Tesis Doctoral/ PhD Thesis

Células Supresoras Derivadas de Mieloides en pacientes trasplantados renales y pulmonares

Myeloid-Derived Suppressor Cells in kidney and lung transplant recipients

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Escuela de Doctorado de la Universidad de Cantabria

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Myeloid-Derived Suppressor Cells in kidney and lung transplant recipients

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PhD Thesis

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CERTIFICA QUE,
Doña María Iglesias Escudero ha realizado, en el Grupo de Trasplante y Autoinmunidad de IDIVAL, bajo su dirección el presente trabajo de tesis doctoral titulado:

"Células Supresoras Derivadas de Mieloides en pacientes trasplantados renales y pulmonares"

"Myelod-Derived Suppressor Cells in kidney and lung transplant recipients"

para optar al grado de Doctor por la Universidad de Cantabria.

Examinado el trabajo considero que está adecuadamente elaborado para su lectura y defensa pública y ante la Comisión que ha de juzgar la Tesis Doctoral.

Y para que conste y surta los efectos oportunos, expido este certificado en Santander a 19 de abril de 2021.

Fdo. Marcos López Hoyos

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Por la presente hago constar que desde el curso académico 2016-2017 he actuado como codirector .de María Iglesias Escudero, Especialista en Inmunología, Licenciada en CC Biológicas, en el Programa de Doctorado de Medicina y Ciencias de la Salud de la Universidad de Cantabria.

Certifico que he dirigido el desarrollo de su proyecto de Tesis Doctoral sobre

"Células Supresoras Derivadas de Mieloides en pacientes trasplantados renales y pulmonares"

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para optar al grado de Doctor por la Universidad de Cantabria.

Examinado el trabajo considero que el trabajo tiene la calidad científica adecuada para proceder a su lectura y defensa pública y ante la Comisión que ha de juzgar la Tesis Doctoral.

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Abbreviations

6-MP 6 Mercaptopurine

7AAD 7-amino-actinomycin D

A

ABMR: Antibody-mediated rejection

ACR: Acute cellular rejection

ADCC: Antibody dependent cellular citotoxicity

AMR: Antibody mediated rejection

APC: Antigen presenting cell

AR: Acute Rejection

Arg-1: Arginase 1

ATG: Anti-thymocyte globulin

AZA: Azatioprine

B

BOS: Bronchiolitis obliterant

C

CAN: Chronic allograft nephropathy

CDC**:** Complement-dependent cytotoxicity

CFSE: Carboxyfluorescein succinimidyl ester

CLAD: Chronic lung allograft dysfunction

CNIs: Calcineurin inhibitors

COPD: Chronic obstructive pulmonar disease

CR: Chronic rejection

CT: Computed tomography

CS: Corticosteroids

D

DAMPs: Danger associated molecular patterns

DIPD: Diffuse interstitial pulmonary disease

DSA: Donor specific antibodies

E

e-MDSCs: Early stage Myeloid-derived suppressor cells

ESLD: End stage lung disease

ESRD: End stage renal disease

EVL: Everolimus

F

FBS: Fetal bovine serum

FEV1: Forced expiratory volume in 1 second

H

HLA: Human leukocyte antigen

HC: Healthy control

HαE: Hematoxylin and Eosin

I

IDO: Indoleamine 2,3-dioxygenase

IL-2R: IL-2 Receptor

IMPDH: Inosine monophosphate dehydrogenase

ImTOR: mTOR inhibitors

ISHLT: International Hearth and LungTransplant Society

ITx: Intestinal transplant

IVIG: Intravenous Immunoglobulin

K

KT: Kidney transplantation

KTR: Kidney transplant recipient

L

LAS: Lung allocation score

LT: Lung transplantation

LTR: Lung transplant recipient

M

MDSCs: Myeloid-derived suppressor cells

MHC: Major histocompatibility complex

MMF: Mycophenolate mofetil

Mo-MDSCs: Monocytic Myeloid-derived suppressor cells

MPA: Mycophenolic acid

MRCs: Myeloid regulatory cells

Mreg: Regulatory macrophages

MSC: Mesenchymal stem cells

Mtor: Mammalian target of rapamycin

N

NFAT: Nuclear factor of activated T cells

NLR: Nucleotide-binding domain oligomerization protein receptors

P

PAS: Periodic acid-Schiff

PBMCs: Peripheral blood mononuclear cells

PGD: Primary graft dysfunction

PI3K Phosphatidyl-inositol-3-kinase

PMN: Polimorphonuclear

PMN-MDSCs: Polimorphonuclear Myeloid-derived suppressor cells

Pmp: Per million population

PRRs: Pattern recognition receptors

R

RAS: Restrictive allograft syndrome

S

SRL: Sirolimus

T

TBBx: Transbronchial biopsies

TCMR: T Cell-Mediated Rejection

TCR: T cell receptor

TLRs: Toll-like receptors

TolDC: Tolerogenic dendritic cells

Treg: T regulatory cell

TX: Transplantation

V

VEGFR-1: Vascular endothelial growth factor receptor 1

Index

Resumen

El trasplante de órganos sólidos es una terapia primaria en pacientes con enfermedades en etapa terminal. Aunque se ha conseguido una notable mejoría gracias a los protocolos inmunosupresores y se ha reducido la incidencia de rechazo agudo, todavía existe un alto porcentaje de receptores de trasplantes renales que padecen rechazo agudo dentro del primer año tras el trasplante. Además, los pacientes que previamente lo han sufrido, suelen mostrar una mayor incidencia de rechazo crónico y, como consecuencia, la supervivencia a largo plazo del aloinjerto no ha mejorado significativamente a lo largo de los años. Asimismo, aunque se ha mejorado la supervivencia a corto y medio plazo en trasplantados de pulmón, la supervivencia a los 5 años postrasplante está alrededor del 50%. Por esta razón, los principales objetivos del trasplante son predecir el riesgo de desarrollar rechazo y encontrar enfoques alternativos relacionados con la tolerancia que permitan minimizar la inmunosupresión con el fin de disminuir los efectos adversos que tienen efectos deletéreos sobre la supervivencia del injerto a largo plazo.

Entre los diferentes enfoques tolerogénicos, las células mieloides desempeñan un papel principal en el control de las respuestas inmunitarias: en determinadas circunstancias, contribuyen al proceso inflamatorio, ampliando la patología de la enfermedad. Sin embargo, las células mieloides con propiedades reguladoras pueden proteger al hospedador de una inflamación incontrolada. Estas células se conocen como células mieloides reguladoras (MRC) y se han descrito dentro de todos los principales linajes de células mieloides. Entre ellos, las células supresoras derivadas de mieloides (MDSCs) se consideran un grupo

heterogéneo de células mieloides que se sabe que se acumulan en condiciones patológicas crónicas.

Las primeras observaciones de células mieloides supresoras se describieron hace más de 20 años en pacientes con cáncer. Sin embargo, su importancia funcional solo se ha apreciado recientemente debido a estudios que informan sobre su contribución a la regulación de las respuestas inmunitarias en otros entornos clínicos, como el trasplante de órganos. En el trasplante, las MDSCs son capaces de suprimir las respuestas inmunitarias adaptativas e innatas y se han sugerido como posibles biomarcadores para la tolerancia al aloinjerto, ya que pueden desempeñar un papel principal en el equilibrio entre la aceptación y el rechazo del injerto.

El efecto de diferentes fármacos inmunosupresores como terapia de mantenimiento, tiene un efecto diferencial sobre el sistema inmunológico. Basándonos en los resultados previos de nuestro grupo, que demuestran que los inhibidores de la calcineurina (CNI) fueron capaces de reducir las células T reguladoras (Treg) circulantes en los pacientes con trasplante renal, es probable que los fármacos inmunosupresores más utilizados estén afectando el fenotipo y la función de las MDSCs de distintas formas. Por esta razón, monitorizar cómo se regulan las MDSCs *in vivo* en receptores de órganos sólidos sería un enfoque adecuado para evaluar su papel en tolerancia.

Además, estudiar el efecto de los distintos regimenes inmunosupresores sobre su función arrojaría luz sobre el terreno y podría ayudar en estrategias futuras para minimizar la inmunosupresión. Como la mayoría

de los estudios publicados se realizaron en modelos animales, el efecto tolerogénico de MDSCs no está bien establecido en los pacientes y el seguimiento de estas poblaciones de células de forma prospectiva podría ayudar.

Las MDSCs humanas se clasifican en tres subpoblaciones principales según sus marcadores fenotípicos, aunque estos marcadores no se expresan exclusivamente por MDSCs y estas poblaciones de células reguladoras se definen mejor por su capacidad para suprimir la proliferación de células T y expandir Tregs. Por un lado, los ensayos de función de MDSCs en humanos son difíciles de implementar debido a su complejidad técnica. Además, el fenotipado convencional de la subpoblación Mo-MDSCs en humanos se basa principalmente en la expresión de HLA-DR y no está claro hasta qué punto la expresión de HLA-DR está influenciada por la inmunosupresión estándar. Por lo tanto, también existe la necesidad de nuevos estudios con respecto al fenotipo de MDSCs y de marcadores sustitutos estables que permitan evaluar su función.

Los objetivos del presente proyecto de tesis son 1) Analizar *in vitro* el fenotipo y la función supresora de MDSCs después de la activación policlonal; 2) Evaluar cómo las MDSCs inducen la diferenciación a células T efectoras o reguladoras; 3) Estudiar si existen diferentes efectos de CNI o imTOR sobre MDSCs obtenidas de pacientes trasplantados renales (PTR); 4) Monitorizar el número y fenotipo de MDSCs de monocitos de sangre periférica después del trasplante renal y pulmonar y estudiar cualquier relación con los resultados del trasplante renal y pulmonar; 5) Buscar nuevos marcadores subrogados fenotípicos y funcionales de MDSCs.

Evaluamos el fenotipo y la función de las diferentes subpoblaciones conocidas de MDSCs en 38 PTR y 82 pacientes trasplantados de pulmón (PTP) en diferentes momentos. Las MDSCS se cuantificaron mediante citometría de flujo siguiendo la estrategia propuesta por Bronte *et al.* para caracterizar subpoblaciones de MDSCs: Mo-MDSCs ($CD33^+$ $CD11b^+$ $HLADR^{-/low}$ $CD14^+$ $CD15^-$), PMN-MDSC ($CD33^+$ $CD11b^+$ $HLADR^-$ $CD15^+$ $CD14^-$) y e-MDSC (Lin^- $CD33^+$ $CD11b^+$ $HLADR^-$ $CD14^-$ $CD15^-$). Las MDSCs totales se definieron como células $CD33^+$ $CD11b^+$ $HLADR^-$. Para aislar Mo-MDSCs, las células $CD33^+$ se separaron primero mediante separación magnética y se realizó un aislamiento adicional de las células $CD33^+$ HLA-DR^- y $CD33^+$ HLA-DR^- $CD14^+$ en un separador celular FACS-ARIA II. Se aislaron células T $CD4^+$ mediante separación magnética y se incubaron con succinimidil éster de carboxifuoresceína (CFSE). Las células T $CD4^+$ marcadas con CFSE ($5x10^5$) se estimularon con Dynabeads Human T-activator CD3/CD28. Para determinar la función supresora de las subpoblaciones de MDSCs, se agregaron MDSCs totales o Mo-MDSCs autólogas al cultivo en una proporción de 1: 2 (Células T $CD4^+$: MDSCs) y se determinó la proliferación a día 5 mediante citometría de flujo. Las condiciones experimentales se repitieron al menos cuatro veces en PTR y controles sanos y 2 veces en PTP. Una eficiencia> 98% se consideró aceptable para el estudio. Para buscar nuevos marcadores subrogados fenotípicos y funcionales, utilizamos una tinción estandarizable en sangre completa para detectar antígenos extra e intracelulares.

Nuestros resultados *in vitro* muestran una reducción en la viabilidad celular que aumenta con el tiempo de cultivo en comparación con la viabilidad basal. También encontramos un predominio de Mo-MDSCs en el

primer día de cultivo, una disminución en la proporción de células Mo-MDSCs a partir del día 3 y la mayoría de las células se vuelven doble negativas con el tiempo de cultivo. Estos resultados sugieren que el fenotipo de las subpoblaciones de MDSCs se ve afectado con el tiempo de cultivo y un análisis fenotípico posterior al tercer día de cultivo podría estar sesgado. Cuando analizamos el efecto *in vitro* de los inmunosupresores sobre las MDSCs nuestros resultados mostraron que la administración *in vitro* de imTOR mantiene el total de MDSC y Mo-MDSC en cultivo, pero las dosis altas de tacrolimus afectan negativamente al número de MDSCs totales y Mo-MDSCs.

Durante la monitorización de PTR, encontramos que los números absolutos de MDSCs totales circulantes aumentaron en PTR y en el corto plazo después del trasplante, mientras que disminuyeron a niveles basales un año después del trasplante. También observamos un aumento en las frecuencias de Mo-MDSCS a corto plazo después del trasplante y 1 año después del trasplante. Estos estudios observacionales sugieren que los números de MDSCs aumentan rápidamente después del trasplante y alcanzan un pico después de la terapia inmunosupresora. Aunque el fenotipo de las Mo-MDSCs parece estar influenciado por la inmunosupresión estándar, necesitamos más investigación para establecer si las MDSCs están reguladas de forma diferencial por las condiciones locales o los tratamientos inmunosupresores.

En relación con otras subpoblaciones celulares con función supresora, observamos un aumento en la expansión de Treg *in vitro* después del co-cultivo con Mo-MDSCs. Sin embargo, no hubo una asociación lineal

significativa entre los porcentajes de MDSCs y Tregs cuando examinamos la relación entre MDSCs y $CD4^+$ $CD25^+$ $Foxp3^+$ Treg *in vivo*.

Debido a la falta de marcadores fenotípicos únicos, se deben realizar estudios funcionales para identificar subpoblaciones de MDSCs. Nuestros resultados demuestran que las las Mo-MDSCs obtenidas de PTR tratados con tacrolimus exhiben una potente función supresora. Además, observamos que esta actividad supresora estaba aumentada en comparación con las Mo-MDSCs obtenidas de pacientes tratados con rapamicina y esta función inhibidora inmunitaria puede estar relacionada con la regulación positiva de IDO.

Los mecanismos por los cuales las MDSCs median la generación de Treg no han sido bien establecidos. En algunos modelos animales portadores de tumores se ha demostrado que el microambiente modula de manera diferencial el desarrollo y la función de las células mieloides. Por lo tanto, una limitación importante en el estudio de MDSCs en PTR en este punto es que los ensayos *in vitro* realizados en sangre periférica pueden no estar replicando las características del entorno del injerto. Debido al número de pacientes incluidos en este proyecto y al seguimiento a corto plazo, tampoco hemos podido establecer el impacto de eventos clínicos como el rechazo en las MDSCs en nuestra cohorte de PTR.

En este trabajo describimos por primera vez subpoblaciones circulantes de MDSCs de PTP en varios puntos de tiempo y evaluamos la relación de las MDSCs con los resultados del trasplante de pulmón a plazo. Encontramos que los porcentajes de MDSCs totales aumentaron en PTP 3 meses después del trasplante hasta un año. Cuando estudiamos el efecto del

trasplante en las subpoblaciones de MDSCs en nuestra cohorte, los porcentajes de Mo-MDSCs aumentaron rápidamente después del trasplante y disminuyeron gradualmente durante el tiempo de seguimiento. Por el contrario, los porcentajes de PMN-MDSCs disminuyen a corto plazo tras el trasplante y aumentan durante el seguimiento aunque no se observaron cambios respecto a los niveles pretrasplante. En comparación con los niveles previos al trasplante, los porcentajes de e-MDSCs aumentaron significativamente a los 7 días, 21 días y 360 días. Obtuvimos resultados similares cuando calculamos los números absolutos de MDSCs.

En experimentos previos, observamos una reducción dosis dependiente en los niveles de expresión de HLA-DR en monocitos después de la exposición a dexametasona *in vitro*, los monocitos eran fenotípicamente indistinguibles de Mo-MDSCs; por tanto planteamos la hipótesis de que los corticosteroides están aumentando las poblaciones de Mo-MDSCs en sangre periférica inmediatamente después trasplante. Sin embargo, en nuestro estudio, las PMN-MDSCs y las e-MDSCs que aumentan 3 meses después del trasplante, no parecen verse afectadas por los corticosteroides y los aumentos sugieren que frecuencia MDSCs no se ve afectada negativamente por la terapia de mantenimiento basada en tacrolimus.

La capacidad supresora de MDSCs obtenidas de PTP tratados con tacrolimus está aumentada en comparación con los resultados supresores cuando se obtuvieron células MDSCs de donantes sanos. Sin embargo, los porcentajes de MDSCs en nuestro estudio no se relacionaron con los niveles de inmunosupresores en sangre periférica. Como nuestra cohorte

de PTP estaba bajo el mismo régimen inmunosupresor, no se pueden determinar las posibles diferencias entre los tratamientos, con respecto a su efecto sobre la frecuencia o función de MDSC, siendo una limitación del estudio.

Aunque no se observó ninguna relación entre las MDSCs y los eventos clínicos a corto plazo, nuestros resultados determinan que las frecuencias de Mo-MDSCs aumentan después del rechazo celular agudo (ACR), lo que sugiere un posible papel de Mo-MDSCs en el desarrollo de la disfunción crónica del aloinjerto pulmonar (CLAD). Con estos resultados, en este momento, se desconoce si las MDSCs desempeñan un papel como biomarcadores de rechazo crónico o no, y se requieren más investigaciones.

Como se mencionó anteriormente, los marcadores de MDSCs no son expresados exclusivamente por ellos; los ensayos para evaluar función de MDSCs son difíciles de implementar y no está claro hasta qué punto la expresión de HLA-DR está influenciada por la inmunosupresión estándar. Por este motivo, durante mi estancia en el laboratorio de inmunomonitorización (Hospital Klinikum, Regensburg, Alemania) implementamos nuevos paneles de citometría de flujo que permiten comprobar una amplia gama de marcadores funcionales y fenotípicos de sangre periférica. Primero diseñamos un ensayo de citometría de flujo para la detección rápida y reproducible de subpoblaciones de MDSCs en pequeños volúmenes de sangre periférica humana. Este ensayo se basa en la definición fenotípica convencional de Mo-MDSCs en humanos.

Las MDSCs humanas ejercen sus acciones supresoras de células T a través de una amplia variedad de mecanismos, incluida la producción de

citocinas antiinflamatorias y la regulación positiva de moléculas inmunoreguladoras, incluida la arginasa 1 (Arg1) y la indolamina 2,3-dioxigenasa (IDO). Como se demostró anteriormente en este proyecto, la rapamicina bloquea parcialmente el potencial supresor de Mo-MDSCs *in vitro* al prevenir la inducción de IDO. A continuación desarrollamos un ensayo estandarizado para controlar la expresión de fosfo-mTOR, fosfo-S6, IRF1 e IDO por Mo-MDSCs en muestras de sangre periférica. Las Mo-MDSCs circulantes generalmente no expresan niveles detectables de IDO, pero su expresión es fácilmente inducida por factores proinflamatorios, incluido IFN-γ. La capacidad de Mo-MDSCs para expresar de forma inducible IDO es un marcador sustituto útil de su función supresora. Por lo tanto, desarrollamos un ensayo para la expresión de IDO inducible en Mo-MDSC mediante citometría de flujo.

El fenotipado convencional de Mo-MDSCs se basa principalmente en la expresión de HLA-DR y como hemos mencionado anteriormente, no está claro hasta qué punto la expresión de HLA-DR está influenciada por la inmunosupresión estándar, especialmente los glucocorticoides. En cultivo, observamos una rápida reducción dosis dependiente en los niveles de expresión de HLA-DR en monocitos tras la exposición a dexametasona; los monocitos eran fenotípicamente indistinguibles de Mo-MDSCs. Para identificar los marcadores de Mo-MDSCs no afectados por la exposición a glucocorticoides, analizamos la expresión diferencial en los monocitos y Mo-MDSCs de varios marcadores y observamos que CD35 (CR1) y CD326 (Ep-CAM) se expresaban más en Mo-MDSCs que en los monocitos. Además la expresión de CD35 no se vio afectada por la dexametasona. CD35 es un receptor para los componentes del complemento C3b y C4b.

Anteriormente se identificó el Receptor 1 del Complemento 5a (C5aR1; CD88) como un marcador de la subpoblación de monocitos humanos no clásicos que es un probable precursor de los macrófagos reguladores que se infiltran en los aloinjertos. La expresión de C5aR1 y CCR2 (CD192) parece estar contrarregulada en monocitos humanos, lo que es consistente con experimentos de trasplante en ratones que muestran C5aR1 en lugar de CCR2 controla la migración de precursores de Mreg en aloinjertos. Por lo tanto, diseñamos un nuevo panel de citometría de flujo de 10 colores que incorpora CD35, CD88, CD192 y CD326.

Para comprobar nuestra nueva definición de Mo-MDSCs basada en la expresión de CD35, realizamos ensayos de supresión y observamos que las células Mo-MDSCs con alta expresión de CD35 eran más supresoras en comparación con los monocitos $CD35^{low}$. A pesar de esto, estos ensayos de aislamiento se siguieron basando en el marcador HLA-DR para aislar Mo-MDSCs. Los nuevos 4 paneles propuestos tomados en conjunto podrían ayudar a identificar subpoblaciones de MDSCs, fenotípicamente y funcionalmente, pero CD35 por sí solo no es lo suficientemente específico para identificar Mo-MDSCs.

Las conclusiones obtenidas de este trabajo fueron las siguientes:

1. Hay un incremento en la frecuencia de Mo-MDSCs 6 meses y un año post-trasplante renal.
2. Las células Tregs se expanden *in vitro* tras co-cultivo con Mo-MDSCs obtenidas de PTR.
3. Mo-MDSCs obtenidas de PTR tratados con tacrolimus a largo plazo tienen mayor actividad supresora *in vitro* comparados con pacientes tratados con rapamicina.

4. La pérdida de función supresora en Mo-MDSCs expuestas a rapamicina está relacionada con una expresión de IDO disminuida.
5. Los porcentajes de MDSCs totales aumentan en PTP 3 meses después del trasplante hasta un año.
6. Los porcentajes de Mo-MDSCs aumentaron inmediatamente después del trasplante y disminuyeron gradualmente durante el tiempo de seguimiento.
7. Las poblaciones de Mo-MDSCs obtenidas de PTP tratados con tacrolimus pueden suprimir eficazmente la proliferación de células T *in vitro*.
8. Las frecuencias de Mo-MDSCs en PTP aumentan después del rechazo celular agudo (ACR).
9. La expresión de HLA-DR en monocitos muestra una reducción dependiente de la dosis después de la exposición a dexametasona, por tanto los monocitos son fenotípicamente indistinguibles de Mo-MDSCs.
10. CD35 (CR1) y CD326 (Ep-CAM) se expresaron más en Mo-MDSCs HLA-$DR^{-/low}$ que en monocitos HLA-DR^{+}.
11. En cultivos *in vitro*, la expresión de CD35 no se vio afectada por la dexametasona.
12. Las células Mo-MDSCs $CD35^{high}$ fueron más supresoras en comparación con los monocitos $CD35^{low}$.

Abstract

Solid organ transplantation is a primary therapy in patients with end-stage diseases. Although remarkable improvement has been achieved due to the immunosuppressive protocols and the reduction in the incidence of acute rejection (AR) has been reduced, there is still a high percentage of transplant recipients that suffer from AR within the first year after transplantation. Moreover patients that have previously suffered an AR in kidney transplantation, usually show a higher incidence of chronic rejection and as a consequence long-term allograft survival has not improved significantly throughtout the years. Likewise, despite the improvement in the management of lung transplant recipients (LTR), 5-year survival for LTR remains low. An increasing field of research is focused on the study of dysregulation of immune mechanisms underlying allograft failure. In this regard, myeloid-derived suppressor cells (MDSCs) represent a heterogeneous group of myeloid regulatory cells that were originally described in cancer. Several studies in animal models point to them as important players in the induction of allograft tolerance, due to their immune modulatory function, but there is a lack of studies regarding their role in human transplantation.

To monitor the number and phenotype of MDSCs and to perform *in vitro* studies we collected blood from 38 kidney transplant recipients (KTR) and 82 LTR at different time points. We observed an increase in Mo-MDSCs frequencies in the short term after transplantation and 1 year after transplantation in KTR. We report an increase in Treg expansion after Mo-MDSCs co-culture. Furthermore, we observed that Mo-MDSCs from KTR receiving tacrolimus had a higher suppressive activity compared to those receiving rapamycin and we attributed the loss of suppressive function to

the decreased expression of IDO in Mo-MDSCs exposed to rapamycin. We describe for the first time circulating subsets MDSCs from LTR at several time points and we evaluated the relationship of MDSCs with sort-term lung transplant outcomes. Although no effect of MDSCs subsets on short-term clinical events was observed, our results determine Mo-MDSCs frequencies are increased after acute cellular rejection (ACR), suggesting a possible role for Mo-MDSC in the development of chronic lung allograft dysfunction (CLAD). Therefore, whether MDSCs subsets play a role as biomarkers of chronic rejection or not, remains unknown and requires further investigations. Finally, the effects of different immunosuppressive drugs on the development and function of MDSCs need to be better characterized and further prospective studies are required to establish whether long-term tolerance to immune modulation transplantation is dependent on MDSCs. In collaboration with the immunomonitoring lab from the group of Experimental Surgery in the Hospital Klinikum of Regensburg (Germany) we set up 4 new cytometry panels. This new strategy for identifying MDSCs in human peripheral blood combines surface markers, function markers and new markers not affected by corticoids.

1 Introduction

1.1- Kidney Transplantation

1.1.1- Background

Kidney transplantation (KT) is a primary therapy in patients with end-stage renal diseases (ESRD). In the last 10 years the total number of kidney transplants has increased worldwide (Figure 1). In 2019 in America a number of 39515 kidney transplants were performed, followed by 28053 in Europe, 15601 in Western Pacific, 10403 in South East Asia, 2086 in Eastern Mediterranean and 470 in Africa. In spite of the pandemic, the data colected from Gobal data Base in donation and transplantation registred a number of 25582 kidney transplants performed in America and 17366 in Europe in 2020.

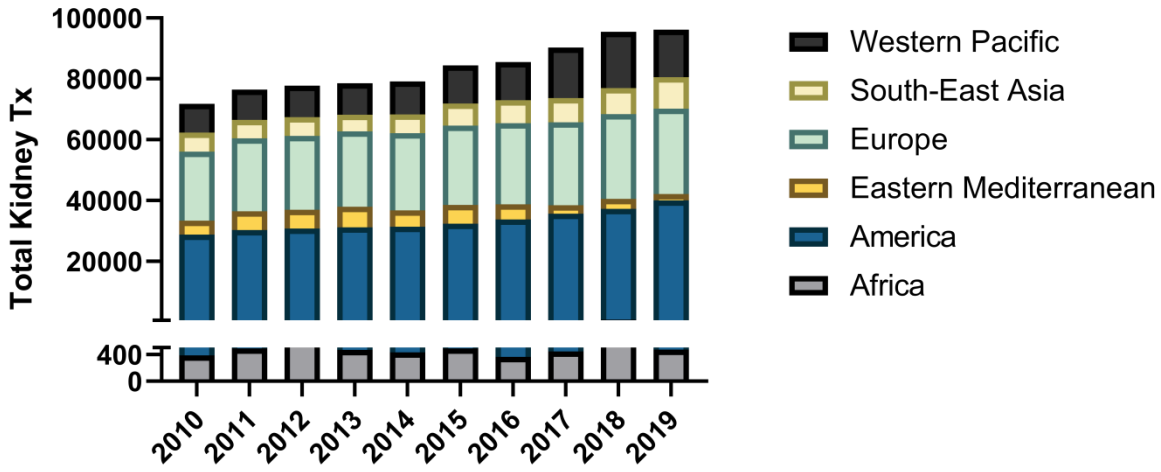


Figure 1| Number of kidney transplants performed each year in the world by region, from 2010 to 2019.

During the last few years the number of interventions in Spain has progressively increased achieving all-time highs in 2019 (3423 number of kidney transplants). In 2019, Spain performed the 3-4% of kidney transplants worldwide and reached the top rate of kidney

transplant patients per million population (pmp) (Figure 2) (1). In 2020, Spain resists impact of Covid-19 and performs a number of 2700 kidney transplants (Figure 3).

The number of kidney transplants performed in the different regions of Spain has been collected in **Table 1** (2). In 2019, Cantabria led the transplantation (Tx) ranking in Spain, with a rate of 89.7 donors pmp. In 2020, the rate was 65.5 donors pmp. Thanks to the increase in the performance of kidney transplants, the waiting lists of patients with chronic kidney failure has stabilized.

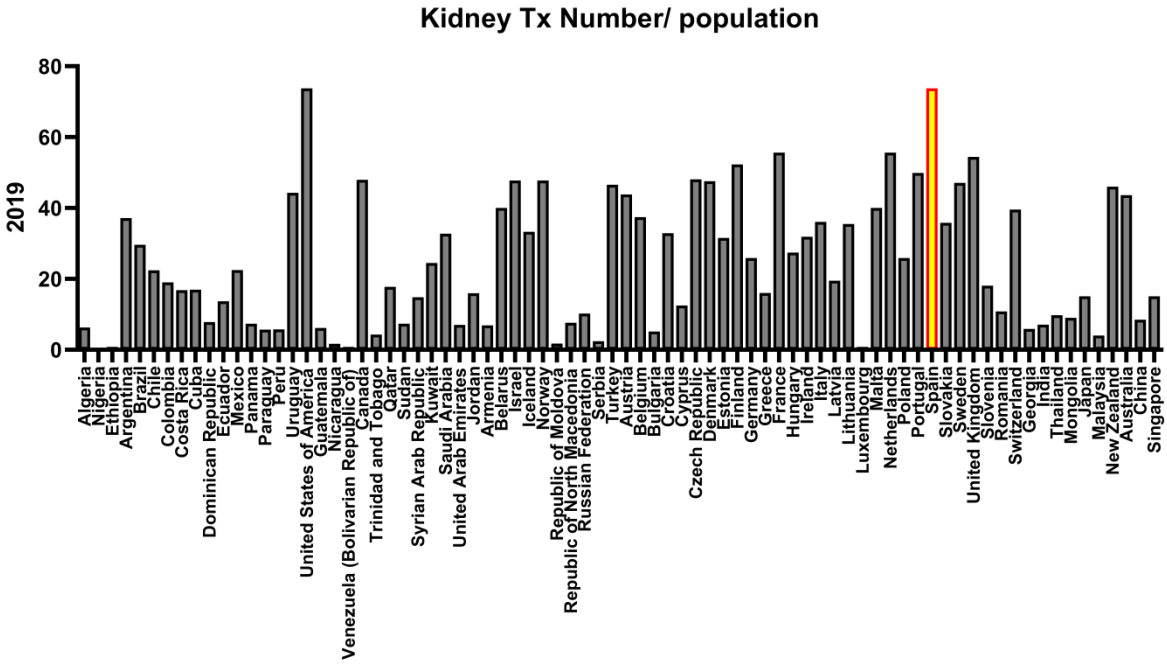


Figure 2| Number of KT pmp performed in 2019 in each country.

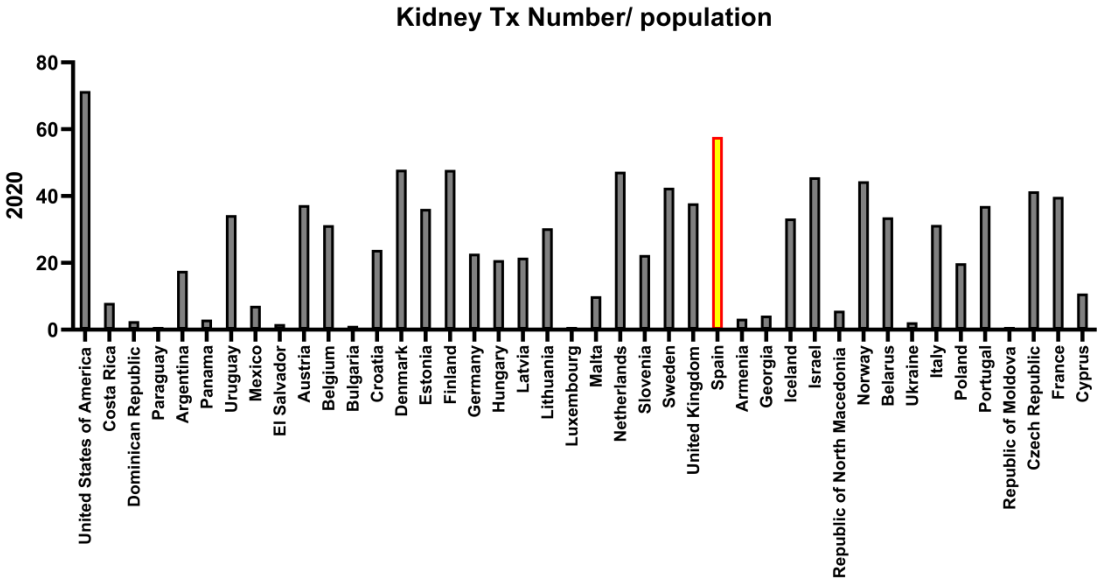


Figure 3| Number of KT pmp performed in 2020 in each country.

	2016	2017	2018	2019	2020
CC.AA					
Andalucía	531	603	609	575	417
Aragón	104	85	77	88	55
Asturias	57	72	77	84	82
Baleares	54	70	80	83	72
Canarias	142	123	156	152	148
Cantabria	**39**	**44**	**45**	**48**	**63**
Castilla La Mancha	95	97	82	120	45
Castilla y León	109	106	148	147	99
Cataluña	708	780	773	882	676
C. Valenciana	267	335	323	299	235
Extremadura	51	54	69	39	43
Galicia	135	160	170	176	141
La Rioja	12	20	21	16	11
Madrid	399	404	420	426	359
Murcia	70	93	82	86	69
Navarra	56	45	38	33	33
País Vasco	168	178	143	169	152

Table 1| Number of KT performed in Spain from 2016 to 2020 subdivided by regions.

Data colected from Spanish Ministry of Health, Consumer Affairs and Social Wellfare (2).

The incorporation of complement-dependent cytotoxicity tests (CDC) for the screening of transplant candidates, has been useful to avoid hyperacute rejection (3). Despite this, acute rejection (AR) used to be one of the main problems until recently (4).

In the 1980s, up to 60% of patients suffered an episode of AR (5). In the 1990s, the use of new immunosuppressive treatments reduced the number of transplant patients who presented an episode of AR (6).

1.1.2- Acute rejection of the graft

AR can be defined as an acute impairment in graft function associated with specific pathological changes in the graft. Although biopsies are considered “the imperfect Gold Standard” by some experts, the biopsy of the transplanted organ is the gold standard for the diagnosis of AR (7). In the Kidney Week (Washington 2019) it was proposed that not only biopsies but emerging biomarkers may help with management of kidney disease in several circumstances.

AR usually occurs within days to weeks after transplantation and it can be classified into antibody-mediated rejection (AMR) and acute celular rejection (ACR).

In kidney transplantation, AR consists in a mononuclear cell infiltrate that affects to the tubules (tubulitis) or the endothelium (endothelitis) of the graft (8). Based on this, in the past, it was infered that the AR of the allograft was caused only by cells and that the humoral response was responsible for hyperacute rejection. In the 90s Feucht demonstrated C4d deposits in the peritubular capillaries as a trace of the activation of the complement by anti-HLA antibodies (9). After that, Terasaki and other´s

works shed light in the diagnosis of humoral AR and current knowledge confirms the secretion of *de novo* donor specific antibodies (DSA) in AMR (10).

1.1.2.1- Acute celular rejection

On the other hand, in the ACR there is usually evidence of a lymphocytic infiltration of the tubules, interstitium (acute tubule-interstitial rejection) (Figure 4) and sometimes the arterial intima (acute vascular rejection) (Figure 5).

Graft dysfunction can lead to graft failure and lost. When biopsies are performed during the first year after Tx, it was observed that the most frequent diagnosis is celular or borderline rejection. However, in biopsies performed after the first year post Tx, AMR is the main cause of graft failure after KT (11). The study by Sellarés *et al*., demonstrates that most episodes of graft failure show features of AMR in biopsies.

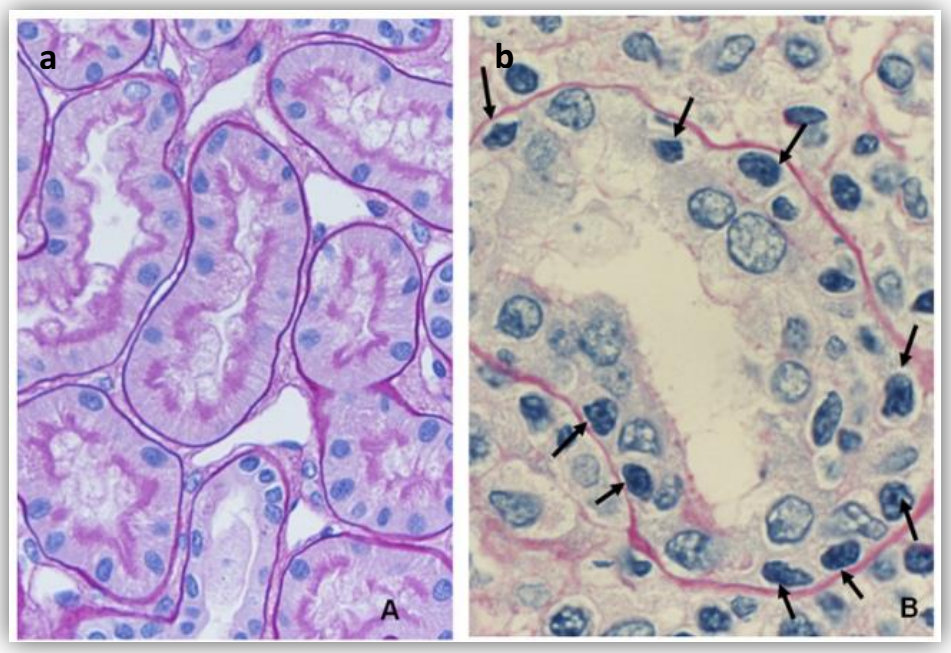


Figure 4| (a) Normal tubules (periodic acid Schiff, PAS). Note that there are no lymphocytes in normal tubules. **(b)** Acute cellular rejection with tubulitis (mononuclear cell infiltration). Mononuclear cells (CD8+ T lymphocytes) migrate from peritubular capillaries attracted by chemokines (PAS). Obtained from Online course in Organ Transplantation (2019), Spanish Society of Transplantation (12).

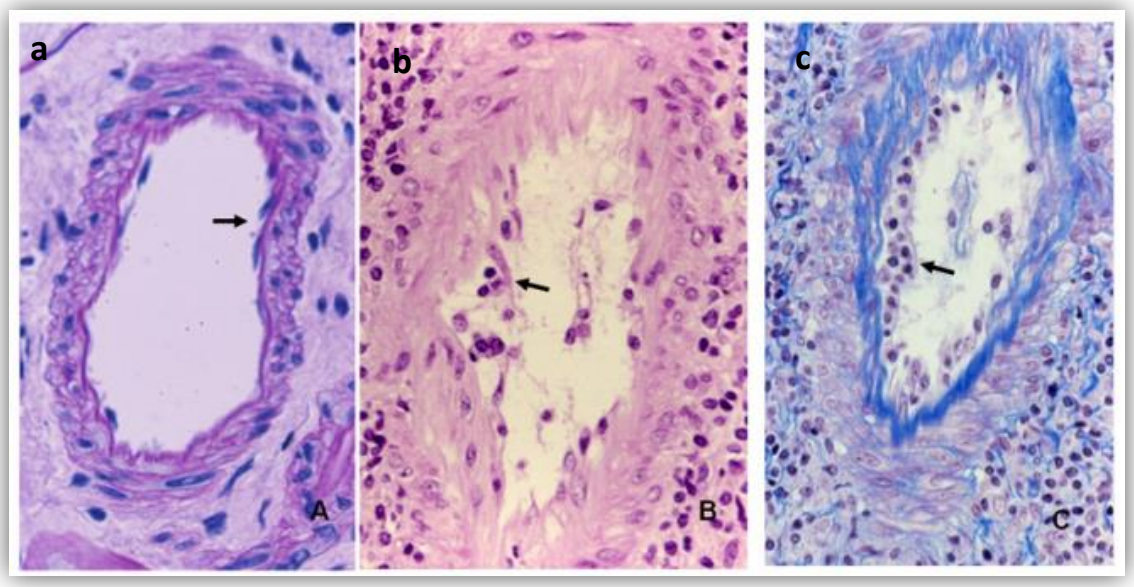


Figure 5| (a) Regular artery lined with endothelial cells (PAS). **(b)** Acute vascular celular rejection. Mononuclear cell infiltration under the vascular endothelium–endothelitis- (hematoxylin and eosin, H&E). **(c)** Multiple subendothelial mononuclear cells (lymphocytes) **(**Masson's trichrome) (12).

1.1.2.2- Antibody-mediated rejection

In AMR there is evidence of circulating DSA and immunological evidence of injuries to the kidney due to the binding of DSAs to human leukocyte antigens (HLA) expressed in the membrane of endothelial cells in the graft. This process activates complement and generates C3a and C5a which are opsonins that attract inflammatory cells to the peritubilar capillaries (capillaritis) and glomerular (glomerulonephritis) capillaries. Furthermore, inflammatory cells will be further activated by the binding of C3a and C5a to their receptors. On the other hand, IgG antibodies attached to the

endothelium molecules bind to Fc receptors expressed on inflammatory cells and cause cellular lysis, a process known as antibody-dependent cellular cytotoxicity (ADCC), causing kidney allograft vasculophaty (8) (Figure 6).

Although remarkable improvement has been achieved due to the treatment of AR , 10% of the kidney transplant recipients still develop AR within the first year (13).

For this reason, a number of research groups have been interested in searching for biomarkers of AR. The development of new technologies, which quantify proteins, mRNA and metabolites in cell extracts or fluids has opened up new windows of opportunities in the non-invasive diagnosis of AR (13).

Moreover, it has been demonstrated (14) in patients who have previously suffered an AR, that they present a higher incidence of chronic rejection (CR) and long-term allograft survival has not improved significantly throughout the years (15).

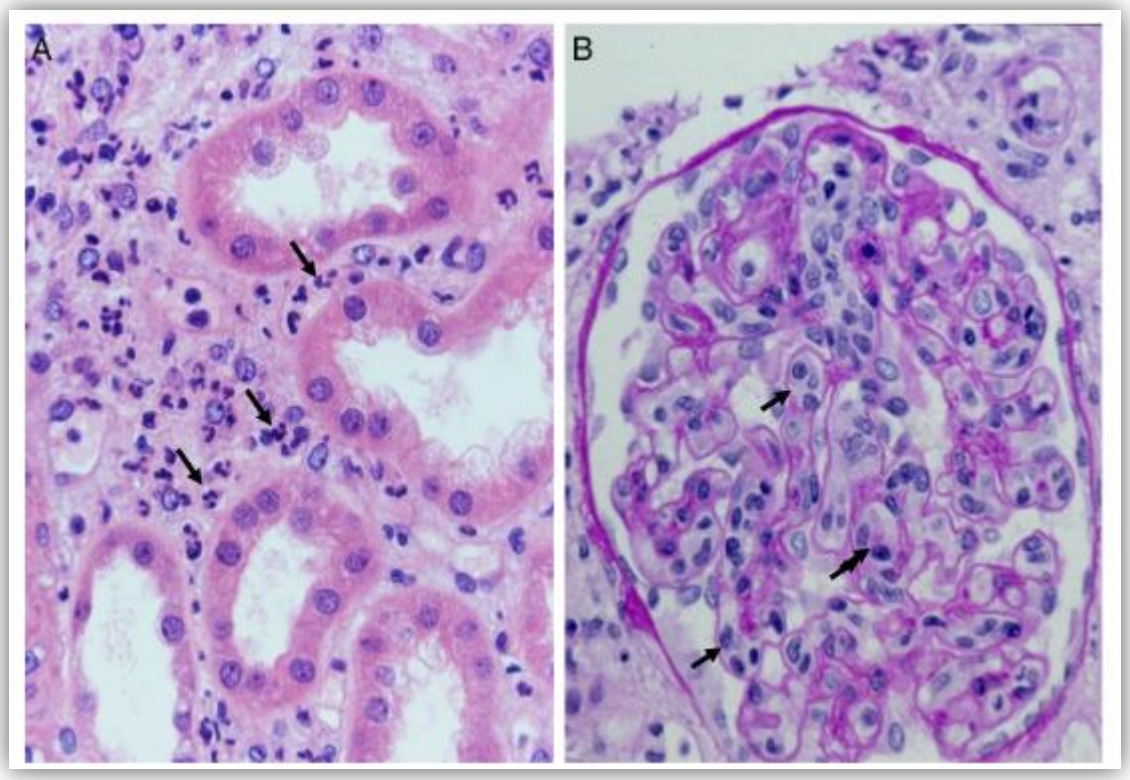


Figure 6| Acute humoral rejection. **(A)** Capillaritis. Polymorphonuclear leukocytes present in peritubular capillaries (H&E). **(B)** Glomerulitis. Inflammatory macrophages in glomerular capillaries can be observed (PAS). Images obtained from M. González Molina et al., 2016 (8).

1.1.3- Chronic rejection of the graft

Chronic tubulo-interstitial rejection is characterized by progressive deterioration of renal function, arterial hypertension, proteinuria, and edema in more advanced situations. Histologically, it is characterized by interstitial fibrosis, tubular atrophy and infiltration of lymphocytes and plasma cells that is accompanied by glomerular sclerosis of variable severity and double contour and lamination of the peritubular capillaries (Figure 7 and 8). These changes are responsible for the impairment in the kidney function as well (16).

The processes that trigger the chronic rejection of KT involve both humoral and cell-mediated immunity. There are other factors that predispose to long-term graft rejection including HLA-mismatching, previous episodes of AR, differences in donor-recipient age, and smoking (16).

Unfortunately, there is little knowledge regarding the improvement of long-term survival in renal transplants and the searching of different drug targets can improve survival of both graft and patient.

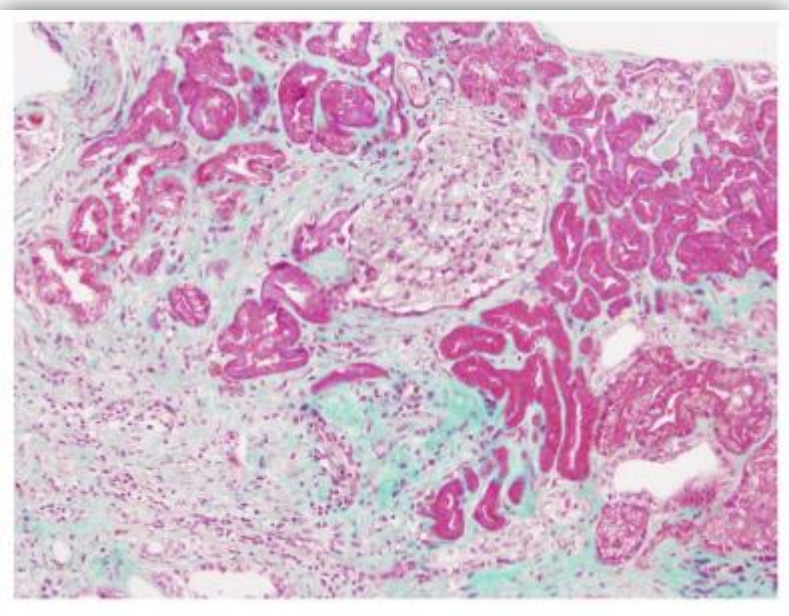


Figure 7| Interstitial fibrosis, tubular atrophy, and glomerular sclerosis in chronic rejection **(**Masson's trichrome) (12)**.**

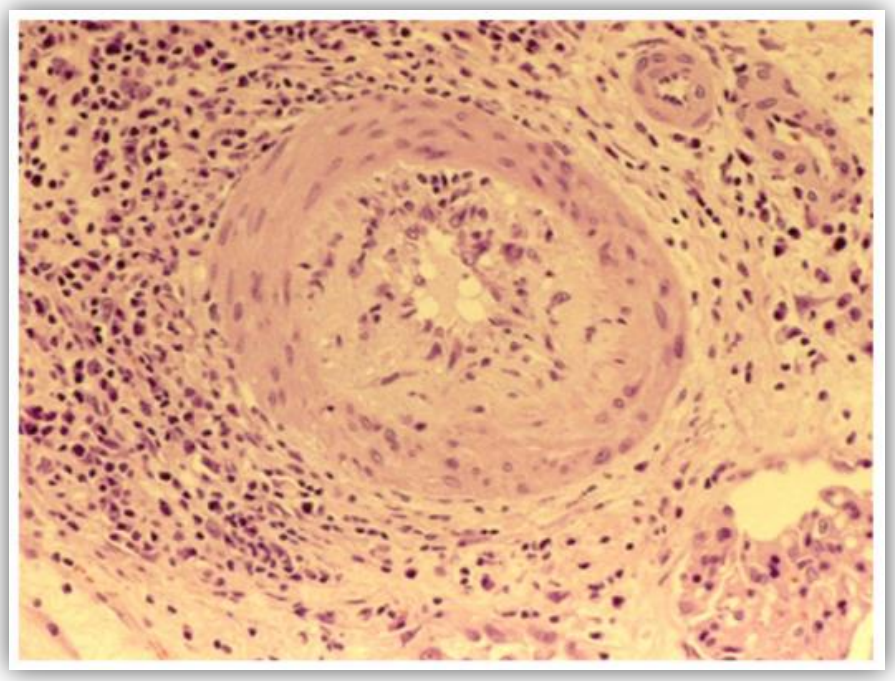


Figure 8| Active chronic rejection. In addition to the fibrosis of the vascular wall, an intimal and interstitial infiltration of mononuclear cells is observed (H&E). Taken from Online course in Organ Transplantation, Spanish Society of Transplantation (12).

1.2- Lung Transplantation

1.2.1- Background

Lung transplantation (LT) is a treatment option that provides quantity and quality of life in patients with end stage lung diseases. The first lung transplant was reported by Hardy and Webb in 1963, followed by others, with short survival only except for one case that lived for 10 months. In the 1980s, the prevention of rejection improved dramatically with the incorporation of immunosuppressive drugs such as cyclosporine and the improvement in surgical techniques (17).

After 50 years and more than 40,000 procedures worldwide, lung transplantation is now a consolidated therapeutic option. In the last 10

years the number of LT has increased worldwide (Figure 9). In 2019 in America 3369 lung transplants were performed, followed by 2327 in Europe, 791 in Western Pacific, 114 in South East Asia, 42 in Eastern Mediterranean and 0 in Africa (1). In spite of the pandemic, the data colected from Gobal data Base in donation and transplantation registred a number of 2629 lung transplants performed in America and 1799 in Europe in 2020.

During the last few years the number of interventions in Spain has progressively increased achieving its maximal rate all-time highs in 2019 (419 lung transplants). In 2019 Spain performed the 6-7% of lung transplants worldwide and reached the third top position in the annual rate of number of lung transplant patients pmp (Figure 10) (1,18). In 2020, Spain resists impact of Covid-19 and performs a number of 336 lung transplants (Figure 11). The number of lung transplants performed in the different comunities of Spain has been collected in **Table 2** (2).

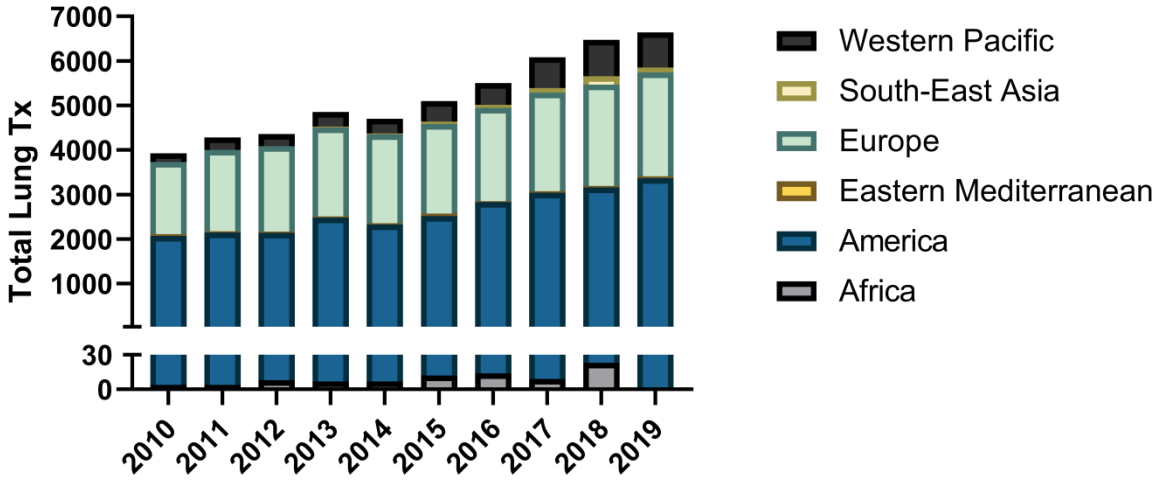


Figure 9| Number of lung transplants performed each year in the world by region, from 2010 to 2019.

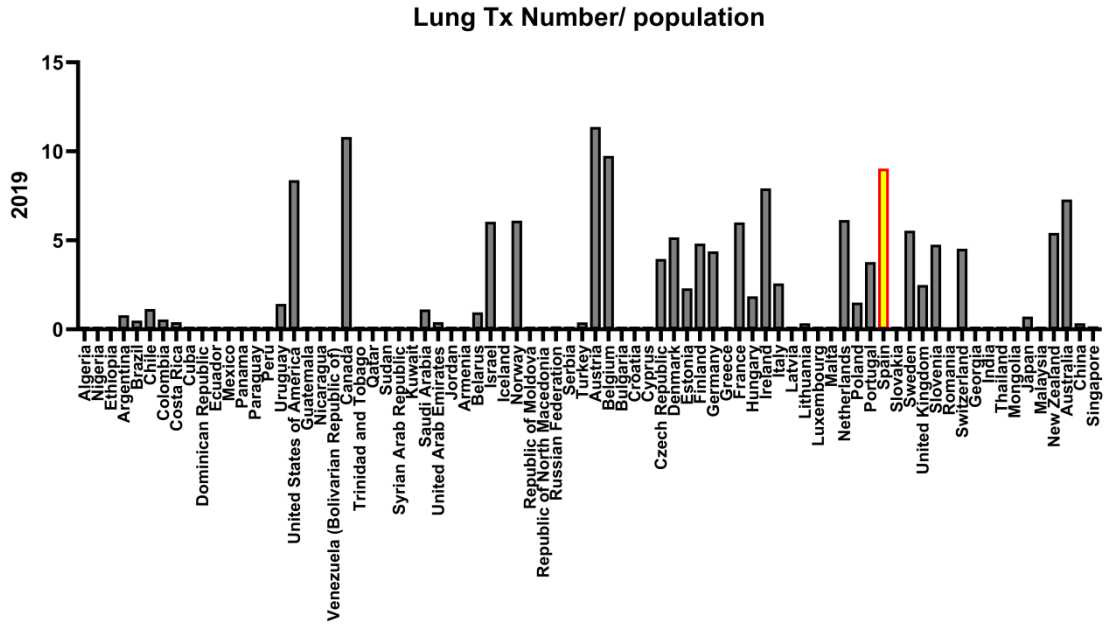


Figure 10| Number of lung transplants pmp performed in 2019 in each country.

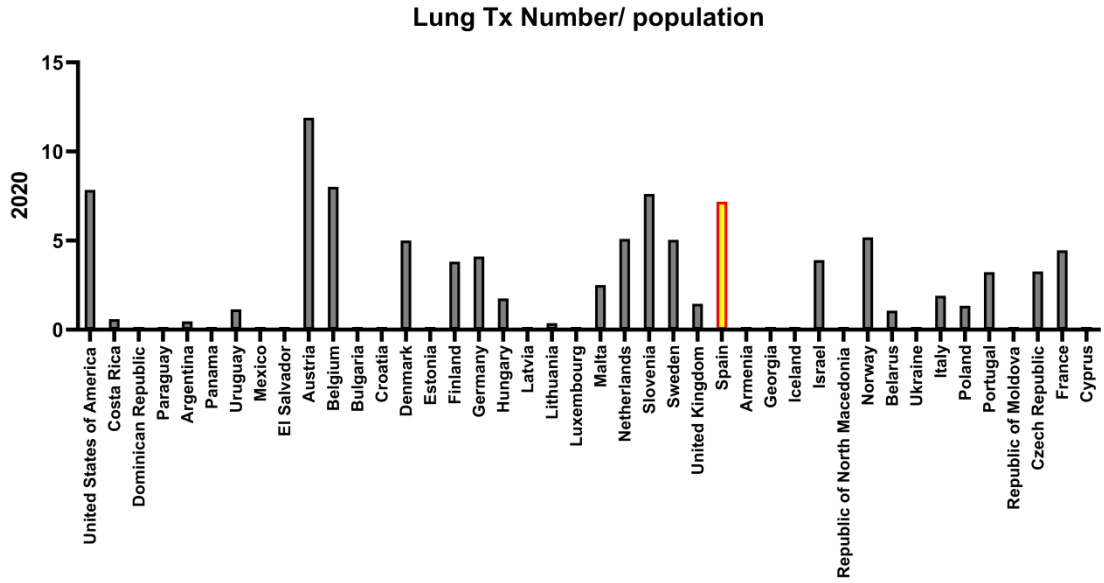


Figure 11| Number of lung transplants pmp performed in 2020 in each country.

		2016	2017	2018	2019	2020
CC.AA	**Hospital**					
Andalucía	H. Reina Sofía	33	43	41	48	49
		(11)	(20)	(18)	(15)	(25)
Cantabria	H. Marqués Valdecilla	**39**	**43**	**45**	**40**	**41**
		(24)	**(29)**	**(32)**	**(32)**	**(41)**
Cataluña	H. Vall d´Hebron	73	89	104	120	72
		(52)	(66)	(78)	(105)	(67)
C. Valenciana	H. La Fe	49	65	55	74	50
		(38)	(44)	(41)	(65)	(49)
Galicia	C.H. Univ. A Coruña	39	48	33	41	53
		(14)	(20)	(14)	(16)	(19)
Madrid	H. Puerta del Hierro	43	34	48	54	34
		(37)	(31)	(42)	(51)	(32)
	H. La Paz infantil	1		1		1
		(1)		(1)		(1)
	H.Doce de Octubre	30	41	42	42	36
		(21)	(33)	(37)	(36)	(25)

(): Bi-Lung Transplantation, including Heart-Lung Transplantation and combinations

Table 2| Number of lung transpants performed in Spain from 2016 to 2020 by region. Data colected from Spanish Ministry of Health, Consumer Affairs and Social Wellfare (2).

In spite of the progression in the management of lung transplant recipients (LTR), 5-year survival remains low (19). Survival is limited by post transplant development of bronchiolitis obliterans syndrome (BOS) which is the main cause of chronic allograft failure (20,21). As a consequence, one of the most importants aims in LT is to predict the risk of developing chronic allograft failure. On the other hand, dysregulation of immune mechanisms underlying chronic allograft failure has not been well defined and the use of prognostic biomarkers is necessary to achieve this objective.

Post-transplant management requires a high load of immunosuppression to avoid acute and chronic lung rejection without causing an increase in

respiratory infections. The achievement of an adequate balance between immunosuppression and infection is the most difficult equilibrium in lung transplantation. In fact, the most frequent causes of post-transplant mortality are rejection and infections (22).

A fundamental issue is the low number of grafts, since only 9% of multiorgan donations have an optimal lung to be implanted. On the other hand, both lung donors and recipients are becoming older as the life expectancy of the population increases. It is necessary to increase the number of donors and for this reason the criteria allowing older patients or the use of suboptimal donors are being implemented in donation programs (23,24).

In fact, one of the biggest problems that transplant units have to deal with is the death of patients that are included in the waiting list, due to the low number of donors (25). For that reason, a precise selection of candidates according to the regulations of the International Heart and LungTransplant Society (ISHLT) must be accomplished (26).

The use of the lung allocation score (LAS) helped to identify those receptors that can achieve the maximum profit from a lung, depending on the severity of the disease and their general state. Regulations in Spain were also published in order to establish criteria indication for the receptors (27). Survival after Lung transplantation in our country is similar to the ISHLT registry (28).

Although the survival rate has been improved in short and medium-term, the survival rate at 5 years post-transplantation is around 50%, in part due

to the persistence of obliterating bronchiolitis, which is the expression of CR.

A high mortality in the first months is conditioned mainly due to respiratory infections. In Spain, diffuse interstitial pulmonary disease (DIPD) is the main cause of transplantation, followed by chronic obstructive pulmonar disease (COPD). The results of lung transplantation may be optimized by choosing the appropriate recipient, the right time and the proper care before and after transplantation. The unsolved issues in LT are low number of available organs and the prevention of AR and CR and infection (29).

1.2.2- Acute Rejection of the graft

AR is an important issue in LT. The registry of the ISHLT reports 28% of lung transplant recipients experience at least one episode of treated AR within the first year following transplantation (30).

1.2.2.1- Acute Humoral rejection

The first description of AMR in lung transplantation was based on hiperacute rejection. However due to the incorporation of new test in HLA screening, the indicende of hiperacute rejection has disminished. AMR, is associated with: DSA, evidence of complement deposition on transbronchial biopsies (TBBx), histologic tissue injury and clinical signs of lung dysfunction (31,32). The diagnosis of AMR in LT is challenging since there are no generally accepted diagnosis criteria.

Although evidence of C4d deposits by immunohistochemistry (Figure 12), is generally pathognomonic for AMR in heart and renal transplantation,

lung transplant C4d staining is rarely seen on routine TBBx; C4d immunostaining can be seen with infection and primary graft dysfunction (PGD), both processes can also activate the complement cascade (33).

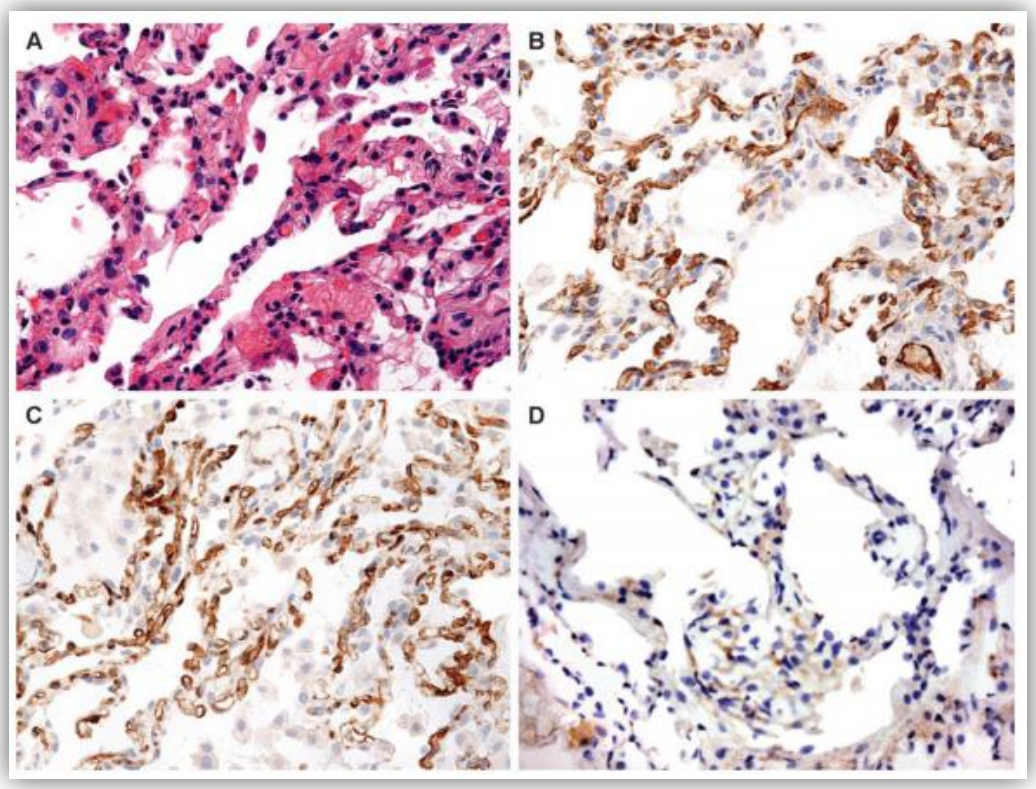


Figure 12| Acute humoral rejection. (A) Transbronchial biopsy at high-power magnification showing no cellular rejection (hematoxylin– eosin stain). **(B)** C4d staining showing diffuse, strong, linear staining of the interstitial alveolar capillaries. **(C)** Persistent C4d staining 6 weeks after study (B). **(D)** Absence of C4d staining 3 months after study in (B) (34).

1.2.2.2- Acute cellular rejection

Acute cellular rejection is characterized by a lymphocytic perivascular infiltrate (Figure 13), which is quantified according to its intensity and perivascular extension, airway inflammation, obliterative bronchiolitis, chronic vascular involvement-accelerated vascular sclerosis of the graft.

The immune response in ACR is driven by T cell priming due to the recognition of alloantigens (35).

ACR is an important risk factor in the development of BOS. The degree of ACR, specially the pathologic evidence of lymphocytic bronchiolitis is associated with the risk of BOS (36).

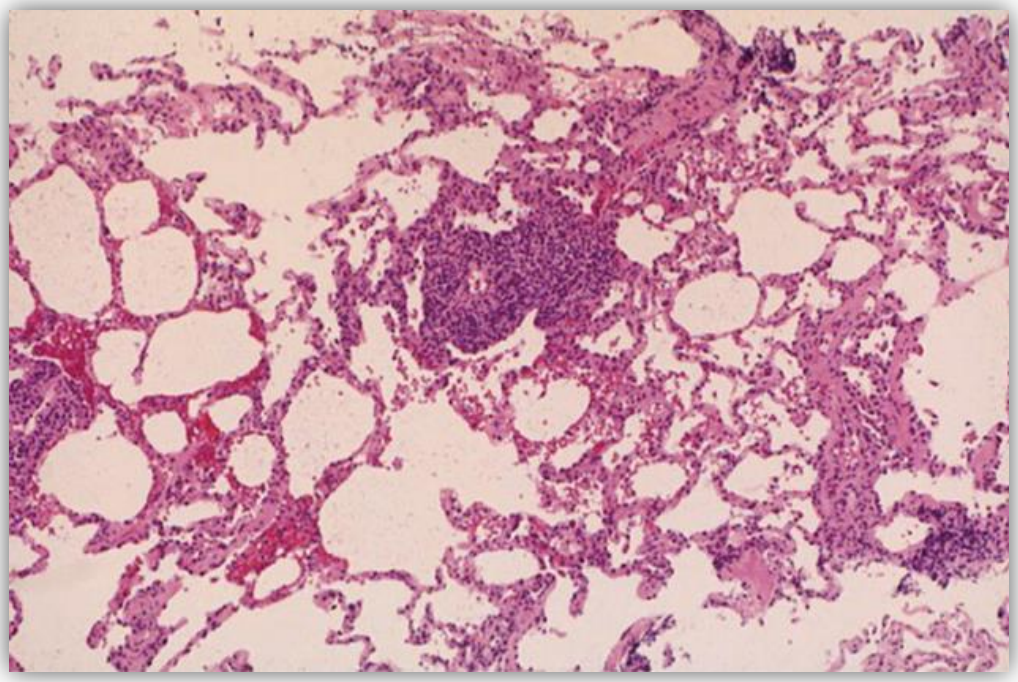


Figure 13| Acute cellular rejection. This transbronchial lung biopsy shows a case of mild ACR. A mononuclear cell infiltrate that expands the vascular adventitia is easily visible. (H&E staining) (37).

1.2.3- Chronic rejection

Long term survival has improved but remains limited by chronic lung allograft dysfunction (CLAD) with the persistence of bronchiolitis obliterans syndrome (BOS) as the main cause of late mortality. Although BOS was considered the main manifestation of chronic rejection for many years, recently another phenotype of CLAD, known as restrictive allograft

syndrome (RAS) or restrictive CLAD (rCLAD) has been described. BOS can be defined as an obstruction of airflow and it is measured as a reduction in forced expiratory volume in 1 second (FEV1) compared to the mean of the two best postoperative values in the absence of AR, infection or mechanical obstruction caused by dehiscence of the bronchial anastomosis without any other identifiable cause (38,39). The biopsy is characterized by lymphocytic inflammation in the submucosa of respiratory bronchioles leading to late proliferation of dense fibromyxoid granulation tissue (Figure 14)(40).

On the other hand RAS/rCLAD patients show a restrictive pulmonary function, persistent pleuro-parenchymal infiltrates on computed tomography (CT) and pleuroparenchymal fibro-elastosis on biopsies. Importantly, the patients with RAS/rCLAD have a severely limited survival post diagnosis of 6–18 months compared to 3–5 years after BOS diagnosis (41).

About 50% of lung recipients will experience BOS within 5 years following transplant, with a median survival after diagnosis between 3 and 5 years (38). Some of the mechanisms suggested to play a role in the development of BOS are damage due to PGD, infection, airway ischemia and gastroesophageal reflux (42).

Other studies have demonstrated cytomegalovirus (43), bacterial airway colonization (44), pseudomonas aeruginosa colonization, (45), fungal pneumonia and aspergillus colonization as important risk factors for the development of BOS (46).

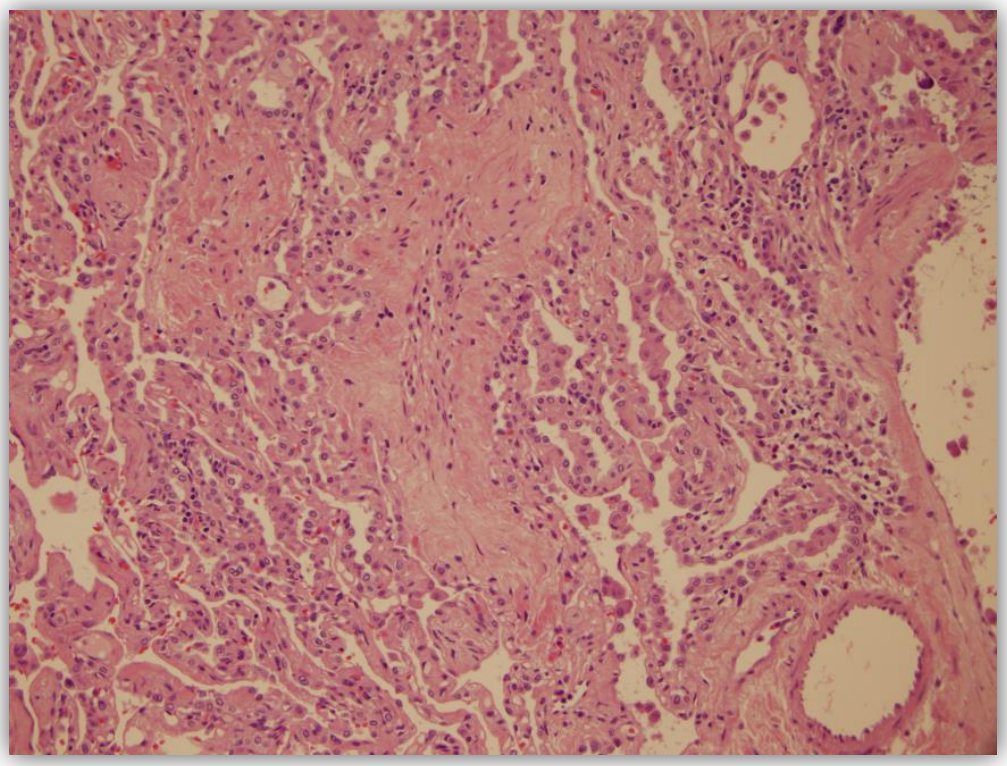


Figure 14| Chronic rejection. Lung biopsy showing bronchiolitis obliterans in the setting of chronic lung transplant rejection (H&E staining) (40).

Besides the most commonly known BOS phenotype, the rCLAD seems to be gaining great interest in the last few years. Some authors have used a 10% decrease in total lung capacity to diagnose patients suffering from a restrictive pulmonary function defect, while others have used a forced vital capacity decrease higher than 20%. There has also being described a combination of total lung capacity and forced vital capacity. However, at the moment, diagnostics of rCLAD remains troublesome. On biopsies pathological pleuroparenchyma fibro-elastosis is the most common histological pattern of rCLAD but nowadays diagnostic guidelines for rCLAD are still lacking (41).

The identification of humoral markers, of both innate and specific immunity would be very useful in the clinical context of transplantation.

1.3- Immunosuppressive treatment in transplantation

The management of immunosuppression in transplant patients is a complex practice in which there are many factors that influence the selection of a therapy, such as age, race, HLA mismatches, DSA antibodies, prevous transplants and pregnancies.

In KT the refinement in the use of immunosuppression combinations of these immunotherapies has improved rejection rates (10-15%) and graft survival (95%) in the first year post transplantation (47). The most widely used strategy in kidney transplantation is the combination of antibodies against T.-cell antigens: thymoglobulin or basiliximab as induction therapy in combination with low doses of conventional immunosuppressive agents (corticosteroids, tacrolimus and mycoplenolate) as maintenance therapy (48,49).

Immunosuppressive treatment is based on the combination of different drugs whose suppressive activity comes from different mechanisms of action, (50) giving rise to protocols grouped into thee categories: induction, which is used pre transplant or within the first days post-transplant, maintenance, and treatment of rejection (51).

In transplantation, dendritic cells from the donor and receptor move to the secondary lymphoid organs where they present the alloantigens to naïve and central memory T cells. Naïve T cells are efficiently triggered by dendritic cells but memory cells can be activated by other cell types, such as graft endothelium. The recognition of the alloantigen by T cells generates “signal 1” transduced through the CD3 molecule. The “Signal 2” is based on the recognition of coestimulatory molecules on dendritic cells

(CD80 and CD86) and T cells (CD28). Signals 1 and 2 activate transduction pathways that boost the expression of many new molecules, such as interleukin-2, CD154, and CD25. Interleukin-2 (IL-2) and other cytokines (e.g., interleukin-15) activate the "target of rapamycin" pathway to provide "signal 3," the trigger for cell proliferation. Proliferation and differentiation lead to a large number of effector T cells. When B cells are activated by the antigen engaging their antigen receptors and by interleukins such as IL-3, IL-4, IL-5 and interferon gamma (IFN-γ) they transforme into plasma cells that produce alloantibodies against donor antigens (50). The immunosuppressive therapy is mainly based on the combination of drugs that block the activation of $CD4^+$ T lymphocytes. According to their mechanism of action, these drugs are classified as: a) antilymphocyte antibodies (polyclonal or monoclonal) anticalcineurinic b) antimetabolites c) mTOR inhibitors d) and e) steroids (52).

1.3.1- Induction therapy

Induction therapy is indicated in those patients with a higher immunological risk such as sensitized patients. Induction treatments against T lymphocytes are administered pre-transplant or within the first days after transplantion; they can be administered in combination with other drugs used as maintenance treatment, such as corticosteroids at higher doses that are gradually reduced. There are polyclonal antibodies such as thymoglobulin, which are anti-human thymocyte antibodies from rabbit and anti-CD25 monoclonal antibodies including basiliximab or daclizumab, which are mouse antibodies that bind to the α component of the IL-2 receptor. Alentuzumab is an anti-CD52 antibody that causes the lysis of T lymphocytes, B lymphocytes, monocytes and macrophages. Due

to the nephrotoxic potential of calcineurin inhibitors (CNI) strategies have been sought to delay the onset of CNI without increasing the risk of acute rejection.

Despite the scarce evidence in the latest international registry (2) it was found that approximately 50% of lung transplant recipients receive induction therapy (most of them treated with anti-CD25, 10% treated with polyclonal antibodies and 8% were receiving anti-CD52) with statistically significant improvement in relation to those who do not receive induction (51).

Although it has been reported the negative impact of basiliximab (53) and the preservation of regulatory T cells (Treg) by thymoglobulin,the effect of induction therapy (54) on myeloid immunoregulatoy cells remains under study.

1.3.2- Maintenance immunosuppressive therapy

Maintenance therapy is a chronic treatment that is adjusted according to the patient's evolution and is less aggressive than induction therapy. Calcineurin inhibitors, such as tacrolimus and cyclosporine, or mTOR (mammalian Target of Rapamycin) inhibitors, such as sirolimus and everolimus, and antiproliferatives, such as mycophenolate mofetil and azathioprine, combined or not with corticosteroids, are regularly used (55) based on the association of calcineurin inhibitors with inhibitors of lymphocyte proliferation and glucocorticoids. For certain patients, they can also be combined with rituximab.

1.3.2.1- Calcineurin Inhibitors

Cylosporin A and tacrolimus enter the cell and bind to immunophilin in the cytoplasm. The CNIs immunophilin complex binds to and inhibits calcineurin. Calcineurin is a phosphatase that desphosphorylates multiple molecules, including nuclear factor NT (NFAT). Dephosphorylated NFAT translocates to the nucleus and binds to DNA regions that are promoters for cytokine synthesis in the T lymphocyte, such as IL-2. The inhibition of calcineurin by CNIs causes the inhibition of IL-2 synthesis, preventing the activation, proliferation, expansion, differentiation and the expression of pro-inflammatory molecules of the T cell. Nephrotoxicity is one of the main adverse effects. Neurological symptoms may appear. CNIs decrease insulin secretion, which can cause hepatotoxicity (more frequent with cyclosporine) and gastrointestinal effects. Although they are not mutagenic by themselves, there have been described cases of tumors (56,57) particularly lymphomas and lymphoproliferative disorders, as well as and increased risk of developing infections.

1.3.2.2- mTOR inhibitors

ImTOR are macrolides with immunosuppressive and antiproliferative activity whose mechanism of action is based on the blockade of the mammalian target of rapamycin (mTOR).

In regular conditions, IL-2 or cell growth factor receptors start the phosphatidyl-inositol-3-kinase (PI3K) cascade that leads to the activation of Akt or protein kinase B, which directly activates mTOR. Once activated, mTOR regulates proteins involved in mRNA translation, promoting the protein synthesis necessary for cell proliferation. The blockade of mTOR

inhibits the kinase p70 S6 and blocks all these translation processes, preventing cells from progressing from G1 to S phase. As a consequence it inhibits the cell cycle of T, B cells and hematopoietic cells in the G1 phase intracellular signal that regulates cell growth and division (58). In B lymphocytes they inhibit the synthesis of antibodies promoted by interleukins and in non-immune cells inhibit the production of growth factors. Everolimus inhibits the growth and proliferation of tumor cells that overexpress mTOR and has demonstrated antineoplastic efficacy in various types of tumors.

The main side effects are alterations in the lipid profile, as well as myelosuppression, mucositis, serositis and edema. Some cases of pneumonitis and proteinuria associated with its use have been described and its use as a basic immunosuppressant is contraindicated in lung transplantation, due to the risk of dehiscence of the bronchial suture during the first 3-6 months post-transplantation (59). On the other hand, it is the antiproliferative capacity of imTORs on fibroblasts (27) that is considered to reduce the development of BOS (60). The role of imTOR inhibitors in lung transplant is still being identified. They may be used in conjunction with or substituted for either calcineurin inhibitors or other antiproliferative agents. The most common reasons for using imTOR include kidney dysfunction due to calcineurin inhibitors, onset of BOS, and malignancy (61–63). For those patients that exhibit kidney dysfunction, adding an imTOR and reducing the CNI dose has been shown to improve kidney function (61,64,65). Additionally, due to their antiproliferative and anti-fibroblast effects (60), imTOR have been used in LTR with BOS to help slow progression.

The effect of different immunosuppressive drugs as maintenance therapy, has a differential effect on the immune system. Our group demonstrated more than a decade ago that CNI were able to reduce Treg in blood of renal transplant patients (66). On the other hand those patients under mTOR treatment showed similar levels of Treg cells in peripheral blood compared to healthy controls. On the other hand the election of CNI or imTOR is also influencing the humoral response, as it has been demonstrated that CNI are more powerful inhibitors of the antibody production and are more efficient in treating the humoral rejection than Everolimus (67).

1.3.2.3- Antimetabolite drugs

Mycophenolic acid is the most widely used antimetabolite in immunosuppression regimens in solid organ transplantation (68). Mycophenolaty mofetil (MMF) is a pro-drug easily absorbed from the gastrointestinal tract and hydrolysed in the liver to its active form or mycophenolic acid (MPA) a non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), avoiding the conversion of inosine monophosphate to guanine monophosphate. This process blocks the *de novo* synthesis of purines, a key process for the proliferation of lymphocytes and DNA replication, unlike other cells that can use other pathways for purine synthesis. This makes MMF a specific inhibitor of lymphocyte proliferation, reducing the production of antibodies by the B lymphocyte as well as the cytotoxicity of the T lymphocyte (69).

The most frequent adverse effects are gastrointestinal, cholecystitis, hemorrhagic gastritis, intestinal perforation and pancreatitis have

occasionally been described. It can cause leukopenia. The association with other immunosuppressants increases the risk of opportunistic neoplasms and viral and fungal infections, particularly cytomegalovirus and candidiasis. In addition, mycophenolate is teratogenic and is contraindicated in pregnancy.

Azathioprine is a derivative of 6-mercaptopurine (6-MP) that releases 6-MP into the tissues. It inhibits DNA synthesis and reduces the proliferation of T and B lymphocytes, in response to the antigenic stimulus. T lymphocytes are more sensitive than B lymphocytes. It is useful in preventing rejection of grafts or organ transplants but not to treat rejection. The main side effect of azathioprine is myelosuppression. Similarly to other immunosuppressants, they increase the risk of infections and neoplasms, particularly skin cancer, by increasing sensitivity to sun exposure.

1.3.2.4- Corticosteroids

Corticosteroids are one of the main pillars of immunosuppression in induction and maintenance treatment during the first years after transplantation and during rejection episodes. They have a powerful and nonspecific immunosuppressive effect that affects all leukocytes. They cross the cell membrane and bind to cytoplasmic receptors, forming a complex that translocates to the nucleus where it modifies the transcription of genes involved in the inflammatory response. Corticosteroids affect the number, distribution, and function of B and T lymphocytes, granulocytes, macrophages, monocytes, and endothelial cells (70,71). They also modify the expression of cytokines, growth factors,

CD-40 ligand, adhesion molecules, chemotactic factors, and proteolytic and lipolytic enzymes. Steroids are very important in the induction of immune tolerance. The great problem with corticosteroids are their side effects in maintenance therapy: arterial hypertension, diabetes, hypercholesterolemia, hydrosaline retention, growth retardation, osteopenia and osteoporosis, nervousness, emotional lability, cataracts, hirsutism, proximal myopathy, acne, capillary fragility, weight gain and central obesity (72).

The effects of corticosteroids on Treg cell number have been studied in animal models and humans but their role appears to be context-dependent. In several *in vitro* studies corticosteroids favour the expansion of activated Treg, but the effects of corticosteroid treatment on Treg seem to be depending on the disease and tissue conditions (73).

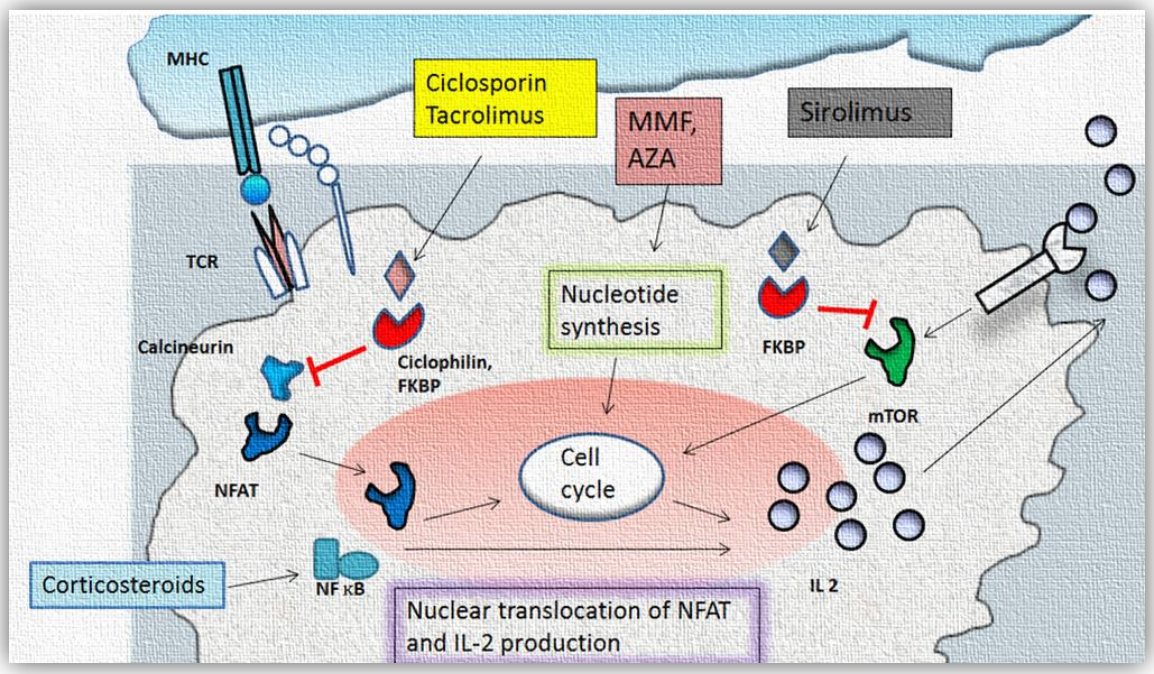


Figure 15| Maintenance immunosuppressive therapy and mechanisms of action.

1.3.3- Humoral rejection therapy

In general, immunosuppressive drugs are used to suppress T cell mediated immunity. However the role of humoral response in solid organ transplant recipients has become more evident over the years and new strategies have been designed to suppress the humoral response. Rituximab, a monoclonal anti-CD20 antibody that induces B cell depletion(74) Plasmapheresis is mainly used for antibody removal from circulation in humoral rejection (75). Bortezomib, an inhibitor of 26S proteasome that leads to plasma cell apoptosis, has been used successfully in case reports to treat possible acute humoral rejection in LTRs (76,77). Intravenous immunoglobulin (IVIG) is used in transplantation in highly sensitized patients as it reduces the levels of HLA antibodies and inhibits their capacity to bind to the graft (78). Treatment with IVIG, plasmaphresis, rituximab, antithymocyte globulin and eculizumab have been described in various case reports with variable results (79–81).

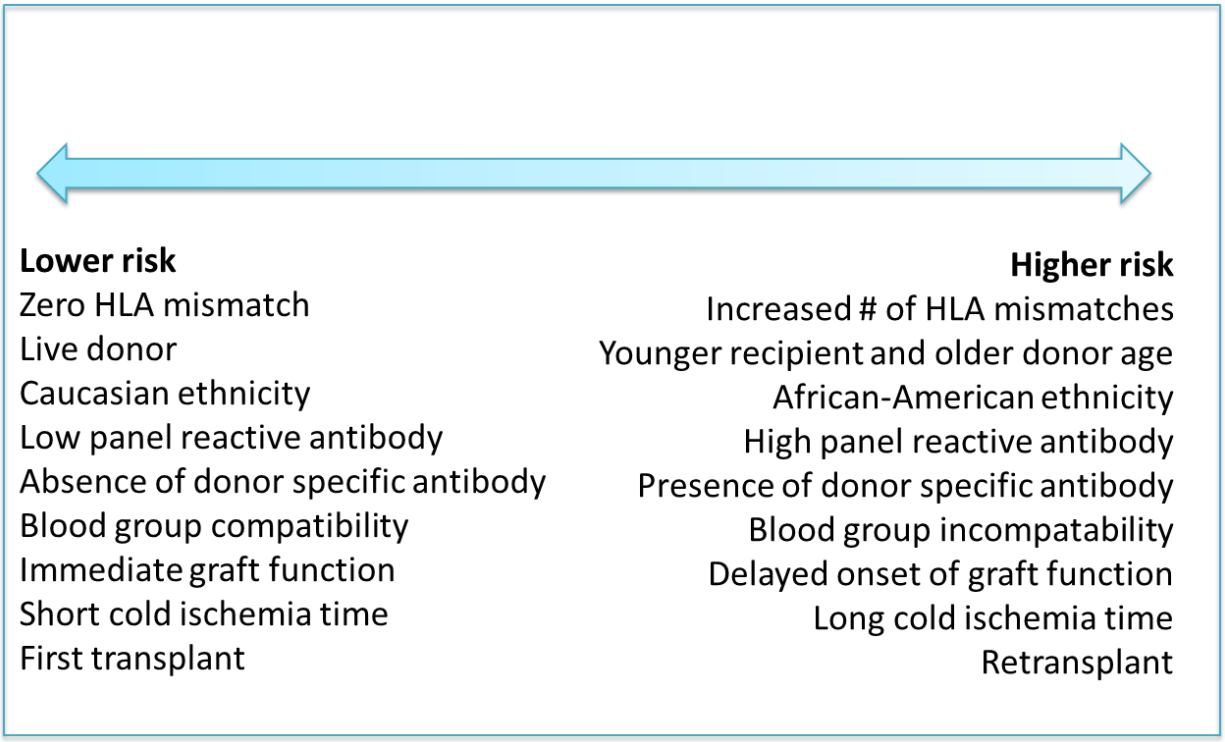


Figure 16| Induction therapy choice based on risk assessment. (Adapted from Hardinger et al., 2013)(48).

1.4- The immune system in transplantation

1.4.1- The role of innate immune response in transplantation

The donor brain death increases the risk of inflammation in the graft. When organs are obtained from brain-dead donors, the ischemia induced in the organs develop an inflammatory response featured by an infiltration of mononuclear and polymorphonuclear cells and increased expression of adhesion molecules and complement factors, which directly activate graft infiltrating T lymphocytes and dendritic cells (82). Brain death is accompanied by hemodynamic changes characterized by an initial phase of arterial hypertension, followed by hypotension (83). The decrease in perfusion pressure causes a storm of catecholamine secretion with a potent release of pro-inflammatory cytokines, which activate cells of the immune system (IS).

The expression of MHC, MCP-1 and RANTES molecules increases in endothelial and dendritic cells. Additionally, in KT, the expression of IL-1β and TNF-α in the interstitium and the renal tubules increases. These findings are found in a significantly lower proportion in the living donor graft.

Ischemia increases the risk of inflammation, delayed graft function, and rejection (84). The injured cells release intracellular material that stimulates the innate immune response. This material is known as « damage associated molecular patterns» (DAMPs).

DAMPs are endogenous molecules released from injured cells, and modulate immune responses via pattern recognition receptors (PRRs). Dying and stressed cells release many DAMPs, but living cells can still expose DAMPs on their plasma membranes (85). They are ligands for Toll-like receptors (TLRs) and cytosol pattern recognition receptors (nucleotide-binding domain oligomerization protein receptors, NLRs). Once stimulated, they initiate a biochemical signal pathway that activates the innate immune response, including the production of cytokines and the expression of costimulatory molecules (86). Organs from brain-dead donors show a higher expression of TLRs than those from living donors (87).

Dendritic cells are specialized phagocytes present in most tissues. In the graft, they are generally resting and are activated by secreted cytokines due to the effect of brain death and by the action of DAMPs on TLRs. Once activated, they acquire mobility and phagocytic capacity of peptides from the medium; such as, those derived from MHC molecules, which process and express them in their HLA (88).

1.4.2- The role of the adaptive immune response in graft rejection

Vascular unclamping connects the transplanted organ and the recipient IS; amplifies the innate immune response in the graft and initiates the stimulation of the adaptive immune response (alloantigen-specific response).

Dendritic cells, DAMPs, cytokines, and chemokines from the transplanted organ enter the recipient's circulation; and on the contrary, cells of the receptor's innate immune response (macrophages, neutrophils and NK

cells) are attracted to the graft. In addition, activated dendritic, endothelial, and stromal cells secrete IL-6 and TNF-α, which stimulate the expression of CXCL8 (IL-8) chemokines in epithelial cells, which attract neutrophils with CXCR2 receptors (89).

Recirculating naïve lymphocytes that are primed in the secondary lymphoid tissues ,once activated can migrate into the transplant (89).

Priming of recipient T cells requires presentation of alloantigens to naive T lymphocytes by dendritic cells. To facilitate it, dendritic cells migrate, guided by chemokines, to naïve T-lymphocyte-rich areas of secondary lymphoid organs. The association between both cells (immune synapse) is carried out between the TCR and the HLA molecule of the dendritic cell (carrier of the alloantigen), with the participation of adhesion and costimulation molecules (elaborated in section 1.3 Immunosuppressive treatment in transplantation).

One of the main characteristics of HLA molecules is their high polymorphism (there are multiple alleles of each gene present in the population). This impressive HLA variability constitutes the molecular basis for rejection. Thus, the recipient's immune system recognizes the differences in HLA between donor and recipient, which triggers an allospecific response, whose purpose is to destroy the graft.

The direct pathway is the predominant in initiating the adaptive immune response to an allogenic mayor histocompatibility complex (MHC) transplant. However, because the number of passenger leukocytes transferred within a transplanted organ is limitated, over the years it is

likely that these donor antigen presenting cells (APC) will disappear and that the predominant response becomes an indirect response (89).

Once activated B lymphocytes differentiate into plasma cells and produce soluble immunoglobulins. For their activation they require contact with the antigen and the collaboration of the cytokines produced by $CD4^+$ Th2.

1.5- Mechanisms of tolerance in transplantation

One of the main objectives in the field of transplantation is the reduction or withdrawal of immunosuppressive drugs. Thus, in liver transplantation there is a number of cases of tolerance after total withdrawal of immunosuppression that have been described (90). This phemomenon is called operational tolerance.

Regarding tolerance in transplantation there are several mechanisms that can favour a state of tolerance of the graft. Clonal deletion is the elimination of alloantigen-reactive T cells from the donor that can be achieved centrally or peripherally. In animal models it has been shown that the ablation of specific T cells in the thymus and the periphery can be accomplished with non-myeloablative conditioning therapies and the tolerance state achieved is systemic (91). In the periphery, tolerance can be stimulated by alloantigen recognition under suboptimal conditions, costimulatory blockade for example (92). The consequence is the elimination or functional inactivation of the cells that are responding to the donor alloantigen. Clonal depletion can occur after liver transplantation, where a large number of donor-derived antigen-presenting cells migrate from the liver to lymphoid tissues after transplantation, being able to activate this response (93).

The concept of anergy consists of a hiporesponse of the T-cell to alloantigen estimulation. It was observed *in vitro* that the lack of signals, the recognition of antigens with low affinity or the presentation by immature APC which secrete suppressor cytokines induce anergy (94). Other forms of T-cell anergy involve the development of regulatory activity due to competition with alloreactive T cells for the surface of APC as well as for locally produced IL-2 (95).

Ignorance is a mechanism for the induction of non-response to alloantigens. Encapsulated cells, such as Langerhans islets prior to implantation, should allow the immune system to ignore the presence of the graft. It has been described that pre transplant donor splenocyte infusion after transplantation induce tolerance (96). Similarly, lack of support at allorecognition would also facilitate tolerance (97,98). The antigen expression level, the amount of proinflammatory cytokines and the presence/absence of costimulatory signals in the graft are some of the factors that influence this process.

Immunoregulation is a process based on one or several populations of cells that regulate the activity of others. Different subpopulations of leukocytes have been described to have the ability to control immune responses to alloantigenic stimulation. This immunoregulatory cells display different mechanisms to regulate both innate and acquired immune responses.

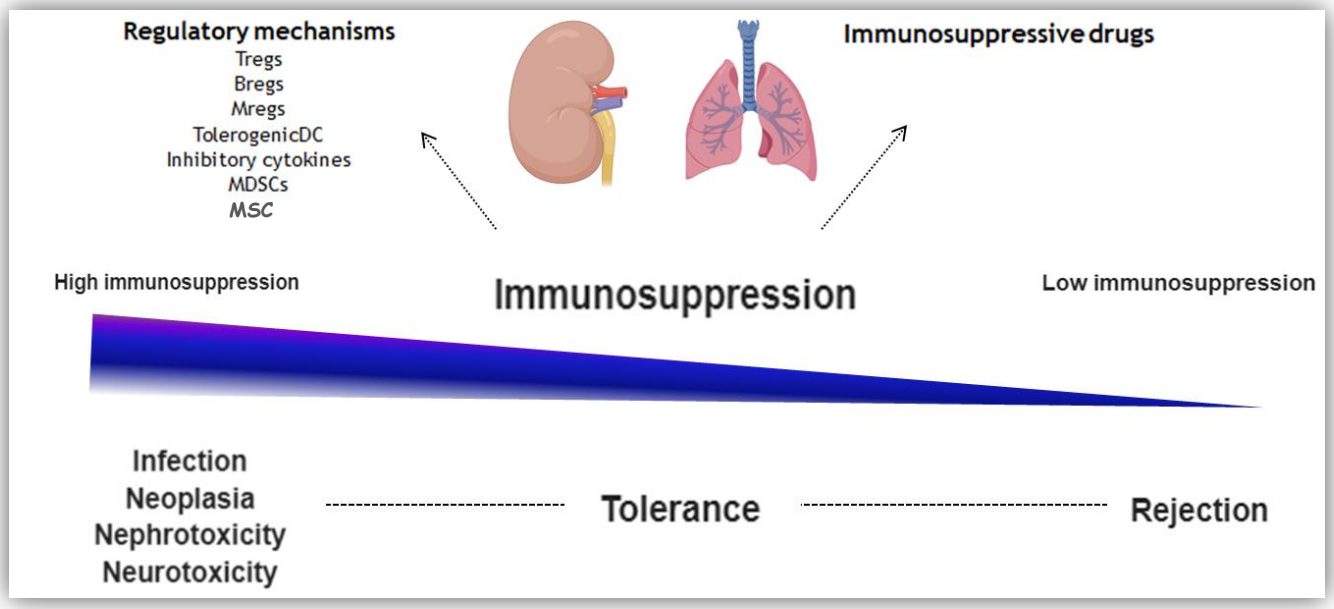


Figure 17| One of the most important goals in transplantation is to achieve a balanced state of tolerance or immunoregulation in which immunosuppressive drugs are able to prevent rejection but the side effects are avoided. Regulatory mechanisms seem to play an important role in this process and may help to accomplish this aim.

1.6-Regulatory mechanisms of the immune response

1.6.1- T, NKT and B regulatory cells

Within the different regulatory mechanisms of the immune response the T regulatory cells are the most studied, the can be classified into natural Tregs originated in the thymus and it has been demonstrated that they exert a main role in controlling autoimmune diseases. IL-10 producing type 1 Tregs (Tr1) can be distinguished from other T CD4+ subsets (Th1/Th2) due to their low expression of IL-2 and IL-4. An important difference between nTregs and Tr1 is that Tr1 do not express high levels of CD25 on their surface (99). TGFβ producing Th3 cells were described from oral tolerance experiments (100,101). Other lymphoid cells such as T CD8+ cells, T γδcells , NK cells, NKT cells, have also been described due to their

immunorregulatory properties (102–106). B regulatory cells (Breg) are considered as a population able to suppress immune responses independent of antibody secretion, but they can be identified by IL-10 secretion (107).

1.6.2- Mesenchymal Stem Cells

Mesenchimal stem cells (MSC) have been studied in the clinical setting due to their immunomodulatory features. MSC were shown to stimulate the polarization of macrophages towards a regulatory phenotype and to inhibit the differentiation into the proinflamatory phenotype and dendritic cells (DCs) (108–110). MSCs suppress T cell proliferation (111) and increase the expansion of Tregs *in vitro* and *in vivo* (112,113). MSC are also strong inhibitors of natural killer cell (NK cell) proliferation. (114) and able to reduce plasmablast formation as well as to promote the induction of Bregs (115).

1.6.3- Myeloid Regulatory Cells

Myeloid cells, which are involved both in non-specific reactions and donor-specific adaptive responses during allograft rejection, play a main role starting immune responses. Under certain circumstances, they contribute to the inflammatory process, enhancing disease pathology. However, myeloid cells with regulatory properties can protect the host from uncontrolled inflammation. These cells, known as MRC, have been described within all the major myeloid cell lineages.

1.6.3.1- Tolerogenic Dendritic cells (TolDC)

TolDC are semi-immature cells (featured by their low HLA-DR expression and costimulatory molecules). At baseline they are able to present autoantigens, but under low costimulation they induce anergy in autorreactive T cells or promote T reg generation. TolDC are induced in anti inflammatory conditions in the local milieu (TGF-β o IL-10, retinoic acid o vitamine D3) (116). In this way TolDC have been generated *in vitro* and have been used as pasive therapy in some transplant models. The increase on the expression of PD-L1 and PD-L2 is among the mechanisms implied in Treg induction from TolDC. Moreover, Tregs are able to reduce costimulatory molecules from the dendritic cell surface an induce TolDC (117).

1.6.3.2- Regulatory Macrophages (Mreg)

Macrophages exhibit considerable plasticity and they can modify their phenotype and functions in response to different microenviroments. (118)Classical macrophages known as M1 are well known for their role in promoting immune responses. On the other hand, M2 macrophages are activated in different conditions and they are associated with the reduction of tissue inflammation. Among M2 macrophages several populations can be classified depending on their specific functions: M2a induced by Th2 cytokines, IL4, IL13 and responsible for mediating tissue repair and Th2 responses; M2b induced by immune complexes, TLRs and IL-1R are responsible for immunoregulation; M2c induced by anti-inflammatoy cytokines IL-10, TGFβ and exerting their function in

phagocytosis or glucocorticoids and M2d induced by IL-6 like cytokines and participating in angiogenesis (119).

Mreg have been described due to their ability to increase Treg and to modulate immune responses favouring allograft acceptance. Although some M2 subpopulations present regulatory features, transcriptomic studies have shown considerable differences between M2 macrophages and Mreg profiles (120).

On the other hand Mreg have been differentiated *in vitro* from monocytes cultured with M-CSF and INF-γ, in a licensing process. leading to stabilized expression of indoleamine 2,3 dioxygenase (IDO) (121). It has been demonstrated an increase in the expression of DC-SIGN M-CSF dependent in tolerogenic macrophages (122).

Among the different mechanisms proposed above to achieve tolerance, the present work will focus on the role played by MDSCs in the regulation of the immune response to achieve tolerance to alloantigens, and the effect of immunosuppressive drugs on this regulatory population.

1.6.3.3- Myeloid-Derived Suppressor Cells (MDSCs)

Among MRCs, myeloid-derived suppressor cells (MDSCs) have been described as a heterogeneous group of myeloid cells known to accumulate under chronic pathological conditions (123). As a reflection of their biology, these cells had been called ‘‘immature myeloid cells’’ or ‘‘myeloid suppressor cells’’ (MSC) but as neither term was considered as accurate, *Gabrilovich DI, et al.*(124) proposed the term ‘‘myeloid -derived suppressor cells’ considering this term closer to reflect their origin and function. Myeloid cells were described for the first time more than 20

years ago in tumors (125–127) but their important role in the immune system has only recently been appreciated due to the evidence that has demonstrated their contribution to the regulation of immune responses in cancer, organ transplantation, infection and autoimmune diseases (123,128–132). Initially MDSC have been described as immature cells that expand in the bone marrow in response to chronic inflammatory signals but evidence support in certain circumstances MDSC may represent monocytes and neutrophils that have been activated into immunosuppressive populations (133).

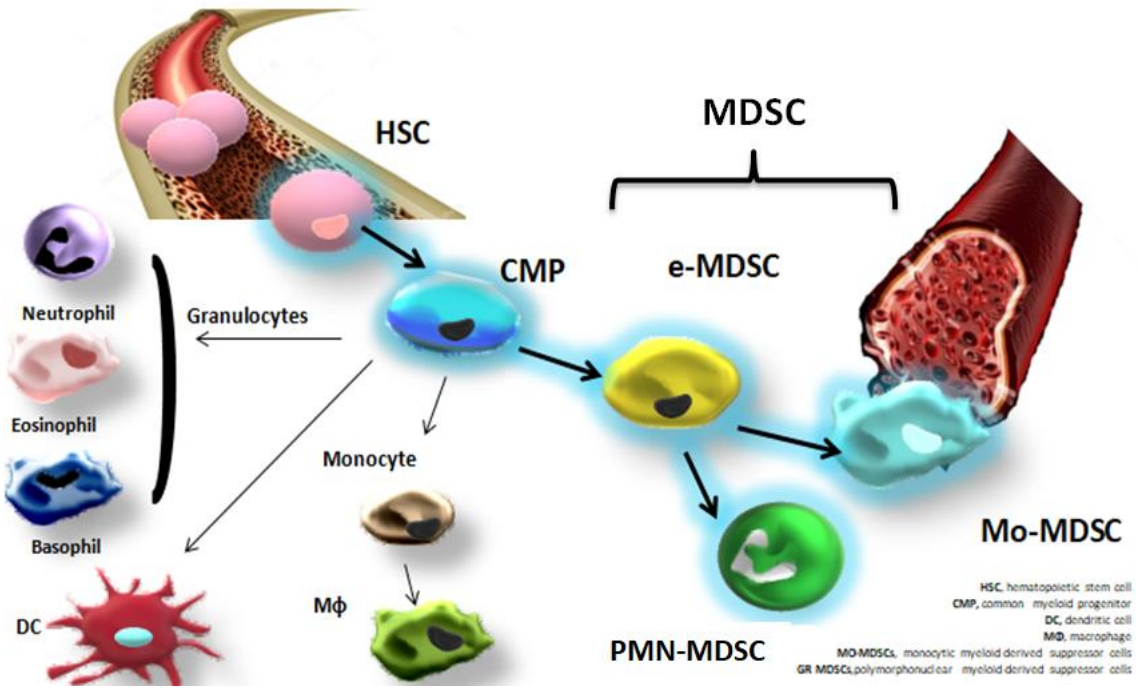


Figure 18| Human MDSCs mainly come from a common myeloid progenitor (CMP) in the bone marrow. They develop under chronic pathological conditions and can be grouped into three main populations: monocytic MDSCs (Mo-MDSCs), polymorphonuclear MDSC (PMN-MDSCs) and early stage MDSC (e-MDSCs).

1.6.3.3.1- Phenotyping of human MDSCs

Human MDSCs in peripheral blood are classified in three main subsets: monocytic-MDSC (Mo-MDSCs: $CD33^{+}$ $CD11b^{+}$ $CD14^{+}$ $HLA\text{-}DR^{-/low}$),

polymorphonuclear-MDSC (PMN-MDSCs:$CD11b^+$ $CD14^-CD15^+$ $HLA\text{-}DR^-$ or $CD11b^+$ $CD14^-$ $CD66b^+$)(134) and a population lacking both differentiation surface markers classified as early-stage MDSC (e-MDSCs: $CD33^+$ $CD15^-$ $CD14^-HLA\text{-}DR^-$)(134). CD33 marker can be swapped with CD11b. cells.While Mo-MDSC are $CD33^+$, PMN-MDSC are $CD33^{dim}$ (135). The features and clinical relevance of e-MDSC are not well stablished but limited suppression of T cell proliferation and cytokine expression was found by some authors (136). Other suggested makers in human MDSCs include high levels of CD66b and low levels of CD62L and CD16, vascular endothelial growth factor receptor 1 (VEGFR1) (Flt-1) (137) and expression of CD124 (138). Initially the term 'granulocytic MDSC' was used to describe PMN-MDSC (139,140) but since PMN-MDSC are phenotypically distinct from steady-state neutrophils lately *V. Bronte et* al proposed the term PMN as more accurate to define this subset (134).

1.6.3.3.2- Suppressive function of human MDSCs

Because these markers are not exclusively expressed by MDSCs, these regulatory cell subsets are best defined by their capacity to suppress T cell proliferation (141), which is associated with their ability to induce T cell apoptosis (142) and expand Treg cells (143)(Figure 19). Moreover, the interaction between MDSC and other immune cells has been described in recent years (144–147).

It is important to remark that assays of human MDSC function are difficult to implement due to their technical complexity and high variability. At the present time, the method to isolate neutrophils from PMN-MDSC is Ficoll gradient. Low-density fraction contains PMN-MDSC and activated

neutrophils. Therefore, $CD11b^{+}CD14^{-}CD15^{+}/CD66^{+}$ cells in low-density fraction may be both subsets (134). For this reason there is a need for reliable markers of human MDSC function since at present the gating criteria is not enough to discriminate monocytes from Mo-MDSCs and neutrophils from PMN-MDSC. Human MDSCs exert their T cell suppressive actions through a wide variety of mechanisms, including production of anti-inflammatory cytokines and up-regulation of immune-regulatory molecules, including arginase 1 (Arg1) and indoleamine 2,3-dioxygenase (IDO) (148,149) (Figure 19).

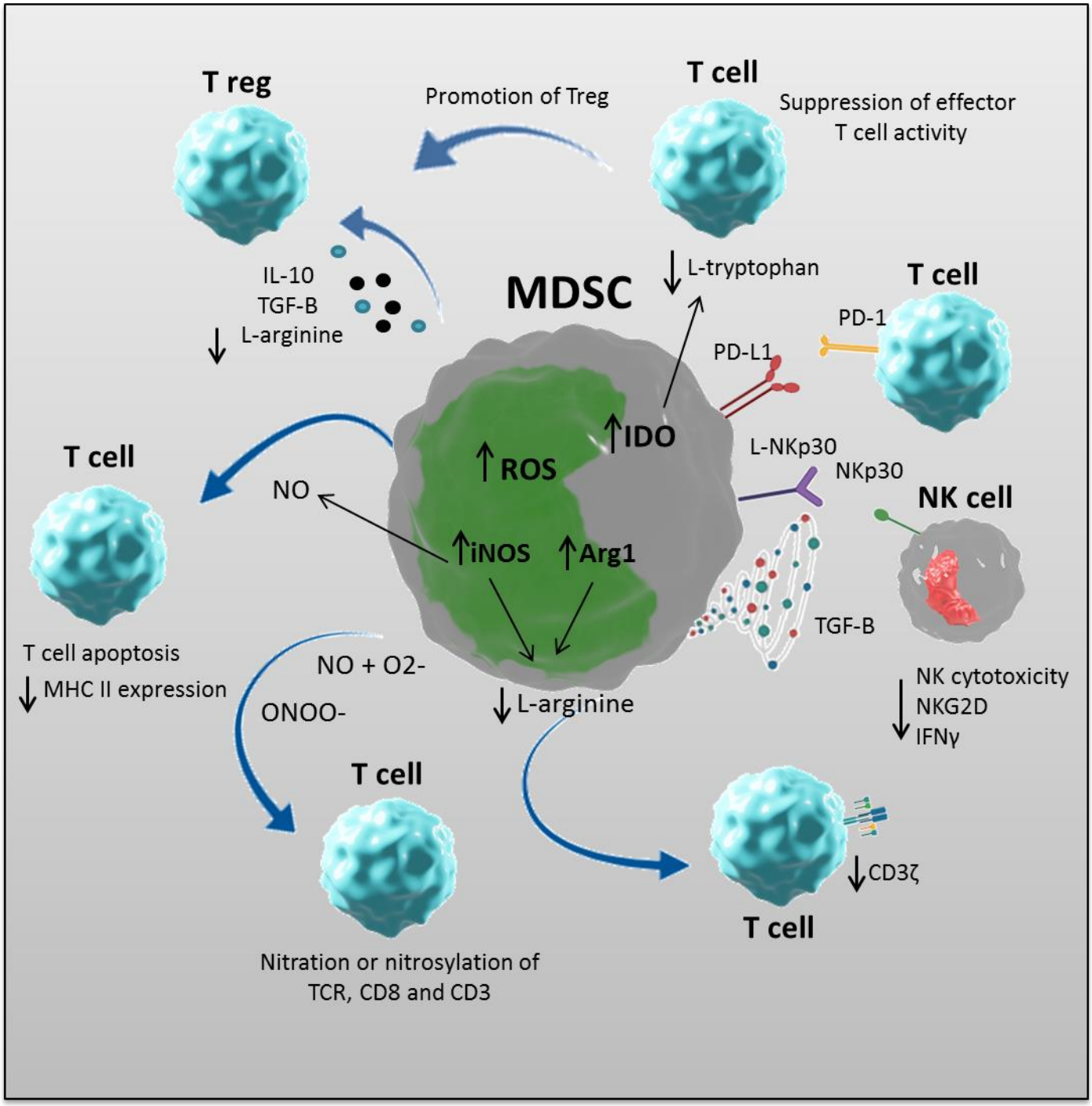


Figure 19| Mechanisms of MDSC suppressive activity. MDSC exert their suppressive function through a variety of mechanisms: (1) secretion of anti-inflammatory mediators, such as IL-10 and TGF-β that promote induction of T-regulatory cells; (2) increased arginase and iNOS: the increased activity of arginase leads to enhanced L-arginine catabolism. The lack of L-arginine inhibits T-cell proliferation through different mechanisms, including decreasing their CD3ζ expression ; on the other hand iNOS generates NO which suppresses T-cell function inhibiting, MHC class II expression and

inducing T-cell apoptosis; (3) increased production of ROS generates peroxynitrite which induces the nitration and nitrosylation of the amino acids and mediate MDSC suppression of T-cell function(4).MDSCs can inhibit NK cell function by interacting with the NKp30 receptor (5) inducing increased PD-1 expressión and (6) increased IDO activity which catabolizes tryptophan and limits T cell proliferation.

1.6.3.3.3- Human MDSCs in Solid Organ Transplantation

In transplantation the MDSCs are able to suppress adaptive and innate immune responses and they have been suggested as potential biomarkers for allograft tolerance as they can play a main role in the balance between graft acceptance and rejection (150,151). The MDSCs were defined in mice as $CD11b^{+}$ $Gr1^{+}$ cells and experimental transplant models demonstrated they play an important role in the induction of tolerance (151,152). Most of the published studies were performed in animal models, hence there is a paucity of data addressing MDSC features and their role in human transplantation.

Conventional phenotyping of human Mo-MDSCs subsets mainly relies upon HLA-DR expression; however, it is unclear to what extent HLA-DR expression is influenced by standard immunosuppression. An important goal in the study of the MDSCs is to find specific surface markers and gating strategies allowing identification of the different populations of MDSCs. On the other hand a mayor challenge in immune monitoring of transplant recipients is distinguishing between changes in biomarkers reflective of underlying alloimmune responses versus changes related to immunosuppressive therapy.

2 Hypothesis

Solid organ transplantation is a primary therapy in patients with end-stage diseases. Although remarkable improvement has been achieved due to the immunosuppressive protocols and the reduction in the incidence of AR has been reduced, there is still a high percentage of transplant recipients that suffer from AR within the first year after transplantation. Moreover patients that have previously suffered an AR in KT, usually show a higher incidence of chronic rejection and as a consequence long-term allograft survival has not improved significantly throughtout the years. Likewise, although the survival rate has been improved in short and medium-term in LTR, the survival rate at 5 years post-transplantation is around 50%. For this reason, the main goals in transplantation are to predict the risk of developing rejection and to find alternative tolerance approaches to allow immunosuppression minimization in order to lower the adverse effects that have deleterious effects on long term graft survival. Among the different tolerogenic approaches, myeloid cells, play a main role in the control of immune responses: under certain circumstances, they contribute to the inflammatory process, expanding disease pathology. However, myeloid cells with regulatory properties can protect the host from uncontrolled inflammation. These cells are known as MRCs and have been described within all the major myeloid cell lineages. Among them, MDSCs are considered as a heterogeneous group of myeloid cells known to accumulate under chronic pathological conditions. The first observations of suppressive myeloid cells were described more than 20 years ago in cancer patients. However, their functional importance has only recently been appreciated due to studies reporting their contribution to the regulation of immune responses in other clinical settings, such as organ transplantation.

In transplantation the MDSCs are able to suppress adaptive and innate immune responses and they have been suggested as potential biomarkers for allograft tolerance as they can play a main role in the balance between graft acceptance and rejection. The effect of different immunosuppressive drugs as maintenance therapy has a differential effect on the immune system. Based on previous results from our group, demonstrating that CNI were able to reduce circulating Tregs in renal transplant patients, it is likely that the immunosuppressive drugs most commonly used are affecting the phenotype and function of MDSCs in distinct manners. For this reason, to monitor how MDSCs are regulated *in vivo* in solid organ recipients would be a certain approach to assess their role in tolerance. Moreover, to evaluate the effect of immunosuppressive regiments on their function would shed light on the field and may help in future strategies in order to minimize immunosuppression. As most of the published studies were performed in animal models, MDSCs tolerogenic effect is not well established in patients and the monitoring of these cell populations prospectively might help. Human MDSCs are classified into three main subsets based on their phenotypic markers, though these markers are not exclusively expressed by MDSCs and these regulatory cell subsets are best defined by their capacity to suppress T cell proliferation and expand Tregs. On the one hand, assays of human MDSCs function are difficult to implement due to their technical complexity. Moreover, conventional phenotyping of human Mo-MDSCs subsets mainly relies upon HLA-DR expression and it is unclear to what extent HLA-DR expression is influenced by standard immunosuppression. Hence, there is also a need for new studies regarding the effect of immunosuppression on

MDSC phenotype and for reliable surrogate markers of human MDSCs function.

3 Objectives

The objectives of the project were the following:

1) To analyze *in vitro* the phenotype and suppressor function of MDSCs after polyclonal activation.

2) To evaluate how MDSCs induce differentiation to effector or regulatory T cells.

3) To study whether there are different effects of CNI or imTOR on MDSCs obtained from KTR.

4) To monitor the number and phenotype of MDSCs from peripheral blood monocytes after renal and lung transplantation and to find out any relationship with kidney and lung transplant outcomes.

5) To search for new phenotypic and functional subrogate markers of MDSCs.

4 Materials and Methods

4.1- Study design

A total of 38 consecutive KTR were enrolled in the prospective study of KTR after consent given while listed for kidney transplantation in the Hospital Universitario Marqués de Valdecilla during 2016. The study was approved by the Hospital Universitario Marqués de Valdecilla Ethics Committee. The mean follow-up time was 459 days. The clinical and immunological features of the KTR are summarized in **Table 3**. Clinical data were collected from patient records and blood was drawn at baseline/ day 0, 180 and 360 days after transplantation. To perform the prospective study in LTR a total of 82 consecutive patients were enrolled in the study after consent given while listed for lung transplantation in the Hospital Universitario Marqués de Valdecilla since 2016. The study was approved by the CEIC. The mean follow-up time was 239 days. The clinical and immunological features of the LTR are summarized in Table 4. A protocol biopsy at day 21 after lung transplantation is performed in our institution. Acute rejection was assigned based on ISHLT guidelines. Clinical data were collected from patient records and blood was drawn at day 0 (n=82), 7 (n=52), 21 (n=73), 90 (n=67), 180 (n=61) and 360 (n=50) days after transplantation. Importantly, all the LTR were receiving Tacrolimus (**Table 4**) as main immunosuppressant during the first 360 days after transplantation.

Table3. Main features of study population (KTRs)	**N=38**
Recipients Age, mean, years	51.88 (SD 13.23)
Donors Age, mean, years	49.61 (SD 12.63)
Healthy Controls Age, mean, years	46.17 (SD 11.85)
Recipient Sex (% Female)	18 (47.37%)
Donor sex (%Female)	19 (50%)
Dialysis post kidney transplant	10 (26%)
Preexisting anti-HLA antibodies	13 (34.21%)
Class I antibodies	10 (26%)
Class II antibodies	8 (21.05%)
Rejection	6 (15.78%)
RT	11 (28.94%)
ATN	10 (26.32%)
Induction treatment	
None	21 (55.26%)
ATG	12 (31.57%)
Basiliximab	5 (13.15%)
Both	0(0.00%)
Immunosupressive protocol	
Calcineurin inhibitor	33 (86.84%)
mTOR inhibitor	0 (0.00%)
Both	5 (13.15%)
ABDR Mismatches	
>3	24 (63.15%)
≤3	14 (36.84%)
Class II Mismatches	
0	8 (21.05%)
1	17 (44.73%)
2	13 (34.21%)
Renal disease	
Glomerular	11 (28.94%)
Others	1(2.63%)
Congenital	7(18.42%)
Sistemic	10 (26.31%)
Vascular	2(5.26%)
Interstitial	5(13.15%)
Unknown	2(5.26%)
Peripheral Blood Creatinine	
Cr 7 days post trasplant	2.28 (SD 1.70)
Cr 30 days post transplant	1.90 (SD 1.39)
Cr 120 days post transplant	1.40 (SD 0.45)
Cr 180 days post transplant	1.40 (SD 0.48)

SD, standard deviation ESRD, end stage renal disease 1stT, first transplant RT, retransplant patients **ATN,** ***Acue tubular necrosis***

Table 4. Main features of study population (LTRs) LTPN=82

Age, mean, years	56.38 (SD 10.34)
Female	27 (32.93%)
PGD	22 (26.82)
Preexisting anti-HLA antibodies	22 (26.83%)
Class I antibodies	22(26.83%)
Class II antibodies	3 (3.65%)
Rejection	30 (36.58%)
Basal Disease	
Bronchiectasis/Cystic fibrosis	8 (9.74 %)
In-tur-STISH-ul	44 (53.65 %)
COPD	22 (26.8 %)
PPH	5 (6.09 %)
Others	3 (3.65 %)
Intubation time	
≤3 days	66 (80.48 %)
>3 days	16 (19.51 %)
Infection (first month)	30 (36.58 %)
Induction treatment	
Basiliximab	82(100%)
Immunosupressive protocol	
Calcineurin inhibitor	82 (100 %)
ABDR Mismatches	
>3	68 (82.92 %)
≤3	14 (17.08 %)
Class II Mismatches	
0	3(3.66%)
1	35(42.68%)
2	44 (53.66%)

SD, standard deviation PGD, primary graft dysfunction *In-tur-STISH-ul diffuse intersticial COPD , chronic obstructive pulmonary disease PPH, primary pulmonary hypertension*

4.2- Monoclonal Antibodies and Flow cytometry analysis

The Peripheral Blood Mononuclear Cells (PBMCs) or isolated MDSCs were stained with the following monoclonal antibodies: Anti-CD33-Allophycocyanin (APC) (clone D3HL60.251), anti-CD3-Fluorescein isothiocyanate (FITC) (clone UCHT1), anti-CD14- Phycoerytrin- Texas Red-(ECD) (clone RMO52) and anti-CD11b-Phycoerytrin (PE)-cyanin 7 (Cy7) (clone Bear1) (Beckman Coulter, Marseille, France); anti-CD16- (APC)- Cy7 (clone 3G8), anti-CD56- FITC (clone HCD56 and, anti-HLA-DR-Brilliant Violet 510 (BV510) (clone L243) (Biolegend, San Diego, CA); anti-CD19-FITC (clone 4G7) and anti-CD14-FITC (clone MϕP9) (BD Biosciences); anti-CD25-PE (clone 2A3) and anti-FoxP3-Pacific Blue (clone 206D) (BD Biosciences); anti-CD15-CF Blue (clone MCS-1) (Inmunostep, Salamanca, Spain); and anti-CD4-APC-Vio770 (clone REA623) from Miltenyi Biotech. The cells were incubated during 20 min, washed with Phosphate Buffer Saline (PBS), acquired in a Cytoflex® flow cytometer (Beckman Coulter) and analyzed using Kaluza version 1.3. MDSC were quantified by flow cytometry following the gating strategy proposed by Bronte et al. (134) to characterize MDSCs subsets: Mo-MDSCs ($CD33^+CD11b^+HLADR^-$ $CD14^+$ $CD15^-$), PMN-MDSC ($CD33^+CD11b^+HLADR^-$ $CD15^+$ $CD14^-$) and e-MDSC Lin^- ($CD14^+CD56^+CD3^+CD19^+$) $CD33^+CD11b^+HLADR^-$ $CD14^-CD15^-$. Total MDSC were defined as $CD33^+CD11b^+HLADR^-$ cells. Fluorescence minus one control was used to identify $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ cells.

To test new flow cytometry panels for MDSCs phenotyping (**Table 5**), peripheral blood samples were collected into EDTA-vacutainers by venopuncture and then delivered to the immune monitoring (Clinikum

Regensburg, Germany) laboratory at room temperature. Pre-analytical samples were stored for up to 4 hours at 4°C until before processing. Whole blood was stained with the antibodies listed in table 5and the PerFix EXPOSE (Phospho-Epitopes Exposure kit) protocol described by Beckman Coulter was followed. Data were recorded with a Navios™ ytometer running Cytometry List Mode Data Acquisition and Analysis Software or NaviosTM Cytometer, version 1.3 from Beckman Coulter. Analyses were performed using Kaluza version 1.3.

4.3- Analysis of cell viability

To assess the frequencies of cell death in *in vitro* cell culture conditions, collected cells from the plates were incubated for 10 minutes with 7-amino-actinomycin D (7AAD) (Tonbo Biosciences before acquisition on a Cytoflex®. All the results showed in the present work are based on gated live cells ($7AAD^-$).

Table 5

		488 nm					633 nm			405 nm	
		FITC	PE	ECD	PerCp-Cy5.5	PE-Cy7	APC	APC-AlexaFluor 700	APC-AlexaFluor 750	Pacific Blue	Krome Organge
Panel 1	**ANTIGEN**	**CD11b**	**LOX1**	**LIN***	**CD15**	**CD124**	**CD16**	**CD14**	**CD33**	**HLA-DR**	**CD45**
	Clone	Bear1	15C4		W6D3	G077F6	3G8	RMO52	D3HL60.251	G46.6	J.33
	Isotype	mIgG1	mIgG2a		mIgG1	mIgG2a	mIgG1	mIgG2a	mIgG1	mIgG2a	mIgG1
	Amount (µl)	5	5		5	5	5	6	4	8	8
	Supplier	BC	BioLeg.		BioLeg.	BioLeg.	BC	BC	BC	BD	BC
	Cat.#	IM0530	359604		323020	355008	B00845	A99020	A70200	562805	B36294
	Status	CE	RUO		RUO	RUO	ASR	ASR	ASR	RUO	CE
Panel 2	**ANTIGEN**	**pS6**	**IRF1**	**LIN***	**CD15**	**IDO**	**pmTOR**	**CD14**	**CD33**	**HLA-DR**	**CD45**
	Clone	N7-548	20/IRF-1		W6D3	Eyedio	O21-404	RMO52	D3HL60.251	G46.6	J.33
	Isotype	mIgG1	mIgG1		mIgG1	mIgG1	mIgG1	mIgG2a	mIgG1	mIgG2a	mIgG1
	Amount (µl)	20	6		5	5	5	6	4	8	8
	Supplier	BD	BD		BioLeg.	Biosciences	BD	BC	BC	BD	BC
	Cat.#	560434	566322		323020	25-9477-42	564242	A99020	A70200	562805	B36294
	Status	RUO	RUO		RUO	RUO	RUO	ASR	ASR	RUO	CE
Panel 3	**ANTIGEN**	**ARG1**	**LOX1**	**LIN***	**CD15**	**IDO**	**CD16**	**CD14**	**CD33**	**HLA-DR**	**CD45**
	Clone	A1exFS	15C4		W6D3	Eyedio	3G8	RMO52	D3HL60.251	G46.6	J.33
	Isotype	rIgG2a	mIgG2a		mIgG1	mIgG1	mIgG1	mIgG2a	mIgG1	mIgG2a	mIgG1
	Amount (µl)	5	5		5	5	5	6	4	8	8
	Supplier	invitrogen	BioLeg.		BioLeg.	Biosciences	BC	BC	BC	BD	BC
	Cat.#	53-3697-82	359604		323020	25-9477-42	B00845	A99020	A70200	562805	B36294
	Status				RUO	RUO	ASR	ASR	ASR	RUO	CE
Panel 4	**ANTIGEN**	**CD35**	**CCR2**	**LIN***	**CD15**	**CD88**	**CD326**	**CD14**	**CD33**	**HLA-DR**	**CD45**
	Clone	J3D3	K036C2		W6D3	S5/1	VU1D9	RMO52	D3HL60.251	G46.6	J.33
	Isotype	mIgG1	mIgG2a		mIgG1	mIgG2a	mIgG1	mIgG2a	mIgG1	mIgG2a	mIgG1
	Amount (µl)	5	5		5	5	5	6	4	8	8
	Supplier	BEC	BioLeg.		BioLeg.	BioLeg.	BC	BC	BC	BD	BC
	Cat.#	RUO	357206		323020	344308	B90408	A99020	A70200	562805	B36294
	Status	IM1836	RUO		RUO	RUO	ASR	ASR	ASR	RUO	CE

*** LIN**

ANTIGEN	CD3	CD19	CD20	CD56
Clone	UCHT1	J3-119	B9E9	N901
Isotype	mIgG1	mIgG1	mIgG2a	mIgG1
Amount (µl)	10	10	15	10
Supplier	BC	BC	BC	BC
Cat.#	A07748	A07770	B92433	B49214
Status	CE	CE	CE	CE

4.4- Myeloid cell subsets changes in *in vitro* culture

To determine the changes of MDSCs subsets *in culture,* sorted 10^5 $CD33^+$ cells/well were cultured with R10 medium (RPMI, 10% fetal bovine serum (FBS), 2% L-Glutamine,1% non essential amino acids, 0.5% Penicillinn/ Streptomycin, 1% Sodium Bicarbonate, 1% Na Pyruvate). The $CD33^+$ cells were sorted by magnetic-automated cell sorting using CD33 positive separation Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer´s instructions. Cells were collected and their phenotype changes were analyzed at baseline, 1, 3, 5 and 7 days after culture. The frequencies of each MDSC subset at each time collection were quantified according to the profiles described above.

4.5- Effect of Immunosuppressant drugs *in vitro*

Upon MACS sorting, 10^5 $CD33^+$ cells/well were cultured in the presence/ absence of different concentrations of tacrolimus, rapamycin and everolimus (Sigma Aldrich, St Louis, MO) in a U-bottomed 96-well plate. The working drug concentration ranges were: tacrolimus (0–50ng/ml), rapamycin (0–50 ng/ml) and everolimus (0–50 ng/ml). Phenotypic changes were tested by flow cytometry at day 3.

4.6- *In vitro* evaluation of MDSCs suppressor function: Cell isolation and sorting of MDSCs

$CD4^+$ T cells were isolated from healthy donors, KTR or LTR PBMCs by immunomagnetic depletion using EasySep™ Human $CD4^+$ naïve T Cell Isolation Kit (Stemcell Technologies, Grenoble, France) according to the manufacturer´s instructions and incubated with CarboxyFluorescein Succinimidyl Ester (CFSE) 5nM. The CFSE-labeled T $CD4^+$ cells ($5x10^5$) were

stimulated with Dynabeads Human T-activator CD3/CD28 (Life Technologies AS, Oslo, Norway) in U-bottomed 96-well plates with complete RPMI media supplemented with 10% human AB+ serum. In order to determine the suppressive function of MDSCs subsets, autologous Mo-MDSCs were added to the culture at 1:2 ratio ($CD4^+$ T cells: MDSCs) and proliferation was determined at day 5 by using flow cytometry. Proliferation assays from blood donors were performed five times. These same functional assays were also carried out with MDSCs from four KTR under calcineurin inhibitor (tacrolimus) and four KTR under imTOR treatment (rapamycin) with at least 24 months of IS treatment and with MDSCs from two LTRs under calcineurin inhibitor treatment with at least 24 months of IS treatment.

4.7- *In vitro* expansion of Treg

PBMC were obtained from KTR under maintenance immunosuppression with tacrolimus. $CD4^+$ T cells were sorted from the PBMC as described above. $CD4^+$ T cells ($5 \cdot 10^5$) were polyclonally stimulated and cultured with Mo-MDSCs at different concentrations. Treg generation was determined at day 5 by staining with the monoclonal antibodies indicated above and flow cytometry analysis.

4.8-Whole blood cultures

Whole blood culture was performed as follows: Fresh blood anticoagulated with Lithium-Heparin was diluted 1:4 in Gibco™ DMEMF/12 GlutaMAX™ Supplement medium (ThermoFisher Scientific, Waltham, Massachusetts, EEUU) containing 100 U/mL penicillin (Lonza) and 100 mg/mL streptomycin (Lonza). Cells were stimulated throughout

cultures with 5 ng/ml recombinant human CSF-1 (rhM-CSF; R&D Systems, Wiesbaden- Nordenstadt). To evaluate if Mo-MDSCs can come from monocytes in peripheral blood human $CD14^+$ monocytes were isolated from Ficoll density gradient centrifugation of PBMC followed by positive-selection using anti-CD14 microbeads (Miltenyi, Bergisch-Gladbach, Germany). Isolated $CD14^+$ monocytes were stained with Cell Tracker™ Green CMFDA Dye (ThermoFisher Scientific, Waltham, Massachusetts, EEUU) at 2nM and then added back into whole blood cultures at 10^5 cells/ tube (Falcon® 5 mL Round Bottom Polystyrene Test Tube) diluted ¼ in Gibco™ DMEMF/12 GlutaMAX™ Supplement medium (ThermoFisher Scientific, Waltham, Massachusetts, EEUU) and supplemented with 100 U/mL penicillin (Lonza), 100 mg/mL streptomycin (Lonza, Basilea, Switzerland), and rhM-CSF (R&DSystems, Wiesbaden-Nordenstadt, Germany) at 5 ng/mL carried on 0.1% human albumin. Purity of sorted cells was tested after isolation and > 95% efficiency was considered acceptable for the study. Cells were collected and location was analyzed at baseline, 1 and 2 days after culture.

4.9- Western blot Gel electrophoresis and immunoblotting were performed as described elsewhere (153).

4.10-Statistical analysis

Non-parametric Mann-Whitney U test and Student´s t test were used to compare two independent groups as appropriate. To test if the variables followed a Gaussian distribution we performed Kolmogorov Smirnoff test. More than two groups were compared using the parametric analysis of variance (ANOVA), the non-parametric Kruskal-Wallis not matching or

Friedman (repeated measures) test. Comparisons between two paired groups were performed using the Student´s t-test for paired data or the Wilcoxon signed-rank test when data were or not normally distributed, respectively. Multiple comparisons were assessed using Dunn or Tukey´s tests. To measure the impact of calcineurin and mTOR inhibitors on phenotypic changes of MDSCs the interaction between each treatment and control was assessed by repeated-measures two-way analysis of variance test. Statistical analyses were performed using Graphpad software version 8.4.3 (GraphPad Inc. San Diego, CA). To examine the relationship between bivariate variables, the Pearson correlation was calculated by using SPSS Statistics version 24.

5 Results

5.1-Phenotypic changes of MDSCs subsets *in vitro*

MDSCs were purified from blood donors PBMCs and cultured for 7 days to test phenotypic changes *in vitro*. Cells were collected at 1, 3, 5 and 7 days and their phenotype was assessed by flow cytometry. The gating strategy and MDSCs phenotyping are described in figure 20. A significant decrease in cell viability of total MDSCs was observed during *in vitro* culture (p=0.0051), especially from day 5 (Figure 21 a). At day one, there was a predominance of Mo-MDSCs (median 86.86%, IQR 62.87-93.47) comparing to PMN-MDSCs (median 10.01%, IQR 1.64-10.71) and e-MDSCs (median 4.46%, IQR 2.05-26.02) within total MDCSs. The MDSCs subsets were affected by time of culture: while the proportion of Mo-MDSCs cells clearly decreased from day 3, the PMN-MDSCs fraction was maintained proportionally and most of the cells become double negative (e-MDSCs-like phenotype) (Figure 21 b,c).

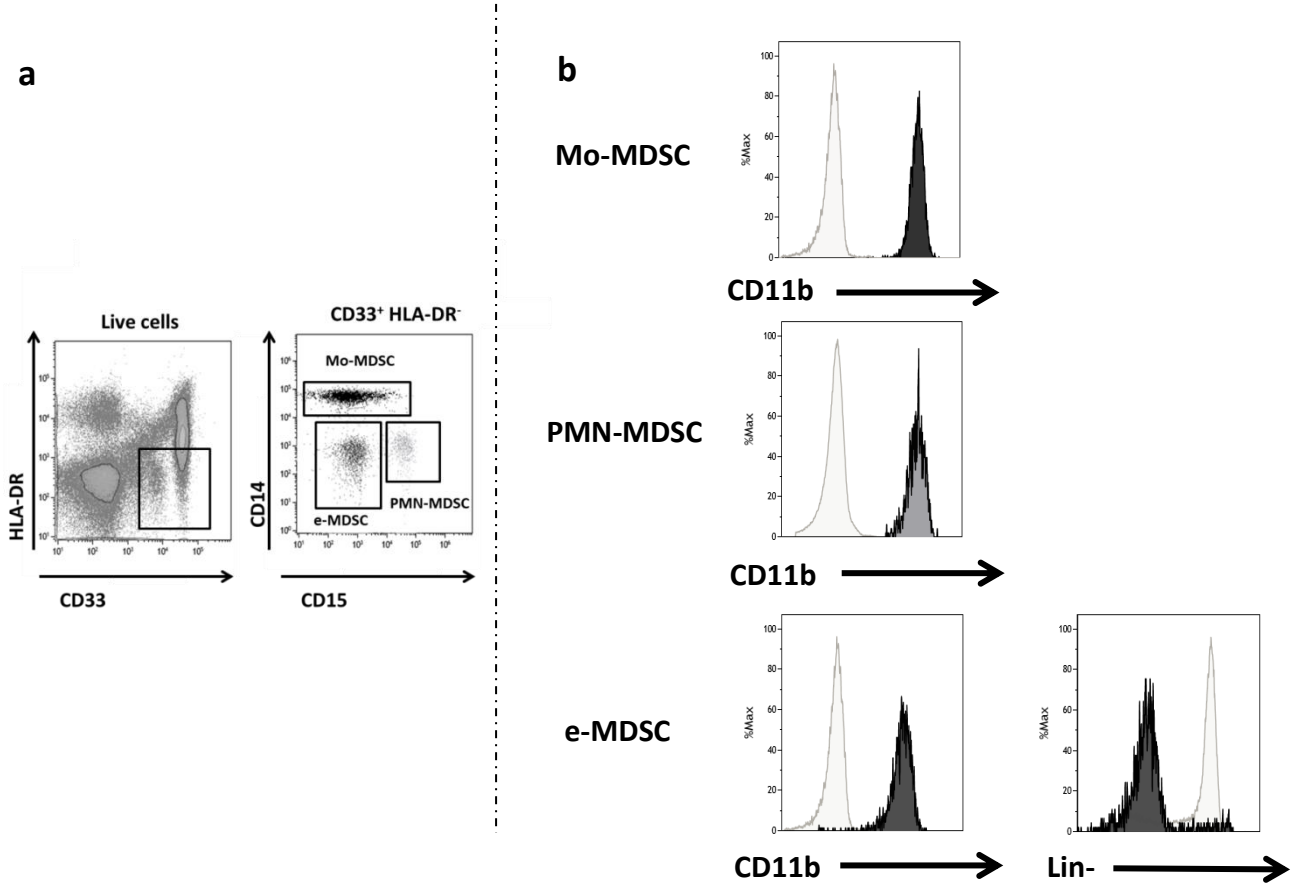
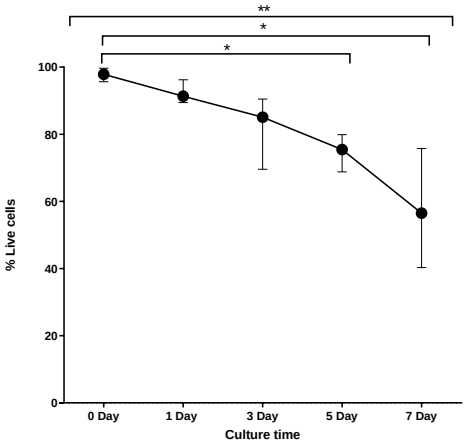
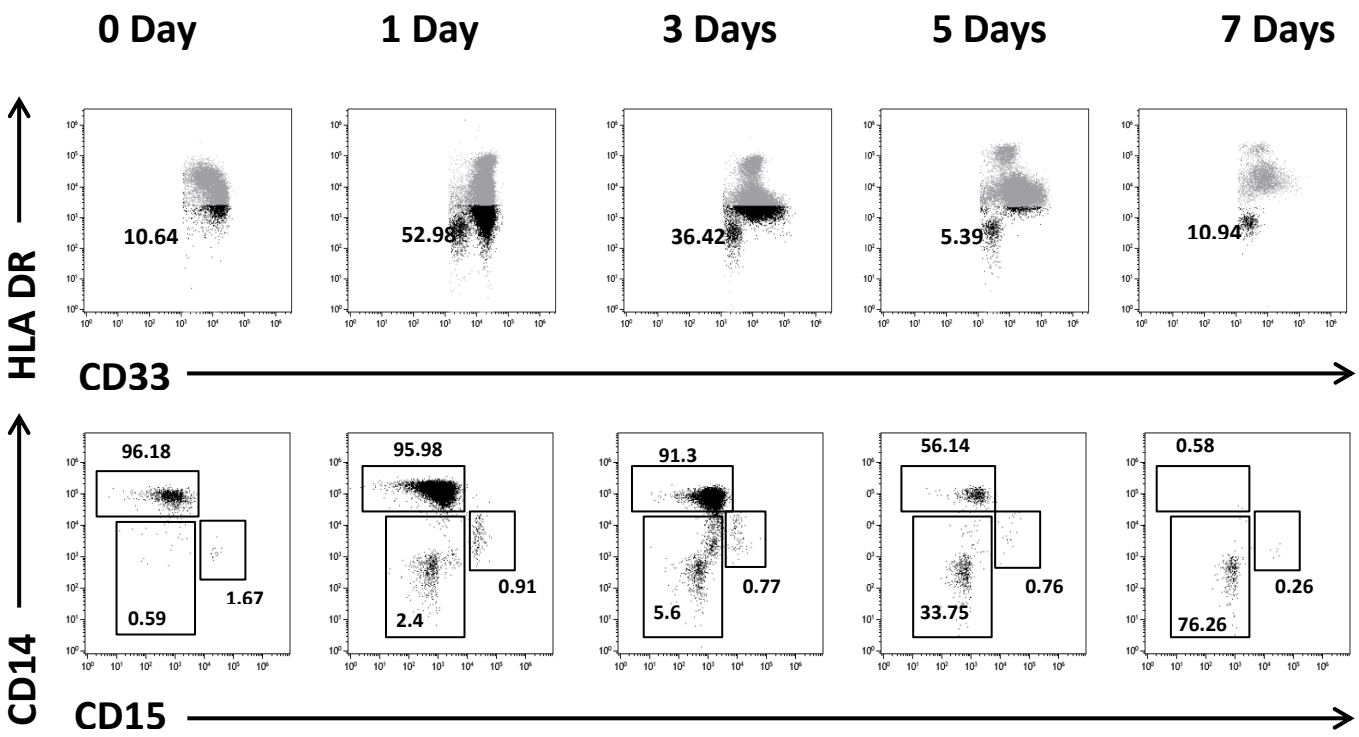


Figure 20| Characterization of MDSCs subsets by flow cytometry. **(a)** $CD33^{+}$ $HLA\text{-}DR^{-}$ cells were selected from live cells gate after doublets and debris exclusion. To define monocytic (Mo-MDSCs), early-stage (e-MDSCs) and polymorphonuclear (PMN-MDSCs) MDSCs, the CD14 and CD15 expression was analyzed on cells selected from the total-MDSCs gate ($CD33^{+}HLA\text{-}DR^{-}$). **(b)** Expression levels of different antigens (CD11b and Lin-) defining MDSCs subsets are depicted.

a



b



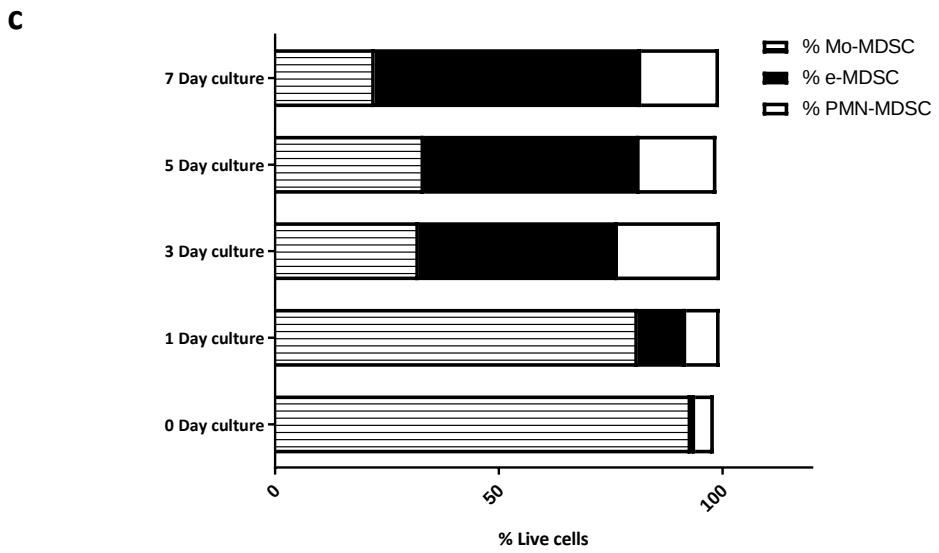


Figure 21| Phenotypic changes of MDSCs from healthy donors after *in vitro* culture. $CD33^{+}$ cells were isolated from PBMCs. MDSCs numbers and percentages were assessed by flow cytometry at 0, 1, 3, 5 and 7 days after culture. Percentage of live cells on total MDSCs after *in vitro* culture, comparison of frequency in live cells at different timepoints is depicted, * $p<0.05$, ** $p<0.01$ **(a)**. Flow cytometry analysis of immunophenotypic changes after culture on total MDSCs (top panel, black dots) and MDSCs subsets from a representative experiment **(b)**. Mean percentage of MDSCs subsets from total-MDSCs after culture is shown for four independent experiments. Monocytic-MDSCs (Mo-MDSCs, stripped box), early stage-MDSCs (e-MDSCs, black box) and polymorphonuclear-MDSCs (PMN-MDSCs, white box) **(c).**

5.2-Impact of calcineurin and mTOR inhibitors on phenotypic changes of MDSCs *in vitro*

To investigate whether MDSCs differentiation and viability are affected by different immunosuppressant drugs of clinical use in transplantation, we treated MDSCs *in vitro* with either CNI or imTOR at different dosages. To avoid the bias of cell viability after long periods of culture, we selected 3 days after culture as the time to test such effect. When we analyzed total MDSCs, we found that the highest dose of tacrolimus induced a reduced frequency of total MDSCs compared with the highest of rapamycin or everolimus (Figure 22 a) 53.4% $\pm$ 29.4 vs 73.3% $\pm$ 17.2 and 71.72% $\pm$ 18.1

respectively (p=0.044 for rapamycin vs tacrolimus, p=0.014 for everolimus vs tacrolimus). When comparing differences between each dose treatment on MDSC subsets, we found maintained Mo-MDSCs viability with imTOR (rapamycin, mean 85.29% SD 12.75 and everolimus, 73.15% SD 18.78) whereas viability was reduced under tacrolimus treatment (mean 48.85% SD 31.04), but we only found significant differences when tacrolimus and rapamycin high doses (50ng/ml) were compared (p=0.0041) (Figure 22 b). To reduce variability, results were normalized to the drug-free MDSC control. The effect was also observed in PMN-MDSCs and e-MDSCs although the differences were not significant.

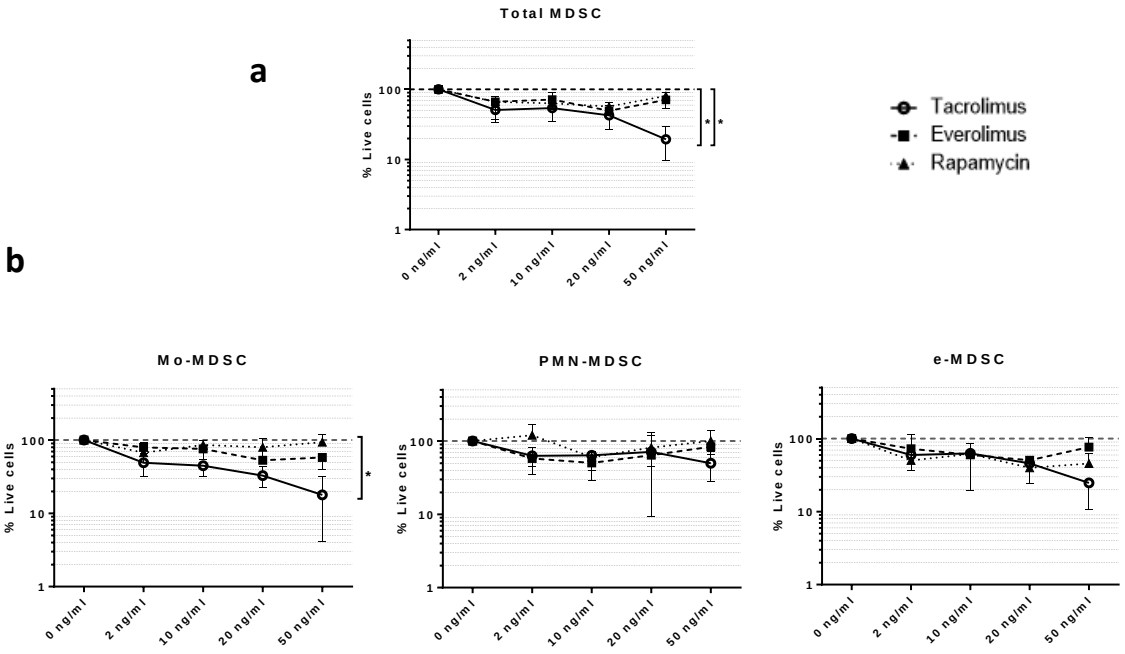


Figure 22| Effect of immunosuppressants on MDSCs population viability. The frequency of total live myeloid-derived suppressor cells (MDSCs) was assessed by 7-amino-actinomycin D (7AAD) negative staining. MDSCs were treated with different doses (ranged 2-50ng/mL) of tacrolimus (open circles), everolimus (black squares) and Rapamycin (black triangles) **(a)**. The effect of immunosuppressor agents on different MDSCs subsets is shown, monocytic-MDSCs (Mo-MDSCs; bottom left), polymorphonuclear-MDSCs (PMN-MDSCs; botton center) and early stage-MDSCs (eMDSCs; bottom right) **(b)**. To reduce inter experiment variability, the results were

normalized to the control drug-free (0 ng/ml=100%) represented with a dotted line in each plot. Results are shown for 4 independent experiments. PBMCs, peripheral blood mononuclear cells, Mo-MDSCs, monocytic myeloid derived suppressor cells; PMN-MDSCs, Polymorphonuclear myeloid derived suppressor cells; e-MDSCs, early stage myeloid derived suppressor cells.

5.3- Monitoring MDSCs in kidney transplant recipients

We hypothesized that MDSCs frequencies might serve as useful biomarkers of clinical outcome after kidney transplantation. Therefore, we first quantified Mo-MDSCs, PMN-MDSCs, and e-MDSC in peripheral blood from KTRs at 0, 180, and 360 days after transplantation (Figure 23, 24, 25, 26). We found a not significant increase in total MDSCs frequency at 180 days after transplantation (median, 11.5%; interquartile range (IQR), 6.2–17.0%) and at 360 days posttransplant (median, 11.2%; IQR, 4.9–17.8%) in comparison with patients on the day of transplantation (median, 8.8%; IQR, 5.0–16.4%) (Figure 24 a). Next, we examined changes in MDSCs subsets distribution after transplantation. Mo-MDSCs frequencies were significantly increased at 180 and 360 days posttransplant (median, 22.71%; IQR, 6.75–57.56% and median, 25.48%; IQR, 8.85–56.58%) in comparison to patients on the day of transplantation (median, 10.56%; IQR, 3.18–37.55%) (Figure 24 b). PMN-MDSCs and e-MDSCs frequencies were lower at 180 days after transplantation (median, 41.71%; IQR, 12.67–62.79% and median, 5.5%; IQR, 1.9–10.87%) compared to patients on the day of transplantation (median, 54.6%; IQR, 29.4–84.95% and median, 6.15%; IQR, 3.9–13.5%), and they remained lower 360 days posttransplantation (median, 43.14%; IQR, 10.28–63.02% and median, 4.09%; IQR, 2.11–8.2%) (Figures 24 c, d).The decreases observed in PMN-MDSCs and e-MDSCs were not significant. Despite these changes, we did not find any association between the MDSCs subsets, and the clinical data

(Figure 27). Importantly, all the KTRs were receiving tacrolimus (**Table 3**) as main immunosuppressant during the first 360 days after transplantation shown.

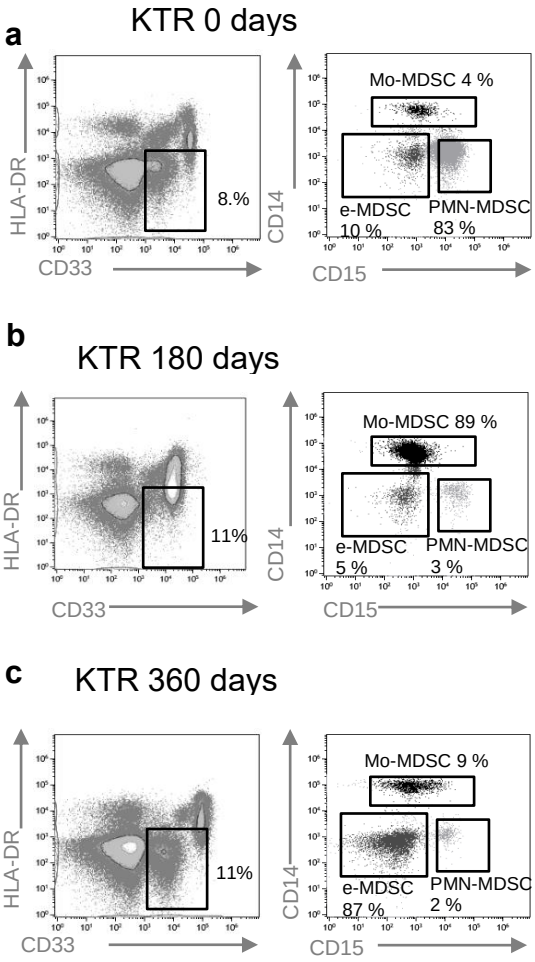


Figure 23| Characterization of MDSCs subsets by flow cytometry. $CD33^{+}$ $HLA\text{-}DR^{-}$ myeloid cells were selected from live cells after doublets and debris exclusion. To define monocytic (Mo-MDSCs), early-stage (e-MDSCs) and polymorphonuclear (PMN-MDSCs) MDSCs, the CD14 and CD15 expression was analyzed on cells selected from $CD33^{+}HLA\text{-}DR^{-}$ MDSCs. Representative flow cytometry data of MDSCs from **(a)** patients on the day of transplantation (day 0); **(b)** kidney transplant recipients on days 180 and **(c)** 360 post-transplantation is shown**.**

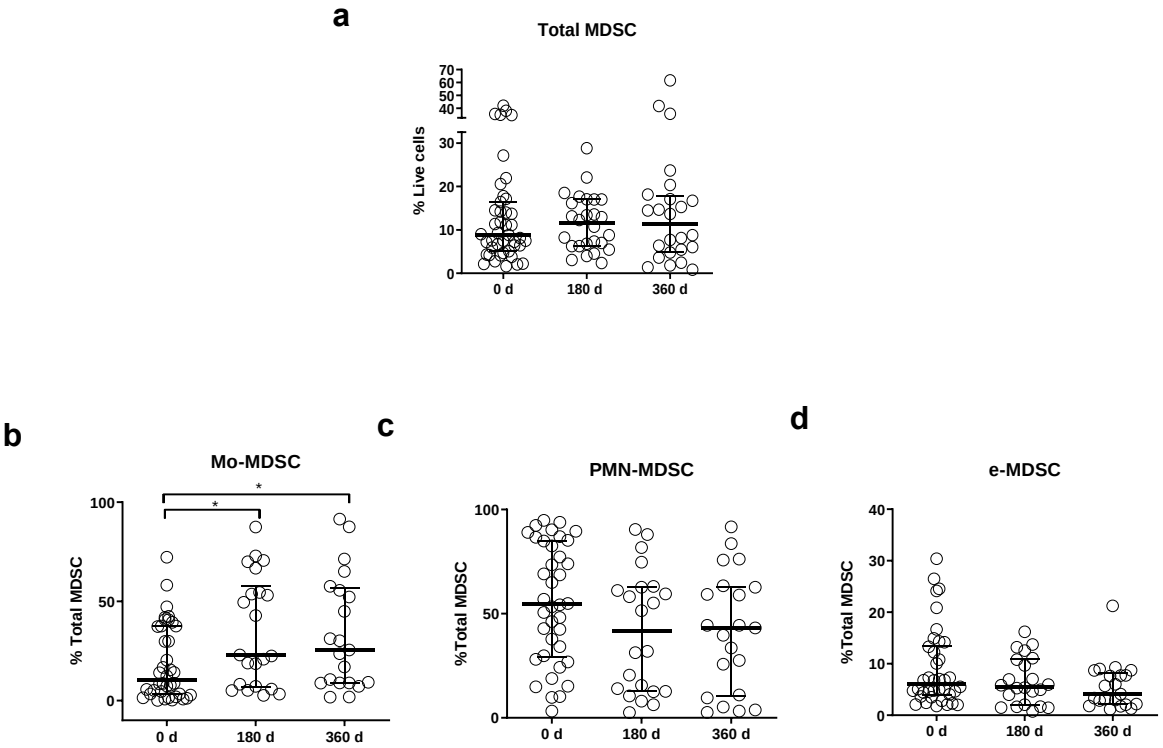


Figure 24| MDSCs frequencies in KTR. (a) Frequencies of total myeloid-derived suppressor cells (t-MDSCs) in peripheral blood mononuclear cells (PBMC); **(b)** monocytic-MDSCs (Mo-MDSCs); **(c)** early stage-MDSCs (eMDSCs) and **(d)** polymorphonuclear MDSCs (PMN-MDSCs) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney U test. (*p<0.05).

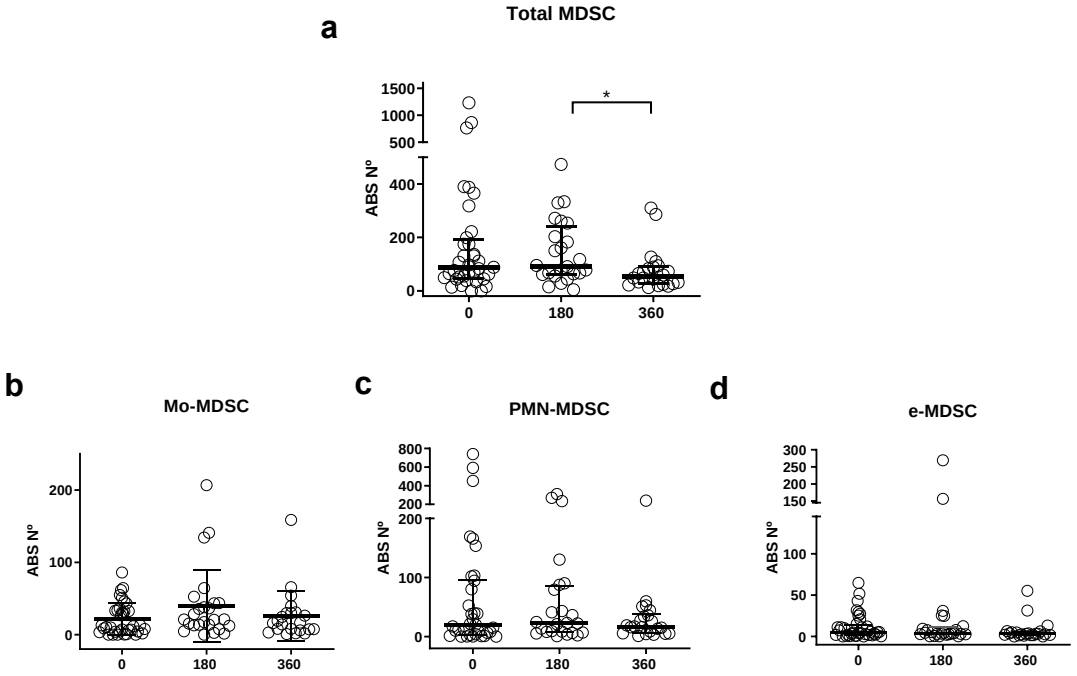


Figure 25| MDSCs absolute numbers in KTR. (a) Frequencies of total myeloid derived suppressor cells (MDSCs) in peripheral blood mononuclear cells (PBMCs); **(b)** monocytic-MDSCs (Mo-MDSCs); **(c)** early stage-MDSC (eMDSCs) and **(d)** polymorphonuclear MDSCs (PMN-MDSCs) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney U test (*p<0.05).

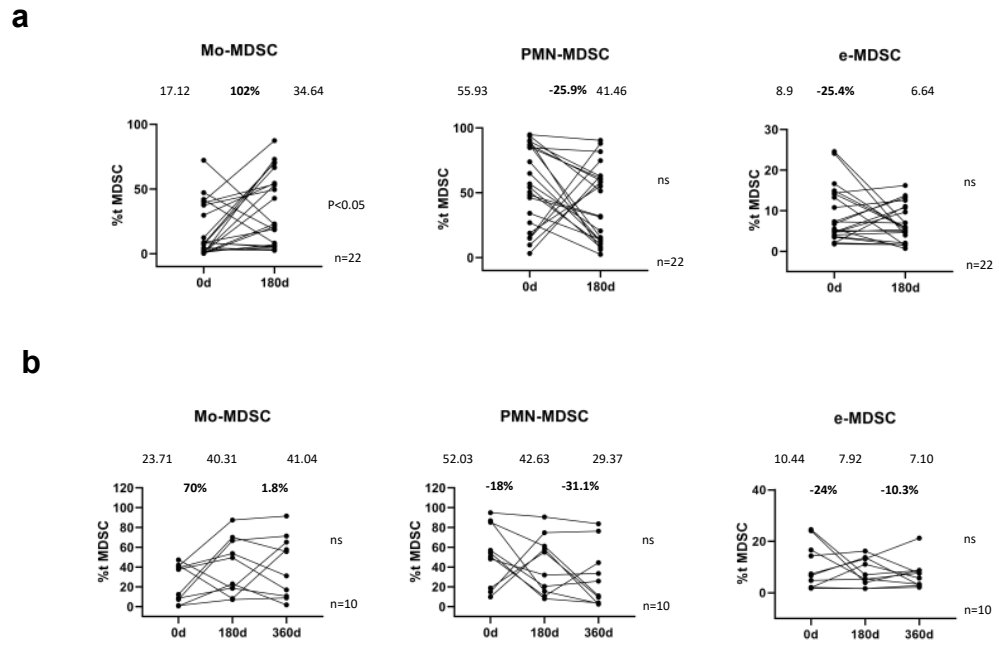
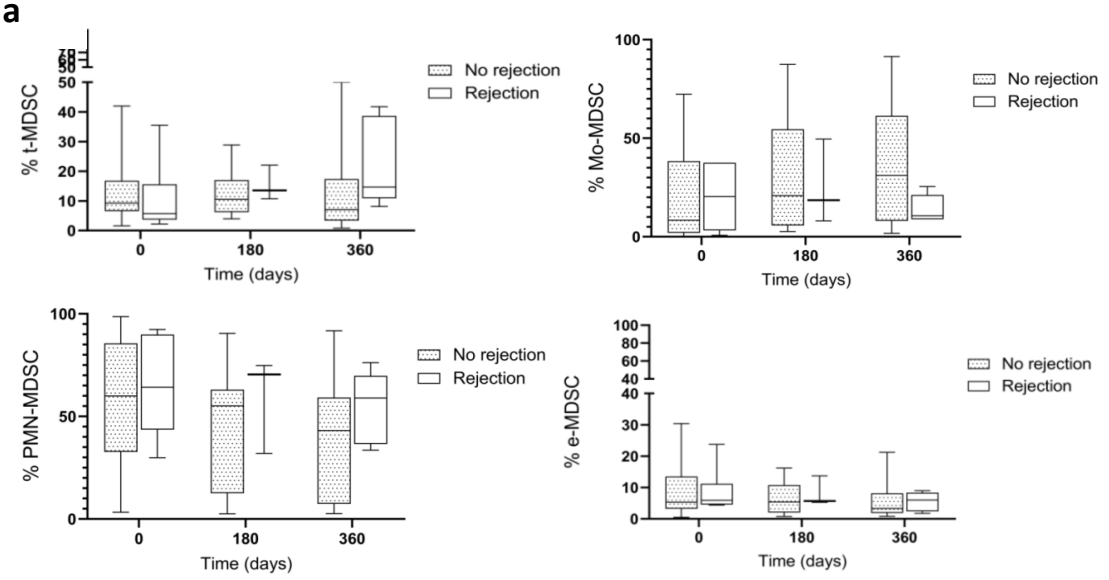


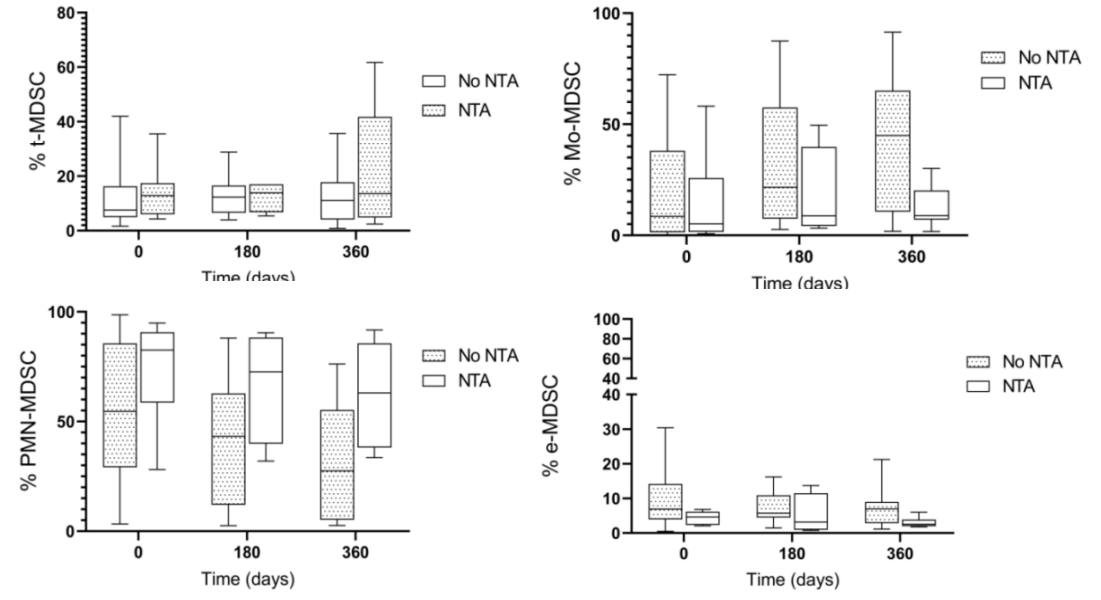
Figure 26| Comparison of MDSCs subsets: Mo-MDSCs, PMN-MDSCs and e-MDSCs at day 0 and 180 days after transplant **(a)** and at day 0, day 180 and 360 after transplant

(b). Levels of Mo-MDSC 180 days after transplant were significantly increased compared to day 0.The central number is the difference (in percent) between the means of the two time points **(a)** and the three time points **(b).** Differences between time points were calculated using the following formula: (mean posTx-mean preTx) / mean preTx and paired analisis were performed to compare differences.

a



b



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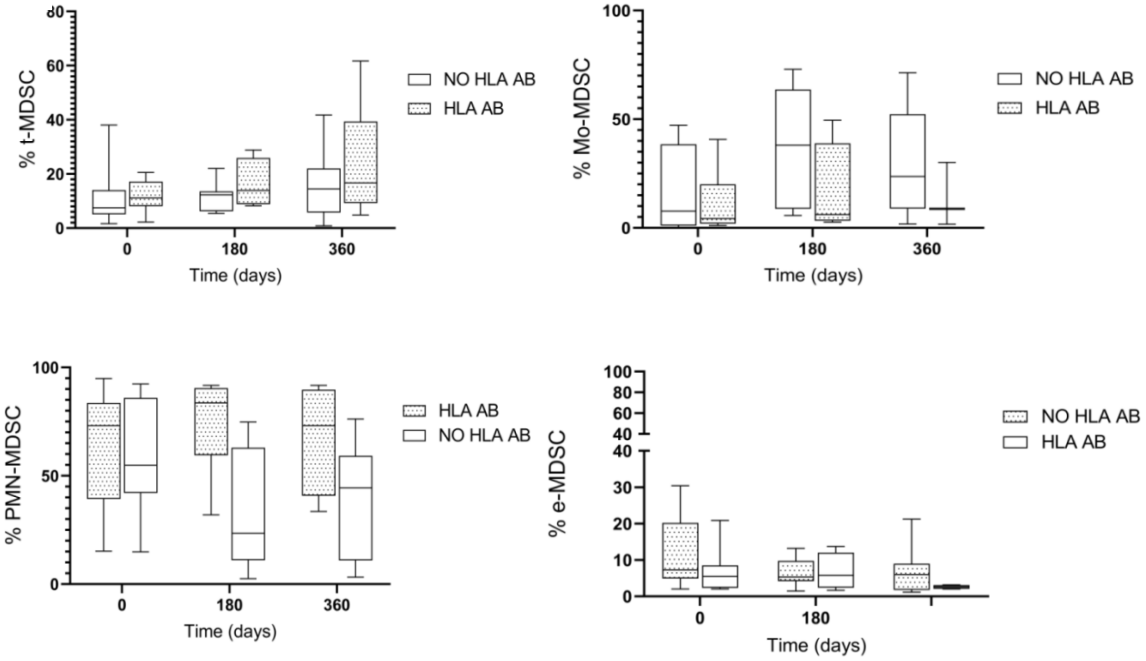
% t-MDSC
NO HLA AB
HLA AB
Time (days)
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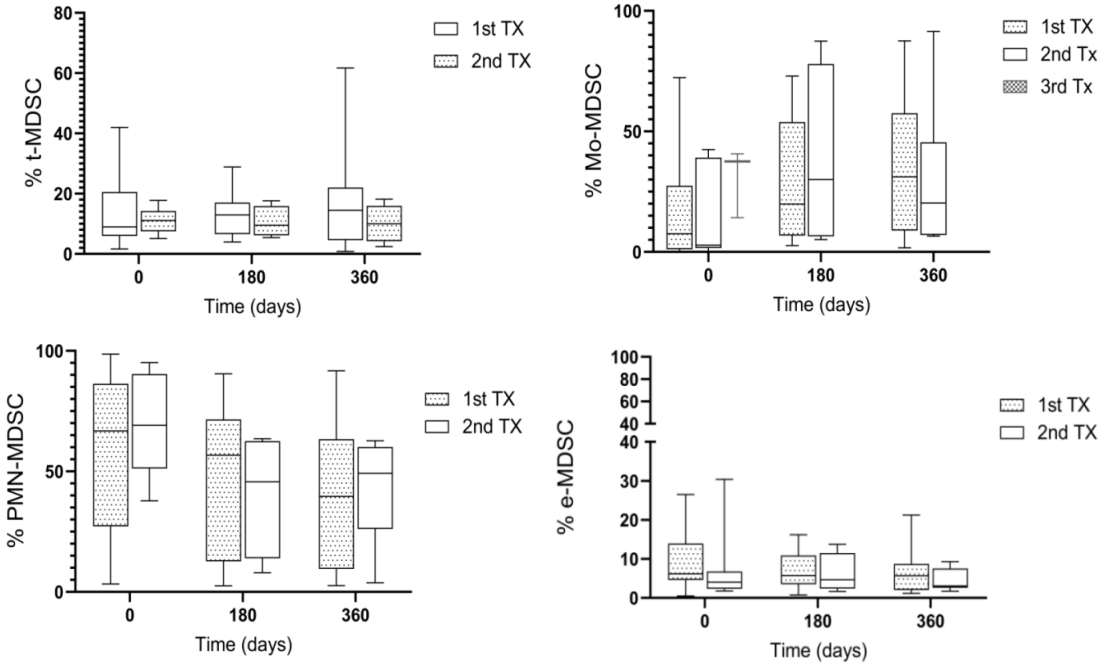
d


% t-MDSC
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Time (days)
% Mo-MDSC
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Time (days)

e

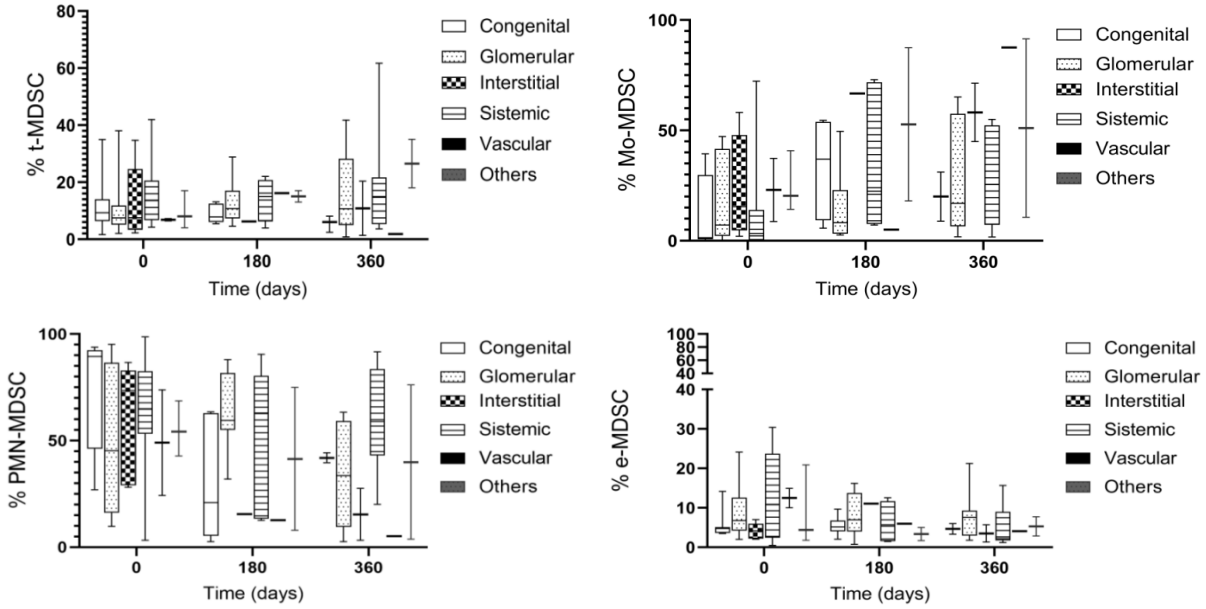


Figure 27| MDSC frequencies in KTR and clinical events Frequencies of t-MDSCs, Mo-MDSCs, PMN-MDSCs, eMDSCs in PBMC were studied and compared with clinical events such as **(a)** rejection **(b)** acute tubular necrosis (NTA), **(c)** HLA antibodies, **(d)** number of transplants and **(e)** basal disease. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney U test. (*p<0.05). The number of patients in each group is indicated in table 3.

5.4- MDSCs from Kidney transplant recipients induce the expansion of Tregs *in vitro*

Treg expansion is one of the main mechanisms by which MDSCs exert suppressive function. Hence, we evaluated the capacity of Mo-MDSCs from healthy donors and KTR to boost Tregs *in vitro*. We observed a significant increase in the frequency of Tregs recovered from the culture when $CD4^+$ T ells were stimulated with Mo-MDSC from cells from KTR at

360 days after transplantation, confirming their suppressive function (Figure 28).

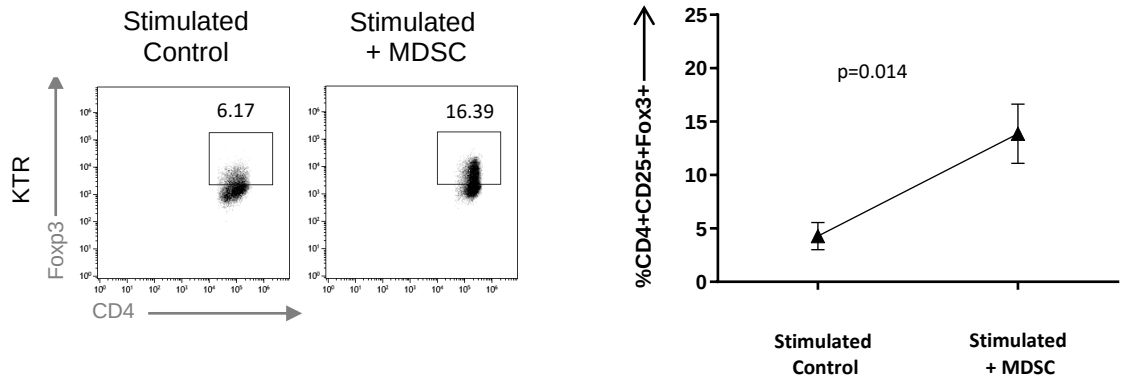


Figure 28| Mo-MDSCs from KTR expand Treg *in vitro*. MDSCs dependent $CD4^+FoxP3^+$ Treg expansion was analyzed by flow cytometry. Naïve $CD4^+$ T cells co-cultured under polyclonal activation with autologous Mo-MDSCs obtained from KTR 360 days after transplantation are shown (n=3, paired t-test).

5.5- MDSCs from tacrolimus treated KTR effectively suppress T cell proliferation *in vitro*

To evaluate the effect of immunosuppressive regiments on MDSCs function we tested the suppressive function Mo-MDSCs obtained from healthy controls, tacrolimus and rapamycin treated patients.The T-cell-suppressive capacity of Mo-MDSCs from healthy controls, tacrolimus, and rapamycin-treated KTR was compared using an in vitro assay of polyclonally activated T cell proliferation. Sorted Mo-MDSCs were added at a 1:2 ratio to autologous CD3/CD28-stimulated CD4+ T cells. Four patients under long-term tacrolimus treatment and four patients under long-term rapamycin maintenance therapy were analyzed (Figure 29). Results indicate that Mo-MDSCs obtained from tacrolimus treated KTR were significantly suppressive in comparison with rapamycin treated KTR.

This suggests that Mo-MDSCs from transplant patients exhibit different suppressive function *in vitro*, according to the immunosuppressive therapy that KTRs receive.

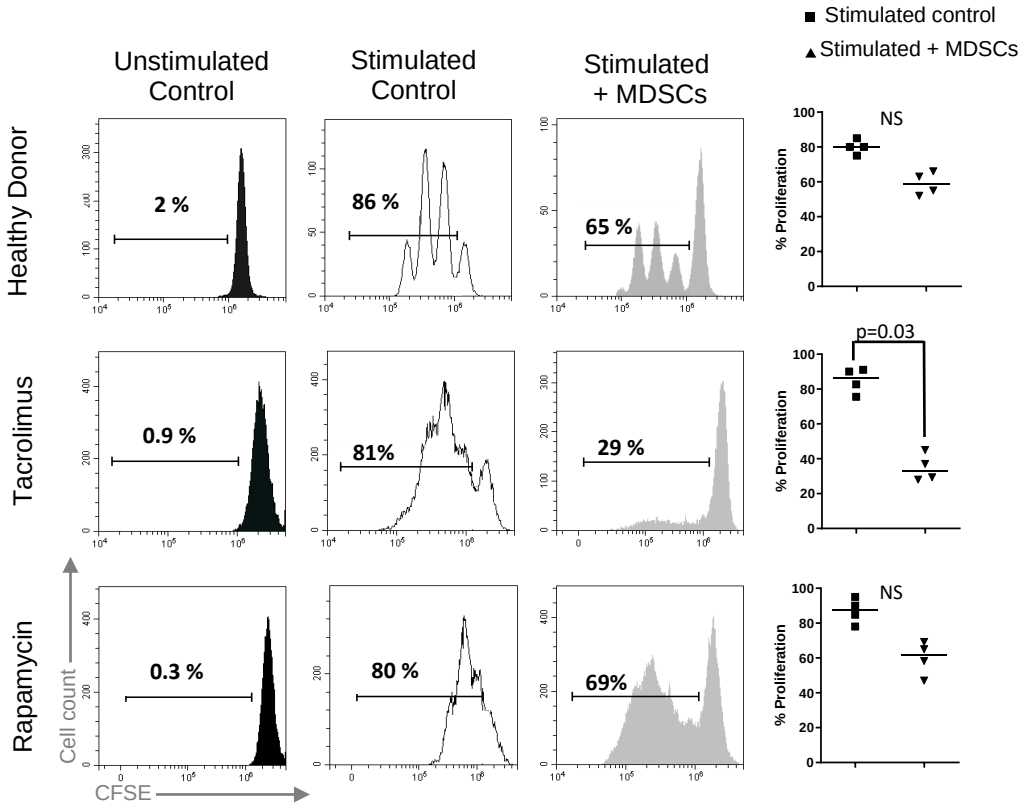


Figure 29| Suppressive function of MDSCs. Sorted $CD4^+$ T cells were stained with carboxyfluorescein-succinimidyl ester (CFSE) and cultured under polyclonal activation alone or with autologous Mo-MDSCs. Representative flow cytometry plots of four independent experiments with Mo-MDSCs from healthy volunteers; kidney transplant patients under tacrolimus treatment and rapamycin are shown. Individual data of experiments are displayed in the right plot graphs where stimulated control cells are represented as black squares and stimulated cells + Mo-MDSCs are represented as black triangles. Differences between groups were assessed by Mann-Whitney test and only indicated when differences were significant.

5.6- Rapamycin Inhibits the Function of *in vitro* Generated Myeloid Suppressor Cells

Following-up our observation of Mo-MDSCs obtained from rapamycin-treated KTRs, we next investigated the effect of rapamycin on myeloid suppressor cells that were generated *in vitro* from whole blood cultures. Using whole blood cultures, we investigated whether CSF1-stimulated human monocytes acquire a Mo-MDSCs phenotype *in vitro*. When cultured for 48 h, we observed an increase in Mo-MDSCs frequency in whole blood cultures from healthy donors (Figure 30 a, b). Next, we investigated the effect of rapamycin on Mo-MDSCs in whole blood cultures and observed that rapamycin led to accumulation of Mo-MDSCs over 48 h (Figure 30 c). These results suggest that mTOR inhibition promotes a Mo-MDSC phenotype. But on the other hand and according to our previous results in KTR, we found that rapamycin exposure substantially reduced the T-cell suppressive capacity of Mo-MDSCs (Figure 30 d). It has been previously shown that T cell suppression by human Mo-MDSCs is in part mediated by the expression of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) (121). Our results confirm that rapamycin blocked the expression of IDO (Figure 30 e), suggesting that the suppressive effect of Mo-MDSCs from rapamycin-treated KTR may be compromised due to the impaired expression of IDO.

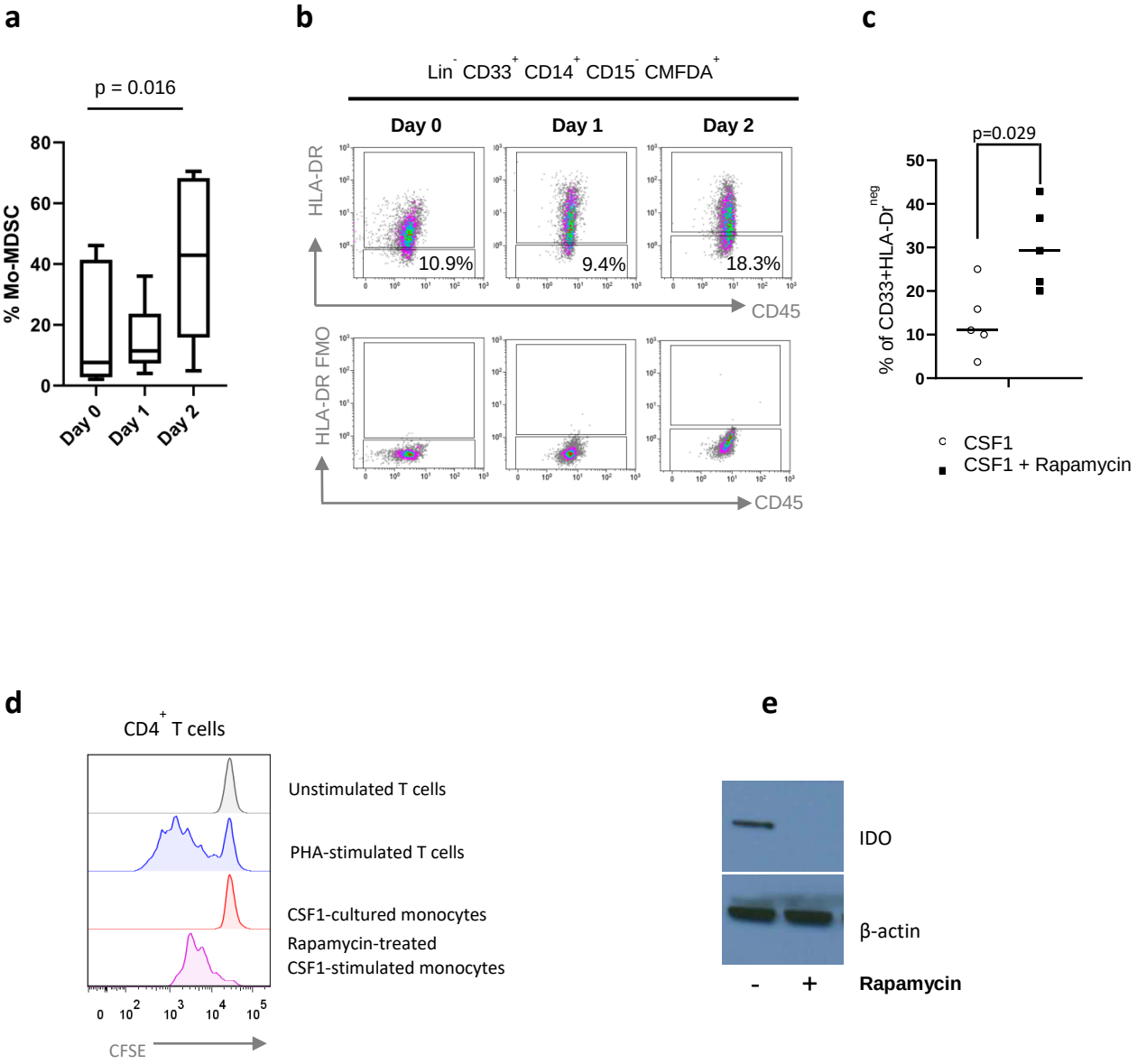


Figure 30| Rapamycin prevents the suppressive function of Mo-MDSCs *in vitro* generated. Colony stimulating factor 1 (CSF1) induces a Mo-MDSCs phenotype *in vitro*. **(a)** CD14+cells were isolated from peripheral blood, labeled with CFDMA and cocultured with CSF1 for 2 days. The phenotype was analyzed in CFDMA+cells at day 0, 1, and 2 after culture. FMO controls were used to define HLA-DR expression **(b)**. Mo-MDSC frequencies after 48 h in WB cultures treated with or without rapamycin. Differences between groups were assessed by pairedt-test **(c)**. Rapamycin-treated CSF1-stimulated monocytes are less effective than untreated monocytes in suppressing phytohemagglutinin (PHA)-stimulated proliferation of allogeneic human CD4+T cells in 1:1 direct cocultures (n= 3) **(d)**. Western Blot analyses indicate that

rapamycin-treated CSF1-derived CD33+HLA-DR−/low myeloid cells prevents the expression of IDO **(e)**.

5.7-Monitoring MDSCs in Lung Transplant Patients

To evaluate the changes on MDSCs subsets after transplant we quantified total MDSC and MDSC subsets: Mo-MDSCs, PMN-MDSCs and e-MDSCs in peripheral blood from end-stage lung disease (ESLD) and lung transplant recipients (figures 31, 32, 33, 34, 35).

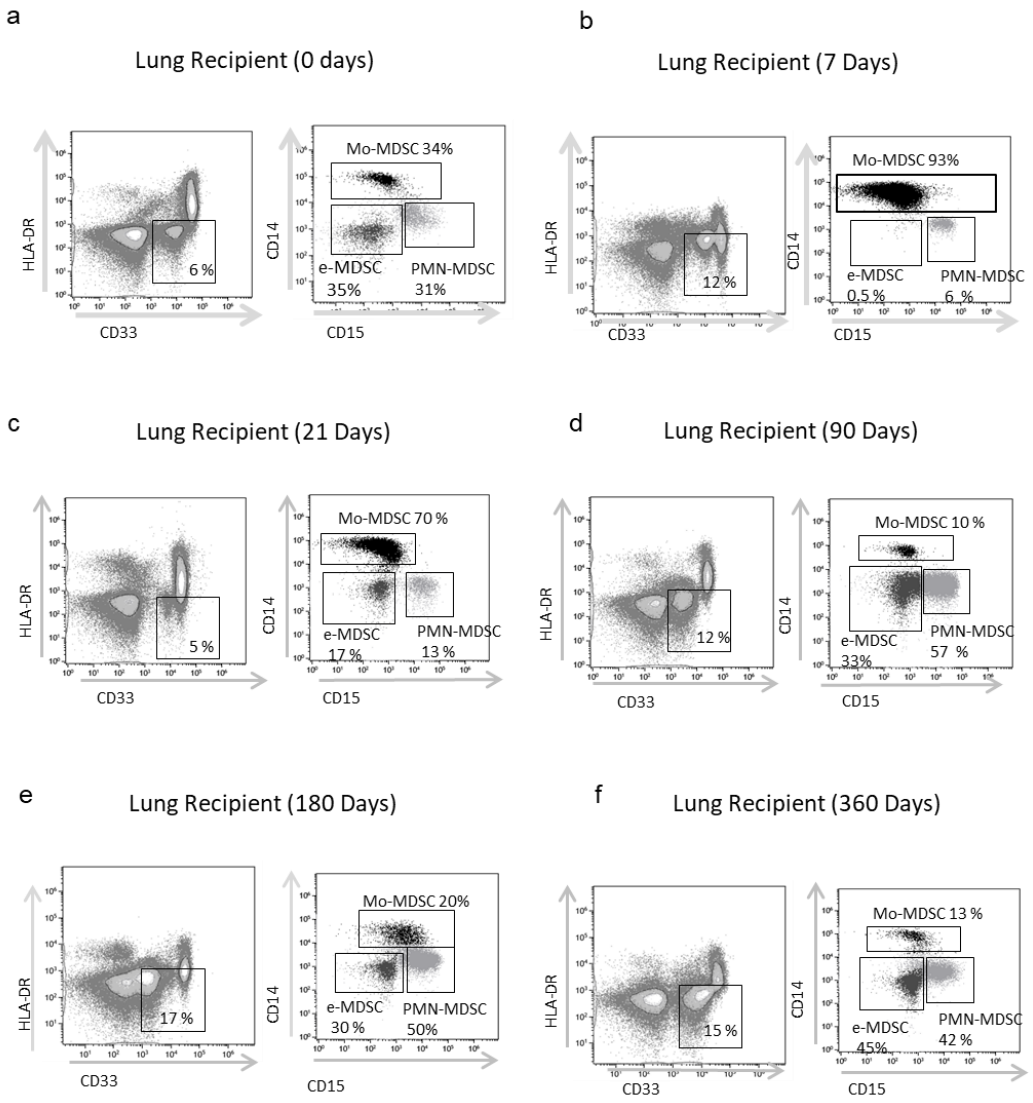


Figure 31| Characterization of MDSCs subsets by flow cytometry. $CD33^{+}$ $HLA\text{-}DR^{-}$ myeloid cells were selected from live cells after doublets and debris exclusion. To

define monocytic (Mo-MDSCs), early-stage (e-MDSCs) and polymorphonuclear (PMN-MDSCs) MDSCs, the CD14 and CD15 expression was analyzed on cells selected from $CD33^{+}HLA\text{-}DR^{-}$ MDSC. Representative flow cytometry data of MDSC from **(a)** patients on the day of transplantation (day 0); lung transplant recipients on days **(b)** 7, **(c)** 21, **(d)** 90, **(e)** 180 and **(f)** 360 post-transplantation are shown.The % subsets calculated from total MDSC.

When we compared MDSCs frequencies from ESLD and healthy controls (HC) matched by sex and age, no significant differences were observed. We found total MDSCs frequencies from ESLD patients and short-term after transplantation remain at baseline levels but they are increased 90 days after transplantation up to a year follow up (ESLD: median 8.49% IQR 4.05%-21.05%; 90 days after transplantation: median 18.21%, IQR 12.41%-33.60%; 180 days after transplantation: median 22.29, IQR 12.83%-30.21%; 360 days after transplantation: median 22.25% IQR 11.06%-39.14%) ($p=0.0002$, $p<0.0001$, $p<0.0001$, figure 32 a; paired tests in figures 33 a and 34 a).

Likewise, we next examined changes in MDSCs subsets distribution after transplantation. The analysis of Mo-MDSCs frequencies showed percentages increased promptly after transplantation and decreased gradually, recovering the basal levels during the time course follow up. (7 days post transplantation median 61.16% IQR 34.12%-79.39% vs ESLD median 26.45%, IQR 4.96%-67.41%; 90 days after transplantation median 13.56% IQR 5.41%-34.47%; 180 days median 12.63% IQR 5.22%-31.02%; 360 days median 15.27%, IQR 6.26%-33.39%) ($p=0.0002$, $p<0.0001$ and $p<0.0001$, $p<0.0001$, figure 32 b; paired tests in figures 33 b and 34 b).

On the contrary, PMN-MDSCs frequencies on the short-term after transplantation were significantly lower up to 90 days; then they remain increased during the time course follow up (7 days after transplantation:

median 29.03%; IQR 12.22%-49.03% vs 90 days: median 69.95% IQR 39.99%-81.04% and 180 days: median 58.28% IQR 33.93%-81.47%; p< 0.0001 p=0.0007, figure 32 c). 21 days: median 26.19% IQR 11.95%-45.58% vs 90 days, 180 days and 360 days: median 59.38% IQR 22.34%-73.13% (p<0.0001, p<0.0001, p=0.0184, figure 32 c; paired tests in figure 33 c and 34 c).

Lastly, the effect of transplantation on e-MDSCs frequencies was calculated as well. We observed e-MDSCs basal levels are low at baseline and post transplant compared to PMN-MDSCs and Mo-MDSCs. Nevertheless, 21 days after transplantation there is a increase (median 7.742%; IQR 2.5%-19.63%) compared to pre transplant levels (median 3.92%; IQR 1.35%-8.06%) and 7 (median 2.04%; IQR 0.84%-3.93% (p=0.0375, p<0.0001, figure 32 d; paired tests in figures 33 d and 34 d).

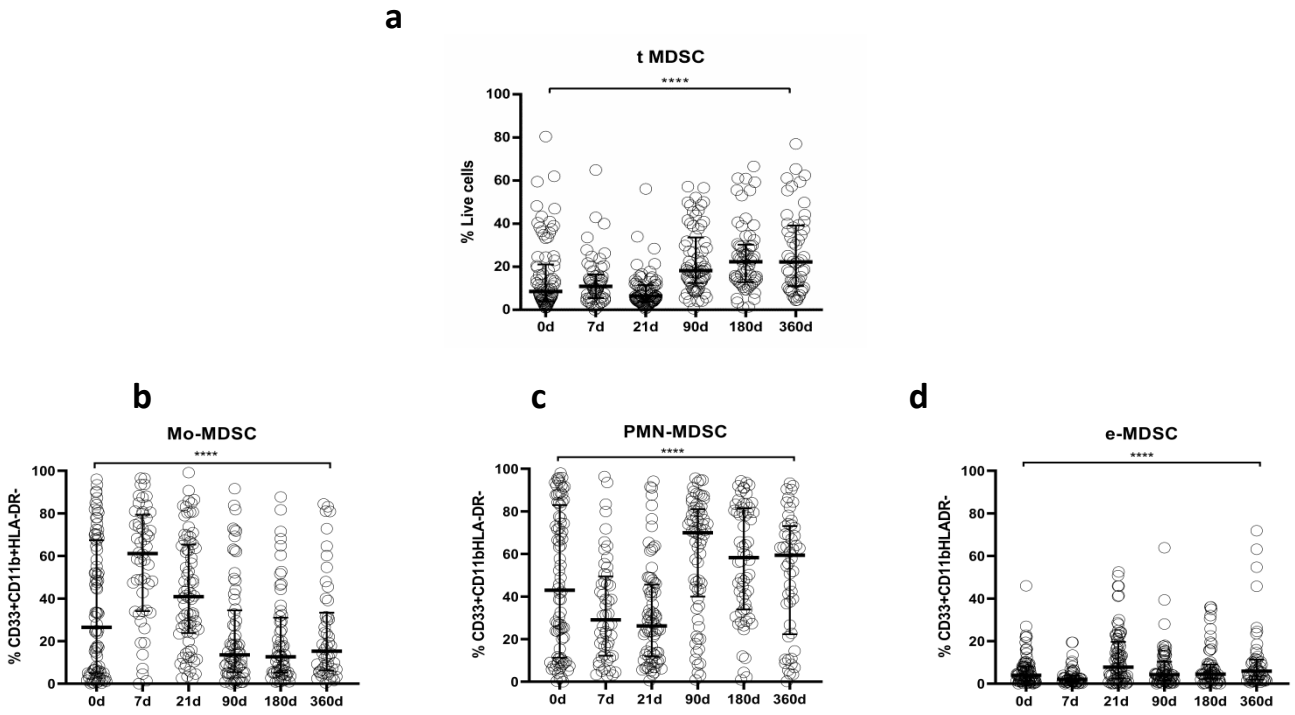


Figure 32| MDSCs frequencies in LTR. (a) The percentajes of total myeloid-derived suppressor cells (MDSCs) in peripheral blood mononuclear cells (PBMC); **(b)** monocytic-MDSC (Mo-MDSCs); **(c)** early stage-MDSC (eMDSCs) and **(d)**

polymorphonuclear MDSC (PMN-MDSCs) were determined using flow cytometry. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney U test. (**** and p<0.0001). % of Subsets was calculated from total MDSCs.

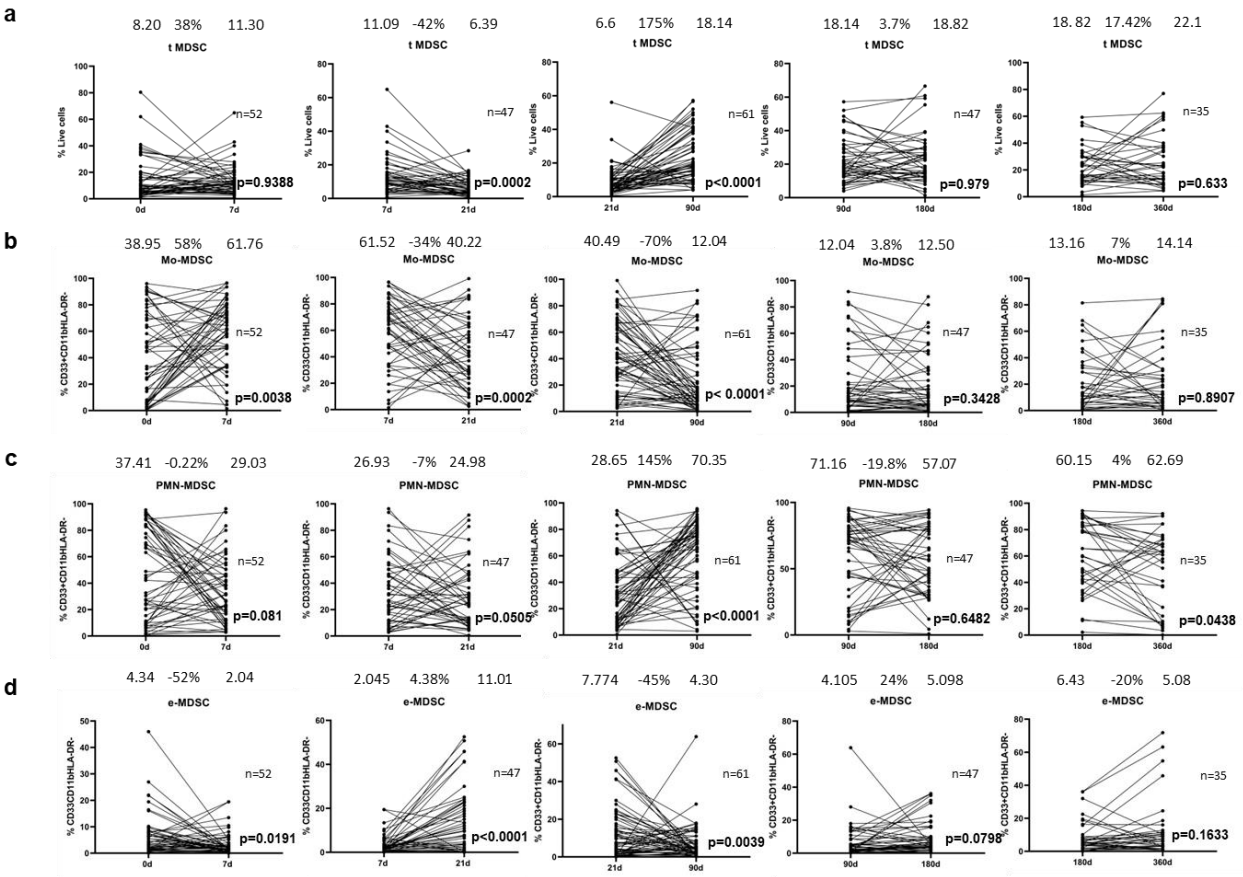


Figure 33| Comparison of MDSCs subsets Paired analysis of **(a)** t-MDSCs, **(b)** Mo-MDSCs, **(c)** PMN-MDSCs and **(d)** e-MDSCs at day 0 and 7 days after transplant, at day 7 and day 21, at day 21 and 90, at day 90 and 180and 180 and 360. Levels of Mo-MDSCs 7 days after transplant were significantly increased compared to day 0 **(b)**. Levels of t-MDSCs, Mo-MDSCs and PMN-MDSCs 21 days after transplant were significantly decreased compared to day 7 **(a, b, c)**. Levels of t-MDSCs, PMN-MDSCs 90 days after transplant were significantly increased compared to day 21 **(a, c)** while Mo-MDSCs and e-MDSCs levels were significantly decreased **(b, d).** The central number is the difference (in percent) between the means of the two time points. Differences between time points were calculated using the following formula: (median posTx-median preTx) / median preTx.

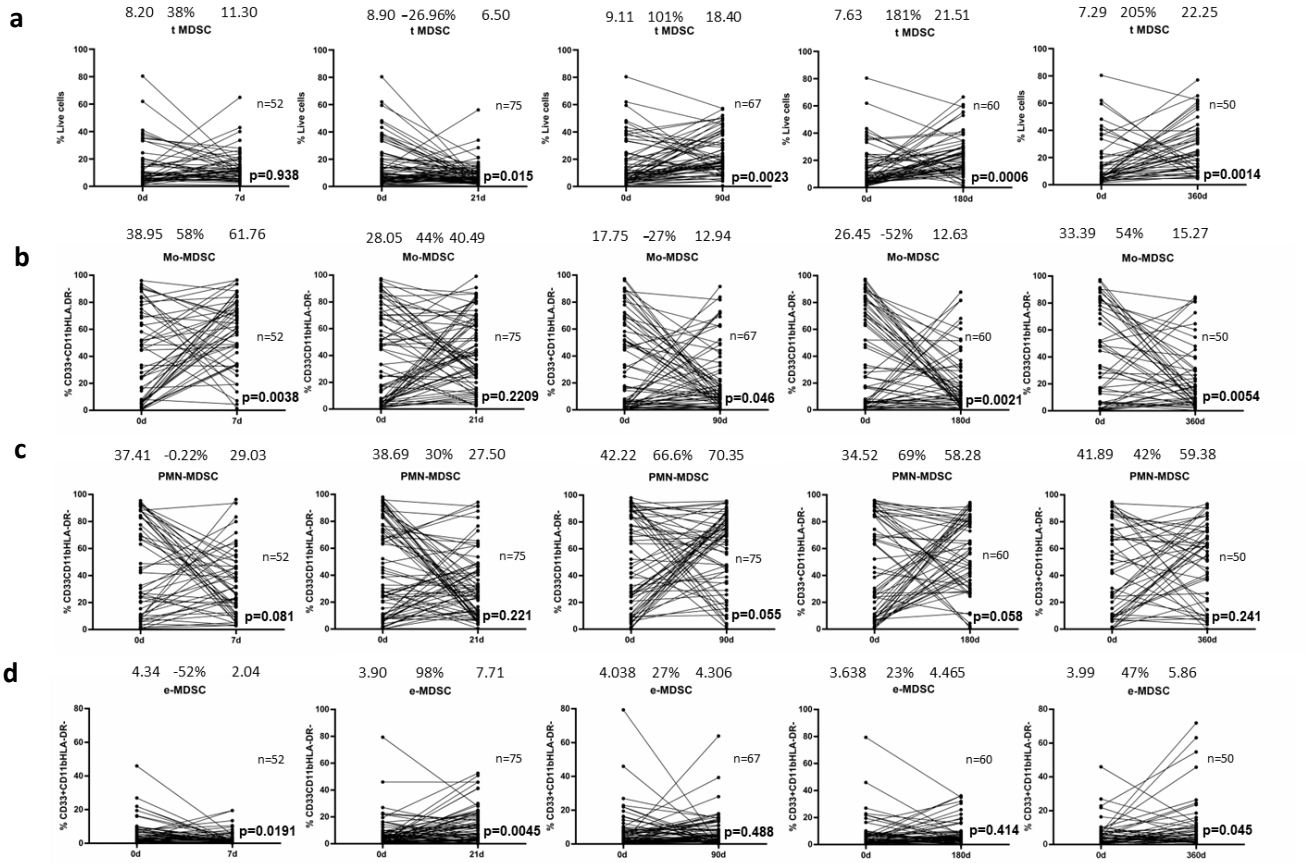


Figure 34| MDSCs subsets frequencies changes compared to pre-lung transplant levels t-MDSCs **(a)**, Mo-MDSCs **(b),** PMN-MDSCs **(c)**, and e-MDSCs **(d)** at day 0 and 7; 0 and 21; 0 and 90; 0 and 180; 0 and 360 days after lung transplantation. t-MDSCs frequency 7 days after transplant significantly decreased 21 days post transplantatation; after that, percentages increased 3 months after transplantation up to a year **(a)**. Mo-MDSCs percentages increased promptly after transplantation and decreased 3, 6 and 12 months after transplantation **(b)**. Levels of PMN-MDSCs significantly increased 3 and 6 months post-transplantation **(c).** e-MDSCs significantly decrease 7, 21 and 360 days post-transplantation compared to pre-transplant levels **(d)**. The central number is the difference (in percent) between the median of the two time points. Differences between time points were calculated using the following formula: (median posTx-median preTx) / median preTx.

The absolute numbers of MDSCs subsets before and after transplantation were calculated as well, as indicated in figure 35.

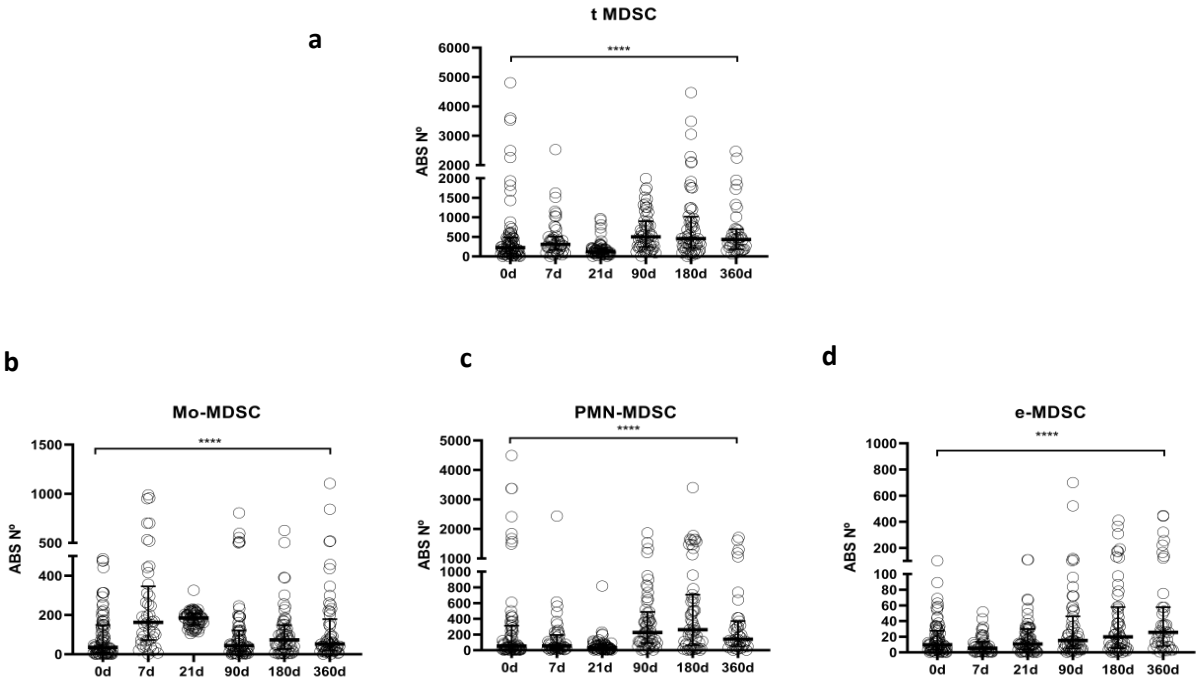


Figure 35| MDSCs absolute numbers in LTR. (a) Frequencies of total myeloid-derived suppressor cells (MDSCs) in peripheral blood mononuclear cells (PBMCs); **(b)** monocytic-MDSCs (Mo-MDSCs); **(c)** polymorphonuclear MDSCs (PMN-MDSCs) and **(d)** early stage-MDSC (eMDSCs) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney U test. (*p<0.05). **p<0.01 ***p<0.001 **** p<0.0001.

5.8- MDSCs from Tacrolimus treated LTR effectively suppress T cell proliferation *in vitro*

Since MDSCs need to be defined upon demonstration of their suppressive function due to the lack of specific markers, we assesed the cell-suppressive capacity of MDSCs from healthy controls and tacrolimus treated LTR. Moreover, as LTR were under Tacrolimus treatment, it is important to check the effect of the immunosuppressive treatment on MDSCs.

The suppressive capacity of Mo-MDSCs was analysed using an *in vitro* assay of polyclonally-activated T cell proliferation. Sorted Mo-MDSCs were added at a 1:2 ratio to autologous CD3/CD28-stimulated $CD4^+$ T cells. Two patients under long-term tacrolimus treatment and four healthy controls were tested (Figure 36). Results indicate that Mo-MDSCs obtained from tacrolimus treated LTR were significantly more suppressive in comparison with HC. This suggests that Mo-MDSCs from transplant patients exhibit potent suppressive function *in vitro* despite the fact that they are under immunosuppressive therapy.

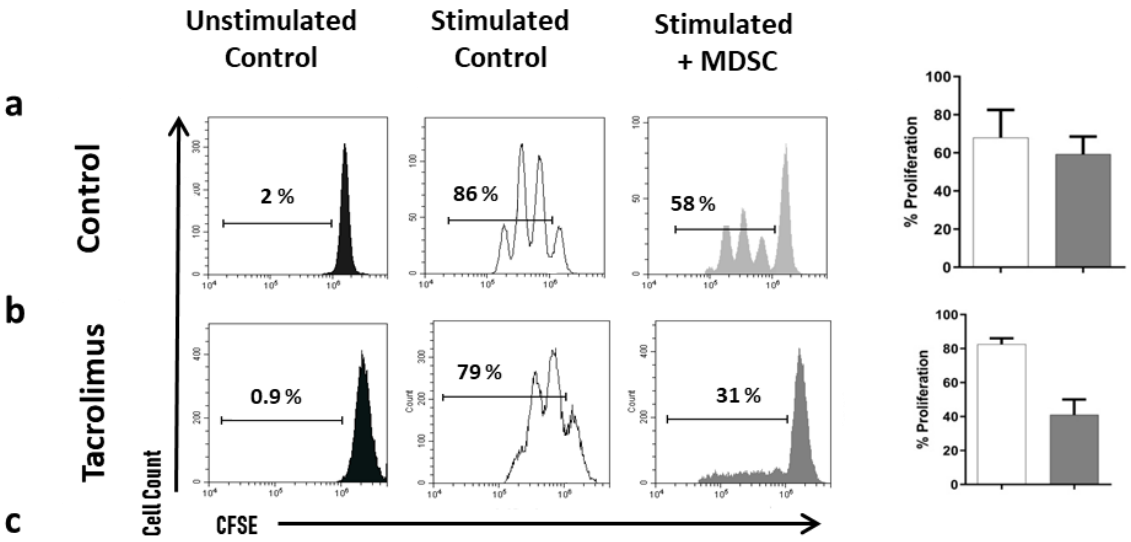


Figure 36| Suppressive function of MDSCs. Sorted $CD4^+$ T cells were stained with carboxyfluorescein-succinimidyl ester (CFSE) and cultured under polyclonal activation alone or with autologous monocytic-myeloid-derived suppressor cells (Mo-MDSCs). Representative flow cytometry plots of two independent experiments with Mo-MDSCs from two healthy volunteers and two lung transplant recipients under tacrolimus treatment are shown.

5.9- MDSCs and clinical events in LTR

Following-up our objectives, to evaluate if MDSCs can play a main role in the balance between graft acceptance and rejection, we next examined the effect of MDSCs subsets frequency on clinical events. In our cohort, we found no association between MDSCs levels and ACR. On the other hand, we observed and inmediate increased of Mo-MDSCs post ACR (90 days postransplant ACR: n=23, median 22.58 IQR 8.96- 83.74; No ACR : n=44, median 10.63 IQR 5.15-20.63) (p=0.0336) and 180 days post-transplant: ACR: n=23, median 17.8 IQR 6.82- 46.28; No ACR n=36, median 8.6 IQR 4.53-20.02) (p=0.0342) (Figure 37). Whether this effect is a consequence of the rejection itself or it is produced by the treatment, remains unknown. We found no differences when we studied MDSCs subsets from patients previously sensitized,, primary graft disfunction (PGD), and base disease (Figure 38). In contrast with one of our prior hypothesis, no correlation was observed when we studied tacrolimus levels in peripheral blood and MDSC frequencies.

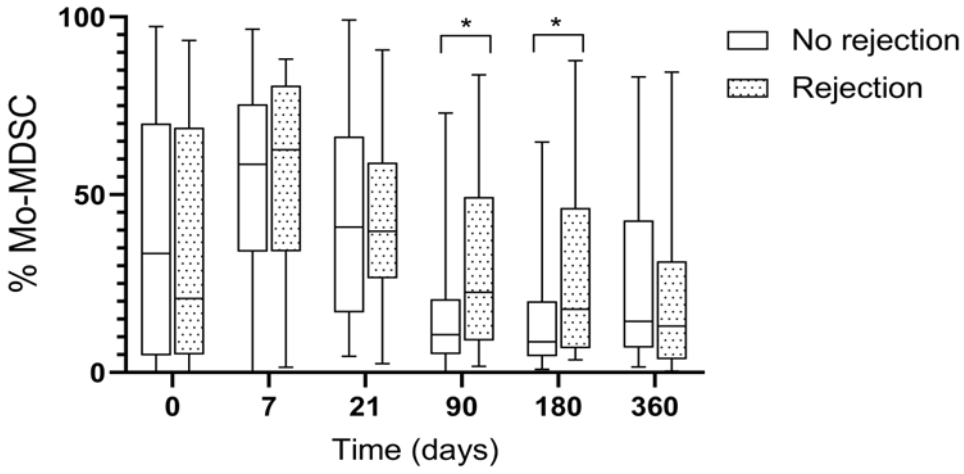
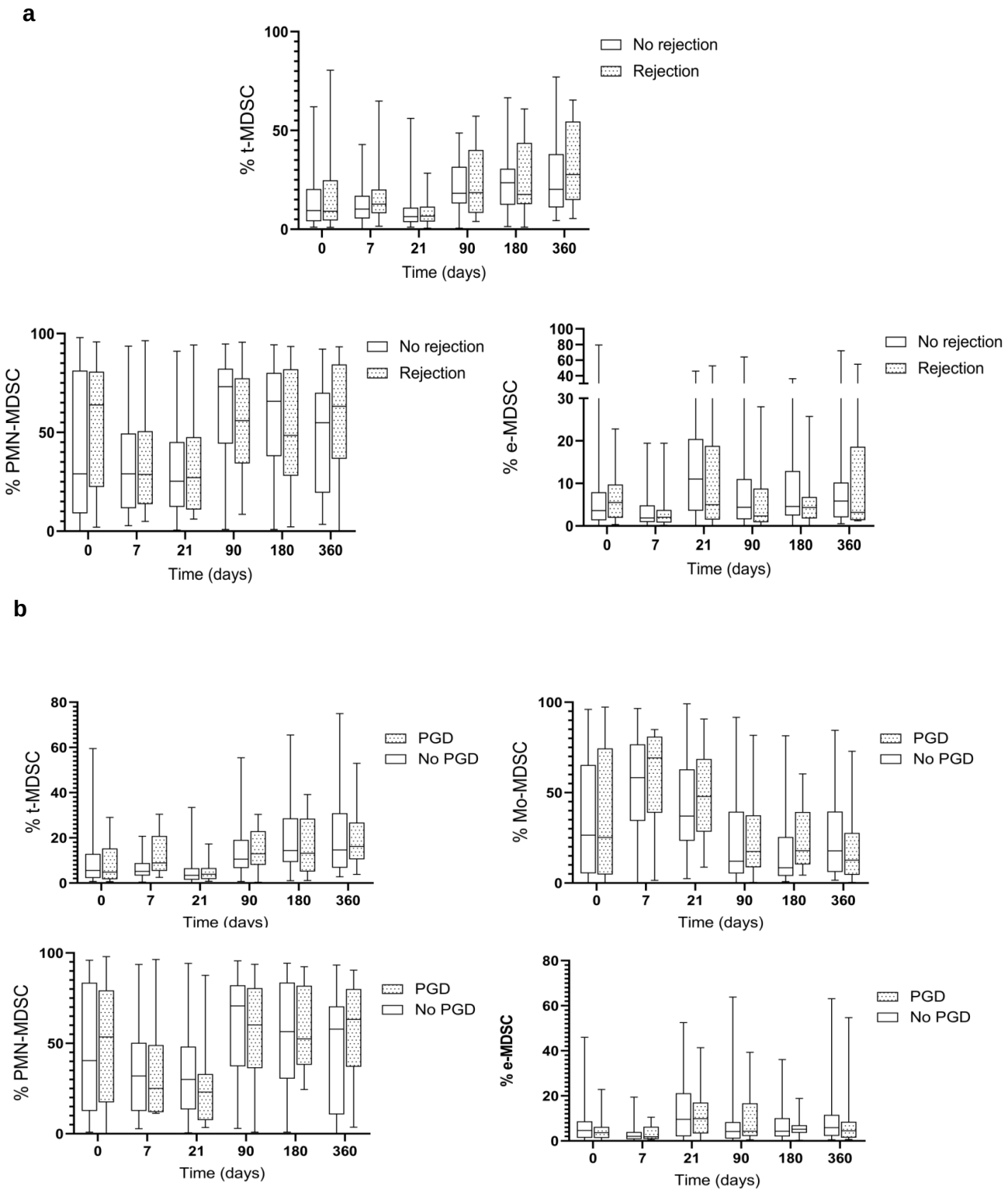


Figure 37| MDSCs and clinical data in ACR in LTR. 90 and 180 days post-transplant Mo-MDSCs percentages were lower in patients who do not reject (n=44, n=36) compared to those who reject (n=23, n=23). Box represents median and 25th and 75th percentiles and whiskers were calculated by the Tukey method (* p<0.05).



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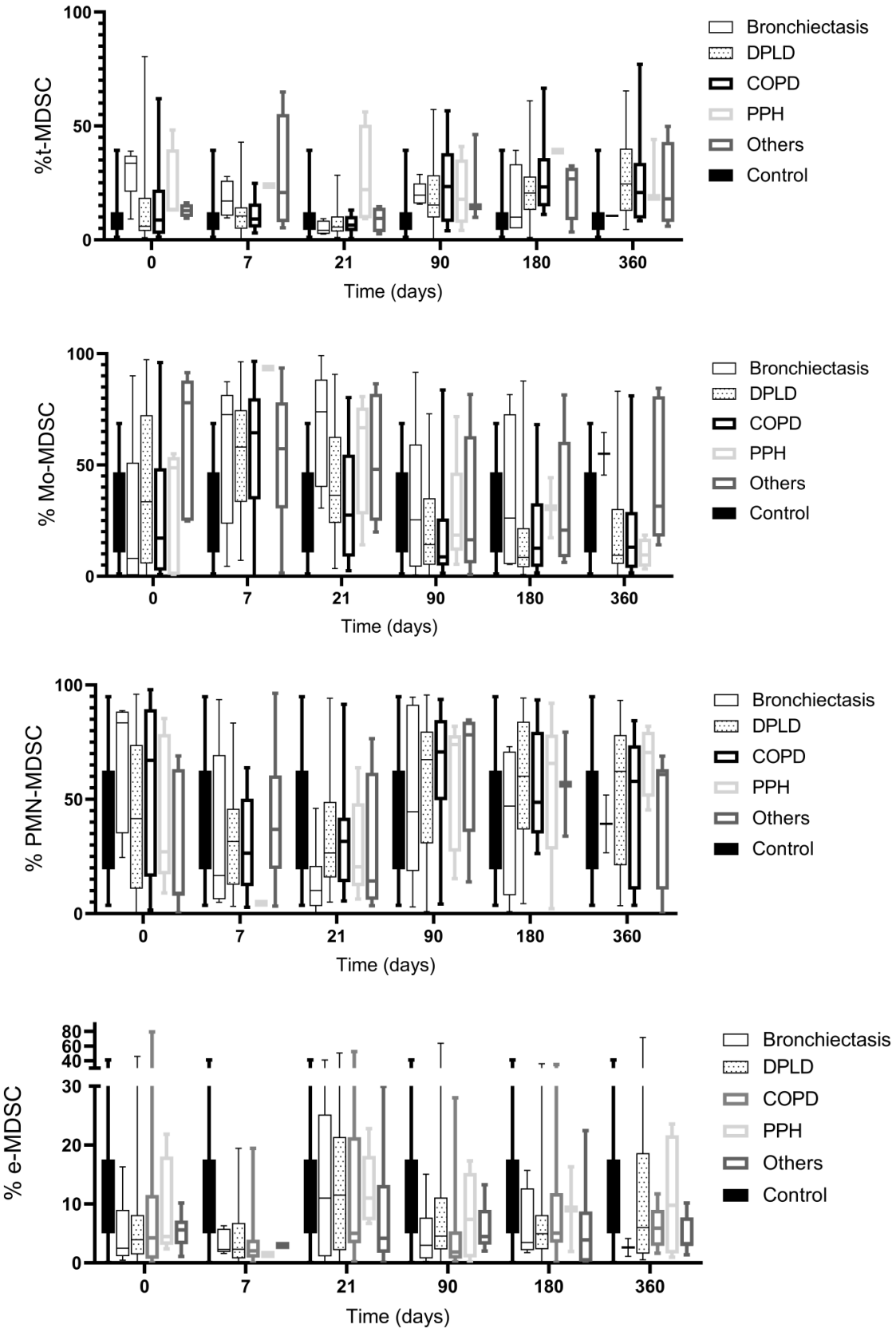
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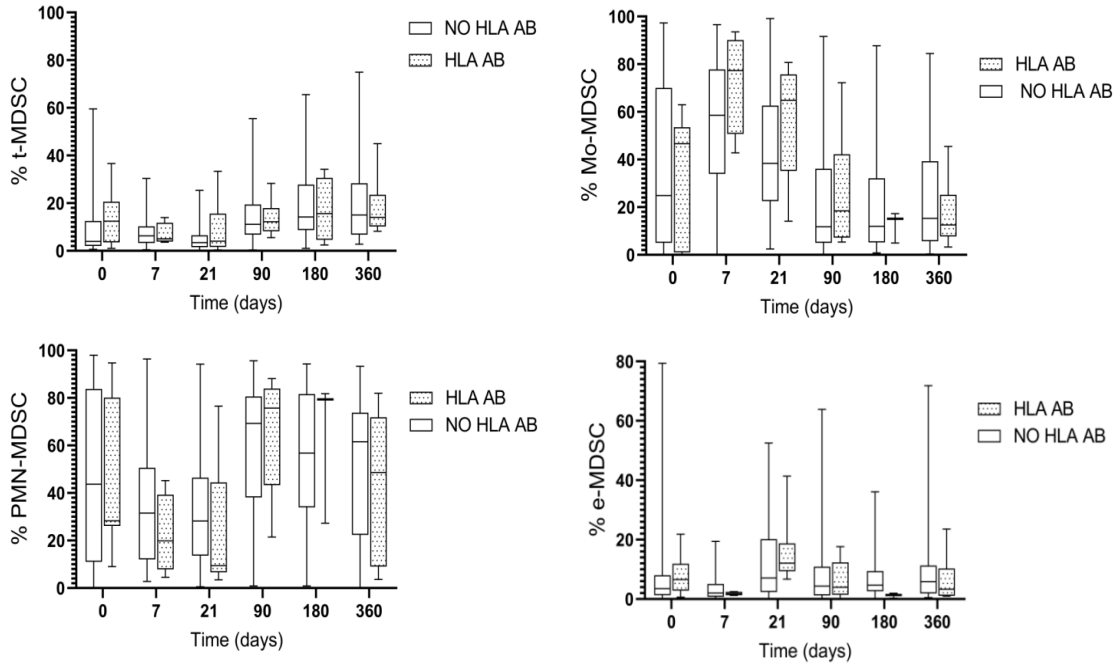


Figure 38| MDSCs frequencies in LTR and clinical events Frequencies of t-MDSCs, Mo-MDSCs, PMN-MDSCs, eMDSCs in PBMC were studied and compared with clinical events such as **(a)** rejection **(b)** primary graft dysfunction (PGD), **(c)** basal disease, **(d)** HLA antibodies. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney U test (*p<0.05).

As we mentioned before, MDSCs markers are not exclusively expressed by them; assays of human MDSC function are difficult to implement and it is unclear to what extent HLA-DR expression is influenced by standard immunosuppression. For this reasons, during my stay in the immunomonitoring laboratory (Hospital Klinikum, Regensburg, Germany) we implement new flow cytometry panels allowing to check a wide range of fuctional and phenotypic markers from peripheral blood.

5.10- Panel 1: Detection of Mo-MDSCs, PMN-MDSCs and e-MDSCs in whole blood samples

We first designed a flow cytometry assay for rapid and reproducible detection of MDSCs subsets in small volumes of human peripheral blood. This assay relies on conventional phenotypic definitions of human Mo-MDSCs ($CD45^+$ Lin^- $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$/low $CD14^+$ $CD15^-$), PMN-MDSCs ($CD45^+$ Lin^- $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$/low $CD14^-$/low $CD15^+$ $LOX1^+$) and e-MDSCs ($CD45^+$ Lin^- $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$/low $CD14^-$/low $CD15^-$ $CD124^+$). Monoclonal antibodies (mAb) against the specified antigens were selected according to regulatory status (with a preference for CE-labeled reagents) and availability of suitable fluorochrome conjugates (**Table 5**). We adopted a convenient and highly standardisable whole blood staining method for detecting extracellular antigens, which is widely used for other human immune monitoring panels. mAbs were titrated to determine optimal staining conditions. Figure 39 shows our recommended gating strategy.

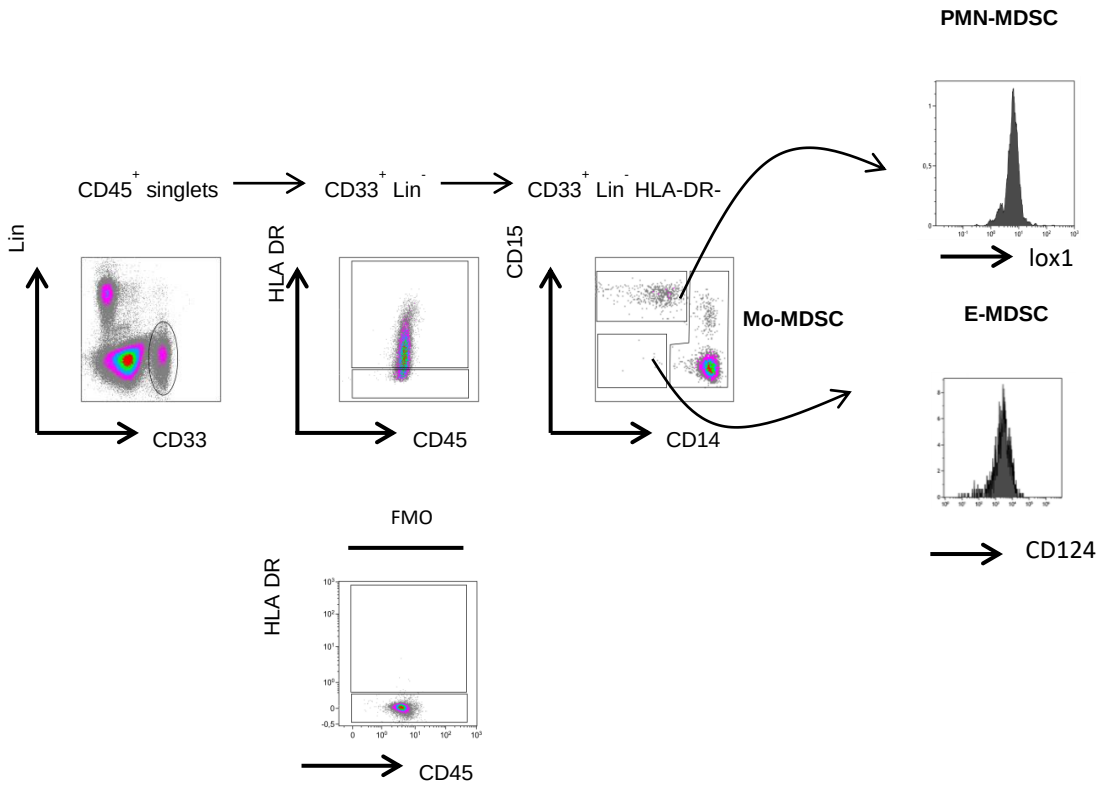


Figure 39| Characterization of MDSCs subsets by flow cytometry. Gating strategy for the identification of MDSCs from peripheral blood. $CD33^{+}$ Lin^{-} myeloid cells were selected from CD45+ cells after doublets and debris exclusion. FMO controls were used to define HLA-DR expression.To define monocytic (Mo-MDSCs), early-stage (e-MDSCs) and polymorphonuclear (PMN-MDSCs) MDSCs, the CD14 and CD15 expression was analyzed on cells selected from $CD33^{+}$ Lin-HLA-DR^{-} MDSC. Representative flow cytometry data of MDSC from a melanoma patient is shown.

5.11- Panel 2: Relative quantification of phospho-mTOR and phospho-S6 in Mo-MDSCs

Ribechini and colleagues (121) previously reported that Mo-MDSCs originate from peripheral blood monocytes through an mTOR-dependent pathway initiated by GM-CSF receptor signaling.

This mechanism appears to rely upon post-transcriptional stabililisation of IDO expression through phosphophorylation of S6, which is a downstream event in the mTOR signaling pathway. We recently discovered that MDSCs

from rapamycin-treated kidney transplant were less suppressive than MDSC from tacrolimus-treated transplant recipients or healthy individuals. We further showed that rapamycin, an mTOR-inhibition, abrogates the suppressive potential of Mo-MDSCs *in vitro* by preventing IDO induction Therefore, we next developed a standardised assay to monitor phospho-mTOR, phospho-S6, IRF1 and IDO expression by human Mo-MDSCs in peripheral blood samples. In this assay, Mo-MDSCs were defined as $CD45^{+}$ Lin^{-} $CD33^{+}$ HLA-$DR^{-/low}$ $CD14^{+}$ $CD15^{-/low}$ cells. mAb against the specified antigens were selected according to regulatory status (with a preference for CE-labeled reagents) and availability of suitable fluorochrome conjugates. We introduced a standardisable method for detecting phosphorylated intracellular antigens in whole blood samples, which also allows for reliable detection of cytoplasmic proteins, such as IDO, and nuclear antigens, such as IRF1. mAbs were titrated to determine optimal staining conditions (**Table 5**). Figure 40 shows our recommended gating strategy.

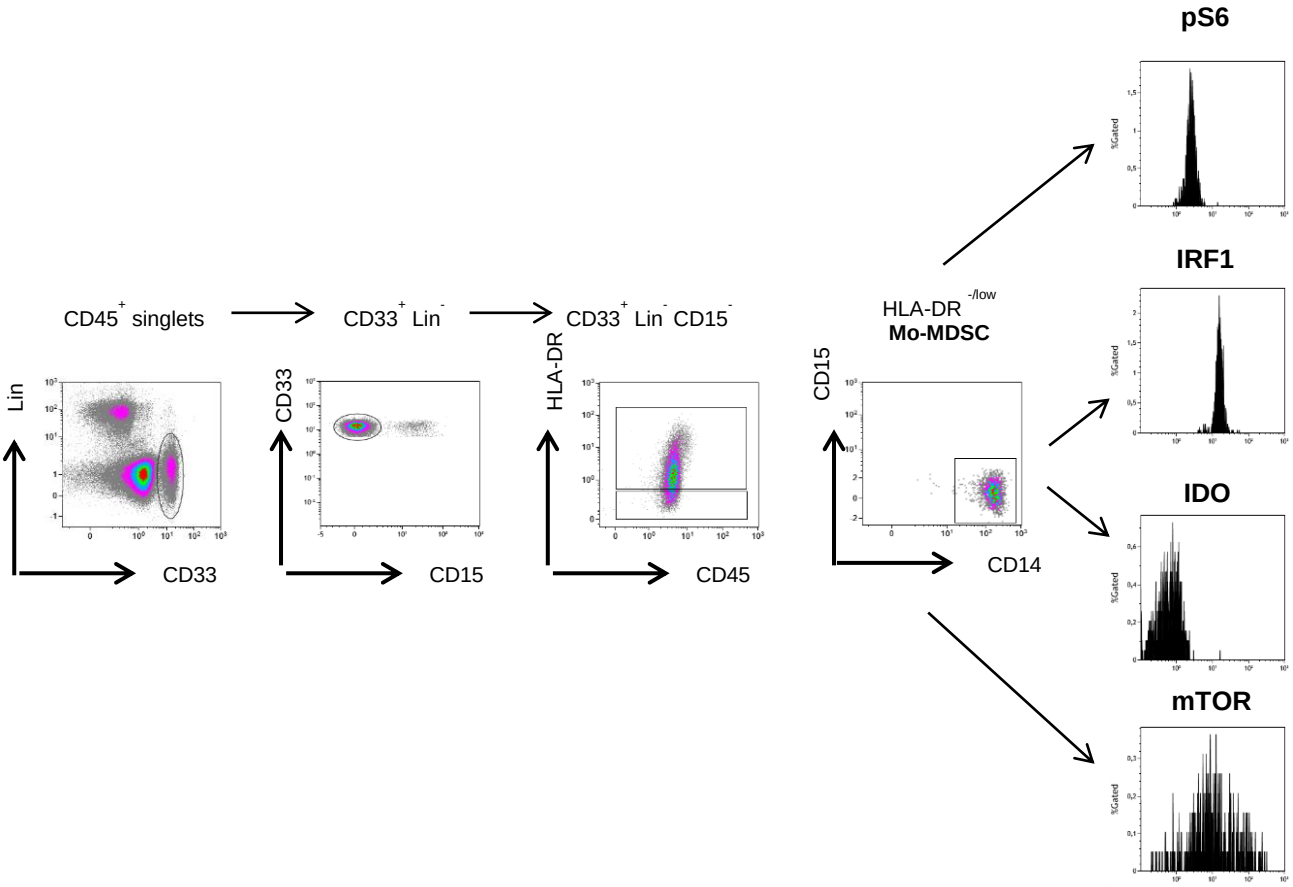


Figure 40| Relative quantification of phospho-mTOR, phospho-S6, IRF-1 and IDO in Mo-MDSCs. Mo-MDSCs were defined as $CD45^{+}$ Lin^{-} $CD33^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{+}$ $CD15^{-/low}$ baseline expression of the indicated markers in Mo-MDSCs is shown.

5.12- Panel 3: Assessment of IFN-γ-inducible IDO expression in Mo-MDSCs

Circulating Mo-MDSCs do not generally express detectable levels of IDO, but is expression is readily induced by proinflammatory factors, including IFN-γ. The capacity of Mo-MDSCs to inducibly express IDO is a useful surrogate marker of their suppressive function. Therefore, we developed a convenient assay for inducible IDO expression based on a whole blood culture method and intracellular staining of Mo-MDSCs for analysis by flow cytometry. This panel identifies Mo-MDSCs as $CD45^{+}$ Lin^{-} $CD33^{+}$ $CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{+}$ $CD15^{-/low}$ cells and enables us to quantify expression of IRF1 and IDO. mAb against the specified antigens were selected according to regulatory status (with a preference for CE-labeled reagents) and availability of suitable fluorochrome conjugates (**Table 5**, figure 41).

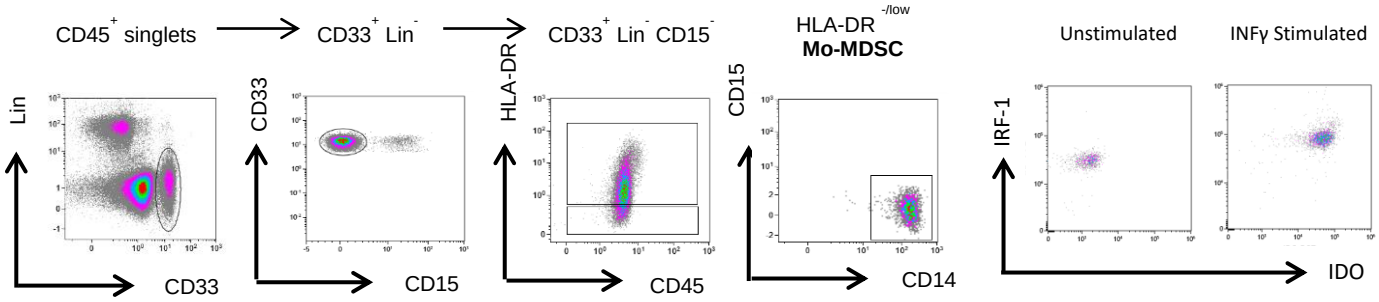
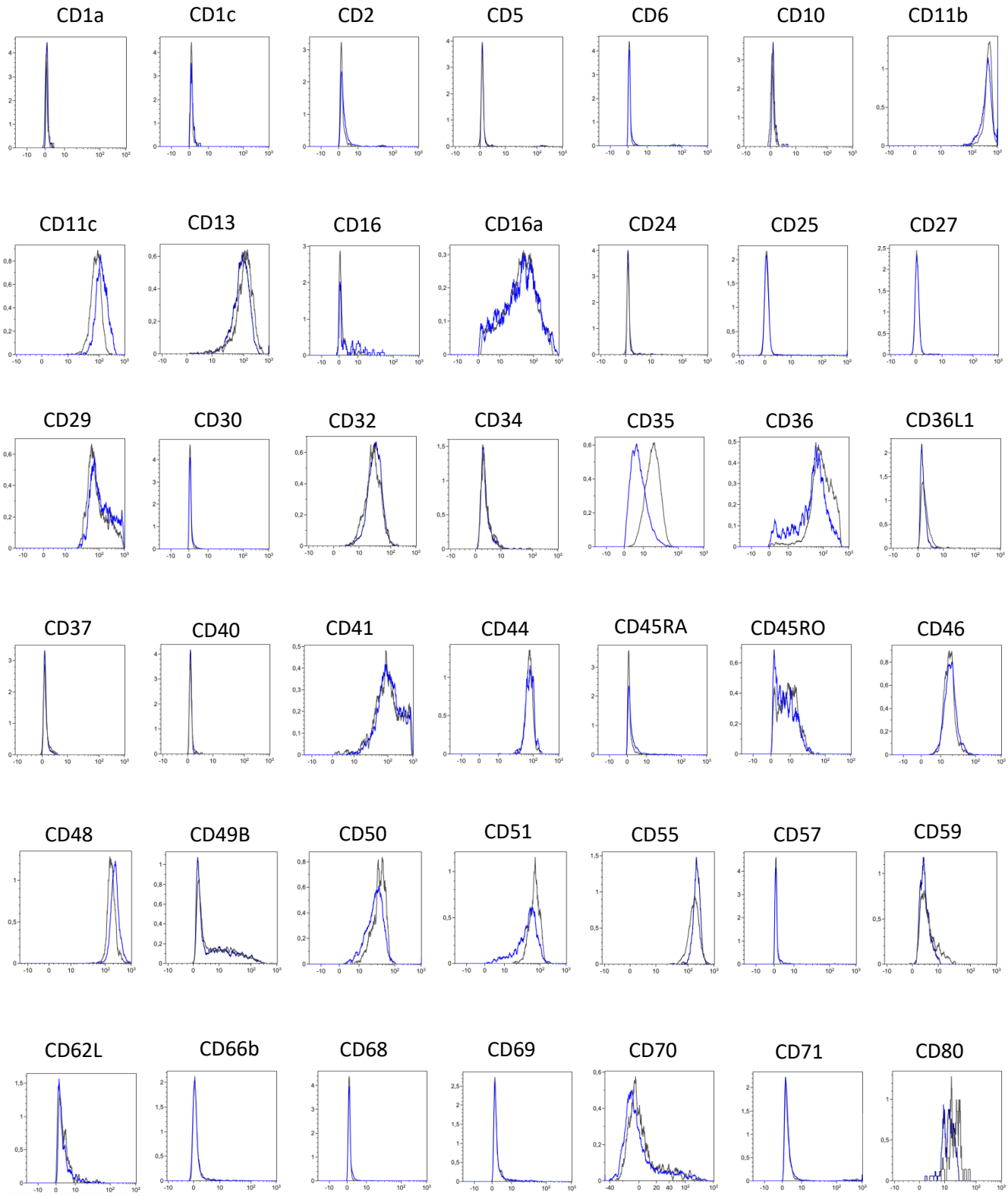


Figure 41| Assessment of INF-γ-inducible IDO expression in Mo-MDSCs. Whole blood was treated with INF for 24 hours. Mo-MDSCs were defined as $CD45^{+}$ Lin^{-} $CD33^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{+}$ $CD15^{-/low}$. Expression of the indicated markers in Mo-MDSC after INF treatment is shown.

5.13- Panel 4: Markers of human Mo-MDSCs unaffected by glucocorticoid treatment

To assess whether down-regulation of HLA-DR might affect our ability to discriminate between monocytes and Mo-MDSCs, isolated $CD14^+$ monocytes were stimulated in culture for 24 h or 48 h with dexamethasone over a wide range of concentrations. We observed a rapid, dose-dependent reduction in HLA-DR expression levels in monocytes (Figure 43 a). We and others previously reported increased Mo-MDSCs frequencies during the early post-transplant period that later subside to baseline values. Solid organ transplant recipients are typically treated with high-dose glucocorticoids immediate after transplantation and then tapered over 8 - 12 weeks to maintenance doses or a steroid-sparing regimen. Hence, post transplant changes in Mo-MDSCs frequency might simply reflect changes in glucorticoid treatment, not a biological response to the allograft.

To identify Mo-MDSCs markers unaffected by glucocorticoid exposure, we next screened 100 cell surface antigens for differential expression in Mo-MDSCs versus $CD14^+$ monocytes from patients with advanced melanoma (figure 42).


CD1a
CD1c
CD2
CD5
CD6
CD10
CD11b
CD11c
CD13
CD16
CD16a
CD24
CD25
CD27
CD29
CD30
CD32
CD34
CD35
CD36
CD36L1
CD37
CD40
CD41
CD44
CD45RA
CD45RO
CD46
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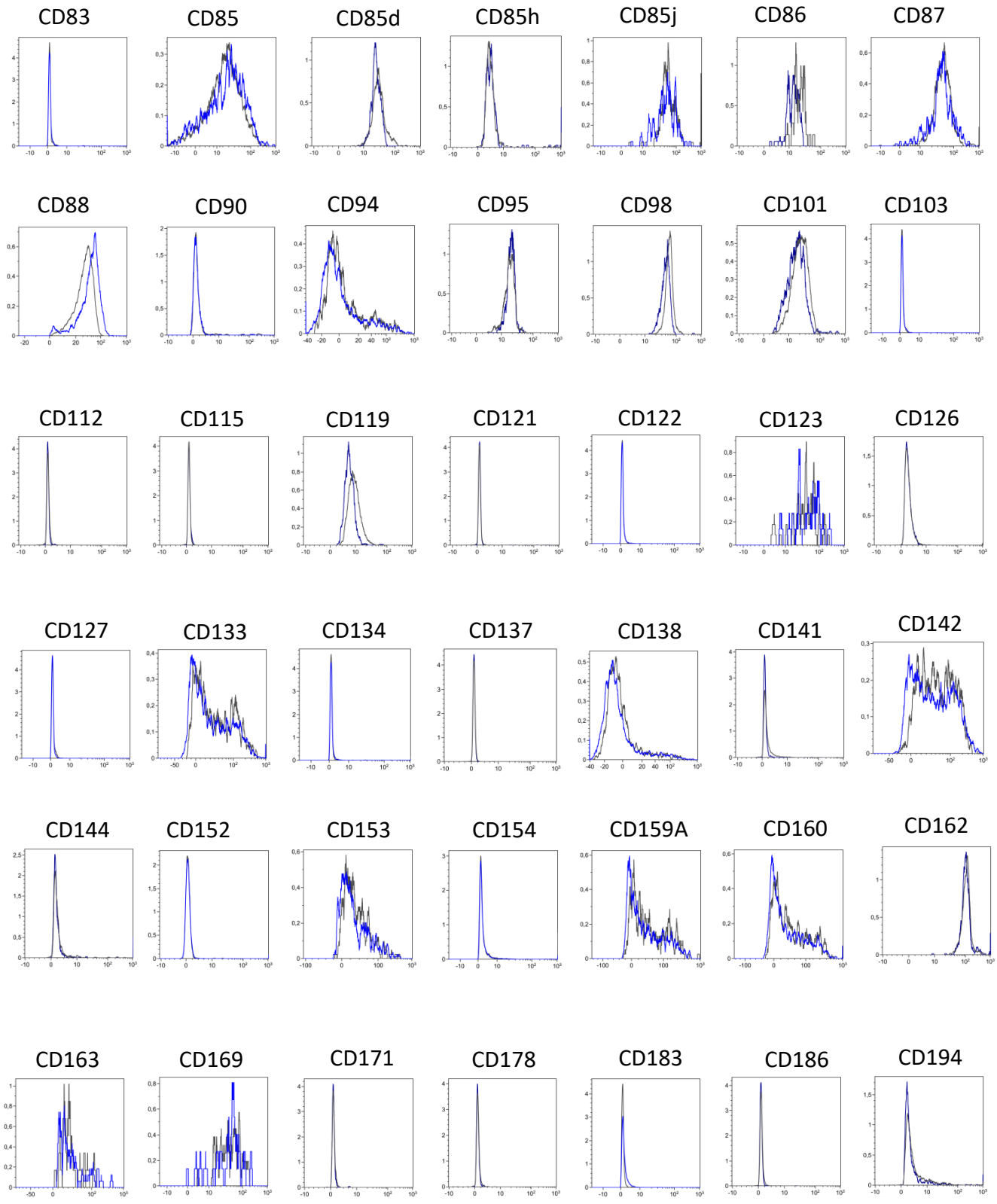
CD71
CD80


CD83
CD85
CD85d
CD85h
CD85j
CD86
CD87
CD88
CD90
CD94
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CD101
CD103
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CD115
CD119
CD121
CD122
CD123
CD126
CD127
CD133
CD134
CD137
CD138
CD141
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CD171
CD178
CD183
CD186
CD194

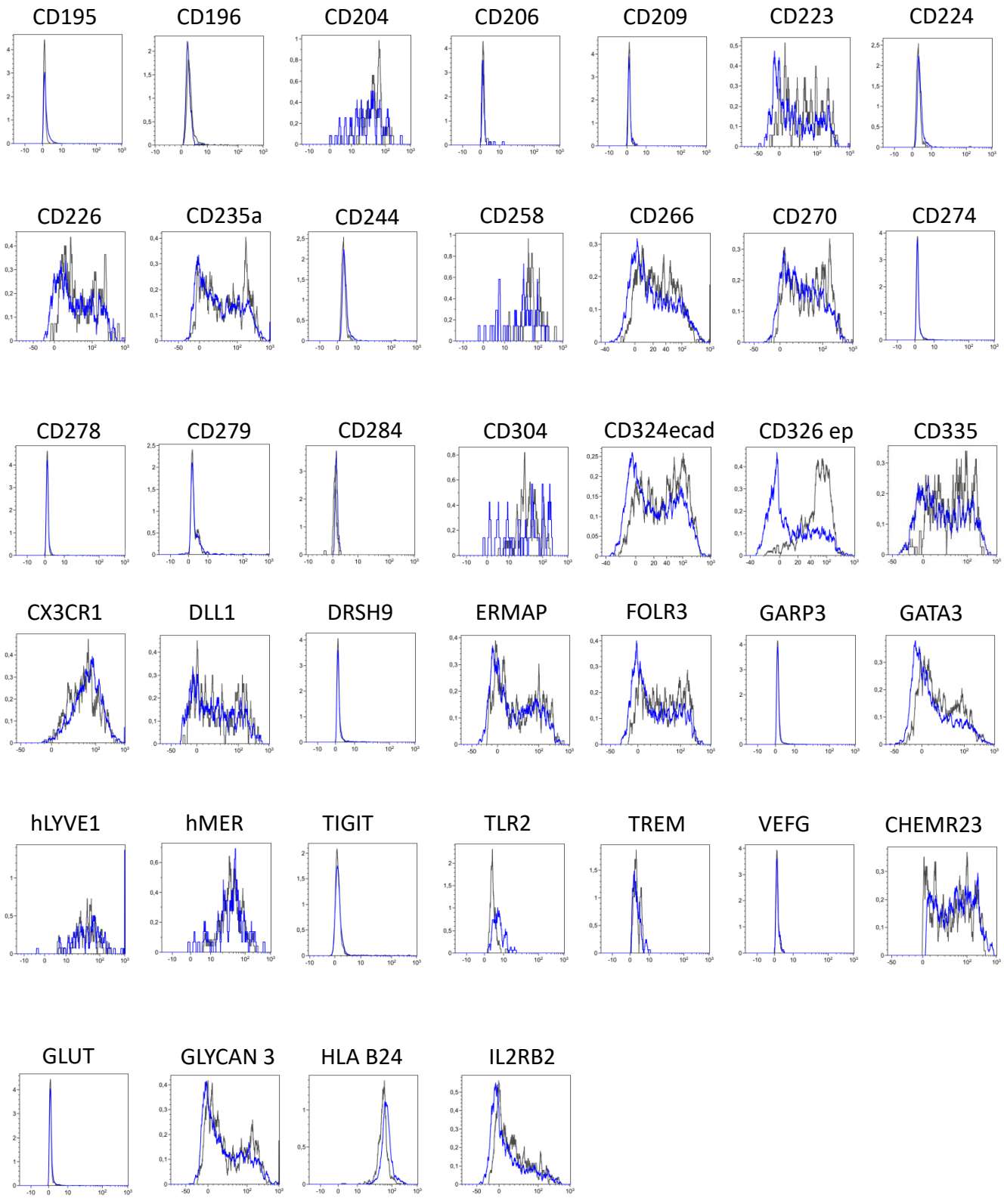
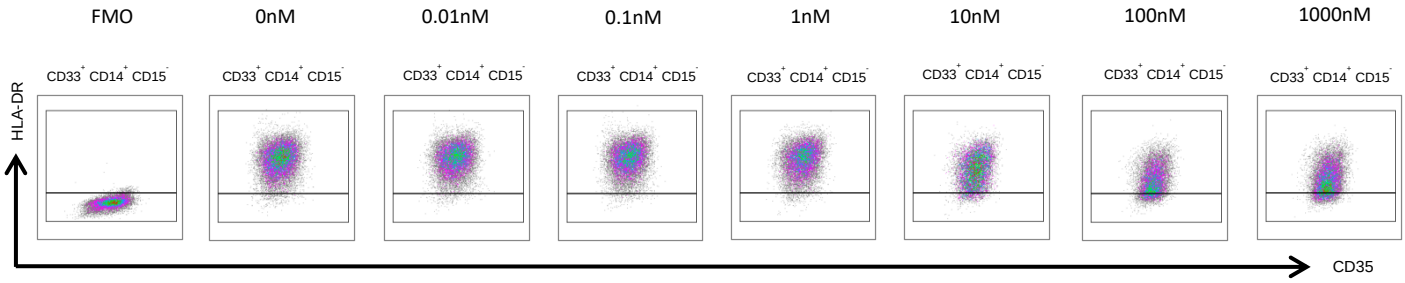

CD195
CD196
CD204
CD206
CD209
CD223
CD224
CD226
CD235a
CD244
CD258
CD266
CD270
CD274
CD278
CD279
CD284
CD304
CD324ecad
CD326 ep
CD335
CX3CR1
DLL1
DRSH9
ERMAP
FOLR3
GARP3
GATA3
hLYVE1
hMER
TIGIT
TLR2
TREM
VEFG
CHEMR23
GLUT
GLYCAN 3
HLA B24
IL2RB2

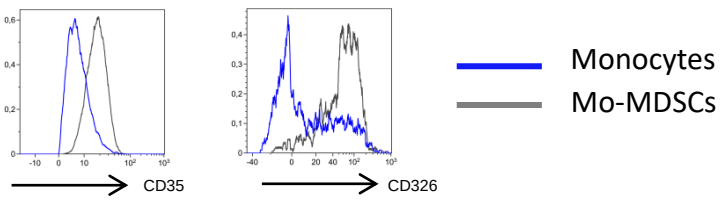
Figure 42| More than 100 cell surface antigens screened for differential expression in Mo-MDSC versus $CD14^+$ monocytes from patients with advanced melanoma.

We observed that CD35 (CR1) and CD326 (Ep-CAM) were more highly expressed in HLA-$DR^{-/low}$ Mo-MDSCs than HLA-DR^+ monocytes (Figure 43 b). We next examined the stability of CD35 and CD326 expression in glucocorticoid-treated monocytes from healthy volunteers. Expression of CD326 increased in a dose-dependent fashion in response to dexamethasone but CD35 expression was not affected (Figure 43c).

a



b



c

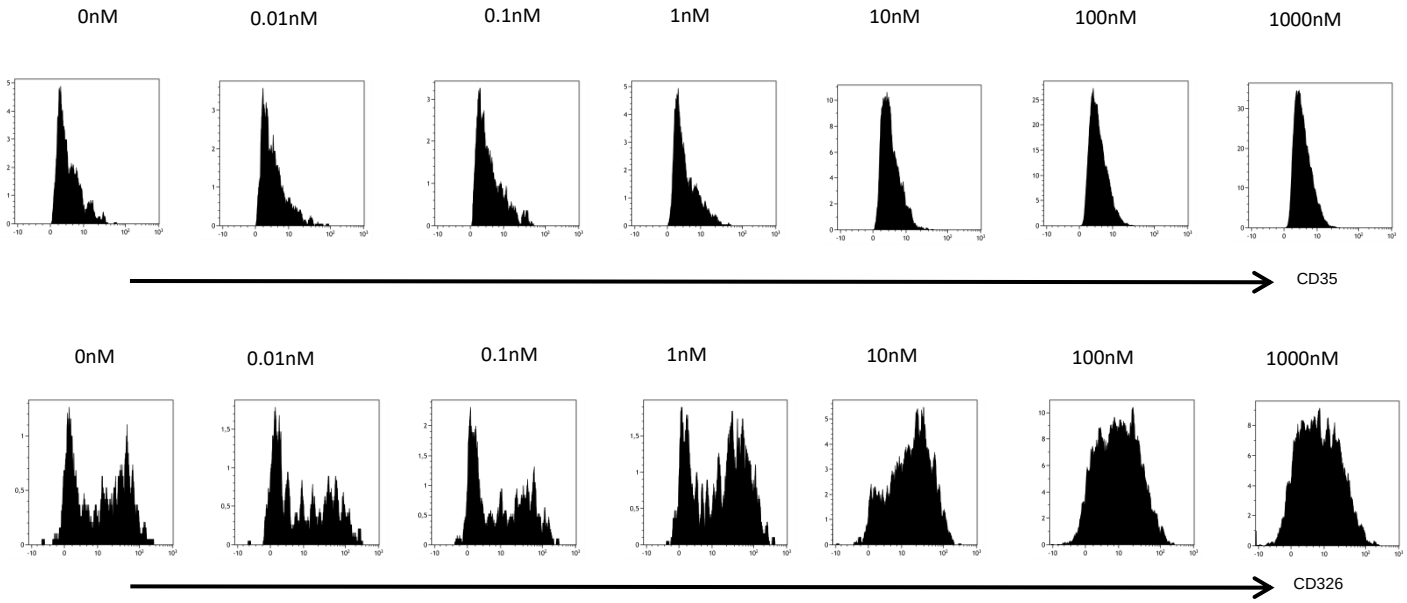


Figure 43| Mo-MDSCs markers unaffected by glucocorticoid exposure. **(a)** Isolated $CD14^+$ monocytes were stimulated in culture for 48 h with dexamethasone over a wide range of concentrations. HLA-DR expression disminished after dexamethasone treatment. **(b)** CD35 (CR1) and CD326 (Ep-CAM) were more highly expressed in HLA-$DR^{-/low}$ Mo-MDSC than HLA-DR^+ monocytes. **(c)** Stability of CD35 and CD326 expression in glucocorticoid-treated monocytes from healthy volunteers.

To test our new definition of human Mo-MDSCs based on CD35 expression, we isolated $CD35^{high}$ Mo-MDSCs cells obtained from healthy donors by FACS sorting. Sorted Mo-MDSCs $CD35^{high}$ were added at a 1:2 ratio to autologous CD3/CD28-stimulated CD4+ T cells. Results indicate that Mo-MDSCs $CD35^{high}$ cells were more suppressive in comparison with $CD35^{low}$ monocytes.

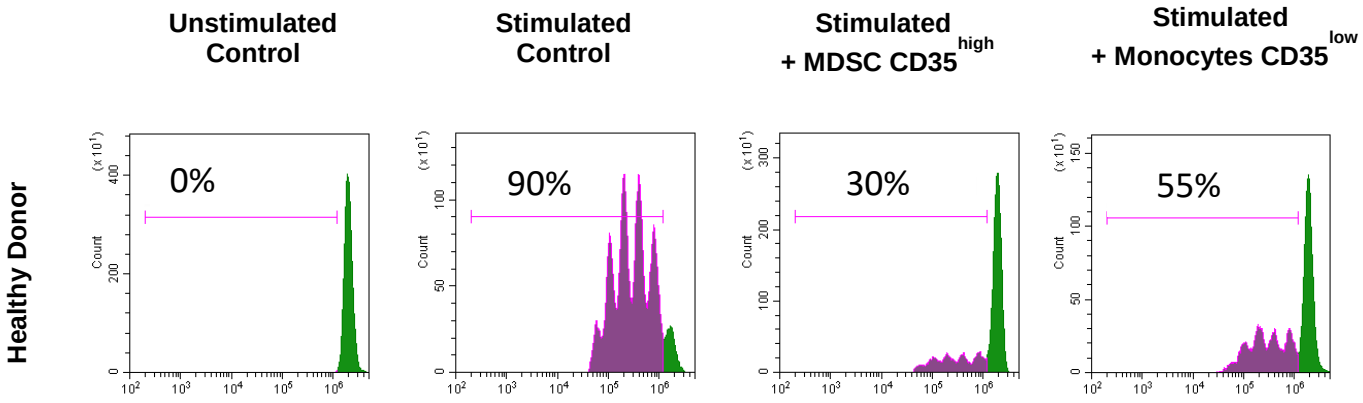


Figure 44| Suppressive function of MDSCs $CD35^{high}$. Sorted $CD4^+$ T cells were stained with CFSE and cultured under polyclonal activation alone or with autologous Mo-MDSCs $CD35^+$ or $CD35^-$ monocytes. Representative flow cytometry plots of one experiment from healthy donors are shown.

6 Discusion

MDSCs represent a varied group of myeloid regulatory cells that were originally studied in cancer (154,155). In transplantation the MDSCs are able to suppress adaptive and innate immune responses and they have been suggested as potential biomarkers for allograft tolerance as they can play a main role in the balance between graft acceptance and rejection(156,157). The earliest study of MDSCs in a kidney transplantation rat model indicated that MDSCs had no antigen-specific immunosuppressive function *in vivo* and *in vitro* (158). Results found by Dilek *et al.* showed that MDSCs contributed to establish a graft-to-periphery CCL5 gradient in tolerant kidney allograft recipients, which controlled the recruitment of Tregs to the graft (159). As most of the published studies were performed in animal models, there is a paucity of data addressing MDSCs features and their role in human transplantation.

Grützner *et al.* (160) analyzed the impact of short time storage of PBMC before analyses of MDSCs. Their results show the first 4 hours after blood sampling as ideal time point for analysis of Mo-MDSCs, but allowing processing of PMN-MDSCs within the same day. Our results show a reduction in cell viability increasing with time of culture compared to baseline. We also found a predominance of Mo-MDSCs at day one of culture, a decrease in the proportion of Mo-MDSCs cells from day 3 and most of cells become double negative with time of culture. These results suggest MDSCs subsets phenotype is affected with time of culture and the analisis of the phenotype could be biased after day 3 of culture.

To date, there is special interest on the effect of immunosuppressants on T cell subsets in transplantation (161) and understanding the effect of immunosuppressive drugs on MDSCs in transplantation might be important to develop strategies to promote tolerance. It has been previously reported that the number of splenic MDSCs decrease significantly in a dose-dependent manner by calcineurin inhibition (162). Acording to these data, our results showed that *in vitro* administration of imTOR maintains total MDSCs and Mo-MDSCs in culture, but high doses of tacrolimus negatively affect the number of total MDSCs and Mo-MDSCs.

When we monitorized MDSCs from peripheral blood in KTR, we found that absolute numbers of circulating total MDSCs were increased in KTR and in the short-term after transplantation, whereas they declined to baseline levels one year after transplantation. We also observed an increase in Mo-MDSCs frequencies in the short-term after transplantation and 1 year after transplantation. *Luan et at.,* found that the overall MDSCs frequencies were elevated at 3, 6 and 12 months post-transplant (163). *Utrero-Rico et al*., observed Mo-MDSCs cells counts rapidly increase after kidney transplantation and remain high one year after transplantation (164). Hock *et al*. showed that renal transplant recipients (RTR) had elevated frequencies of circulating MDSCs (129), but they further found MDSCs numbers had returned to normal levels 12 months post-transplantation (165) . However, in their previous study long-term KTR, elevated MDSCs numbers were detected in the majority of patients, suggesting that MDSCs expand in the long-term, as the graft acceptance progresses. These observational studies suggest that MDSCs numbers increase rapidly after transplantation and peak following

immunosuppressive therapy. Moreover, analysis of the changes in MDSCs obtained from donors, provided strong evidence that the changes occurring in RTRs were likely due to the immunosuppressive regimens rather than the acute inflammation from surgery itself (165). Although Mo-MDSCs phenotype seems to be influenced by standard immunosuppression, whether MDSCs subsets are differentially regulated by local conditions or treatments require further investigations.

Studies developed in mice suggest that MDSCs have an important role to induce Tregs after transplantation (166,167), but their role in human transplantation is under investigation. In KTR, *Luan et al.,* observed that $CD33^{+}$ $CD11b^{+}$ HLA-DR^{-} MDSC are capable of expanding Treg, and they correlate with Treg increases *in vivo* (163). Consistent with this view, *Meng et al*., (168) found that MDSCs isolated from transplant recipients were also able to expand regulatory T cells and were associated with longer allograft survival. Okano S *et al.* also found a positive correlation between MDSCs and Treg in intestinal transplant patients and we report here an increase in Treg expansion after Mo-MDSCs co-culture. However, there was no significant linear association between MDSCs and Treg percentages when we examined the relationship between MDSCs subsets and $CD4^{+}CD25^{+}Foxp3^{+}$ Treg *in vivo*.

Due to the lack of unique phenotypic markers functional studies have to be performed to identify MDSCs subsets (141). We tested the suppressive capacity of MDSCs from KTR under calcineurin (tacrolimus) or mTOR (rapamycin) inhibition after 360 days of immunosuppressant maintenance therapies. Our results demonstrate that MDSCs from healthy donors display marginal suppression of polyclonal T $CD4^{+}$ responses compared to

Mo-MDSCs from KTR under tacrolimus treatment, that exhibit potent suppressive function. Similarly, previous data showed that $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ myeloid cells from human KTR inhibit T cell proliferation but no inhibition was found when $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ cells were obtained from healthy donors (163). Moreover, we observed that Mo-MDSCs from KTR under tacrolimus treatment had increased suppressive activity compared to rapamycin and this immune inhibitory function may be related to the upregulation of iNOS (169).

Previous studies demonstrated that expression of FK binding protein FKBP in Mo-MDSCs and PMN-MDSCs from tumor-bearing mice is increased and regulates their suppressive function (170). On the other hand, rapamycin downregulates iNOS expression in MDSCs and the suppressive activity and MDSCs numbers are significantly reduced after rapamycin treatment in an allogeneic skin transplant model (171). Ribechini et al., showed that IDO protein expression and suppressor function of human suppressor monocytes was also impaired by rapamycin treatment (121). Our results are consistent with this hypothesis and we attribute loss of suppressive function to diminished IDO expression in rapamycin-exposed Mo-MDSCs.

However, other studies demonstrated that rapamycin prolongs cardiac allograft survival through the enhancement of MDSCs migration and suppressive activity (172). Chen X *et al.* showed that mTOR signaling is a negative determinant of MDSCs function in immune-mediated hepatic injury (IMH) diseases. In the context of IMH, the blocking of mTOR with rapamycin or mTOR-deficient $CD11b^{+}Gr1^{+}$ MDSCs mediated the protection against IMH (173). Another study addressing the murine MDSCs response to acute kidney injury demonstrated that MDSCs reduced the injury and

the effect was potentiated by MDSCs induction and enhancement of the immunosuppressive activity promoted by mTOR (174).

More recently, a previously unrecognized mechanistic pathway associated with organ rejection identifies the expression of mTOR by graft infiltrating macrophages at the center of epigenetic and metabolic changes that correlate with graft loss (175). This novel functional mechanism involves non-permanent reprogramming of macrophages and has been termed “trained immunity” (176). Therefore, it seems that, while mTOR inhibition may prevent trained immunity and inflammatory pathways in myeloid cells (177,178) it may also interfere with tolerogenic programing and the ability of myeloid cells to expand Treg and suppress T cell mediated immune responses. This dual effect of mTOR inhibition (immunogenic vs tolerogenic) and the resulting dominant pathway in vivo is likely to determine the outcome of the transplanted organ. Taken together, the effects of distinct immunosuppressive drugs on MDSCs development and function need to be better characterized in KTR.

The mechanisms by which MDSCs mediate Treg generation have not been well established. In some tumor-bearing animal models it has been demonstrated that the microenvironment modulates differentially myeloid cell development and function (179). Therefore, an important limitation in the study of MDSCs in KTR at this point is that *in vitro* assays performed in peripheral blood could not replicate the features of graft environment. Due to the number of patients included in this project and the short term follow-up, the impact of clinical events such as rejection on MDSCs can not be well established.

Although some studies about MDSCs in human organ transplantation have been reported and previously mentioned (163–165,168,180) this is the first study concerning the monitoring of MDSCs in human LTR. In the current project, MDSCs frequencies in 82 LTR were analysed at multiple time points over the first year after transplantation.

We found that percentages of total MDSCs were increased in LTR 3 months after transplantation up to a year. When we studied the effect of transplantation on MDSCs subsets in our cohort, Mo-MDSCs percentages increased promptly after transplantation and decreased gradually during the timecourse follow up. On the contrary, PMN-MDSCs percentages decrease in the short term after trasplantation, and increase during the follow up although no changes compared to pre-transplant levels were observed. Compared to pre-transplant levels, e-MDSCs percentages were significantly increased at 7 days, 21days and 360 days**.** We obtained similar results when we calculated MDSCs absolute numbers.

Our previous data indicate that Mo-MDSCs increase in KTR at 6 months and 12 months post-transplantation (181). Consistently with our results, *Luan et at.* reported that the overall MDSCs frequencies were elevated at 3, 6 and 12 months post-transplant (163). *Utrero-Rico et al*., observed Mo-MDSCs cells counts rapidly increase after kidney transplantation an remain high one year after transplantation(164). Hock *et al*. showed that renal transplant recipients (RTR) had elevated frequencies of circulating MDSCs (129), but they further found MDSC numbers had returned to normal levels 12 months post-transplantation (165). All of these observational studies suggest that MDSCs numbers increase rapidly after transplantation and peak following immunosuppressive therapy and such kinetics seems

to be equivalent in both KTR and LTR.

The release of neutrophils from the bone marrow in response to glucocorticoids is well established (182) and it has been reported that glucocorticoids can induce anti-inflammatory monocytes resembling MDSCs (183,184). In previous experiments from our group we observed a dose-dependent reduction in HLA-DR expression levels in monocytes after dexamethasone exposition, monocytes were phenotypically indistinguishable from Mo-MDSCs, then we hypothesize that corticosteroids are increasing Mo-MDSCs populations in peripheral blood immediately after transplantation. However, in our study, PMN-MDSCs and e-MDSCs that increase 3 months after transplantation, do not seem to be affected by corticosteroids and the increase suggests that MDSCs are not affected by the tacrolimus-based maintenance therapy.

In our previous experiments we tested the suppressive capacity of MDSCs from KTR under calcineurin (tacrolimus) or mTOR (rapamycin) inhibition at 360 days of immunosuppressant maintenance and we observed that tacrolimus, but not rapamycin treated KTR, was able to inhibit $CD4^+$ T cell proliferation *in vitro* (181). Calcineurin inhibitors are potent immunosuppressive drugs used in lung transplantation to block the immune response; hence, understanding their effect on MDSCs is important to develop strategies to promote tolerance in transplantation. Here we report the suppressive capacity of MDSC from Tacrolimus treated LTR is increased compared to the suppressive results when $CD14^+CD11b^+CD33^+HLA\text{-}DR^-$ cells were obtained from healthy donors. Heigl *et al*, characterized MDSCs in lung transplant recipients to assess if MDSCs can serve as a potential new research target in the field (185) .

They observed that G-MDSCs obtained from LTR were functionally suppressive and showed a modest correlation with increasing CNI trough levels, a previously reported phenomenon (169,186). Previous studies demonstrated that expression of FK binding protein FKBP in Mo-MDSCs and PMN-MDSCs from tumor-bearing mice is increased and regulates their suppressive function (170).

These results taken together indicate that MDSCs activity and numbers are modulated by immunossupressive treatments, such as CNI. In contrast, MDSCs percentages in our study were not related to immunosuppressant levels in peripheral blood. As our cohort of LTR was under the same immunosuppressive regimen, potential differences between treatments, with respect to their effect on MDSC frequency or function can not be determined.

When we evaluated the effect of MDSCs subsets on clinical events no effect of MDSCs subsets on short-term clinical events in LTR was observed. In contrast with previous reports (164,168), we observed that 90 and 180 days post-transplant Mo-MDSCs frequencies were higher after acute cellular rejection (ACR), suggesting a possible role for Mo-MDSCs in the development of chronic lung allograft dysfunction (CLAD). In the same way, reduced MDSCs numbers have been found in intestinal transplant recipients with acute cellular rejection (187).

Similarly to our previous results when Mo-MDSCs were treated with dexamethasone, *Okano et al.* reported on day 3 after MP-pulse treatment for ACR in an ITx (Intestinal Trasplant) patient, the number of MDSCs in PBMCs increased to 6 times the levels observed before treatment.

Interestingly, In a long term retrospective study, patients with more than 10-year functional, stable kidney grafts and low doses of immunosuppression, Mo-MDSCs were significantly superior than in short-term renal recipients, and Mo-MDSCs levels correlated with survival rates (188). Therefore, the use of glucocorticoid drugs to maintain immunosuppression and treat ACR may drive MDSCs accumulation in patients following transplantation and whether MDSCs subsets play a role as biomarkers of chronic rejection or not, remains unknown and require further investigations.

Hoffman et al. monitored HLA-DR expression weekly after transplantation in 13 pediatric lung transplant recipients (LRT) (189). Six out of seven patients who developed post-transplant pneumonia demonstrated lack of monocyte HLA-DR expression within the first two weeks of monitoring and those who developed pneumonia had lower monocyte HLA-DR expression over the four-week study period than those who remained infection-free. The authors propose that monitoring HLA-DR expression in monocytes may be useful to identify risk of infection and stratifying patients into higher and lower risk groups. *Alingrin et al.* assessed the influence of early post-operative sepsis on T cell and monocyte reconstitution in anti-thymocyte globulin (ATG)-treated lung transplant recipients. Peripheral blood T-lymphocytes counts and monocyte HLA-DR (mHLA-DR) expression within 60 days post-transplant were analyzed. The authors found that sepsis is negatively correlated with the HLA-DR expression in monocytes (190). These findings taken together highlight the importance of immunomonitoring after lung transplantation. *Deshane et al.* found high numbers of $CD11b^{+}$ $CD14^{+}$ $CD16^{-}$ HLA-DR^{-} NO-producing myeloid derived

regulatory cells, in the airways of patients with asthma but not in patients with chronic obstructive pulmonary disease (COPD) or in healthy control subjects (191). On the other hand *Scrimini et al.* observed elevated levels of circulating-lineage HLA-DR^{-} CD33^{+} CD11b^{+} MDSCs in patients with COPD (192). Other researchers demonstrated that CCR2^{+} M-MDSCs inhibit collagen degradation and promote lung fibrosis by producing transforming growth factor-β1 (TGF-β1) (193). The number of circulating activated MDSCs was found to be significantly increased in patients with pulmonary hypertension (PH) compared to control subjects, and was correlated with an increase in mean pulmonary artery pressure (194). Sharma et al., described the association of distinct MDSCs sub-populations with the lung microbiome in LTRs. Their results suggested a functional link between the local microbiome and MDSC phenotype, which may play a role in the pathogenesis of BOS (195).

As it was mentioned before, MDSCs markers are not exclusively expressed by them; assays of human MDSCs function are difficult to implement and it is unclear to what extent HLA-DR expression is influenced by standard immunosuppression. For this reasons, during my stay in the immunomonitoring laboratory (Hospital Klinikum, Regensburg, Germany) we implement new flow cytometry panels allowing to check a wide range of fuctional and phenotypic markers from peripheral blood.Human MDSCs in peripheral blood have been classified by the experts into three main subsets previously studied in this project: Mo-MDSCs, PMN-MDSCs (134) and a population lacking both differentiation surface markers classified as early-stage MDSCs. CD33 marker can be used instead of CD11b since very few CD15^{+} cells are CD11b^{-}.While Mo-MDSCs are CD33^{+}, PMN-MDSCs are

CD33^{dim} (135). We first designed a flow cytometry assay for rapid and reproducible detection of MDSCs subsets in small volumes of human peripheral blood. This assay relies on conventional phenotypic definitions of human Mo-MDSCs.

At the present time, the technique allowing for separation of neutrophils from PMN-MDSCs is Ficoll gradient regularly used for the isolation of mononuclear cells. Low-density fraction contains PMN-MDSCs and activated neutrophils. Therefore, CD11b^{+}CD14^{-}CD15^{+}/CD66^{+} cells in low-density fraction contain both PMN-MDSCs and neutrophils(134). As PMN-MDSCs share expression markers with mature neutrophils it is not clear whether they are different cell types whether one cell type that can functionally polarize depending on environmental conditions (196). Therefore, to appropriate characterize PMN-MDSCs, functional analysis of these cells is required. However, when we isolated PMN-MDSCs from KTR we were not able to demonstrate consistently their suppressive capacity *in vitro*.

Human MDSCs exert their T cell suppressive actions through a wide variety of mechanisms, including production of anti-inflammatory cytokines and up-regulation of immune-regulatory molecules, including arginase 1 (Arg1) and indoleamine 2,3-dioxygenase (IDO) (148). As it was previously shown in this proyect, rapamycin, abrogates the suppressive potential of Mo-MDSCs in vitro by preventing IDO induction. Therefore, we next developed a standardised assay to monitor phospho-mTOR, phospho-S6, IRF1 and IDO expression by human Mo-MDSCs in peripheral blood samples. Circulating Mo-MDSCs do not generally express detectable levels of IDO, but is expression is readily induced by proinflammatory

factors, including IFN-γ. The capacity of Mo-MDSCs to inducibly express IDO is a useful surrogate marker of their suppressive function. Therefore, we developed an assay for inducible IDO expression in Mo-MDSCs by flow cytometry.

Moreover conventional phenotyping of human Mo-MDSCs subsets mainly relies upon HLA-DR expression; however, it is unclear to what extent HLA-DR expression is influenced by standard immunosuppression, especially glucocorticoids. The release of neutrophils from the bone marrow in response to glucocorticoids is well established (183) and it has been reported that glucocorticoids can induce anti-inflammatory monocytes resembling MDSCs (184,197). In culture, we observed a rapid, dose-dependent reduction in HLA-DR expression levels in monocytes after dexamethasone exposition, monocytes were phenotypically indistinguishable from Mo-MDSCs. To identify Mo-MDSCs markers unaffected by glucocorticoid exposure, we screened cell surface markers for differential expression in Mo-MDSCs versus $CD14^{+}$ monocytes and we observed that CD35 (CR1) and CD326 (Ep-CAM) were more highly expressed in HLA-$DR^{-/low}$ Mo-MDSCs than in HLA-DR^{+} monocytes and CD35 expression was not affected by dexamethasone. CD35 is a receptor for complement components C3b and C4b. We previously identified Complement 5a Receptor 1 (C5aR1; CD88) as a marker of non-classical human monocyte subpopulation that is a likely precursor of allograft-infiltrating regulatory macrophages (Mregs). Expression of C5aR1 and CCR2 (CD192) appear to be counter-regulated in human monocytes, which is consistent with transplant experiments in mice that show C5aR1 instead of CCR2 controls migration of Mreg-precursors into allografts. Therefore,

we designed a new 10-colour flow cytometry panel incorporating CD35, CD88, CD192 and CD326.

To check our new definition of human Mo-MDSCs based on CD35 expression, we performed suppression assays and we observed that Mo-MDSCs CD35^{high} cells were more suppressive in comparison with CD35^{low} monocytes. In spite of this, these isolation essays still rely in the marker HLA-DR to isolate Mo-MDSC, then the new 4 proposed panels taken together could help in identifying MDSCs subpopulations, phenotypically and functionally, but CD35 alone is not specific enough to identify Mo-MDSCs.

7 Conclusions

The conclusions obtained from this work were the following:

1. There is an increase in Mo-MDSCs frequencies 6 months and 1 year after kidney transplantation.
2. There is and expansion of Treg after Mo-MDSCs co-culture.
3. Mo-MDSCs from KTR under tacrolimus treatment have increased suppressive activity compared to rapamycin.
4. Loss of suppressive function in rapamycin-exposed Mo-MDSCs is related to diminished IDO expression.
5. Percentages of total MDSCs are increased in LTR 3 months after transplantation up to a year.
6. Mo-MDSCs percentages increased promptly after lung transplantation and decreased gradually during the timecourse follow up.
7. Mo-MDSCs subsets from tacrolimus treated LTR are able to efectively suppress T cell proliferation.
8. Mo-MDSCs frequencies increase after acute cellular rejection (ACR) in LTR.
9. HLA-DR expression in monocytes show a dose-dependent reduction after dexamethasone exposition, then monocytes are phenotypically indistinguishable from Mo-MDSCs.
10. CD35 (CR1) and CD326 (Ep-CAM) were more highly expressed in HLA-$DR^{-/low}$ Mo-MDSC than in HLA-DR^{+} monocytes.
11. CD35 expression was not affected by dexamethasone.
12. Mo-MDSCs $CD35^{high}$ cells were more suppressive in comparison with $CD35^{low}$ monocytes.

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9 Annex

frontiers in Immunology

ORIGINAL RESEARCH
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Myeloid-Derived Suppressor Cells in Kidney Transplant Recipients and the Effect of Maintenance Immunotherapy

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Myeloid-derived suppressor cells (MDSC) represent a heterogeneous group of myeloid regulatory cells that were originally described in cancer. Several studies in animal models point to MDSC as important players in the induction of allograft tolerance due to their immune modulatory function. Most of the published studies have been performed in animal models, and the data addressing MDSCs in human organ transplantation are scarce. We evaluated the phenotype and function of different MDSCs subsets in 38 kidney transplant recipients (KTRs) at different time points. Our data indicate that monocytic MDSCs (Mo-MDSC) increase in KTR at 6 and 12 months posttransplantation. On the contrary, the percentages of polymorphonuclear MDSC (PMN-MDSC) and early-stage MDSC (e-MDSC) are not significantly increased. We evaluated the immunosuppressive activity of Mo-MDSC in KTR and confirmed their ability to increase regulatory T cells (Treg) *in vitro*. Interestingly, when we compared the ability of Mo-MDSC to suppress T cell proliferation, we observed that tacrolimus, but not rapamycin-treated KTR, was able to inhibit $CD4^+$ T cell proliferation *in vitro*. This indicates that, although mTOR inhibitors are widely regarded as supportive of regulatory responses, rapamycin may impair Mo-MDSC function, and suggests that the choice of immunosuppressive therapy may determine the tolerogenic pathway and participating immune cells that promote organ transplant acceptance in KTR.

Keywords: kidney transplantation, mTOR inhibition, myeloid-derived suppressor cells, tacrolimus, immunosuppression

INTRODUCTION

Kidney transplantation is a treatment option for patients with end-stage renal disease (ESRD). Although immunosuppressive protocols have clearly reduced the incidence of acute rejection, transplant patients continue at high risk of treatment side effects, and long-term allograft survival has not improved significantly (1). As a consequence, the main goals in transplantation are to predict the risk of developing rejection and to find biomarkers of tolerance to allow immunosuppression withdrawal in order to minimize the adverse effects of the currently available immunosuppressive regimens.

An increasing field of research is focused on the study of immune cells with regulatory and/or suppressive function. Among them, myeloid-derived suppressor cells (MDSCs) have gained attention in the last years. The MDSCs are a heterogeneous group of myeloid cells able to suppress adaptive and innate immune responses and have been suggested as potential biomarkers for allograft tolerance. They were initially described in cancer, and several studies have pointed out MDSC to play an important role in the regulation of immune responses in other clinical setting, such as organ transplantation, infection, and autoimmune diseases (2–4).

Myeloid-derived suppressor cells were first described in mice as $CD11b^+$ $Gr1^+$ cells, and experimental transplant models demonstrated that MDSCs have an important role in the induction of tolerance (5). On the contrary, evidence on their role in human transplantation is scarce and non-conclusive. In renal transplant patients, Luan et al. observed MDSC, defined as $CD33^+$ $CD11b^+HLA\text{-}DR^-$, able to expand T regulatory cells (Treg) *in vitro* and correlate with Treg cell numbers *in vivo* (6). These data were confirmed by Meng et al. who associated MDSC numbers with less tissue injury and longer allograft survival (7). Human MDSCs are divided into three main subsets: monocytic MDSC (Mo-MDSCs: $CD33^+CD11b^+CD14^+HLA\text{-}DR^-$), polymorphonuclear MDSC (PMN-MDSCs: $CD33^+CD11b^+CD15^+HLA\text{-}DR^-$), and a population lacking both differentiation surface markers classified as early-stage MDSC (e-MDSCs: $CD33^+HLA\text{-}DR^-CD15^-CD14^-$) (8). Since these phenotypic markers are not exclusive of MDSCs and they are present in other myeloid cells such as monocytes, macrophages, and granulocytes, MDSC cells are further defined upon demonstration of their suppressive function (9).

Due to the paucity of the MDSC data in clinical organ transplantation and that different immunosuppressants may have a distinct effect on MDSC, we monitored circulating MDSC subset frequencies in kidney transplant recipients (KTRs). The main goal of the study was to compare transplant recipients receiving standard triple therapy to those maintained on a regimen including rapamycin and evaluate the effect of each therapeutic arm on MDSC in relation to kidney transplant outcomes.

Abbreviations: 7AAD, 7-amino-actinomycin D; CNI, calcineurin inhibitors; e-MDSC, early-stage MDSCs; HC, healthy controls; KTRs, kidney transplant recipients; MDSCs, myeloid-derived suppressor cells; Mo-MDSCs, monocytic MDSCs; mTOR, mammalian target of rapamycin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMN-MDSCs, polymorphonuclear MDSCs.

MATERIALS AND METHODS

Study Design

A total of 38 consecutive KTRs were enrolled in the study after giving consent while they were listed for kidney transplantation in the Hospital Universitario Marqués de Valdecilla in 2016. The study was approved by the Hospital Universitario Marqués de Valdecilla Ethics Committee. The mean follow-up time was 459 days. The clinical and immunological features of the KTR are summarized in **Table 1**. Clinical data were collected from patient records, and blood was drawn at baseline/day 0, 180, and 360 days after transplantation. The clinical and immunological features of the KTR are summarized in **Table 1**.

Monoclonal Antibodies and Flow Cytometry Analysis

The PBMCs or isolated MDSCs were stained with the following monoclonal antibodies: anti-CD33-APC (clone D3HL60.251), anti-CD3-FITC (clone UCHT1), anti-CD14-ECD (clone RMO52), and anti-CD11b-PE-cyanin (clone Bear1) (Beckman Coulter, Marseille, France); anti-CD16-APC-Cy7 (clone 3G8) and anti-CD56-FITC (clone HCD56 and anti-HLA-DR-Brilliant Violet 510 (clone L243) (Biolegend, San Diego, CA, United States); anti-CD19-FITC (clone 4G7), anti-CD14-FITC (clone MφP9), anti-CD25-PE (clone 2A3), and anti-FoxP3-Pacific Blue (clone 206D) (BD Biosciences); anti-CD15-CF Blue (clone MCS-1) (Inmunostep, Salamanca, Spain); and anti-CD4-APC-Vio770 (clone REA623) from Miltenyi Biotech. The cells were incubated for 20 min, washed with phosphate-buffered saline (PBS), and acquired in a Cytoflex® flow cytometer (Beckman Coulter). MDSCs were quantified by flow cytometry following the gating strategy proposed by Bronte et al. (8) to characterize MDSC subsets: Mo-MDSCs ($CD33^+CD11b^+HLADR^-$ $CD14^+$ $CD15^-$), PMN-MDSC ($CD33^+CD11b^+HLADR^-$ $CD15^+$ $CD14^-$), and e-MDSC Lin^- ($CD14^+CD56^+CD3^+CD19^+$) $CD33^+CD11b^+HLADR^-$ $CD14^-CD15^-$. Total MDSCs were defined as $CD33^+CD11b^+HLADR^-$ cells. Fluorescence minus one control was used to identify $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ cells.

Isolation and Sorting of MDSC

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy donors and from KTR by Ficoll density gradient centrifugation. To isolate $CD33^+$ $HLA\text{-}DR^-$ and $CD33^+$ $HLA\text{-}DR^-$ $CD14^+$ cells (Mo-MDSC), the $CD33^+$ cells were first sorted by magnetic-automated cell sorting using CD33-positive separation microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Further isolation of $CD33^+HLA\text{-}DR^-$ cells and $CD33^+HLA\text{-}DR^-$ $CD14^+$ was performed by sorting enriched cells on a FACS-ARIA II (BD Biosciences, San Jose, CA, United States). The purity of the cell sorting was tested after

TABLE 1 | Main features of study population (*N* = 38).

Recipients: Age, mean, years	51.88 (SD13.23)
Donors: Age, mean, years	49.61 (SD12.63)
Healthy controls: Age, mean, years	46.17 (SD11.85)
Recipient Sex (% female)	18 (47.37%)
Donor sex (% female)	19 (50%)
Dialysis post kidney transplant	10 (26%)
Preexisting anti-HLA antibodies	13 (34.21%)
Class I antibodies	10 (26%)
Class II antibodies	8 (21.05%)
Rejection	6 (15.78%)
RT	11 (28.94%)
Induction treatment	
None	21 (55.26%)
ATG	12 (31.57%)
Basiliximab	5 (13.15%)
Both	0 (0.00%)
Immunosupressive protocol	
Calcineurin inhibitor	33 (86.84%)
mTOR inhibitor	0 (0.00%)
Both	5 (13.15%)
ABDR mismatches	
>3	24 (63.15%)
=3	14 (36.84%)
Class II mismatches	
0	8 (21.05%)
1	17 (44.73%)
2	13 (34.2%)
Renal disease	
Glomerular	11 (28.94%)
Others	1 (2.63%)
Congenital	7 (18.42%)
Sistemic	10 (26.31%)
Vascular	2 (5.26%)
Interstitial	5 (13.15%)
Unknown	2 (5.26%)
Peripheral blood creatinine	
Cr 7 days post trasplant	2.28 (SD1.70)
Cr 30 days post transplant	1.90 (SD1.39)
Cr 120 days post transplant	1.40 (SD0.45)
Cr 180 days post transplant	1.40 (SD0.48)

SD, standard deviation; ESRD, end stage renal disease; 1stT, first transplant; RT, retransplant patients.

each experiment, and >98% efficiency was considered acceptable for the study. The experimental conditions were replicated at least four times.

Whole Blood Cultures

Whole blood culture was performed as follows: fresh blood anticoagulated with lithium-heparin was diluted 1:4 in Gibco^{TM} DMEMF/12 GlutaMAX^{TM} supplement medium (Thermo Fisher Scientific) containing 100 U/ml penicillin (Lonza) and 100 mg/ml streptomycin (Lonza). Cells were stimulated throughout the cultures with 5 ng/ml recombinant human monocyte colony stimulating factor (rhM-CSF; R&D Systems, Wiesbaden-Nordenstadt). For some experiments, human CD14$^+$ monocytes were isolated from Ficoll density gradient centrifugation of PBMC followed by positive selection using anti-CD14 microbeads (Miltenyi, Bergisch-Gladbach, Germany). Isolated CD14$^+$ monocytes were stained with Cell Tracker^{TM} Green CMFDA Dye (Thermo Fisher Scientific) at 2 nM and then added back into whole blood cultures at 10^5 cells/tube (Falcon® 5 ml round bottom polystyrene test tube) diluted 1/4 in Gibco^{TM} DMEMF/12 GlutaMAX^{TM} supplement medium (Thermo Fisher Scientific) and supplemented with 100 U/ml penicillin (Lonza), 100 mg/ml streptomycin (Lonza), and rhM-CSF (R&D Systems, Wiesbaden-Nordenstadt) at 5 ng/ml carried on 0.1% human albumin. Purity of sorted cells was tested after isolation, and >95% efficiency was considered acceptable for the study. Cells were collected, and location was analyzed at baseline and 1 and 2 days after culture.

In vitro Evaluation of MDSC Suppressor Function

CD4$^+$ T cells were isolated from healthy donors or KTR PBMC by immunomagnetic depletion using EasySep^{TM} Human CD4$^+$ naive T Cell Isolation Kit (Stemcell Technologies, Grenoble, France) and incubated with carboxyfluorescein succinimidyl ester (CFSE). The CFSE-labeled T CD4$^+$ cells (5×10^5) were stimulated with Dynabeads human T-activator CD3/CD28 (Life Technologies AS, Oslo, Norway) in U-bottomed 96-well plates with complete Roswell Park Memorial Institute (RPMI) media supplemented with 10% human AB + serum. Proliferation was determined using flow cytometry. Autologous Mo-MDSCs were added to the culture at 1:2 ratio (CD4$^+$ T cells: MDSCs), and proliferation was determined at day 5. Proliferation assays from blood donors were performed five times. These same functional assays were also carried out with MDSC from four renal transplant receptors: four patients under calcineurin inhibitor (tacrolimus) and four patients under mTOR inhibitor treatment (rapamycin) with at least 24 months of IS treatment.

In vitro Expansion of Treg Generation

peripheral blood mononuclear cells were obtained from KTR under maintenance immunosuppression with tacrolimus. CD4$^+$ T cells were sorted from the PBMC as described above. CD4$^+$ T cells (5×10^5) were polyclonally stimulated and cultured with CD33$^+$HLA-DR$^-$CD14$^+$ (Mo-MDSC) at different concentrations. Treg generation was determined at day 5 by staining with the monoclonal antibodies indicated above and flow cytometry analysis.

Western Blot

Gel electrophoresis and immunoblotting were performed as described elsewhere (10).

Statistical Analysis

Non-parametric Mann–Whitney *U* test and Student's *t*-test were used to compare two groups, as appropriate. More than

two groups were compared using the parametric analysis of variance (ANOVA), the non-parametric Kruskal–Wallis (not matching), or Friedman (repeated measures) test. Comparisons between two paired groups were performed using the Student's *t*-test for paired data or the Wilcoxon signed-rank test when data were or not normally distributed, respectively. Multiple comparisons were assessed using Dunn or Tukey's tests. Statistical analyses were performed using GraphPad software version 6.01 (GraphPad Inc., San Diego, CA, United States). To examine the relationship between bivariate variables, the Pearson correlation was calculated using SPSS Statistics version 24.

RESULTS

Monitoring MDSC in Kidney Transplant Patients

We hypothesized that MDSC subset frequencies might serve as useful biomarkers of clinical outcome after kidney transplantation. Therefore, we first quantified Mo-MDSC, PMN-MDSCs, and e-MDSC in peripheral blood from KTRs at 0, 180, and 360 days after transplantation. We found an increase in total $CD33^{+}HDL\text{-}DR^{lo}$ MDSC frequency at 180 days after transplantation [median, 11.5%; interquartile range (IQR), 6.2–17.0%] (**Figures 1B**, **2A**) in comparison with patients on the day of transplantation (median, 8.8%; IQR, 5.0–16.4%) (**Figures 1A**, **2A**). MDSC frequency at 360 days posttransplant was also increased but not significantly (median, 11.2%; IQR, 4.9–17.8%; **Figures 1C**, **2A**). Next, we examined changes in MDSC subset distribution after transplantation (**Figure 2** and **Supplementary Figures S1, S2**). Mo-MDSC frequencies were significantly increased at 180 and 360 days posttransplant (median, 22.71%; IQR, 6.75–57.56% and median, 25.48%; IQR, 8.85–56.58%) in comparison to patients on the day of transplantation (median, 10.56%; IQR, 3.18–37.55%) (**Figures 1A–C**, **2B**). PMN-MDSC and e-MDSC frequencies were lower at 180 days after transplantation (median, 41.71%; IQR, 12.67–62.79% and median, 5.5%; IQR, 1.9–10.87%) compared to patients on the day of transplantation (median, 54.6%; IQR, 29.4–84.95% and median, 6.15%; IQR, 3.9–13.5%), and they remained lower 360 days posttransplantation (median, 43.14%; IQR, 10.28–63.02% and median, 4.09%; IQR, 2.11–8.2%) (**Figures 1A–C**, **2C,D**). Despite these changes, we did not find any association between the MDSC subsets, and the clinical data are summarized in **Table 1** for patients included in the present work. Importantly, all the KTRs were receiving tacrolimus (**Table 1**) as main immunosuppressant during the first 360 days after transplantation shown.

MDSC From Transplant Patients Induce the Production of Tregs *in vitro*

Treg expansion is one of the main mechanisms by which MDSCs exert suppressive function (11, 12). Hence, we evaluated the capacity of Mo-MDSC from healthy donors and KTR to boost Tregs *in vitro*. We observed a significant increase in the frequency of Tregs recovered from the culture when $CD4^{+}$ T cells were stimulated with Mo-MDSC from cells from KTR at 360 days after transplantation, confirming their suppressive function (**Figure 3**).

MDSC From Tacrolimus Treated KTR Effectively Suppress T Cell Proliferation *in vitro*

The T-cell-suppressive capacity of Mo-MDSC from healthy controls, tacrolimus, and rapamycin-treated KTR was compared using an *in vitro* assay of polyclonally activated T cell proliferation. Sorted Mo-MDSC were added at a 1:2 ratio to autologous CD3/CD28-stimulated $CD4^{+}$ T cells. Four patients under long-term tacrolimus treatment and four patients under long-term rapamycin maintenance therapy were analyzed (**Figure 4**). Results indicate that Mo-MDSC obtained from tacrolimus treated KTR were significantly suppressive in comparison with rapamycin treated KTR. This suggests that Mo-MDSC from transplant patients exhibit different suppressive function *in vitro*, according to the immunosuppressive therapy that KTRs receive.

Rapamycin Inhibits the Function of *in vitro* Generated Myeloid Suppressor Cells

Following-up our observation of Mo-MDSC obtained from rapamycin-treated KTRs, we next investigated the effect of rapamycin on myeloid suppressor cells that were generated *in vitro* from whole blood cultures. First, we developed a flow cytometry panel that allowed us to reliably detect Mo-MDSC from human whole blood cultures according to their $CD45^{+}$ $CD33^{+}$ Lin^{-} $HLA\text{-}DR^{lo}$ $CD14^{+}$ $CD15^{-}$ phenotype (**Figure 5A**). Using whole blood cultures, we next investigated whether CSF1-stimulated human monocytes acquire a Mo-MDSC phenotype ($CD33^{+}$ Lin^{-} $HLA\text{-}DR^{lo}$ $CD14^{+}$ $CD15^{-}$) *in vitro*. When cultured for 48 h, we observed an increase in Mo-MDSC frequency in whole blood cultures from healthy donors (**Figure 5B**). Next, we investigated the effect of rapamycin on Mo-MDSC in whole blood cultures and observed that rapamycin led to accumulation of $HLA\text{-}DR^{lo}$ $CD14^{+}$ Mo-MDSC over 48 h (**Figure 5C**). This suggests that mTOR inhibition promotes Mo-MDSC development. Surprisingly, we found that rapamycin exposure substantially reduced the T-cell-suppressive capacity of Mo-MDSC (**Figure 5D**). It has been previously shown that T cell suppression by human-monocyte-derived Mo-MDSC is in part mediated by the expression of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) (13). Our results confirm that rapamycin blocked the expression of IDO (**Figure 5E**), suggesting that the suppressive effect of Mo-MDSC from rapamycin-treated KTR may be compromised due to the impaired expression of IDO.

DISCUSSION

Myeloid-derived suppressor cells represent a varied group of myeloid regulatory cells that were originally studied in cancer

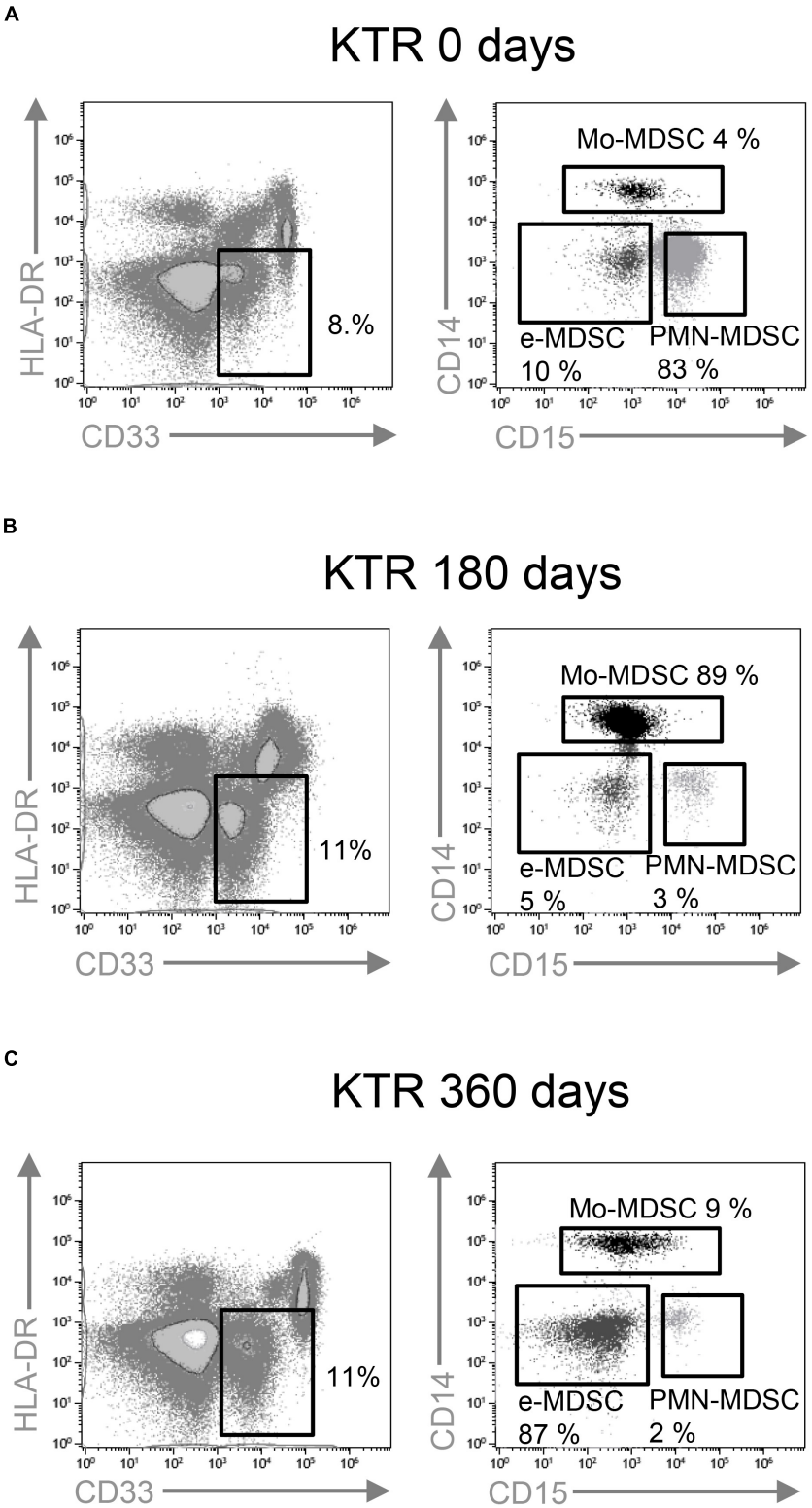

FIGURE 1 | Characterization of myeloid-derived suppressor cell (MDSC) subsets by flow cytometry. $CD33^{+}$ $HLA\text{-}DR^{-}$ myeloid cells were selected from live cells after doublets and debris exclusion. To define monocytic (Mo-MDSC), early-stage (e-MDSC), and polymorphonuclear (PMN-MDSC) MDSC, the CD14 and CD15 expression was analyzed on cells selected from $CD33^{+}HLA\text{-}DR^{-}$ MDSC. Representative flow cytometry data of MDSC from **(A)** patients on the day of transplantation (day 0), **(B)** kidney transplant recipients on day 180, and **(C)** day 360 posttransplantation is shown.

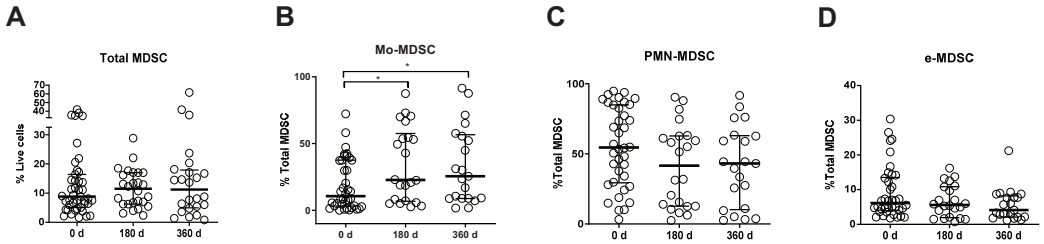

FIGURE 2 | Myeloid-derived suppressor cell (MDSC) frequencies in kidney transplant recipients (KTRs). **(A)** Frequencies of total myeloid-derived suppressor cells (MDSC) in peripheral blood mononuclear cells (PBMC); **(B)** monocytic MDSC (Mo-MDSC); **(C)** early-stage MDSC (eMDSC); and **(D)** polymorphonuclear MDSC (PMN-MDSC) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney *U* test. (*$p < 0.05$).

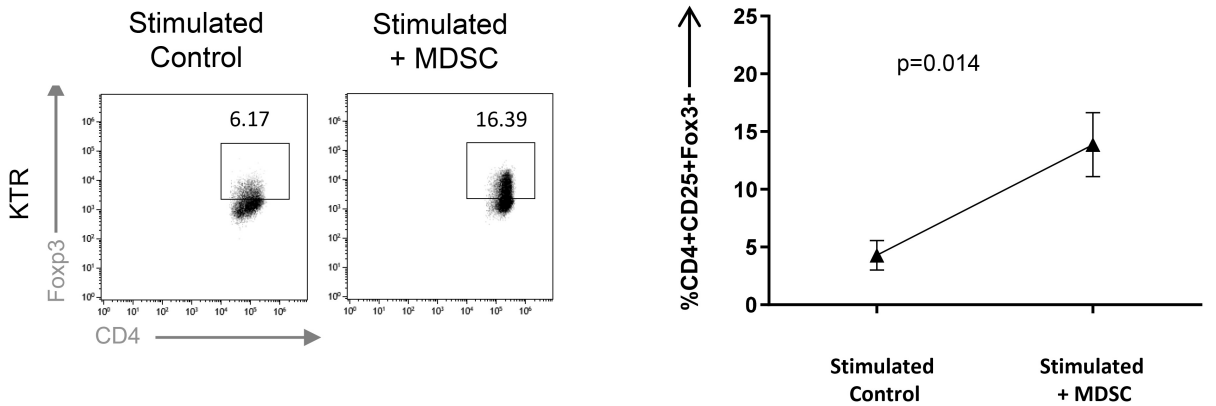

FIGURE 3 | Monocytic myeloid-derived suppressor cell (Mo-MDSC) from kidney transplant recipients (KTRs) expand Treg *in vitro*. MDSC dependent $CD4^+FoxP3^+$ Treg expansion was analyzed by flow cytometry. Naive $CD4^+$ T cells cocultured under polyclonal activation with autologous Mo-MDSC obtained KTR at day 360 are shown ($n = 3$, unpaired *t*-test).

(14). Several studies demonstrating their immunoregulatory action in animal models point to a potential role of MDSC in the induction of tolerance after transplantation (2). As most of the published studies were performed in animal models, there is a paucity of data addressing MDSC features and their role in human transplantation. We found that absolute numbers of circulating total MDSC were increased in KTR and in the short term after transplantation, whereas they declined to baseline levels 1 year after transplantation. We also observed an increase in Mo-MDSCs frequencies in the short term after transplantation and 1 year after transplantation. Luan et al. found that peripheral blood MDSCs were increased in KTR (6). Hock et al. also reported that renal transplant recipients had elevated frequencies of circulating MDSC (15), but they further found that MDSC numbers had returned to normal levels 12 months posttransplantation (16). However, in their previous study, long-term KTR had increased MDSC numbers, suggesting that MDSC recover and even expand in the long term, as graft acceptance progresses. These observational studies suggest that MDSC numbers increased rapidly and peaked following immunosuppressive therapy. Whether these increases are the result of potential differences between the two immunosuppressive regimens used (tacrolimus and mTOR inhibitors) or whether MDSC subsets are differentially regulated by local conditions or treatments is still a matter of debate.

Studies developed in mice suggest that MDSCs have an important role to induce T regulatory cells (Treg) after transplant (11, 12), but their role in human transplantation is still unclear. In KTR, Luan et al. observed that $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ MDSC are capable of expanding Treg, and they correlate with Treg increases *in vivo* (6). Consistent with this view, Meng et al. (7) found that MDSCs isolated from transplant recipients were also able to expand regulatory T cells and were associated with longer allograft survival. Okano S. et al. also found a positive correlation between MDSC and Treg in intestinal transplant patients (17), and we report here an increase in Treg expansion after Mo-MDSC coculture. However, there was no significant linear association between MDSC absolute numbers and percentage Treg when we examined the relationship between total MDSC subsets and $CD4^+CD25^+Foxp3^+$ Treg *in vivo*.

Myeloid cell surface markers define potential MDSC, but the lack of unique phenotypic markers obliges to perform functional studies to identify MDSC subsets. We tested the suppressive capacity of MDSCs from KTR under calcineurin (tacrolimus) or mTOR (rapamycin) inhibition at 360 days of immunosuppressant maintenance therapies. Our results demonstrate that MDSC from healthy donors display marginal

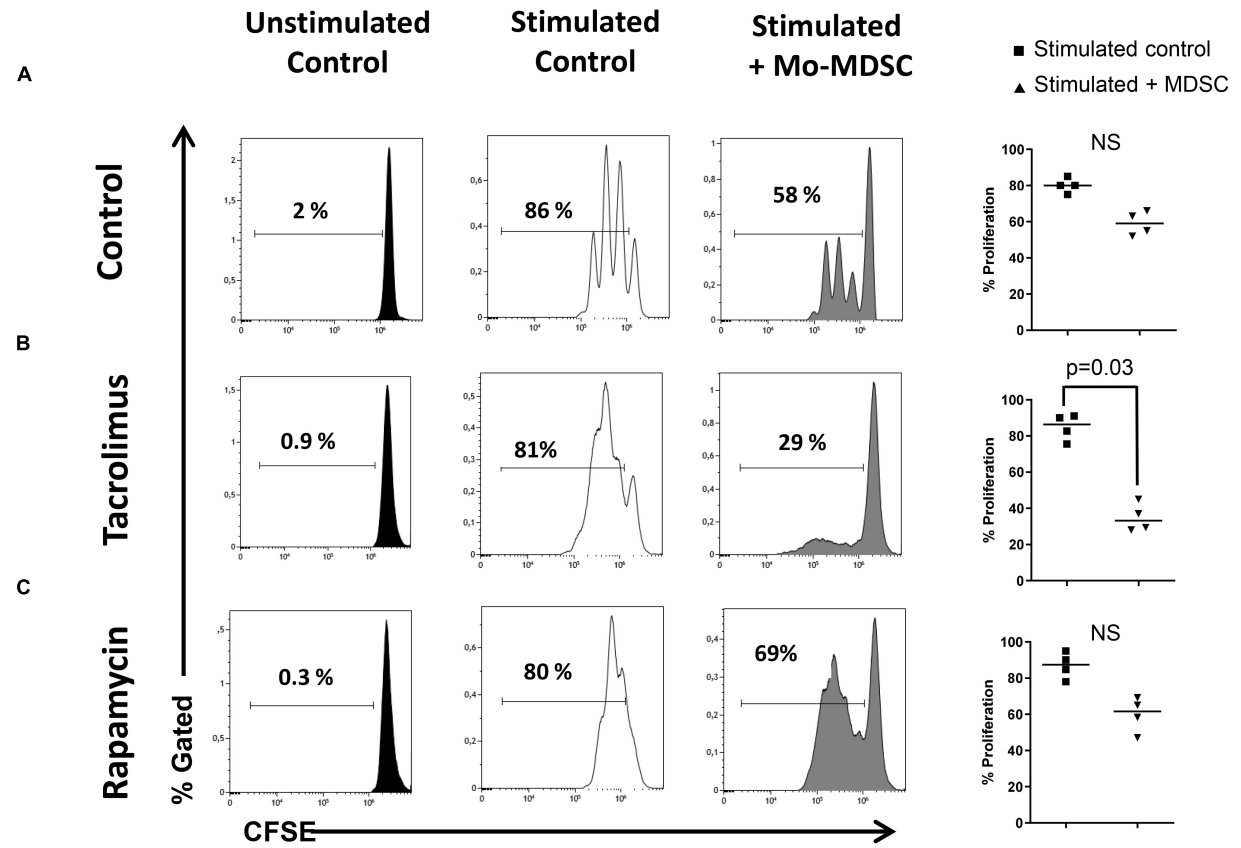

FIGURE 4 | Suppressive function of myeloid-derived suppressor cells (MDSCs). Sorted $CD4^+$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and cultured under polyclonal activation alone or with autologous monocytic myeloid-derived suppressor cells (Mo-MDSCs). Representative flow cytometry plots of four independent experiments with Mo-MDSCs from healthy volunteers; kidney transplant patients under tacrolimus treatment and rapamycin are shown. Individual data of experiments are displayed in the right plot graphs where stimulated control cells are represented as black squares and stimulated cells + Mo-MDSC are represented as black triangles. Differences between groups were assessed by Mann–Whitney test and only indicated if differences were significant.

suppression of polyclonal T $CD4^+$ responses. In contrast, Mo-MDSCs from KTR exhibit potent suppressive function. The results are consistent with previous data demonstrating that $CD11b^+CD33^+HLA\text{-}DR^-$ myeloid cells from human KTR inhibit T cell proliferation, but they found no inhibition when $CD11b^+CD33^+HLA\text{-}DR^-$ cells were obtained from healthy donors (6). Moreover, we observed that Mo-MDSC from KTR under tacrolimus treatment had increased suppressive activity compared to rapamycin, and this immune inhibitory function may be related to the upregulation of inducible nitric oxide synthase (iNOS) (18).

On the other hand, rapamycin downregulates iNOS expression in MDSC, and the suppressive activity and MDSC numbers are significantly reduced after rapamycin treatment in an allogeneic skin transplant model (19). Our results are consistent with this hypothesis, and we attribute loss of suppressive function to diminished IDO expression in rapamycin-exposed Mo-MDSC. However, other studies demonstrated that rapamycin prolongs cardiac allograft survival through the enhancement of MDSC migration and suppressive activity (20). Chen X. et al. showed that mTOR signaling is a negative determinant of MDSC function in immune-mediated hepatic injury (IMH) diseases. In the context of IMH, the blocking of mTOR with rapamycin or mTOR-deficient $CD11b^+Gr1^+$ MDSC mediated the protection against IMH (21). Another study addressing the murine MDSC response to acute kidney injury demonstrated that MDSC reduced the injury, and the effect was potentiated by MDSC induction and enhancement of the immunosuppressive activity promoted by mTOR (22). More recently, a previously unrecognized mechanistic pathway associated with organ rejection identifies the expression of mTOR by graft infiltrating macrophages at the center of epigenetic and metabolic changes that correlate with graft loss (23). This novel functional mechanism involves non-permanent reprogramming of macrophages and has been termed "trained immunity" (24). Therefore, it seems that, while mTOR inhibition may prevent trained immunity and inflammatory pathways in myeloid cells (25, 26), it may also interfere with tolerogenic programming and the ability of myeloid cells to expand Treg and suppress T-cell-mediated immune responses. This dual effect of mTOR inhibition (immunogenic vs. tolerogenic) and the resulting

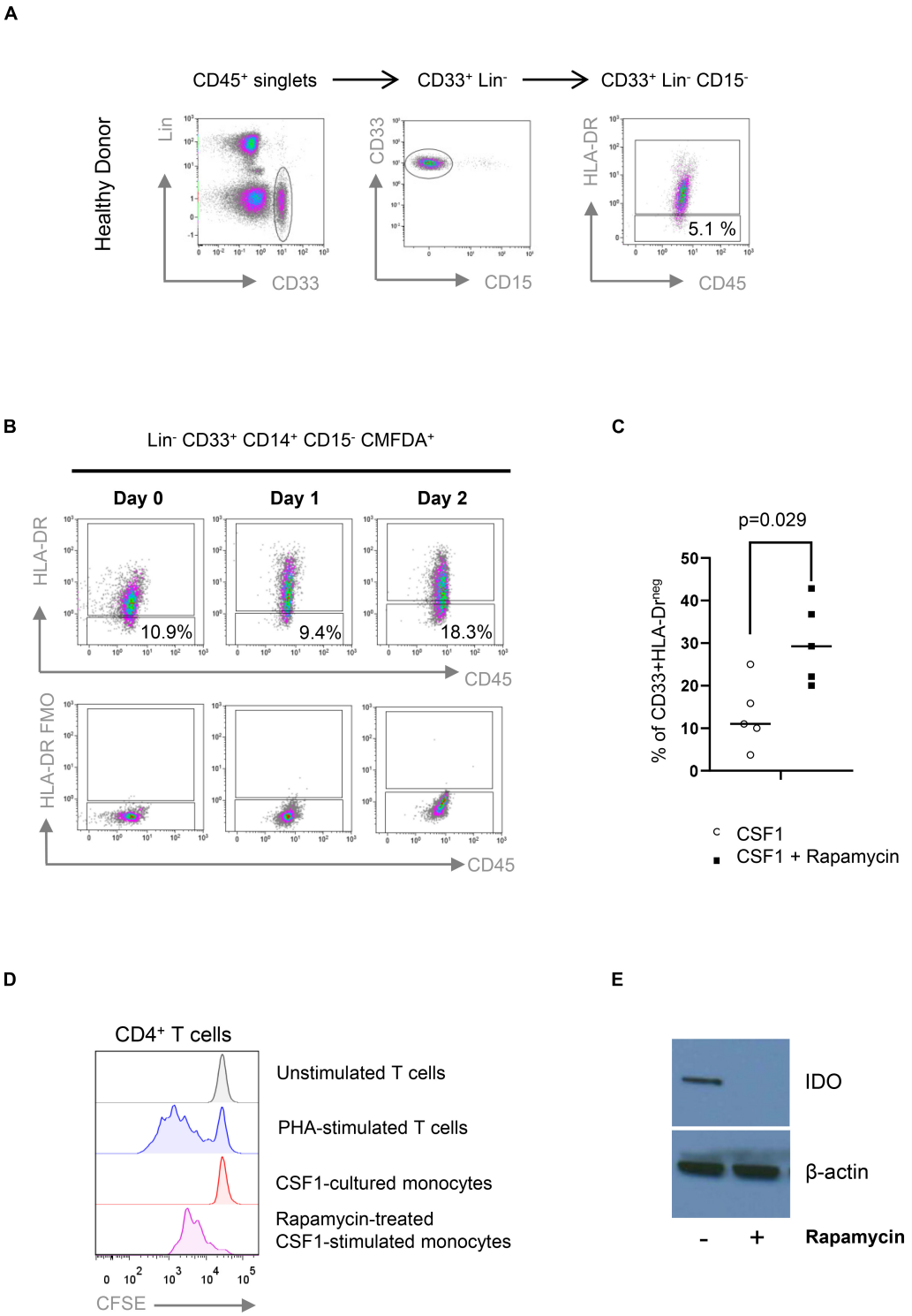

FIGURE 5 | Rapamycin prevents the suppressive function of CD33$^+$HLA-DR$^-$/low myeloid cells. **(A)** Gating strategy for the identification of CD33$^+$HLA-DR$^-$/low myeloid cells obtained from healthy control (HC). Fluorescence minus one (FMO) controls were used to define HLA-DR expression (not shown). **(B)** Colony stimulating factor 1 (CSF1) induces the accumulation of CD33$^+$HLA-DR$^-$/low myeloid cells *in vitro*. CD14$^+$ cells were isolated from peripheral blood, labeled with CFDMA and cocultured with CSF1 for 2 days. CD33$^+$HLA-DR$^-$/low phenotype was analyzed in CFDMA$^+$ cells at day 0, 1, and 2 after culture. FMO controls were used to define HLA-DR expression. **(C)** CD33$^+$HLA-DR$^-$/low myeloid cell frequencies after 48 h in WB cultures treated with or without rapamycin. Differences between groups were assessed by paired *t*-test. **(D)** Rapamycin-treated CSF1-stimulated monocytes are less effective than untreated monocytes in suppressing phytohemagglutinin (PHA)-stimulated proliferation of allogeneic human CD4$^+$ T cells in 1:1 direct cocultures (n = 3). **(E)** Western Blot analyses indicate that rapamycin-treated CSF1-derived CD33$^+$HLA-DR$^-$/low myeloid cells prevents the expression of IDO.

dominant pathway *in vivo* is likely to determine the outcome of the transplanted organ. Taken together, the effects of distinct immunosuppressive drugs on MDSC development and function need to be better characterized in KTR.

Understanding the effect of immunosuppressive drugs on MDSC in clinical transplantation is important to develop strategies to promote tolerance. While there are many unanswered questions regarding the development and function of MDSC human transplantation, we conclude that MDSCs are increased in KTR early after transplantation and that Mo-MDSC subsets from KTR are able to suppress immune responses *in vitro*. How immunosuppressive therapy may enhance or impair MDSC numbers and function is not clear, and additional prospective studies in KTR are required to establish if the long-term transplant tolerance by immune modulation is dependent on MDSC.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Hospital Universitario Marqués de Valdecilla Ethics Committee (CEIC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MI-E: data acquisition, analysis, interpretation, investigation, methodology, writing, and original draft. DS-A and PR: conceptualization, formal analysis, supervision, writing, and review. DM-F: data acquisition, analysis, interpretation, investigation and methodology. SG-F, CP, and PL-P: investigation, and methodology. RV, JR, and ER: patient recruitment and clinical data analysis. JH, JO, and ML-H: conceptualization, project administration, funding acquisition, formal analysis, writing, reviewing, and editing.

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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.2020.00643/full#supplementary-material

FIGURE S1 | Comparison of MDSC subsets: Mo-MDSC, PMN-MDSC, and e-MDSC at day 0 and 180 days after transplant **(A)** and at day 0, day 180, and 360 after transplant **(B).** Levels of Mo-MDSC 180 days after transplant were significantly increased compared to day 0. The central number is the difference (in percent) between the means of the two time points **(A)** and the three time points **(B)**. Differences between time points were calculated using the following formula: (mean posTx-mean preTx)/mean preTx.

FIGURE S2 | MDSC absolute numbers in KTR. **(A)** Frequencies of total myeloid-derived suppressor cells (MDSCs) in peripheral blood mononuclear cells (PBMCs); **(B)** monocytic-MDSCs (Mo-MDSCs); **(C)** early stage-MDSC (eMDSCs), and **(D)** polymorphonuclear MDSCs (PMN-MDSCs) are shown. Differences between groups were assessed by Kruskal-Wallis and Mann-Whitney *U* test. ($^*p < 0.05$).

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Conflict of Interest: The 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.

Trends in Transplantation

Review Article ISSN: 1887-455X

Human Myeloid-Derived suppressor cells in solid organ transplantation

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Abstract

The balance between effector and regulatory immune cells needs a very exquisite balance in the context of solid organ transplantation to avoid the rejection of the organ while maintaining the more immune competence as possible. In the last two decades the role of regulatory immune cells has been extensively studied, mainly with regulatory T cells (Tregs), and in the last years another subset of regulatory cells, named myeloid derived suppressor cells (MDSC), has gained importance. These cells have different mechanisms of action and some of them are differentially regulated in a number of immune-mediated processes. Thus, MDSC, which play important roles in tolerance of experimental models of solid organ transplantation, have been proposed as biomarkers of the degree of immunosuppression and risk of rejection. Besides, they are also thought as therapeutic approach for the establishment of tolerance in human transplantation.

Introduction

Solid organ transplantation is a primary therapy in patients with end-stage disease. Throughout the years immunosuppressive protocols have clearly reduced the incidence of acute rejection, but current pharmacological protocols still result in undesirable side effects, such as infection and cancer among others, what results in a moderate long-term allograft survival [1,2]. As a consequence, the main goals in transplantation are to predict the risk of developing rejection and to find alternative tolerance approaches to allow immunosuppression withdrawal in order to minimize the adverse effects that have deleterious effects on long term graft survival. In this regard, myeloid cells, which are involved both in non-specific reactions and donor-specific adaptive responses during allograft rejection, play a main role starting and controlling immune responses. Under certain circumstances, they contribute to the inflammatory process, expanding disease pathology. However, myeloid cells with regulatory properties can protect the host from uncontrolled inflammation. These cells, known as myeloid regulatory cells (MRCs), have been described within all the major myeloid cell lineages. Among them, myeloid-derived suppressor cells (MDSCs) have been described as a heterogeneous group of myeloid cells known to accumulate under chronic pathological conditions [3]. As a reflection of their biology, these cells had been called "immature myeloid cells" or "myeloid suppressor cells" (MSC) but as neither term was considered as accurate, Gabrilovich DI, *et al.* [4] proposed the term "myeloid -derived suppressor cells' considering this term closer to reflect their origin and function. The first observations of suppressive myeloid cells were described more than 20 years ago in patients with cancer [5-7]. However, their functional importance in the immune system has only recently been appreciated due to the evidence that has demonstrated their contribution to the negative regulation of immune responses in cancer and other clinical settings, such as organ transplantation, infection and autoimmune diseases [3,8-12]. Initially MDSC have been described as immature cells that expand in the bone marrow in response to chronic inflammatory signals but evidence support in certain circumstances MDSC may represent monocytes and neutrophils that have been activated into immunosuppressive populations [13].

In transplantation the MDSCs are able to suppress adaptive and innate immune responses and they have been suggested as potential biomarkers for allograft tolerance as they can play a main role in the balance between graft acceptance and rejection [14,15]. The MDSCs were first described in mice as $CD11b^+$ $Gr1^+$ cells and experimental transplant models demonstrated they have an important role in the induction of tolerance [15,16]. As most of the published studies were performed in animal models, there is a paucity of data addressing MDSC features and their role in human transplantation. Human MDSCs in peripheral blood are classified in three main subsets: monocytic-MDSC (Mo-MDSCs: $CD33^+$ $CD11b^+$ $CD14^+$ HLA-DR-/low), polymorphonuclear-MDSC (PMN-MDSCs:$CD11b^+$ $CD14^-$ $CD15^+$ HLA-DR^- or $CD11b^+$ $CD14^-$+ $CD66b^+$) [17] and a population lacking both differentiation surface markers classified as early- stage MDSC (e-MDSCs: $CD33^+$ $CD15^-$ $CD14^-$HLA-DR^-) [17]. CD33 marker can be used instead of CD11b since very few $CD15^+$ cells are $CD11b^-$. While Mo-MDSC are $CD33^+$, PMN-MDSC are $CD33^{dim}$ [18]. The features and clinical relevance of e-MDSC are not well stablished but limited suppression of T cell proliferation and cytokine expression was found by some authors [19]. Other suggested makers in human MDSCs include high levels of CD66b and low levels of CD62L and CD16, vascular endothelial growth factor receptor 1 (VEGFR1) (Flt-1) [20] and expression of CD124 [21]. Initially the term 'granulocytic MDSC' was used to describe PMN-MDSC [22,23] but since PMN-

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MDSC are phenotypically distinct from steady-state neutrophils lately V. Bronte, *et al.* proposed the term PMN as more accurate to define this subset [17]. Because these markers are not exclusively expressed by MDSCs, these regulatory cell subsets are best defined by their capacity to suppress T cell proliferation [24], which is associated with their ability to induce T cell apoptosis [25] and expand Treg cells [26] (Figure 1). Moreover, the interaction between MDSC and other immune cells has been described in recent years [27–30]. It is important to remark that assays of human MDSC function are difficult to implement due to their technical complexity and high variability. At the present time, the technique allowing for separation of neutrophils from PMN-MDSC is Ficoll gradient regularly used for the isolation of mononuclear cells. Low- density fraction contains PMN-MDSC and activated neutrophils. Therefore, $CD11b^{+}CD14^{-}CD15^{+}/CD66^{+}$ cells in low-density fraction contain both PMN-MDSC and neutrophils [17]. Hence, there is a need for reliable surrogate markers of human MDSC function as gating criteria cannot discriminate monocytes from Mo-MDSCs and neutrophils from PMN-MDSC since at present there are no combinations of markers exclusive to MDSC. Human MDSCs exert their T cell suppressive actions through a wide variety of mechanisms, including production of anti-inflammatory cytokines and up-regulation of immune- regulatory molecules, including arginase 1 (Arg1) and indoleamine 2,3-dioxygenase (IDO) [31,32] (Figure 1). Conventional phenotyping of human Mo-MDSC subsets mainly relies upon HLA-DR expression; however, it is unclear to what extent HLA-DR expression is influenced by standard immunosuppression, especially glucocorticoids. The release of neutrophils from the bone marrow in response to glucocorticoids is well established [33] and it has been reported that glucocorticoids can induce anti-inflammatory monocytes resembling MDSC [34,35]. In previous experiments from our group (data not published) we observed a dose-dependent reduction in HLA-DR expression levels in monocytes

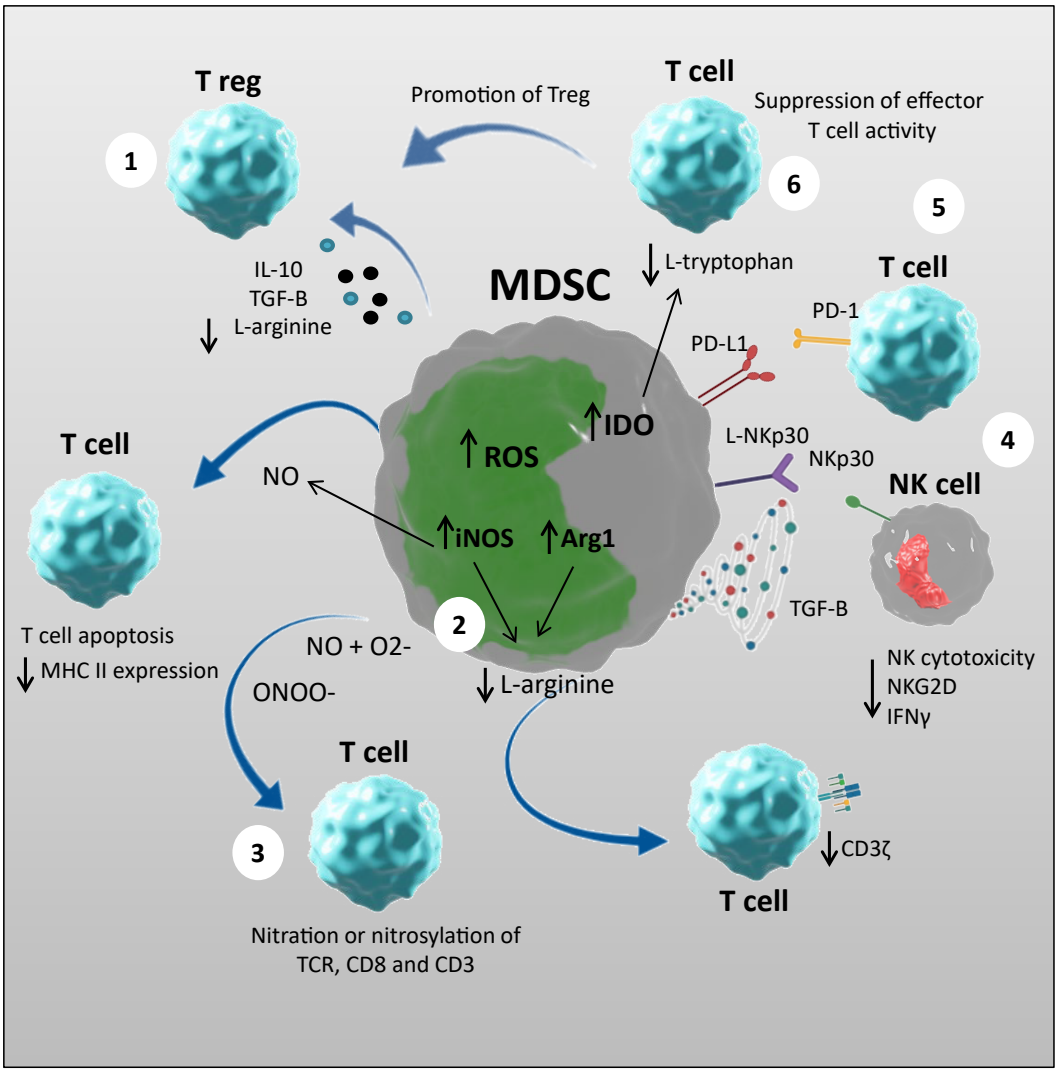

Figure 1. Mechanisms of MDSC suppressive activity

MDSC exert their suppressive function through a variety of mechanisms: **(1)** secretion of anti--inflammatory mediators, such as IL--10 and TGF--β that promote induction of T--regulatory cells; **(2)** increased arginase and iNOS: the increased activity of arginase leads to enhanced L--arginine catabolism. The lack of L--arginine inhibits T--cell proliferation through different mechanisms, including decreasing their CD3ζ expression; on the other hand iNOS generates NO which suppresses T--cell function inhibiting, MHC class II expression and inducing T--cell apoptosis; **(3)** increased production of ROS generates peroxynitrite which induces the nitration and nitrosylation of the amino acids and mediate MDSC suppression of T--cell function; **(4)** MDSCs can inhibit NK cell function by interacting with the NKp30 receptor; **(5)** inducing increased PD--1 expression; and **(6)** increased IDO activity which catabolizes tryptophan and limits T cell proliferation.

after dexamethasone exposure, monocytes were phenotypically indistinguishable from Mo-MDSC. In the critical illness context, Le Tulzo, *et al*. studied 48 septic patients and found an association between high levels of circulating cortisol and reductions in monocyte HLA-DR expression on day 6 of illness [36]. The authors then demonstrated *in vitro* that dexamethasone caused a down-regulation of a key transcription factor for HLA-DR in normal monocytes. They suggest that glucocorticoid action may represent another mechanism for the development of innate immune dysfunction. Similarly, Volk, *et al.* demonstrated that the administration of methylprednisolone in the setting of cardiopulmonary bypass resulted in an exacerbation of innate immunosuppression over that obtained with bypass alone [37]. An important goal for future studies is to define cell-surface markers and gating strategies that uniquely identify the different populations of MDSC. On the other hand, a major challenge in immune monitoring of transplant recipients is distinguishing between changes in biomarkers reflective of underlying alloimmune responses versus changes related to immunosuppressive therapy.

Kidney Transplantation

In kidney transplant models Dugast, *et al.* reported the role of MDSC in kidney transplant recipient rats [38]. In this model, MDSC in the recipient allograft were described for the first time in organ transplantation and their suppressive mechanism of tolerance was in part mediated by iNOS. In concordance, the role of NO (nitric oxide) in MDSC mediated suppression was first described by Mazzoni [39]. Another report from Vanhove's laboratory reported that secretion of CCL5 by MDSC was responsible for the accumulation of Treg into tolerized kidney allografts [40]. In subsequent studies the results indicate that a gradient of CCL5 might contribute to the intra graft localization of Treg in tolerant recipients controlled by MDSC [41]. In human kidney transplantation Luan, *et al.* found that the overall MDSC frequencies were elevated at 3, 6 and 12 months post-transplant [42]. Utrero-Rico, *et al*., observed Mo-MDSC cells counts rapidly increase after kidney transplantation and remain high one year after transplantation [43]. Hock *et al*. showed that renal transplant recipients (RTR) had elevated frequencies of circulating MDSC [8], but they further found MDSC numbers had returned to normal levels 12 months post-transplantation [44]. However, in their previous study of RTRs with longer term transplants elevated MDSC numbers were detected in the majority of patients, suggesting that MDSC expand in the long-term, as the graft acceptance progresses. In a previous report from our group we evaluated the phenotype and function of different MDSCs subsets in 38 kidney transplant recipients (KTR) at different time-points and our data indicated that Mo-MDSC increase in KTR at 6 months and 12 months post-transplantation [45]. Moreover, the MDSCs were shown to expand early after transplantation, independently of using basiliximab or thymoglobulin during induction [44] and almost tolerant kidney transplant recipients (ATKTRs) had significantly higher levels of monocytic MDSCs and CD4+CD25+FoxP3+ Tregs than short-term graft survival kidney transplant recipients and healthy donors [46].

These observational studies suggest that MDSC numbers increase rapidly after transplantation and peak following immunosuppressive therapy. Moreover, analysis of the changes in MDSCs obtained from donors, provided strong evidence that the changes occurring in RTRs were likely due to the immunosuppressive regimens rather than the acute inflammation from surgery itself [44]. Although Mo-MDSC phenotype seems to be influenced by standard immunosuppression, especially glucocorticoids, whether MDSC subsets are differentially regulated by local conditions or treatments require further investigations.

Studies developed in mice suggest that MDSCs have an important role to induce T regulatory cells (Treg) after transplantation [16,47], but their role in human transplantation is under investigation. In KTR, Luan, *et al.* observed that $CD33^{+}$ $CD11b^{+}$ $HLA\text{-}DR^{-}$ MDSC are capable of expanding Treg, and they correlate with Treg increases *in vivo* (42). Consistent with this view, Meng, *et al.* [48] found that MDSCs isolated from transplant recipients were also able to expand regulatory T cells and were associated with longer allograft survival, and we also reported an increase in Treg expansion after Mo-MDSC coculture [45]. Further, the Mo-MDSC levels correlated positively with the survival rates, estimated glomerular filtration rates (eGFRs) of grafts, and the levels of CD4+CD25+FoxP3+ Treg in ATKTRs [46]. In two cohorts of patients with acute rejection the mRNA levels of S100A8 and S100A9 in biopsies predicted improved graft outcome. Expression of both proteins correlated with MDSC markers in PBMC and renal biopsies and higher expression of immune regulatory molecules [49]. Due to the lack of unique phenotypic markers functional studies have to be performed to identify MDSC subsets [24]. Murphy, et al. evaluated the capacity of blood derived $CD11b^{+}CD33^{+}HLADR^{-}$ MDSC from human KTRs to suppress CD4+T cells proliferation in vitro [42] demonstrating that $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ myeloid cells from human KTR inhibit T cell proliferation, but no inhibition was found when $CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ cells were obtained from healthy donors [42]. Moreover, we observed that Mo-MDSC from KTR under tacrolimus treatment had increased suppressive activity compared to rapamycin [45] and we attribute loss of suppressive function to diminished IDO expression in rapamycin-exposed Mo-MDSC. However, another study addressing the murine MDSC response to acute kidney injury demonstrated that MDSC reduced the injury, and the effect was potentiated by MDSC induction and enhancement of the immunosuppressive activity promoted by mTOR [50]. More recently, a previously unrecognized mechanistic pathway associated with organ rejection identifies the expression of mTOR by graft infiltrating macrophages at the center of epigenetic and metabolic changes that correlate with graft loss [51]. This novel functional mechanism has been termed "trained immunity" [52]. Therefore, it seems that, while mTOR inhibition may prevent trained immunity and inflammatory pathways in myeloid cells [53,54], it may also interfere with tolerogenic programming and the ability of myeloid cells to expand Treg and suppress T-cellmediated immune responses. This dual effect of mTOR inhibition in vivo is likely to determine the outcome of the transplanted organ.

Liver Transplantation

In the 1990s, Settmacher, *et al.* described an association between aggressive calcineurin inhibition and a reduction HLA-DR expression in monocytes in the setting of induction therapy in adults following liver transplantation [55]: among 91 patients, those whose monocyte HLA-DR expression dropped below 30% experienced increased rates of bacteremia, viremia, and fungemia compared to those whose HLA-DR levels remained > 30%. In the same manner, Haveman JW, *et al.* monitored 20 liver transplantation recipients during the first month after transplantation and measured the expression of HLA-DR in monocytes. Seven out of 20 patients developed sepsis after a median of 15 days post-transplantation and HLA-DR expression was significantly lower in these patients. The expression of HLA-DR in monocytes remained low before onset of sepsis. On day 7 after transplantation, HLA-DR expression on 50% or less of monocytes had a positive predictive value for sepsis of 71%, whereas the negative predictive value was 85%. Furthermore, patients who received significantly more prednisolone

developed sepsis. The authors conclude that low HLA-DR expression on monocytes is a marker for a high risk of subsequent sepsis in liver transplantation patients and this high risk may be related to the dose of prednisolone [56]. It is known that under inflammation and fibrosis, MDSC are induced in the liver due to the local conditions [57]. MDSC are recruited in the liver and they differenciate by mechanisms that depend on contact between several cell types and on soluble mediators. For example, hepatic stellate cells promote MDSCs in mice and humans and mesenchymal stromal cells in human [58]. Bernsmeier, *et al.* reported that immunosuppressive $CD14^{+}HLA\text{-}DR^{-}$ Mo-MDSCs, are expanded in patients with acute-on- chronic liver failure (ACLF) and TLR-3 agonists reversed Mo-MDSC expansion [59]. In a murine model, rapamycin induced the recruitment of MDSC and protected against immunological hepatic injury. Downregulating the mTOR activity in MDSCs induced iNOS and NO, and the pharmacological inhibition of iNOS completely eliminated the recruitment of MDSCs [60]. In another model of allogeneic liver transplantation, the authors observed an increase of regulatory T cell phenotypes and accumulation of MDSC in spleen [61].

Lung Transplantation

Hoffman, *et al.* monitored HLA-DR expression weekly after transplantation in 13 pediatric lung transplant recipients (LRT) [62]. Six out of seven patients who developed post-transplant pneumonia demonstrated lack of monocyte HLA-DR expression within the first two weeks of monitoring and those who developed pneumonia had lower monocyte HLA-DR expression over the four-week study period than those who remained infection-free. The authors propose that monitoring HLA-DR expression in monocytes may be useful to identify risk of infection and stratifying patients into higher and lower risk groups. Alingrin, *et al.* assessed the influence of early post-operative sepsis on T cell and monocyte reconstitution in anti- thymocyte globulin (ATG)-treated lung transplant recipients. Peripheral blood T-lymphocytes counts and monocyte HLA-DR (mHLA-DR) expression within 60 days post-transplant were analyzed. The authors found that sepsis is negatively correlated with the HLA-DR expression in monocytes [63]. These findings taken together highlight the importance of immunomonitoring after lung transplantation. Deshane, *et al.* found high numbers of $CD11b^{+}$ $CD14^{+}$ $CD16^{-}$ $HLA\text{-}DR^{-}$ NO-producing myeloid derived regulatory cells, in the airways of patients with asthma but not in patients with chronic obstructive pulmonary disease (COPD) or in healthy control subjects [64]. On the other hand Scrimini, *et al.* observed elevated levels of circulating-lineage $HLA\text{-}DR^{-}$ $CD33^{+}$ $CD11b^{+}$ MDSCs in patients with COPD [65]. Other researchers demonstrated that $CCR2^{+}$ Mo-MDSCs inhibit collagen degradation and promote lung fibrosis by producing transforming growth factor-β1 (TGF-β1) [66]. The number of circulating activated MDSCs was found to be significantly increased in patients with pulmonary hypertension (PH) compared to control subjects, and was correlated with an increase in mean pulmonary artery pressure [67]. Sharma, *et al.* described the association of distinct MDSC sub-populations with the lung microbiome in LTRs. Their results suggested a functional link between the local microbiome and MDSC phenotype, which may play a role in the pathogenesis of BOS [68].

One from our group (unpublished) analyzed MDSC frequencies in 82 LTR were analyzed during the first year after transplantation. Percentages of total MDSC were increased in LTR 3 months after transplantation up to a year. When we studied the effect of transplantation on MDSC subsets in our cohort, Mo-MDSC percentages increased promptly after transplantation and decreased gradually during follow up. On the contrary, PMN-MDSC percentages decreased in the short term after transplantation, and increased during follow up although no changes compared to pre-transplant levels were observed. Compared to pre-transplant levels, e-MDSC percentages were significantly increased at 7 days, 21days and 360 days**.** We obtained similar results when we calculated MDSC absolute numbers. In previous experiments we observed a dose-dependent reduction in HLA-DR expression levels in monocytes after dexamethasone exposition, then monocytes were phenotypically indistinguishable from Mo-MDSC. On concordance with these results, some studies previously published [33,37,56] point corticosteroids are modulating MDSC levels then we hypothesize that corticosteroids are increasing Mo-MDSC populations in peripheral blood immediately after transplantation. We observed (unpublished data) the suppressive capacity of MDSC from Tacrolimus treated LTR is increased compared to the suppressive results when $CD14^{+}CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ cells were obtained from healthy donors. Heigl, *et al.* characterized MDSCs in lung transplant recipients to assess if MDSCs can serve as a potential new research target in the field [69]. They observed that G-MDSCs obtained from LTR were functionally suppressive and showed a modest correlation with increasing CNI trough levels, a previously reported phenomenon [70,71]. Previous studies demonstrated that expression of FK binding protein FKBP in Mo-MDSCs and PMN- MDSCs from tumor-bearing mice is increased and regulates their suppressive function [72]. Altogether these results indicate that MDSC activity and numbers are modulated by immunossupressive treatments, such as CNI. In contrast, MDSC percentages in our study were not related to immunosuppressant levels in peripheral blood. As our cohort of LTR was under the same immunosuppressive regimen, potential differences between treatments, with respect to their effect on MDSC frequency or function cannot be determined which still remains a limitation. In contrast with previous reports [23,24] we observed that 90 and 180 days post-transplant Mo-MDSCs percentages were higher in patients who reject compared to those who do not reject.

Heart, Corneal, Pancreatic islets and Skin Transplantation

Ling Zhou, *et al.* found a cardioprotective role of MDSCs in heart failure [73] although the human MDSC response in heart transplantation remains unstudied. In murine models, several studies demonstrated MDSC were required for the induction of transplantation tolerance [47,74,75]. Some authors reported the development of MDSC and induction of tolerance after treating recipients with rapamycin and costimulatory blockade with anti-CD40L mAb [76,77] in contrast to mice treated with either rapamycin or anti-CD40L mAb alone [77]. Nakamura T, *et al.* further observed that rapamycin increased PD-L1 expression on MDSC that accumulate in the cardiac allograft [78]. The effect of dexamethasone for the induction of MDSC was also reported [79] by Zhao, *et al.* In corneal allograft animal models it was observed an increase in allograft survival after MDSC infusion [80]. These data suggest that transplantation may lead to recipient derived MDSCs able to suppress anti-donor responses [81]. Further, it was observed an expansion of Mo-MDSC after dexamethasone administration [82] and rapamycin nano-micelle (RNM) ophthalmic solution treatment delayed rejection and expanded MDSC in allografts [83]. In pancreatic Islet transplantation MDSC infusion prolonged allograft survival and increased the number of Tregs within the graft [84,85]. MDSC generated by hepatic stellate cells (HCS) increased islets allograft survival [84]. As well as heart, corneal and pancreatic islet transplantation there is a lack of studies regarding skin human transplantation but several animal models pointed the ability of MDSC to increase skin graft

survival [86,87]. Yang, *et al.* described that TNFα induced MDSC *in vitro* and the expression of iNOS was necessary for suppression of T cell proliferation [88]. The role of iNOS in MDSC function was also described by Wu, et al. [89]. Liao, *et al.* reported induced iNOS expression and NO production in MDSC after dexamethasone treatment [90]. Rapamycin downregulates iNOS expression in MDSC, and the suppressive activity and MDSC numbers are significantly reduced after rapamycin treatment in an allogeneic skin transplant model [91]. The results confirm the administration of glucocorticoids as a therapeutic approach by increasing the development of MDSC and point mTOR as an intrinsic factor essential for the differentiation and immunosuppressive function of MDSCs.

Conclusions and future directions

MDSC are a group of immunoregulatory myeloid cells that are gaining attention throughout the years in the field of transplantation. Several animal models have point them as important regulators in transplantation but in human transplantation their role as a biomarkers and their potential use as immunotherapy to promote tolerance remains under investigation.

A major challenge in immune monitoring of transplant recipients is distinguishing between biomarkers changes as a consequence of underlying alloimmune responses from those related to immunosuppressive therapy. Conventional phenotyping of human Mo-MDSC subsets relies upon HLA-DR expression [17]. Although it may be possible MDSC increase naturally after transplantation [15] provided evidence supports that HLA-DR expression is influenced by standard immunosuppression, especially glucocorticoids [33,34,37,56] which supports the need for new and specific markers to identify human MDSC. Then one of the more important goals for future studies is to define specific cell-surface markers and gating strategies that uniquely identify MDSC subpopulations [24]. The lack of specific markers also obliges to perform functional assays to check human MDSC function. In addition functional assays are difficult to implement due to their technical complexity and high intra-assay variability. In this regard, both the definition of specific markers and identification of the transcriptomic profile of human MDSC may shed light on the field [92]. Even though there are some studies regarding the effect of immunsuppressive drugs on human MDSC function, the effect of the current main immunosuppressive regiments on MDSCs should be further studied.

MDSC represent a promising therapeutic approach in solid organ transplantation but additional investigations are needed to fully understand their role in tolerance and to achieve immunosuppression withdrawal or minimization.

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