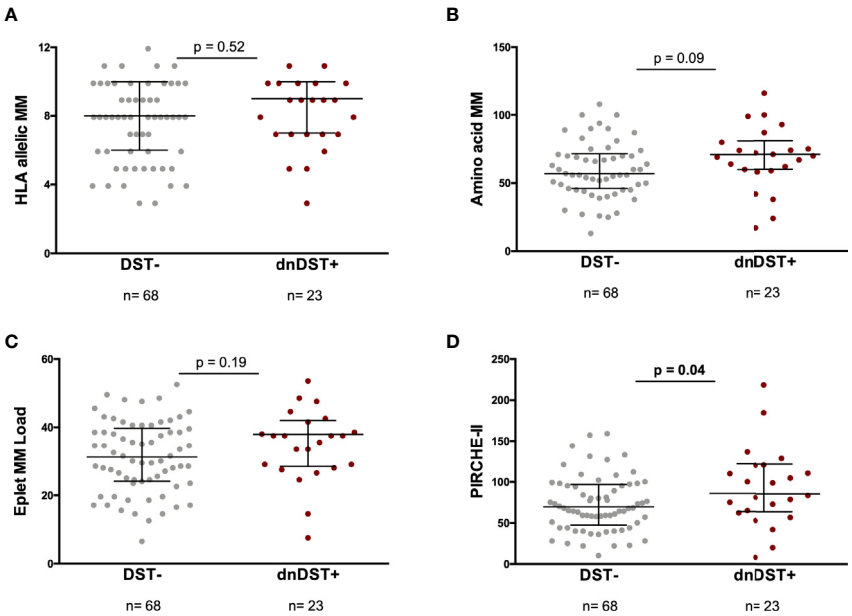

FIGURE 4 | HLA allelic MM, amino acid MM, eplet MM load, global PIRCHE-II and *de novo* DST. Comparisons between HLA MM scores and dnDST- or dnDST+ patients. **(A)** HLA allelic MM 7.66 ± 2.3 vs 8.05 ± 2.2, p=0.52. **(B)** Amino acid MM 60.52 ± 20.6 vs 69.47 ± 25.8, p=0.09. **(C)** eplet MM load 31.62 ± 10.5 vs 35 ± 10.5, p=0.19. **(D)** PIRCHE-II 74.1 ± 33.2 vs 94.8 ± 48.1, p=0.04. MM, Mismatch; DST, donor specific.

TABLE 2 | Univariate and multivariate logistic regression for the risk of *de novo* donor-specific T-cell (dnDST).

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	p	OR	95% CI	p
Recipient age (y)	1.02	0.99–1.06	0.21			
Donor age (y)	1.02	0.98–1.05	0.42			
Recipient gender (m)	0.96	0.35–2.59	0.93			
Donor gender (m)	1.52	0.55–4.21	0.42			
Transplant number (>1)	1.02	0.19–5.42	0.98			
Race (Caucasic)	0.48	0.08–3.1	0.44			
Type of donor (deceased	**4.96**	**1.34–18.3**	**0.02**	1.04	0.05–20.5	0.99
Cold ischaemia time (hours)	**1.08**	**1.02–1.15**	**0.01**	1.05	0.91–1.21	0.51
DGF	**4.67**	**1.64–13.24**	**0.004**	**4.11**	**1.18–14.3**	**0.03**
Type of induction IS (no rATG)	1.36	0.49–3.76	0.55			
Steroid withdrawal	1.40	0.45–4.29	0.56			
Type of maintenance IS (CNI)	0.85	0.26–3.07	0.85			
Tacrolimus CV%	1.004	0.98–1.03	0.76			
Previous BPAR	3.14	0.42–23.70	0.27			
HLA allelic MM	1.08	0.86–1.36	0.52			
Amino acid MM	1.02	0.99–1.04	0.13			
Eplet MM load	1.03	0.98–1.08	0.18			
Global PIRCHE-II	**1.014**	**1.001–1.03**	**0.03**	**1.015**	**1.001–1.03**	**0.04**

rATG, rabbit anti thymoglobulin; DGF, delayed graft function; CNI, calcineurin inhibitor; CV, coefficient of variation (CV = σ/μ × 100); BPAR, biopsy-proven acute rejection; HLA, human leukocyte antigens; MM, mismatches; IS, immunosuppression; DST, donor-specific T-cell alloimmunity.
In bold are statistically significant variables.

De Novo DST Predicts Subsequent Development of dnDSA

While postDST+ showed a higher risk of subsequent dnDSA formation (HR 2.66, 95% CI=1.19–5.95, p=0.017), when stratifying postDST in either persistent or *de novo*, dnDST displayed a stronger risk of dnDSA than persistDST (HR 2.64, 95% CI=1.08–6.44, p=0.03 and HR 1.62, 95% CI=0.63–4.13, p=0.31, respectively). Kaplan-Meier dnDSA-free survival curves illustrate the cumulative dnDSA rates among different postDST groups (**Figure 5**).

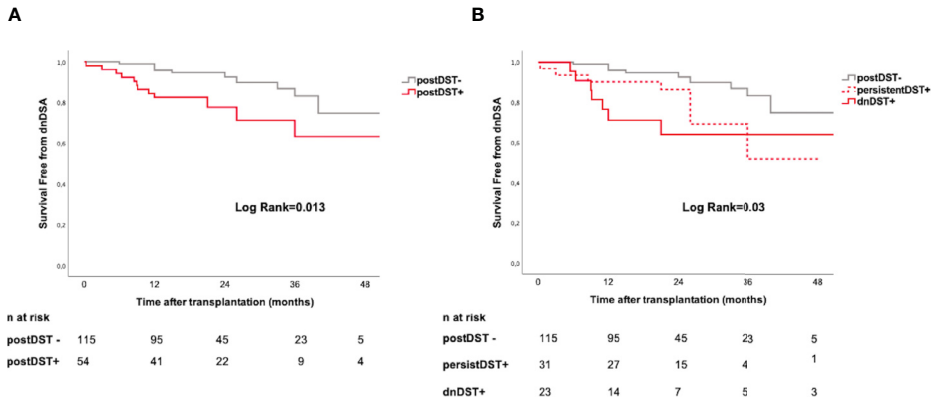

FIGURE 5 | Posttransplant donor-specific T-cell alloreactivity and *de novo* DSA formation. Kaplan–Meier curves illustrating the cumulative incidence of dnDSA stratified according to: **(A)** postDST– *vs*. postDST+ **(B)** postDST+ further stratified in dnDST or persistDST. postDST– *vs*. persistDST+= log rank 0.07; postDST– *vs*. dnDST+ log rank=0.01; persistDST+ *vs*. dnDST+ log rank=0.36. dnDSA, *de novo* donor-specific antibody; DST, donor specific T-cell alloreactivity; postDST, post-transplant donor specific T-cell alloreactivity; dnDST, *de novo* donor specific T-cell alloreactivity; persistDST, persistent donor specific T-cell alloreactivity.

In addition, transplant patients with both dnDST+ and dnDSA+ showed significantly higher PIRCHE-II global score as compared to patients with either dnDST or dnDSA or those without dnDSA nor dnDST (101±49 *vs*. 78.9±38, p=0.04). No differences were observed with any of the other HLA molecular MM algorithms at this level.

While we did not observe any correlation between posttransplant IFN-γ ELISpot frequencies and MFI of dnDSA (Rho −0.7, p=0.75), a weak but statistically significant inverse correlation with 12 and 24-month graft function was observed (eGFR 12months r=−0.25, p=0.01; eGFR 24 months r=−0.20, p=0.01).

Higher Involvement of CD4+ T Cells in *De Novo* T-Cell Alloreactivity as Compared to Pretransplantation

The contribution of CD8+ and CD4+ T cells to donor-reactive T-cell responses were investigated using the TCR dependent activation-induced cell marker (AIM) assay in a subset of patients. CD4+ and CD8+ AIM+ T cells varied among different DST groups, which were detected both within preDST+ and dnDST+ patients (**Supplementary Figure 5**).

The percentages of CD8+ and CD4+ AIM+ T cells, were significantly higher among DST+ as compare to DST–

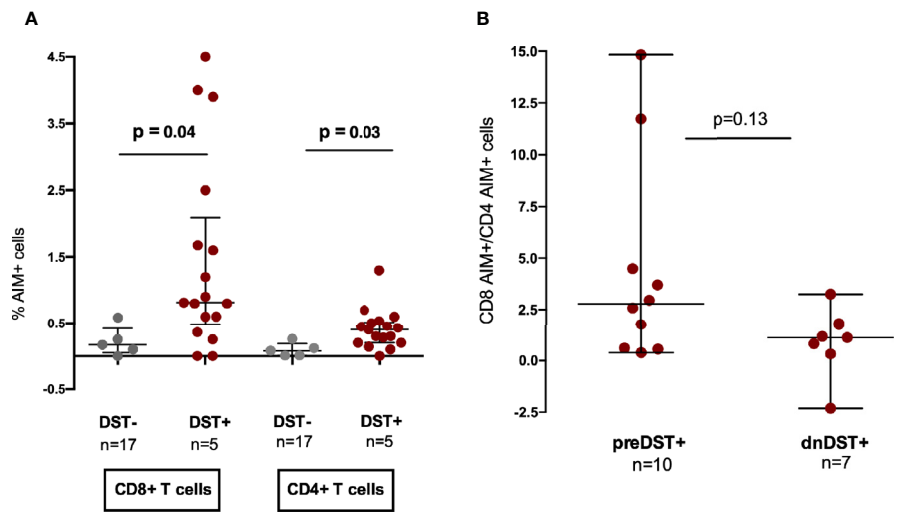

FIGURE 6 | Contribution of CD8 and CD4 T cell subsets to pre- and posttransplant donor-specific alloreactivity assessed by T-cell receptor dependent activation-induced cell markers (AIM) by flow cytometry analysis. **(A)** Comparison of % of CD8+ AIM + T cells (CD69+CD137+) and AIM+ (OX40+CD137+) CD4+ T cells after allogenic (donor-specific stimulation) in DST– or DST+ patients. CD8+ AIM+: median 0.17% (0.05–0.42) vs 0.81% (0.48–2.09), p=0.041; CD4+AIM+: median 0.08% (0.008–0.19) vs 0.40% (0.2–0.56), p=0.029 in non alloreactive versus alloreactive patients, respectively. **(B)** CD8+/CD4+ AIM+ T-cell ratio in preDST+ and dnDST+ samples, respectively. Median 2.77 (0.6–6.3) in preDST+ *vs*. 1.13 (–2.3–1.79) in dnDST+, p=0.13. AIM, T-cell receptor dependent activation-induced cell markers; DST, donor specific T-cell alloreactivity; preDST, pretransplant donor specific T-cell alloreactivity; dnDST, *de novo* donor specific T-cell alloreactivity.

independently of the time of the IFN-γ ELISpot test assessment, either before or after transplant (**Figure 6A**), confirming that the two assays are concordant detecting the same donor-reactive T cells. Notably, when we stratified for time of assessment, *dn*DST+ showed a numerically lower CD8+/CD4+ AIM+ T-cell ratio than preDST+ suggesting an increased contribution of CD4+ alloreactive T cells after transplantation among dnDST+ patients (**Figure 6B**).

Contribution of Distinct Alloantigen Presentation Pathways Priming Posttransplant Donor-Specific T-Cell Alloreactivity

In order to characterize the contribution of the two main alloantigen presenting pathways, both direct (DP) and indirect (IP), priming circulating donor-reactive T cells, we functionally characterized them *in vitro*. When using whole recipient PBMC, different cell subsets other than T cells such as B cells, monocytes and dendritic cells were present, whereas only T cells were detectable when recipient PBMC were enriched for T cells (**Supplementary Figure 1**).

While most circulating preDST+ responses [7/9, (78%)] were driven by donor-reactive T cells primed by the DP, an important proportion of patients with postDST+ responses, either dnDST+ or persistDST+, were also primed by the IP (5/9, 55% in both groups) (**Figure 7A**). While no differences were found at the HLA allelic, aminoacidic and eplet MM scores, patients with IP_postDST+ (either dnDST+ or persistDST+) showed a trend toward higher PIRCHE-II scores than those with only DP_DST+ (**Figures 7B–E** and **Supplementary Table 3**).

DISCUSSION

Primary humoral alloimmune activation through dnDSA production is a well-characterized deleterious factor inducing chronic ABMR and accelerated graft loss (1, 2) and recent reports have recently shown that it may be predicted by quantifying the donor/recipient HLA MM at the molecular level (6, 31). However, for B-cell activation in absence of preformed immune memory, cognate T-cell help is required thus, previous *de novo* T-cell alloimmune priming (dnDST) against donor antigens might also occur, subsequently driving anti-donor humoral immune activation.

In our study, we first confirm that HLA matching at the molecular level using distinct algorithms outperforms allelic MM assessment predicting primary humoral alloimmunity by means of dnDSA formation. Furthermore, we report that a relevant number of kidney transplant recipients develop dnDST after transplantation, which ultimately predicts the advent of dnDSA. Notably, unlike pretransplant DST, an important proportion of posttransplant DST patients, either those with persistent or *de novo* DST, display high frequencies of donor-reactive CD4+ T cells primed by the indirect antigen presentation pathway, which contributes to their global DST response. Most interestingly, and similarly to dnDSA, our data suggest that patients at risk of dnDST seem to also show a poor donor/recipient HLA molecular matching, and in particular, at the Predicted indirectly Recognizable HLA Epitopes II (PIRCHE-II) score level, emphasize the contribution of the indirect antigen presenting pathway driving DST development. These data highlight a continuous increased risk of dnDST and dnDSA for each

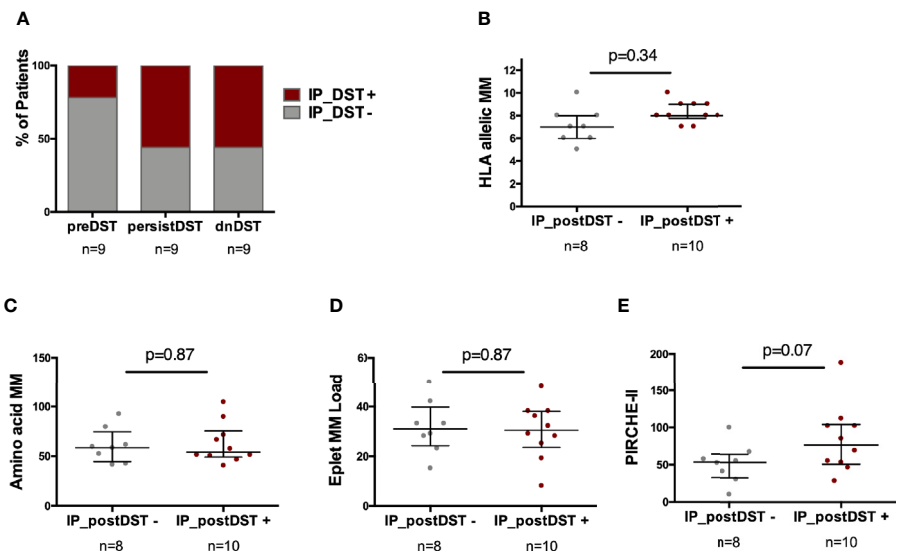

FIGURE 7 | Predominance of the type of alloantigen presenting pathway priming DST according to the time of DST assessment and association with distinct HLA MM scores. **(A)** Proportion of patients showing any degree of IP_ DST+ according to timing of DST assessment (preDST n=2/9, 22%; persistDST n=5/9, 56%; or dnDST n=5/9, 56%). **(B)** Distribution of HLA allelic MM between IP_postDST– (n=8) and IP_postDST+ patients (n=10): 7.12 ± 1.5 *vs*. 8.3 ± 0.95, p=0.34 **(C)** Amino acidic MM 60.5± 17.5 *vs*. 62.5 ± 20.4, p=0.87 **(D)** Eplet MM load 31.6 ± 10.8 *vs*. 30.1 ± 11.2, p=0.87 **(E)** PIRCHE-II 51.5 ± 26 *vs*. 83.9 ± 45, p=0.07. MM, mismatch; DST, donor specific T-cell alloreactivity; preDST, pretransplant DST; postDST, posttransplant donor specific T-cell alloreactivity; dnDST, *de novo* donor specific T-cell alloreactivity; IP DST, indirect pathway donor specific T-cell alloreactivity (with recipient APCs).

individual predicted peptide presented by recipient APC through indirect presentation. This is, to our knowledge, the first report showing the impact of HLA molecular incompatibility on the development of primary adaptive alloimmunity, not only at the humoral but also at the cellular level in solid organ transplantation.

In order to track the presence of donor-reactive T-cell responses, we used the IFN-γ donor-specific T-cell ELISpot, a sensitive and reproducible immune-assay tracking circulating donor-reactive IFN-γ-producing memory/effector T cells (21, 32). Most studies using this test have focused on the pretransplant setting and have shown its capacity identifying transplant candidates at higher risk of BPAR, regardless preformed donor-specific humoral immune sensitization (22–24, 29). Here, while we confirm this observation, pretransplant DST was not associated with any HLA MM score thus, strongly suggesting that its presence may arise from either antigen cross-reactivity amid heterologous immunity or prior transient alloantigen recognition triggering a low immune sensitization state, predominantly at the T-cell compartment. Notably, it has recently been reported the impact of HLA class-II mismatching predicting not only the advent of dnDSA and ABMR but also TCMR (14, 33, 34). In this regard, our findings support a mechanistic explanation of incompatibility at the DR and DQ molecules being especially associated to the risk of *de novo* T-cell activation. Although intuitively, a specific threshold would be of high relevance to help stratifying patients into high or low risk for either dnDSA or dnDST, from the biological point of view these thresholds might not represent the potential impact for alloimmune activation. Indeed, despite the strong correlation between the load of molecular MM and risk of *de novo* alloimmunity, even a small amount of mismatched antigens may be sufficient to activate an immune response, thus application of specific cut-offs may be misleading in clinical practice (34, 35).

Another important observation of our study is that up to 50% of transplant recipients with preDST maintained a strong DST response after kidney transplantation, which seems to be mainly influenced by pretransplant anti-donor T-cell frequencies and the absence of T-cell depletion induction therapy. Interestingly, a strong association was observed between postDST and subsequent dnDSA formation, particularly among dnDST patients. While we cannot confirm whether patients with persistent DST show the same pretransplant donor-reactive T-cell clones after transplantation, we observed that an important proportion of them did also display DST primed by the IP, similarly to patients with dnDST thus, suggesting that DST responses among persistDST may have also been developed *de novo*. Interestingly, dnDST was also influenced by the development of delayed graft function, which could possibly be explained by an inflamed milieu with increased class II HLA antigen expression on graft cells ultimately driving T-cell alloantigen recognition. The higher presence of alloreactive CD4+ T cells in dnDST+ samples as compared to pretransplantation does also support that posttransplant anti-donor alloreactivity is driven, at least also in part, by the IP of antigen presentation. While the presence of the IP after transplantation has been widely described (16, 36, 37), a body of evidence has also shown the potential relevance of a semi-direct or third pathway of antigen presentation (38–40). In this line, we also found circulating postDST responses primed by the DP when assessed *in vitro*, most likely representing the presence of such semidirect pathway of antigen presentation *in vivo*.

Our study has some limitations. The retrospective design may hamper achieving robust conclusions. Nonetheless, the use of high-resolution HLA typing and the significant associations observed together with the concomitant mechanistic *in vitro* experiments performed, counterbalance this drawback. Also, both DPA and DRB3/4/5 typing could not be assessed, leaving undetermined the impact of molecular MM at those loci on dnDST generation as well as their peptide presenting role. Nevertheless, the accurate prediction of dnDST by donor-derived DRB1 peptides and also when evaluating DQ presentation strengthens the consistency of our findings. Notably, dnDST was accurately predicted by donor-derived DRB1 peptides but not by the global peptide burden if DQ presentation is evaluated. The expression of DQ molecules in recipient APC or different activation capacity of CD4+ T cells according to distinct HLA class-II molecules may explain this observation.

In conclusion, we here show the impact of novel HLA molecular matching scores, also influencing a higher risk of primary anti-donor cellular alloimmune activation after kidney transplantation, which seems to precede the subsequent development of *de novo* humoral alloreactivity. Importantly, the value of implementing these novel donor/recipient HLA matching scores in kidney transplantation to refine current immune-risk stratification needs to be further explored in larger studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Investigator Research Board Bellvitge University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OB: conceptualization of the study and supervision. OB, JG, MM, EM, AF, NM, AM, JC: clinical patients' follow-up. MM, EC, AT, NL, VP, PF: performed the experiments. MM, EC, OB: collected and organized the data and performed the statistical

analysis. MN, EP, JM: supervised the HLA molecular MM analysis. MM and OB wrote the first draft of the manuscript. OB and JG reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.623276/full#supplementary-material

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Conflict of Interest: MN is an employee of PIRCHE AG that runs the PIRCHE web-portal.

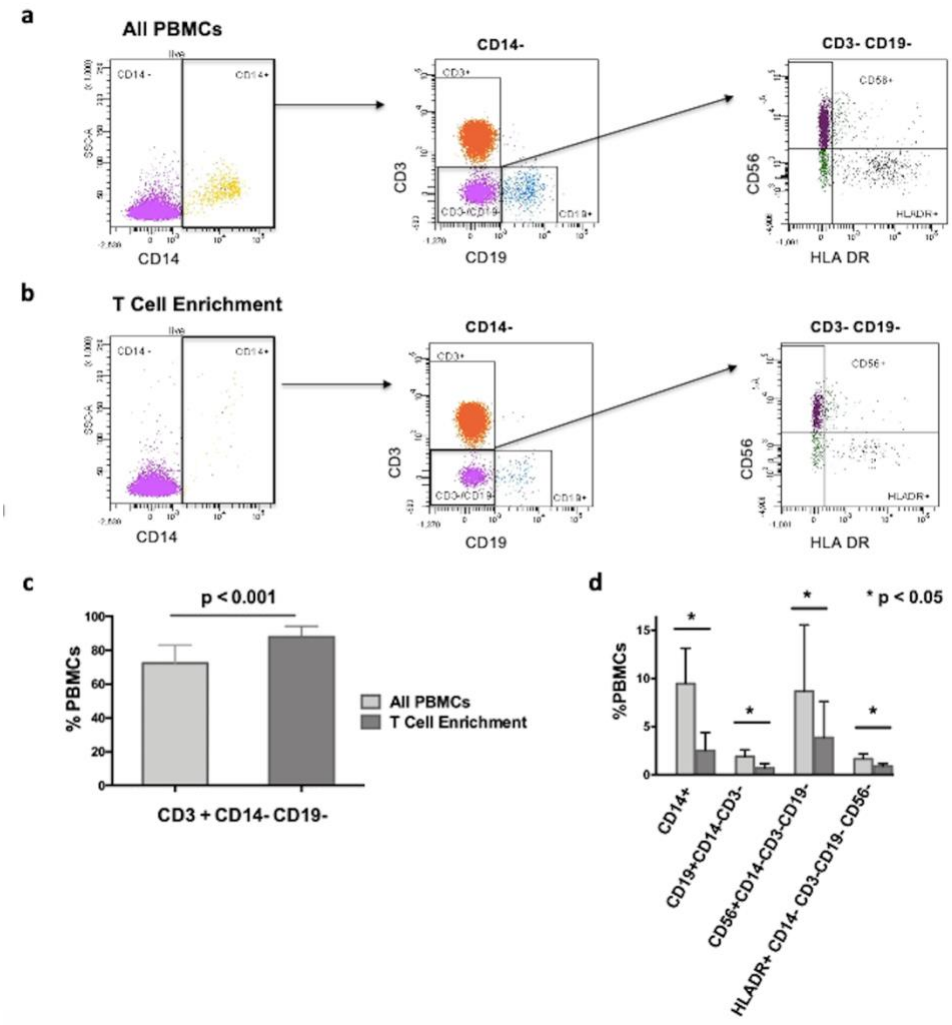
The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Supplementary Figures

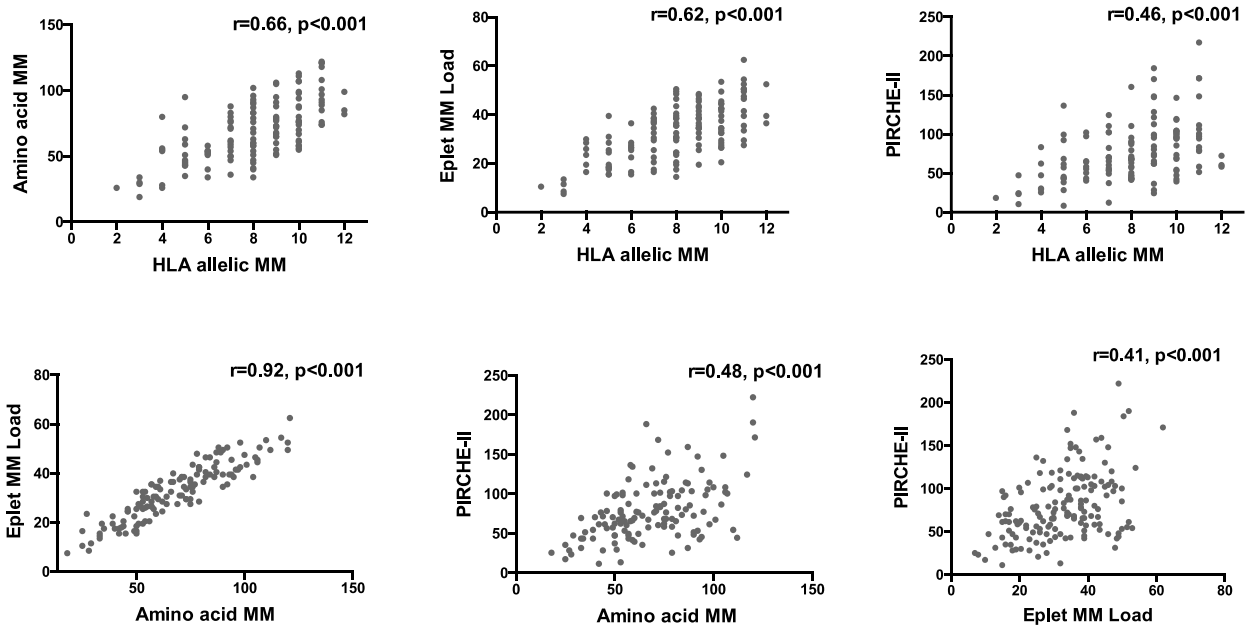
Supplementary figure 1.

1. Peripheral blood mononuclear cell populations before and after T cell enrichment. Representative FACS plot gating strategy used to characterize the PBMC population (**1a**) and after T cell Enrichment (**1b**). **1c.** Variation in % of CD3+CD14-CD19- cells: 65.68±9.0 vs 87.9±5.7 in all PBMCs and T cell Enrichment samples respectively, p<0.001. Results from 25 independent experiments. **1d.** Variation in % of the other cells assessed: CD14+: 9.44±3.7 vs 2.5±1.9, p=0.002; CD19+CD14-: 2.08±0.84 vs 0.96±0.3, p=0.04; CD56+ CD14- CD3-CD19-: 8.68±6.8 vs 3.85±3.7, p=0.03; HLADR+CD14-CD19-CD3-CD56-: 1.650.5 vs 0.87±0.3, p=0.03 in All PBMCs and T cell



Abbreviations: PBMC: Peripheral blood mononuclear cell

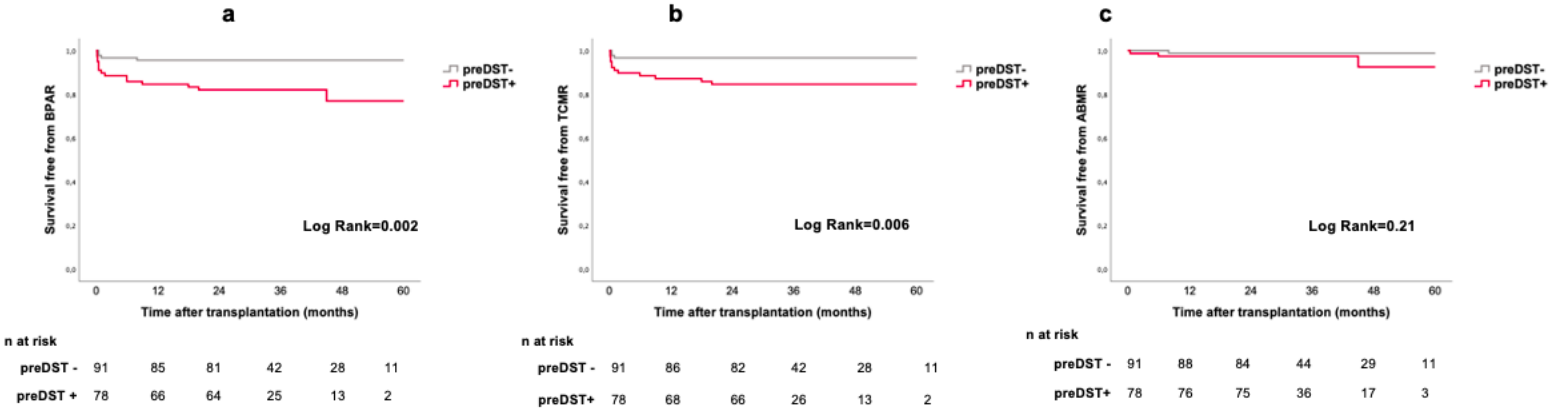
Supplementary figure 2. Association between HLA allelic MM, amino acidic MM, Eplet MM load and PIRCHE-II scores



Abbreviations: MM: mismatches

Supplemental figure 3. Pretransplant DST and risk of BPAR

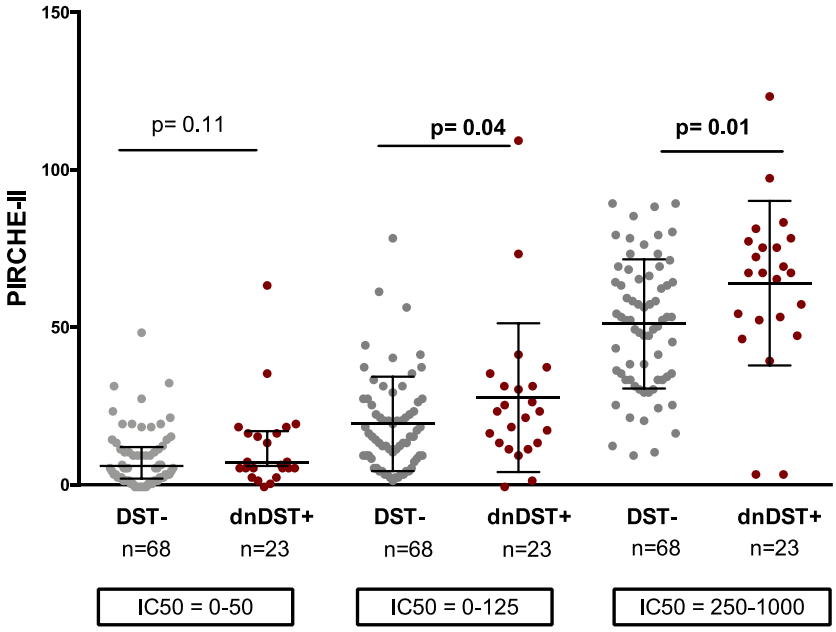
Kaplan–Meier curves illustrating the cumulative incidence of BPAR (**3a**), TCMR (**3b**) and ABMR (**3c**) stratified according to pretransplant DST.



Abbreviations: BPAR: biopsy-proven acute rejection; TCMR: T-cell mediated rejection; ABMR: antibody-mediated rejection; preDST: pretransplant donor-specific T-cell alloimmune response

Supplementary figure 4. Relationship between PIRCHE-II score and dnDST for different peptide affinity thresholds.

Relation between PIRCHE-II and dnDST at different IC50 thresholds. IC50 0-50: 8.76±9.4 vs 12.6±13.9, p=0.11; IC50 0-125: 19.2±15 vs 27.6±23.6, p=0.04; IC50 250-1000: 51.07±20.5 vs 64±26.1, p=0.01 for DST- and dnDST, respectively.



Abbreviations: DST: donor-specific; dnDST *de novo* donor-specific T-cell alloimmune response

Supplemental figure 5. Representative FACS plot gating strategy for negative control, Allo DST negative test, pretransplant DST positive test and *de novo* posttransplant DST test.

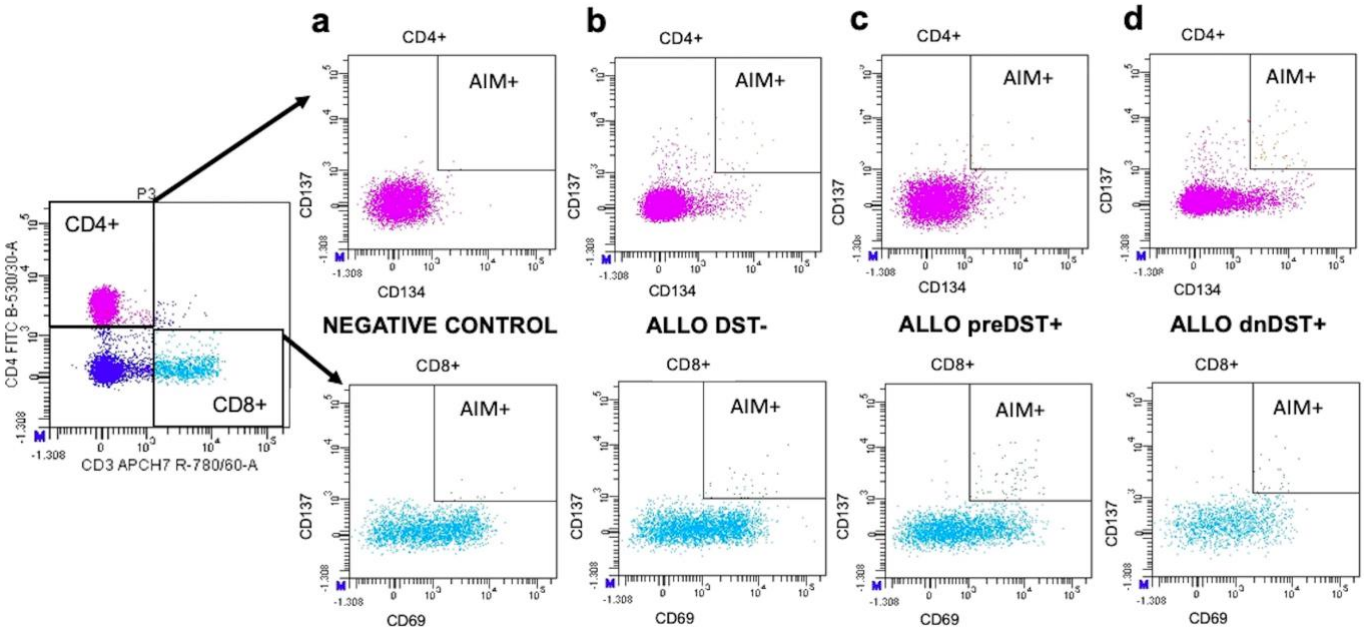
AIM+ Cells were defined as % of living cells expressing CD69 and CD137 for CD8+T cells and CD134-OX40 and CD137 for CD4+T cells, respectively. All results are expressed subtracting the percentage of AIM+ cells after stimulation with medium (negative control) from % of AIM+ cells after allogenic stimulation

5a. Negative control (medium): median 0.02 [0.012-0.076] for CD4+AIM+ and 0.45 [0.29-0.96] for CD8+AIM+.

5b. Allo DST negative test: median 0.08% [0.008-0.19] for CD4+AIM+ and 0.17% [0.05-0.42] for CD8+AIM+

5c. pre transplant DST positive test: median 0.25% [0.14-0.62] for CD4+AIM+ and 1.2% [0.65-3.55] of CD8+AIM+

5d. *de novo* posttransplant DST test: median 0.4% [0.14-0.53] for CD4+AIM+ and 0.60% [0.25-1.2] for CD8+AIM+.



Abbreviations: AIM: T-cell receptor dependent activation-induced cell markers (AIM); DST: donor-specific T-cell alloimmune response; preDST: pretransplant donor-specific T-cell alloimmune response; dnDST *de novo* donor-specific T-cell alloimmune response

Supplementary Tables

Supplementary table 1. Donor/recipient HLA allelic, amino acidic MM, HLAMatchmaker Eplet and PIRCHE-II of all patients of the study.

Donor/Recipient HLA MM scores	All patients N=169
HLA allelic MM	8 (7-10)
Class I (A, B, C)	4 (3-5)
Class II (DRB1, DQB1; DPB1)	4 (3-5)
Amino acidic HLA MM / Solvent accessible	69.5 (51-87)
A	15 (7,5-23) / 11 (5-17)
B	14 (9-18) / 6 (4-9)
C	11 (6-15) / 6 (2-9)
DRB1	11 (4-17) / 8 (3-14)
DQB1	12 (3-18) / 8 (2-13)
DPB1	4 (1-10.25) / 2 (1-7)
Eplet MM Load / Antibody verified	33 (24-40)
Eplet Class I (A+B+C)	15 (11-19) / 9 (6-11)
Eplet Class II	19 (11-25) / 7 (4-9)
Eplet DRB1	8 (3-12) / 3 (1-5)
Eplet DQB1	7 (2-10) / 2 (1-4)
Eplet DPB1	2 (1-5) / 1 (0-2)
PIRCHE-II score *(originated peptides per locus)*	71 (51-102)
PIRCHE-II A	14 (6-25)
PIRCHE-II B	14 (6-25)
PIRCHE-II C	12 (8-22)
PIRCHE-II DRB1	10 (4-16)
PIRCHE-II DQB1	17 (7-26)
PIRCHE-II DPB1	3 (0-7)

Data are median (Inter-quartile Range: IQR).

Abbreviations: MM: mismatch

Supplementary table 2. Comparison of main baseline characteristics and clinical outcomes according to pretransplant DST.

Main baseline characteristics	preDST- (n= 91)	preDST+ (n=78)	P value
Recipient age (Years)	51.5±14	51.9±14	0.84
Recipient gender (Male)	60 (66)	50 (64)	0.80
Race (Caucasic)	86 (95)	72 (92)	0.56
Cause of End Stage disease			0.77
Vascular	10 (11)	10 (13)	
Diabetes	4 (4)	4 (5)	
Glomerular	28(31)	20 (26)	
Polycystic Kidney disease	15 (16)	8 (10)	
Interstitial disease	12 (13)	12 (15)	
Others/unknown	22 (24)	24 (31)	
Time on dialysis (months)	26.32±35	24.19±33	0.69
Transplant type (deceased)	59 (65)	56 (72)	0.33
Transplant number (1)	83 (91)	69 (88)	0.55
Cold ischemia time (Hours)	12.34±10	13.25±9.4	0.55
Pre-transplant anti-HLA antibodies (cPRA)			
- Class I	10 (12)	7 (9)	0.51
- Class II	22 (27)	16 (21)	0.35
Main Clinical Outcomes			
DGF	22 (24)	24 (31)	0.36
BPAR	4 (4)	15 (19)	**0.002**
TCMR	3	12	**0.006**
ABMR	1	3	0.24
De novo DSA	13 (14)	11 (14)	
HLA Class I	3 (23)	3 (27)	0.97
HLA Class II	11 (85)	8 (73)	
HLA Class I&II	0	1 (9)	
eGFR (ml/min/1.73m^2)			
12 months (n=160)	54.52±17.7	55.31±18.7	0.77
24 months (n=157)	53.38±17.7	52.49±19.0	0.76
36 months (n=146)	52.32±19.2	50.73±17.3	0.60
Death-censored graft loss	5 (6)	4 (5)	0.87
Patient death	7 (8)	4 (5)	0.50

Abbreviations: cPRA: calculated panel of reactive antibodies; rATG: rabbit anti thymoglobulin; CNI: Calcineurin inhibitor; DGF: delayed graft function; eGFR: estimated glomerular filtrate rate (CKD-EPI); BPAR: Biopsy-proven acute rejection; TCMR: T cell mediated rejection; ABMR: antibody-mediated rejection; DSA: donor-specific antibodies.

Supplementary table 3. DST frequencies primed by the direct and indirect pathway of antigen presentation

	Global postDST	Frequencies of DP_DST	Frequencies of IP_DST	Presence of IP	HLA allelic MM	Amino acid MM	Eplet MM Load	Global PIRCHE-II
preDST+								
1	108	80	28	Yes	8	50	32	46
2	85	50	35	Yes	6	25	37	141
3	69	78	0	No	6	60	33	41
4	32	32	0	No	9	51	19	26
5	36	40	0	No	7	79	42	57
6	31	35	0	No	7	87	40	112
7	234	234	0	No	8	59	23	30
8	95	100	0	No	8	40	8	69
9	26	30	0	No	4	55	17	65
dnDST+								
1	54	35	20	Yes	7	46	25	55
2	68	6	60	Yes	8	89	48	102
3	52	40	12	Yes	9	63	36	187
4	91	70	21	Yes	8	51	29	102
5	250	30	200	Yes	7	71	38	112
6	45	65	0	No	6	41	28	55
7	41,5	45	0	No	5	42	15	10
8	79,3	80	0	No	7	52	29	100
9	33	33	0	No	8	92	50	52
persistDST+								
1	44	35	10	Yes	9	51	19	26
2	53	19,5	30	Yes	10	57	28	53
3	80.5	38	40	Yes	9	104	38	85
4	65	45	20	Yes	8	50	32	46
5	79	51	30	Yes	8	40	8	69
6	71	80	0	No	10	61	33	67
7	60.8	70	0	No	6	58	33	41
8	36	55	0	No	7	79	42	57
9	234	230	0	No	8	59	23	30

Abbreviations: DST: donor specific T cell alloreactivity (DP_DST: direct pathway; IP_DST indirect pathway; postDST: post-transplant DST; dnDST: *de novo* DST; persistDST: persistent DST). MM: mismatch

Article 3.

Preformed T cell alloimmunity and HLA eplet mismatch to guide immunosuppression minimization with tacrolimus monotherapy in kidney transplantation: Results of the CELLIMIN trial

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Objective: *to evaluate the value of assessing preformed donor-reactive IFN-γ-producing T cells and donor/recipient Molecular HLA mismatching to identify kidney transplant recipients at low risk of developing allograft rejection when receiving reduced immunosuppression based on tacrolimuns monotherapy.*

The CELLIMIN trial was a multicenter biomarker-driven randomized controlled trial assessing if immunosuppression minimization with Tacrolimus monotherapy is feasible in patients with low pre-transplant immune risk as defined by the absence of both humoral and T-cell alloreactivity (negative pretransplant DSA and negative donor-specific IFN-γ T cell ELISPOT).

Results were limited by early trial termination due to slow recruitment and we analyzed the outcomes of 167 patients: 101 ELISPOT negative (E-) randomized to either standard of care triple maintenance therapy (E-/SOC) or Tacrolimus monotherapy (E-/LI), and 66 ELISPOT positive (E+) receiving standard of care treatment. All patients received no T-cell depleting induction therapy with Basiliximab.

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ORIGINAL ARTICLE

AJT

Preformed T cell alloimmunity and HLA eplet mismatch to guide immunosuppression minimization with tacrolimus monotherapy in kidney transplantation: Results of the CELLIMIN trial

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Abbreviations: ABMR, antibody-mediated rejection; BL, borderline lesions; BPAR, biopsy-proven acute rejection; DSA, donor-specific alloantibodies; DSMB, Data Safety Monitoring Board; E−, ELISPOT negative; E+, ELISPOT positive; eGFR, estimated glomerular filtration rate; HLA, Human Leukocyte Antigens; IFN-γ ELISPOT, Interferon gamma Enzyme-linked ImmunoSpot; ITT, intention-to-treat; LI, low immunosuppression (tacrolimus monotherapy); MFI, mean fluorescence intensity; MM, mismatches; MMF, mycophenolate mofetil; PP, per protocol; PVAN, polyoma-virus-associated nephropathy; sc-BPAR, subclinical BPAR; SOC, standard of care; SOP, standard operating procedures; TAC, tacrolimus; TCMR, T cell–mediated rejection.

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Personalizing immunosuppression is a major objective in transplantation. Transplant recipients are heterogeneous regarding their immunological memory and primary alloimmune susceptibility. This biomarker-guided trial investigated whether in low immunological-risk kidney transplants without pretransplant DSA and donor-specific T cells assessed by a standardized IFN-γ ELISPOT, low immunosuppression (LI) with tacrolimus monotherapy would be non-inferior regarding 6-month BPAR than tacrolimus-based standard of care (SOC). Due to low recruitment rates, the trial was terminated when 167 patients were enrolled. ELISPOT negatives (E−) were randomized to LI (n = 48) or SOC (n = 53), E+ received the same SOC. Six- and 12-month BPAR rates were higher among LI than SOC/E− (4/35 [13%] vs. 1/43 [2%], p = .15 and 12/48 [25%] vs. 6/53 [11.3%], p = .073, respectively). E+ patients showed similarly high BPAR rates than LI at 6 and 12 months (12/55 [22%] and 13/66 [20%], respectively). These differences were stronger in *per-protocol* analyses. Post-hoc analysis revealed that poor class-II eplet matching, especially DQ, discriminated E− patients, notably E−/LI, developing BPAR (4/28 [14%] low risk vs. 8/20 [40%] high risk, p = .043). Eplet mismatch also predicted anti-class-I (p = .05) and anti-DQ (p < .001) *de novo* DSA. Adverse events were similar, but E−/LI developed fewer viral infections, particularly polyoma-virus-associated nephropathy (p = .021). Preformed T cell alloreactivity and HLA eplet mismatch assessment may refine current baseline immune-risk stratification and guide immunosuppression decision-making in kidney transplantation.

KEYWORDS
biomarker, clinical decision-making, clinical research/practice, clinical trial, immunobiology, immunosuppression/immune modulation, immunosuppressive regimens - minimization/withdrawal, kidney transplantation/nephrology, rejection: acute

1 | INTRODUCTION

Kidney transplantation is the best treatment for end-stage kidney failure as it improves both quality of life and survival, and it is cost-effective.[1] However, despite optimal short-term outcomes, long-term graft and patient survival remain almost unchanged and unsatisfactory, [2] mainly due to chronic immune-mediated graft injury in addition to the adverse effects related to chronic immunosuppressive therapy.[3,4]

Transplant recipients are not a homogeneous population both in terms of immunological experience and susceptibility for *de novo* alloimmune activation against mismatched donor human leukocyte antigens (HLAs).[5] Hence, the implementation of novel immune tools identifying the distinct anti-donor immune risk is warranted to enable safe individualized immunosuppressive strategies while avoiding unnecessary toxic treatments.[6,7]

Current immunological risk assessment prior to transplantation is exclusively based on the detection of preformed circulating donor-specific alloantibodies (DSA), assuming that humoral allosensitization relates to the allospecific T cell memory immune compartment. However, cellular alloreactivity may occur without humoral activation[8] and plays a major role in initiating and mediating allograft rejection.[9-11] Among different attempts to monitor alloreactive T cell memory *ex vivo*, measuring the frequencies of circulating donor-specific IFN-γ-secreting memory T cells using Enzyme-linked ImmunoSpot (ELISPOT) assays has been shown to be feasible[12,13] and capable of assessing the risk of T cell–mediated rejection (TCMR) both in non-human primates[14] and kidney transplant patients.[15-17] Overall, these studies have shown the potential to specifically rule out the rejection risk among transplant candidates without detectable anti-donor T cell alloimmune responses. The data suggest that the IFN-γ ELISPOT assay is a valuable tool that can be used to guide decision-making regarding the rejection risk and the type and burden of immunosuppressive therapy.[18] To date, most of the studies reported are retrospective and based on small, single-center cohorts and no prospective, randomized trials with treatment interventions guided by the ELISPOT assay have been conducted. Therefore, most biomarkers have no direct impact on guidance of immunosuppression.

Within the European FP7 BIO-DrIM (*BIOmarker-Driven personalized IMmunosuppression*) consortium, the CELLIMIN trial (*Prospective donor-specific Cellular alloresponse assessment for Immunosuppression Minimization in de novo renal transplantation*) was designed to evaluate the usefulness of assessing pretransplant donor-reactive T cell memory, using an IFN-γ ELISPOT assay with a validated standardized operational procedure in each center, to identify kidney transplant

candidates that could safely benefit of receiving lower immunosuppressive burden with tacrolimus (TAC) monotherapy soon after transplantation. The feasibility of implementing a new immune assay in clinical transplantation, and a non-inferior hypothesis regarding the incidence of biopsy-proven acute rejection (BPAR) as compared to recipients with the same immune-risk profile receiving current standard of care (SOC) therapy based on TAC, mycophenolate mofetil and prednisone, was tested. The main hypothesis of the trial was that by excluding preformed anti-donor immune memory, both cellular and humoral, TAC monotherapy would be effective enough to abrogate primary anti-donor immune activation while reducing drug-related toxicity within the first year after transplantation.

2 | METHODS

2.1 | Study design

The CELLIMIN trial was a prospective, multi-center, biomarker-driven, randomized trial performed within the European BIO-DRIM research consortium, sponsored by the European Union Seventh Framework Program (FP7-HEALTH-2012-INNOVATION-1, grant agreement nº 305147). Eight kidney transplant centers across Europe participated in the trial, Bellvitge University Hospital (Barcelona, Spain), Charité (Berlin, Germany), Amsterdam University Medical Centers (Amsterdam, the Netherlands), Universitätsklinikum Hamburg-Eppendorf (Hamburg, Germany), Institute for Clinical and Experimental Medicine (Prague, Czech Republic), Centre Hospitalier Universitaire Nantes (Nantes, France), University Hospital Regensburg (Regensburg, Germany), and University Hospital Marqués de Valdecilla (Santander, Spain). Each center participated under the approval of the Europe-wide voluntary harmonization process (VHP). An external Data Safety Monitoring Board (DSMB) was responsible for periodic safety review and guided by predetermined protocol–defined stopping criteria.

The study protocol is available online at https://clinicaltrials.gov/ct2/show/NCT02540395.

2.2 | Participants

Low immunological risk subjects were eligible to participate if >18 years of age and receiving a primary single kidney transplant (inclusion and exclusion criteria are described in Data S1). Enrolment was targeted to 673 patients, with 302 E− transplant patients randomized to low or SOC immunosuppression. However, due to slow patient enrolment, the trial was terminated when 167 were recruited. In all, 101 patients were randomized and followed for 12 months.

All subjects freely gave written informed consent prior to participation, including informed consent for the screening procedures to establish subject eligibility.

2.3 | Procedures

2.3.1 | Study treatments

Transplant patients were first allocated into two groups according to their pretransplant donor-specific IFN-γ ELISPOT result (flow chart of the study in Figure 1).

Group I. ELISPOT negative (E−) candidates were randomized to receive:

- **Standard of care immunosuppression (SOC):** Based on current standard of care therapy consisting in TAC to achieve a 4–8 ng/ml plasma trough levels, mycophenolate mofetil (MMF) initially 1gr bid and subsequently adjusted according to the subjects tolerance, and 500 mg methylprednisolone perioperatively to continue with oral prednisone (20 mg/day the first 2 weeks and tapered not less than 5 mg/day at 4 weeks posttransplant).
- **Low immunosuppression (LI):** Based on TAC monotherapy to achieve TAC 8–10 ng/ml plasma trough levels during the first 4 weeks and 6–8 ng/ml thereafter, MMF (1 g bid) during the first week posttransplant and stopped thereafter, and 500 mg methylprednisolone perioperatively to continue with oral prednisone 20 mg/day the first 2 weeks and tapered to 5 mg/day from month 1 to month 2 when finally discontinued.

Group II. ELISPOT positive (E+) transplant candidates received the same current standard of care immunosuppressive regimen than group E−/SOC.

All patients received two doses of basiliximab (20 mg) at days 0 and 4 after transplantation.

Patients were followed up for a total of 12 months for secondary outcome measures.

Types of BPAR rescue therapies were provided according to the respective standard of care in each center: for TCMR: Banff <IIA TCMR, 3 doses of 500 mg of 6-Methyl prednisolone; Banff >IB TCMR, 3–5 doses of 1 mg/kg Thymoglobulin. For ABMR: plasmapheresis/immunoadsorption with IVIG or Rituximab. MMF and prednisone were reintroduced in all patients developing rejection under TAC monotherapy.

2.3.2 | Histology assessment

For cause biopsies were performed in case of either lack of graft function improvement or sudden graft dysfunction by means of serum creatinine, estimated glomerular filtration rate (eGFR) or proteinuria and rejection was defined as *clinical BPAR*. *Surveillance* biopsies were planned at 3 and 12 months after transplantation and were defined as graft biopsies performed in patients with serum creatinine <300 μmol/L; proteinuria <1 g/24 h and stable renal function (variability of serum creatinine of <15% during 2 weeks before and after biopsy) and rejection was defined as *subclinical BPAR*. All core biopsy samples were analyzed by expert transplant pathologists from each

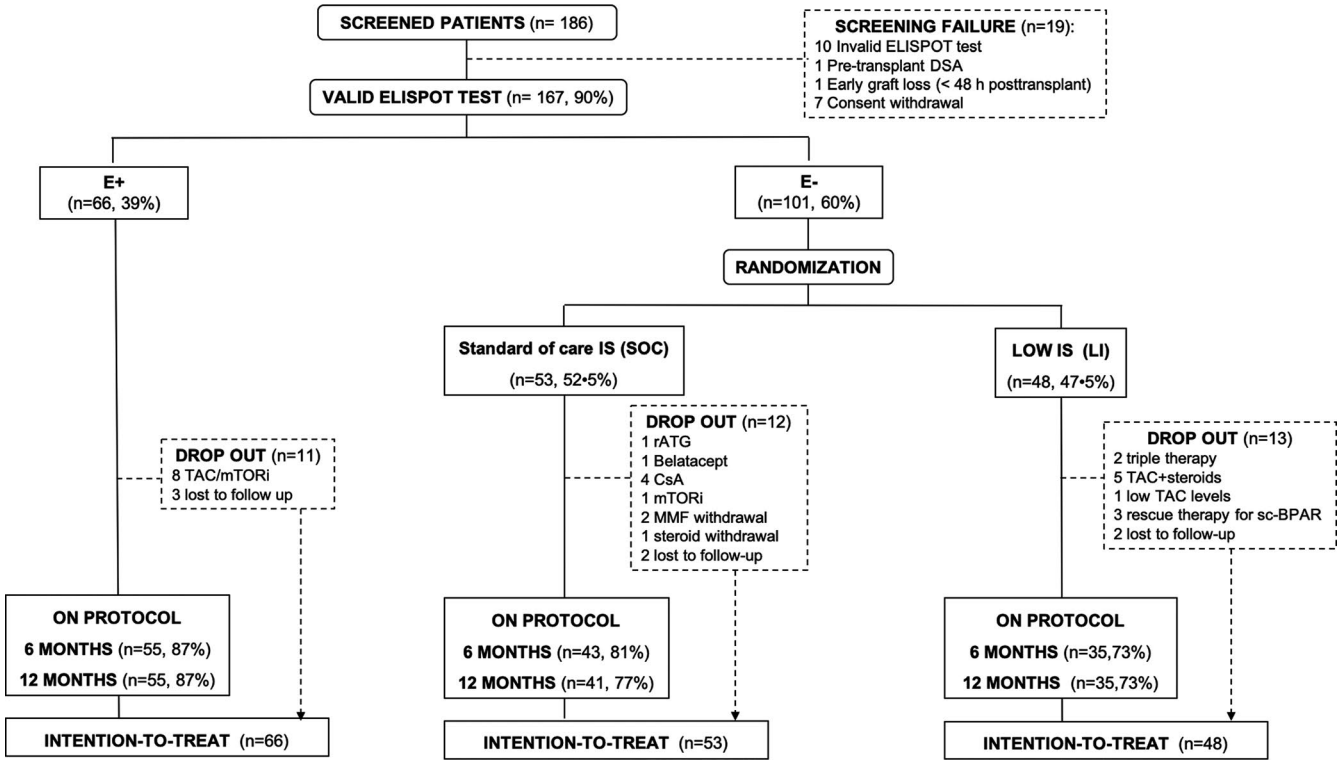

FIGURE 1 Flow chart of the study

participating center and graded following the Banff 2017 classification.[19] In total, 113 (69%) patients underwent a surveillance biopsy at 3 and/or 12 months and 106 (63.5%) were evaluable for its diagnosis; 35 (66%), 38 (79%), and 33 (50%) in the E−/SOC, E−/LI, and E+ groups, respectively.

2.3.3 | Laboratory studies

Donor-specific IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) assays

Supplemental methods report recipient and donor peripheral blood mononuclear cell and splenocytes standard operating procedures (SOP) used as well as a detailed description of the donor-specific IFN-γ ELISPOT assays, which was extensively cross-validated between centers.[12] A result of ≥25 IFN-γ ELISpots/3x10^5 PBMC was considered as a POSITIVE test, whereas <25 as NEGATIVE.

HLA typing and molecular mismatches

Donor and recipient HLA class-I (A, B, and C) and class-II (DRB1, DQB1, and DQA1) high-resolution typing was performed with NGS technology in 154/167 (92%) donor/recipient pairs on a MiSeq platform (Illumina, San Diego, California). In the remaining patients, DNA-based low-resolution HLA typing was performed with sequence-specific primers (SSP) and were extrapolated to high-resolution using a previously validated computational method based on haplotype frequency Tables.[20,21] Donor/recipient HLA eplet mismatches (both non-verified and antibody-verified) were determined by the last versions of the HLAMatchmaker software (HLA-ABC Eplet Matching V3.1 and DRDQDP Eplet Matching Program V3.1). Results were also compared with the previous HLA-Matchmaker software version (HLA-ABC Eplet Matching Version 2 and DRDQDP Eplet Matching Program V2.2).[22]

Anti-HLA antibody determination

A Single-Antigen Class-I and Class-II flow beads-assay kit was used (Lifecodes, Immucor, Stanford, CA) to monitor serum anti-HLA antibodies at baseline and at 12 months after transplantation. All beads showing a normalized mean fluorescence intensity (MFI) >500 were considered positive if (MFI/MFI lowest bead)>5.

2.4 | Outcomes

The primary study endpoint was to demonstrate in a per-protocol analysis, non-inferiority rates of BPAR, excluding borderline lesions, in *for cause* biopsies at 6 months after transplantation, allowing a non-inferiority margin of 10% (full description in Data S1).

Secondary outcomes analyzed as a post-hoc analysis were as follows: incidence of clinical and subclinical BPAR both per protocol and intention-to-treat, also taking into account the E+ group of patients, differences in eGFR, dnDSA, graft and patient survival and impact of donor/recipient HLA molecular mismatches on BPAR and dnDSA between groups at 12 months of follow-up.

Incidence of adverse events, serious adverse events, infections, and malignancies was recorded in each center.

TABLE 1 Main clinical and demographic characteristics of the patients of the study

	E−/SOC (*n* = 53)	E−/LI (*n* = 48)	E+ (*n* = 66)	*p* value
Recipient age (years)	53.51 ± 12.81	54.68 ± 14.11	53.88 ± 13.97	.907
Recipient sex				
Female	12 (22.6)	16 (33.3)	19 (29.2)	.481
Male	41 (77.4)	32 (66.7)	46 (70.8)	
Recipient ethnicity				
Caucasian	50 (94.3)	45 (93.75)	46 (97.9)	.574
No Caucasian	3 (5.7)	3 (6.25)	1 (2.1)	
Cause of end-stage renal disease				
Glomerulonephritis	10 (18.9)	15 (31.9)	17 (26.2)	.514
Vascular	3 (5.7)	3 (6.4)	8 (12.3)	
Diabetes Mellitus	12 (22.6)	4 (8.5)	7 (10.8)	
Polycystic kidney disease	12 (22.6)	10 (21.3)	13 (20)	
Unknown	9 (17)	10 (21.3)	15 (23.1)	
Others	7 (13.2)	5 (10.4)	5 (7.5)	
Type of donor				
Living	28 (52.8)	26 (54.2)	35 (53)	.990
Living-related, yes	11 (20.8)	11 (22.9)	25 (37.9)	.384
Preemptive transplantation	13 (24.5)	36 (75)	40 (60.6)	.133
Time on dialysis (months)	41.20 ± 50.44	34.50 ± 51.06	23.06 ± 28.30	.088
CMV prophylaxis, yes	15 (28.8)	14 (31.1)	23 (39)	.492
Baseline Panel Reactive Antibodies	0.45 ± 2.43	0.0 ± 0	0.23 ± 1.14	.469
Preformed DSA	0 (0)	0 (0)	0 (0)	1.000
HLA allelic MM	5.58 ± 2.59	6.77 ± 1.77	7.24 ± 2.3	.001*
Class I	3.57 ± 1.69	4.33 ± 1.19	4.24 ± 1.59	.03
Class II	2.02 ± 1.29	2.44 ± 1.09	3.00 ± 1.07	<.001
Pretransplant donor-specific IFN-γ ELISpots (per 3 x 10^5 PBMC)	7.75 ± 6.82	7.67 ± 7.03	80.02 ± 84.13	<.001**
Delayed graft function	14 (26)	7 (15)	7 (11)	.137
Kidney graft loss	3 (5.8)	0 (0)	1 (1.6)	.155
Patient death	2 (3.8)	1 (2.1)	1 (1.5)	.704

Abbreviations: CMV, cytomegalovirus; E−/LI, donor-specific ELISPOT negative/low immunosuppression; E−/SOC, donor-specific ELISPOT negative/standard of care immunosuppression; E+, donor-specific ELISPOT positive; HLA, human leukocyte antigen; MM, mismatches.

Data are mean ± SD or n (%).

*Total HLA allelic MM: E−/SOC vs. E−/LI $p = .036$; E−/SOC vs. E+ $p = .001$; E−/LI vs. E+ $p = .55$. Class I HLA allelic MM: E−/SOC vs. E−/LI $p = .043$; E−/SOC vs. E+ $p = .058$; E−/LI vs. E+ $p = .95$. Class II HLA allelic MM: E−/SOC vs. E−/LI $p = .19$; E−/SOC vs. E+ $p < .001$; E−/LI vs. E+ $p = .038$. ; **Pretransplant donor-specific IFN-γ ELISpots: E−/SOC vs. E−/LI $p = 1.000$; E−/SOC vs. E+ $p < .001$; E−/LI vs. E+ $p < .001$.

2.5 | Statistical analysis

The study design and sample size calculation are depicted in detail in Data S1. Since the primary study endpoint could not be achieved, a number of clinically relevant outcomes were analyzed as a post-hoc analysis. Comparisons of the primary and secondary outcomes across ELISPOT subgroups were done using a chi-square test for qualitative data and T-test or Wilcoxon signed-ranked test for the comparison of continuous secondary outcomes. The time-dependent association of the variables assessed with BPAR was

studied using Kaplan–Meier plots and log-rank test. Receiver operating characteristic (ROC) curve analysis was used to evaluate most sensitive and specific donor/recipient HLA molecular mismatch cutoffs predicting BPAR. The statistical significance level was defined as two-tailed $p < .05$. Statistical analyses were performed with IBM SPSS Statistics, version 26 and GraphPad Prism version 6.0 (GraphPad Software).

3 | RESULTS

3.1 | Patients of the study and main clinical outcomes

As described in Figure 1, a total of 186 patients were screened and 167 enrolled between December 8, 2015 and October 23, 2018; 66 (39%) were Elispot positive (E+), whereas 101 (60%) E− and were subsequently randomized to receive either lower immunosuppression (LI) with TAC monotherapy (n = 48, 47.5%) or current SOC (n = 53, 52.5%). Despite the high recruitment priority established in each center, the stringent low immunological risk inclusion criteria led to insufficient recruitment rates. Thus, in agreement with the DSMB, the trial was terminated.

Main baseline clinical characteristics were not different between groups (Table 1), but E+ showed higher HLA allelic mismatches and, as per study design, higher donor-reactive IFN-γ ELISpots. There were four (2.3%) graft losses, three within the E−/SOC group (two because of obstructive nephropathy and one for polyoma-virus-associated nephropathy), and one in the E+ because of chronic antibody-mediated rejection (cABMR), and there were four (2.3%) deaths (two E−/SOC patients because of a bacterial sepsis and lung cancer, one in the E−/LI group due to multiple myeloma and one in the E+ group because of sudden cardiac arrest). At 6 months, 133 (80%) patients remained on protocol and 131 (78%) at 12 months; 41 (77%) in the E−/SOC, 35 (73%) E−/LI, and 55 (87%) E+. Main causes of dropout are described in Table S1.

As per study protocol, plasma TAC trough levels were significantly higher among E−/LI than E−/SOC and E+ patients until month 2, whereas at 3, 6, and 12 months, all groups showed similar exposure (Table S2).

3.1.1 | Incidence of BPAR in the trial

At 6 months, 21 (12.5%) patients developed clinical BPAR, 28 (17%) when including Banff borderline lesions. At 12 months, three additional clinical BPAR occurred (Banff≥IA); thus, a total of 31 (18.5%) patients developed BPAR during the 12-month follow-up (Table S3). While all BPAR within the E− groups were TCMR, there were six ABMR among E+ patients. Of the total BPAR, six occurred in patients not on protocol (three E−/LI arm [1 BL and 2 Banff≥IA] and three among E−/SOC group, all Banff≥IA). In total, 106 patients underwent a 3/12 months protocol biopsy with evaluable material. In all, 17 (16%) patients developed Banff≥IA subclinical BPAR (sc-BPAR) and 10 (9.4%) showed BL changes (Table S4). 6/17 (35.3%) patients with sc-BPAR and two out of 10 (20%) showing sc-BL changes had previously developed clinical BPAR. TAC trough levels and intrapatient variability (IPV) prior to clinical or subclinical BPAR was not associated with higher rejection rates, both globally and within each study group.

3.1.2 | Primary study endpoint

The analysis of the primary study endpoint evaluating the incidence of BPAR at 6 months between E−/SOC and E−/LI groups, excluding BL lesions, showed no statistically significant differences between groups (1/43 [2%] vs. 4/35 [13%], p = .16, respectively) (Table 2). Six-month cumulative incidences of BPAR were not different between the two E− groups both in PP and ITT analyses (Figure 2A-B).

3.2 | Post-hoc analysis of main clinical outcomes between all study groups

3.2.1 | Incidence of clinical and subclinical BPAR

When E+ patients were also analyzed, at 6 months, E+ showed significantly higher BPAR (both with and without BL lesions) than E−/SOC patients (Table 2). Similarly, at 12 months, BPAR rates were significantly higher within E+ and E−/LI patients as compared to E−/SOC, especially in patients remaining on protocol. 12-month cumulative BPAR between the three groups showed the same differences both when assessed PP or ITT (Figure 2C-D).

Likewise clinical BPAR, both E+ and E−/LI groups developed significantly higher incidence of sc-BPAR than E−/SOC (Table 2).

3.2.2 | Twelve-month de novo DSA (dnDSA)

At 12 months, 149 (89%) patients were tested for anti-HLA antibodies; 47 (88%) among E−/SOC, 43 (89%) within E−/LI and 59(89%) among E+ patients (Table S4). In all, 17 dnDSA were detected among 11 (7.4%) patients, 6 class I (3 anti-A and 3 anti-B), and 11 class II (7 anti-DQ and 4 anti-DR). As shown in Table 2, while no differences were observed regarding total dnDSA between the three groups, E+ patients displayed higher class-II dnDSA than the other groups.

3.2.3 | Kidney graft function progression

After month 2, E−/LI patients displayed lower eGFR than E−/SOC and E+ recipients until month 12 after transplantation (Figure 3), although these differences were not significant when only patients

TABLE 2 Main study outcomes between the different study groups

	E−/SOC	E−/LI	E+	E−/LI vs. E−/SOC	E+ vs. E−/LI	E+ vs. E−/SOC
6-mo PP (*n* = 133)	*n* = 43	*n* = 35	n = 55	*p* values		
BPAR (excluding BL)[a]	1 (2)	4 (13)	12 (22)	.158[b]	0.394	0.006
BPAR	3 (7)	8 (23)	12 (22)	.056	0.908	0.051
12-mo PP (*n* = 131)	*n* = 41	*n* = 35	*n* = 55			
BPAR	3 (7)	9 (26)	13 (24)	.055	0.823	0.051
BPAR ITT (*n* = 167)	*n* = 53	*n* = 48	*n* = 66	*p* values		
6-mo BPAR	5 (9.5)	11 (23)	12 (18)	.064	0.534	0.175
12-mo BPAR	6 (11.3)	12 (25)	13 (20)	.073	0.499	0.213
Sc-BPAR	1 (2.9)	10 (26.3)	6 (18.2)	.005	0.413	0.038
Sc-BL	4 (11.4)	4 (10.5)	2 (6.1)	.902	0.500	0.435
De novo DSA	*n* = 47	*n* = 43	*n* = 59	*p* values		
Total dnDSA	1 (2)	3 (7)	7 (12)	.345	0.513	0.074
Class-I dnDSA	1 (2)	3 (7)	2 (3.4)	.345	0.648	1.000
Class-II dnDSA	0	1 (2)	7 (12)	.478	0.134	0.017

Abbreviations: BL, Banff borderline lesions; BPAR, biopsy-proven acute rejection; dnDSA, *de novo* donor-specific antibodies; E−/LI, donor-specific ELISPOT negative/low immunosuppression; E−/SOC, donor-specific ELISPOT negative/standard of care immunosuppression; E+, donor-specific ELISPOT positive; ITT, intention-to-treat; mo, months; PP, per protocol; Sc-BPAR, subclinical biopsy-proven acute rejection.

All BPAR analyses include Banff borderline (BL) lesions but the primary study endpoint.

Data are mean ± SD or n (%).

[a]Patients having received rescue therapy due to borderline BPAR prior to 6 months (*n* = 4, in the E−/LI and *n* = 2 in the E−/SOC) were excluded of this per protocol analysis.

[b] Statistical comparison of the primary endpoint of the CELLIMIN trial.

on protocol were analyzed. 12-month eGFR was lower among E−/LI patients developing BPAR as compared to those that did not. These differences were not observed in the other two groups. Subclinical BPAR did not impact on 12-month eGFR in any study group (Figure S1).

3.3 | HLA eplet mismatching and *de novo* alloimmune activation

We next assessed the impact of donor/recipient HLAMatchmaker eplet mismatches on main immune-mediated events between the distinct study groups. Similar to HLA allele mismatches, E− patients showed lower eplet mismatches as compared to E+ (Table S5).

3.3.1 | HLA eplet mismatching and incidence of BPAR

Mean class-II eplet mismatches (MM) (DRB1+DQ), and particularly at DQ locus, were significantly higher in patients developing BPAR than in those that did not (Figure 4

). However, these differences were only observed among the two E− study groups. A threshold of DQ (A1/B1) eplet mismatches ≥10 defined high eplet risk for BPAR with the highest accuracy within all E− patients (AUC = 0.733; 95% CI 0.612–0.853) (Figure S2). As illustrated in Figure 5A, high-risk DQ eplet mismatching was associated with higher BPAR rates only among E− patients, and particularly among E−/LI (6/28 [21%] in E+, 1/28 [4%] in E−/SOC and 4/28 [13%] in E−/LI, $p = .137$ in low-risk eplet patients, whereas 7/38 [18%] in E+, 5/25 [20%] in E−/SOC and 8/20 [40%] in E−/LI, $p = .16$ within the high-risk eplet group). When we analyzed the association between eplet MM risk score and global BPAR rates (clinical and/or subclinical), similarly higher rates of BPAR and/or sc-BPAR were observed among E− patients, especially within E−/LI patients, with high-risk eplet score ($p = .07$) (Figure 5b).

DQ eplet MM risk score at the single donor molecule identified three risk groups (low risk: 0 DQ MM, intermediate: 1–5; high: ≥6 DQ MM), although with lower predictive accuracy (AUC = 0.684; 95% CI 0.59–0.78, $p < .001$). High-risk patients did also display significantly higher BPAR rates than low and intermediate-risk groups within E−/SOC and E−/LI patients (1/25 [4%] vs. 5/28 [18%], $p = .19$ in E−/SOC and 1/14 [7%] vs. 11/34 [32%], $p = .06$ in E−/LI) but not in E+ (4/21 [19%] vs. 9/45 [20%], $p = 1$].

3.3.2 | HLA eplet mismatching and de novo DSA

Patients with anti-class-I and anti-DQ dnDSA displayed significantly higher class I and DQB1 single molecule eplet mismatches

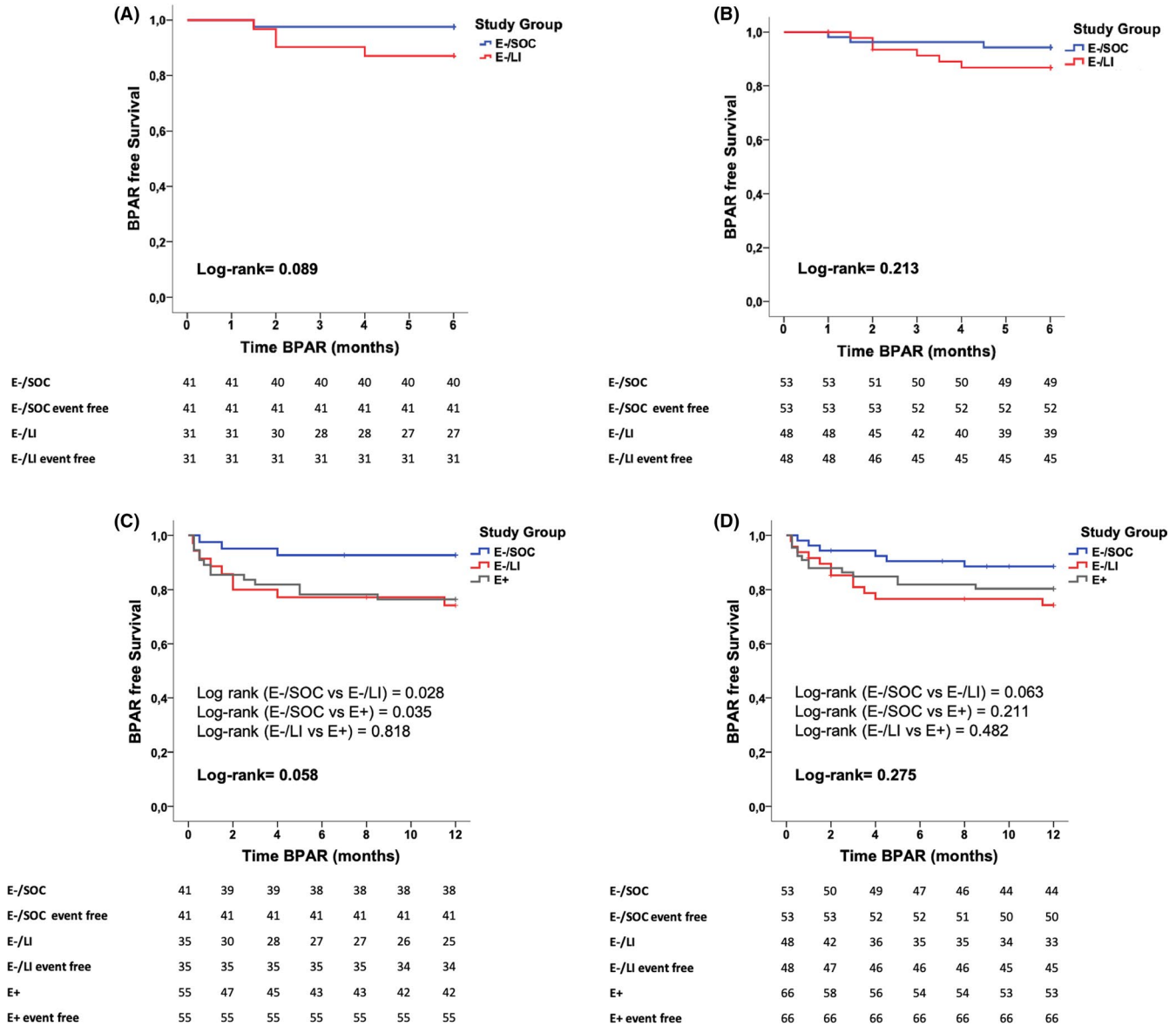

FIGURE 2 BPAR rates between the study groups in all patients and in patients on protocol at 6 and 12 months. (A) Six-month Kaplan–Meier BPAR-free (excluding BL lesions) survival curves in patients on protocol (primary endpoint) (n = 72) in the two E− groups (log rank = 0.089). (B) Six-month Kaplan–Meier BPAR-free (excluding BL lesions) survival curves in all patients (intention to treat) (n = 101) in the two E− groups (log rank = 0.213). (C) Twelve-month Kaplan–Meier BPAR-free (including BL lesions) survival curves in patients on protocol (n = 131) according to the three different study groups (log rank = 0.058). Log rank (E−/SOC vs. E−/LI) =0.028; log rank (E−/SOC vs. E+) =0.035; log rank (E−/LI vs. E+) =0.818. (D) Twelve-month Kaplan–Meier BPAR-free (including BL lesions) survival curves in all patients (intention-to-treat) (n = 167) according to the three different study groups (log rank = 0.275). log rank (E−/SOC vs. E−/LI) =0.063; log rank (E−/SOC vs. E+) =0.211; log rank (E−/LI vs. E+) =0.482

than patients that did not, respectively (14.74 ± 7.04 vs. 19.00 ± 2.89, p = .050 for class I and 5.19 ± 5.16 vs. 13.33 ± 5.09, p < .001for DQ) (Figure S3). Eplet mismatches at the DR locus were not assessed because only four patients developed anti-DR dnDSA.

A high correlation between the number of eplet MM detected with the two most recent HLAMatchmaker algorithms versions (V2 and V3.1) was observed (Spearman Rho >0.9 and p < .001 at all loci). The same impact on main clinical outcomes both BPAR and dnDSA was similarly observed with the two algorithms (data not shown).

3.4 | Safety

The number of adverse and serious adverse events did not differ between the three study groups (Table 3). While the incidence of any kind of infection equally occurred across the three groups, a

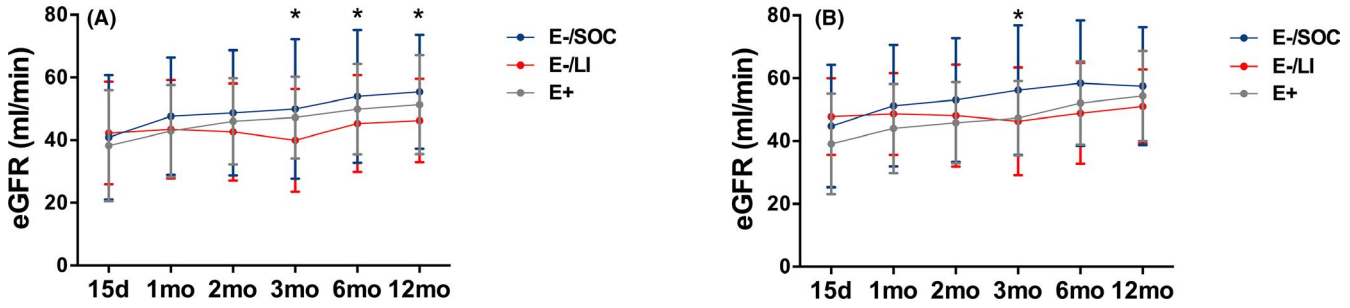

FIGURE 3 Twelve-month eGFR progression between study groups. (A) Twelve-month eGFR progression between study groups in all patients (intention to treat), n = 167. eGFR were 40.88 ± 19.88 vs. 42.26 ± 16.36 vs. 38.21 ± 17.74 ml/min, p = .549 at 15 days; 47.66 ± 18.71 vs. 43.46 ± 15.69 vs. 42.93 ± 14.70 ml/min, p = .266 at 1 month; 48.72 ± 19.98 vs. 42.62 ± 15.51 vs. 46.00 ± 13.79 ml/min, p = .202 at 2 months, 49.95 ± 22.27 vs. 39.97 ± 16.41 vs. 47.20 ± 13.03 ml/min, p = .019 at 3 months, 53.95 ± 21.16 vs. 45.31 ± 15.44 vs. 49.91 ± 14.41 ml/min, p = .078 at 6 months, and 55.44 ± 18.21 vs. 46.25 ± 13.29 vs. 51.36 ± 15.81 ml/min, p = .030 at 12 months in E−/SOC vs. E−/LI vs. E+, respectively. (B) Twelve-month eGFR progression between study groups in patients that were on protocol at 12 months (n = 106). eGFR were 44.77 ± 19.49 vs. 47.80 ± 12.15 vs. 39.11 ± 15.99 ml/min, p = .155 at 15 days; 51.25 ± 19.34 vs. 48.65 ± 12.99 vs. 44.01 ± 14.17 ml/min, p = .135 at 1 month; 53.08 ± 19.71 vs. 48.01 ± 16.23 vs. 45.84 ± 12.95 ml/min, p = .157 at 2 months, 56.25 ± 20.60 vs. 46.32 ± 17.13 vs. 47.28 ± 11.82 ml/min, p = .029 at 3 months, 58.43 ± 20.00 vs. 48.84 ± 16.07 vs. 52.10 ± 13.26 ml/min, p = .069 at 6 months, and 57.48 ± 17.86 vs. 51.04 ± 11.76 vs. 54.36 ± 14.32 ml/min, p = .296 at 12 months in E−/SOC vs. E−/LI vs. E+, respectively

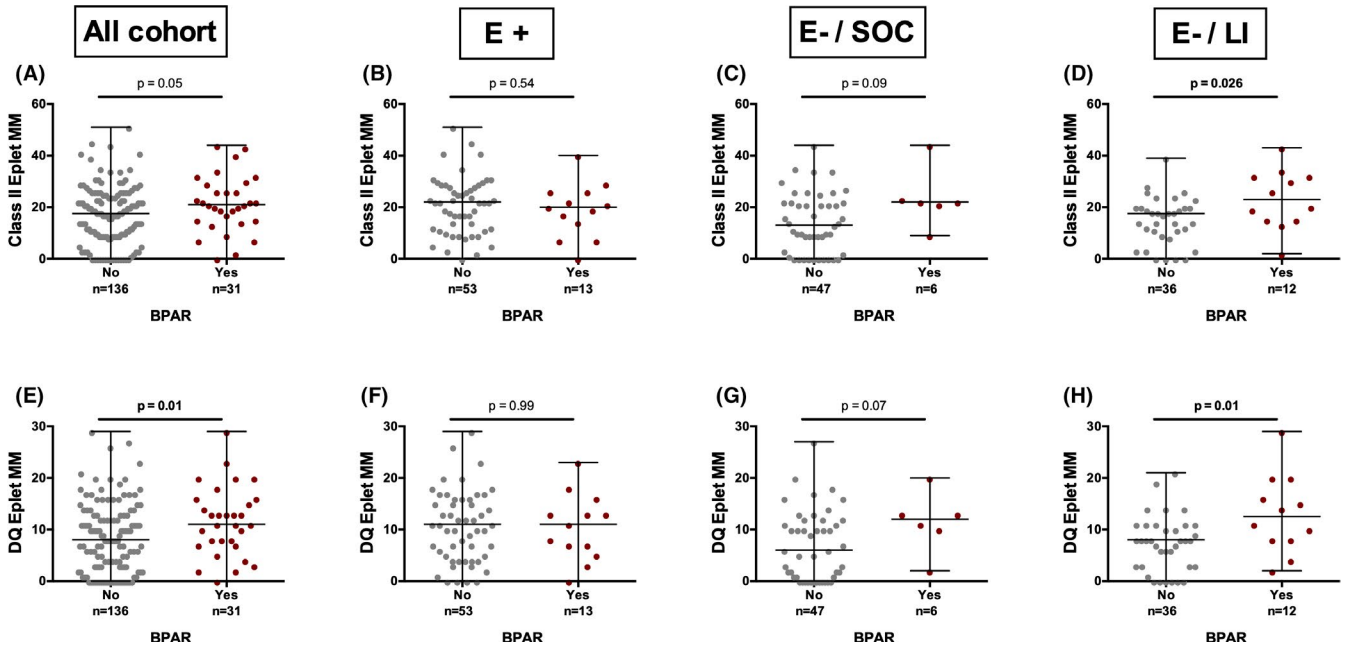

FIGURE 4 Mean donor/recipient HLA class II and DQ eplet MM between patients with or without BPAR. (A) Mean donor/recipient HLA class-II eplet MM and BPAR in all patients: 21.61 ± 10.88 in BPAR patients vs. 17.12 ± 11.16 in patients not experiencing BPAR, p = .05. (B) Mean donor/recipient HLA class-II eplet MM and BPAR in E+ patients: 19.08 ± 10.53 in BPAR patients vs. 20.91 ± 11.10 in patients not experiencing BPAR, p = .529. (C) Mean donor/recipient HLA class-II eplet MM and BPAR in E−/SOC patients: 23.50 ± 11.32 in BPAR patients vs. 14.25 ± 11.57 in patients not experiencing BPAR, p = .089. (D) Mean donor/recipient HLA class-II eplet MM and BPAR in E−/LI patients: 23 ± 42 in BPAR patients vs. 15.31 ± 9.22 in patients not experiencing BPAR, p = .026. (E) Mean donor/recipient HLA DQ eplet MM and BPAR in all patients: 11.71 ± 6.66 in BPAR patients vs. 8.54 ± 6.63 in patients not experiencing BPAR, p = .015. (F) Mean donor/recipient HLA DQ eplet MM and BPAR in E+ patients: 10.53 ± 6.38 in BPAR patients vs. 10.69 ± 6.87 in patients not experiencing BPAR, p = .987. (G) Mean donor/recipient HLADQ MM and BPAR in E−/SOC patients: 11.50 ± 5.82 in BPAR patients vs. 6.96 ± 6.72 in patients not experiencing BPAR, p = .07. (H) Mean donor/recipient HLA DQ MM and BPAR in E−/LI patients: 13.08 ± 7.59 in BPAR patients vs. 7.42 ± 5.31in patients not experiencing BPAR, p = .015

significantly lower incidence of viral infections, particularly BK viremia and polyoma-virus-associated nephropathy (PVAN) was observed among E−/LI patients (16 [30.2%], 6 [12.5%], and 11 [16.9%], p = .06 for BK viremia and 5 [9.4%], 0 (0%), and 1 [1.5%], p = .02 for PVAN, in E−/SOC, E−/LI, and E+ patients, respectively). No other differences were observed regarding main hematological, cardiovascular or metabolic disorders, or in the incidence of malignancies between study groups.

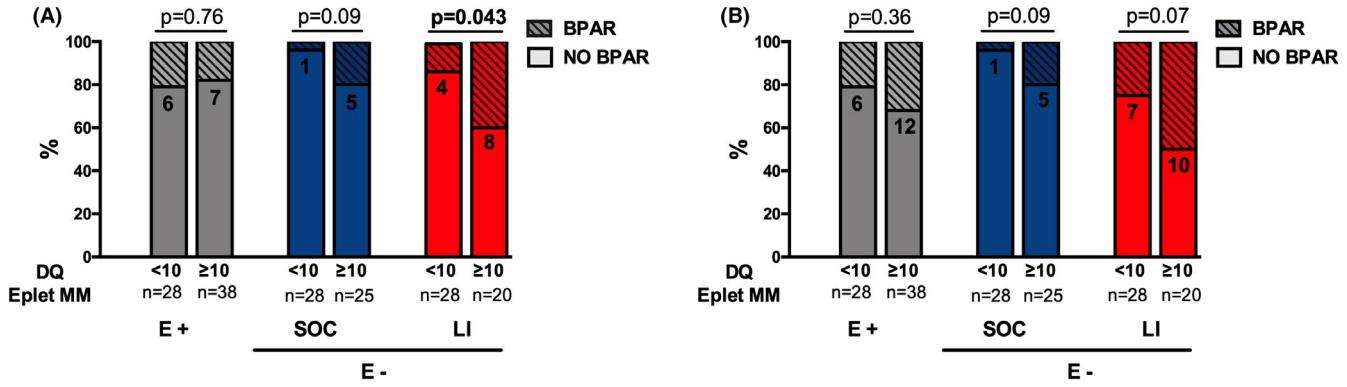

FIGURE 5 Donor/recipient HLA DQ eplet MM risk score for clinical and subclinical BPAR between study groups. (A) Donor/recipient HLA DQ eplet MM risk score for clinical BPAR between study groups. E+ patients: 6/28 (21%) low eplet risk vs. 7/38 (18%) high eplet risk, $p = .76$. E−/SOC patients: 1/28 (4%) low eplet risk vs. 5/25 (20%)high eplet risk, $p = .09$. E−/LI patients: 4/28 (14%) low eplet risk vs. 8/20 (40%) high eplet risk, $p = .043$. (B) Donor/recipient HLA DQ eplet MM risk score for clinical+subclinical BPAR between study groups. E+ patients: 6/28 (21%) low eplet risk vs. 12/38 (31%) high eplet risk, $p = .36$. E−/SOC patients: 1/28 (4%) low eplet risk vs. 5/25 (20%) high eplet risk, $p = .09$. E−/LI patients: 7/28 (25%) low eplet risk vs. 10/20 (50%) high eplet risk, $p = .07$

4 | DISCUSSION

The CELLIMIN trial was designed to evaluate the hypothesis of whether immune-monitoring preformed anti-donor T cell immune memory, posttransplant immunosuppression minimization with TAC monotherapy would be effective enough while reducing drug-related toxicities. Although we were unable to reach the statistical power required to evaluate our primary hypothesis, our findings reveal interesting novel information. First, we show that implementing a novel cellular-based immune assay measuring donor-reactive memory/effector IFN-γ-producing T cells is safe and feasible in real clinical practice. However, the higher BPAR rates observed among the low immunologic risk group receiving TAC monotherapy (E−/LI) as compared to low-risk patients receiving current standard of care therapy (E−/SOC), especially when also taking into account Banff BL lesions (25% vs. 11%), outweighs any potential benefit of maintaining E− kidney transplant recipients on TAC monotherapy on the solely basis of monitoring pretransplant anti-donor T cell memory and serum DSA. Nonetheless, we found that among patients receiving the same SOC therapy, E− transplants outperformed significantly the E+ group regarding BPAR rates, *dn*DSA formation and eGFR, suggesting the value of the ELISPOT immune-risk stratification. Moreover, and as hypothesized, patients on TAC monotherapy did benefit of lower viral infection rates as compared to patients on a triple drug-based regimen.

Since the exceeding BPAR rates among E−/LI patients as compared to E−/SOC could not be explained by preformed anti-donor T cell memory, we hypothesized whether they could rather be due to poor donor/recipient HLA eplet matching in the context of low immunosuppression. Unlike E+ transplants, E− patients with high-risk DQ eplet mismatch score more frequently developed BPAR, an effect that was even more evident within E− patients on TAC monotherapy. Indeed, while only 4/28 (14%) and 7/28(25%) of E−/LI patients with low-risk eplet score developed clinical and subclinical BPAR, respectively, up to 8/20 (40%) and 10/20 (50%) of those with a high-risk eplet score did. These findings are in agreement with previous and recent studies showing the capacity of HLA molecular mismatching predicting primary alloimmune activation, and especially in patients receiving low or insufficient immunosuppression.[23-26] Moreover, and as previously reported,[27,28] we found a close association between a poor donor/recipient HLA eplet matching at each respective locus and *dn*DSA formation. Altogether, these data suggest that adding the analysis of HLA eplet mismatching to preformed anti-donor T- and B-cell memory seems to have the potential to identify a relevant proportion of transplant recipients (25%) that could successfully receive lower immunosuppression with TAC monotherapy until 1 year after transplantation.

The assessment of preformed anti-donor T cell memory discriminated transplant patients receiving the same SOC immunosuppression who were at higher risk of BPAR. These findings corroborate previous retrospective studies[18,29] and highlight the importance of monitoring preformed T cell memory as these patients could not have been identified using current clinical and epidemiologic factors indicative of low immunological risk, such as first transplant recipients with low cPRA and no DSA. Interestingly, ABMR did only occur within E+ patients and relatively soon after transplantation, a finding suggesting the concomitant presence of anti-donor alloreactive memory B cells despite the absence of detectable DSA in serum.[30] Nevertheless, while the high BPAR rates within E+ patients seem to be predominantly driven by preformed anti-donor T cell memory, the poorer HLA matching of this group of patients, raises concerns on whether these patients might also be at high risk of subsequent primary alloimmune activation in the long term. While we cannot exclude that higher donor/recipient HLA mismatching among E+ patients may be coincidental, our data also suggest that since the ELISPOT assay used in the trial exclusively assessed donor-specific T cell responses, in the presence of a higher HLA mismatch burden, there may be a higher

TABLE 3 Adverse events (safety population) between the three study groups

	E−/SOC (*n* = 53)	E−/LI (*n* = 48)	E+ (*n* = 66)	*p* value
Any AE	53 (100)	4 (97.9)	59 (95.2)	.242
Any SAE	14 (26.4)	7 (14.6)	NA	.143
Infections				
Any infection	33 (62.3)	26 (54.2)	42 (67.7)	.347
Any viral infection	25 (47.2)	16 (33.3)	35 (56.5)	.054[a]
CMV infection	12 (22.6)	12 (25)	25 (40.3)	.079
CMV disease	4 (7.5)	1 (2.1)	4 (6.7)	.442
BKV infection	16 (30.2)	6 (12.5)	11 (16.9)	.063[b]
PVAN	5 (9.4)	0 (0)	1 (1.5)	.021[c]
Other (EBV, HSV, VZV)	5 (9.4)	1 (2.1)	6 (11.8)	.178
Any bacterial infection	19 (35.8)	19 (39.6)	15 (29.4)	.560
Any fungal infection	0 (0)	1 (2.1)	1 (2)	.580
Hematological disorders				
Hemoglobin (g/dL)	12.40 ± 4.04	11.50 ± 4.30	13.55 ± 1.86	.196
Leukocytes (1/nL)	7.62 ± 2.79	6.86 ± 2.09	7.28 ± 1.98	.355
Thrombocytes (1/nL)	229.02 ± 57.78	214.31 ± 59.47	204.11 ± 41.60	.236
Metabolic disorders				
NODAT	8 (15.1)	7 (14.6)	9 (17.6)	.903
Cholesterolemia (mmol/L)	4.86 ± 1.35	4.69 ± 0.98	4.45 ± 1.02	.450
Triglyceridemia (mmol/L)	1.86 ± 1.78	1.69 ± 0.96	1.55 ± 0.54	.766
Cardiovascular disorders				
Hypertension	43 (84.3)	40 (85.1)	39 (76.5)	.463
Cardiovascular events	3 (5.7)	3 (6.3)	5 (13.5)	.345
Cancer of any grade	3 (5.7)	4 (8.5)	5 (9.8)	.726

Abbreviations: AE, adverse event; BKV, BK virus; CMV, cytomegalovirus; E−/LI, donor-specific ELISPOT negative/low immunosuppression; E−/SOC, donor-specific ELISPOT negative/standard of care immunosuppression; E+, donor-specific ELISPOT positive; EBV, Epstein-bar virus; HSV, Herpes simplex virus; NODAT, New onset diabetes mellitus; PVAN, Polyomavirus virus nephropathy; SAE, serious adverse event; VZV, varicella-zoster virus.

Data are mean ± SD or n (%).

[a]Any viral infection: E−/SOC vs. E−/LI $p = .157$; E−/SOC vs. E+ $p = .321$; E−/LI vs. E+ $p = .016$.

[b]BKV infection: E−/SOC vs. E−/LI $p = .031$; E−/SOC vs. E+ $p = .088$; E−/LI vs. E+ $p = .516$.

[c]PVAN: E−/SOC vs. E−/LI $p = .029$; E−/SOC vs. E+ $p = .052$; E−/LI vs. E+ $p = .388$.

likelihood that patients with the same immunological alloreactive background could display a positive test against a specific donor than against others with better HLA matching.[17,31]

The CELLIMIN trial was safe, as patient and graft survival were comparable across the three different groups. However, although no differences were observed in patients remaining on protocol, E−/LI displayed the lowest kidney graft function until month 12, which could be influenced by the slightly higher TAC trough exposure and higher BPAR rates. Remarkably, a significantly lower incidence of viral infections, particularly BK viremia and PVAN, was detected only among patients receiving TAC monotherapy. These data suggest that early MMF and prednisone withdrawal leads to a lower global immunosuppressive burden.

A main limitation of the CELLIMIN trial was its premature termination due to insufficient recruitment rates, which illustrates the complexity of conducting large, prospective randomized trials using

novel biomarkers. The stringent inclusion criteria used, reducing the number of potential candidates with a more limited economical support accounted for this main drawback. Nevertheless, we could prospectively analyze an important number of patients allocated in three study groups after the biomarker intervention, thus providing unique biological and clinical information which will help designing future clinical trials further expanding on this hypothesis. We did not randomize E+ patients into LI or SOC therapy due to ethical concerns, so while we cannot rule out the possibility that E+ with a low-risk eplet mismatch score could safely receive TAC monotherapy, the higher BPAR rates among E+ than E−/SOC patients, both receiving the same immunosuppressive regimen, strongly discourages this option. Importantly, all ELISPOT assays were performed in each participating center using the same validated SOP, thus demonstrating for the first time the safety and feasibility of implementing this technology in clinical practice. Last, typing DP and DRB3/4/5 HLA loci was unfortunately not feasible, thus precluding the study of their impact on clinical outcomes. However, the consistent differences observed between groups using high-resolution HLA typing at all other class I and II locus counterbalance this constraint.

In conclusion, the results of the CELLIMIN trial strongly suggest the value of refining current immune-risk stratification by monitoring preformed T cell memory and primary alloimmune activation using the IFN-γ ELISPOT assay and HLA eplet mismatching. While the benefits of *de novo* TAC monotherapy as compared to current triple SOC therapy seem not to be supported even in low immunological risk patients, the combined risk assessment of preformed memory and *de novo* alloimmune activation seems to have the potential to help decision-making regarding immunosuppression therapy. Patients with low preformed donor-specific memory and low HLA-eplet mismatch seem to benefit from immunosuppression minimization with TAC monotherapy, which is about a quarter of first kidney transplant patients. This must be confirmed in prospective multicenter trials. In addition, new immunosuppressive approaches are warranted to increase the pool of low-risk patients, ultimately allowing safe immunosuppression minimization.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Bestard O, Meneghini M, Crespo E, et al. Preformed T cell alloimmunity and HLA eplet mismatch to guide immunosuppression minimization with tacrolimus monotherapy in kidney transplantation: Results of the CELLIMIN trial. *Am J Transplant*. 2021;00:1–13. https://doi.org/10.1111/ajt.16563

SUPPLEMENTAL MATERIAL

Table of Contents

Study design and statistical analysis

In this study, first, non-sensitized, kidney transplant patients were assessed for pretransplant donor-specific IFN-γ ELISPOT prior to transplant surgery. Pretransplant ELISPOT negative patients were randomized 1:1 to either low (Tacrolimus monotherapy) or standard of care (SOC) immunosuppression, whereas all pretransplant ELISPOT positive patients received the same SOC immunosuppressive regimen. The enrichment study tested non-inferiority of low immunosuppression regimen compared to high immunosuppression regimen, assuming 10% of BPAR (excluding borderline lesions) at 6-months in the control group, allowing a non-inferiority limit of maximum 10%. The following assumptions were used for the sample size calculation:

- power: 1-β=0.80
- cumulative probability of type I error: α=0.05
- probability of BPAR in the control (standard "high" immunosuppression treatment) group: π=01
- non-inferiority limit - maximum 0.10 (10%)
- probability of BPAR in the "low" immunosuppression treatment group, under the null hypothesis: πt0=0.20 (20%)
- probability of BPAR in the "low" immunosuppression treatment group, under the alternative hypothesis: πt1=0.10 (10%)
- drop-out – sample size increased with 10% to account for patients lost to follow-up or withdrawn from the trial
- proportion of ELISPOT negative patients in the population 45%

The trial needed to recruit 302 patients allowing for 10% drop-out rate, having 272 patients with complete follow-up for primary outcome. Taking into account previous retrospective studies evaluating the pretransplant donor-specific IFN-γ ELISPOT, we considered that approximately 55% of patients are ELISPOT positive prior to transplantation,[1,2] thus 672 patients were needed to be screened. Patients were followed up for a total of 12 months for secondary outcome measures. Given the large sample size and cost of follow up, an interim analysis of primary outcome was planned when 122 randomized patients complete 6 months follow-up. The trial would stop if sufficient evidence to conclude non-inferiority using a Lan De Mets spending function with Obrian-Fleming Parameters would be observed.

Testing of the non-inferiority hypothesis for the primary outcome would be carried out Per-Protocol (PP).

Randomization of E- patients was performed by a randomization tool incorporated into secuTrial. The randomization output was either "SOC: Standard of care" or "LI: Low Immunosuppression regimen", and was unblinded, with output of a center specific ID for laboratory assignation. The enrollment and assignment of the patients was an automated process provided by secuTrial, in which the list was incorporated.

Inclusion and exclusion criteria for patient enrolment.

Inclusion criteria

1) Men and women, age ≥18 years.

2) Subject must be a recipient of a first renal transplant from a deceased or living donor.

3) Subject must have a current documented PRA <20% and no detectable anti-class I and II HLA antibodies by solid phase assay (Luminex®).

4) Subject is willing to provide signed written informed consent.

5) Women of Childbearing Potential (WOCBP) must be using a highly effective method of contraception to avoid pregnancy throughout the study in such a manner that the risk of pregnancy is minimized. WOCBP include any female who has experienced menarche and who has not undergone successful surgical sterilization (hysterectomy, bilateral tubal ligation, or bilateral oophorectomy) or is not postmenopausal [defined as amenorrhea ≥ 12 consecutive months; or women on hormone replacement therapy (HRT) with documented serum follicle stimulating hormone (FSH) level > 35 mIU/mL]. WOCBP must have a negative serum or urine pregnancy test (minimum sensitivity 25 IU/L or equivalent units of HCG) within 72 hours prior to the start of clinical trial.

Exclusion criteria

1) Subjects undergoing renal transplant with a current documented PRA >20% and/or detectable anti-class I and II HLA antibodies by solid phase assay (Luminex®).

2) CDC positive cross match.

3) Subjects receiving an allograft from a donor older than 65 years with elevated creatinine levels and/or treated diabetes.

4) Subjects receiving an allograft from a donor after cardiac death (DCD).

5) Cold ischemia time (CIT) higher than 24h.

6) Subjects with a prior solid organ transplant (SOT), including renal re-transplantation, or receiving a concurrent SOT.

7) Patients previously treated with daclizumab or basiliximab.

8) Subjects with underlying renal disease of:

a. Primary focal segmental glomerulosclerosis.

b. Type I or II membranoproliferative glomerulonephritis

c. Atypical Haemolytic uremic syndrome (HUS) / thrombotic thrombocytopenic purpura syndrome.

9) Subject with Hepatitis B chronic infection and/or active infection by Hepatitis C virus (positive PCR result) at the moment of transplant.

10) Subjects with known human immunodeficiency virus (HIV) infection.

11) Patients with active systemic infection that requires the continued use of antibiotics.

12) Patients with neoplasia except localized skin cancer receiving appropriate treatment.

13) Patients with severe anemia (hemoglobin < 6g/dl), leucopenia (WBC <2500/mm3), thrombocytopenia (platelets <80.000/mm3).

14) Hemodynamically instable patients even if their hemoglobin level counts > 6 g/dl.

15) Patients with intestinal pathology or severe diarrhea that can hinder absorption according to medical criteria.

16) Subjects with a known hyper sensibility to any of the drugs used in this protocol.

17) Subjects who have used any investigational drug within 30 days prior to enrolment in this clinical trial.

18) WOCBP who are unwilling or unable to use an acceptable method to avoid pregnancy for the entire study period, women who are pregnant or breastfeeding or women with a positive pregnancy test on enrolment.

19) Subjects who are legally detained in an official institution

Laboratory techniques

Donor and recipient sample collection and processing

Recipient blood samples were obtained in citrate tubes from renal transplant recipients before kidney transplantation. Donor cells were harvested before transplantation from spleens or peripheral blood samples in deceased or living donors, respectively. Peripheral blood mononuclear cells (PBMCs) and splenocytes were isolated by standard Ficoll density gradient centrifugation. Deceased-donor splenocytes were CD2-depleted (Easysep® Human CD2 Selection kit, StemCell, France) and living-donor PBMCs were CD3-depleted (human CD3+ Cell Depletion Cocktail, RosetteSep® kit, StemCell, France). Cells were counted with trypan

blue by hematocytometer and cell concentration was adjusted to have cell suspensions of 3x106 cells/ml, which were used to perform the IFN-γ ELISPOT assays in each participating center.

Donor-specific IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) assays

All centers followed the same standardized operating protocol. Trainings and four different cross-validations among the different centers were done before the beginning of the recruitment of patients. Donor-specific (d-sp) IFN-γ ELISPOT assays were performed following recently described standard operating procedures.[3] Briefly, $3x10^5$ responder cells were placed in quintupled wells with $3x10^5$ CD2-depleted splenocytes or CD3-depleted living-donor PBMCs. Recipient cells were stimulated with complete medium alone in triplicates (RPMI 1640, GE Healthcare Life Sciences, USA; with 10% heat inactivated FBS, Biochrom AG, Germany; antibiotics and L-glutamine, Merck Millipore, USA) and Pokeweed (AID, Autoimmune Diagnostika) as negative and positive controls, respectively. Donor cells were also stimulated with complete medium alone in triplicates as negative controls. After 24 hours incubation without vibration in a CO2-incubator (5%CO2) plates were washed and secondary antibody was added to the wells and incubated for 2 hours at room temperature in a humid chamber. Substrate solution was added to the wells after washing steps and reaction was stopped after 5 minutes. Resulting spots were counted in all centers using the same computer assisted Bioreader with the same software (software, AID, Autoimmune Diagnostika). Results were given as frequencies of IFN-γ producing d-sp T-cells/3x105 PBMCs, subtracting responses of the negative control wells. All different centers sent all output data files to a central lab where results were validated, and patients proceeded to randomization.

References:

1 Crespo E, Lucia M, Cruzado JM, et al. Pre-transplant donor-specific T-cell alloreactivity is strongly associated with early acute cellular rejection in kidney transplant recipients not receiving T-cell depleting induction therapy. PLoS One 2015; 10: e0117618.

2 Crespo E, Cravedi P, Martorell J, et al. Posttransplant peripheral blood donor-specific interferon-γ enzyme-linked immune spot assay differentiates risk of subclinical rejection and de novo donor-specific alloantibodies in kidney transplant recipients. Kidney Int 2017; 92: 201—213.

3 Bestard O, Crespo E, Stein M, et al. Cross-validation of IFN-γ Elispot assay for measuring alloreactive memory/effector T cell responses in renal transplant recipients. Am J Transplant 2013; 13: 1880—1890.

Supplemental tables

Supplemental table 1. Main causes of dropout in each study group.

E-/SOC (n=12)
Prior to 6 months:
1 received ATG for DGF.
1 switched to Belatacept due to BK viremia.
3 switched to CsA due to PVAN.
1 switched to mTORi due to CNI nephrotoxicity.
1 MMF withdrawal due to CMV disease.
1 steroid withdrawal because of severe cataracts.
2 were lost to follow-up.
Between 6-12 months:
1 switched to CsA due to hair loss.
1 MMF withdrawal due to PVAN.
E-/LI (n=13)
Prior to 6 months:
2 remained on TAC-based triple therapy due to suboptimal graft function.
5 prednisone was not withdrawn, 3 due to prolonged DGF and 2 because of suboptimal graft function.
1 remained on TAC trough levels below the predetermined target exposure.
3 received rescue therapy for 3-month sc-BPAR.
2 were lost to follow-up.
E+ (n=11)
Prior to 6 months:
In 8 patients MMF was switched to a mTOR-inhibitor; 4 because of persistent CMV viremia, 2 because of gastrointestinal intolerance and 2 due to leucopenia.
3 were lost to follow up.

Abbreviations: E-/SOC: donor-specific ELISPOT negative/standard of care immunosuppression; E-/LI: donor-specific ELISPOT negative/Low Immunosuppression; E+: donor-specific ELISPOT positive; ATG: Anti-

thymocyte globulin; DGF: delayed graft function; CsA Cyclosporine A; PVAN: polyoma virus Nephropathy; mTORi: mammalian target of rapamycin inhibitors; CNI: calcineurin inhibitors; MMF: mycophenolate mofetil; CMV cytomegalovirus; TAC: Tacrolimus; sc-BPAR subclinical biopsy-proven acute rejection

Supplemental table 2. Mean plasma tacrolimus trough levels during the study period.

	E-/SOC (n=53)	**E-/LI (n=48)**	**E+ (n=66)**	**p value**
15 days (ng/ml)	8.2±3.3	9.9±3.5	9.7±4.9	**0.028***
1 month (ng/ml)	8.5±3.9	10.9±3.1	9.3±2.5	**<0.001****
2 months (ng/ml)	7.6±2.2	9.1±2.7	8.6±2.5	**0.012*****
3 months (ng/ml)	7.0±1.7	8.3±3.3	8.1±2.7	0.092
6 months (ng/ml)	7.3±2.2	8.1±2.6	7.3±2.5	0.194
12 months (ng/ml)	6.6±1.9	7.6±2.6	6.8±2.1	0.286

Data are mean (SD).

*15 days: E-/SOC vs E-/LI p=0.034; E-/SOC vs E+ p=0.117; E-/LI vs E+ p=1.000
**1 month: E-/SOC vs E-/LI p<0.001; E-/SOC vs E+ p=0.036; E-/LI vs E+ p=0.050
***2 months: E-/SOC vs E-/LI p=0.014; E-/SOC vs E+ p=0.093; E-/LI vs E+ p=1.000

Abbreviations: E+: donor-specific ELISPOT positive; E-/SOC: donor-specific ELISPOT negative/standard of care Immunosuppression; E-/LI: donor-specific ELISPOT negative/Low Immunosuppression; TAC: tacrolimus.

Supplemental table 3. Histological findings in *for cause* biopsies during the 12-month study follow-up.

	E-/SOC (n=53)	**E-/LI (n=48)**	**E+ (n=66)**	**Total (n=167)**
6-month BPAR				
BL changes	2 (3.7)	5 (10.4)	0 (0)	7 (4.2)
TCMR	3 (5.7)	6 (12.5)	7 (10.6)	16 (9.5)
- IA	1	1	2	
- IB	1	1	3	
- IIA	0	3	1	
- IIB	1	1	1	
- III	0	0	0	
ABMR	0 (0)	0 (0)	4 (6)	4 (2.3)
Mixed (TCMR + ABMR)	0 (0)	0 (0)	1 (1.5)	1 (0.6)
Total (including BL)	5 (9.5)	11 (23)	12 (18)	28 (17)
12-month BPAR				
BL changes	2 (3.7)	5 (10.4)	0 (0)	7 (4.2)
TCMR	4 (7.5)	7 (18.3)	7 (10.6)	18 (10.7)
- IA	1	1	2	
- IB	2	1	3	
- IIA	0	4	1	
- IIB	1	1	1	
- III	0	0	0	
ABMR	0 (0)	0 (0)	4 (6)	4 (2.3)
Mixed (TCMR + ABMR)	0 (0)	0 (0)	2 (3)	2 (1.2)
Total (including BL)	6 (11)	12 (25)	13 (19.6)	31 (18.5)

Data are n (%).

Abbreviations: E+: donor-specific ELISPOT positive; E-/SOC: donor-specific ELISPOT negative/standard of care Immunosuppression; E-/LI: donor-specific ELISPOT negative/Low Immunosuppression; BPAR: biopsy proven acute rejection; TCMR: T-cell mediated rejection; ABMR: antibody-mediated rejection; BL: Banff borderline lesions.

Supplemental table 4. Incidence of subclinical BPAR and *de novo* DSA in the study.

	E-/SOC	E-/LI	E+	Total
3/12-month sc-BPAR	**(n=35)**	**(n=38)**	**(n=33)**	**(n=106)**
BL changes (%)	4 (11.4)	4 (10.5)	2 (6.1)	10 (9.4)
TCMR (%)	1 (2.9)	9 (23.6)	4 (12.1)	14 (13.2)
- IA	1	5	4	
- IB	0	3	0	
- IIA	0	0	0	
- IIB	0	0	0	
- III	0	1	0	
ABMR (%)	0 (0)	1 (2.6)	2 (6.1)	3 (2.8)
12-month dnDSA	**(n=47)**	**(n=43)**	**(n=59)**	**(n=149)**
Patients with dnDSA (%)	1 (2)	3 (7)	7 (12)	11 (7.4)
dnDSA specificities	1	4	12	17
Class I dnDSA	1	3	2	6
- Anti-A	1	1	1	3
- Anti-B	0	2	1	3
Class II dnDSA (%)	0	1	10	11
- Anti-DR	0	0	4	4
- Anti-DQ	0	1	6	7
Class I and II (%)	0	1	2	3

Data are n (%).

Abbreviations: E+: donor-specific ELISPOT positive; E-/SOC: donor-specific ELISPOT negative/standard of care immunosuppressive treatment; E-/LI: donor-specific ELISPOT negative/low immunosuppressive treatment; Sc-BPAR: subclinical biopsy-proven acute rejection; BL: Banff borderline lesions; dnDSA: de novo donor-specific antibodies.

Supplemental Table 5. Description of HLA eplet mismatches.

	E-/SOC (n=53)	**E-/LI (n=48)**	**E+ (n=66)**	**p value**
Global HLA Eplet MM	28.24±17.10	33.33±11.81	36.15±15.19	**0.012***
HLA class I eplet MM	12.94±7.56	16.00±5.84	15.62±7.12	**0.039****
HLA class II eplet MM	15.30±11.81	17.33±10.3	20.54±10.93	**0.035*****
- DRB1	7.83±6.20	8.50±5.32	9.87±5.44	0.098
- DQB1	5.64±5.53	6.69±5.33	8-07±5.43	**0.033**
-DQA1	1.83±1.95	2.15±1.99	2.59±1.98	0.078
-DQ (A1+B1)	7.47±6.73	8.83±6.37	10.67±6.73	**0.028**

Data are mean±SD.

*Global Eplet MM: E-/SOC vs E-/LI p=0.237; E-/SOC vs E+ p=0.018; E-/LI vs E+ p=0.612

**HLA class I eplet MM: E-/SOC vs E-/LI p=0.089; E-/SOC vs E+ p=0.112; E-/LI vs E+ p=0.959

***HLA class II eplet MM: E-/SOC vs E-/LI p= 0.654; E-/SOC vs E+ p=0.039; E-/LI vs E+ p=0.312

DQB1: E-/SOC vs E-/LI p= 0.628; E-/SOC vs E+ p=0.055; E-/LI vs E+ p=0.406.

DQ (A1+B1): E-/SOC vs E-/LI p= 0.589; E-/SOC vs E+ p=0.035; E-/LI vs E+ p=0.348.

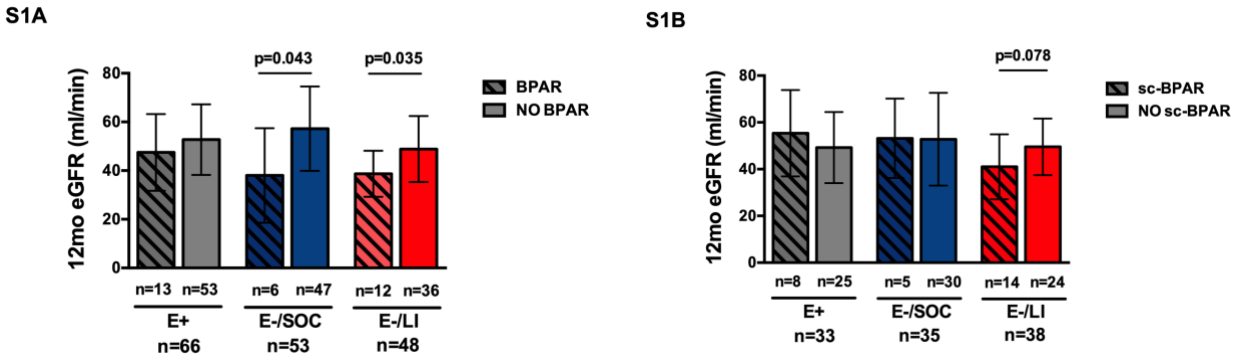
Abbreviations: E+: donor-specific ELISPOT positive; E-/SOC: donor-specific ELISPOT negative/standard of care immunosuppression; E-/LI: donor-specific ELISPOT negative/Low immunosuppression; MM: mismatches. Data is presented as Mean±SD.

Supplemental figures

Supplemental figure 1. 12-month eGFR between patients with or without BPAR and sc-BPAR.

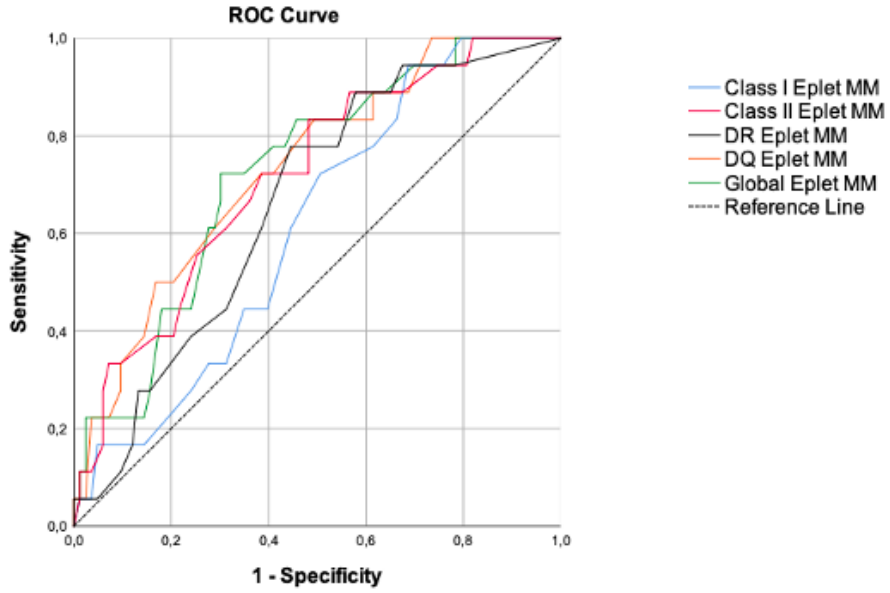
S1a. 12-month eGFR between patients developing BPAR and no BPAR in each study group (n=167). eGFR in E-/SOC patients not displaying BPAR were 57.23±17.36 ml/min vs 38.00±19.44 ml/min in patients with BPAR, p=0.043. eGFR in E-/LI patients not displaying BPAR were 48.86±13.54 ml/min vs 38.70±9.47 ml/min in patients with BPAR, p=0.035. eGFR in E+ patients not displaying BPAR were 52.74±14.51 ml/min vs 47.48±15.77 ml/min in patients with BPAR, p=0.291.

S1b. 12-month eGFR between patients developing sc-BPAR and no sc-BPAR in each study group (n=106 patients with evaluable protocol biopsies at 3 and 12 months). eGFR in E-/SOC patients not displaying sc-BPAR were 53.18±16.98 ml/min vs 52.80±19.86 ml/min in patients with sc-BPAR, p=0.964. eGFR in E-/LI patients not displaying sc-BPAR were 49.54±12.09 ml/min vs 41.00±13.92 ml/min in patients with sc-BPAR, p=0.078. eGFR in E+ patients not displaying sc-BPAR were 49.22±15.22 ml/min vs 55.37±18.49 ml/min in patients with sc-BPAR, p=0.359.



Abbreviations: E+: donor-specific ELISPOT positive; E-/SOC: donor-specific ELISPOT negative/standard of care Immunosuppression; E-/LI: donor-specific ELISPOT negative/Low Immunosuppression; BPAR: biopsy proven acute rejection; sc-BPAR: subclinical biopsy proven acute rejection; mo: months; eGFR: estimated glomerular filtration rate.

Supplemental figure 2. ROC Curve analysis of different Eplet MM scores for prediction of 12 months clinical BPAR among E- patients.



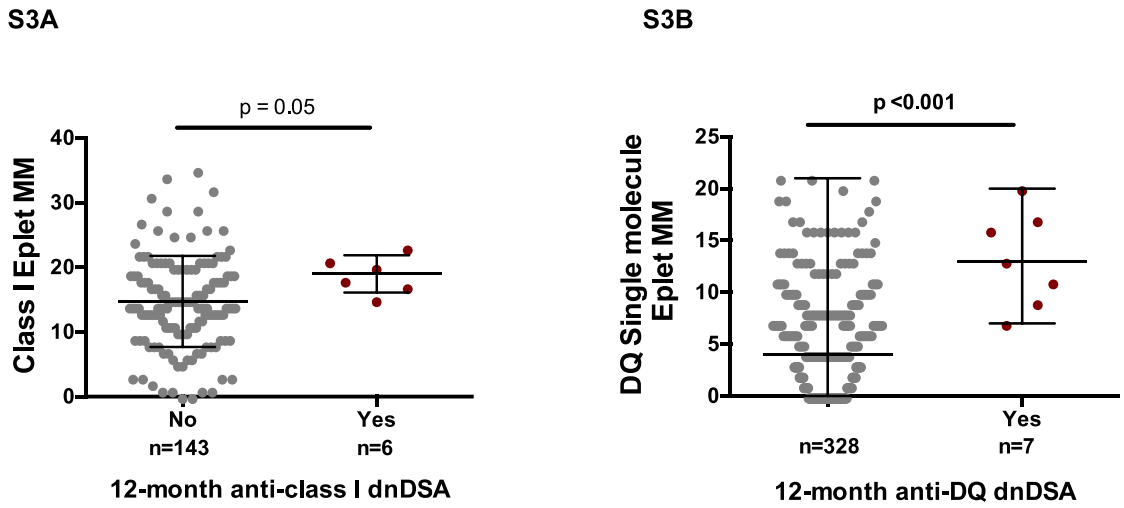
Variable	AUC	SE	p	95% Confidence Interval
Class I Eplet MM	0.614	0.065	0.129	0.488 - 0.741
Class II Eplet MM	0.716	0.064	**0.004**	0.591 - 0.841
DR Eplet MM	0.662	0.064	**0.031**	0.538 - 0.787
DQ Eplet MM	0.733	0.062	**0.002**	0.612 - 0.853
Global Eplet MM	0.724	0.061	**0.003**	0.605 - 0.843

Abbreviations: MM: mismatches.

Supplemental figure 3. Donor/recipient HLA molecular mismatches and *de novo* DSA.

S3a. Mean class I eplet mismatches and anti-class I dnDSA. Mean: 14.74±7.04 vs 19.00±2.89, p=0.050.

S3b. Mean DQ single molecule eplet mismatches and anti-DQ dnDSA. Mean: 5.19±5.16 vs 13.33±5.09, p<0.001.



Abbreviations: MM: mismatches; dnDSA: *de novo* donor-specific antibody.

V. DISCUSSION

The specific risk of every transplant recipient is inevitably determined by the combination of both its preformed alloimmune ***memory*** together with the immunogenicity of the allograft that can trigger a ***primary*** alloimmune response after transplantation.

Current transplant immune-risk stratification is fundamentally based on the assessment of serological memory by means of circulating anti-HLA antibodies and specifically DSA at the time of transplantation[81], whereas the risk of primary alloimmune activation is indirectly estimated by the assessment of donor/recipient HLA incompatibility through the number of class I and II HLA antigen mismatches. Importantly, the implementation of these two approaches has helped on the one hand, to significantly reduce the incidence of acute rejection, and especially ABMR and on the other hand, to guide current organ allocation policies by assigning kidney organs to most compatible transplant recipient candidates while avoiding incompatible donor antigen mismatches. However, there are a number of critical questions that still remain to be better clarified to establish the most optimal immune-risk stratification strategy, namely; i) defining the biological concordance and clinical interpretation of different currently available immune assays assessing serum DSAs prior to transplantation in order to delineate the serological anti-donor immune memory of kidney transplant candidates; ii) whether a refined evaluation of donor/recipient HLA mismatching at the molecular level using different biological algorithms may improve the assessment of the degree of incompatibility, ultimately increasing the susceptibility to primary alloimmune activation and furthermore iii) describing the biological role, clinical impact and usefulness of tracking anti-donor T-cell alloimmunity besides the humoral alloimmune response, in clinical kidney transplantation.

This doctoral thesis aimed at investigating these distinct issues within three different studies.

In the first study[217], evaluating a large cohort of living-donor kidney transplant recipients from 2 different transplant programs, we showed that although all currently available immunoassays are capable of identifying transplant candidates with different degrees of humoral sensitization, they provide different insights regarding the biological mechanisms by which the humoral immune response might be activated and thus, ultimately displaying a rather poor overlap among them. In agreement with previous works[218,219,106, 109, 220, 104] in our cohort all immunoassays were associated with a higher incidence of acute rejection, however the respective discrimination capacity significantly varies among them. Therefore, we assessed in multivariate models the different immunological tests individually. Indeed, although a positive result of any of the tests was

associated with a higher incidence of acute rejection, only DSAC3d+ independently predicted this risk, highlighting the greater aggressiveness of such preformed DSAs.

We observed that patients displaying a positive test result at baseline shared similar clinical backgrounds such as a longer dialysis time, previous transplantations, female sex with previous pregnancies, and receipt of a transplant from donors with whom they had previously been exposed to alloantigens, such as husband or child to wife/mother, respectively. However, although all tests had similarly high negative predictive value, FC-XM+ and DSA-C3d+ showed the greatest specificity in predicting acute rejection. This observation strongly suggests the greater aggressiveness of such preformed DSAs as compared to those not fixing complement despite similar immunosuppression; thus, guided preventive strategies would be highly recommended.[156] In line with contemporaneous works, high MFI-DSAs more likely fixed complement and displayed a stronger risk of acute rejection[119], particularly ABMR. However, the presence of a pretransplantation DSA, regardless of its complement-binding capacity, together with a positive FC-XM, were the strongest correlates of allograft loss, suggesting the presence of persistent alloimmune activation over time despite chronic immunosuppression.

In our study, patients receiving desensitization therapy because of any positive pretransplantation immunoassay result appeared to be at high risk for acute rejection, even if they achieved a negative CDC-XM after such a preventive strategy. However, despite this greater risk, desensitization therapy *per se* did not have an impact on graft or patient survival, thus highlighting the relevance of the result of the immunoassay performed prior to desensitization, which could guide the decision to go further into transplantation or to reconsider alternative approaches such as paired exchange donation programs.

Although pretransplantation sensitization, regardless of the type of immunoassay used, was associated with poorer graft survival, only low 6-month eGFR, previous acute rejection, and FC-XM+/DSA+ were independent predictors of graft loss. These data suggest the need for considering these two tests as main immunoassays for immune risk stratification before transplantation.

In this first study we also investigated the impact of donor/recipient HLA eplet mismatching in the context of hypersensitized kidney transplant candidates. Interestingly, we found that despite that no differences were observed regarding HLA allele matching and rejection risk, most sensitized individuals were poorly matched at the eplet level. This might be of great importance, particularly among this high-risk population, as poor matching at this level might increase the likelihood of DSA

binding to true immunogenic donor epitopes and leading to allograft rejection. In this regard, we found that the higher the mean number of donor/recipient HLA eplet mismatches, the higher the incidence of acute rejection, particularly ABMR. Remarkably, the importance of optimal matching at this level, was also replicated among non-sensitized transplant recipients, in whom a higher donor/recipient HLA-DR eplet mismatch correlated with a significantly higher incidence of ABMR, thus confirming the importance of an optimal molecular matching to reduce the risk of primary alloimmune activation after kidney transplantation.

This study had some methodological limitations. As previously reported, not all DSA with high MFI fix complement, and conversely, some low MFI-DSA are capable of binding complement *in vitro*, due to a prozone effect that may lead to falsely low MFI in the presence of a high load of antibodies per bead. A titration or DSA IgG subclass characterization may overcome such a limitation, although this is costly and labor intensive for daily clinical practice.[97, 111, 112] In three cases, patients displayed a very mild but positive FC-XM without any detectable DSA at solid phase assay. Although our first hypothesis is that they were all false-positive test results, one patient displayed a single anti-C HLA antibody with a very low MFI (500); thus, in the absence of the HLA C antigen donor type, we cannot exclude the presence of a potential DSA in this patient.

In fact, the main limitation of this study is that high-resolution HLA typing was not available to calculate donor/recipient eplet mismatches and was inferred using a local frequency table typed by sequence-based typing, which may entail a certain bias in the interpretation of the data.[221] Nonetheless, a good correlation between high- and low-resolution typing predicting the development of *de novo* DSA has been previously shown, thus suggesting that immunogenic epitope mismatches might also be inferred by using low-resolution HLA typing.[222] Importantly, and to counterbalance this important constraint, our patient population was highly homogeneous in terms of ethnicity, thus significantly reducing the difference in this estimation approach.

While pretransplant humoral alloimmune memory may be accurately assessed with a plethora of immune assays (and as shown in the first study), a major cause of graft loss is derived from a primary or *de novo* alloimmune activation occurring after kidney transplantation. Importantly, such alloimmune activation may be driven by the two main effector mechanisms of adaptive immunity, both T and B-cell anti-donor responses. In this regard, several recent studies have shown the

association between a poor donor/recipient HLA matching, especially at the molecular level and the advent of *de novo* humoral alloactivation by means of dnDSA.[223,224]

However, for B-cell activation in absence of preformed immune memory, cognate T-cell help is required thus, previous *de novo* T-cell alloimmune priming (dnDST) against donor antigens might also occur, subsequently driving anti-donor humoral immune activation.

In the second study of this thesis[225], we aimed at investigating whether primary anti-donor T-cell alloimmune activation assessed with an IFN-γ donor-specific T-cell ELISpot assay, may arise after kidney transplantation, due to a poor donor/recipient HLA matching and ultimately impact on the development of *de novo* humoral alloimmune activation and poorer graft outcomes. Importantly, in this study we assessed donor and recipient HLA typing using high-resolution NGS to accurately assess molecular differences between pairs with different algorithms.

Here, we first confirmed that HLA matching at the molecular level using distinct approaches outperforms allelic mismatches assessment predicting primary humoral alloimmunity by means of dnDSA formation.[224] Notably, and as for the first time, we reported that a relevant number of kidney transplant recipients develop dnDST over the course of the first 2 years after transplantation, and development of dnDST ultimately predicts the subsequent advent of dnDSA. In this line, recent reports showed the impact of HLA class-II mismatching predicting not only the advent of dnDSA and ABMR but also TCMR.[202,193,199] Most interestingly, and similarly to dnDSA, our data suggest that patients at risk of dnDST seem to also show a poor donor/recipient HLA molecular matching, and in particular, at the PIRCHE-II score level, emphasizing a possible contribution of the indirect antigen presenting pathway driving DST development. Indeed, unlike pretransplant DST, an important proportion of posttransplant DST patients, either those with persistent or *de novo* DST, displayed high frequencies of donor-reactive CD4+ T cells primed by the indirect antigen presentation pathway, which contributed to their global DST response. Altogether, these data highlight a continuous increased risk of dnDST and dnDSA for each individual predicted peptide presented by recipient APC through indirect presentation.

Although intuitively, a specific threshold would be of high relevance to help stratifying patients into high or low risk for either dnDSA or dnDST, from the biological point of view these thresholds might not represent the potential impact for alloimmune activation. Indeed, despite the strong correlation between the load of molecular mismatches and risk of *de novo* alloimmunity, even a small amount of mismatched antigens may be sufficient to activate an immune response, thus application of specific cut-offs may be misleading in clinical practice.[199, 192,158]

Another important observation of our study is that up to 50% of transplant recipients with preDST maintained a strong DST response after transplantation (persistDST), which seemed to be mainly influenced by pretransplant anti-donor T-cell frequencies and the absence of T-cell depletion induction therapy.

We observed a strong association between postDST and subsequent dnDSA formation, that was particularly evident among dnDST patients. In this study we cannot confirm whether patients with persistent DST show the same pretransplant donor-reactive T cell clones after transplantation without a TCR sequencing analysis. We observed that an important proportion of patients with preDST+ responses did also display DST primed by the IP, similarly to patients with dnDST thus, suggesting that DST responses among persistDST may have also been developed *de novo*.

Interestingly, dnDST was also influenced by the development of delayed graft function, which could possibly be explained by an inflamed milieu increasing class II HLA antigen expression on graft cells and ultimately driving T-cell alloantigen recognition.[226] The higher presence of alloreactive CD4+ T cells in dnDST+ samples as compared to pretransplantation samples does also support that posttransplant anti-donor alloreactivity is driven, at least also in part, by the IP of antigen presentation. While the presence of the IP after transplantation has been widely described[25, 35, 227], a body of evidence has also shown the potential relevance of a semidirect or third pathway of antigen presentation.[36, 37, 41] In this line, we also found circulating postDST responses primed by the direct pathway when assessed *in vitro*, most likely representing the presence of such semidirect pathway of antigen presentation *in vivo*.

This second study has also some limitations. In particular, even if high resolution typing was here available, both DPA and DRB3/4/5 typing could not be assessed, leaving undetermined the impact of molecular mismatch at those loci on dnDST generation as well as their peptide presenting role. Nevertheless, the accurate prediction of dnDST by donor-derived DRB1 peptides and also when evaluating DQ presentation strengthens the consistency of our findings. Notably, dnDST was accurately predicted by donor-derived DRB1 peptides but not by the global peptide burden if DQ presentation is evaluated. The expression of DQ molecules in recipient APC or different activation capacity of CD4+ T cells according to distinct HLA class-II molecules may explain this observation.

Of note, in order to track the presence of donor-reactive T-cell responses, we used the IFN-γ donor-specific T-cell ELISPOT, a sensitive and reproducible immune-assay tracking circulating donor-reactive IFN-γ-producing memory/effector T cells.[139,140]

Most previous studies using this test have focused on the pretransplant setting and have shown its capacity identifying transplant candidates at higher risk of BPAR, regardless preformed donor-specific humoral immune sensitization.[133, 135-137] Indeed, in this study we also confirmed the strong association between pretransplant DST and higher risk of acute rejection, and especially TCMR. However, pretransplant DST was not associated with any HLA of the mismatch scores thus, strongly suggesting that its presence may arise from either antigen cross-reactivity amid heterologous immunity or prior transient alloantigen recognition triggering a low immune sensitization state, predominantly at the T-cell compartment.

While pretransplant immune-risk stratification in clinical practice is exclusively focused on the humoral effector arm of adaptive immunity, by means of circulating DSA, the presence of preformed T-cell alloreactivity is not currently assessed because of lack of validated and reproducible immune assays. Therefore, in the third study of this thesis[228], we took into account abundant previous retrospective data suggesting the value of measuring preformed frequencies of circulating donor-reactive IFN-γ-producing memory/effector T cells with an IFN-γ ELISpot assay to rule out the risk of post-transplant acute rejection, and especially TCMR[133], to design the CELLIMIN trial. In this study we aimed to evaluate the hypothesis of whether in the absence of immune-monitoring preformed anti-donor T cell immune memory, post-transplant immunosuppression minimization with tacrolimus monotherapy would be effective enough while reducing drug-related toxicities.

Although we were unable to reach the statistical power required to evaluate our primary hypothesis, our findings revealed interesting novel information. First, we showed that implementing the IFN-γ donor-specific T-cell ELISPOT assay is safe and feasible in real clinical practice. Notably, all different European transplant centers performed the assay in real time using the same standard operating procedure after a thorough prior validation study[139], thus demonstrating for the first time the safety and feasibility of implementing this technology in clinical practice.

First, we observed significantly higher BPAR rates among the low immunologic risk group ELISPOT negative patients (E-) receiving tacrolimus monotherapy (E−/LI) as compared to low-risk patients receiving current standard-of-care therapy (E−/SOC), especially when also taking into account Banff borderline (BL) lesions (25% vs. 11%), thus outweighing any potential benefit of maintaining E− kidney transplant recipients on tacrolimus monotherapy on the solely basis of monitoring pretransplant anti-donor T cell memory and pretransplant serum DSA. Nonetheless, we found that among patients receiving the same SOC therapy, E− transplants significantly outperformed the E+

group regarding BPAR rates, dnDSA formation and graft function, suggesting the value of the ELISPOT immune-risk stratification.

These findings corroborate previous retrospective studies[141, 137] and highlight the importance of monitoring preformed T cell memory as these patients could not have been identified using current clinical and epidemiologic factors indicative of low immunological risk, such as first transplant recipients with low cPRA and no DSA.

Moreover, and as hypothesized, patients on tacrolimus monotherapy did benefit of lower viral infection rates as compared to patients on a triple drug-based regimen.

Since the exceeding BPAR rates among E−/LI patients as compared to E−/SOC could not be explained by preformed anti-donor T cell memory, we hypothesized whether they could rather be due to poor donor/recipient HLA matching at the molecular level in the context of low immunosuppression, which was also here evaluated using high-resolution HLA typing. Unlike E+ transplants, E− patients with high DQ eplet mismatch score more frequently developed BPAR, an effect that was even more evident within E− patients on tacrolimus monotherapy. Indeed, while only 4/28 (14%) and 7/28(25%) of E−/LI patients with low-risk eplet score developed clinical and subclinical BPAR, respectively, up to 8/20 (40%) and 10/20 (50%) of those with a high-risk eplet score did. These findings are in agreement with previous studies showing the capacity of HLA molecular mismatching predicting primary alloimmune activation, and especially in patients receiving low or insufficient immunosuppression.[165, 142, 202]

Moreover, and as previously reported [208, 194] we found a close association between a poor donor/recipient HLA eplet matching at each respective locus and dnDSA formation. Altogether, these data suggest that adding the analysis of HLA eplet mismatching to preformed anti-donor T- and B-cell memory seems to have the potential to identify a relevant proportion of transplant recipients (25%) that could successfully receive lower immunosuppression with TAC monotherapy until 1 year after transplantation.

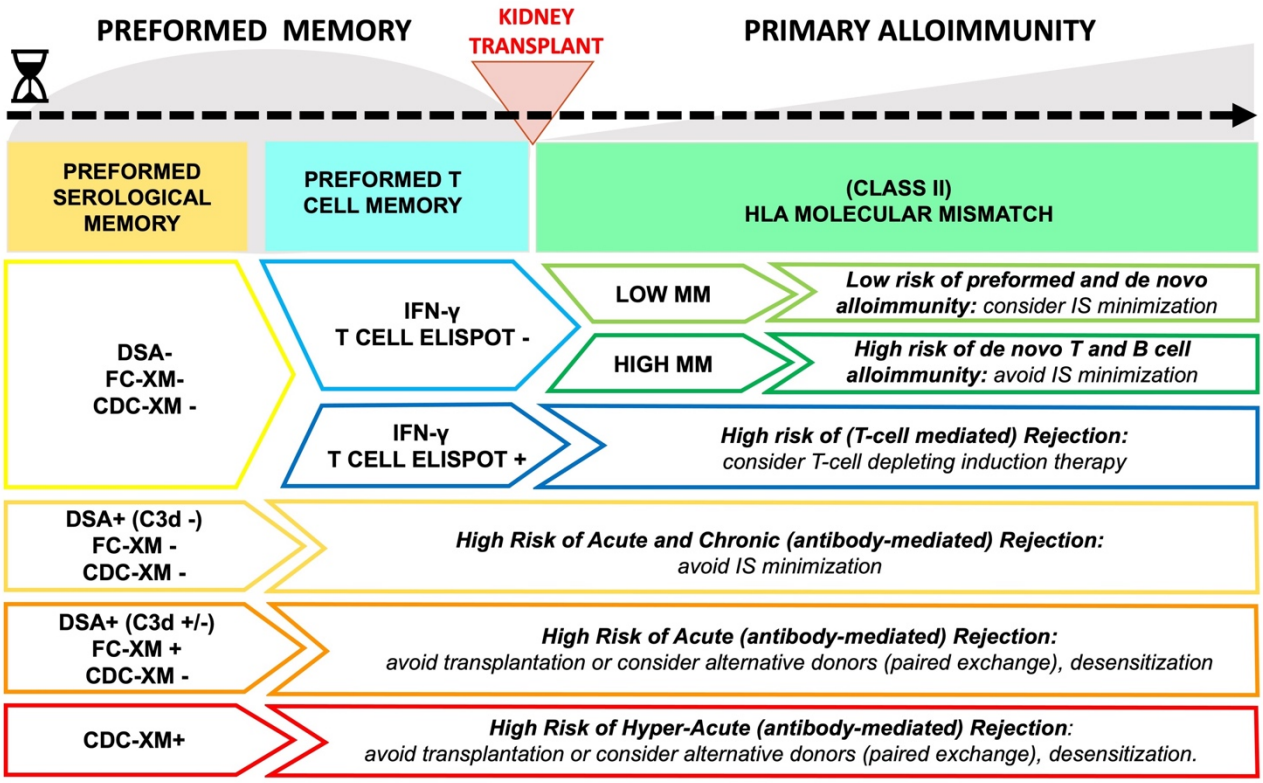
Nevertheless, while the high BPAR rates within E+ patients seem to be predominantly driven by preformed anti-donor T cell memory, the poorer HLA matching of this group of patients, as observed in the two previous studies, also for preformed humoral sensitization, raises concerns on whether these patients might also be at high risk of subsequent primary alloimmune activation in the long term. While we cannot exclude that higher donor/recipient HLA mismatching among E+ patients may be coincidental, our data also suggest that since the ELISPOT assay used in the trial exclusively

assessed donor-specific T cell responses, in the presence of a higher HLA mismatch burden, there may be a higher likelihood that patients with the same immunological alloreactive background could display a positive test against a specific donor than against others with better HLA matching.[143, 229]
It should be noted that the CELLIMIN trial was safe, as patient and graft survival were comparable across the three different study groups. However, although no differences were observed in patients remaining on protocol, E−/LI displayed the lowest kidney graft function until month 12, which could be influenced by the slightly higher tacrolimus trough exposure and higher BPAR rates. Remarkably, a significantly lower incidence of viral infections, particularly BK viremia and PVAN, was detected only among patients receiving tacrolimus monotherapy. These data suggest that early mycophenolate and prednisone withdrawal leads to a lower global immunosuppressive burden.
The main limitation of the CELLIMIN trial was its premature termination due to insufficient recruitment rates, which illustrates the complexity of conducting large, prospective randomized trials using novel biomarkers. The stringent inclusion criteria used, reducing the number of potential candidates with a more limited economical support accounted for this main drawback. Moreover, we did not randomize E+ patients into LI or SOC therapy due to ethical concerns, so while we cannot rule out the possibility that E+ with a low-risk eplet mismatch score could safely receive tacrolimus monotherapy. However, the higher BPAR rates among E+ than E−/SOC patients, both receiving the same immunosuppressive regimen, strongly discourages this option.
To confirm these data, a randomized, multicenter, interventional trial relying on these two biomarkers is currently ongoing (Clinical Trial.gov:NCT03465397).

In summary, throughout the three studies of this doctoral thesis, we have shown that while all currently available immune assays assessing serological memory provide reliable biological information about the degree of humoral anti-donor sensitization, solid-phase DSA identification, with special caution to those displaying high MFI values, and flow cytometry crossmatch assays are the two main tests providing the most compelling immune-risk stratification prior to transplantation. Furthermore, while we confirm the value of different donor/recipient HLA molecular matching scores predicting the advent of primary humoral alloimmune activation, we show for the first time that it also directly influences the development of primary anti-donor T-cell alloimmunity, and especially at the Predicted indirectly Recognizable HLA Epitopes II (PIRCHE-II) score level, emphasizing the contribution of the indirect antigen presenting pathway, which in turn seems to precede the subsequent appearance of *de novo* DSA. Finally, and despite the premature

termination of the CELLIMIN trial, the results of the study strongly emphasize the need of refining current baseline immune-risk stratification by adding to current serological assays, the assessment of preformed T-cell memory and risk of primary alloimmune activation using the IFN-γ ELISPOT assay and HLA molecular mismatching. While the benefits of *de novo* tacrolimus monotherapy as compared to current triple SOC therapy seem not to be supported even in low immunological risk patients defined as no preformed serological nor T-cell immune memory, the combined risk assessment of preformed memory and *de novo* alloimmune activation might have the potential to help decision-making regarding immunosuppression therapy. Patients without preformed donor-specific memory and low HLA-eplet mismatch seem to benefit from immunosuppression minimization with tacrolimus monotherapy, which is about a quarter of first kidney transplant patients. Importantly, the clinical value of implementing these novel biomarkers must be in prospective multicenter, interventional trials.

Figure 3: Suggested decision algorithm taking into account both preformed memory and risk of *de novo* alloimmunity after kidney transplantation



VI. CONCLUSIONS

- Identification of preformed donor-specific antibodies by solid-phase assay and flow-cytometry crossmatch in combination is highly warranted for a compelling serological immune-stratification prior to kidney transplantation.

- Pretransplant high MFI-DSAs may more likely fix complement and display a stronger risk of antibody-mediated rejection.

- There are different algorithms capable of measuring the degree of donor/recipient HLA mismatch at a molecular level, all of them accurately predicting the advent of primary humoral alloimmune activation.

- A better donor/recipient HLA matching at the molecular level seems to minimize the risk of posttransplant primary alloimmune activation, both humoral and cellular level

- *De novo* donor-reactive T-cell immunity (DST) may also arise after kidney transplantation and seems to be directly influenced by the degree of donor/recipient HLA molecular mismatch, especially at the Predicted indirectly Recognizable HLA Epitopes II (PIRCHE-II) score level.

- *De novo* DST seems to be driven by both indirectly and directly primed circulating CD4+ alloreactive cells, the latter suggesting the presence of a semidirect antigen presenting pathway

- Implementation of preformed donor-reactive memory/effector T cells using a validated IFN-γ ELISPOT assay seems to be feasible and reliable in current clinical practice.

- The evaluation of preformed anti-donor T-cell immune memory may refine current immune-risk stratification on top of current serological immune-risk assessment.

- Patients without preformed donor-specific serological and T-cell memory together with low class II eplet mismatch would benefit of participating in novel prospective, interventional trials

to investigate the safety and efficacy of new immunosuppression minimization strategies to ultimately reduce short and long-term drug-related toxicities.

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