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New Approaches for the Induction of Tolerance in Transplantation: Evaluation of Exosomes and Tolerogenic Dendritic Cells

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that the thesis entitled "New approaches for the induction of tolerance in transplantation: Evaluation of exosomes and tolerogenic dendritic cells" has been performed by Patricia Bastos Amador under their direction and guidance and is able to be presented and defended in order to apply for the PhD degree in Immunology by the "Universitat Autònoma de Barcelona" (UAB).

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Dr. Francesc E. Borràs Serres

Dra. Inmaculada Herrero Fresneda

A mis padres

Ab: Antibody
ALS: Anti-lymphocyte serum
APC: Allophycocyanin
APC: Antigen presenting cell
APRIL: A proliferation inducing ligand

BAFF: B-cell activating factor
BDCA: Blood dendritic cell antigen
BMDCs: Bone marrow-derived dendritic cells
BST-2: Bone marrow stromal cell antigen (also known as PDCA-1)

CCR7: Chemokine (C-C motif) receptor-7
CD: Cluster of differentiation
CD40-L: CD40-ligand
cDCs: Conventional dendritic cells
CFSE: Carboxyfluorescein succinimidyl ester
CIA: collagen-induced arthritis
CLRs: C-type lectin receptors
CTL: Cytotoxic T lymphocyte
CTLA-4: Cytotoxic T-lymphocyte antigen-4, also known as CD152

DAMPs: Damage-associated molecular patterns
DCs: Dendritic cells
DC-SIGN: DC-specific ICAM-3-grabbin non-integrin
DEC-205: Dendritic and epithelial cells, also known as CD205
DMEM: Dubelcco's Modified Eagle Medium
DNT: CD4-CD8- TCR$\alpha\beta$+ T cells

EBV: Epstein–Barr virus
ELISA: Enzyme-linked immunosorbent assay
ESCRT: Endosomal sorting complex required for transport

FAS-L: Fas-ligand
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
FLT3L: FM- Like Tyrosine Kinase 3 Ligand
Foxp3: Forkhead box P3

GM-CSF: Granulocyte macrophage colony stimulating factor
GPI: Glycosylphosphatidylinositol

HIV: Human immunodeficiency virus
HMGB-1: High mobility group protein B-1
HRP: Horseradish Peroxidase
HSPs: Heat shock proteins

i.p.: Intraperitoneal
i.v.: Intravenous
ICAM-1: Intercellular Adhesion Molecule 1, also known as CD54
ICOS: Inducible T-cell co-stimulator
IDO: Indoleamine-2,3-dioxygenase
IFN: Interferon
Ig: Immunoglobulin
IL-: Interleukin
ILT: Immunoglobulin-like transcript

KLH: Keyhole limpet hemocyanin

LAMP: Lysosome-associated membrane protein
LFA-1: Lymphocyte function-associated antigen 1
LN: Lymph nodes
LPS: Lipopolysaccharide

MAC-1: Macrophage-1 antigen, also known as complement receptor 3
MDDCs: Monocyte-derived DCs
MFGE-8: Milk fat globule-EGF factor 8 protein, also known as lactadherin
MFI: Median Fluorescent Intensity
MHC: Major histocompatibility complex
miRNA: Micro RNA
mRNA: Messenger RNA
MVB: Multivesicular bodies

NFκB: Nuclear factor kappa B
NK: Natural killer
NLRs: NOD-like receptors

ODNs: Oligodeoxyribonucleotides

PAMPs: Pathogen-associated molecular patterns
PBMCs: Peripheral blood mononuclear cells
PBS: Phosphate buffer saline
PD-1: Programmed cell death protein 1
pDCs: Plasmacytoid dendritic cells
PE: Phycoerythrin
PGE-2: Prostaglandin E2
PI3K: Phosphatidylinositide 3-kinase
PLP: Proteolipid protein
PMA: Phorbol myristate acetate
Poly I:C: Polyinosinic-polycytidylic acid
PRRs: Pathogen recognition receptors
PS: Phosphatidylserine

RAPA: Rapamycin
RLHs: RIG-like helicases
RNA: Ribonucleic acid
RPMI: Roswell Park Memorial Institute medium

SD: Standard deviation
Siglec: Sialic acid binding immunoglobulin-like lectin
SIRP-α: signal regulatory protein α, also known as CD172
SLO: Secondary lymphoid organs

TfR: Transferrin receptor
TGF-β: Transforming growth factor-β
Th 1: Type 1 T helper cell
TLRs: Toll-like receptors
TNF: Tumour necrosis factor
Tr1: Regulatory type 1 T cells
Treg: Regulatory T cell

Following transplantation, the immune system is triggered to induce an immune response to donor histocompatibility antigens expressed by the graft (allo-antigens), leading to organ rejection. Dendritic cells (DCs) are the most potent antigen presenting cells and have a fundamental role in the initiation of the immune response to the graft by presenting allo-antigens to T cells through several pathways. However, DCs are also key players in the induction of tolerance. One of the main targets in transplantation is the induction of donor-specific tolerance thereby avoiding chronic administration of immunosuppression, which has many side effects. Understanding the immunological mechanisms involved in transplant rejection has allowed the generation of alternative therapies to conventional immunosuppression. In different animal models, several strategies have been employed to induce graft tolerance such as the injection of tolerogenic DCs. In humans, there are several possible sources of alloantigens including exosomes, which can be isolated from several biological fluids. The aim of this thesis is to investigate the potential use of plasma-derived exosomes as a source of alloantigens, the ability of human peripheral blood dendritic cells to capture allogeneic exosomes and finally, evaluate the effect of recipient tolerogenic DCs on allograft survival in a model of allogeneic kidney transplantation in rats.

Our results provide valuable information about plasma-derived microvesicles. By proteomic analysis, we have detected 161 microvesicle-associated proteins, including many related to the complement and coagulation signal-transduction cascades. However, when exosomes-enriched preparations were analysed the number of proteins identified was much reduced, suggesting that under healthy conditions there are limited amounts of exosomes and, therefore, are not a feasible source of alloantigens. Moreover, our results provide some insight in the interaction of human peripheral blood DCs subsets with allogeneic exosomes. In vitro analyses show that both conventional DCs (cDCs) and plasmacytoid DCs (pDCs) capture exosomes from a T-cell line, although with different ability. The uptake of exosomes does not modify the activation state of pDCs. In addition, exosomes-loaded pDCs are able to stimulate autologous T cells suggesting that this subset could have a role in allo-antigen presentation. Finally, we have generated tolerogenic DCs in the presence of dexamethasone to evaluate their effect in a model of kidney transplantation in rats. Donor alloantigens were obtained from immature BMDCs-derived

exosomes. After donor exosomes capture, tolerogenic DCs present a semi-mature phenotype and an anti-inflammatory cytokine profile. Although these tolerogenic DCs do not improve allograft survival, after intravenous injection in kidney recipients are able to modify the number of peripheral blood B cells. In addition, in vitro experiments show that tolerogenic DCs are able to inhibit LPS-dependent proliferation of B cells. These results indicate that tolerogenic DCs, in vitro loaded or not with donor exosomes, may have a biological role in transplant rejection through the modulation of B cell responses.

Tras el trasplante, el sistema inmunitario se activa para inducir una respuesta inmunitaria contra los antígenos de histocompatibilidad del donante expresados por el injerto (alo-antigenos), que conduce al rechazo del órgano. Las células dendríticas (CD) son las células presentadoras de antígeno más potentes y tiene un papel fundamental en la iniciación de la respuesta inmunitaria contra el injerto mediante la presentación de aloantígenos a las células T a través de varias vías. No obstante, las CD son también participantes clave en la inducción de tolerancia. Uno de los principales objetivos en el trasplante es la inducción de tolerancia específica de donante evitando de este modo la administración crónica de inmunosupresión, que tiene muchos efectos secundarios. La comprensión de los mecanismos inmunológicos implicados en el rechazo del trasplante ha permitido la generación de terapias alternativas a la inmunosupresión convencional. En diferentes modelos animales, se han empleado varias estrategias para inducir la tolerancia del injerto tales como la inyección de CD tolerogénicas. En humanos, existen varias posibles fuentes de aloantígenos incluyendo los exosomas, que se pueden aislar de diferentes fluidos biológicos. El objetivo de esta tesis es investigar el potencial uso de los exosomas derivados de plasma como fuente de aloantígenos, la capacidad de las células dendríticas humanas de sangre periférica para capturar exosomas alogénicos y, finalmente, evaluar el efecto de las CD tolerogénicas en la supervivencia del injerto en un modelo de trasplante renal alogénico en rata.

Nuestros resultados proporcionan información valiosa sobre las microvesículas derivadas del plasma. Mediante análisis proteómico hemos detectado 161 proteínas asociadas a las microvesículas, incluyendo muchas relacionadas con el complemento y con las cascadas de transducción de señales de la coagulación. Sin embargo, cuando las preparaciones enriquecidas en exosomas fueron analizadas el número de proteínas identificado fue muy reducido, lo que sugiere que en condiciones saludables hay cantidades limitadas de exosomas y, por tanto, no son una fuente viable de aloantígenos. Por otra parte, nuestros resultados proporcionan nueva información sobre la interacción de las células dendríticas humanas de sangre periférica con los exosomas alogénicos. Los experimentos in vitro muestran que tanto las CD convencionales como las plasmacitoides capturan exosomas derivados de una línea de células T aunque con diferente capacidad. La captura de los exosomas por las CD plasmacitoides no modifica su estado de activación.

Además, las CD plasmacitoides cargadas con los exosomas son capaces de estimular linfocitos T autólogos lo que sugiere que esta población podría tener un papel en la presentación de aloantígenos. Por último, hemos generado CD tolerogénicas en presencia de dexametasona con el fin de evaluar su efecto en un modelo de trasplante renal en rata. Los aloantígenos donantes fueron obtenidos a partir de exosomas derivados de células dendríticas inmaduras derivadas de médula ósea. Tras la captura de los exosomas donantes, las CD presentan un fenotipo semi-maduro y un perfil de citocinas anti-inflamatorio. Aunque estas DC tolerogénicas no mejoran la supervivencia del aloinjerto tras su inyección vía intravenosa en las ratas receptoras del riñón, son capaces de modificar el número de células B en sangre periférica. Además, los experimentos in vitro muestran que las CD tolerogénicas son capaces de inhibir la proliferación dependiente de LPS de las células B. Estos resultados indican que las CD tolerogénicas, cargadas o no con exosomas donantes in vitro, pueden tener un papel biológico en el rechazo del trasplante a través de la modulación de la respuesta de células B.

INTRODUCTION

INTRODUCTION I: DENDRITIC CELLS AND TRANSPLANTATION

Solid-organ transplantation is often the most effective therapy for end-stage organ failure. However, the disparity of major histocompatibility antigens between donor and recipient promotes the activation of the immune system in the host which results in graft destruction and rejection. To avoid such deleterious response, transplant recipients receive immunosuppressive therapies. Unfortunately, these therapies often cause a general suppression of the immune system inducing side effects such as opportunistic infections and malignancy. Understanding the immunological mechanisms involved in transplant rejection has permitted the development of alternative therapies to classical immunosuppression.

1. IMMUNOLOGICAL MECHANISMS IN TRANSPLANT REJECTION

Transplant rejection is a complex and coordinated immune response against the different major histocompatibility complex (MHC) class I and class II molecules (allo-antigens) and, to a lesser extent, minor histocompatibility antigens expressed by the graft. Effector mechanisms responsible for injury and destruction of the transplanted organ involve activation and differentiation of alloantigen-specific T cells and B cells. Such effector pathways of transplant rejection can be divided according to clinical/pathological manifestations [1–3].

a) Hyperacute rejection. It occurs a few minutes/hours after transplantation, when preformed antibodies against donor MHC molecules or ABO system are present at the moment of transplantation.

b) Acute cellular rejection (T cell-mediated). This type of rejection develops between the first week and a month. It is mediated by T cells after alloantigen recognition.

c) Acute humoral rejection (B cell-mediated). It takes place at the first/second week after transplantation and is mediated by allo-antibody reactivity to donor antigens.

d) Chronic rejection. It is a progressive process of deterioration of the graft function. Both T and B cells are involved.

1.1. T cells in allorecognition and graft rejection

The central role of T cells in transplant rejection has been demonstrated by experimental models in which T lymphocytes were sufficient to reject a graft [4–6]. The priming and activation of T cells takes place through three different pathways: direct allorecognition, indirect allorecognition and semi-direct allorecognition **(Figure 1)** [7–10].

- **Direct allorecognition.** This pathway involves direct recognition of donor peptide-MHC complexes by T cells on donor antigen presenting cells (APCs) such as dendritic cells (DCs). This mechanism is responsible for the acute rejection and diminishes with time due to the progressive loss of donor APCs.
- **Indirect allorecognition.** After the capture and processing of donor antigen by APCs, donor-derived peptides are presented on recipient MHC molecules to alloantigen-specific T cells. This is the main mechanism in chronic rejection.
- **Semi-direct allorecognition.** This process occurs when donor MHC molecules are recycled and presented as intact molecules on recipient APCs and presented to antigen-specific T cells [11].

The activation of T cells starts with the traffic of donor APCs - mainly DCs - from the graft to the draining lymph nodes where they activate CD4+ and CD8+ effector T cells through the direct pathway of allorecognition. Donor DCs also function as suppliers of donor alloantigens that can be captured and presented by recipient DCs in the context of self-MHC and activate antigen-specific CD4+ and CD8+ T cells through the indirect pathway. After priming, T cells proliferate and differentiate into effector cells and migrate to the graft where they initiate the process of rejection [8,12]. The effector mechanisms by which T cells mediate graft destruction are lymphocyte-mediated cytotoxicity triggered by CD8+ cytotoxic T cells (CTLs) and delayed-type hypersensitivity mediated by CD4+ T. During the lymphocyte-mediated cytotoxicity, CD8+ CTLs are primed and activated by recognition of allogeneic class I MHC molecules. Following activation, CD8+ CTLs induce the lysis of target cells by secreting cytotoxic granules, which contain perforin and granzyme B, or by

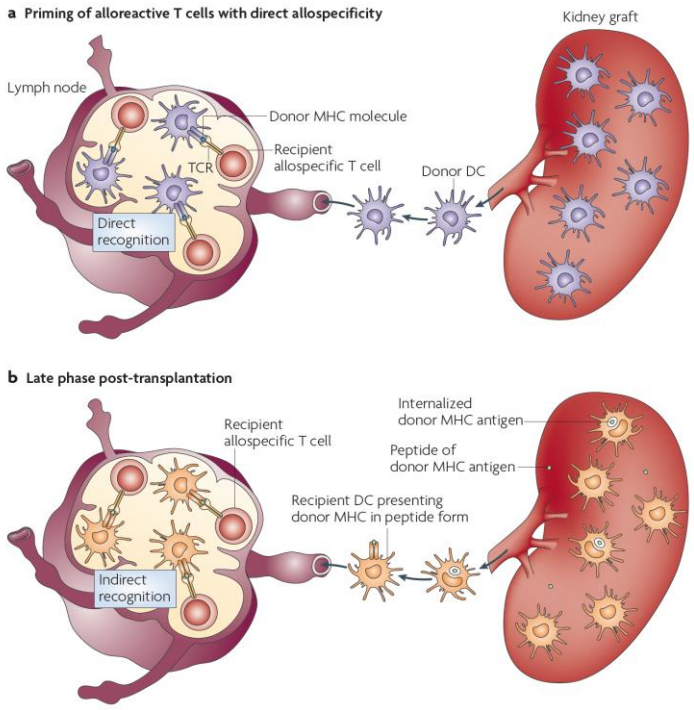


Figure 1. Pathways of alloantigen recognition by T cells. After engraftment donor DCs migrate to secondary lymphoid organs (SLO) and activate recipient T cells through the presentation of intact donor MHC molecules **(a)**. Then, recipient DCs migrate into the graft and capture and process donor allogeneic MHC molecules. These DCs circulate to draining lymph nodes and present donor allopeptides on self-MHC molecules to alloantigen-specific T cells **(b)**. A third mechanism has been described named semi-direct pathway (not depicted). Through this pathway, recipient T cells recognize intact donor MHC molecules presented on the surface of recipient DCs. The transfer of these intact donor MHC molecules occurs via cell-cell contact or transference of exosomes. Illustration from [13], Nature Reviews Immunology.

upregulating Fas ligand (FAS-L), triggering caspase-mediated apoptosis on the target cell. On the other hand, delayed-type hypersensitivity is a mechanism orchestrated by donor-specific CD4+ helper 1 T cells (Th1). Upon alloantigen recognition, Th1 cells secrete cytokines, such as interferon (IFN)-γ, interleukin (IL)-1 and tumour necrosis factor (TNF)-α, and other proinflammatory mediators that recruit monocytes and macrophages that become activated and secrete proteolytic enzymes, nitric oxide, reactive oxygen species and products of the metabolism of arachidonic acid, that further amplify the response

against the graft [1,3,14]. Additionally to these two major effector mechanisms, T cells control B cell responses by expressing co-stimulatory molecules and secreting cytokines which modulate the proliferation, differentiation and alloantibody production by B cells [10].

It is worth to mention that there are an elevated proportion of memory T cells in humans as consequence of heterologous immunity [15], a process by which memory T cells specific for a microbial antigen, cross-react with allogeneic molecules [16]. Memory T cells have a lower threshold of activation, being less dependent on co-stimulation than naïve T cells. Therefore, non-professional APCs such as donor endothelial cells, after receiving a proinflammatory stimulus, can up-regulate MHC II molecules and activate memory T cell via direct presentation.

1.2. B cell-mediated transplant rejection

B cells are important mediators of hyperacute, acute and chronic humoral rejection [10,17]. The principal effector mechanism of B cells in transplant rejection involves antibody production against MHC molecules (both class I and class II) expressed by the graft –alloantibodies-. In addition, B cells act as APCs by presenting alloantigens through self-MHC II molecules to antigen-specific T cells. During these cognate interactions, helper T cells become activated and, in turn, provide cytokines and co-stimulatory molecules to B cells that modulate their proliferation, differentiation and antibody production -including class switching- [2,18]. Allo-antibodies can participate in graft rejection by two major mechanisms, antibody-mediated activation of complement and antibody-mediated cellular cytotoxicity. Complement activation is initiated by interaction of the complement component C1q with the immunoglobulins (Ig)G and IgM, deposited on donor endothelial cells. This interaction triggers the activation of complement cascade which promotes the formation of the membrane attack complex and the lysis of the target cell. On the other hand, natural killer (NK) cells and macrophages express the low-affinity Fc receptor for IgG FcγRIII (CD16) and are able to induce apoptosis of antibody-coated cell, mechanism referred as antibody-mediated cellular cytotoxicity [2].

1.3. Innate immune system and rejection

There is no doubt that cells of the adaptive immune system, both T and B cells, are the central players of the graft rejection. However, elements of the innate immune system also play an important role in the initiation of early inflammatory responses and in the modulation of the graft rejection [19].

1.3.1. Complement

Complement is a key component of innate immune system presents in the plasma that participates in graft rejection. As mentioned previously, classical pathway of complement activation is triggered by antigen-antibodies complexes. There are two other pathways of activation that are antibody-independent, lectin and alternative pathways, which are triggered by damage-associated molecular patterns (DAMPs). In addition to the aforementioned formation of membrane attack complex which promotes the lysis of target cell, the activation of complement induces the release of soluble effectors. The anaphylotoxins C3a and C5a cause vasodilation, chemotaxis of neutrophils and macrophages and degranulation of neutrophils, amplifying the response to the graft. The opsonins C3b and C4b bind to target cells (process known as opsonisation) allowing the recognition by complement receptors on phagocytes, thereby increasing antigen presentation. The complement system also activates endothelial cells which produce pro-inflammatory molecules such as cytokines and chemokines and increases the expression of adhesion molecules, thus contributing to graft damage and destruction [1,2,12,20].

1.3.2. Innate immune cells

Innate immune cells are gaining importance in transplant rejection because of their role in modulating adaptive immune responses [21,22]. Moreover, in some experimental models, depletion or inhibition of T cells is not sufficient to induce allograft acceptance, suggesting the participation of other cell types [23,24]. Innate immune cells are not able to reject an organ by their own but can participate in the outcome of the rejection. They generate pro-inflammatory factors promoting graft damage, secrete chemokines which attract other leukocytes, produce cytokines which regulate differentiation of T effector cells and can act

as APCs. The activation of innate immune system is an antigen-independent process not related with the genetic similarities or differences between donor and recipient. Instead, the manipulation of the graft and the ischemia-reperfusion procedure, result in tissue injury [25] and the release of DAMPs [26]. DAMPs induce the activation of innate immune system through the recognition of these molecules by pathogen associated molecular patterns receptors (PRRs) [16]. DAMPs, such as heat shock proteins (HSPs), hyaluronan, high-mobility group box protein-1 (HMGB1), biglycan and heparan sulphate, are released by necrotic cells and extracellular matrix components under degradation. PRRs are transmembrane and soluble receptors that link innate and adaptive immunity in response to pathogens, although they also react to host-derived danger signals. PRRs are composed by a set of family receptors that include Toll-like receptors (TLRs), RIG-like helicases (RLHs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), as well as scavenger receptors and complement receptors [26–28]. Recognition of dangers signals by innate immune cells through PRRs triggers an inflammatory response by the secretion of proinflammatory cytokines, chemoattractant chemokines and type I interferons. As a result, donor APCs get activated and migrate to draining lymph nodes to initiate direct pathway of allorecognition. Another consequence is the recruitment of leukocytes into the graft, where they can amplify the immune response against the transplant. Some of the cell types of the innate immune system that have a role in allograft rejection are NK cells, macrophages and dendritic cells, among others. NK cells kill donor cells that lack self MHC I molecules including donor DCs, which increase the availability of donor alloantigens. In addition, NK cells through the secretion of IFN-γ and TNF-α induce the maturation of DCs and by producing IFN-γ promote the polarization of CD4+ T helper cells toward Th1 profile. [18,29–31]. Macrophages are rapidly recruited to rejecting allografts. Macrophages participate in acute responses by producing proinflammatory cytokines (IL-1, IL-6, TNF-α), reactive nitrogen and oxygen species and proteolytic enzymes therefore amplifying tissue damage. Macrophages can also phagocytose necrotic debris increasing antigen presentation to effector T cells [12,22]. Dendritic cells play an important role in initiating immune responses linking innate and adaptive immune systems. Both donor and recipient DCs participate in alloantigen presentation to antigen-specific T cells and modulate B cell responses by direct and indirect mechanisms. However, DCs also regulate immune

responses. This dual ability to activate and modulate immune system situates DCs as a fundamental key in transplantation.

2. DENDRITIC CELLS

2.1. Dendritic cells: a heterogeneous population

DCs constitute a heterogeneous population that arise from bone marrow hematopoietic progenitor cells. DCs can be divided into subtypes according to their phenotype, functions and localization [32–34]. In mice, under homeostatic conditions (steady-state), DCs can be defined as lymphoid tissue-resident DCs and tissue-resident DCs [35].

1) **Lymphoid tissue-resident DCs**. DCs residing in lymphoid organs (spleen or lymph nodes) do not traffic from peripheral tissues. Instead, they enter directly from blood through high endothelial venules. Both conventional and plasmacytoid DCs are present in lymphoid organs.
    1. **Conventional DCs (cDCs)**. Conventional DCs express high levels of CD11c and can be subdivided according to the expression of CD8α into CD8+ CD4- CD11b- DEC205 (CD205)+ DCs **(CD8+ DCs)**, CD8- CD4+ CD11b+ DEC205- DCs **(CD4+ DCs)** and **CD8- CD4- DCs** (known as double negative DCs) [33]**.** Whereas CD4+ DCs are found in marginal zones of the spleen, and the subcapsular sinuses of the lymph nodes, CD8+ DCs are located at T-cell rich areas of lymphoid organs and play and important role in antigen cross-presentation to CD8+ T cells [36].
    2. **Plasmacytoid DCs (pDCs)**. Mice pDCs in the steady state do not migrate to peripheral tissues, a characteristic shared with human pDCs. In mice pDCs express intermediate level of CD11c, low levels of MHC II and are negative for CD11b. This population is positive for B220 (CD45RA) and LY6C and selectively express SIGLEC-H and BST2 (also known as CD317 or PDCA-1).
2) **Migratory DCs**. Migratory DCs reside in peripheral non-lymphoid tissues and migrate to draining lymph nodes. All of them express CD11c.

1. **Langerhans cells** reside in the epidermis and express high levels of langerin (CD207).
2. **Epithelial DCs**. Epithelial DCs can be subdivided as CD11b+ CD103- DCs (also known as dermal or interstitial DCs) and CD11b- CD103+ DCs.

Moreover, under inflammatory conditions, blood monocytes can differentiate into DCs which express CD11c, MHCII and DC-SIGN (CD209a) [37], although recently it has become a matter of controversy.

In human, the best characterized DCs are peripheral blood DCs and peripheral tissue-resident DCs [38].

1. **Peripheral blood DCs** comprise two main populations that can be differentiated by CD11c expression, conventional DCs (CD11c+) and plasmacytoid DCs (CD11c-). Both subsets lack lineage markers such as CD3, CD14, CD19 and CD56. Both populations can be found in spleen [39].
   - 1.1. **Conventional DCs (cDCs).** The cDC population is positive for the myeloid markers CD13 and CD33 and also express CD11c and MHC II. cDCs can be further subdivided in CD1c (BDCA1), CD16 and CD141 (BDCA3) [35,39–41].
   - 1.2. **Plasmacytoid DCs (pDCs).** pDC express CD4 and CD123 (IL-3Rα) and the specific markers CD303 (BDCA2), CD304 (BDCA4) and ILT7 [42,43].
2. **Peripheral tissue-resident DCs.** This population can be subdivided in Langerhans cells which express langerin and CD1a, and Interstitial DCs which are positive for DC-SIGN and CD11b [40]. Of note, in humans this two populations can be differentiated from CD34+ hematopoietic progenitors cells in presence of GM-CSF and TNF-α [40].

Due to the low number of DC in vivo, in many studies DCs are differentiated in vitro from monocytes [34]. This type of DCs is commonly used for immunotherapy.

Finally, as stated by Ziegler-Heitbrock et al. [44] there is not available data about rat blood DCs. However rat splenic DCs subtypes have been well defined as conventional DCs [45] and plasmacytoid DCs [46]. Conventional DCs are OX62+ (CD103) and CD11b+ and can be further subdivided into CD4+ SIRPα+ (CD172a) DCs and CD4- SIRPα- DCs [45]. These two

CD4+ and CD4- DCs populations can be also purified from afferent lymph [47]. Rat splenic pDCs are negative for OX62 and CD11b, express high levels of CD4 and are positive for CD45R and SIRPα [46]. Additionally to the freshly isolated splenic DCs, the generation of rat DCs from bone marrow progenitors in presence of different combinations of growth factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), GM-CSF, IL4 and fms-like tyrosine kinase 3 ligand (FLT3L), has been well documented [48–55]. Rat bone marrow derived dendritic cells (BMDCs) express high levels of MHC II, are positive for SIRPα and CD11b and express variable levels of OX62 and CD4.

2.2. Dendritic cells: antigen presenting cells and linkers of innate and adaptive immune responses

As described before, DCs are widely distributed throughout the body in both lymphoid and non-lymphoid tissues. DCs are highly efficient antigen-presenting cells specialized in sampling the environment for capture, processing and presentation of antigens. DCs subsets are endowed with different endocytic mechanisms to capture antigens [56]. Moreover, DC subsets cooperate between each other to mount adequate responses [33,56]. DCs are capable of responding to pathogens that invade the organism, and they are also able to sense tissue damage by recognizing pathogen-associated molecular patterns (PAMPs) and molecular-associated molecular patterns (DAMPs) through pattern recognition receptors (PRRs) [57]. DCs express several PRRs such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors. After encounter the antigen, DCs mature and migrate to lymphoid organs where they present antigens and prime naïve T cells efficiently thanks to their unique ability to upregulate MHCII molecules and co-stimulatory molecules such as CD40, CD80, CD83 and CD86. Furthermore, DCs secrete a broad array of cytokines and chemokines which attract and modulate innate and adaptive immune cells [40]. In addition, DCs have the ability to present exogenous antigens (such as virus that do not infect DCs or apoptotic bodies) via MHC class I molecules to CD8+ T cells, a process known as cross-presentation [58]. These features together with their migratory capacity [59] make DCs key players in induction and regulation of innate and adaptive immune responses.

Besides its role in the activation of T cells, DCs also modulate B cells responses **(Figure 2)**. In vivo, it has been shown that antigen-loaded injected DCs modulate antibody production suggesting a role for DCs in antigen presentation to B cells [60–62]. B cell priming requires the recognition of antigens in a native (unprocessed) state [63]. DCs can transfer antigens directly to B cells in the spleen [60] by a mechanism in which antigens are internalized and recycled to the cell surface as native antigens [64]. In addition, DCs influence B cell responses through the production of soluble factors. DCs secrete BAFF and APRIL which enhance B cell survival, proliferation, differentiation and antibody production [60,65,66]. DC-mediated production of cytokines such as IL-12, IL-6 or type I interferons also appears to influence the differentiation of B cells to plasma cells and class switching [67,68]. Moreover, DCs can regulate B cells through cell contact-dependent such as CD40-CD40 ligand (CD40-L) or CD70-CD27 interactions [69,70].

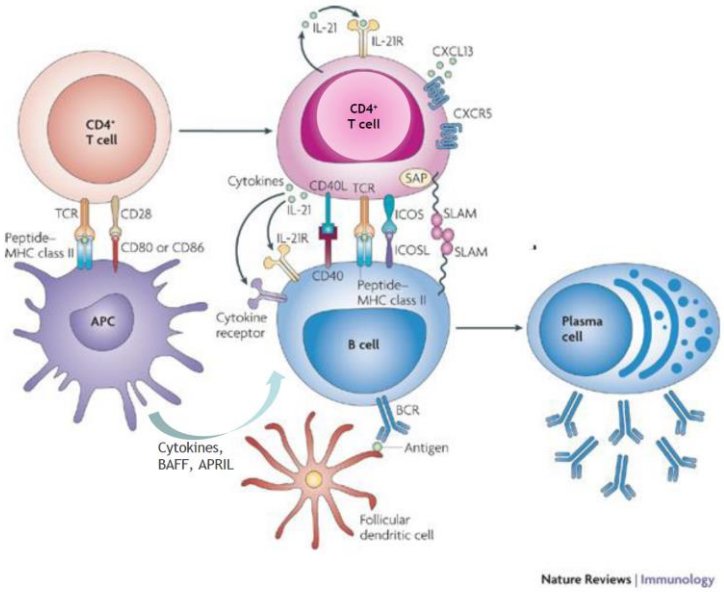


Figure 2. Dendritic cells modulate T and B cell responses. Illustration adapted from [71], Nature Reviews Immunology.

In transplantation, allo-antigen presentation and T cell priming by DCs occurs through direct pathway and indirect pathway initiated by donor and recipient DCs respectively [7]. As a consequence of ischemia/reperfusion-mediated injury, graft releases pro-inflammatory mediators and TLRs ligands which activate donor DCs [25]. After activation, donor DCs migrate to lymph nodes and spleen where they present intact allo-MHC molecules to specific T cells [72,73]. Moreover, donor DCs may function as suppliers of allo-MHC molecules for lymphoid organs-resident DCs [8]. On the other hand, chemokines released by the graft and the up-regulation of adhesion molecules expressed by endothelial cells induce the migration of circulating recipient DCs into the graft [14]. Then, recipient DCs capture, process and present donor alloantigens in the context of self-MHC molecules to CD4+ T cells and CD8+ T cells after migration to lymphoid organs [10]. The profile of cytokines secreted by DCs will further influence the type of response generated against the graft [40,74].

2.3. Dendritic cells: regulators of immune responses

Tolerance can be defined as a partial or total inhibition of an adaptive immune response. The immune system is provided by several mechanisms to induce tolerance to self-antigens and control excessive responses against foreign antigens. Central tolerance is the mechanism by which self-reactive lymphocytes are eliminated in the thymus to avoid autoimmunity. Peripheral tolerance serves as a mechanism to delete any remaining self-reactive lymphocyte and to prevent immune responses against harmless antigens. DCs participate in both central and peripheral tolerance. This section will focus on induction and maintaining of peripheral tolerance by DCs.

2.3.1. Which DCs participate in peripheral tolerance?

Dendritic cells in the steady state express low levels of MHC II and co-stimulatory molecules, displaying an immature phenotype. These immature DCs present self-antigens to self-reactive T cells leading to T-cell tolerance [75]. Indeed, in vivo experiments have shown that delivering antigen to quiescent DCs in absence of inflammation induces T cell anergy, deletion of antigen-specific T cells or the induction of regulatory T cells (Treg) [76,77]. However, mature DCs are also involved in tolerance induction. In vitro

experiments have shown that activated DCs expand Treg efficiently [78]. Interestingly, disruption of E-cadherin-mediated contact induces an alternative form of maturation in DCs, triggering up-regulation of MHC II and costimulatory molecules and chemokines receptors. Yet, these E-cadherin-activated DCs do not secret inflammatory cytokines and induce regulatory T cells [79]. Moreover, activated pDCs through TLR or CD40 ligation induce regulatory T cells [43]. Thus, the tolerogenic function of DCs depends on several factors such as a certain maturation state, the nature of microbial stimuli and tissue microenvironment. Indeed, various subsets of DCs located in lymphoid organs and mucosal surfaces induce T-cell tolerance. These specialized DCs subsets in the mucosal compartments avoid excessive reaction to commensal bacteria, food antigens or environmental antigens. For example, CD103+ DCs subsets in gut and lungs and CD103- DCs in the skin induce Treg by different mechanisms [80–83]. In mice, splenic CD11b+ CD8- DCs at the steady state induce tolerance to self-antigens [84]. Like myeloid DCs, plasmacytoid DCs induce tolerogenic T-cell responses [85]. For instance, liver pDCs contribute to oral tolerance [86] whereas lung pDCs prevent excessive immune responses to harmless antigens [87].

2.3.2. Mechanisms of tolerance induction

DCs induce T-cell tolerance by several mechanisms which promote T-cell anergy, deletion and induction/de novo differentiation of Treg [75,88] **(Figure 3)**. T cell anergy can be defined by functional inactivation of antigen-specific T cells in response to antigen presentation in absence of co-stimulation [89]. Deletion/apoptosis of T cells is a mechanism of activation-induced cell death and involves Fas-FasL interactions [90,91]. Also, DCs induce the expansion of natural Foxp3 regulatory T cells or de novo differentiation of Foxp3-expressing T cells. In addition, tolerogenic DCs promote the induction of Foxp3-negative T cells such as regulatory type 1 T cells (Tr1) [92].

As mentioned before, DCs promote tolerance through the presentation of antigens in the absence of potent co-stimulation. Moreover, tolerogenic DCs generate and maintain regulatory responses through the secretion of several anti-inflammatory and suppressive factors such as IL-10, transforming growth factor (TGF)-β, indoleamine-2,3-dioxygenase

(IDO) and retinoic acid, among others. IL-10 is an important anti-inflammatory cytokine involved in limiting/controlling immune responses against pathogens and in the maintenance of homeostasis [93]. DCs secrete IL-10 to promote antigen-specific T cell anergy and the induction of regulatory T cells [94,95]. TGF-β plays an important role in the differentiation of Foxp3+ T cells by DCs during antigen presentation [77]. In addition, TGF-β maintains the suppressive function and survival of Treg and the expression of Foxp3 [96]. Moreover, this cytokine seems to be involved in maintaining IDO in tolerogenic DCs [97]. IDO is an immunoregulatory enzyme that degrades the amino acid tryptophan, which is crucial for the development of effective T cell responses. Different subsets of IDO-expressing DCs supress and regulate T cell responses [98–101]. Retinoic acid, a metabolite of vitamin A, plays a fundamental role in the regulation of immune responses in the intestinal tract and in the skin. The induction of Foxp3 regulatory T cells by CD103+ DCs from intestine lamina propria and by dermis-derived CD103- DCs relies on retinoic acid [81], being necessary the presence of TGF-β [80–82].

Besides the secretion of anti-inflammatory products, tolerogenic DCs express several ligands and receptors that allow them to control immune responses. Inducible T-cell co-stimulator (ICOS) is an inducible molecule of the CD28 family expressed by T cell after antigen recognition. ICOS-ligand (ICOS-L/B7-H2) is expressed by tolerogenic DCs, and after interaction with its receptor on T cells mediates the suppression and anergy of T cells [102] or the generation of regulatory T cells [103].The molecule programmed death-1 (PD-1) is another member of the CD28 family expressed by activated T cells. The interaction of PD-1 with their ligands, the co-inhibitory receptors PDL-1 (B7-H1) and PDL-2 (B7-DC) expressed on DCs provide negative signals to T cells [104] or generate antigen-specific regulatory T cells [105]. The inhibitory receptors Immunoglobulin (Ig)-like transcript (ILT)-3 and ILT-4 are expressed by tolerogenic DCs [106]. It has been reported that tryptophan deprivation and suppressor CD8+ CD28- T cells induce the up-regulation of ILT-3 and ILT-4 on DCs, providing them with tolerogenic properties which translates in the induction of Foxp3+ regulatory T cells [107] or the ability to promote anergy in CD4+ T cells [108].

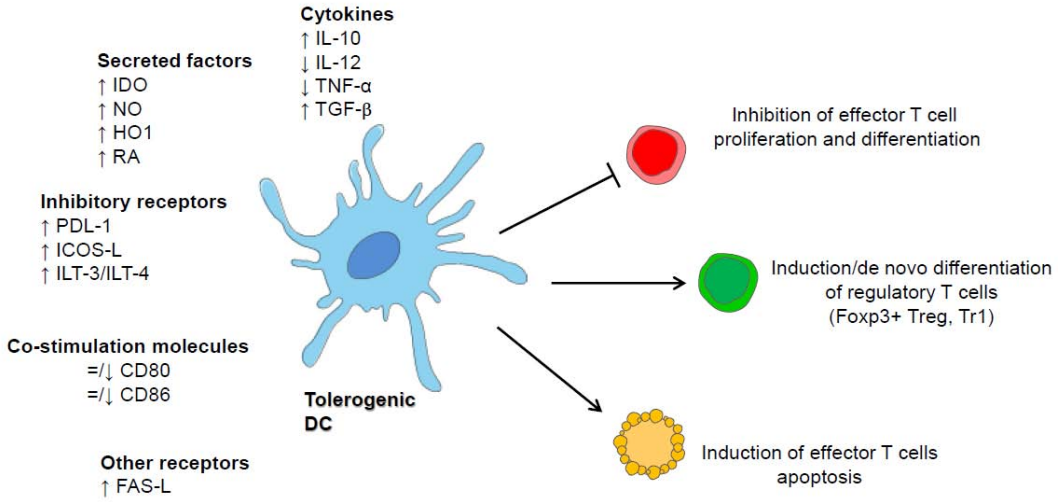


Figure 3. Mechanisms involved in tolerance induction by tolerogenic DCs.

3. STRATEGIES FOR TOLERANCE INDUCTION IN TRANSPLANTATION

3.1. Long term immunosuppressive therapies in transplantation

Transplant patients require the administration of immunosuppressive drugs to avoid transplant rejection. The classical immunosuppressive therapies (corticosteroids, calcineurin inhibitors, mTOR inhibitors, antiproliferatives) are generally non-specific and their chronic administration causes drug toxicities, opportunistic infections and malignancies [109]. Novel strategies have been developed and include monoclonal antibodies or fusion receptor proteins that target co-stimulatory receptors and co-receptors affecting more specific populations. Some examples are blocking antibodies against CD154 or anti-CD80/CD86, and fusion proteins such as cytotoxic T-lymphocyte antigen-4(CTLA-4)-Ig that binds to CD80/CD86 [110]. Unfortunately, some of these therapies caused serious complications such as thromboembolic events [111]. Other approaches consider the use of depleting agents which target alloreactive effector lymphocytes such as OKT3 antibodies, polyclonal antibodies preparations and anti-CD52 [109]. However these therapies do not deplete memory T cells because of their resistance

to depletion [112] or to classical co-stimulation blockade [113]. Regarding B cells, there are new strategies for both depletion/inhibition of B cells and elimination of circulating antibodies. Some of these therapies come from treatments for autoimmune diseases and include antibodies against CD20 and CD22, or against B cell activating factors such as BAFF and APRIL [114]. Although current therapies induce long-term immunosuppression, there still exists the need for allograft tolerance, defined as lack of immune responses to the graft in the absence of maintained immunosuppression. Some novel strategies to induce donor-specific tolerance include targeting and infusion of specific immunoregulatory cells such as DCs, regulatory T cells, regulatory macrophages, mesenchymal stem cells and myeloid-derived suppressor cells [115–118].

3.2. Tolerogenic dendritic cells in transplantation

One of the possible approaches to induce long term allograft tolerance is the use of tolerogenic DCs. However, implementation of tolerogenic DC therapy in clinical transplantation needs the consensus about several parameters that include the origin of DCs (donor or recipient), the tolerogenic agent, the dose, time and frequency of injection, the route, the type of combined immunosuppression (if needed) and the origin of donor allo-antigens (when recipient DCs are chosen). Different strategies have been developed to test all these factors in animal models **(Figure 4)**.

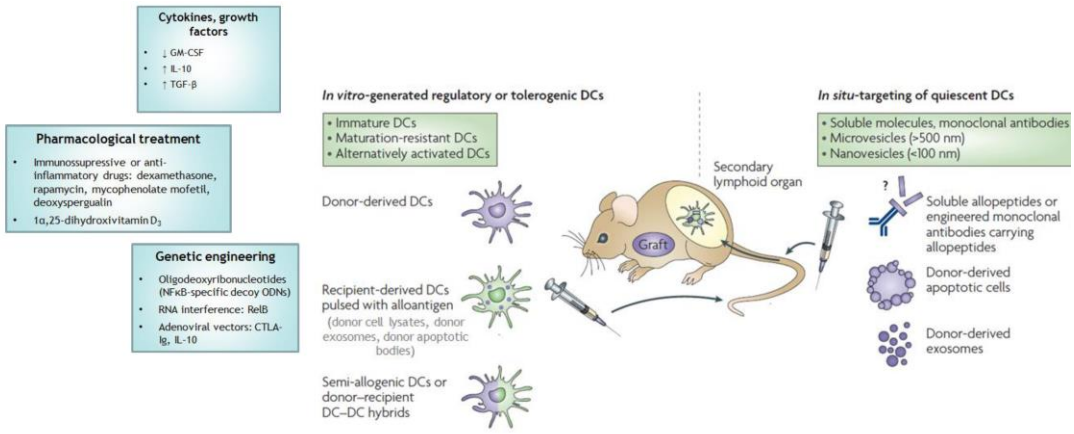


Figure 4. Different approaches using DCs for tolerance induction in transplantation. Illustration adapted from [119].

The ideal tolerogenic dendritic cell should be capable of capturing antigens, capable to migrate to secondary lymphoid organs and, once injected, be resistant to further maturation [120]. Another important feature would be the capacity to inhibit memory T cells responses due to its great proportion in humans and its resistance to conventional therapies [121].

3.2.1. Generation of tolerogenic DCs

During the last decades, the ability of tolerogenic dendritic cells to control immune responses has been exploited to treat autoimmune disorders and to induce long lasting tolerance in transplantation. First studies reported the successful use of immature DCs in tolerance induction [122–124]. However, the risk of further maturation of injected DCs has prompted the development of a variety of strategies to maintain DCs in an immature state and generate stable tolerogenic DCs.

Several approaches have been developed to regulate DCs biology. Pharmacological drugs, classically used in transplantation such as rapamycin (RAPA) and glucocorticoids have been employed to induce tolerogenic DCs. These immunosuppressive agents interfere with DCs biology at different stages: differentiation, antigen uptake, maturation and migration [125]. The serine/threonine kinase mammalian target of rapamycin mTOR, besides playing a role in T cell proliferation, is also involved in DCs maturation and function. The macrolide rapamycin (Sirolimus) interferes with mTOR signalling in DCs resulting in the inhibition of differentiation and maturation, the impairment of antigen uptake, the alteration of cytokine production and the modulation of migratory abilities [126]. Glucocorticoids are steroid hormones that negatively regulate the canonical NFκB pathway through its binding to the glucocorticoid receptor [127]. Dexamethasone, a synthetic member of this family, exerts its immunosuppressive effects on DCs by altering differentiation, maturation and migration of these cells [128]. Other agents that also interfere with NFκB signalling, and therefore with DC maturation, are deoxyspergualin and its analogue LF15-095 [119]. In addition, various biologic agents such as cytokines (IL-10, TGF-β, PGE-2) and growth factors have been exploited to generate tolerogenic DCs [129]. Moreover, it has been reported the use of low concentrations of GM-CSF to generate

immature, resistant to maturation DCs [124]. The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3], is a steroid hormone that regulates calcium/phosphate metabolism [130]. Vitamin D3 affects differentiation, maturation and antigen presentation in DCs [131]. Gene therapy, through the interference with NFκB pathway –responsible for DCs maturation- or through the blockade of co-stimulatory molecules, is another approach in the generation of tolerogenic dendritic cells [132].

3.2.2. Origin of DCs: Donor versus recipient DCs

Transplantation is a unique immune scenario where the antigens can be presented by three pathways as mentioned before: direct allorecognition, indirect allorecognition and semi-direct allorecognition. Different strategies using donor, recipient or semi-allogeneic DCs have been developed to modulate these pathways.

3.2.2.1. Donor DCs

Donor DCs are responsible for the direct pathways of allo-antigen presentation and, consequently, of acute transplant rejection. The use of donor DCs have been broadly studied in experimental models with success [123,124,133,134]. Initial studies showed that differentiation of bone marrow precursors with GM-CSF, in the absence of IL-4, generated DCs with an immature phenotype capable of induce weak allostimulatory responses [122] and with the ability to prolong heart grafts survival in mice [123,124]. In a rat cardiac transplantation model, the use of low doses of GM-CSF plus IL-4 in combination with anti-lymphocyte serum (ALS) transient immunosuppression has been shown to prolong allograft survival in rats [135].

Not only conventional myeloid DCs are able to regulate immune responses in transplantation, several studies have reported the successful use of donor plasmacytoid DCs [85]. pDCs precursors propagated from bone marrow cultures in the presence of FLT3L show an immature phenotype and are inefficient allogeneic-T cells stimulators. Moreover, a single injection of pDCs precursors prior to heart transplantation prolongs graft survival in mice, although not in a donor-specific way because the injection of third party pDCs precursors also prolonged allograft survival [133].

However, once injected donor DCs may mature and lose their immunomodulatory properties promoting the induction of immunogenicity. For that reason, strategies directed to generate stable immature/tolerogenic donor DCs have been developed. It has been reported the prolongation of allograft survival of recipients injected with donor BMDCs treated with NFκB oligodeoxyribonucleotides (ODNs), which results in blockade of co-stimulatory molecules expression and nitric oxide production after TLR-4 ligation [136]. This therapy is more effective when BMDCs are treated with NFκB ODNs in combination with adenoviral vectors encoding CTLA4-Ig [132]. Another approach targeting NFκB pathway involves the silencing of RelB protein by small interfering ribonucleic acid (siRNA) in BMDCs. The RelB-silenced dendritic cells showed maturation resistance to CD40L-mediated activation and prolonged heart allografts survival in mice [137].

The generation of donor tolerogenic DCs in the presence of different growth factors has also been described. Differentiation of BMDCs in presence of TGF-β and IL-10 produces alternatively activated DCs that, after stimulation with lipopolysaccharide (LPS), are maturation resistant. They secrete IL-10 but show little IL-12 production and induce poor allostimulatory responses expanding regulatory Foxp3+ T cells after mixed leukocyte reaction. After infusion in heart allograft recipient they are able to prolong graft survival, effect potentiated by a single administration of CTLA4-Ig [138]. This strategy has also be proven successful in corneal grafts [139].

Alternatively activated or semi-matured DCs can be generated in the presence of immunosuppressive drugs. Conditioning of bone marrow progenitors with a 1α,25 dihydroxyvitamin D3 analogue generates donor immature DCs, resistant to maturation by TLR-4 and CD40 ligation and with the ability to prolongs graft survival in a mouse model of skin transplantation [140]. In another study, the pre-treatment of BMDCs with the glucocorticoid dexamethasone prior to LPS stimulation generated donor tolerogenic DCs. These DCs displayed a semi-mature phenotype, showed a high IL-10 production while IL-12 secretion was reduced and, after intravenous injection, were able to prolong heart allograft survival in mice [141].

However, it has recently been demonstrated that tolerogenic donor-derived DCs not only failed to induce tolerance but also accelerated graft rejection [142]. Alternatively, it

has been shown that despite of inducing tolerance, the mechanism depends on recipient DCs, which are able to capture and present donor allo-antigens to T cells by indirect pathway [143]. In a similar way, it has been reported that recipient NK cells can eliminate donor DCs contributing to transplant tolerance [144]. All these studies suggest that the use of donor dendritic cells may not be the ideal strategy for the induction of tolerance. Other disadvantage is the impossibility to differentiate tolerogenic DCs from deceased donors, although this situation can be overcome in living transplantation.

3.2.2.2 Recipient DCs

Recipient DCs may represent a more safety strategy for recipients than donor DCs and can be prepared at any time. As in the case of donor DCs, several approaches have been investigated to generate tolerogenic DCs in order to induce tolerance in transplantation. Most strategies involve loading the recipient DCs with donor-alloantigens prior to injection. It has been reported that treatment of heart allograft recipient with bone marrow-derived or thymic host DCs loaded with donor peptide, in combination with anti-lymphocyte serum injection, results in permanent allograft acceptance [145]. Significantly, some groups have described the use of non-pulsed recipient DCs to induce transplant tolerance [52]. After differentiation of BMDCs in presence of GM-CSF plus IL-4, adherent DCs showed resistance to maturation after polyinosinic-polycytidylic acid (poly I:C), LPS and CD40L stimulation. These maturation-resistant DCs, once intravenously injected in heart recipient rats, migrated to spleen and were able to prolong graft survival by a mechanism partially dependent on nitric oxide [52]. The same group reported that the administration of sub-therapeutic doses of rapamycin and LF15-095 together with these tolerogenic DCs was necessary to achieve indefinite survival [146]. The mechanism by which these immature DCs induce tolerance involves the generation of double negative CD4 and CD8 TCR$\alpha\beta$+ T cells (DNT), the expression of EBI3 by DCs and IFN-γ secretion by this DNT population [147].

The generation of tolerogenic DCs through conditioning of recipient BMDCs with rapamycin has been described by different groups. RAPA-DCs do not show an impaired ability to capture allo-antigens or to migrate to secondary lymphoid organs, are resistant

to maturation by various stimuli, produce high doses of IL-10 and expand naturally regulatory T cells. After loading with donor spleen cells lysates and infusion intravenously in recipients, RAPA-DCs are able to induce long-term survival of skin and limb grafts, and to induce indefinite survival of heart grafts [148–151]. In all these studies animals received a short rapamycin treatment [148], sub-therapeutic doses of FK506 [151] or a combination of cyclosporine A and anti-lymphocyte serum [149,150].

Expanded pDCs from spleen and lymph nodes of FLT3L-treated animals are able to prolong heart allograft in a mouse model deficient for CCR7, under CD40 co-stimulatory molecule blockade [152]. In fact, it has been described that lymph nodes-resident pDCs are responsible for tolerance induction after heart transplantation. In this model, pDCs were able to migrate to the graft, capture allo-antigens and induce the generation of donor-specific regulatory Foxp3 T cells in the lymph nodes. Moreover, the infusion of pDCs from tolerized mice injected in naïve recipients prolonged graft survival [153]. By contrast, it has been reported that treatment of heart graft recipients with CD40-Ig resulted in pDCs accumulation in spleen and allograft, but not in lymph nodes, effect that correlated with the generation of CD8+ regulatory T cells and tolerance induction [154].

3.2.2.2.1 In situ targeted DCs.

Because of the dogma that capture of antigens in absence of danger/inflammatory signals program immature DCs to be tolerogenics, some groups have studied the induction of tolerance through the delivery of allo-antigens to quiescent host DCs. [75]. It has been reported the administration of alloantigen-coupled antibody to induce allograft tolerance [155]. In this study, targeting of MHC class I peptide to the 33D1 antibody deletes alloantigen-specific T cells, inhibiting indirect alloresponse against the graft and the production of alloantibodies. Nevertheless, in transplantation there is more than one allo-peptide, so it would be difficult to administer the entire repertoire of alloantigens by this way. Therefore, other strategies that include a broader repertoire of donor alloantigens have been experimented, for instance the administration of apoptotic bodies or exosomes [156,157]. In a fully-mismatched aortic allograft model, apoptotic donor splenocytes intravenously injected were captured by splenic conventional, but not plasmacytoid, DCs.

After apoptotic cells internalization, these DCs remained quiescent, were maturation resistant ex vivo and, although presented allopeptides for a limited period of time, down-regulated the indirect pathway of presentation and reduced the levels of alloantibodies [156]. Similarly, treatment of heart transplant recipients with donor exosomes prolonged allografts survival [157] that resulted in long-term allograft survival when combined with short term LF15-095 treatment [158].

3.2.2.3 Semi-allogeneic DCs

Because both the direct and indirect presentation are important in transplantation, a third approximation has been exploited by some groups which consists in the generation of semi-allogeneic DCs that express recipient and donor MHC molecules at the same time. (Recipient x Donor) F1 BMDCs differentiated in vitro in presence of dexamethasone prolonged graft survival in a model of kidney transplantation in rats. Whereas pre-treatment of animals with these tolerogenic DCs alone failed to prolong kidney allografts, when a single injection of CTLA-4-Ig was administered the recipient accepted the graft after a short course of cyclosporine A to inhibit direct presentation. Although (recipient x donor) BMDCs expressed both MHC molecules from donor and host, only indirect pathway of allo-presentation was inhibited, showed by ex vivo challenge of splenic T cells from immunized animals cells with donor DCs and donor antigens-pulsed syngeneic DCs [159]. A similar strategy using (recipient x donor) F1 BMDCs, transduced to express IL-10 and CCR7 and injected one week before transplantation has been demonstrated to induce indefinite heart allografts survival in mice. In this approach, CCR7 expression seems to be essential for tolerance induction thanks to the ability of CCR7-transduced DCs to migrate to secondary lymphoid organs [160].

3.2.3 Other parameters to consider

Regardless to the source of DCs, there are no reports that show permanent graft acceptance using only DCs. The best results have been observed in studies where tolerogenic DCs were combined with the administration of immunosuppressive agents, to prevent host and donor DCs maturation or to deplete T cells [145–148,151,161]. However, it should be considered that these agents may interact with the injected cells and have

some impact on them [162,163], so the choice of the immunosuppressive agent must be done carefully.

Also, an important parameter is the half-life of injected DCs, which may vary depending on the source of DCs. Whereas some groups have reported the presence of syngeneic DCs in lymphoid organs for at least two weeks [52,164,165], others have reported the elimination of donor DCs within 3 days [143]. Therefore, it is important to establish what frequency and time of injection are most adequate. Regarding the frequency of administration, contradictory results have also been shown. It has been reported that repetitive injections do not improve allograft survival [146] but other study has shown better results with multiple injections [151]. Concerning the time of vaccination, most of the studies have evaluated the administration of DCs prior to transplantation, usually one week before. However, very few studies have addressed DCs injection post-transplantation [149,150]. Administration of tolerogenic DCs post-transplantation seems feasible when combined with transient immunosuppression to avoid initial acute rejection and the loss of immunomodulatory properties of injected DCs.

3.2.4 Source of alloantigens

Due to the high variability of the MHC antigens, many studies have chosen donor cell-free lysates as a source of donor allo-antigen [148–151,166,167]. However, alternative sources of alloantigens such as exosomes can be considered. Exosomes derived from APCs are nanovesicles enriched in class I and class II MHC molecules [168]. In contrast to cell lysate or apoptotic bodies, exosomes represent a more controlled source, are relatively stable and can be cryopreserved. Additionally, the production of clinical grade exosomes have been developed [169]. Moreover, exosomes-derived allo-MHC molecules may be processed and presented as allopeptides in the context of self-MHC molecules by DCs or, alternatively, can be directly recycled to the cell surface and presented as intact donor MHC molecules [170]. This scenario would be similar to what happen in semi-allogeneic DCs as mentioned before. Thus, exosomes-loaded tolerogenic DCs could modulate both direct and indirect pathways through the direct and indirect presentation of allo-antigens.

INTRODUCTION II. EXOSOMES

Cellular communication is a tightly controlled event to assure correct coordination among different cell types. Cells use different mechanisms to communicate such as soluble molecules, nanotubes, exchange of membrane patches, and the release of microvesicles.

Exosomes were first described by two independent groups as a mechanism to eliminate obsolete proteins during reticulocytes maturation [171] [172], although the term exosomes was not used until 1987 [173]. These works suggested an alternative mechanism to lysosomal degradation by which transferrin receptor (TfR), together with other membrane proteins, was removed from plasma membrane bound to these vesicles. Ten years later, Graça Raposo described that B cells secreted vesicles with antigen presenting functions [174]. Since then, an increasing number of publications have demonstrated that exosomes are secreted by a variety of cell types. In addition, these vesicles can be found in several biological fluids such as urine, plasma, malignant effusions, etc.

2.1. BIOGENESIS

The most accepted theory for exosomes biogenesis is that these vesicles arise from endosomal compartments by inward budding. Exosomes are formed by inward budding of the limiting membrane of late endosomes called multivesicular bodies (MVB). This process generates nanovesicles (30-100 nm) with the same topological orientation that the plasma membrane [172]. Upon fusion of MVB with plasma membrane, they are released to the extracellular milieu and termed exosomes. The mechanisms by which proteins and lipids are incorporated into these vesicles are not well understood but it seems that involve the endosomal sorting complex required for transport (ESCRT), lipid metabolism and tetraspanins-enriched microdomains **(Figure 5)**.

ESCRT machinery. Because exosomes arise from MVB [172,174], it was postulated that machinery involved in the formation of internal vesicles of MVB also participated in exosomes generation [175]. This mechanism proposes that ubiquitinated proteins at plasma membrane are recognized and sorted into endosomal membrane of MVB and then

internalized into exosomes. ESCRT is a heterogeneous complex composed of various set of proteins whose function is to recognize and sort (mono)-ubiquitinated proteins into MVB. Hrs protein, member of ESCRT-0 complex, binds to ubiquitinated cargo and recruits Tsg101, member of ESCRT-I machinery. Tsg101 interacts with ESCRT-II that, in turn, associates with ESCRT-III complex which is thought to be responsible for membrane budding.

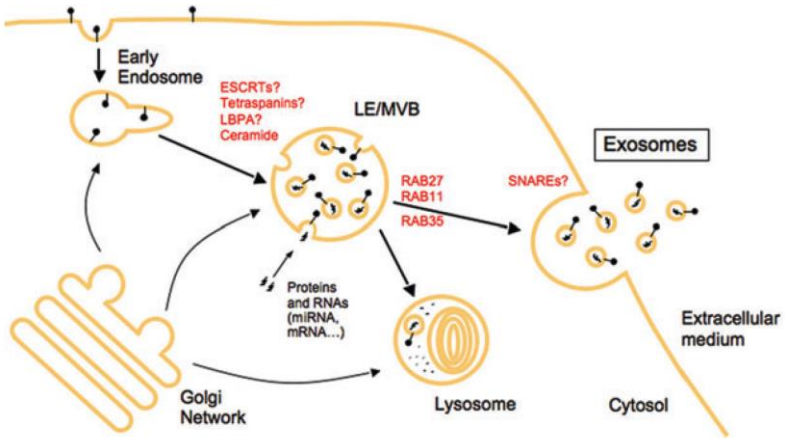


Figure 5. Mechanisms proposed for exosomes biogenesis and secretion. Illustration from [176], Traffic. Exosomes are formed by inward budding of the limiting membrane of MVB through different mechanisms such as the ESCRT complex, lipid metabolism and tetraspanins-enriched microdomains. Upon fusion of the MVB with the plasma membrane, exosomes are secreted to the extracellular medium through a process that is still unclear.

Finally, the ESCRT machinery is dissociated from MVB through VPS4 protein [177]. The presence of different members of the ESCRT machinery in exosomes suggests an important role for this system in exosomes biogenesis. The first evidence of ESCRT involvement in exosomes biogenesis was the detection of Tsg101 and Alix/AIP-1, components of this system, in DC-derived exosomes [178]. Tsg101 was also found in T cell exosomes, together with c-Cbl, a ubiquitin ligase [179]. Moreover, exosomes from dendritic cells and B cells are enriched in ubiquitinated proteins [180], the essential signal for cargo recognition and

sorting by the ESCRT complex. However, ubiquitination is not always necessary for sorting proteins into exosomes. Other ubiquitin-independent mechanism involving components of ESCRT complex has been described such as Alix/AIP-1, which binds to TfR resulting in its sorting into exosomes [181].

ESCRT-independent machinery. In an oligodendrocyte cell line, the transport of the proteolipid protein (PLP) to exosomes does not depend on ESCRT machinery. Silencing three of their components, Tsg101, Alix or Vps4, does not affect the PLP sorting into exosomes. Conversely, ceramide synthesis by sphingomyelinases from lipid-raft containing sphingolypids seems to be involved [182]. In fact, lipids and lipid microdomains seem to account for an important mechanism in exosomes biogenesis [183]. B lymphocytes-derived exosomes are enriched in cholesterol, sphingomyelin and ganglioside GM3. These lipids are characteristics of raft domains, and their association with tetraspanins CD81, CD63 and MHCII molecules suggests the presence in exosomes of lipid rafts possibly involved in membrane budding and even in membrane fission [184]. In reticulocytes-derived exosomes, lipid raft-associated proteins, such as flotillin-1, Lyn and stomatin, are present. Moreover, molecules such as MHCII are associated with these raft structures, supporting again a lipid-dependent mechanism of sorting [183]. It has been shown that clustering of some molecules, like TfR and acetylcholinesterase (AChE), increases their presence in exosomes [185] maybe mediated by its GPI-anchor, as it occurs in the case of CD55 and CD59 that are also included in exosomes [186]. In accordance with that, higher-order oligomerization is enough to target proteins into exosomes [187], mechanism that looks like to operate in the generation of virus-like particles. Clustering of exosomal cargo has also been described in B cell exosomes; crosslinking of peptide-MHCII complexes results in an increase of exosomes secretion [188]. In a similar way, after cognate interaction with T cells, mature dendritic cells sort MHCII molecules together with CD9 into exosomes by an independent-ESCRT mechanism, different from ubitiquin-dependent sorting for lysosomal degradation [189]. Finally, it has recently been reported a new mechanism involving syntenin and its interaction with the transmembrane protein syndecan and Alix for exosome budding [190].

Regarding the **release of exosomes**, although it has not been completely elucidated, some information is available. Rab11, a protein involved in membranes trafficking, is associated to the formation and release of exosomes in K562 cells [191]. Two members of the Rab family of small GTPases, named Rab27a and Rab27b, have been proposed to participate in the targeting and fusion of multivesicular endosomes, resulting in exosomes secretion in HELA cells [192]. These results have also been demonstrated in a tumour cell line [193] suggesting that this pathway takes place in other cell types. Similarly, Rab35 regulates exosome release in oligodendrial cells [194].

In addition to the generation of exosomes from endosomal compartments, there are evidences of nanovesicles released directly from plasma membrane in T cells, the so-called direct pathway [176]. These nanovesicles, also termed exosomes, can emerge from plasma membrane by direct budding through endosome-like domains which contain some exosomal proteins such as CD63, CD81, TSG101 or AIP1 [195].

2.2. COMPOSITION

Due to the potential use of exosomes in therapy, there is a great interest in the knowledge of their molecular composition. In fact, numerous proteomic studies of cell derived- and biologic fluid derived-exosomes have been performed [178,184,196–198]. As a consequence of their origin, exosomes do not contain proteins from some intracellular compartment such as nucleus, endoplasmic reticulum (ER), Golgi or mitochondria [178]. Exosomes composition varies depending on their cellular origin, although they present some ubiquitous molecules related to their generation. Recently, two web-based databases of exosomal content have been created in order to gather information about exosomes [199,200]. The knowledge about exosomal proteins, lipids and RNA molecules and their interactions will aid the scientific community in understanding the biogenesis, functions and biological significance of these nanovesicles. Exosomes, which are composed of lipid, proteins and nucleic acids, are not a mere reflect of their cellular origin, there is a selective enrichment of certain molecules whereas others are excluded **(Figure 6)**.

2.1. Protein composition

Exosomal proteins are involved in antigen presentation, cellular adhesion, cell structure and motility, trafficking and membrane fusion, lipid rafts, MVB biogenesis, signalling, metabolism and transcription and protein synthesis.

• **Antigen recognition and presentation.** Exosomes contain MHC I molecules [179,201] and those derived from professional antigen presenting cells (APC) such as dendritic cells contain MHC II molecules [202] which are also present in exosomes from other APC such as B cells [174], intestinal epithelial cells [203] and bone marrow derived mast cells [204]. Exosomes also bear co-stimulatory molecules such as CD40 [205–207], CD80 [208,209] and CD86 [188,210,211]. Besides proteins related to activation of immune system, immunomodulatory molecules such as PDL-1 and PDL-2 have also been found [212]. Other examples of molecules involved in antigen recognition are TCR and CD3ε on T lymphoblasts-derived exosomes [179] and CD19 on B cell-derived exosomes [213].

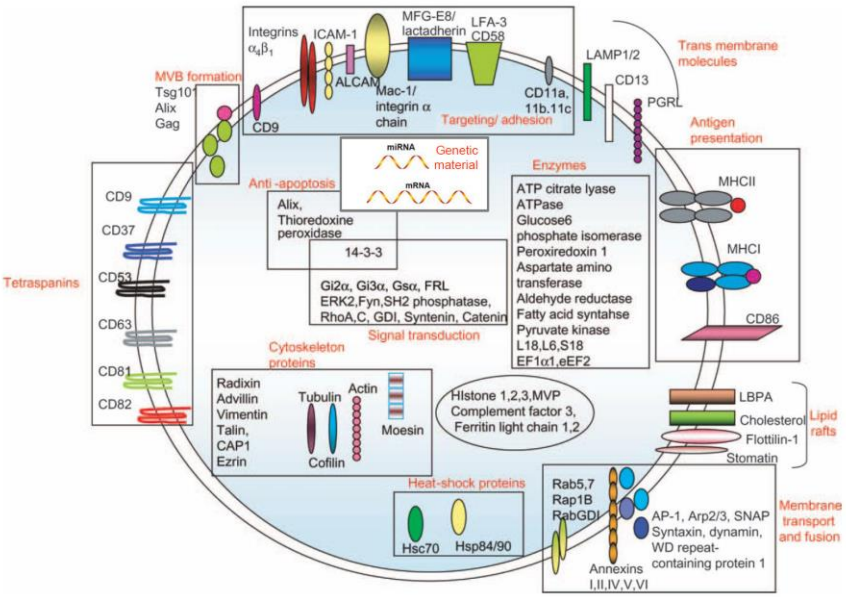


Figure 6. Schematic composition of a canonical exosome. Figure adapted from [214], Traffic.

• **Cellular adhesion**. One important characteristic of exosomes is the presence of surface molecules involved in adhesion and targeting, allowing them to potentially bind to cells or the extracellular matrix. Exosomes contain β2 integrin/CD18 [179,215] which together with integrin α1/CD11a [208] and integrin αM/CD11b [209] form LFA-1 and MAC-1/CR3, both also present in exosomes. Other integrins such as α4/CD49d [184] and β1/CD29 [198] are also expressed on exosomes. LFA-1 binds to other molecule sorted on exosomes, the adhesion molecule ICAM-1/CD54 [188,207,210]. MFGE8/lactadherin is a peripherally associated adhesion molecule also enriched in exosomes [208,216]. Tetraspanins are a family of transmembrane proteins involved in cellular adhesion but also in antigen presentation, motility, etc. Some members of this family enriched in exosomes are CD9 [189,217], CD37[211], CD63 [184,195,218], CD81 [188,219,220] and CD82 [211,221].

• **Cytosolic proteins.** The composition of exosomes includes cytosolic proteins, mainly cytoskeletal components related to **cell structure and motility** such as tubulin, actin, moesin [178,196,203,222]. Other cytosolic components are proteins related to **membrane fusion** and **trafficking** such as annexins and small GTPases family members: Rab11, Rab27 [196,202,223]. They also carry molecules related to **signalling processes** such as Gα2i, syntenin and 14-3-3 [196,202,206] and contain **metabolic enzymes** (α–enolase, kinases, dehidrogenases), [179,184,203] and proteins related to **protein synthesis** such as elongation factor1α [178]. **Chaperones** and **heat shock proteins** are another set of proteins enriched in exosomes, it has been reported the presence of hsc70 [157,196,217] and hsp94 [203,222].

• **MVB biogenesis.** There is a set of proteins related to MVB biogenesis which includes some component of the ESCRT complex such as Tsg101 [210,217,224] and AIP1/Alix [196,223,225].

• **Lipid rafts.** There is a group of proteins associated to lipid rafts that are present in exosomes, the GPI-anchored proteins CD55, CD58 and CD59 [186]; flotillin and stomatin [183,216,220].

• **Other proteins.** It has been reported the presence in exosomes of immunoglobulins [205,213,226]. The molecules CD107a/Lamp1 and CD107b/Lamp2 can also be found in exosomes [204,208,218].

• **Specific proteins.** It is worth mentioning that exosomes contain specific proteins related to their cellular origin. For example, A33 antigen (receptor-like molecule of the immunoglobulin superfamily) is present in epithelial cell-derived exosomes [203] and the specific hepatic marker ASGR receptor is expressed by exosomes derived from hepatocytes [198].

2.2. Lipid composition

The lipid composition of exosomes has not been so extensively studied as proteins. However it is known that there is a specific enrichment on exosomes of certain lipids such as cholesterol, sphingomyelin and the ganglioside GM3 compared to total cell membranes. Other lipids present in exosomes are phosphatidylethanolamine, phosphatidylcoline, phosphatidylserine and phosphatidic acid [184] or the ganglioside GM1 [186].

2.3. Genetic material

One important breakthrough has been the demonstration that exosomes contain messenger RNA (mRNA) and micro RNA (miRNA) [227]. Importantly, the authors showed that mRNA could translate into protein in target cells. As occur with exosomal protein and lipid content, there is a specific set of miRNA loaded into exosomes different from their parental cell [228,229] which varies depending on the activation state of the exosome-producing cell [230]. Conversely, others studies have reported that exosomes carry similar miRNA content compared to parental cell [231,232], highlighting the suitable use of exosomes for diagnosis of several diseases. Importantly, exosomal miRNA can be transferred to recipient cells and repress target mRNA [228,229].

3. ADHESION, CAPTURE AND INTERNALIZATION OF EXOSOMES

Once secreted, exosomes may bind to extracellular matrix components and/or cells. In fact, exosomes express several integrins [179,184,202,206,215] and adhesion molecules

such as ICAM-1 [188,207,208,210] This binding can result in the attachment to the plasma membrane of acceptor cell without internalization [233] or in the engulfment of the nanovesicles. In both situations exosomes have to interact with the acceptor membrane which can be mediated by specific ligand-receptor interactions or via lipid-dependent fusion mechanisms. After capture, exosomes can follow different pathways: fusion with plasma membrane releasing their content to cytosol, engulfment by phagocytosis or enter into the endocytic compartment via receptor-mediated endocytosis. Endocytosed exosomes can enter into the lysosomal route being digested and processed, or can back fuse with the endocytic membrane, possibly recycling some membrane components [170] **(Figure 7)**.

Several studies have demonstrated the uptake of exosomes [208] [234] [235] [236] by different cell types. But how are they delivery into the cells? In vitro experiments have shown that endocytosis of DCs-derived exosomes by BMDCs is an active process (inhibited by cytochalasin D, EDTA or low temperatures) partially regulated by integrins (CD51, CD61, CD11a), CD54, phosphatidylserine, and MFGE8. Blocking these molecules does not account for a total inhibition of exosomes uptake, suggesting that other mechanisms may be involved [208]. Other study has reported that LFA-1 expression on DCs is essential to capture ICAM-1-bearing exosomes derived from mature DCs [237]. Moreover, it has been demonstrated that DCs capture CD8+ T cell-derived exosomes via LFA-1 [238]. Exosomes are rapidly internalized in early recycling endosomes, following the endosomes/lysosomes pathway [208]. Alternatively, DC-derived exosomes can fuse or hemifuse with acceptor membrane, requiring cholesterol-enriched domains, and their content can be released into the cytosol [229].

Other mechanisms independent of endocytosis have been described, for example macrophages from nervous system, microglia, internalize oligodendrocytes-derived exosomes by macropinocytosis, process that is downmodulated after IFN-γ or LPS stimulation [235]. K562 cell- and MT4 (HTLV-transformed T leukemia)-derived exosomes are captured by RAW 264.7 macrophages by phagocytosis. TIM-4 but not TIM-1 (both receptors for phosphatidylserine (PS)) is partially involved in the capture of exosomes by

macrophages. Moreover, Dyn2, necessary for clathrin-, caveolin-dependent endocytosis and phagocytosis, was shown to participate in exosome internalization [239].

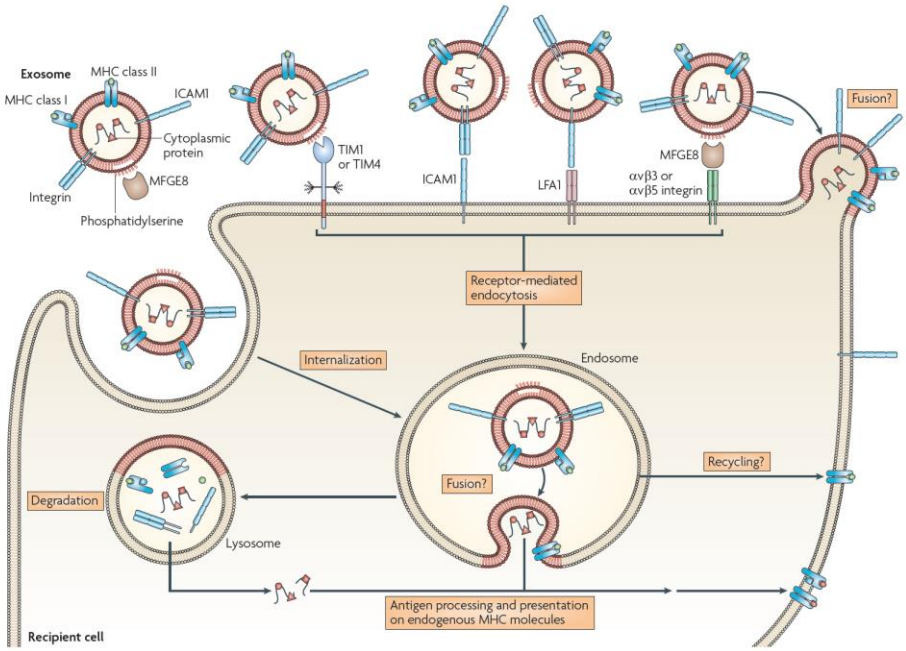


Figure 7. Interaction of exosomes with target cells. Illustration from [170], Nature Immunology Reviews.

All these experiments are performed with highly endocytic/phagocytic cells. But what happens in other cell types? Naïve T cells are not able to capture exosomes [239–241] but upon cognate interactions, activated T cells express high levels of LFA-1, which allow them to recruit DC-derived exosomes, although they are not internalized or fused with the plasma membrane [242]. The requirement of T cell activation to bind exosomes has been demonstrated by others [229]. Similarly, treatment of fibroblasts with TNF-α, which augments the expression of ICAM-1, increases the adhesion of B-cell derived exosomes, process that can be inhibited by blocking integrins β1 and β2 [215].

These results demonstrate the strong relation between the fate of exosomes and the state of activation of the acceptor cell. Another important factor which determines the

selective targeting of exosomes is the expression of specific molecules derived from the parental cells. In vitro experiments comparing the capture of different types of exosomes by various blood cells shows that breast milk and monocyte-derived DCs (MDDCs) exosomes bind preferentially to monocytes whereas Epstein–Barr virus (EBV)-transformed B cell line-derived exosomes target B cells. The binding of EBV-transformed B cell line-exosomes by B cells decreases by the blockade of CD21 and its ligand, gp350 [241]. Interestingly, follicular dendritic cells that also bind B cell exosomes, express CD21 [233].

Recently, the participation of sugar domains in exosome capture has been proposed. Jurkat-derived exosomes capture by mature DCs is almost totally inhibited by blocking Siglec-1, a sugar-binding lectin [243]. Consistent with this, mouse pDCs (which express SIGLEC-H) are able to capture exosomes in vivo [229] and galectin-5, a β galactoside-binding lectin, has been found to participate in the capture of erythrocyte-exosomes by macrophages [236]. Furthermore, exosomes from an ovarian carcinoma cell line contain glycosylated sialic acid and mannose-containing glycoproteins [234].

Using in vitro models is helpful to study isolated events of interaction between exosomes and acceptor cells, but it is also important to know what happens in vivo and what type of cell capture preferentially those exosomes. After in vivo injection, exosomes from dendritic cells are efficiently captured by splenic dendritic cells and macrophages and by hepatic kupffer cells [208,237,244]. In addition, exosomes from serum of transplanted mice, i.v. injected, are captured by splenic DCs and macrophages [244]. Importantly, pDCs although less efficient than conventional DCs, are able to capture exosomes once i.v. injected [229,244].

4. EXOSOMES FUNCTIONS

Exosomes are small vesicles mirroring their cellular origin containing a specific set of proteins, lipids and nucleic acids, which may be related to their specialized function. Since exosomes were discovered as a mechanism to eliminate obsolete proteins in maturing reticulocytes, several additional functions have been proposed. Exosomes serve as intercellular vehicles transferring molecules or delivering signals between cells. They are secreted by cells of different origin and act in immune, nervous, urinary and cardiovascular

systems. In immune system, exosomes functions can grouped into activating and inhibitory effects [170]. Most of the attributed roles have been demonstrated in vitro **(Figure 8)**.

4.1. Antigen presentation

Exosomes secreted by antigen presenting cells bear functional peptide-MHCII and MHCI complexes [174,201] and contain costimulatory molecules such as CD80 and CD86 [202,208], therefore they can possibly present antigens and activate T lymphocytes. In fact, in absence of antigen presenting cells, exosomes derived from antigen-loaded APCs, such as B cells and DCs, can activate T cell lines and T cells clones in an antigen dependent manner [174,188,225,245,246]. However, exosomes require to be captured by DCs to efficiently activate naïve T lymphocytes [210,240]. In vitro experiments have shown that antigen-bearing exosomes derived from DCs require mature DCs to stimulate antigen-specific naïve T cells [240]. In this study, the presence of the costimulatory molecules CD80 and CD86 on recipient DCs was necessary whereas DCs could be MHC II-negative. Conversely, other study has reported that the capture of exosomes, bearing MHC-peptide complexes, by splenic DCs leads to activation of antigen-specific T cells but requires the presence of DC-derived MHC II molecules [244]. So, in summary, DCs are able to use preformed exosomal peptide-MHC II and MHCI or, alternatively, DCs may process MHC II-peptide complexes and load these peptides onto endogenous MHCI and MHCII molecules. Nevertheless, exosomes from dendritic cells carrying functional MHC II-peptide complexes may transfer the ability to activate CD4+ T cells to DCs that have not encountered the antigen [240,247]. Interestingly, exosomes do not require to be internalized by dendritic cells to activate T lymphocytes [237]. Exosomes secreted by mature DCs stimulate T cells more efficiently than those derived from immature DCs [210,244]. Furthermore, mature DCs-derived exosomes carrying MHC molecules can transfer to non-professional APCs, such as B cells, the ability to activate naïve T cells [210].

In addition to MHCII-peptides complexes, exosomes derived from DCs, carry antigens that can be captured and presented by DCs on their own MHC II molecules to induce the activation of antigen-specific T cells [208]. Exosomes secreted by tumour cells [248] or

isolated from malignant effusions [249] also represent a source of antigens to be processed and presented by DCs.

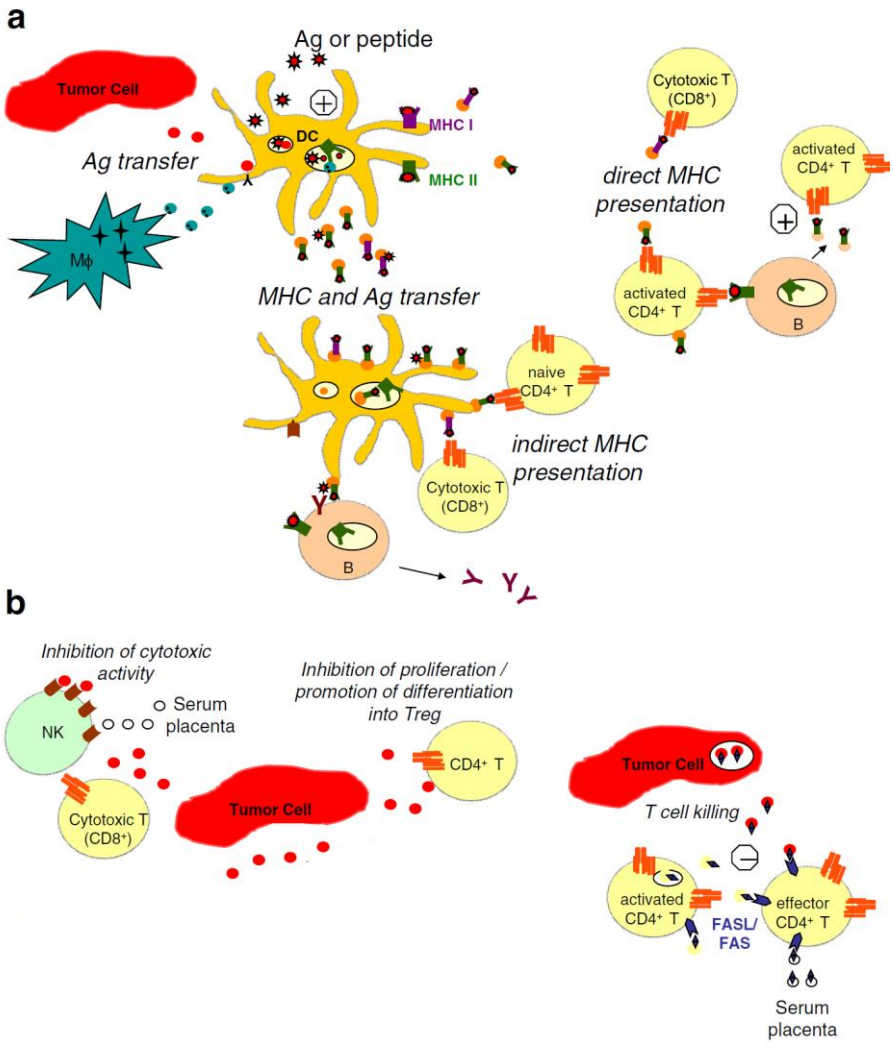


Figure 8. Interaction of exosomes with immune cells. Exosomes from various cellular sources transfer antigens or peptide-MHC complexes to DCs and T cells promoting activating effects (a). Alternatively, through antigen-independent mechanisms exosomes inhibit immune system (c). Illustration adapted from [250].

Exosomes not only boost T cell responses, but also humoral responses. It has been demonstrated that exosomes bear native antigens, thus providing necessary signals to activate B lymphocytes [246]. Diphtheria toxoid pulsed dendritic cells-derived exosomes induce primary and stimulate secondary humoral responses after intravenous injection into mice, being mature exosomes more potent than immature exosomes [62]. In other model, exosomes from ovalbumin loaded, IFN-γ-stimulated intestinal epithelial cell line induced a humoral response after intraperitoneal injection in mice [251]. Moreover, DCs pulsed with *Toxoplasma gondii* antigens secrete exosomes that, once intravenously injected, induce a protective humoral as well as Th1 responses against the parasite [252]. Regarding exosomes secretion by B cells, some authors have hypothesized that B cells that have encounter the antigen, and after interaction with antigen-specific T cells, secrete exosomes bearing peptide-MHC II complexes to sustain long-lasting antigen presentation to T cells [188].

4.2. Inhibition of immune responses

As mentioned previously, exosomes from tumour cells can be used by dendritic cells as a source of antigen and thereby initiate an immune response, but tumours can also exploit these vesicles in their own benefit. Exosomes are secreted by tumours as a mechanism to evade the immune system. Exosomes from pleural effusions of mesothelioma patients, containing TFG-β and NKG2D ligands, inhibit CD8+ T cells and NK cells by down-regulating NKG2D receptor expression [219]. Other mechanisms involve the inhibition of IL2-mediated proliferation of NK cells and T cells [253], expansion of regulatory T cells [254] and induction of T cells-apoptosis through the expression of FAS-L [255]. Recently, it has been reported that tumour-derived exosomes can promote metastasis by educating bone marrow progenitor cells [256].

In a similar way, exosomes from placenta cells which also express NKG2D ligands, such as ULBP1-5 and MIC, modulate surface expression of NKG2D on NK, CD8+ and γδ T cells down-regulating their cytotoxic activity [257]. Moreover, exosomes from placenta contain FAS-L [224] molecules that mediate apoptosis in CD4+ T cells [258].

5. EXOSOMES AS THERAPEUTIC AGENTS

Since it was reported that exosomes from tumour cells could induce an antitumor response once injected into animals [201], great efforts have been made in order to use them as cellular-free vaccines in antitumoral therapies [249,259–261]. Besides their use in antitumoral therapies, they have been studied as alternative therapies in immune regulation due to their stable phenotype that, contrary to cells, is not subject to further changes **(Figure 9)**.

Different strategies have been developed in order to suppress autoimmune disease or induce tolerance in transplantation. DC-derived exosomes expressing FAS-L, or IL-4-transduced BMDC-derived exosomes, subcutaneously injected reduce swelling in a model of delayed-type hypersensitivity (DTH) in mice. The effects depend on syngeneic MHCII molecules, Fas-L expression on exosomes and Fas expression on recipient mice. In addition, systemic injection of these exosomes also delays the onset and severity of collagen-induced arthritis (CIA), probably through interaction with APCs and T cells on recipient mice [262,263]. In a similar way, exosomes from IL-10-treated BMDCs or transduced with an adenovirus expressing IL-10 suppress DTH responses [264]. Of note, some effect has been reported with mock exosomes, so immature dendritic cells may secrete exosomes with regulatory properties. In fact, allogeneic exosomes from immature dendritic cells can modulate the rejection of heart allografts [157]. Immunosuppressive exosomes can also be obtained from plasma of KLH-immunized animals. The anti-inflammatory effect of plasma exosomes is antigen-, Fas-L- and MHC II molecules-dependent [209].

The intranasal administration of exosomes isolated from bronchoalveolar fluid of tolerized mice upon intranasal allergen exposure inhibit allergic reaction [217]. In addition, serum exosomes obtained from tolerized mice after ovalbumin (OVA) feeding, intraperitoneally injected, regulate the allergic response to intranasal administration of OVA antigen [265].

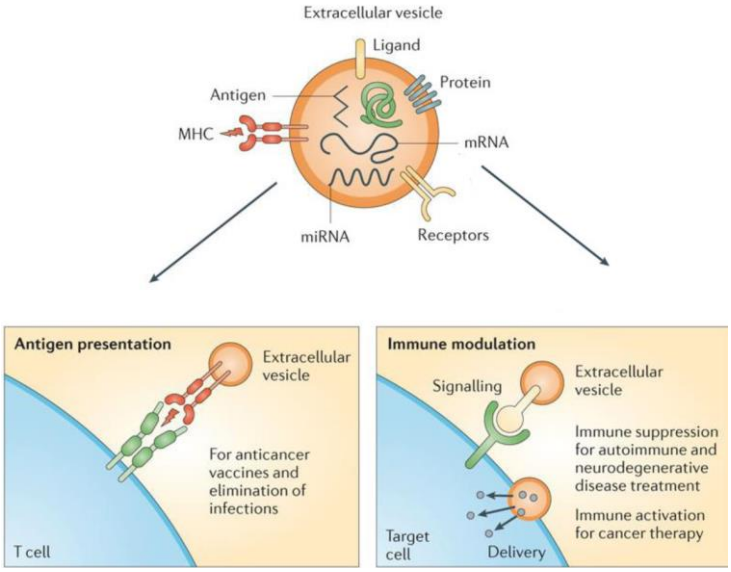


Figure 9. Therapeutic uses of exosomes to promote immunostimulatory responses (anti-cancer therapies) or immune tolerance (autoimmune diseases, transplantation tolerance). Illustration adapted from [266].

In the setting of transplantation, it has been reported that intravenous injection of donor immature BMDC-derived exosomes (bearing donor MHC molecules), prior to heterotopic heart transplantation, prolongs allograft survival [157]. This effect is accompanied by a decrease in graft infiltrating leukocytes, a reduction of IFN-γ mRNA expression in the graft and a decrease in the anti-donor cellular response post-transplantation. In another model, the administration of immature DC-derived exosomes decreases anti-donor cellular response, promotes the generation of regulatory T cells and also prolongs the survival of intestinal allografts [267]. Interestingly, donor immature DC-derived exosomes administered post-transplantation in combination with the immunosuppressive drug LF15-0195 induce donor-specific tolerance and delayed chronic rejection [158]. Furthermore, the combination of rapamycin and donor immature DC-derived exosomes promotes donor-specific tolerance, induces the generation of CD4+ CD25+ T cells and up-regulation of Foxp3 expression in recipient splenic T cells, and prolongs allograft survival in a mouse model of cardiac transplantation [268].

6. EXOSOMES AS BIOMARKERS FOR DIAGNOSIS

Exosomes have been found in several biological fluids such as pleural and peritoneal tumour effusions [249], urine [223], plasma [218], breast milk [269], etc. This fact has prompted the study of vesicles composition under pathological situations. Exosomes may constitute a noninvasive (or minimally invasive) method to obtain information for the diagnosis of diseases thereby avoiding the need for biopsies. The protein composition and genetic material in exosomes vary between normal donors and patients, in fact they can serve as diagnostic and prognostic of clinical stages in melanoma cancer patients [256]. Proteomic analysis of exosomes from hepatocytes identified some proteins related to specific diseases, potentially useful as biomarkers [198]. Consistent with this, in a rat model of hepatic disease, urine exosomes from experimental rats were shown to express a different composition when compared to control animals [220]. In addition, in renal transplant recipients, proteomic analysis of urinary exosomes may serve to discriminate among different causes of allograft rejection [270].

HYPOTHESIS AND OBJECTIVES

Exosomes are nanovesicles present in most biological fluids, including plasma. Among other proteins, exosomes contain major histocompatibility complex class I and class II molecules. These molecules are the most relevant antigens involved in transplant rejection (alloantigens). Moreover, it has been described that exosomes are released by graft-infiltrating DCs and play a role in alloantigen spreading between DCs amplifying the response against the graft. On the other hand, several experimental models have demonstrated that donor alloantigen-loaded tolerogenic DCs may induce transplantation tolerance.

Our hypothesis postulates that exosomes from biological fluids such as plasma may represent a useful source of alloantigens to induce tolerance in organ transplantation. The combination of donor exosomes with recipient, tolerogenic DCs could be a feasible strategy to induce alloantigen-specific tolerance in clinical organ transplantation.

The main objective of this study is to evaluate the use of plasma- or cell culture-derived exosomes as a source of alloantigens in tolerance induction and to verify which of the options, the administration of isolated exosomes or the combination with tolerogenic DCs, is more adequate to promote tolerance in an in vivo model. To achieve these objectives, we have set the following objectives:

1. To evaluate the presence of exosomes in human plasma samples of healthy donors. To this purpose, proteomic and western blot analyses were performed.

2. To verify by in vitro experiments the ability of human peripheral blood DCs to capture exosomes and present exosomal alloantigens to autologous T cells.

3. To perform an in vivo assay, in a fully mismatched kidney transplantation model in rats, to compare the different possibilities to modulate graft rejection by administration of donor exosomes alone or in combination with tolerogenic DCs (exosomes-loaded tolerogenic DCs). With this aim we generated tolerogenic bone marrow-derived DCs (BMDCs) in presence of dexamethasone and donor exosomes were isolated from immature BMDCs.

MATERIAL AND METHODS

SECTION I. Proteomic analysis of microvesicles from plasma of healthy donors reveals high individual variability

1. Culture media and reagents

Media and reagents for tissue culture were purchased from Invitrogen (Carlsbad, CA). All other reagents were from Sigma-Aldrich, unless stated otherwise. Hepatic cell lines AML12 (CRL-2254) and Clone 9 (CRL-1439), the kidney-derived cell line NRK-52E (CRL-1571) and the monocyte/macrophage cell line RAW264.7 (TIB-71) were obtained from the American Type Culture Collection (ATCC). The progenitor hepatic cell line (MLP29) and primary mouse fibroblasts from C57BL/6j have been described previously [198,271]. Monoclonal antibodies were purchased from BD Bioscience, anti-human CD81 (JS81), anti-Flotillin (clone 18) and anti-CD29/Integrin β1 (18/cd29); Santa Cruz Biotech., Inc, anti-Clusterin (H-330), anti-CD13 (3D8) and anti-CD9 (C-4); Abcam, anti-Moesin (38/87), anti-Tsg101 (4A10), anti-Stomatin, anti-Gal3BP and anti-CLIC1; R&D systems, anti-Ficolin3 (296134); Novus Biologicals, CD5L (1C8), Developmental Studies Hybridoma Bank, anti-human CD63 (H5C6). Alexa 488-, Cy3-, and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Molecular Probes, Jackson ImmunoResearch, and GE Healthcare, respectively.

2. Plasma collection from healthy donors

Plasma samples, provided by the Catalan Blood and Tissue Bank (BST), were obtained from healthy blood donors following the Institutional Standard Operating Procedures for blood donation and processing. Information on blood group, gender and age of donors is provided in Table 1.

Sample ID	Gender	Blood Group	Age
1	Male	O-	41
2	Female	A+	50
3	Female	O	34
4	Female	O+	45
5	Female	O+	39
6	Female	AB+	25
7	Female	O+	45
8	Male	A+	57
9	Female	A+	32
10	Female	A+	58
11	Female	A+	50
12	Female	O+	45
13	Female	A+	32
14	Female	B+	44
15	Female	A+	32
16	Female	A+	38
17	Male	A+	43
18	Male	A+	37
19	Female	B+	34
20	Female	A+	45
21	Female	O+	20
22	Male	A+	57
23	Female	A+	30
24	Female	O+	32
25	Male	A+	31
26	Female	O+	51
27	Female	O+	45
28	Female	O+	32
29	Male	A+	38
30	Male	O+	54
31	Female	A+	43
32	Female	O-	39
33	Female	O+	42
34	Female	O+	38
35	Male	A+	41
36	Female	O-	54
37	Male	B+	35
38	Male	O+	47

Table 1. Data of healthy donors used in this study

3. Purification of circulating MVs from plasma samples

MV preparations were isolated as in [272]. In brief, 200 ml of plasma was centrifuged at 2000×g (30 min), followed by 12.000×g (45 min). The supernatant was then ultracentrifuged at 110.000×g for 120 min. The resulting pellet was resuspended in phosphate-saline solution (PBS), filtered through 0.22 µm micropore filters, and ultracentrifugated at 100.000×g (60 min). The final pellet of MVs was resuspended in 200 µl of PBS, aliquoted and stored at -80 °C. MV preparations were immunodepleted of

albumin and immune globulins as indicated, using ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit (Sigma-Aldrich) according to manufacturer's specifications.

4. Exosome-enriched MV preparations from plasma samples

In some cases, MV preparations were further enriched in exosomes by ultracentrifugation on a 30% sucrose cushion as previously described [198]. Briefly, 200 µl PBS-suspended MV sample was diluted in 30 ml of PBS and underlayered on a 20mM Tris/30% sucrose/deuterium oxide (D2O) pH 7.4 (4 ml) density cushion to form a visible interphase. Samples were ultracentrifuged at 100.000×g at 4 °C for 75 min in a SW-32 Ti swinging bucket rotor (Beckman Coulter). The ultracentrifuge tubes were pierced on the side with an 18-gauge needle and 3.5 ml fluid was withdrawn from the bottom. Exosomes from the 30% sucrose/D2O cushion were collected, diluted a minimum of 10 times with PBS, and centrifuged at 100.000×g at 4 °C for 60 min. The final exosome-enriched preparations were suspended in 100 µl of PBS and stored at -80°C.

5. Tryptic digestion

The proteins were extracted from the isolated vesicles by incubating with 0.1% SDS in 0.5 M triethylammonium bicarbonate on ice for 30 min; protein solubilisation was aided by gentle pipetting and brief sonication. Insoluble material was spun down and protein concentration determined using the Bio-Rad protein assay kit. Proteins from MVs, Alb/Ig-depleted MVs or exosome-enriched preparations were lyophilized, incubated in 50 mM ammonium bicarbonate (pH 8.5) with 0.05% Rapigest™, for 15 min at 60□°C, to re-dissolve lyophilized peptides. Samples were reduced in 10 mM dithiothreitol at 60 °C for 30 min followed by alkylation in 50 mM iodoacetamide, for 30 min at room temperature in the dark. Proteins were digested overnight at 37 °C with modified trypsin (1:10), followed by hydrolysis of the Rapigest surfactant with the addition of 2 µl of HCl. Samples were then incubated at 37 °C for 30 min, centrifuged at 10.000xg for 30 min and the supernatant recovered.

6. Liquid chromatography–mass spectrometry (LC–MS 160 ^{E})

The samples prepared as above were analysed using a NanoAcquity UPLC and Q-ToF Premier mass spectrometer (Waters Corporation). All analyses were performed in triplicate on 500 ng of protein. Peptides were trapped and desalted prior to reverse phase separation using a Symmetry C18 5 µm, 5mm x 300 µm precolumn. Peptides were then separated prior to mass spectral analysis using a 10 cm x 75 µm C18 reverse phase analytical column. Mass accuracy was maintained during the run using a lock spray of the peptide glu-fibrinopeptide B delivered through the auxiliary pump of the NanoAcquity at a concentration 200 fmol/µl and at a flow rate 500 nl/min. The LC–MS^{E} method acquires precursor and product ion data on all charge-states of an eluting peptide across its entire chromatographic peak width, providing more comprehensive precursor and product ion spectra. Peptides were analysed in positive ion mode using a Q-ToF Premier mass spectrometer that was operated in v-mode with the resolving power of 10,000 fwhm. Prior to analyses, the ToF analyser was calibrated using the doubly charge of glu-fibrinopeptide B (785.8426m/z). Post calibration data files were corrected using 181 the doubly charged precursor ion of glu-fibrinopeptide B (785.8426m/z) with a sample frequency of 30 s. Accurate mass LC–MS data were collected in a data-independent and alternating lowand high collision energy mode. The spectral acquisition time in each mode was 1 s with a 0.15 s interscan delay. In low energy MS mode, data were collected at constant collision energy of 10 eV. InMS^{E} mode, collision energy was ramped from 15 to 35 eV during each 1 s data collection cycle.

7. Data processing and database searching

ProteinLynx GlobalServer (PLGS) version 2.6 was used to process data. Protein identifications were obtained by using the Swiss-Prot database. Protein identification from the low/high collision spectra required more than three fragment ions per peptide, seven fragment ions per protein and two peptides per protein, to be matched. All proteins with greater than two peptides identified with less than 4% false discovery rate were considered as real hits. Carbamidomethylation was set as fixed modification and oxidation of methionine and N-acetyl terminal as variable modifications, Q3 with no more than one

mis-cleavage being allowed. The ion detection, clustering, and normalization were processed using PLGS as previously described [273].

8. SDS-PAGE and Western blot analysis

Total extracts from AML12 cells were prepared by incubation of 1·10^6 cells for 15 min on ice in the presence of 100 µl of lysis buffer [300 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100 and protease inhibitors]. After centrifugation at 20.000xg, the supernatant was transferred to a fresh Eppendorf tube. The protein concentration of cell extracts and MVs was determined using Bradford protein assay (Bio-Rad) with BSA as standard. SDS-sample buffer was added to 5 µg of protein and samples were incubated for 5 min at 37 °C, 65 °C and 95 °C and separated on 4–12% pre-casted acrylamide gels (Invitrogen). After being transferred to PVDF membranes and blocked overnight (5% milk and 0.05% Tween-20 in PBS), primary antibody was added for 1 h, followed by PBS washing and the application of secondary HRP-conjugated antibody. Chemiluminescent detection of proteins was performed using ECL Plus reagent (Amersham).

9. Electron microscopy

For cryo-electron microscopy, MV preparations were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL). Grids were blotted at 95% humidity and rapidly plunged into liquid ethane with a VITROBOT (Maastricht Instruments BV). For negative staining, 2.5 µl of purified-MVs was adsorbed onto glow-discharged carbon-coated copper grids, washed with distilled water, and stained with freshly prepared 2.0% aqueous uranyl acetate. Samples were imaged using a JEM-1230 transmission electron microscope (JEOL, Japan) equipped with a thermionic tungsten filament and operated at an acceleration voltage of 120 kV. Images were taken with a pixel size of 0.34 nm using the ORIUS SC1000 (4008 x 2672 pixels) cooled slow-scan CCD camera (GATAN).

10. ”In vivo”-capture/internalization assay, and confocal microscopy and flow cytometry analyses

Cell lines AML12, Clone 9, MLP29, RAW246.7, C57BL fibroblasts and NRK-52E were grown in complete medium [DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin]. For confocal microscopy 5·10^5 cells were grown on cover-slips and incubated at 37 °C in complete medium containing 25 µg of pooled MV preparations. After 24 h of incubation cells were washed in PBS three times and fixed in 2% formaldehyde-PBS solution. Subsequently, coverslips were stained using the specie-specific monoclonal anti-human CD81 (JS81) diluted in PBS solution containing 0.1% saponin and 0.1% BSA. After 1-hour incubation, coverslips were PBS-washed and incubated with donkey anti-mouse Cγ3-conjugated secondary antibody. Finally, coverslips were mounted on DAPI containing Fluoromount G and analysed under a 63× objective on a Leica TCS SP multiphoton confocal microscope. For flow cytometry analysis, 1×10^6 cells from AML12 cell line were incubated with complete medium (control) or complete medium containing 5 µg of the indicated MV preparations. Following a 16-hour incubation (37 °C and 5% CO2), cells were trypsinized, washed in PBS and fixed in 2% formaldehyde. Subsequently, cells were stained with monoclonal antibodies against human CD81 (JS81) or CD63 (H5C6) proteins (in PBS+0.1% saponin and 0.1% BSA) for 1 h. Cells were then rinsed in PBS and incubated with donkey anti-mouse Alexa 488-conjugated secondary antibody. Finally, cells were rinsed 3 times in PBS and analysed by flow cytometry using a FACS Canto II instrument. Fluorescence intensities for Alexa-488 and APC channels were read using a minimum of 30.000 cells.

SECTION II. Capture of exosomes by human peripheral blood dendritic cells

1. Culture media and reagents

Culture media was composed by RPMI 1640 (Gibco) supplemented with 10% heat inactivated FBS (Gibco), 2mM L-glutamine (Sigma-Aldrich) 100 U/mL penicillin (Cepa S.L.), and 100µg/mL streptomycin (Laboratorios Normon S.A.). Recombinant human IL-3 (R&D Systems) was added at 10ng/mL to all pDCs cultures. Resiquimod (R848) (Alexis Biochemicals) was used at 5 µM at the indicated times. Murine monoclonal antibodies were purchased from BD Bioscience FITC-CD3, FITC-CD86, PE-CD40, APC-CD83, APC-H7-HLA-DR, PE-Cy5-CD11c; ImmunoTools, FITC-CD4, PE-CD14; R&D Systems, PE-CD25; Miltenyi Biotec, FITC-CD123. Anti-human CD81, anti-human flotillin, anti-human calnexin and anti-human CD63 were from Santa Cruz Biotech. IRDye 680 goat anti-rabbit and IRDye 800CW goat anti-mouse conjugated secondary antibodies were from LI-COR Biosciences. Alexa 488-conjugated rabbit anti-goat and goat anti-rabbit secondary antibodies were from Molecular Probes.

2. Peripheral blood DC isolation

Buffy coats, provided by BST [Badalona (Barcelona), Spain], were obtained from healthy donors following all of the guidelines and standards for blood donation. DC subsets were isolated and cultured as reported before [274]. Briefly, peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Lymphoprep) and CD3 positive cells were depleted by RosetteSep Human CD3 Depletion Cocktail (StemCell). Recovered cells were washed twice in PBS and counted using Perfect count (Cytognos). Then, monocytes were removed by positive selection using Human CD14 Microbeads and autoMACS Columns (Miltenyi). The remaining cells were incubated with monoclonal antibodies (CD4-FITC, CD14-PE, CD3-PE, CD11c-PE-Cy5) and sorted with FACSAria II cell sorter (BD Biosciences). PE-low, double-positive cells for CD4 and CD11c were gated and sorted as cDC. PE-negative, single positive CD4 cells were selected and sorted as pDCs (Figure 10A). The tubes collecting pDC contained human recombinant IL-3 (R&D) at 10

ng/ml to maintain pDC viability during the sorting process. In all samples, purity was over 99% (Figure 10B) and viability over 90%.

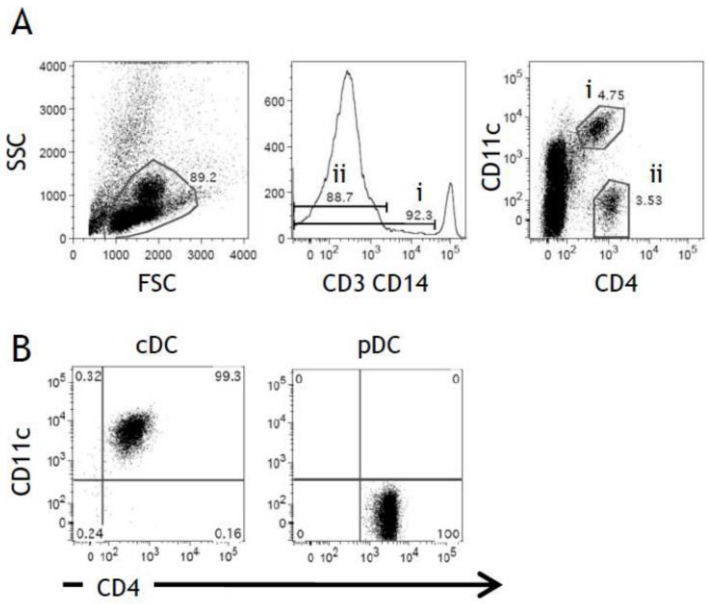


Figure 10. Isolation of human cDCs and pDCs by cell sorting. (A) Following the enrichment process, the remaining population was incubated with mAbs to FITC-CD4, PE-CD14, PE-CD3 and PE-Cy5-CD11c, and sorted. PE negative cells were selected (gate i for cDCs and ii for pDCs). Single CD4+ cells were sorted as pDCs whereas double CD4+ and CD11c+ cells were sorted as cDCs. **(B)** In all samples, the purity of each sorted population was over 99%.

3. Culture of cDCs and pDCs

After sorting, cDCs and pDCs were cultured separately in complete medium in Eppendorf tubes at 1·10^6/ml in a maxim volume of 1 ml. Human recombinant IL-3 (R&D) was added to pDC cultures at 10 ng/mL. To mature pDC, resiquimod (R848; Alexis Biochemicals) was added to pDC cultures at 5 µM.

4. Immunophenotype

For immunophenotype analysis, cells were washed, resuspended in 50 µl of PBS and incubated with monoclonal antibodies for 15 minutes at room temperature. Acquisition was performed in a FacsCanto II flow cytometer using the Standard FacsDiva software (BD

Biosciences). Subsequent analyses were performed using FlowJo software 7 (Tree Star, Inc). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris.

5. Generation of exosomes and apoptotic bodies from Jurkat T cell line

Vybrant DiO (Molecular Probes)-labelled Jurkat T cells were cultured in exosome-depleted medium. Supernatants were collected after 24–48h, and exosomes were isolated as described [272]. Briefly, supernatants were centrifuged at 450xg for 5 min., filtered through 0.22 µm, and ultracentrifuged at 100.000xg for 75 min in a SW-28 swinging bucket rotor (Beckman Coulter). Pelleted exosomes were resuspended in PBS, added on a 30% sucrose cushion, and ultracentrifuged for 75 min. The phase containing the exosomes was washed in PBS and pelleted for 1 h at 100.000xg. Apoptotic bodies were obtained from Vybrant DiO-labeled Jurkat T cells. Apoptosis was induced by UVB irradiation, as described previously [275]. Apoptotic bodies from supernatants were obtained following methods published previously [178]. After UV irradiation, apoptotic bodies were obtained from culture supernatants following successive centrifugation at 450xg (5 min.), 1.200xg (20 min.), and 10.000 xg (30 min). The resulting pellet was washed with PBS and centrifuged at 100.000xg. The final apoptotic-enriched preparation was resuspended in PBS and stored at -80 °C. The protein concentration of exosomes and apoptotic bodies was determined using Bradford protein assay (Bio-Rad) with BSA as standard.

6. SDS-PAGE and Western-blot analysis of Jurkat exosomes

A million cells were lysed with 100 µl of lysis buffer (300mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100 and proteases inhibitors). The protein concentration of cell lysates and exosomes was measured using a Pierce BCA protein assay (Thermo Scientific). Equal amounts of protein were diluted in 0.125M Tris-HCl, 4% v/v SDS, 20% v/v glycerol, 0.004% bromophenol blue, 10% 2-mercaptoethanol (pH 6.8), boiled at 95ºC for 10 min and separated in 12% acrylamide gels (Bio-Rad). Gels were transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Bio-Sciences GmbH) and blocked for 1h (PBS, 5% milk protein). Thereafter membranes were incubated overnight with primary

antibodies followed by IRDye 680 anti-rabbit or IRDye 800CW anti-mouse. Blotted proteins were detected by fluorescence using Odyssey Infrared Imaging System (LI-COR GmbH).

7. Antigen capture by DCs

DC (1·10^5) were pulsed with 25 μg fluorescent exosomes or apoptotic bodies and incubated at different times at 37 °C or 4□°C as control. Thereafter, cells were washed with PBS, and the percentage of fluorescent cells was analysed by flow cytometry. In some experiments, pDCs and cDCs were cocultured at a 1:1 ratio for 3 h or 21 h in the presence of labelled exosomes. Then, cells were washed and stained for CD11c, and the exosome capture was measured by flow cytometry. For uptake inhibition experiments, cells were treated for 30 min at 37□°C with cytochalasin D or wortmannin (Calbiochem) at the indicated concentrations prior to the addition of exosomes.

8. Analysis of exosome capture by confocal microscopy

After capture, DCs were attached to poly-l-lysine-coated slides, fixed with paraformaldehyde 4%, and labelled with HLA-DR mAb (EDU-1; a kind gift from Dr. Ramon Vilella, Hospital Clinic, Barcelona, Spain) or LAMP-1 (H4A3, DSHB), followed by Alexa 546–anti-mouse IgG. Nuclei were stained with Hoechst 33342 (Molecular Probes). Images were analysed with a TCS SP2 AOBS Leica laser-scanning spectral confocal microscope.

9. Assay of antigen presentation

Autologous T cells were isolated from the same donor as the DCs by negative selection using the EasySep human T cell enrichment kit (Stemcell Technologies) and CFSE (Molecular Probes) labelled. Purity was >90% in all experiments. Exosome-loaded pDCs were cultured for 7 days with 1·10^5 CFSE-labelled T cells at a 1:20 ratio (pDC:T) in 96-well round bottom plates in a final volume of 200 μl of complete medium. Cell proliferation of T cells was measured by loss of CFSE fluorescence by flow cytometry.

10. Cytokines analysis by ELISA

Previously to proliferation analysis, 50 µl of supernatants from DC:T coculture were collected and tested for IL-10 and IFN-γ production by ELISA (eBioscience). Similarly, the production of IFN-α by pDC was analysed by ELISA (eBioscience).

11. Statistical analysis

The results are expressed as the mean ± SD, unless otherwise indicated. Comparison among groups was conducted using the paired t test and Wilcoxon signed rank test using GraphPad Prism 4.0 software (GraphPad Software). P values < 0.05 were considered significant.

SECTION III. Effect of donor exosomes-pulsed tolerogenic DCs in a model of renal transplantation in rats

1. Culture media and reagents

Culture media was composed by RPMI 1640 (Gibco) (unless stated otherwise) supplemented with 10% heat inactivated FBS (Gibco), 2mM L-glutamine (Sigma-Aldrich) 100 U/mL penicillin (Cepa S.L.), and 100µg/mL streptomycin (Laboratorios Normon S.A.). Recombinant rat GM-CSF and IL-4 (Peprotech), and human FLT3-ligand (Miltenyi) were added at 10ng/ml, 5ng/ml and 50ng/ml respectively, at the indicated time. Murine monoclonal antibodies were purchased from AbD Serotec, FITC-CD3, FITC-RT1B,FITC-CD11b, PE-CD86, PE-OX62, PE-CD45RA, Alexa Fluor 647-CD25, Alexa Fluor 647-CD86; eBioscience, APC-CD3; BD Bioscience, APC-CD3/FITC-CD45RA/PE-CD161a cocktail.

2. Generation of rat bone-marrow derived dendritic cells

Femur and tibia were extracted from Wistar-Agouti rats and disinfected in 70% ethanol. Muscle was removed and both ends were cut. Bone marrow was flushed with 25 ml of complete medium. Cell suspension was centrifuged at 400 xg for 5 min. The supernatant was decanted and the cells were resuspended in lysis buffer (BD Biosciences) and incubated for 6 min. at room temperature to remove erythrocytes. Cells were washed with PBS and centrifuged at 400xg for 5 min. Then cells were resuspended in 20 ml of complete medium and counted with Perfectcount beads (Cytognos) by flow cytometry. Bone marrow precursors were cultured at 1.5·10^6/3ml of complete medium supplemented with 10 ng/ml of rat GM-CSF (Peprotech), 5 ng/ml of rat IL-4 (Peprotech) and 50ng/ml of human FLT3-L (Miltenyi) in 6 wells plates. Every 2 days half the medium was exchange for fresh medium containing cytokines, and any remaining cell was centrifuged and added to the culture. Conversely to the protocol in mice, the elimination of floating neutrophils and granulocytes from rat bone marrow cultures is not possible because rat DC are also floating cells [276]. After 8 days BMDCs were harvested, cell cultures were centrifuged at 400xg for 5 min. and resuspended in 5 ml of complete medium. To recover the adherent fraction 1 ml of accutase (PAA laboratories GmbH) was added to each well for 30 minutes

at 37 °C, and then cells were obtained by gently pipetting, washed with complete medium, collected with the other fraction, centrifuged, resuspended in complete medium and counted. Viability was determined by labelling cells with 7-AAD (BD Biosciences). In order to obtain mature BMDC, cells were cultured at 1·10^6/ml in 6-wells plates in complete medium supplemented with cytokines and LPS 100ng/ml. To obtain tolerogenic DCs, the glucocorticoid dexamethasone (Fortecortín, Merck Farma y Química, S.L) was added at the specified concentration to cultured cells at days 6 and 8, moment of DCs maturation.

3. Immunophenotype

For immunophenotype analysis, cells were washed, resuspended in 50 µl of PBS and incubated with monoclonal antibodies for 15 minutes at room temperature. Acquisition was performed in a FacsCanto II flow cytometer using the Standard FacsDiva software (BD Biosciences). Subsequent analyses were performed using FlowJo software 7 (Tree Star, Inc). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris.

4. Cytokines analysis by Luminex

IL-10, IL-12 and TNF-α were measured from supernatants of BMDC cultures by Luminex© xMAP© technology (Panomics). The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), dual laser design and digital signal processing to effectively allow multiple assays within a single sample. The minimum detectable concentration (pg/mL) of each protein was 2.48 for IL-12p70, 5.88 for IL-10, and 3.44 for TNF-α. All the cytokines measured were over the detection limit.

5. Splenocytes preparation

Spleen was disaggregated mechanically, cell suspension was filtered with 70-µM cell strainer and centrifuged at 400xg for 5 min. Cells were resuspended in lysis buffer and incubated for 6 min at room temperature to remove erythrocytes. Cells were washed with PBS and centrifuged at 400xg for 5 min. and then resuspended in 20 ml of complete

medium and counted. When splenocytes were used in mixed lymphocyte reactions, 1·10^5 were seeded in 96 wells plates.

T lymphocytes. T cells were obtained from splenocytes by negative selection using the T cell enrichment columns (R&D) following manufacturer's instructions. Purity was higher than 90%.

B lymphocytes. Isolation of B cells was performed by negative selection using the MagCellect Rat B Cell Isolation Kit (R&D) following manufacturer's instructions. Purity was higher than 85%.

6. Allogeneic mixed lymphocyte reaction

Splenocytes or T lymphocytes (1·10^5) from Brown Norway rat were cultured with Wistar Agouti BMDC at a 1:10 ratio (DC:T) in a final volume of 200 µl in 96-well round bottom plates. After 4.5 days, cells were pulsed with 1uCi/well of (^{3}H)-thymidine (Amersham) and cultured for another 16 hours. To quantify proliferation cells were harvested (Harvester 96, Tomtec) and analysed using a scintillation counter (1450 Microbeta reader Trilux Wallac). Data are represented as mean count per minute (cpm).

7. Exosome isolation from BMDCs

To obtain exosomes, the protocol for BMDCs differentiation was slightly modified. Bone marrow cells from Brown Norway rats were seeded in Petri dishes (150 mm) at 1·10^6/ml in complete medium supplemented with 10 ng/ml of rat GM-CSF (Peprotech) and 5 ng/ml of rat IL-4 (Peprotech) in a final volume of 50 ml. Every 2 days half volume of media was replaced by the same volume supplemented with cytokines for the final volume. At day 6, media was replaced by 100 ml of complete fresh medium being FBS ultracentrifuged. At day 8 supernatants were collected and centrifuged at 450xg for five min. to eliminate cells and store at 4 °C. Cells were resuspended in 100 ml and added to the culture for another 48h. At 10 day the supernatants were collected following the same procedure. In order to isolate exosomes, and after the 450xg centrifugation, supernatants were filtered by 0.22 µm low retention filter. Then, supernatants were concentrated using Centricon-70 Plus

units (Millipore) to reduce the volume to ultracentrifuge. The concentrated supernatants were then ultracentrifuged at 100.000xg for 75 min in a SW-28 swinging bucket rotor (Beckman Coulter) at 4 °C. After decanting the supernatants, exosome pellets were then resuspended in PBS and filtered again by 0.22 µm low retention filter to eliminate possible aggregates generated by concentrating the supernatants. Diluted exosomes were then ultrancentrifuged at 100.000xg for 75 min. at 4 °C. Finally, after decanting the supernatant, exosomes were resuspended in the remaining PBS (100-200µl) and the protein content was quantified by Bradford (BioRad).

7.1 Immunophenotype of exosomes

Exosomes were incubated with 4 µm aldehyde/sulphate latex beads (Invitrogen) at 50 µg/µl for 15 min at room temperature. Beads were resuspended in 1 ml of PBS-0,01 % BSA and incubated overnight at room temperature. Exosomes-coated beads were spun down at 1000xg (10 min), washed with PBS-0,01 % BSA and centrifuged again at 1000xg (10 min). Exosomes-coated beads were labelled in the darkness at 4 °C with monoclonal antibodies for 30 min. Then, exosomes-coated beads were washed twice with PBS and incubated at 4°C with Alexa 488-conjugated secondary antibodies for 30 min., washed with PBS and analysed for protein expression. Acquisition was performed in a FacsCanto II flow cytometer using the Standard FacsDiva software (BD Biosciences). Subsequent analyses were performed using FlowJo software 7 (Tree Star, Inc).

7.2 Electron microscopy of exosomes

For cryo-electron microscopy, BMDC-derived exosomes preparations were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL). Grids were blotted at 95% humidity and rapidly plunged into liquid ethane with a VITROBOT (Maastricht Instruments BV). Samples were imaged using a JEM-1230 transmission electron microscope (JEOL) equipped with a thermionic tungsten filament and operated at an acceleration voltage of 120 kV. Images were taken with a pixel size of 0.34 nm using the ORIUS SC1000 (4008 x 2672 pixels) cooled slow-scan CCD camera (GATAN).

7.3 Analysis of size distribution of exosomes by NanoSight

Size distribution of exosomes preparations was analysed by measuring the rate of Brownian motion using a Nanosight LM10HS system. The instrument, which is based on a conventional optical microscope, uses a laser light source to illuminate nano-scale and is equipped with a fast video capture and particle-tracking analysis software (Nano- Sight, Amesbury, U.K.). Results are displayed as a frequency size distribution.

8. Exosome labelling and capture by BMDCs

To analyse exosome capture by BMDCs in some experiments exosomes were labelled with PKH67 (Sigma) as previously described [241]. Briefly, exosomes were resuspended in Diluent C at 600 µg/ml and mixed at 1:1 volume with PKH67 diluted in Diluent C at 8 µM (final concentration 4 µM) and incubated for five minutes at room temperature. Then, the same volume of FBS was added to stop the reaction and incubated for another two min. To eliminate the excess of dye, labelled exosomes were then washed with PBS, filtered through 0,22µm filters and ultracentrifuged at 100.000xg for 75 min at 4° C. Exosomes were resuspended in complete medium to perform the capture assays. To analyse the capacity of BMDCs to capture exosomes, at day 8 BMDCs (5·10^5) were incubated with different doses of PKH67 labelled exosomes for 24h. At this point, immature cells were cultured with GM-CSF, IL4 and FTL3L, mature and tolerogenic DCs were differentiated in presence of LPS at 100 ng/ml while tolerogenic DCs were cultured also in presence of Dexamethasone. Then, after treatment with accutase, cells were recovered, extensively washed with PBS and exosome capture was analysed by flow cytometry.

9. Assay of exosomal antigen presentation

Immature, mature or tolerogenic BMDCs from Wistar Agouti rats were incubated for the last 24 hours with Brown Norway exosomes (5 µg/1·10^5). Then, BMDCs were recovered, extensively washed with PBS, resuspended in complete medium, counted by PerfectCount and analysed for viability and phenotype. Different numbers of DCs were co-cultured with syngeneic splenic T cells in a final volume of 200 µl in 96-well round bottom plates. After 4.5 days thymidine (1 micro Curie) was added and cells were cultured for another 16

hours. To quantify the proliferation, cells were harvested (Harvester 96, Tomtec) and analysed using a scintillation counter (1450 Microbeta reader Trilux Wallac). Data are represented as mean count per minute (cpm).

10. Inhibition of B cell proliferation

Isolated B lymphocytes from spleen of Wistar Agouti rats were labelled with CFSE 5µM. Immature, mature and tolerogenic BMDCs from Wistar Agouti rats were then co-cultured at different ratios with 1·10^5 syngeneic B lymphocytes in IF-12 media (1:1 mixture of Iscove's DMEM and Ham's F12) supplemented with 10% FBS, L-Glutamine, streptomycin/penicillin in 96-well round bottom plates. Cells were cultured in presence or absence of 5 µg/ml LPS (Sigma), 30ng/ml PMA (Sigma) and 100ng/ml Ionomycine (Sigma) or 10 µg/ml of $F(ab')_2$ mouse anti rat-IgM (Acris Antibodies GmbH). After 48-72h proliferation was analysed by flow cytometry.

11. Assay of Exosomes and Tolerogenic DCs migration in vivo

Tolerogenic DCs from Wistar Agouti rats were labelled with Cell Vue® NIR815 dye (Molecular Targeting Technologies) at 4 µM following manufacturer's instructions. Briefly, cells were resuspended in Diluent C at 20·10^6/ml and NIR815 was added to a final concentration of 4 µM and incubated for 5 min. at room temperature. Then, the same volume of FBS was added to stop the reaction and cells were extensively washed with complete medium three times and finally resuspended in saline solution. Finally, cells were resuspended in saline solution, counted and analysed for viability. Exosomes were also labelled with Cell Vue® NIR815 dye following the same protocol as for PKH67 labelling. Briefly, exosomes were resuspended in Diluent C at 600 µg/ml and mixed at 1:1 volume with NIR815 diluted in Diluent C at 8 µM (final concentration 4 µM) and incubated for five minutes at room temperature. Then, the same volume of FBS was added to stop the reaction and incubated for another two minutes. To eliminate the excess of dye, labelled exosomes were then washed with PBS, filtered through 0,22µm filters and ultracentrifuged at 100.000xg for 75 min at 4C. Exosomes were resuspended in saline solution.

Wistar Agouti rats (250g) were anesthetised using 1,5-2% (vol/ vol) isoflurane delivered in medical air at a flow rate of 1 L/min, and intravenously injected with 6.5·10^6 of NIR815-labelled tolerogenic DCs or 400 µg of NIR815-labelled exosomes. Exosome or DC migration was monitored at 5 minutes, 2.5h, 24h, 48h or 120h. At this point rats were sacrificed and different organs were extracted and analysed for the presence of exosomes or tolerogenic DCs. Imaging was performed using Pearl Imager equipment (Licor Biosciences).

12. Rat model of kidney transplantation

12.1 Animals and surgical technique

All animals were purchased from Charles River. Inbred male Wistar-Agouti rats (WAG) (250g) received an allogeneic kidney from Brown-Norway rats (BN) (250g). The surgical technique has previously described [277]. Recipient rats were bilaterally nephrectomised at the moment of transplantation. Animals did not receive any immunosuppressant. They were maintained in accordance with the Guidelines of the Committee on Care and Use of Laboratory Animals and Good Laboratory Practice.

12.2 Groups and Follow-up

Recipient rats (WAG) were intravenously injected (penile vein) one week before and the day of transplantation with 5·10^6 of syngeneic tolerogenic DCs pulsed with 125 µg of donor exosomes (Tolerogenic-DCexos), 5·10^6 of syngeneic tolerogenic DCs (Tolerogenic-DCs) or 125 µg of donor BMDCs-derived exosomes (Donor exosomes). As control, recipient rats were orally administered with rapamycin (dissolve in olive oil) from the day of transplantation (first dose pre-transplantation) until day 15, every 24 h.

12.3 Serum creatinine determination

Serum creatinine (sCr, µmol/L) was measured by Jaffe's reaction on auto-analyser (Beckman) on blood samples collected from the tail vein every 2 days beginning the day after surgery.

12.4 Analysis of peripheral blood cells populations

One week after transplantation, blood samples were collected from tail vein. 100 µl of blood were incubated with monoclonal antibodies for 20 minutes at room temperature. To remove erythrocytes, blood was incubated with lysing buffer for 6 minutes and then washed with PBS. Acquisition was performed in a FacsCanto II flow cytometer using the Standard FacsDiva software (BD Biosciences). Subsequent analyses were performed using FlowJo software 7 (Tree Star, Inc). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris and the percentage of T lymphocytes (CD3+), B lymphocytes (CD45RA+), NKT cells (CD3+ CD161a+) and NK cells (CD161a+) was calculated from the total of peripheral blood mononuclear cells.

13. Statistical analysis

The results are expressed as the mean ± SD, unless otherwise indicated. Results were analysed with GraphPad Prism 4.0 (GraphPad Software). Appropriated statistical tests were used according to the variance, matching pairs, and distribution. P values < 0.05 were considered significant.

RESULTS

SECTION I. Proteomic analysis of microvesicles from plasma of healthy donors reveals high individual variability

Several works have reported the use of DC-derived exosomes as cell-free vaccines for anti-tumour therapies [247,259,261,278]. Alternatively, DC-derived exosomes have been exploited as mediators of tolerogenic responses [279]. Exosomes from immature or tolerogenic DCs have shown to suppress inflammation in a model of delayed-type hypersensitivity and in a model of rheumatoid arthritis [262,263,280]. Furthermore, DC-derived exosomes modulate anti-donor responses [157], prolong allograft survival [267] and delayed the appearance of chronic rejection in a rat model of transplantation [158]. Despite some studies show contradictory results [281], plasma exosomes have a similar composition to those derived from in vitro cell cultures [178,202,218]. Moreover, plasma-derived exosomes suppress inflammation in an antigen-specific manner [209]. The use of plasma exosomes versus cell culture-derived exosomes or apoptotic bodies as a source of alloantigens to induce tolerance transplantation could be advantageous for several reasons. First, purification of exosomes from plasma may be an easier and faster approach since it is not necessary any cellular differentiation. Moreover, it could be a simpler and more safety method since there is not possible variations related to cellular differentiation. Finally, exosomes can be frozen for long time periods, an important fact especially for deceased donors. Therefore, we first evaluated the protein content of plasma exosomes obtained from healthy donors.

1.1. Circulating MVs from healthy donors

A preparation of MVs obtained from a 50-ml sample of plasma of a healthy donor was obtained as described in materials and methods section. Negative-staining and cryo-electron microscopy analyses showed the presence of round-shape membrane limiting vesicles of a size between 50 and 200 nm in the purified material (Figure 11A,B). SDS-PAGE analysis shows a distinct Coomassie blue staining pattern (Figure 11C) to the hepatic cell line extract indicating that regulated secretion of MVs is more likely than their release following cellular breakage. Remarkably, MVs were especially rich in high molecular weight proteins (bigger than 250 kDa) which may correspond to post-translationally modified

proteins as found in MVs from other sources [180,282]. Western-blotting against the MV-associated markers Flotilin, CD63 and CD81 [283] [272] was used to evaluate the reproducibility of MV purification. The procedure is highly reproducible with a plasma donor samples split into three 50-ml aliquots and processed independently, Figure 11D. The presence of these three well-established markers confirms that MVs form a significant component of blood plasma used therapeutically.

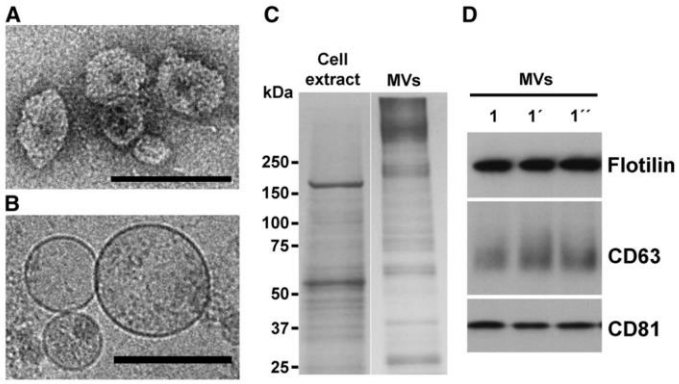


Figure 11. Ultra-structural and biochemical characterization of circulating plasma microvesicles. Representative negative staining **(A)** and cryo **(B)** electron micrographs of microvesicles isolated from human plasma. Bar 200 nm. **(C)** Coomassie staining pattern of protein extracts from cells or circulating plasma microvesicles (MVs). Note that the pattern of bands present in both samples is different. **(D)** To evaluate the reproducibility of the differential centrifugation procedure used in this work to purify MVs, one plasma sample was split into three equal aliquots (1, 1', 1") and 5 µg of the MVs from each was analysed by Western blotting using antibodies recognizing the known MV markers, Flotillin, CD63, CD81.

1.2. Proteomics of plasma MVs from healthy donors

To obtain detailed information on the protein content of the plasma MVs, we made a shotgun LC- MS^E proteomic analysis of 27 independent MV preparations obtained from healthy donors. Eight of these preparations were processed directly, while four were immunodepleted for the two most abundant plasma proteins, albumin and IgG. Finally, fifteen samples were enriched for exosomes to facilitate analysis of this component, by using a sucrose cushion. In total, we have identified 161 proteins in MVs below 220 nm in size. 52 proteins belong to the immunoglobulin protein family (Table 2) and probably are part of immune complexes as recently reported [284]. In addition, we have identified 109

proteins including components of the coagulation and complement cascades, also proteases and protease inhibitors, chaperones, cytoskeleton-associated proteins, enzymes, signalling molecules and proteins involved in the transport and metabolism of nutrients (Table 3).

Remarkably, there is a large quantitative and qualitative variation in preparations from different donors (Table 3, Figure 12). In the majority of MV preparations more than 30 proteins were detected, but only in 2 of the 15 exosome-enriched samples were more than 10 proteins identified. This variation suggests that under normal conditions limited amounts of exosomes are present. Furthermore, only 7 of the 109 MV proteins were detected in each of the directly processed samples (Table 3). This proportion increases slightly (up to 10) when albumin and immune globulin levels were depleted. The ubiquitous proteins included galectin-3-binding protein (Gal3BP), alpha-2-macroglobulin, histidine rich glycoprotein (HRG), C3 complement (CO3), fibrinogen alpha (FIBA), alpha-1-antichymotrypsin (AACT), pregnancy zone protein (PZP), clusterin (CLUS), haemoglobin (HBA) and ceruloplasmin (CERU).

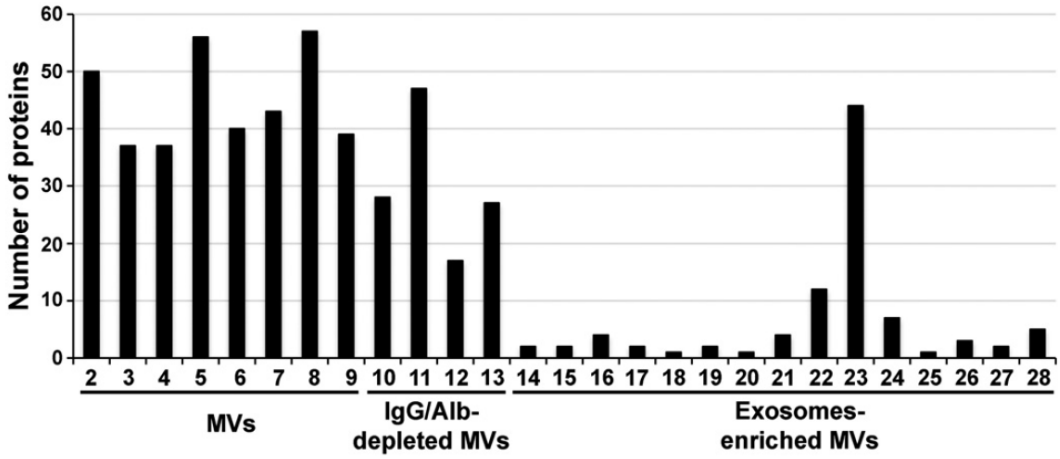


Figure 12. LC–MSE proteomics analysis of circulating MVs isolated from healthy individuals. Independent MV preparations were obtained by differential centrifugation from twenty-seven healthy donors. Eight (numbers 2 to 9) were directly processed for proteomics, four (10 to 13) were immuno-depleted for IgGs and albumin, and fifteen (samples 14 to 28) were exosome-enriched by flotation on a sucrose cushion. The number of proteins detected in each case is depicted in the graph. Note the individual variation and the low number of proteins detected in exosome-enriched preparations.

MVs (a)	MVs (b)	MVs (c)	Protein ID	Protein Description
1	2	1	HV303_HUMAN	Ig heavy chain V III region VH26
4	1	1	HV304_HUMAN	Ig heavy chain V III region TIL
5	2	1	HV305_HUMAN	Ig heavy chain V III region BRO
	2		HV313_HUMAN	Ig heavy chain V III region POM
1	2		HV316_HUMAN	Ig heavy chain V III region TEI
2	2	1	HV318_HUMAN	Ig heavy chain V III region TUR
	1	1	HV320_HUMAN	Ig heavy chain V III region GAL
8	3	1	IGHA1_HUMAN	Ig alpha 1 chain C region
5		1	IGHA2_HUMAN	Ig alpha 2 chain C region
1			IGHD_HUMAN	Ig delta chain C region
8	4	1	IGHG1_HUMAN	Ig gamma 1 chain C region
3	4		IGHG2_HUMAN	Ig gamma 2 chain C region
8	4	1	IGHG3_HUMAN	Ig gamma 3 chain C region
3	4		IGHG4_HUMAN	Ig gamma 4 chain C region
8	4		IGHM_HUMAN	Ig mu chain C region
8	4	13	IGJ_HUMAN	Immunoglobulin J chain
8	4	6	IGKC_HUMAN	Ig kappa chain C region
4	3	10	KV101_HUMAN	Ig kappa chain V I region AG
1	1		KV102_HUMAN	Ig kappa chain V I region AU
7	1		KV106_HUMAN	Ig kappa chain V I region EU
2			KV107_HUMAN	Ig kappa chain V I region Gal
3	1		KV108_HUMAN	Ig kappa chain V I region Hau
	1		KV110_HUMAN	Ig kappa chain V I region HK102 Fragment
	1		KV113_HUMAN	Ig kappa chain V I region Lay
	1	1	KV115_HUMAN	Ig kappa chain V I region Rei
3			KV116_HUMAN	Ig kappa chain V I region Roy
2			KV117_HUMAN	Ig kappa chain V I region Scw
4			KV118_HUMAN	Ig kappa chain V I region WEA
1			KV119_HUMAN	Ig kappa chain V I region Wes
7	1	1	KV201_HUMAN	Ig kappa chain V II region Cum
1	1	1	KV204_HUMAN	Ig kappa chain V II region TEW
1			KV205_HUMAN	Ig kappa chain V II region GM607 Fragment
	1		KV206_HUMAN	Ig kappa chain V II region RPMI 6410
4	3		KV302_HUMAN	Ig kappa chain V III region SIE
	2		KV303_HUMAN	Ig kappa chain V III region NG9
	1		KV305_HUMAN	Ig kappa chain V III region WOL
	2		KV306_HUMAN	Ig kappa chain V III region POM
	2		KV308_HUMAN	Ig kappa chain V III region CLL
2		1	KV309_HUMAN	Ig kappa chain V III region VG Fragment
	3		KV310_HUMAN	Ig kappa chain V III region VH Fragment
4	2	1	KV312_HUMAN	Ig kappa chain V III region HAH
1	1		KV313_HUMAN	Ig kappa chain V III region HIC
5			KV401_HUMAN	Ig kappa chain V IV region Fragment
4	1		KV402_HUMAN	Ig kappa chain V IV region Len
		3	LAC_HUMAN	Ig lambda chain C regions
	1		LAC1_HUMAN	Ig lambda 1 chain C regions
8	2		LAC2_HUMAN	Ig lambda 2 chain C regions
	2		LV102_HUMAN	Ig lambda chain V I region HA
4	3		LV106_HUMAN	Ig lambda chain V I region WAH
4			LV302_HUMAN	Ig lambda chain V III region LOI
1			LV403_HUMAN	Ig lambda chain V IV region Hil
8	3	7	MUCB_HUMAN	Ig mu heavy chain disease protein

Table 2. Immunoglobulins detected in MVs preparations of plasma samples from healthy donors.

(a) MVs preparations in which the indicated protein was detected amongst 8 independent MVs preparations directly analysed by proteomics.

(b) MVs preparations in which the indicated protein was detected amongst 4 independent MVs preparations IgG/Alb-depleted before proteomics.

(c) MVs preparations in which the indicated protein was detected amongst 15 independent MVs preparations, sucrose-enriched before proteomics.

Table 3 – Proteomics analysis of circulating MVs from healthy donors.

MVs [a]	MVs [b]	MVs [c]	Protein ID	Protein description
Coagulation- and complement-involved proteins				
	1	1	KNG1_HUMAN	Kininogen 1
		1	THRB_HUMAN	Prothrombin
		1	ANT3_HUMAN	Antithrombin III
	2	1	IC1_HUMAN	Plasma protease C1 inhibitor
		2	PROS_HUMAN	Vitamin K dependent protein S
	4	2	C1QB_HUMAN	Complement C1q subcomponent subunit B
	5	2	C1QC_HUMAN	Complement C1q subcomponent subunit C
	1	2	C1R_HUMAN	Complement C1r subcomponent
	4	2	C1S_HUMAN	Complement C1s subcomponent
	5	2	C4BPA_HUMAN	C4b binding protein alpha chain
		2	CFAB_HUMAN	Complement factor B
		2	CFAH_HUMAN	Complement factor H
	8	4	CO3_HUMAN	Complement C3
	6	3	CO4A_HUMAN	Complement C4 A
	2	2	CO4B_HUMAN	Complement C4 B
		1	CO8A_HUMAN	Complement component C8 alpha chain
		1	CO8G_HUMAN	Complement component C8 gamma chain
		1	CO9_HUMAN	Complement component C9
		1	FHR1_HUMAN	Complement factor H related protein 1
		1	FHR3_HUMAN	Complement factor H related protein 3
	1	1	ITA2B_HUMAN	Integrin alpha IIb
		1	CIB2_HUMAN	Calcium and integrin binding family member 2
		4	HRG_HUMAN	Histidine rich glycoprotein
	1	1	PLMN_HUMAN	Plasminogen
	1		F13A_HUMAN	Coagulation factor XIII A chain
	7	4	FIBA_HUMAN	Fibrinogen alpha chain
	8	3	FIBB_HUMAN	Fibrinogen beta chain
	8	3	FIBG_HUMAN	Fibrinogen gamma chain
Protease and protease inhibitors				
	2		TRY1_HUMAN	Trypsin 1
	2		TMPSD_HUMAN	Transmembrane protease serine 13
		1	A2AP_HUMAN	Alpha 2 antiplasmin
	8	4	2 A2MG_HUMAN	Alpha 2 macroglobulin
	2	4	AACT_HUMAN	Alpha 1 antichymotrypsin
		1	AMBP_HUMAN	Protein AMBP
		1	ANGL4_HUMAN	Angiopoietin related protein 4
		1	ANGT_HUMAN	Angiotensinogen
	1	2	ITIH1_HUMAN	Inter alpha trypsin inhibitor heavy chain H1
		3	ITIH2_HUMAN	Inter alpha trypsin inhibitor heavy chain H2
		1	ITIH4_HUMAN	Inter alpha trypsin inhibitor heavy chain H4
	7	4	1 PZP_HUMAN	Pregnancy zone protein
Chaperones				
			1 HS71L_HUMAN	Heat shock 70 kDa protein 1L
			1 HSP71_HUMAN	Heat shock 70 kDa protein 1
			1 HSP72_HUMAN	Heat shock related 70 kDa protein 2
			1 HSP76_HUMAN	Heat shock 70 kDa protein 6
			1 HSP77_HUMAN	Putative heat shock 70 kDa protein 7
	1		1 HSP7C_HUMAN	Heat shock cognate 71 kDa protein
	6	4	CLUS_HUMAN	Clusterin
Cytoskeletal- and trafficking-related proteins				
	1		MOES_HUMAN	Moesin
	1		GRAP1_HUMAN	GRIP1 associated protein 1
			1 SDCB1_HUMAN	Syntenin 1
	1		PROF1_HUMAN	Profilin 1
	3	1	2 ACTB_HUMAN	Actin cytoplasmic 1
		1	ACTC_HUMAN	Actin alpha cardiac muscle 1
		2	1 ACTG_HUMAN	Actin cytoplasmic 2
			1 ACTH_HUMAN	Actin gamma enteric smooth muscle
			1 ACTS_HUMAN	Actin alpha skeletal muscle
		1	GELS_HUMAN	Gelsolin
			1 POTEE_HUMAN	POTE ankyrin domain family member E
			1 POTEF_HUMAN	POTE ankyrin domain family member F
	2		3 STOM_HUMAN	Erythrocyte band 7 integral membrane protein
	1		B3AT_HUMAN	Band 3 anion transport protein
	1		VTNC_HUMAN	Vitronectin OS Homo sapiens
			FINC_HUMAN	Fibronectin precursor
		3	K2C1_HUMAN	Keratin type II cytoskeletal 1

continued on next page

Table 3. Proteomics analysis of circulating MVs from healthy donors.

Table 3 *(continued)*

MVs [a]	MVs [b]	MVs [c]	Protein ID	Protein description
Enzymes				
		1	ACSM1_HUMAN	Acyl coenzyme A synthetase ACSM1 mitochondrial
		2	CHLE_HUMAN	Cholinesterase
		1	PON1_HUMAN	Serum paraoxonase arylesterase 1
		1	CPN2_HUMAN	Carboxypeptidase N subunit 2
		1	OAZ3_HUMAN	Ornithine decarboxylase antizyme 3
		1	KDM4D_HUMAN	Lysine specific demethylase 4D
		1	DNPEP_HUMAN	Aspartyl aminopeptidase
Carriers and solute transporters				
	8	1	ALBU_HUMAN	Serum albumin
	2	3	A1AG1_HUMAN	Alpha 1 acid glycoprotein 1
		2	A1AG2_HUMAN	Alpha 1 acid glycoprotein 2
		1	A1BG_HUMAN	Alpha 1B glycoprotein
		3	FETUA_HUMAN	Alpha 2 HS glycoprotein
	8	2	APOA1_HUMAN	Apolipoprotein A I
	3		APOA2_HUMAN	Apolipoprotein A II
		1	APOA4_HUMAN	Apolipoprotein A IV
	1		APOE_HUMAN	Apolipoprotein E
		2	APOL1_HUMAN	Apolipoprotein L1
	6	4	1 LG3BP_HUMAN	Galectin 3 binding protein
	7	4	1 HBA_HUMAN	Hemoglobin subunit alpha
	8		3 HBB_HUMAN	Hemoglobin subunit beta
	1		HBD_HUMAN	Hemoglobin subunit delta
			1 HBE_HUMAN	Hemoglobin subunit epsilon
			1 HBG2_HUMAN	Hemoglobin subunit gamma 2
	4	2	HEMO_HUMAN	Hemopexin
	8	2	1 HPT_HUMAN	Haptoglobin
	6		1 HPTR_HUMAN	Haptoglobin related protein
	1		1 TFR1_HUMAN	Transferrin receptor protein 1
	8	1	1 TRFE_HUMAN	Serotransferrin
	4	4	CERU_HUMAN	Ceruloplasmin
		2	AFAM_HUMAN	Afamin
		3	VTDB_HUMAN	Vitamin D binding protein
			1 GTR1_HUMAN	
	1		CLIC1_HUMAN	Chloride intracellular channel protein 1
Miscellaneous				
	1	1	1433Z_HUMAN	14 3 3 protein zeta delta
		1	TGFB1_HUMAN	Transforming growth factor beta 1
		1	EIF2A_HUMAN	Eukaryotic translation initiation factor 2A
	1		ZBT38_HUMAN	Zinc finger and BTB domain containing protein 38
		1	ZN177_HUMAN	Zinc finger protein 177
	1	1	1 CD5L_HUMAN	CD5 antigen like
	5		2 FCN2_HUMAN	Ficolin 2
	1		FCN3_HUMAN	Ficolin 3
	3		SAMP_HUMAN	Serum amyloid P component
		1	PRS8_HUMAN	26S protease regulatory subunit 8
	1		UBIQ_HUMAN	Ubiquitin
		1	INT11_HUMAN	Integrator complex subunit 11

[a] MV preparations in which the indicated protein was detected amongst 8 independent MV preparations directly analyzed by proteomics.

[b] MV preparations in which the indicated protein was detected amongst 4 independent MV preparations IgG/Alb-depleted before proteomics.

[c] MV preparations in which the indicated protein was detected amongst 15 independent MV preparations, exosome-enriched before proteomics.

Table 3. Proteomics analysis of circulating MVs from healthy donors.

1.3. Western blot analysis

Six additional preparations of MVs obtained from healthy donors were evaluated by Western-blotting. As shown in the Figure 13A, Moesin, CLIC1, Stomatin, Gal3BP, Clusterin, CD5L and Ficolin-3 were detected in MVs, thus validating the proteomic analysis (Table 3). Furthermore individual variability was also present in this new set of samples. Thus, for example, MV preparations obtained from the plasma of donors 31 and 32 were enriched in CLIC1 protein compared to MVs from donors 29 and 30. Conversely these donors 29 and 30 were enriched in Stomatin (Figure 13A). This variability also applied for well-established protein markers of MVs [285] including Tsg101, CD9, CD13, CD81 and CD63 (Figure 13A). In the case of Gal3BP, the band corresponding to the high molecular weight isoform of the protein was higher in donor 34 than in donor 33 (Figure 13A). This variability was also observed in four exosome-enriched MV preparations obtained from independent healthy donors (Figure 13B). The electron micrograph shown in Figure 13B evidences the presence of membrane limiting vesicles in the sucrose enriched preparations. Together, proteomics and Western-blot analysis highlight the existence of a high variability in the protein composition of circulating plasma MVs from healthy donors, both in protein abundance and levels of post-translational modification.

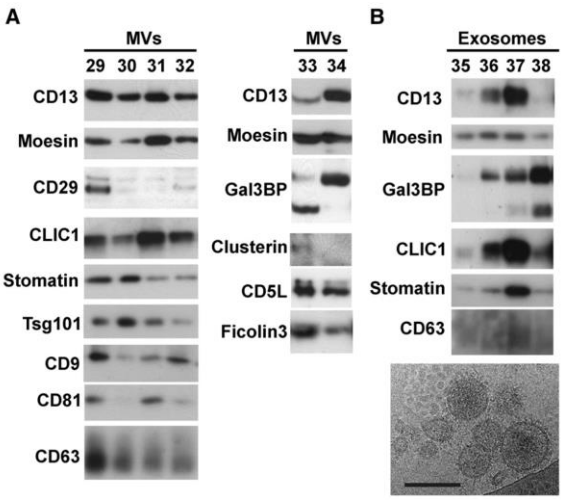


Figure 13. MVs show considerable variation in protein composition. 5 µg of protein from six independent MV preparations **(A)** or four exosome enriched **(B)** preparations obtained from healthy donors were analysed by Western blotting using antibodies against proteins identified in the proteomics analysis (Moesin, Gal3BP, CLIC1, Clusterin, Stomatin, CD5L, Ficolin3) and some markers of MVs (Tsg101, CD9, CD13, CD63 and CD81). Cryoelectron micrograph from exosome-sucrose enriched MVs isolated from human plasma. Bar, 100 nm.

1.4. Interaction of circulating-MVs with different cell lines

The function of circulating-MVs is not well characterized. Besides being involved in different extracellular processes such as blood coagulation [286] and immune modulation [176], MVs have suggested roles in material disposal and intercellular communication [283]. To investigate the interaction of MVs with cellular systems we evaluated the capture capacity of different cell lines using a capture/internalization assay in combination with confocal microscopy (Figure 14) and flow cytometry (Figure 15).

To facilitate the detection of the circulating-MVs inside the cells we incubated MVs with a repertoire of six established non-human cell lines, and subsequently using species-specific antibodies that only recognize the human CD81 or CD63 proteins. Following this approach, we analysed the capture capacity of different cell types towards a pooled preparation of MVs (Figure 14). While a high level of MVs was incorporated in two adult hepatic-derived cell lines, Clone 9 (Figure 14A) and AML12 (Figure 14B), the monocyte/macrophage cell line RAW264.7 (Figure 14D), and in mouse primary fibroblasts (Figure 14E), little or no MV capture was observed in the progenitor hepatic cell line, MLP29 (Figure 14C), or in the kidney derived NRK52 cell line (Figure 14F). This result implies that the interactions between cells and MVs are different in different cell lines and such interaction occurs by regulated mechanisms.

To examine whether the cellular uptake of MVs was affected by their protein content, 4 different MV preparations (from donors 29, 30, 31 and 32) were incubated during 16 h with the hepatic cell line AML12, and MV incorporation into cells was analysed by flow cytometry using the CD81 and CD63 proteins as reporters. As shown in Figure 15 the incorporation of CD63-positive MVs into cells correlates with the CD63 content of the MVs (Figure 13A), with higher incorporation of MVs prepared from donor 29 than from MVs obtained from donor 30. In contrast, although in general very low levels of incorporation of CD81- positive MVs were observed, a trend suggesting that low amount of CD81 could benefit the uptake into AML12 cell line was evidenced. Thus, MVs prepared from donor 30 that contained the lowest amount of CD81 (Figure 13A) were slightly more incorporated than MVs obtained from donors 29 or 31 (Figure 15) that contain higher levels of CD81

(Figure 13A). These results highlight that the variability in the protein composition of circulating-MVs appears to be a relevant feature that may determine the fate of the MV.

Overall, these results show that exosomes derived from plasma healthy donors are not a feasible source of alloantigens. Besides the great variability among donors, the low number of proteins in exosomes-enriched microvesicles preparations indicates low number of vesicles in plasma under non-pathological conditions. Therefore, other sources of alloantigens such as exosomes derived from cellular cultures will be considered.

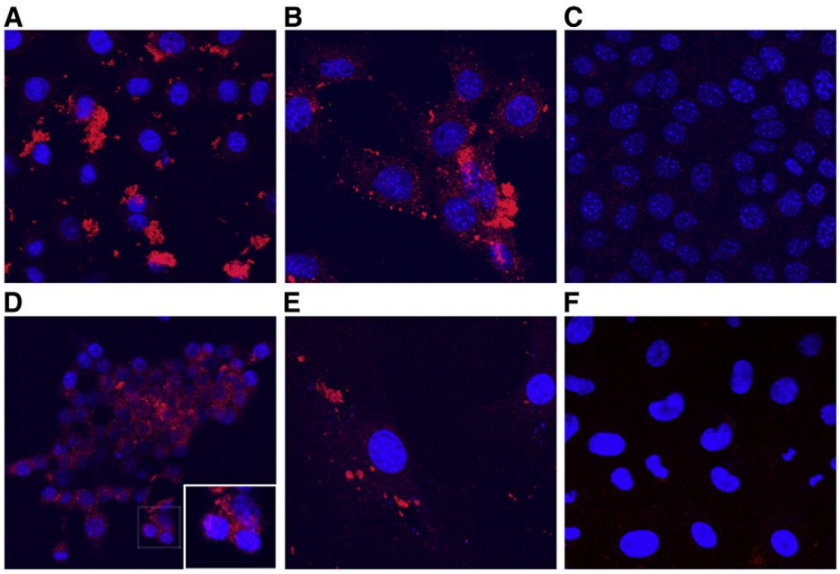


Figure 14. MVs capture by different established cell-lines. Mouse liver derived-cell lines, Clone 9 **(A)**, AML12 **(B)**, MLP29 **(C)**, murine macrophage cell line RAW264 **(D and inset 4×)**, primary mouse fibroblasts **(E)** and rat kidney derived-cell line NRK52 **(F)** were incubated with 25 µg of pooled MVs purified from healthy donors. Cells are stained with a species-specific antibody against human CD81 (red), while nuclei are counterstained with DAPI (blue) and analysed by confocal microscopy. Note that different cell lines showed differential uptake of plasma MVs suggesting the existence of an underlying MV-capturing mechanism.

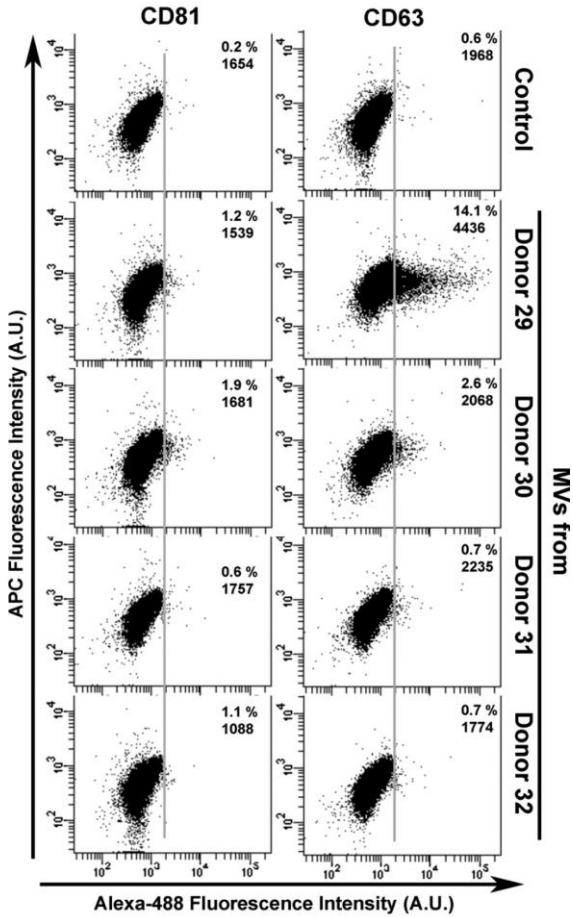


Figure 15. Variability in protein content of MVs affects differential uptake. 1×10^6 cells from AML12 cell line were incubated with complete medium (control) or complete medium containing 5 µg of the indicated MV preparations. Cells were formaldehyde-fixed and stained using the species-specific monoclonal antibodies against human CD81 (JS81) or CD63 (H5C6) proteins, and donkey anti-mouse Alexa 488-conjugated secondary antibody to visualize the incorporated CD81 or CD63-positive material. Background level (grey line) was established on control conditions, and the percentage of Alexa-488-positive cells above this threshold is indicated along with the mean fluorescence intensity cell populations.

SECTION II. Capture of exosomes by human peripheral blood dendritic cells

Dendritic cells (DCs) are critical for activation of donor-reactive T cells during transplant rejection. Through the indirect pathway, recipients DCs present donor alloantigens to T cells. Recipient DCs may acquire donor alloantigens from exosomes released by the graft. It has been demonstrated that following transplantation, recipient DCs migrate to the graft and release exosomes, which contain allopeptides and are captured by different subsets of DCs of secondary lymphoid organs [244]. Moreover, splenic DCs capture intravenously injected allogeneic exosomes [208]. Although it has been previously demonstrated the ability of human MDDCs to capture allogeneic exosomes [287], this capacity has not been addressed in human peripheral blood DCs. In blood, two major subsets of DCs have been described, named conventional (cDC) and plasmacytoid (pDC), which have specialized functions. Conventional DCs have an excellent capacity for antigen capture and presentation based on their high endocytic activity, ability to retain on their surface long-lived MHCII-peptide complexes, and the capacity to cross-present (reviewed in [56]). Meanwhile, pDCs are specialized in the secretion of type I IFNs upon viral challenge, but they may be also as good as cDC in presenting endogenous antigens [288–292]. However, the capacity of pDCs to capture and present exogenous antigens remains controversial. pDCs poorly capture dextran or lucifer yellow, revealing they are not macropinocytic cells [293,294]. However, pDCs may efficiently capture soluble proteins such as ovalbumin or HEL in vitro and in vivo [87,292,295], possibly through micropinocytosis or receptor-mediated endocytosis. In a mouse model of organ transplantation, pDCs were pointed out as the cell population responsible of tolerance induction [153] by capturing donor cells through phagocytosis, and presentation of peptide antigens in a tolerogenic manner. Yet, several studies using pDCs from mouse and human concluded that pDCs cannot phagocytose dead cells, zymosan, or artificial particles [294,296–298]. Others have shown that pDC may engulf microparticles produced by endothelial cells [299] and artificial microparticles [300]. Therefore, to analyse the capacity of both plasmacytoid and conventional DCs to capture allogeneic microvesicles we used as a model exosomes derived from a T-cell line.

2.1. Both cDC and pDC capture enriched exosomes

We first compared the ability to capture and internalize "cell-derived" antigenic material by incubating peripheral blood DCs subsets with exosomes. These vesicles were obtained by ultracentrifugation from vybrant-Dio labelled Jurkat T cells cultures and characterized as shown (Figure 16A). As reported before [195], Jurkat-derived exosomes express flotillin and the tetraspanins CD63 and CD81. We also could observe a residual presence of calnexin, an endoplasmic reticulum molecule which might be consistent with the reported plasma membrane origin of T cells-derived exosomes [176].

Conventional DCs and pDCs were sorted from the same donor as described in material and methods. We then pulsed isolated DCs with fluorescent-labelled exosomes obtained from Jurkat T and capture was analysed by flow cytometry at early (3 hours) and long-time (21 hours) points. We observed that exosomes were rapidly internalized by cDCs, in a dynamic process that, as it is already known, may be inhibited at low temperature (4 °C) (Figure 16B). Time course experiments revealed that approximately 50% of cDCs were already labelled at 3-6h after incubation, reaching saturation after approximately 12 h (Figure 16C). In sharp contrast, exosome capture by pDCs was only detectable after the first 12 h of incubation, but reached higher levels at longer incubation period (51,8% ± 13,8 n=16 at 21 h) (Figure 16B and 16C). At this time point most of cDCs were positively labelled with the fluorescent dye (90,4% ± 12,4 n=11), thus revealing a maximum level of capture (Figure 16B and 16C).

To analyse whether exosome capture by pDCs would still be relevant in the presence of cDCs, sorted DC subsets were mixed 1:1 and incubated with labelled exosomes for 21 h. Interestingly, flow cytometry data showed that both DC subsets captured exosomes at similar proportions than in time-matched isolated cultures (Figure 17), thus suggesting that pDCs can still interact with exosomes even in the presence of the highly endocytic cDCs.

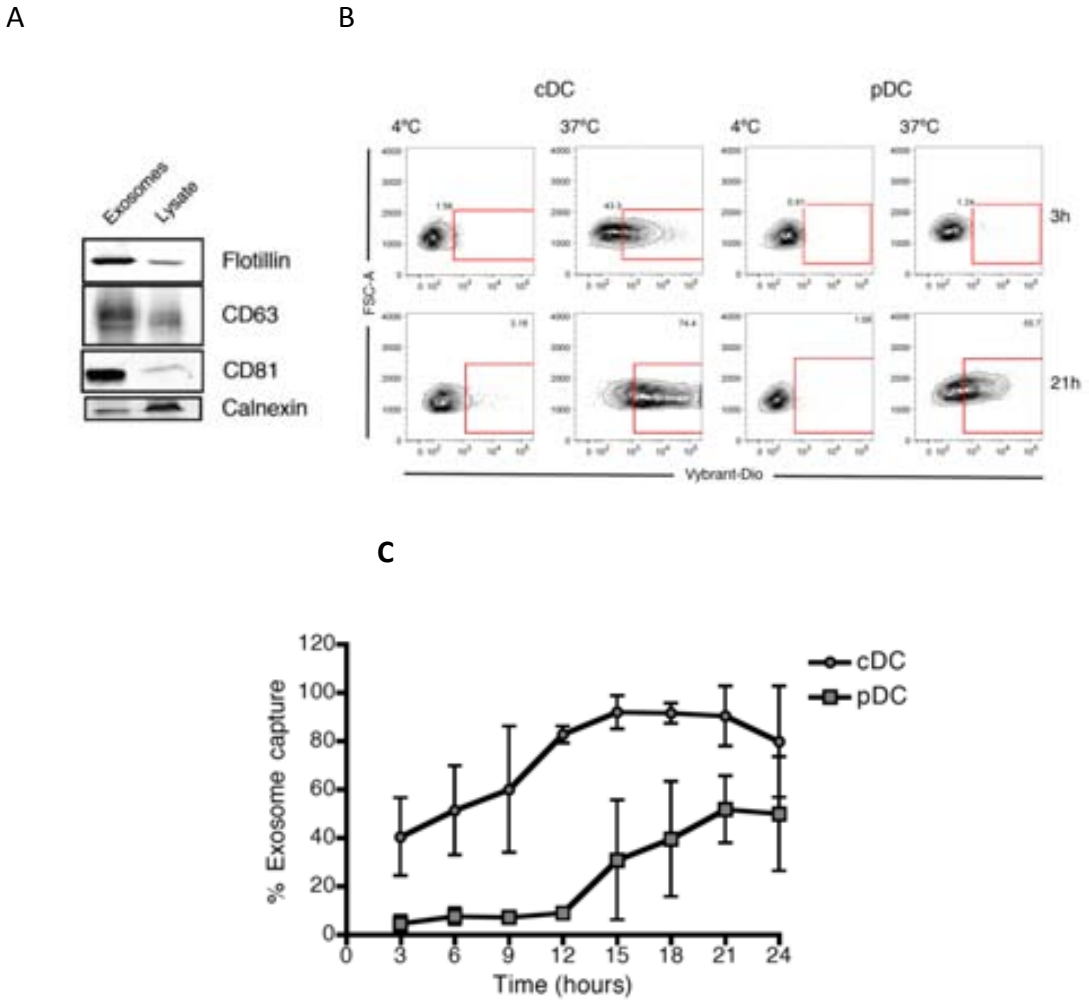


Figure 16. Peripheral blood dendritic cells capture Jurkat exosomes with different ability. **(A)** Western blot analysis was performed with the same amount of protein obtained from exosomes or Jurkat lysate extracts. Exosomal characteristic proteins included tetrastapanins CD81 and CD63 and the membrane protein flotillin, while calnexin (endoplasmic reticulum component) was used as control. **(B-C)** Sorted DCs were cultured with Vybrant Dio-labeled exosomes at different time points. Uptake of exosomes was analysed by flow cytometry. Control experiments were performed at 4ºC. **(B)** Counter plots of capture at 3h and 21h of a single experiment (out of six) are shown. Numbers indicate the percentage of cells. **(C)** Time course experiment showing the kinetics of exosome capture by DC subsets. cDCs (grey circles) and pDCs (grey squares) are depicted. Each time point shows the mean ± SD of at least two independent experiments.

Previous works have demonstrated that exosome internalization can be blocked by actin and PI3K inhibitors [208,239]. So we then investigated the involvement of endocytic mechanisms in the capture of exosomes by DC subtypes. The addition of cytochalasin D and wortmannin to cDC cultures induced a clear dose-dependent reduction in exosome-capture (Fig 18A and 18B), thus confirming the participation of endocytic processes in the exosome-capture by cDCs. In addition, confocal microscopy confirmed that internalized exosomes (Figure 18C) co-localized with markers of the endocytic pathway such as LAMP-1 (Figure 18E), as demonstrated before [208,244]. Unfortunately, as pDCs required longer incubation times to detect exosome capture, the cellular toxicity induced by cytochalasin D and wortmannin in pDCs precluded the possibility to confirm the involvement of endocytic mechanisms. Although fluorescent-labelled vesicles were observed by confocal microscopy in pDCs (Figure 18D), no co-localization with endocytic markers was detected (data not shown).

2.2. pDC capture enriched apoptotic bodies

Having demonstrated the capacity of pDCs to capture exosomes, we aimed to verify whether these cells were able to capture other types cell-derived microvesicles such as apoptotic bodies, as previous data in the literature have provided controversial results [296,301]. Capture of apoptotic bodies was first evaluated in co-culture experiments of each DC subset with UV-induced apoptotic Jurkat T cells. We could detect capture of apoptotic bodies by cDCs but not pDCs (data not shown) even at extended time points (up to 24h). However, when using enriched fluorescent-labelled apoptotic bodies from supernatants of UV-induced apoptotic Jurkat T cells, we could detect that pDCs (as cDCs) were able to capture apoptotic bodies in a similar fashion observed to exosomes (Figure 19). Thus, at least in vitro, using enriched apoptotic bodies rather than apoptotic-induced cells may account for the detection of antigen capture by pDCs.

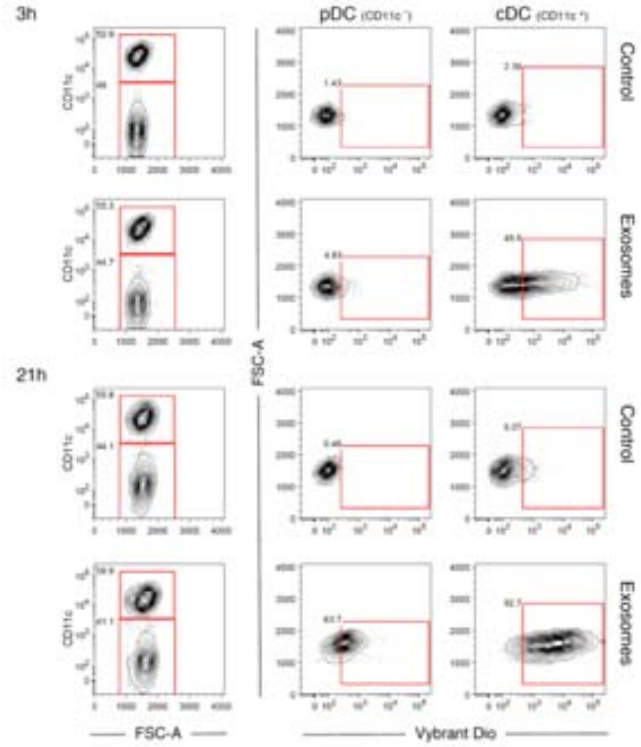


Figure 17. pDCs are able to capture exosomes even in presence of the high endocytic cDCs. After sorting, pDCs and cDCs were cocultured at 1:1 ratio for 3h or 21h in the presence of labelled exosomes. Then, cells were washed, stained for CD11c and the exosome capture was measured by flow cytometry. Numbers indicate the percentage of cells. One representative experiment of two is shown.

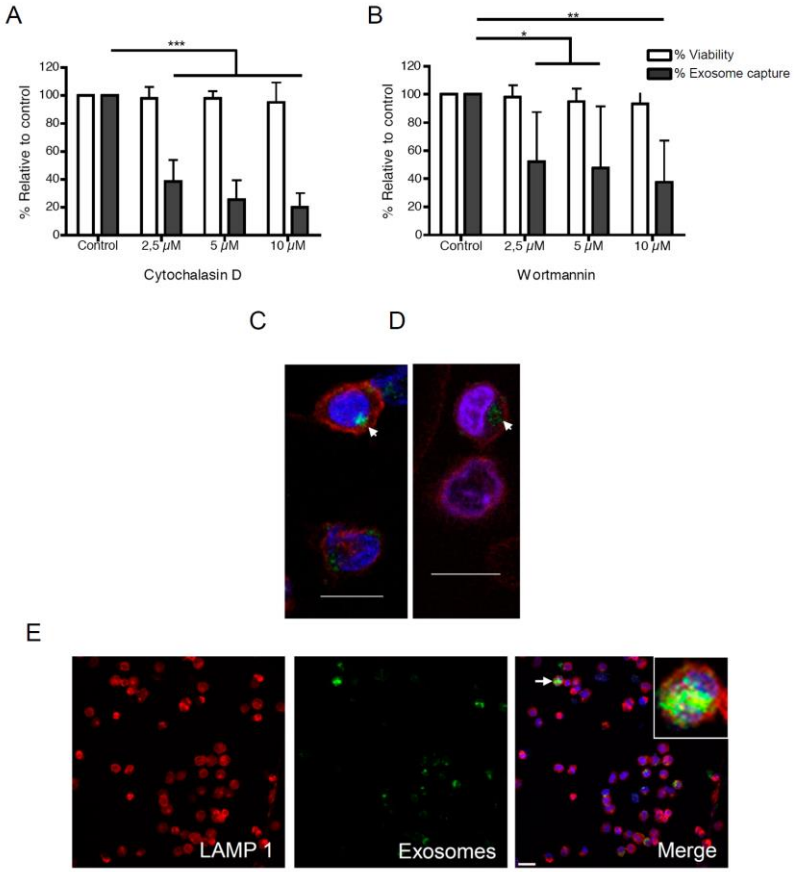


Figure 18. Endocytosis inhibitors block exosome uptake by cDCs. Cells were pre-incubated with cytochalasin D **(A)** or wortmannin **(B)** (2,5 µM, 5 µM, 10 µM) for 30 min before exosomes were added. The percentage of capture was analysed after 3 hours by flow cytometry. Cell viability (white bars) and exosome capture (black bars) was measured in at least 5 independent experiments in each condition. The results are shown as relative to the maximum value obtained in time-matched non-treated cells (Control). *P < 0,05; **P < 0,01; *** P < 0,001. Internalized exosomes follow the endocytic pathway in cDCs. Exosome capture was confirmed in cDCs **(C)** and pDCs **(D)** by confocal microscopy to ascertain the internal localization of captured vesicles (white arrows, one experiment of three is shown). Cell membrane was stained with an anti HL-DR antibody followed by Alexa 546-anti mouse IgG. Nuclei were stained with Hoechst 33342. **(E)** Internalization of exosomes through late endosomes/lysosomes was confirmed in cDCs by colocalization of Vybrant Dio and LAMP-1 in cytospins after 2h of exosome capture. Inner panel shows a magnification of the selected (white arrow) cell. (Bars= 10 µm)

2.3. Jurkat-derived exosomes do not alter the phenotype and maturation of human pDCs

It is considered that uptake of apoptotic cells negatively regulates DC maturation and promotes the generation of tolerogenic DCs [275,302–304]. However, Hoeffel et al [301] showed that purified human pDCs cross-presented vaccinal lipopeptides and HIV-1 antigens from infected apoptotic cells. In that study the authors suggested that tolerance induction by cross-presenting pDCs may be disrupted by recurrent infection leading to autoimmunity or allergy. Much less is known about the effect of exosomes in pDCs. First, exosome capture did not induce any significant modification in the surface expression of CD25 and CD83 in pDCs (Figure 20A). These results are in line with other observations [208]. Moreover, pDCs were still able to respond to R848 stimulation even after exosome capture (Figure 20A). In all the experiments performed, only the expression of CD40 was significantly reduced upon exosome capture (Figure 20B). In addition, exosome capture itself neither induced the secretion of IFNα by pDCs, nor inhibited the secretion of this cytokine induced by TLR ligation in cells that had captured exosomes (Figure 20E).

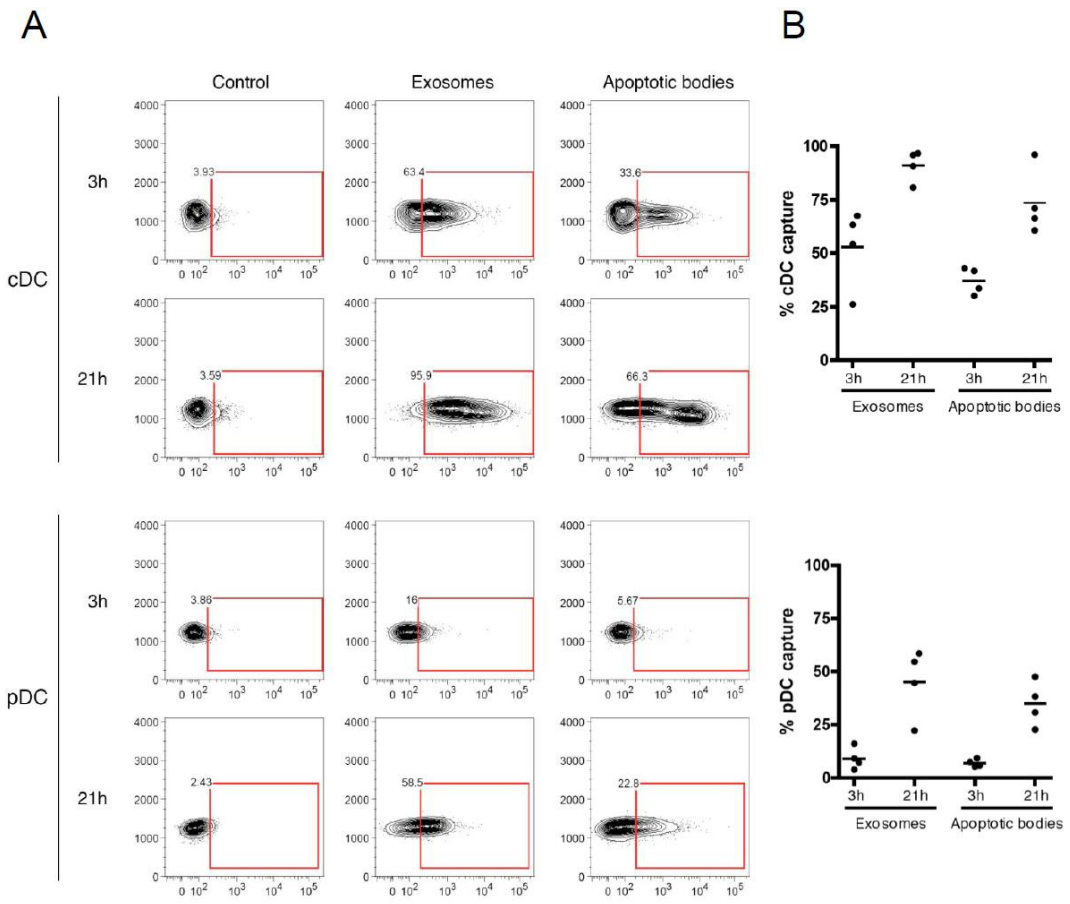


Figure 19. pDC capture enriched apoptotic bodies. Sorted pDCs and cDCs were incubated with fluorescent-labelled exosomes or apoptotic cells. **(A)** Uptake of vesicles was analysed by flow cytometry after 3h (cDC) or 21h (pDC) of incubation. Counter plots show the data from a representative experiment out of 4. Numbers in each panel show the percentage of capture. **(B)** Data from four independent experiments is shown for cDC and pDCs. Lines indicate the mean of the performed experiments.

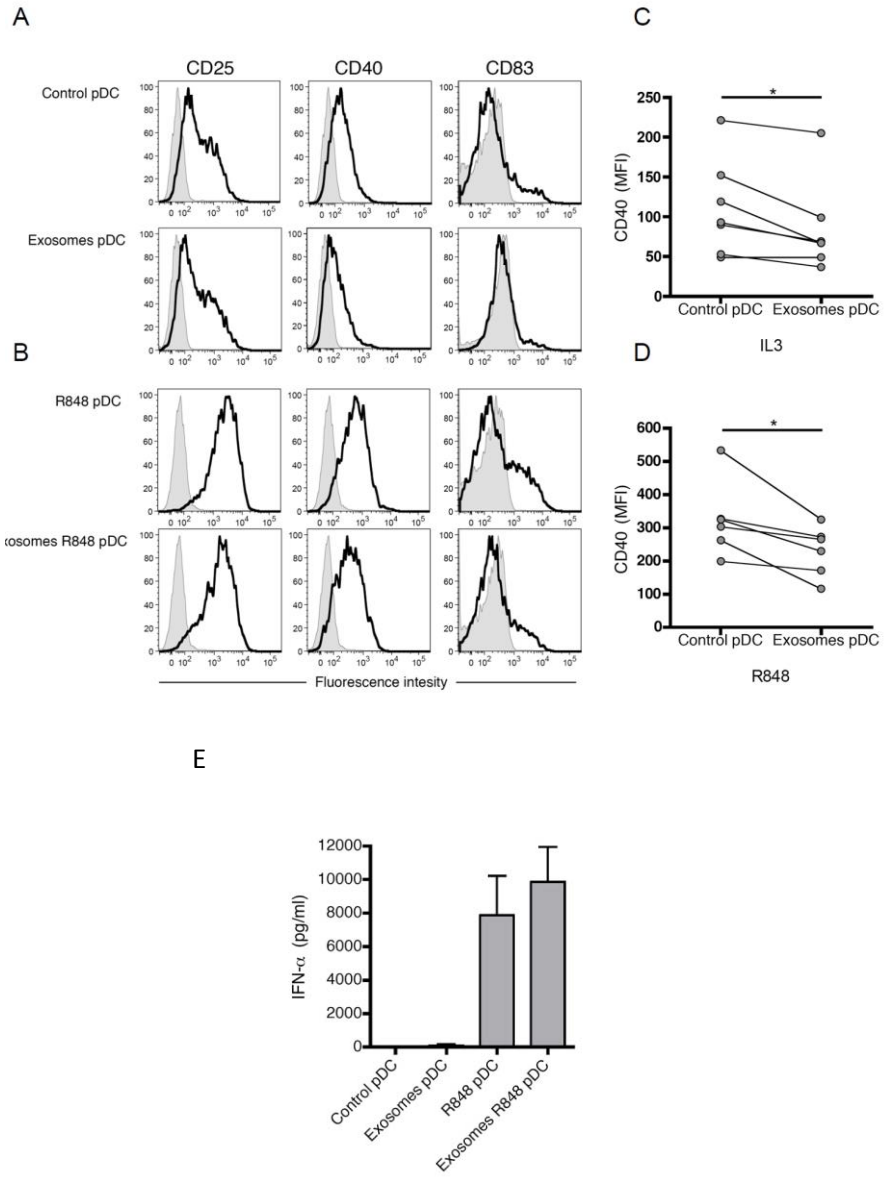


Figure 20. Uptake of Jurkat exosomes does not activate human pDCs. Plasmacytoid DCs were incubated with exosomes for 24h, then activated **(B)** or not **(A)** with R848 (5μM) for 16h. Open histograms show the expression of activation markers and solid histograms represent the isotype controls. **(C, D)** Graphs show the mean fluorescence intensity (MFI) expression of the co-stimulation molecule CD40, represented as paired data obtained from seven (IL3) and six (R848) independent experiments. *P < 0,05. **(E)** Production of INF-γ after exosome capture and incubation of pDCs with R848 was measured by ELISA. Data represent the mean ± SD of two independent experiments.

2.4. Exosome-loaded pDC induce autologous T cell proliferation

Exosomes captured by DCs could be a feasible source of "external" antigens to induce T cell responses. Therefore, we next studied the exosomes-derived antigen presentation by pDCs. The results showed that exosome-loaded pDCs were able to induce proliferation of autologous T cells (Figure 21A). Interestingly, IFN-γ (but no IL-10) was detected in supernatants of T cells stimulated with exosome-loaded pDCs, as expected for an activated T cell phenotype (Figure 21B). Due to the limited quantity of protein-associated exosomes present in the serum of healthy donors [218] (and our own results), appropriate autologous exosome loaded-pDCs could not be used in comparative experiments.

To summarize, human pDC are able to capture “cell-derived” antigenic material in the form of microvesicles such as exosomes and also apoptotic bodies. Although (in vitro) human cDCs appear to be far more efficient than pDCs in capturing these microvesicles, the capacity of pDCs to manage cell-derived microvesicles is of relevance for the homeostasis of immune system.

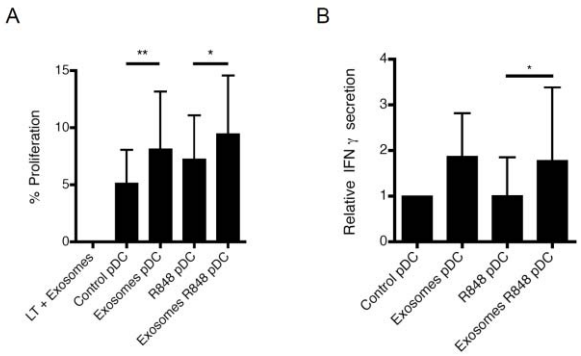


Figure 21. Induction of autologous T cells proliferation by human exosome-loaded pDC. After exosome uptake and activation, human pDCs were co-cultured with CFSE-labelled autologous T cells. Cell proliferation was analysed by flow cytometry at day 7 as determined by loss of CFSE. **(A)** The mean ± SD of 8 independent observations was calculated by subtracting the values observed in control conditions (isolated lymphocytes). **(B)** IFNγ secretion was measured in the supernatants by ELISA. Data are shown as relative to the control (pDCs) in 7 independent experiments. *P < 0,05; **P < 0,01.

SECTION III. Effect of donor exosomes-pulsed tolerogenic DCs in a rat model of renal transplantation

In the previous section, we have shown that human peripheral blood DCs capture exosomes. As DCs play a central role in antigen presentation and induction of tolerance, these results could be of interest in transplantation. So far, different strategies have been developed to induce specific tolerance in transplantation. For instance, the transference of regulatory DCs to recipient animals has been demonstrated to efficiently modulate allograft survival and induction of donor-specific tolerance [115,119,120,305]. Since kidney transplantation is one of the most usual procedures in organ transplantation, we have analysed the effect of donor exosomes-loaded tolerogenic DCs in a rat model of functional kidney transplantation. In contrast to human and mouse DCs, rat DCs have not been studied extensively until recently [52,54,159,306,307], so we first studied the generation of tolerogenic DCs from rat bone marrow precursors.

3.1. Differentiation of rat bone marrow derived dendritic cells

Rat dendritic cells were derived from bone marrow precursors. Although in most protocols DCs are differentiated solely in presence of GM-CSF [276], it has been demonstrated that addition of IL-4 results in a higher yield [50,51,307]. In contrast to the results observed in mice in which IL-4 induces the maturation of BMDCs [124], rats DCs maintained an immature state [145]. Similarly, the use of the Fms-related tyrosine kinase 3 ligand (FLT3L) expands the number of DCs precursors [54,308]. Therefore, we cultured rat BMDCs in presence of GM-CSF, IL-4 and Fms-related tyrosine kinase 3 ligand (FLT3L) as described in material and methods for 8 days. BMDCs were harvested and counted to assess the number of DCs generated. Regarding the yield, we could confirm previous observations in which the addition of FLT3L increased the number of DCs in GM-CSF/IL-4 treated cells (Figure 22A).

At day 8, both BMDCs treated with or without FLT3L were stimulated with LPS to induce maturation. After 24h, LPS-matured DCs increased the expression of MHC II (RT1B) and CD86 (Figure 22C, 22D). As previously reported, the addition of FLT3L to GM-CSF and

IL4 generates DCs with a lower expression of RT1B compared to FLT3L-untreated DCs. We also analysed the expression of the integrin alpha E2, recognized by the monoclonal antibody OX62, that is expressed by some DC subtypes in rats [309]. In spleen, it has been described that OX62 expression corresponded to conventional DC population whereas plasmacytoid DCs were negative for this molecule [46]. Rat BMDCs may express variable levels, or even not express, of OX62 depending on the culture conditions [50,52,276,307]. In this sense, and in contrast to previous studies [54], our BMDCs generated in presence of GM-CSF and IL-4 expressed high levels of OX62. Although positive, OX62 expression was lower in FLT3L-derived BMDCs. Strikingly, only FLT3L-derived BMDCs up-regulated OX62 upon maturation (Figure 22B).

To study the functionality, DCs generated in presence or absence of FLT3L, were co-cultured with allogeneic splenocytes. As expected, while immature BMDCs induced poor mixed lymphocyte responses, mature BMDCs induced the proliferation of allogeneic splenocytes (Figure 22D). FLT3L-DCs (Immature, mean= 5200 cpm ± 3215; LPS, mean= 9785 cpm ± 7918) induced slightly higher allo-responses compared to non-treated FLT3L-DCs (Immature, mean= 2976 cpm ± 1561; LPS, mean= 7207 cpm ± 4623) confirming previous observations [53,54].

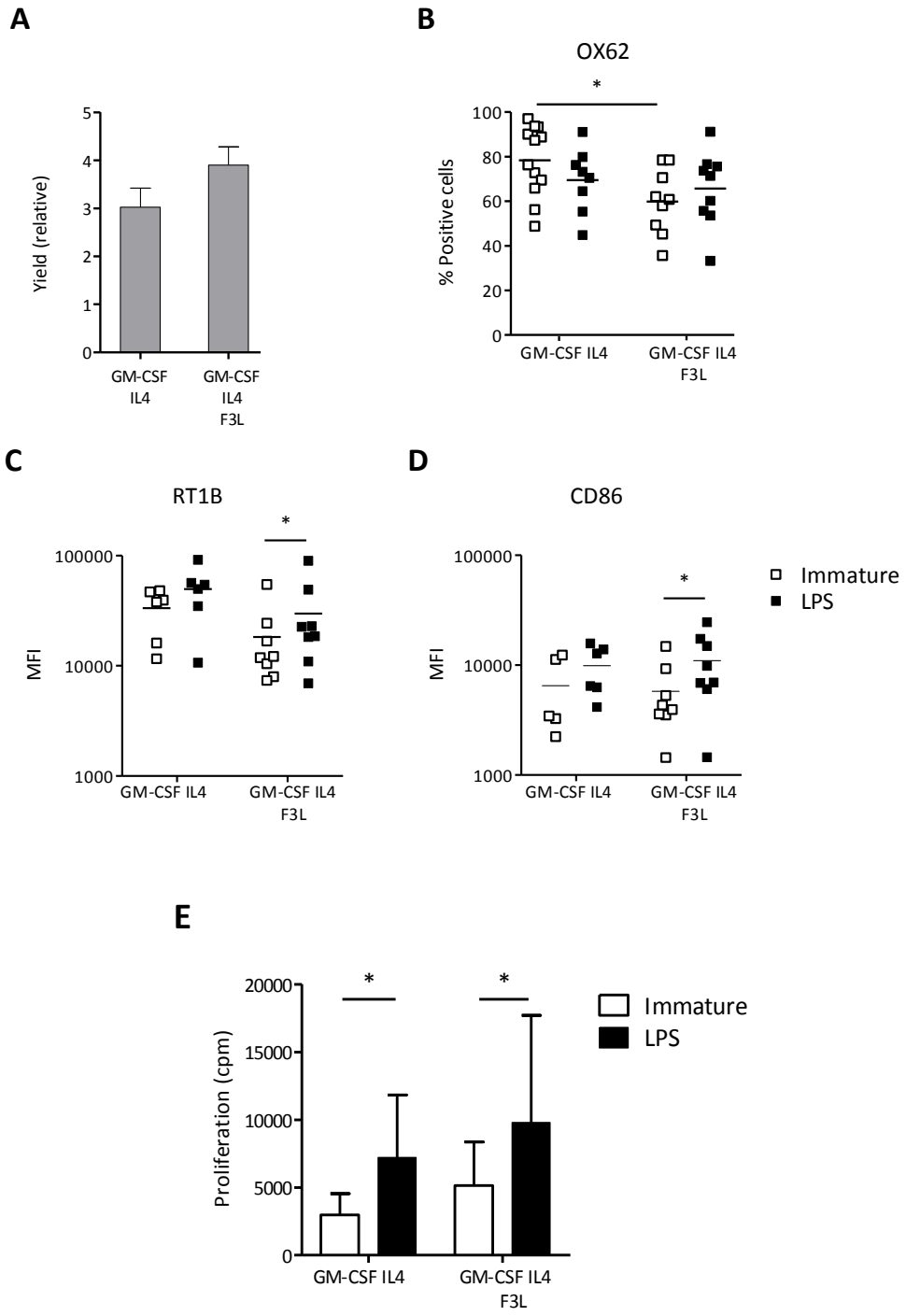


Figure 22. LPS induces the maturation of BMDCs and promotes their allo-stimulatory ability.

Figure 22. LPS induces the maturation of BMDCs and promotes their allo-stimulatory ability. Comparison of the yield, cell surface antigen expression and allo-stimulatory ability of BMDCs differentiated in presence of GM-CSF (10 ng/ml) and IL-4 (5 ng/ml) or GM-CSF (10 ng/ml), IL4 (5 ng/ml) and FLT3L (50 ng/ml). **(A)** At day 8, DCs were harvested and counted. Yield is represented as number of cells at day eight/ number of bone marrow precursors seeded at the beginning of the culture (n=4). **(B-D)** At day 8, GM-CSF + IL4 DCs and GM-CSF + IL4 + FLT3L DCs were stimulated with LPS (100 ng/ml) for 24h. Data are representative of at least 5 independent experiments. **(B)** OX62 expression was analysed to measure differences between the two types of DCs generated (Unpaired t test; $P < 0.05$). **(C-D)** Maturation was assessed by analysing the surface expression of RT1B **(C)** (Paired t test; $P < 0.05$) and CD86 **(D)** (Wilcoxon signed rank test; * $P < 0.05$). **(D)** At day 9, DCs were harvested, counted and co-cultured with allogeneic splenocytes (1:10). After 5 days, proliferation of splenocytes was measured by thymidine incorporation (Paired t test; * $P < 0.05$). Data are representative of at least 5 independent experiments.

3.2 Generation of tolerogenic BMDCs

Our next step was to generate tolerogenic DCs from FLT3L-treated BMDCs. Dexamethasone is a well-known inducer of tolerogenic DCs with a semimature phenotype and tolerogenic functions in human and rodent models [128,141,159,306,310]. Yet, it has been suggested that the generation of tolerogenic DCs in presence of dexamethasone may hamper the differentiation process and decrease the cell viability [311,312].

To evaluate the effect of dexamethasone on rat BMDCs differentiation and generation of tolerogenic DCs, BMDCs were cultured as before in presence of two different doses of dexamethasone and yield and morphology were analysed at day 8 (Figure 23). At 1 µM, dexamethasone decreased significantly the yield and DCs did not form the characteristic large cluster of aggregated cells. However, at 0.01 µM DC yield was as high as for immature DCs and they formed clusters as in control (Figure 23A-C).

These DCs were then activated with LPS and analysed after 24 h (Figure 24). In summary, dexamethasone induced a significant dose dependent reduction of RT1B expression in tolerogenic DCs compared to both immature and mature DCs. However, dexamethasone did not affect the expression of the costimulatory molecule CD86 and of the integrins CD11b and CD103 (OX62) in tolerogenic DCs compared to mature DCs.

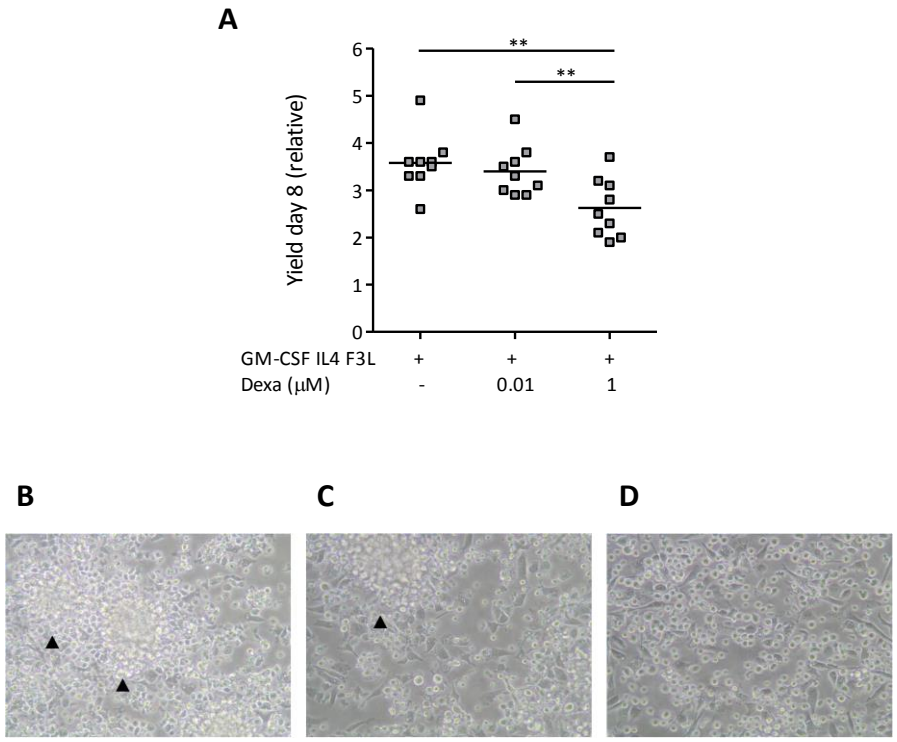


Figure 23. Generation of tolerogenic BMDCs. Addition of dexamethasone at the highest dose significantly impairs the yield and differentiation of DCs. **(A)** At day 8, DCs were harvested and counted. Yield is represented as number of cells at day 8/ number of bone marrow precursors seeded at the beginning of the culture (** $p < 0.01$, paired t test, n=9). **(B-C)** Immature DCs **(B)** and Dexa 0.01 μM-treated DCs **(C)** form aggregates of cells, whereas this morphology was not observed when dexamethasone was added at 1 μM **(D)** Light microscopy 20x. Arrows indicate clusters of DCs.

To further characterize tolerogenic DCs, cytokine production was also evaluated (Figure 25). LPS-treated DCs secreted IL-12 (16,62 ± 11,17 pg/ml) and TNF-α (937,5 ± 407 pg/ml) as previously reported [51]. In addition, DCs stimulated with LPS secreted IL-10 (mean=178,7 ± 49,94 pg/ml). Conversely, LPS-stimulated tolerogenic DCs failed to secrete IL-12 (Dexa 0,01μM, mean=4,67±1,51 pg/ml; Dexa 1 μM, mean=3,97±1,49 pg/ml) but secreted similar levels of IL-10 (Dexa 0,01 μM; mean=180,1 ± 54,81 pg/ml; Dexa 1 μM; mean=231,8 ± 35,61 pg/ml). Finally, these tolerogenic DCs produced lower levels of the pro-inflammatory cytokine TNF-α when compared to LPS-DCs and this reduction was more pronounced with the higher dose of dexamethasone (Dexa 0,01 μM, mean=695 ± 326 pg/ml; Dexa 1 μM,

mean=478,7 ± 203,4 pg/ml). Overall, these results showed that tolerogenic DCs generated with 0.01 μM dexamethasone exhibited typical morphology and phenotype of DCs, with high viability and impaired production of pro-inflammatory cytokines.

To gain further insight into the tolerogenic properties of Dexamethasone-treated DCs we decided to analyse their ability to stimulate allogeneic T cells. Immature, mature (LPS) or tolerogenic DCs (Dex-LPS DCs) were co-cultured with allogeneic splenocytes at 1:10 ratio. After 5 days, splenocytes proliferation was measured by thymidine incorporation (Figure 26). Mature DCs induced a potent stimulation of allogeneic T cells whereas both immature DCs and Dex-treated DCs showed a 30-40% of reduction of the stimulatory capacity.

In conclusion, Dexamethasone induces a regulatory phenotype and the inhibition of pro-inflammatory cytokines (IL-12, TNF-α) production without the alteration of IL-10 secretion. These alternative activated DCs also present reduced ability to promote activation of allogeneic T lymphocytes.

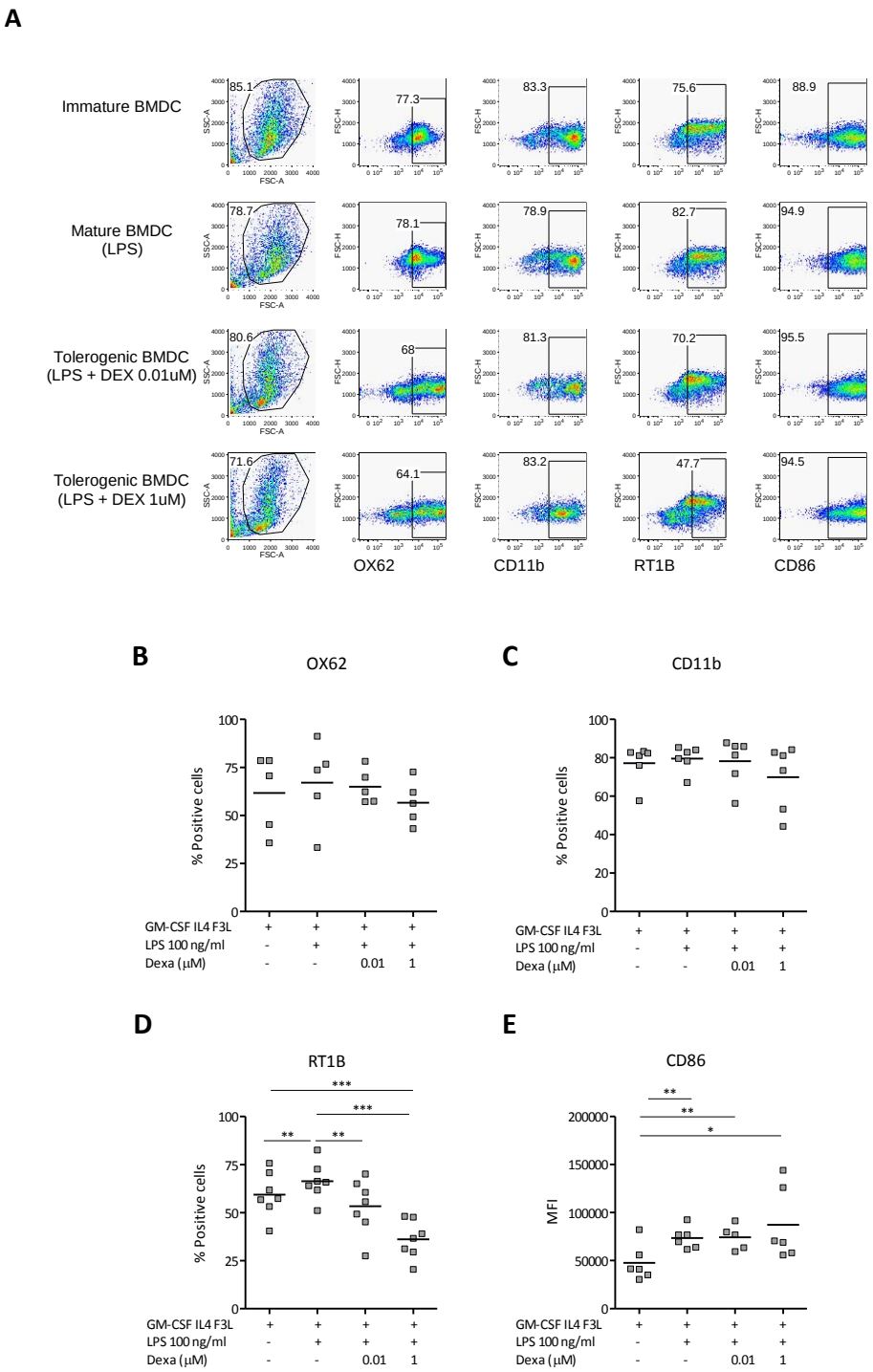


Figure 24. Tolerogenic BMDCs display a semi-mature phenotype.

Figure 24. Tolerogenic BMDCs display a semi-mature phenotype. Bone marrow precursors were cultured in presence of GM-CSF, IL4 and FLT3L. Dexamethasone was added to cultures at the dose indicated. To generate mature DCs, at day 8 cells were stimulated with LPS. Tolerogenic DCs were also activated with LPS in presence of dexamethasone. After 24h, DCs were harvested and the surface expression of the indicated molecules was analysed by flow cytometry. **(A)** One representative experiment is shown. Numbers indicate the percentage of positive cells. **(B-E)** Data are represented as the mean of the results and each dot corresponds to an individual experiment (* $P < 0.05$, ** $P < 0.01$, paired t test, $n \geq 5$).

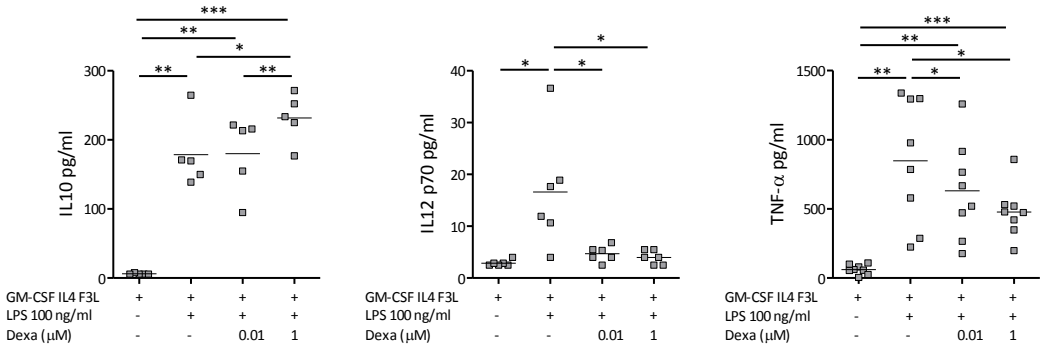
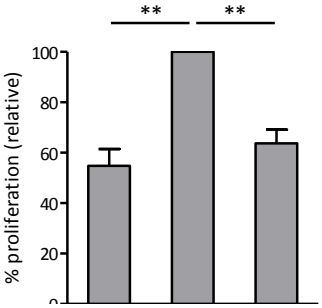


Figure 25. **Cytokine secretion by tolerogenic BMDC.** At day 9, BMDCs were harvested, centrifuged and supernatants were collected to measure the indicated cytokines by Luminex (from left to right IL-10, IL12p70 and TNF-α). Data are represented as the mean of the results and each dot corresponds to an individual experiment. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, paired t test, $n \geq 5$).

Figure 26. Tolerogenic BMDCs induce a hyporesponse of allogeneic T cells. At day 9, immature, mature and tolerogenic BMDCs were harvested, counted and co-cultured with allogeneic T lymphocytes (1:10). Cells were cultured for 5 days and proliferation of T cells was measured by thymidine incorporation. Data are expressed as relative to mature DC (** $P < 0.01$, paired t test, n=3).



3.3 Antigen presentation by rat tolerogenic BMDCs

Having observed the tolerogenic profile of Dex-treated DCs, and aiming their use in the model of kidney transplantation, in vitro analyses were performed to study the ability of Dex-treated DCs to present donor antigens to syngeneic T cells using exosomes as a source of alloantigens. As DC-derived exosomes are enriched in class I and class II MHC molecules, we obtained exosomes from DCs supernatants. Depending on their maturation state, BMDCs secrete exosomes with different phenotype [210,229]. Exosomes from immature DCs express lower levels of MHC I and II molecules and co-stimulatory molecules compared to mature DCs-derived exosomes [268]. Moreover, RNA content may vary between exosomes derived from immature versus mature DCs [229]. To avoid any undesirable effect derived from maturation of BMDCs and, thereby, from exosomes we decided to isolate exosomes from immature BMDCs.

3.3.1. Exosome isolation and characterization

First, we characterized exosomes derived from BMDCs cultures obtained as described in material and methods. On average, we purified 1.25 µg of protein per million of cell (Figure 27A). A concise analysis of the exosome phenotype by flow cytometry showed the expression of RT1B as well as typical exosomal markers such as the tetraspanins CD81 and CD63 (Figure 27B). Using the Nanosight technology, we also determined the distribution and size of the BMDC-derived exosomes. Exosomes preparations contained vesicles ranging 90-180 nm of diameter with an average concentration of 26·10^8 vesicles/ml (Figure 27C). Finally, cryoelectron microscopy of the purified exosomes revealed the presence of vesicles with the characteristic morphology and size (Figure 27D).

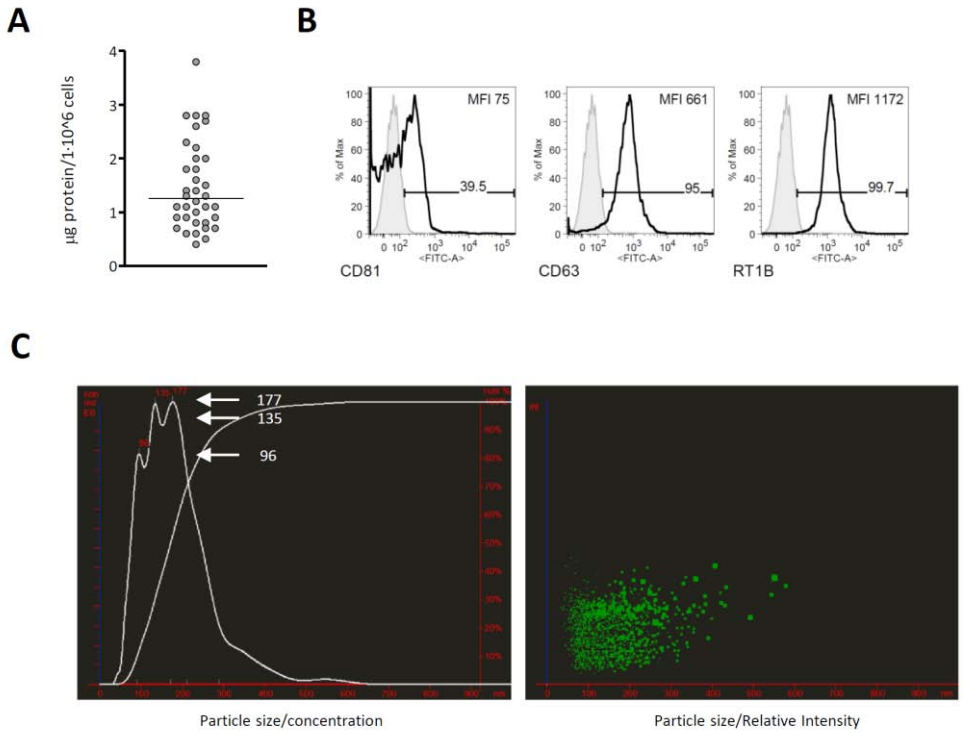


Figure 27. Characterization of BMDC-derived exosomes. BMDCs were cultured in presence of GM-CSF and IL-4 for 10 days. At days 8 and 10 supernatants were collected and exosomes were isolated. **(A)** Protein was quantified by Bradford assay. Bar represent the mean of 36 independent experiments. **(B)** Phenotype analysis of exosomes by flow cytometry. Equal amounts of exosomes were incubated overnight with sulphate aldehyde-beads and label with the indicated antibodies. **(C)** Nanosight analysis of exosomes. The left panel indicates the frequency size distribution (mean=186 nm) of particles and the right panel shows the relative intensity of particle size. One representative experiment is shown. **(D)** Cryoelectron microscopy showing the characteristic round-shape morphology and size of exosomes. One representative experiment is shown. Bar 200 nm.

3.3.2. Capture of exosomes by BMDCs

To study the ability of rat BMDCs to capture exosomes, immature, mature and tolerogenic BMDCs were incubated with allogeneic BMDC-derived exosomes. In order to evaluate the capture, exosomes were labelled with PHK67 dye to allow their detection (Figure 28). At day 8, BMDCs were counted and pulsed with equal amounts of donor labelled exosomes. After 24h, DCs were recovered, washed extensively with PBS, counted and exosome capture was analysed by flow cytometry. A dose-dependent increase of exosome capture was similarly detected in immature, mature and tolerogenic DCs. In fact, at this time point no significant difference was observed in the ability of exosome uptake between immature and mature DCs. Regarding tolerogenic DCs, exosome capture was not as efficient as for immature and mature DCs. However, more than 50 % of cells were positively labelled with the medium dose of exosomes, revealing exosome capture.

3.3.3. Analysis of yield, phenotype and cytokine production of exosome-loaded tolerogenic BMDCs

It has been reported that exosome uptake does not substantially modify the phenotype of DCs [208]. However, in our experimental model the expression of allogeneic MHC molecules in exosomes derived from immature cells could alter the tolerogenic state of dexamethasone-treated DCs. To verify this, at day 9, immature-, mature-, tolerogenic- and exosome-loaded tolerogenic BMDCs were harvested and counted (Figure 29A). LPS-induced maturation of BMDCs significantly decreased the yield compared to immature DCs. This effect was not so pronounced for tolerogenic BMDCs, even when exosomes were present. In fact, it seems that dexamethasone attenuates LPS-induced reduction of recovered cells. These results are in concordance with previous data, where mature but not tolerogenic BMDCs presented a lower viability measured by annexin-V and propidium iodide staining [141].

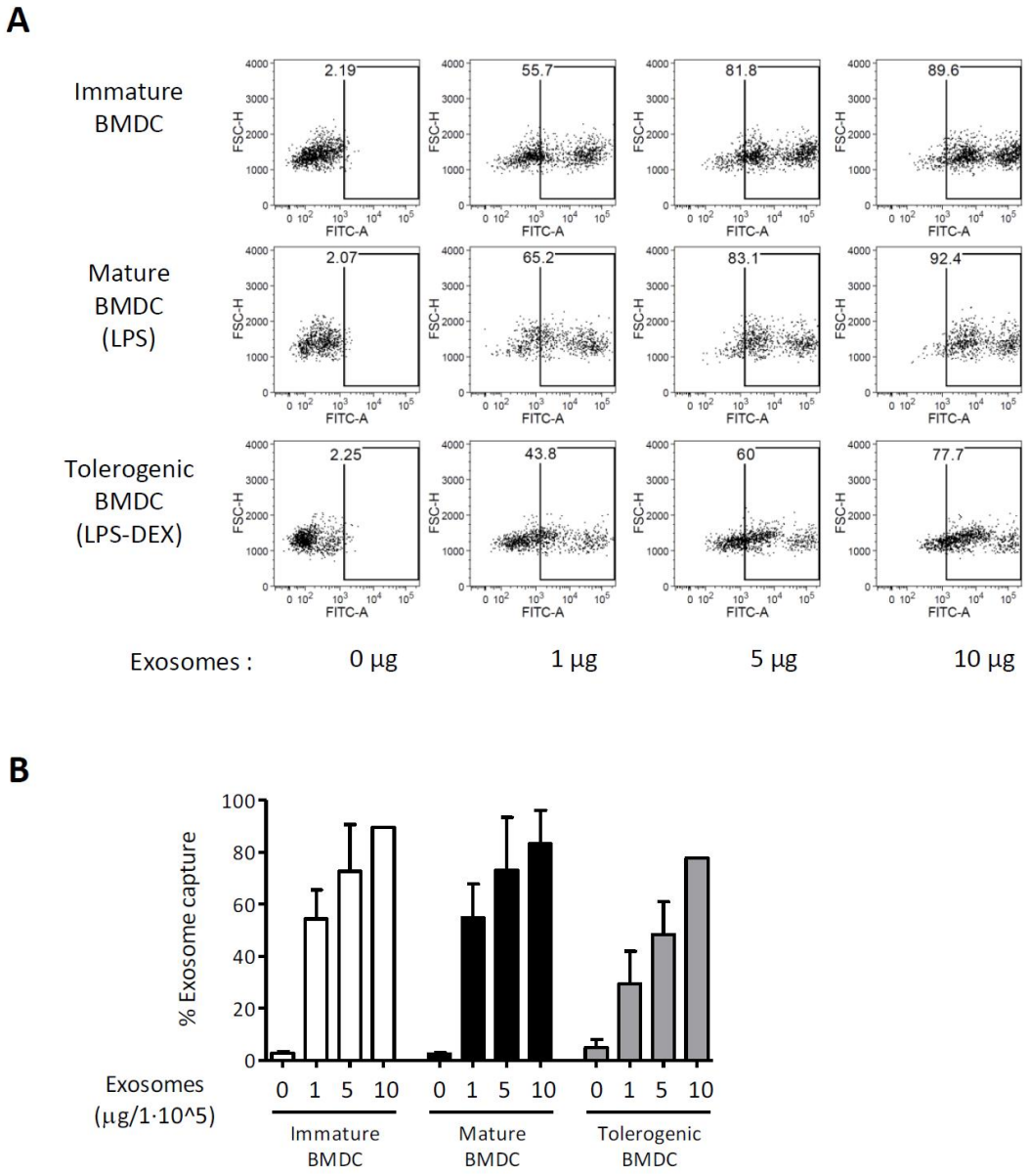


Figure 28. Tolerogenic BMDCs capture donor exosomes efficiently. At day 8, Wistar Agouti BMDCs were harvested, counted and 0.5·10^6 of cells were pulsed with increasing doses of PKH67-labeled donor (Brown-Norway) exosomes. Mature and tolerogenic BMDCs were activated in presence of LPS and tolerogenic BMDCs were treated with LPS and dexamethasone. After 24h, cells were extensively washed and exosomes uptake was analysed by flow cytometry. **(A)** Plots show an example of one representative experiment. **(B)** Bars show the media and standard deviation of three independent experiments.

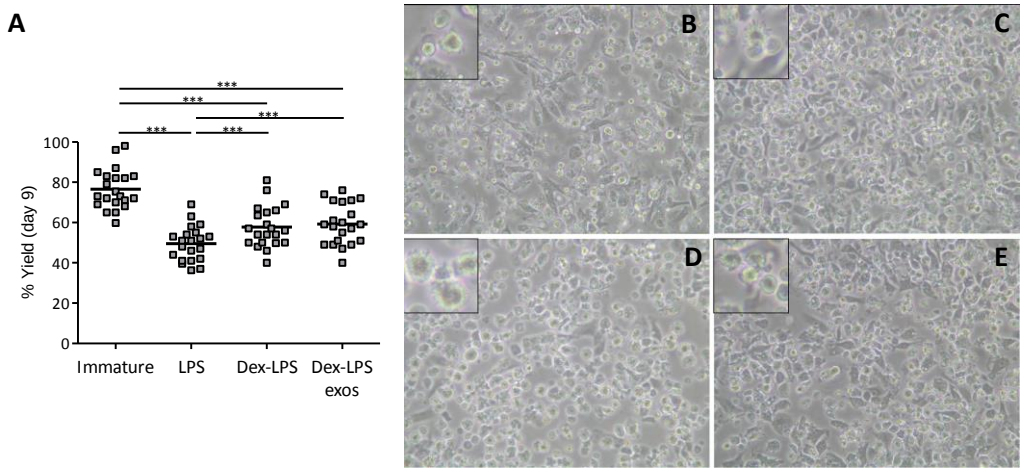
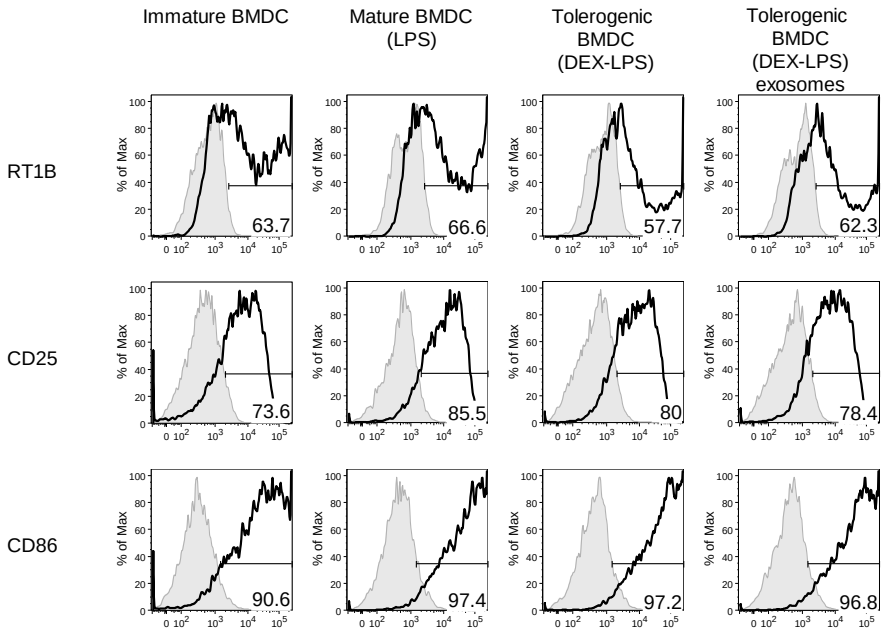


Figure 29. Generation of donor exosome-loaded tolerogenic BMDC. (A) At day 9, immature, mature, tolerogenic and exosome-loaded BMDCs were harvested, counted and yield was analysed. Each dot corresponds to one independent experiment (*** $P < 0.001$, paired t test, n=21). **(B-E)** Light microscopy (20x) showing the morphology of BMDCs (B: Immature DCs, C: Mature DCs, D: Tolerogenic DCs, E: Exosome-loaded tolerogenic DCs). One representative experiment is shown. Inner panels show a magnification of a selected area.

Phenotypically, mature DCs increased the expression of RT1B and the co-stimulatory molecule CD86 compared to immature DCs. Moreover, mature DCs up-regulated the expression of the activation molecule CD25 [313]. When dexamethasone was present at the time of maturation, RT1B expression was significantly reduced compared to mature DCs. However, tolerogenic DCs did not reduce the levels of CD86 as we observed before. Conversely, CD25 expression was moderately reduced compared to mature DCs. Exosomes-loaded tolerogenic BMDCs, did not show any phenotypically difference with their non-pulsed counterparts (Figure 30A-C). Moreover, the cytokine secretion profile of exosome-loaded tolerogenic BMDCs was not different to that observed in the non-pulsed tolerogenic DCs (Figure 31), an equivalent to the result obtained in previous experiments (Figure 25).

A



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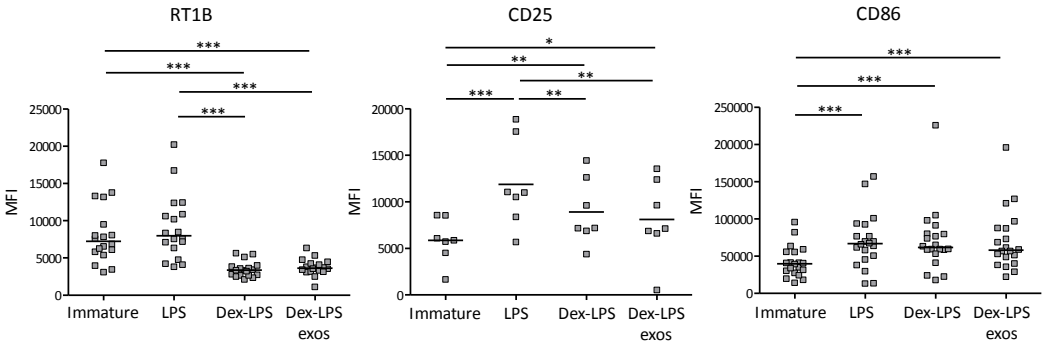


Figure 30. Effect of exosomes on the phenotype of tolerogenic BMDC. Flow cytometry analysis at day 9 of immature, mature, tolerogenic and exosome-loaded tolerogenic BMDCs. **(A)** Histograms show one representative experiment. Grey histograms show the isotype controls. Numbers indicate the percentage of positive cells. **(B)** Data are represented as the mean of the results and each dot corresponds to an individual experiment (RT1B, n=18 and CD25, n=7, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, paired t test; CD86, n=20 *** $P < 0.001$ Wilcoxon signed rank test).

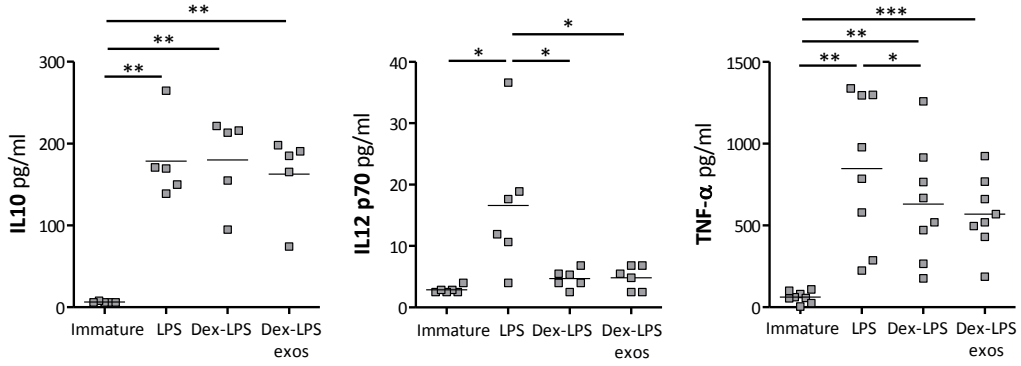


Figure 31. Donor exosomes do not alter the cytokine profile of tolerogenic BMDC. IL-10, IL12p70 and TNF-α were measured in supernatants of immature, mature, tolerogenic and exosome-loaded tolerogenic BMDCs by Luminex. Bars represent median values of at least five experiments and each plot represents an independent experiment. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, paired t test).

To summarize, LPS-Dex treated DCs show a modestly reduced ability for exosome capture compared to immature and mature DCs. Importantly, the combination of LPS with dexamethasone to generate tolerogenic BMDCs increases the yield of DC that otherwise is reduced after LPS stimulation. Finally, incubation of tolerogenic BMDCs with donor exosome does not modify their phenotype or cytokine profile.

3.3.4. Antigen presentation by exosome-loaded BMDCs

To examine the potential of Dex-treated DCs to induce tolerance to alloantigens, we performed an assay of exosomes-derived allo-antigens presentation to syngeneic T cells. As detailed in the previous section, DCs were pulsed at day 8 with donor exosomes and then cultured in presence of LPS to obtain mature DCs, or in presence of LPS and dexamethasone to generate tolerogenic DCs. As control, DCs were rendered immature without adding LPS. Syngeneic T cells were purified from splenocytes and co-cultured with different numbers of exosome-loaded DCs. After 5 days, proliferation of T cells was measured by thymidine incorporation (Figure 32). As expected, immature (grey circles) and tolerogenic DCs (grey diamonds) did not induce proliferation of syngeneic T cells. Similarly, proliferation was not observed when they were pulsed with allogeneic exosomes (black symbols). Conversely, LPS-activated DCs (grey squares) showed a background level of T cell

proliferation that was clearly increased when exosomes-loaded mature DCs were used (black squares) indicating that antigen presentation was taking place. The small differences observed account for a primary response.

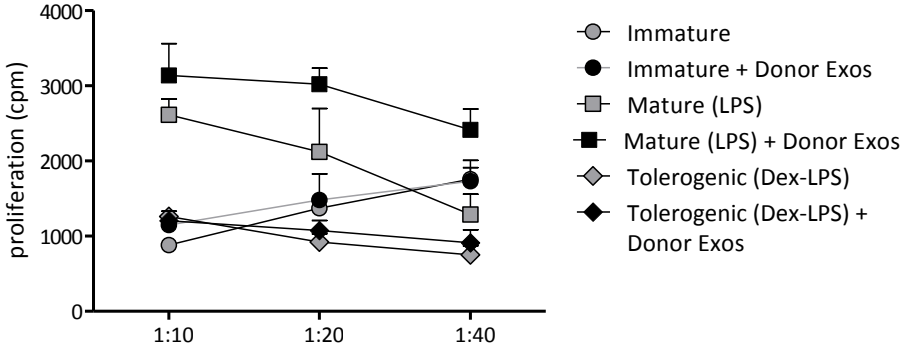


Figure 32. Tolerogenic DCs induce alloantigen-specific hyporesponse of T cell in vitro. BMDCs were pulsed or not at day 8 with allogeneic exosomes for 24h. Mature DCs were activated in presence of LPS, tolerogenic DCs were generated in presence of LPS and dexamethasone and immature DCs did not receive any stimuli. At day 9, DCs were recovered, extensively washed with PBS and co-cultured with syngeneic T lymphocytes at different ratios. After 5 days, T cell proliferation was analysed by thymidine incorporation. Data are represented as mean ± SD of four replicates.

Overall, these data demonstrate that treatment of BMDCs with dexamethasone generates alternative activated DCs that, after receive a maturation stimulus such as LPS, present a semimature phenotype. Moreover, these tolerogenic DCs show a hampered secretion of pro-inflammatory cytokines while the production of the anti-inflammatory cytokine IL-10 is not affected. Finally, albeit Dex-DCs are able to capture exosomes as do immature and mature DCs, do not stimulate syngeneic T cells after exosomal alloantigen presentation.

3.4. Effect of donor exosomes-pulsed tolerogenic BMDCs in a model of kidney transplantation in rats

To evaluate the possible role of exosomes-loaded Dex-DCs in modulating allo-responses in vivo, we set up a model of kidney transplantation in rats. It has been previously shown that treatment of heart graft recipients with donor exosomes results in prolongation of graft survival [157]. However, indefinite survival was not achieved unless immunosuppression was administered [158,268]. One plausible reason is that in the

absence of immunosuppressant drugs, host DCs may mature and present the injected exosomes inducing an immunostimulatory response. Nevertheless, although exosomes have been localized in secondary lymphoid organs (SLO) such as the spleen, once intravenously injected they circulate to the liver where can be eliminated. Therefore, using donor alloantigen-loaded tolerogenic DCs may overcome these situations by active migration of DCs to SLO where they can present donor antigens to resident lymphocytes in a tolerogenic way.

3.4.1. In vivo migration of tolerogenic DCs and donor exosomes

To assess this point we investigated the migratory ability of tolerogenic DCs in vivo in healthy animals. As detailed in material and methods, tolerogenic DCs cells were labelled with the fluorescent dye NIR815. Animals were anesthetized and 6.5·10^6 of syngeneic labelled DCs were intravenously injected. Monitoring of DCs migration in vivo was carried out at different time points. Five minutes after injection, tolerogenic DCs were faintly located in the lungs (Figure 33a). Thereafter, DCs were mainly localized in liver (Figure 33c) where they persist until day 6 (Figure 33e-g). However, at this time point the fluorescent signal seemed to have shifted to the splenic zone (Figure 33g). Interestingly, from day 1 until day 6 we detected a weak signal in lower limbs (Figure 33e, red triangle), indicating that probably DCs also migrated to bone marrow. At day 6 rats were sacrificed and different organs were isolated and analysed ex vivo for the presence of labelled DCs (Figure 34a, d, f-g). Although DCs were still localized in the liver, a potent signal was shown in the spleen (Figure 34a). Ex vivo imaging of lungs also showed that DCs were localized in these organs, despite no signal was detected in vivo from day 1 (Figure 33e). No positive signal was detected in kidney, mesenteric lymph nodes or heart at this time point (data not shown). Interestingly, the presence of DCs in bone marrow could be confirmed when tibias and femurs were extracted and compared to control (Figure 34g –iii- and 34g -iv-).

In the same experiment, we also studied the localization of intravenously injected donor exosomes that were labelled in the same way that tolerogenic DCs (Figure 33b-g). From early time points exosomes were mostly localized in liver and, probably due to the high signal observed, it was not possible to identify exosomes in other anatomical sites (Figure 33b-g). When animals were sacrificed at day 6, we could observe a strong signal in

spleen and liver (Figure 34b), compared to DCs that were found in spleen (Figure 34a). As in DCs, exosomes were found in lungs (Figure 34e) and bone marrow (Figure 34g –iv-) but not in mesenteric lymph nodes, heart or kidney (data not shown).

Thus, since tolerogenic DCs are able to migrate to SLO and persist there for at least 5 days, they can potentially present donor alloantigens from graft and/or exosomes to recipient lymphocytes in a tolerogenic manner. Similarly, exosomes localized in the spleen, in the absence of danger signals or under immunosuppressive regimen, may be presented by host DCs and could induce an immunomodulatory response.

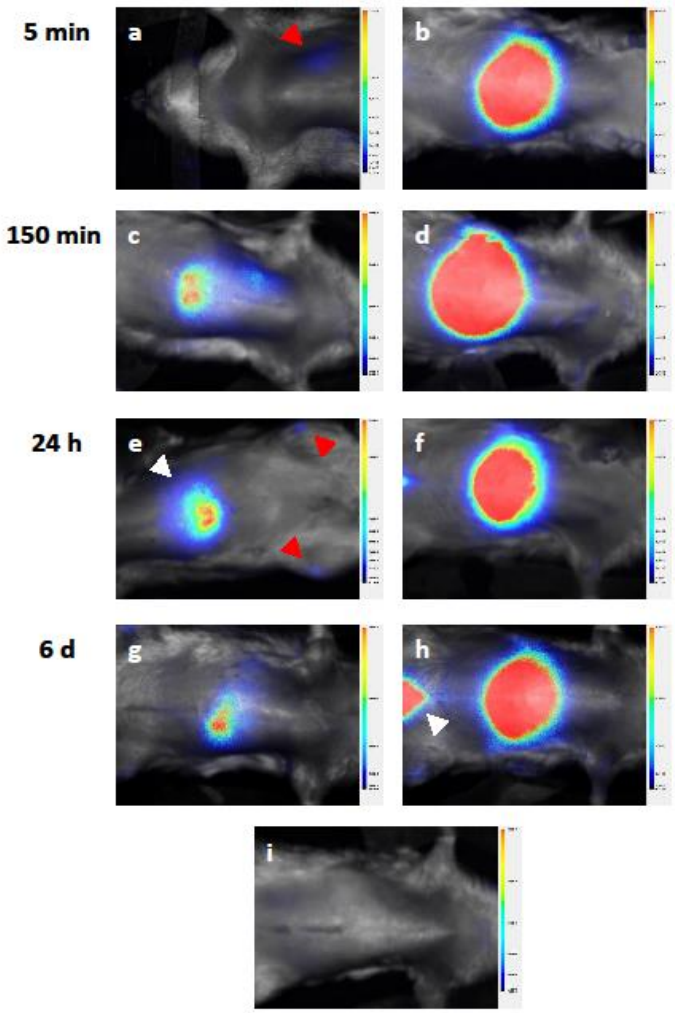


Figure 33. Migration of tolerogenic BMDC and donor exosomes. In vivo imaging of intravenously injected tolerogenic BMDCs and donor exosomes labelled with the fluorescent dye NIR815. **(a, c, e, g)** Homing of BMDCs at different time points. **(a)** Five minutes after i.v. injection, DCs migrated to lungs (red triangle). **(c)** Two hours and a half later they were mainly localized in liver. **(e)** One day after injection, DCs were still localized mostly in liver (white triangle). A weak signal was detected in both lower limbs (red triangles). The same results were observed at days 3 and 5 (data not show). **(g)** At day 6 DCs were still localized in liver, however there was a shift of the signal towards the splenic zone (see Figure 14). **(b, d, f, h)** Traffic of donor exosomes in vivo. Unlike DCs, exosomes were quickly localized in liver at early time points **(b)**. At later time points, 2.5h **(d)**, 24h **(f)** and 6

days **(h)** exosomes persisted in liver. Signal from any other localization was not observed probably due to the strong fluorescence in liver. White triangle indicates residual exosomes at the injection site. (i) In vivo imaging of control animal in absence of DCs or donor exosomes.

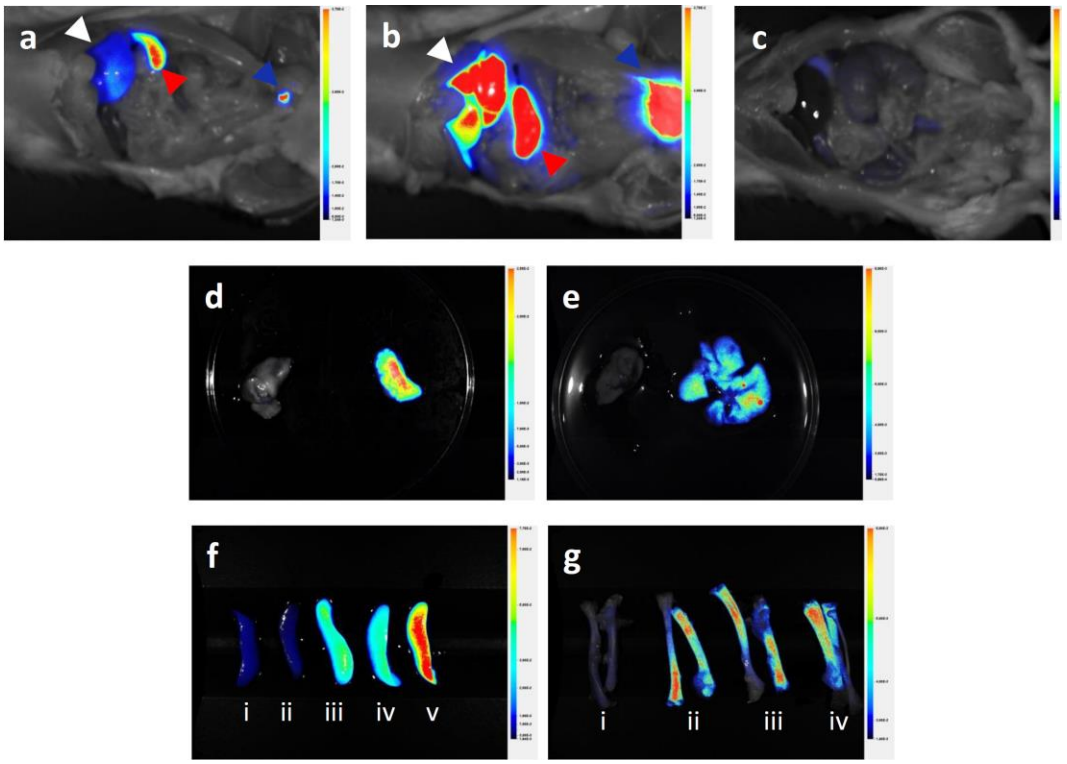


Figure 34. Ex vivo analysis of tolerogenic BMDCs and donor exosomes traffic. At day 6, animals were sacrificed, and organs were extracted to compare injected animals to non-injected animal control. **(a-c)** Tolerogenic BMDCs **(a)** were localized mainly in the spleen (red triangle) with some of them still in the liver (white triangle). Blue triangle indicates residual BMDCs in the injection site. Conversely, donor exosomes **(b)** were localized with the same intensity in the liver and the spleen. Blue triangle indicates residual exosomes in the injection site. **(c)** Background signal from the control rat. **(d-g)** Isolated organs from injected animals compared to control. **(d-e)** Tolerogenic BMDCs **(d)** and donor exosomes **(e)** were localized at lungs. **(f)** The image shows isolated spleens from all animals injected, from left to right: control (i), control (ii), tolerogenic BMDCs (animal 1) (iii), tolerogenic BMDCs (animal 2) (iv) and donor exosomes (v).**(g)** The image shows isolated femur and tibias from all animals injected, from left to right: control (i), tolerogenic BMDCs (animal 1) (ii), tolerogenic BMDCs (animal 2) (iii) and donor exosomes (iv).

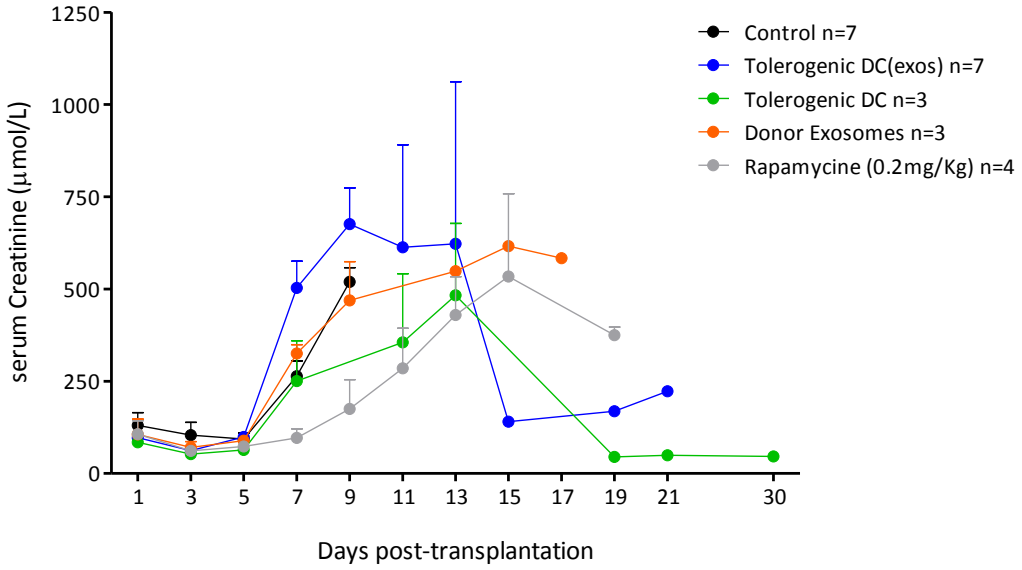
3.4.2. Tolerogenic BMDCs treatment does not prolong graft survival in a fully mismatched kidney transplantation model

To evaluate the tolerogenic capacity of exosomes-loaded tolerogenic DCs in vivo a rat model of functional kidney transplantation was set up. In this model Wistar Agouti rats are "recipient" and Brown Norway rats are "donors". In absence of treatment, kidneys are rejected within 9 days after transplantation [314], mainly due to acute humoral rejection.

Recipient animals received an allogeneic kidney and were bi-nephrectomised at the moment of transplantation. Therefore, these animals only maintain the function of the allogeneic transplanted kidney. Rats were injected with 5·10^6 of donor exosomes-loaded syngeneic tolerogenic-DCs (Tolerogenic DCexos) or non-pulsed tolerogenic DCs (Tolerogenic DC). The third group was treated with 125 µg of donor-exosomes (Donor Exosomes), the same quantity used for loading tolerogenic DCs. Animals were intravenously injected one week and the same day of the transplantation. As control, recipient animals did not receive any immunosuppressive therapy (Control). Finally, one additional group was treated with subtherapeutic doses of rapamycin (0.2 mg/kg).

To assess the evolution of the renal function, serum creatinine was measured every 2 days being the first measure taken the day after transplantation (Figure 35). In untreated groups, there is a progressive increase in creatinine levels from day 5 until the moment of sacrifice. Similarly, rats that received an injection of exosomes alone did not show any difference from untreated animals. Tolerogenic DCs-treated animals showed an interesting response. Despite the majority of rats were not able to control their increasing levels of creatinine it is important to note that those animals which survived over 9 days controlled the creatinine values for a long period. These observations were similar in both tolerogenic DCs and exosomes-loaded tolerogenic DCs. Specific attention must be done to a rat that survived for 30 days with no signs of rejection which was sacrificed for further analyses. Finally, and unexpectedly, creatinine was reduced in animals treated with sub-therapeutic dose of Rapamycin (0.2mg/kg) compared to control group.

A



B

	Control			Tolerogenic DC(exos)			Tolerogenic DC			Donor Exosomes			Rapamycine (0.2mg/Kg)		
Days	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
1	130,7	34,1	7	97,2	13,1	7	84,6	12,7	3	105,8	41,7	3	106,4	36,6	3
3	104,1	34,7	7	61,9	4,5	7	52,6	4,0	2	70,7	15,9	3	61,0	3,5	3
5	93,1	16,6	7	98,7	7,5	6	63,6	5,5	3	88,7	11,5	3	73,4	11,5	2
7	263,4	41,6	7	503,3	72,1	6	250,2	109,3	3	325,0	24,4	3	96,7	24,6	3
9	519,3	38,6	7	675,7	98,2	6				469,1	104,8	3	174,7	79,6	3
11				613,1	278,0	2	355,7	186,0	3				284,9	108,7	3
13				622,3	439,3	2	483,1	194,9	2	548,1		1	429,3	104,2	3
15				140,6		1				616,1		1	533,9	224,5	2
17										583,4		1			
19				168,8		1	45,08		1				374,8	22,1	2
21				222,8		1	49,5		1						
27															
30							45,97		1						

Figure 35. Serum creatinine levels in kidney transplanted rats. Serum creatinine was determined on blood samples collected from the tail vein every 2 days beginning the day after surgery. **(A)** Results are expressed as mean ± standard error for each day and group. Control n=7, Tolerogenic DC(exos) n=7, Tolerogenic DC n=4, Donor exosomes n=3, Rapamycin (0.2mg/Kg) n=4. (1 way Anova, P=ns). **(B)** Summary table indicates the mean, error standard and number of surviving rats included in each day.

Regarding the overall survival rate, control group rejected kidneys at day 9 (n=7; days 9,9,9,9,9,10,10). All other treatments assessed increased the survival rate [DC(exos) n=7, median=10 (days 7,9,10,10,10,12,26); DC n=4, median=14 (days 11,14,15,30); Exos n=3, median=11 (days 11,11,17); Rapamycin n=4, median= 16 (days 12,13,19,20)], but none of them showed significant differences to control group (Figure 36). Strikingly, one animal of each group showed prolonged survival (Donor Exosomes=17 days, Tolerogenic DC(exos)=26 days, Tolerogenic DC=30 days), although the exosomes-treated rats did not show reduced levels of creatinine. Rats injected with either donor exosomes-loaded DCs and not loaded DCs showed a decrease in serum creatinine. This observation might reflect an improvement of renal function that could be due to DCs treatment and indicate a biological response to the cellular therapy.

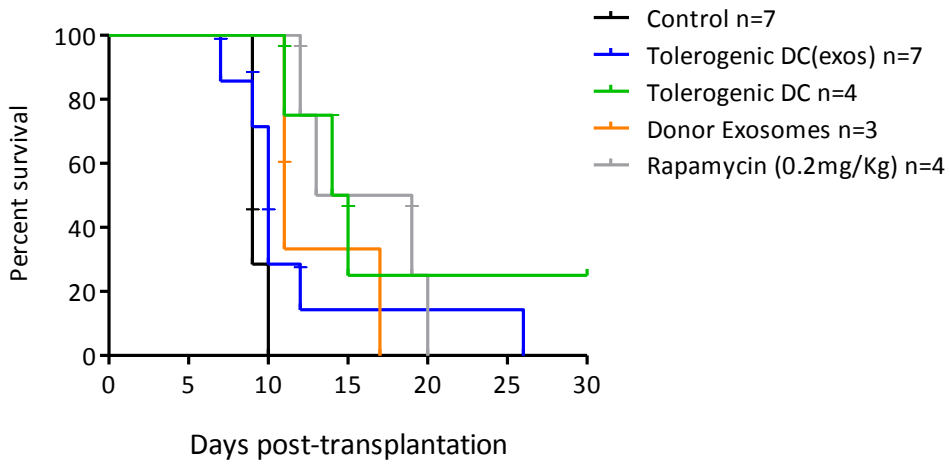


Figure 36. Effect of tolerogenic DCs and donor exosomes on kidney graft recipients survival. Bilateral nephrectomised Wistar-Agouti recipients were transplanted with Brown Norway kidney. Recipients were injected i.v. with syngeneic tolerogenic-DCs pulsed with exosomes [Tolerogenic DC(exos), n=7] or non-pulsed tolerogenic DCs [Tolerogenic DC, n=4]. Alternatively, allograft recipients were injected with donor exosomes [Donor exosomes, n=3]. All treatments were administered twice, one week before and at the day of transplantation. As control, kidney allograft recipients received subtherapeutic doses of rapamycin (orally administered) every 24h [Rapamycin] or were not treated [Control] (Log-rank (Mantel-Cox) Test, P=ns).

3.4.3 Treatment of kidney allografts recipients with tolerogenic DCs modifies peripheral blood cells populations

Due to the acute rejection model used, it is possible that the tolerogenic DCs treatment could be inducing donor alloantigen tolerance but not sufficiently to modify the graft survival. Yet, the creatinine levels observed and the survival of certain animals was indicative of a biological effect. To investigate a possible effect on peripheral blood cells, blood was obtained from tail vein at day 7 of transplantation and different populations were evaluated (Figure 36). As control, we analysed blood from untreated recipient animals. While NKT cells or T lymphocytes did not reflect any change, two other important populations reflected modifications in peripheral blood. First, there was an increased proportion of NK cells in both groups treated with tolerogenic DCs. This increase was significant when comparing Tolerogenic DC group with Rapamycin group (Tolerogenic DC n=4, median=20,28 ± 1,8 vs. RAPA n=6, median=8,9 ± 5,5). Unexpectedly, we also observed a marked reduction in B lymphocytes that was significant for donor exosomes-loaded tolerogenic DCs group compared to RAPA-treated group (RAPA n=5, median=26,4 ± 5,1 vs. Tolerogenic DC(exos) n=6, median=10,93 ± 4,9).

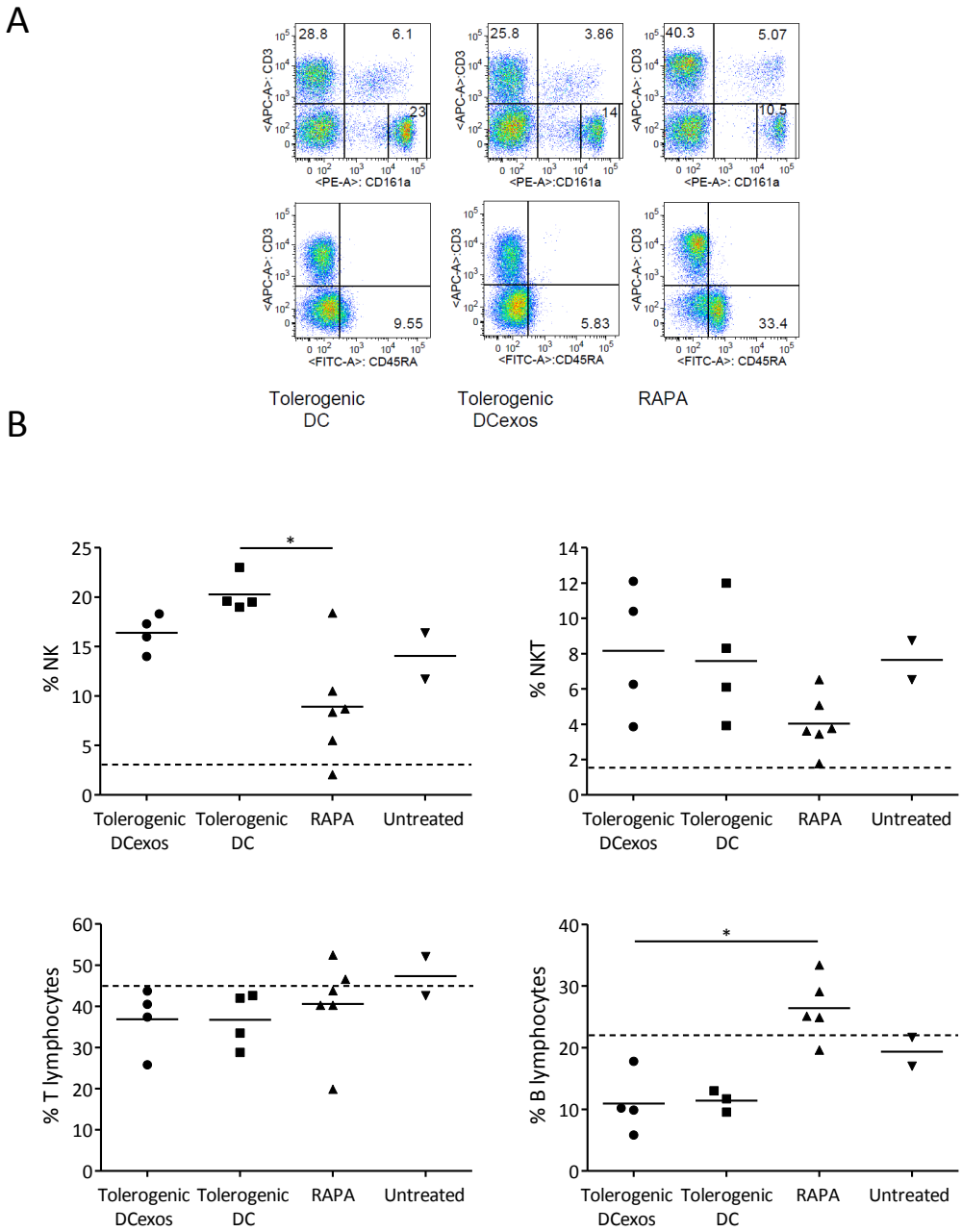


Figure 36. Injection of Tolerogenic DC modulates the percentages of peripheral blood NK cells and B lymphocytes in kidney graft recipients. On day 7 post-transplantation, peripheral blood from donor exosomes-pulsed tolerogenic DCs, non-pulsed tolerogenic DCs or rapamycin treated kidney allograft recipients was collected. As control, peripheral blood from untreated allograft recipients was obtained. The percentage of NK cells (CD161a^{high}), NKT cells (CD161a^{high} CD3+), T lymphocytes

(CD3+) and B lymphocytes (CD45RA+) was analysed by flow cytometry. **(A)** One representative experiment from each treatment is shown. Numbers indicate the percentage of each population. **(B)** Symbols indicate individual rats in each group (bar indicate the mean). Dotted line represents values from healthy rats. (*P <0.05, Kruskal-Wallis test, Dunn's Multiple Comparison Test, n≥2).

3.5. Tolerogenic BMDCs modulate B cell activation

Since injection of autologous tolerogenic DCs induced a reduction of peripheral blood B lymphocytes on grafted animals, we further investigated the role of DEX-LPS BMDCs in modulating B cell responses. It has been described that immature but not mature bone marrow-derived DCs inhibit B cell proliferation in a contact-dependent manner, upon stimulation via BCR and TLR ligands such as LPS and poly I:C [315,316].

To further confirm the results observed in vivo, we co-cultured CFSE-labelled syngeneic splenic B lymphocytes with different ratios of immature, mature and tolerogenic BMDCs in the presence of LPS (TLR4 stimulation), PMA and ionomycin or anti-IgM (BCR stimulation). After 48-72h we analysed the effect of DCs on B cells proliferation (Figure 37A). As previously reported [316], immature but not mature BMDCs suppressed TLR4-induced B cells proliferation. This inhibition was more pronounced when higher numbers of immature DCs were present in the co-cultures. Interestingly, similar results were obtained when tolerogenic DCs were used indicating that, even after receive an activation stimulus such as LPS, these cells are able to control B cell activation. This suppression was specific for TLR4-mediated proliferation because when B cells were activated in presence of PMA and ionomycin, we did not observe any changes in B cell proliferation with any DCs type or any ratio analysed. Moreover, contrary to what has been published before [315], we neither observed any inhibitory effect of immature or tolerogenic DCs on BCR-activated B cells. Additionally, we also analysed the B cell viability measured as percentage of cells for forward and side scatter (Figure 37B). We could observe a decrease in the viability of B cells, which is higher with increasing number of immature DCs. However tolerogenic DCs, which decreased B cell proliferation as efficiently as immature DCs, did not modify the number of B cells. It has been reported that the mechanism by which BMDCs induce B cell inhibition is the induction of B cell apoptosis. We could confirm partially these data for immature BMDCs but not for tolerogenic BMDCs, suggesting that other mechanisms may be involved in tolerogenic DC-mediated B cell inhibition.

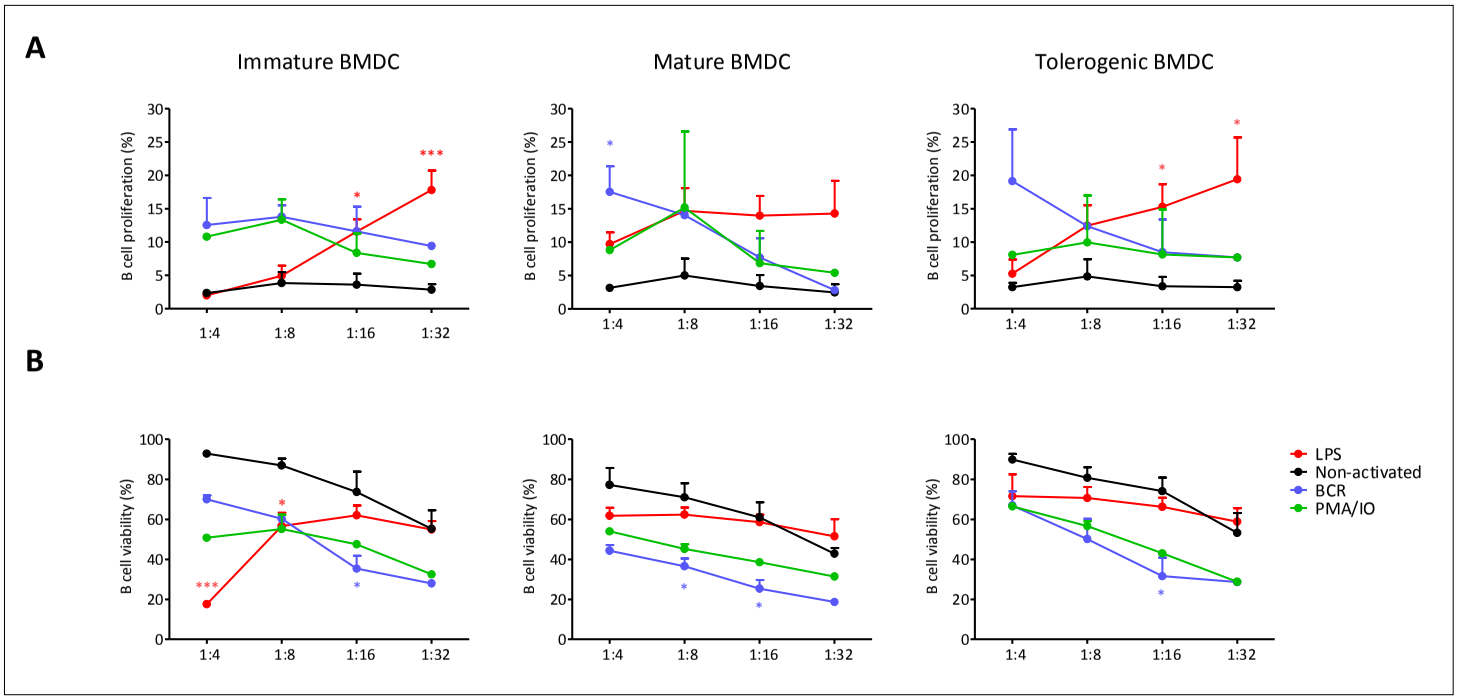


Figure 37. Immature and tolerogenic BMDC inhibit LPS-mediated B cell proliferation. Splenic purified CFSE-labelled B cells were co-cultured with immature, tolerogenic or mature BMDCs at different ratios and activated with the indicated stimuli: LPS (red), anti-IgM (blue), PMA/IO (green), non-activated (black). After 48-72h, the percentage of viability and CFSE loss of B cells were analysed by flow cytometry. Results are presented as mean ± SD of three independent experiments. *P < 0.05, *** P < 0.001, 2 way Anova, Bonferroni post-test comparing each condition of B cell stimulation with non-stimulated B cells (the significance for each condition is depicted in the corresponding colour).

DISCUSSION

General overview

This thesis has addressed the study of exosomes and tolerogenic DCs as a therapeutic strategy to induce transplantation tolerance. To this aim, we first evaluated plasma of human healthy donors as a possible source of exosomes for immunotherapeutic purposes. Then, we assessed the capacity of human peripheral blood dendritic cells to capture and present allogeneic exosomes. We also studied the ability of tolerogenic DCs to modulate the T cell response to allo-antigens. To this effect, we obtained rat tolerogenic DCs and characterized their tolerogenic properties in vitro. Moreover, we analysed their ability to capture and present alloantigens derived from exosomes. And finally, to obtain more insight into the therapeutic effect of recipient tolerogenic DCs and donor exosomes, we analysed the biological implications of these two treatments, alone or in combination, in the modulation of allograft outcome in a rat model of renal transplantation.

Regarding the first objective, proteomic and western blot analyses of plasma-derived microvesicles revealed a high variability between donors with a low number of proteins detected in sucrose cushion exosomes-enriched preparations. Importantly, these microvesicles have abundant immunoglobulins and proteins related to the complement system and coagulation processes with important implications in transplantation. These two features seem to preclude the use of plasma-derived exosomes as a source of alloantigens. Then, as an alternative source to plasma samples, we obtained exosomes from Jurkat T cells lines and DCs cultures.

In vitro analyses confirmed the ability of human peripheral blood DCs to capture allogeneic exosomes. pDCs were less efficient than cDCs in internalizing exosomes, suggesting that the two subsets may use different mechanisms to capture exosomes or exhibit a different density of molecules involved in this process. Uptake of exosomes by DCs is blocked at 4 °C. Moreover, exosomes uptake was inhibited by pretreatment of cDCs with the actin polymerization inhibitor, cytochalasin D, and the PI3K inhibitor, wortmannin, in a dose-dependent manner. These results demonstrated the involvement of endocytic mechanisms in exosomes internalization. Partial colocalization with the lysosomal marker LAMP-1 revealed that, once internalized, exosomes are in part targeted to lysosomes. The

internalization of exosomes by pDCs does not modify their phenotype and correlates with the ability of pDCs to induce the activation of autologous T cells.

Rat tolerogenic BMDCs conditioned with dexamethasone and LPS displayed a semi-mature phenotype and an anti-inflammatory cytokine profile. After exosomes capture, rat tolerogenic BMDCs showed a decrease in their ability to present exosomes-derived alloantigens to syngeneic T cells. In a model of kidney transplantation, the infusion of donor exosomes-loaded tolerogenic BMDCs did not show any improvement compared to non-loaded BMDC or isolated donor exosomes regarding the graft survival and organ function. However, peripheral blood population analyses revealed a significant decrease in the number of B cells in transplanted rats receiving tolerogenic BMDCs (both donor exosomes-loaded and not loaded). In vitro analyses demonstrated the ability of tolerogenic BMDCs to inhibit TLR-4-dependent proliferation of B cells.

The results obtained in each section will be discussed separately in order to obtain the final conclusions.

SECTION I. Proteomic analysis of microvesicles from plasma of healthy donors reveals high individual variability

Previous reports have shown that DCs-derived exosomes are enriched in MHC class I and class II molecules [157,201,202,208,267,268] and can prolong graft survival and induce donor-specific tolerance when injected in recipient animals [157,158,267,268]. Alternatively, plasma exosomes which have been shown to present a similar composition to those derived from in vitro cell cultures [218] may play an important role in immune regulation [209]**.** These immunoregulatory properties together with the fact that the purification of exosomes from plasma may represent a faster alternative protocol to cellular culture-derived exosomes for therapeutic purposes prompted us to investigate the quality and quantity of plasma-derived exosomes.

We have performed a gel-free LC–MSE-based proteomic analysis on the whole population of plasma-circulating MVs focused on the exosomes population using large number of individual donors. We have identified 161 proteins of which 52 belonged to the immunoglobulin protein family. In agreement with previous reports, immunoglobulins form a high proportion of the protein content of the MV subpopulation and are also found in exosomes-enriched preparations [226]. These immunoglobulins probably form part of immune complexes [284]. Such immune complexes induce inflammatory reactions [317,318] which may have deleterious effect on transplantation. In agreement with the previous reports, our proteomic analysis also detected an elevated number of proteins involved in the complement and coagulation processes [319]. Remarkably, and contrary to other works, these proteins were not detected in exosome-enriched preparations [226]. Among the other proteins, we detected cytosolic proteins (e.g. enzymes, heat shock proteins, albumin) and cytoskeleton-associated proteins (e.g.moesin, actins and ankyrin-related proteins, such as POTE). All these proteins have been detected previously in MVs, and have been postulated to accumulate in MV lumens during their formation [285]. We also detected ubiquitin, suggesting the presence of ubiquitinated proteins which is consistent with the characterization of MVs from other sources [285]. Other proteins identified such as lectin Gal3BP, protease inhibitor alpha-2-macrobulin, histidine rich glycoprotein or component 3 of the complement, were related to coagulation processes.

For example, an increased amount of the lectin Gal3BP and the protease inhibitor alpha-2-macroglobulin, both associated to circulating-MVs, occurs with deep venous thrombosis [320] and a role in amplifying thrombus progression has been proposed for these MVs. Histidine rich glycoprotein (HRG) is another MV-associated protein with an important role in the modulation of coagulation [321]. The implication of this protein in other processes such as immune complex/necrotic cell/pathogen clearance, cell adhesion and angiogenesis has also been reported [322]. Another important protein associated to circulating-MVs with important implications in the modulation of coagulation and platelet activation is the component 3 of the complement (C3). Thus, high levels of this protein in plasma have been pointed as one of the factors responsible for some thromboembolic adverse reactions reported after transfusion [323]. In addition to C3, which is an essential component in the activation of the complement cascade, many other complement factors have been found. Complement system plays an important role in transplant rejection [20]. Whether the presence of these proteins and those related to coagulation process in plasma-derived microvesicles could have deleterious effects in transplantation requires further investigation.

With the exception of 2 out of the 15 exosome-enriched preparations, no more than 10 proteins were identified in our study suggesting that under healthy conditions exosomes content of plasma MVs is low. In fact, the exosome-enriched preparation in which most proteins were identified contained several members of heat shock protein family implying that this donor may have been under stress.

In this study, we provide data that indicate the variable uptake of MVs in different cell types. In our study, not all the cell lines assayed were able to incorporate MVs indicating that a regulated mechanism mediates uptake of these vesicles. Macrophages and endothelial cells are able to capture plasma MVs [324,325]. Here we show that fibroblasts and hepatocyte-like cells can also take up MVs. Indeed hepatocytes probably have important functions in both secretion and clearance of plasma components. In addition, we show differential incorporation of MVs from different healthy donors into cultured cell-lines indicating that the variability in MV protein content could regulate cellular uptake. Consistent with this, it has been reported that CD81 modulates the inclusion of a selective

repertoire of molecules into exosomes including the adhesion molecule ICAM-1 [326]. We are aware that the capture experiments described in our study have been performed in a cross-species fashion to facilitate the detection of the incorporated material, and the result may not correspond completely with the actual situation however they provide basis for future deeper studies.

In summary, this pilot study establishes that the exosome content of plasma is low under normal, healthy conditions. These data demonstrate that despite the feasible use of plasma exosomes for disease diagnostic they cannot be used as a source of alloantigen in transplantation procedures. In addition, the protein content of plasma MVs from healthy donors is highly variable. Variation in MV protein content may affect their differential uptake in different cell-types. Nevertheless, this difference in protein composition confirms the feasible use of plasma-derived microvesicles/exosomes in the diagnostic of diseases. In fact, there has been an increasing number of works reporting the potential use of exosomes as non-invasive biomarkers for the diagnostic in cancer, autoimmune diseases and kidney-related diseases. [196,226,256,327–329]. In transplantation medicine, exosomes are emerging as potential candidates to detect possible immune rejections and to monitor the graft function [330].

SECTION II. Human pDCs capture and present Jurkat-derived exosomes

In vitro and in vivo experiments have shown that several types of DCs efficiently capture exosomes from DCs and other cell types [208,237,244,287]. DCs have a unique ability to induce potent immune responses. Indeed, it has been demonstrated in different animal models that after the injection of exosomes, DCs from lymphoid nodes or spleen are able to capture them and recycle exosome-derived MHC-peptide complexes resulting in naïve/effector T cells priming and initiation of immune responses [201,240,331]. Importantly, graft infiltrating DCs release exosomes to the blood [244] which potentially may be captured by circulating blood DCs.

In order to test this hypothesis, we analysed the ability of human peripheral blood dendritic cells to capture allogeneic exosomes. While the high capacity of conventional dendritic cells to capture any type (soluble, particulate) of antigen is well established, until

now the ability of pDCs to capture particulate antigens has been a matter of controversy [294,296–301]. We first demonstrated by in vitro experiments that both human peripheral blood cDCs and pDCs may capture Jurkat T cell line-derived exosomes. Nevertheless, pDCs were not as efficient as cDCs in capturing microvesicles. However, a recent report has demonstrated that despite human pDCs are less efficient in antigen capture, they cross-present antigens to CD8+ T cells as efficiently as myeloid DC subsets [332]. Moreover, the time required by pDCs to capture exosomes was quite longer compared to cDCs. A long incubation time has also been observed in the capture of synthetic poly(lactic-coglycolic acid) microparticles by pDCs [300]. The absence of fluorescence in pDCs before 12h of co-incubation with fluorescent exosomes may be due to a limited sensitivity of the cytometer that does not allow detection of very few fluorescent molecules, or to a different sensitivity of the dye to the endosomal pH of pDCs. Also, confocal microscopy only allows visualization of objects larger than 200 nm, hence accumulations of exosomes, such as observed in endosomal compartments of cDCs, but not individual vesicles [229]. Several molecules such as ICAM1 [237] and the integrins CD51 and CD61 [208] have been shown to promote exosome capture by DCs. Thus, probably, a differential expression of the molecules involved in such processes would rather account for such differential ability in exosome capture by each DC subset.

We have also demonstrated that exosomes are captured by cDCs through endocytic mechanisms and co-localize within late endosomal/lysosomal compartments. These results are in concordance with previous works [208,244]. Unfortunately, the long time required for exosome uptake by pDCs did not allow us to analyse the mechanisms involved in exosome capture. DCs capture antigens through pinocytosis, phagocytosis and receptor-mediated endocytosis [333,334], which one of these mechanisms are involved in exosomes capture by pDCs requires further investigation.

Remarkably, the same kinetic of exosome capture by both subsets was observed during co-culture experiments, thus suggesting that pDCs can still interact with exosomes even in the presence of the highly endocytic cDCs. Whether cDCs rapidly capture exosomes until saturation and pDCs are less efficient and take longer periods, or whether cDCs accumulate the vesicles while pDCs promote their rapid degradation (thus hampering their

detection) require further investigation. Importantly, mouse spleen pDCs capture intravenously injected exosomes in vivo [229,244]. pDCs have been shown to mediate tolerance to allografts after alloantigens capture and presentation [153]. Therefore, exosomes secreted by graft-infiltrating DCs could be a potentially source of alloantigens [244].

Regarding pDC activation, we could observe that Jurkat-derived exosomes do not interfere with the maturation process triggered after TLR7/TLR8 signalling analysed by co-stimulation molecules expression and IFN-α secretion. Furthermore, exosomes do not modify the phenotype of pDCs. These results are in line with other observations [208]. The only molecule that was significant reduced was CD40 but it could be attributed to the expression of CD40-L on exosomes from Jurkat T cells. It remains to be determined whether these effects are inherent to the source (and therefore the composition) of exosomes. In a physiological way, exosomes may represent an early signal alerting of malfunction or infection of a given organ or tissue, thus providing signals trying to induce tolerogenic or immunogenic responses when necessary.

Finally, we have shown that exosome-loaded pDC induce autologous T cell proliferation. It is not clear whether the autologous T cell proliferation induced by exosome-loaded pDCs is due to a mechanism of antigen processing, due to direct recycling of alloantigens to the cell surface of the antigen presenting cell, or both [208,237,240], or induced by other molecules (such as heat shock proteins) that may be present in the exosome preparation. Hence, this antigen presenting capacity must be taken carefully.

Overall, human pDCs are able to capture microvesicles such as exosomes and also apoptotic bodies. These results suggest that, although cDCs are the unique DC subset with the capacity to rapidly internalize a complete range of soluble and particulate antigens, the capacity of human pDCs to capture and present exogenous cell-derived antigens may be also relevant.

SECTION III. Effect of tolerogenic DCs in a rat model of kidney transplantation

Our final goal was the induction of tolerance in a model of kidney transplantation in rats. To this aim we have administered recipient DCs pulsed with donor-derived exosomes into

transplant recipients. Because of their capacity to modulate immune responses, DCs can be used as therapeutic agents. Isolation of peripheral blood DCs for clinical trials is a challenge since they are at relative low number in vivo. Therefore, most studies have used in vitro-generated DCs. During the last two decades, several works have demonstrated that administration of donor- or recipient-derived tolerogenic DCs, either alone or in combination with suboptimal doses of immunosuppression, prolongs allografts survival in several animal models. We have optimized the culture conditions for generation of rat BMDCs with tolerogenic properties.

Previous studies have shown that differentiation of rat DCs from bone marrow cultures in the presence of different combinations of GM-CSF, IL-4 and FLT3L generates different subsets of DCs [48,53,54,307,335]. We first compared the yield, phenotype and allo-stimulatory ability of BMDCs differentiated in the presence of GM-CSF, IL-4 with or without the addition of FLT3L. The addition of FLT3L to BMDC cultures increased the yield as previously reported [54,308]. Phenotypically, both types of DCs express OX62 as shown before [307,308,335]. This molecule is present in some subsets of DCs in vivo [45,46,309]. However, the expression of OX62 on in vitro differentiated BMDCs may depend on culture conditions [48,52–54,307].

After maturation with LPS, RT1B and CD86 expression was up-regulated in both GM-CSF and IL4 and GM-CSF, IL4 and FLT3L treated BMDCs although only was significant in the latter. This could be due to a more immature state of FLT3L-treated BMDCs prior to maturation. MLR experiments showed that both types of BMDCs are able to stimulate allogeneic splenocytes being more potent after receive the maturation stimuli. In conclusion, the addition of FLT3L besides improve the BMDCs yield, generates BMDCs with a similar phenotype and allo-stimulatory ability to BMDCs differentiated just with GM-CSF and IL-4.

Rat BMDCs differentiated in presence of LPS and dexamethasone present a semi-mature phenotype with an anti-inflammatory cytokine profile and reduced ability to stimulate allogeneic T lymphocytes

We have generated alternatively activated, semi-mature tolerogenic DCs in presence of dexamethasone and LPS. Dexamethasone is a synthetic glucocorticoid that upon binding to

the nuclear glucocorticoid receptor impairs several signalling pathways such as STAT, AP-1 and NFκB among others resulting in the blockade of differentiation and/or maturation in DCs [127,128]. It has been described that dexamethasone impairs the fully differentiation of human MDDCs [312] in a dose and time dependent manner [311]. In fact, when dexamethasone was added at the higher concentration and/or earlier time points the differentiation of BMDCs was affected (data not shown). However, the addition of dexamethasone at later time points results in good yields and the expression of OX62 and CD11b on BMDCs demonstrating the fully differentiation of these cells.

Regarding the phenotype, we have observed an alteration of MHCII (RT1B) and CD86 expression in tolerogenic BMDCs. Pre-treatment with dexamethasone blocks the up-regulation of MHCII molecules in activated DCs as it has been widely shown [141,306,310,336]. We observed a decrease in RT1B expression that was dependent on dexamethasone concentration with the highest dose causing more pronounced reduction. Concerning the expression of CD86 by dexamethasone-treated DCs, contradictory results have been reported. On one hand, CD86 expression is down-modulated in alternatively activated mouse and rat BMDCs treated with dexamethasone and LPS [141,306]. Similar results have been reported in dexamethasone-conditioned human monocyte-derived DCs after maturation with TNF-α [311], CD40L [311,312] or LPS [312,336]. On the other hand, exposure of dexamethasone-treated MDDCs to LPS does not decrease CD86 expression [310]. The authors attributed this effect to glucocorticoid-induced leucine zipper (GILZ)-dependent CD86 induction [310]. This is in agreement with our results as we observed similar levels of CD86 in tolerogenic BMDCs compared to mature DCs. The differences observed could be related to the dose and time of dexamethasone treatment [337,338]. Nevertheless, it has been reported the CD86-dependent expansion of regulatory T cells by mature pDCs in rats and BMDCs in mice [339,78].

Concerning cytokine production, we have shown that tolerogenic DCs do not secrete IL-12, but secrete similar levels of IL-10 to mature DCs and lower levels of TNF-α. This cytokine profile has been described before in alternatively activated BMDCs treated with dexamethasone prior to LPS maturation [141,306] and dexamethasone-pretreated MDDCs activated with LPS, TNF- α or CD40L [311,312]. In fact, it seems that the presence of

dexamethasone induces IL-10 production in alternatively activated DCs, as shown for the IL-10 secretion of CD40L-mediated [338] and cytokine cocktail-mediated activation [310] of DCs after dexamethasone treatment. Moreover, LPS-activated BMDCs treated with the higher dose of dexamethasone secrete significantly more IL-10. This effect may be mediated by the induction of the target gene of the glucocorticoid receptor GC-inducible leucine zipper (GILZ), after interaction with dexamethasone [340]. IL-12p70 is the bioactive form of IL-12, a cytokine important for Th1 polarization. Although IL-12p70 is detected in low amounts we were able to observe differences between mature and tolerogenic DCs. Importantly, while DCs stimulated with LPS secrete high levels of IL-12, tolerogenic DCs produce lower amounts, at levels comparable to immature DCs. The importance of cytokine secretion in T cell modulation lies on the whole of the cytokines secreted. The ratio of IL-10/IL-12 is important for anergy or tolerance induction of T cells [119].

Finally, while mature DCs induced potent alloproliferative T cell responses, tolerogenic DC presented a decreased T cell-stimulatory capability, similar to immature DC as previously reported [141,306,311,312]. Although we did not analyse the mechanism by which dexamethasone-conditioned DCs impairs the proliferative ability of T cells, other works have reported that these cells induce T cell anergy or promote the generation of regulatory T cells in vitro [336,338,341].

In summary, we have generated semi-mature, alternatively activated BMDCs with an anti-inflammatory cytokine profile and with the ability to induce hypo-response of allogeneic T lymphocytes.

Rat tolerogenic BMDCs capture allogeneic exosomes but are hampered in their ability to present alloantigens to syngeneic T lymphocytes

Donor immunodominant peptide has been used as source of alloantigen for DC-mediated tolerance induction in transplantation [145]. However, there is a great variability in histocompatibility antigens. For that reason, the translation of this approximation into clinically-applicable procedures is not feasible due to the difficulty in generating specific peptides for each donor-recipient combination in addition to the need for a wider (the entire or at least the immune-dominant) repertoire of peptides. Therefore, other studies have investigated the use of cell-free donor lysate [148–150,167]. We have chosen DC-

derived exosomes as source of alloantigens for our study for several reasons. First, exosomes express MHC I and II molecules. Second, the range of alloantigens available is greater than using a single immunodominant peptide. Third, when captured by DCs, exosomal MHC molecules can be digested and presented as peptides on host DCs-derived MHC molecules thus contributing to the indirect via of alloantigens presentation, or alternatively, intact MHC molecules can be directly presented on the receptor cell surface, which could contribute to direct presentation pathway [170]. And finally, it is worth mentioning that the generation of large batches of clinical grade exosomes has been developed for cancer therapy [169,260,261].

We first analysed the ability of immature, mature and tolerogenic rat BMDCs to capture exosomes. It has been demonstrated that mature splenic DCs capture exosomes less efficiently than immature DCs in vitro [208]. Conversely, mature monocyte-derived DCs capture T cell-derived exosomes more efficiently than immature DCs in vitro [287]. Our results showed that both immature and mature BMDCs are able to capture allogeneic exosomes with similar efficiency. Differences related to the type of DCs/source of exosomes could be responsible for these contradictory results. Regarding tolerogenic DCs, previous works have reported that dexamethasone does not interfere with endocytic and macropinocityc activity of BMDCs [338] and even increases the expression of CD32 and thereby the endocytic activity of MDDCs [311]. However, we did not observe an increased ability in dexamethasone-treated DCs to capture exosome. Conversely, these cells were less efficient in taking up exosomes than mature and immature DCs. Exosomes uptake is mediated by different mechanisms such as receptor-mediated endocytosis, phagocytosis or lipid-dependent fusion events [170]. Further experiments will be required to explore which type of mechanism is mediating the internalization of exosomes by tolerogenic DCs.

Concerning the phenotype and cytokines secreted by donor-exosomes loaded-DCs, we did not observe any changes in any parameters indicating that exosomes, still carrying allogeneic MHC molecules, do not activate DCs. It is important to stress that exosomes are derived from immature DCs. This could be a decisive factor considering that there are differences in RNA content between mature and immature DC-derived exosomes [229] that could somehow promote alterations in the target DCs. Nevertheless, these results are

in agreement with previous works where i.v. injected donor-exosomes do not activate splenic receptors DCs [208]. Interestingly, i.v. injection of donor-exosomes prolongs allograft survival in two rat models of heart [157] and intestinal transplantation [267].

Finally, in contrast to mature DCs, tolerogenic DCs pulsed with allogenic exosomes were not able to activate syngeneic T lymphocytes. This effect can be related with a lower capacity to capture exosomes. However, in the case of immature DCs, which present the same ability to capture exosomes than mature DCs, the capability to stimulate T-cells is related with DC maturation state.

Syngeneic tolerogenic DCs and allogeneic exosomes localize in liver and spleen after intravenous injection

Several authors argue that while intradermal injection of dendritic cells elicits immune responses, the intravenous route promotes the induction of tolerance [115,120,305]. In fact, in a murine model of transplantation it has been demonstrated that intravenous infusion of tolerogenic DCs prolonged graft survival whereas this effect was not observed when subcutaneous injection was performed [141]. In addition to the route of injection, another important feature of DC-based therapies is their proper localization after injection to promote the desirable effects. Therefore, we studied the localization of syngeneic, tolerogenic DCs after intravenous injection in healthy rats. Tolerogenic DCs were localized in lungs at very early time points (5 minutes) from where they disappeared very quickly migrating to liver and probably spleen. The rapid disappearance from lungs and preferentially migration to liver and secondary lymphoid organs such as spleen has been previously observed [342]. In vivo analyses of DC homing did not allow us to affirm that tolerogenic DCs migrate to the spleen because the strong signal observed in liver mask other localizations. However, when rats were sacrificed we could observe a strong signal in spleen, even greater than in liver. Importantly, in a rat model of transplantation it has been reported that after intravenous injection, syngeneic (and allogeneic) DCs are localized in spleen rather than in mesenteric lymph nodes one day after the administration, where they persist until day 7 [52]. Interestingly, from the first day after injection we could observe a weak signal in posterior limbs. When animals were sacrificed and bones were

extracted we could confirm that tolerogenic DCs had migrated to bone marrow as previously described [343].

Regarding allogeneic exosomes, we just detected a positive signal in the liver at every time point analysed. As for tolerogenic DC, when animals were sacrificed we could also detect exosomes in spleen although the strong signal in liver still persisted. Moreover, when bones from posterior limbs were extracted, we could also detect exosomes in bone marrows. Exosomes trafficking into liver and spleen has been shown by ex vivo internalization analysis of splenic DCs and macrophages and hepatic kupffer cells [208]. Significantly, melanoma cell lines-derived exosomes have been localized in bone marrow one day after i.v. injection where they educate bone marrow cells to promote metastasis and cancer progression [256].

Effect of tolerogenic rat BMDCs and donor exosomes alone or in combination in a model of kidney transplantation

We have analysed the effect of recipient-derived, exosomes-loaded, dexamethasone-treated DCs in a fully mismatched kidney transplantation model (Brown Norway to Wistar Agouti) on graft survival and organ function. As control, non-loaded tolerogenic DC or isolated donor exosomes have been used. We have shown in our model that injection of dexamethasone-treated DCs in kidney recipient rats has no effect on allograft survival. Since our model is characterized by an acute response we performed two separately injections being the first one week before transplantation. We hypothesized that exosomes loaded-tolerogenic DC injected one week prior to transplantation could present exosomal alloantigens in a non-inflammatory environment modulating the response to these allo-antigens. Then, this response would be further reinforced by the second injection on the day of transplantation. To facilitate the discussion, the results derived from donor exosomes treatment of transplanted rats will be discussed first separately.

Exosomes in allograft survival

Our initial hypothesis was that treatment with donor exosomes alone would induce worse responses than exosomes-pulsed tolerogenic DCs. Indeed, although it was reported the prolongation of allograft survival by using donor exosomes [157,267,268] it has been suggested that exosomes require to be captured by DCs to induce an immune response

[240]. In fact, after i.v. injection donor exosomes are uptaken and presented by splenic DCs [208]. Treatment of graft recipients with donor exosomes did not result in a prolonged graft survival or a decrease of serum creatinine levels compared to control group. We only analysed one dose of exosomes (125 μg per dose) and one regimen of injection (two doses, at day -7 and day 0). Several rodent models of transplantation have shown that injection of low doses of exosomes (from immature DCs) before transplantation induces an increase in graft survival. However, this effect was abrogated with higher doses of exosomes [157,267,268]. Therefore, the high dose used in our study could explain the lack of effect on graft survival in the present study. Alternatively, differences related to the transplant model could account for this discrepancy. Significantly, the combination of donor exosomes with immunosuppression has been shown to improve graft survival [158,268]. In a mice model of heterotopic heart transplantation the injection of donor exosomes, before and after transplantation, in combination with sub-therapeutic doses of rapamycin induced long-term survival [268]. Moreover, the administration of donor exosomes post transplantation in combination with suboptimal doses of LF 15-0195 to inhibit DC maturation induced indefinite survival in the same model of heterotopic heart transplantation performed in rats [158]. In both studies the response to alloantigens was inhibited in a donor-specific manner. Furthermore, the tolerance induction was transferable to naïve recipient receiving an allograft through the injection of splenocytes from tolerant recipients.

Tolerogenic DCs therapy in transplantation

Because we obtained slightly better effects by treating rats with tolerogenic DCs we decided to focus in these groups. Our results showed that treatment of graft recipients with non-loaded tolerogenic DCs did not improve allograft outcome regarding graft survival and creatinine levels. Similar results were observed with donor exosomes-loaded tolerogenic DCs. Nonetheless, it has been shown that while non-pulsed tolerogenic DCs, even in combination with anti-lymphocyte serum and cyclosporine A, do not prolong graft survival [150], the injection of tolerogenic DC pulsed with donor alloantigens promote indefinite survival of skin grafts [150]. Similar results have been observed in other models [149,166]. Therefore, several factors can be responsible for the lack of effects in our study.

Perhaps the most important fact is that kidney transplantation was performed in a model of acute rejection. Since graft recipients did not receive any concomitant immunosuppressive therapy, the treatment with tolerogenic DCs may not be of sufficient magnitude to induce any observable response. Importantly, two animals (one treated with loaded DCs and the other with non-loaded DCs) survived more than 25 days (15 days more than the control group) with low levels of serum creatinine. Probably in a less stringent model, the combination of any of these treatments (tolerogenic DCs or donor exosomes-pulsed tolerogenic DCs) with low doses of immunosuppressant (i.e. Rapamycin) would induce better responses.

Regarding the immunomodulatory agent selected to generate tolerogenic DCs, in vitro experiments have shown that dexamethasone-treated DCs have a stable phenotype [310,341,344] which would prevent their subsequent maturation once injected in vivo. Remarkably, cytokine cocktail-stimulated tolerogenic DCs generated in the presence of dexamethasone (but not vitamin D3 or rapamycin) secrete IL-10 after restimulation with LPS [344]. Our study is, to our knowledge, the first report that evaluates the effect of recipient tolerogenic DCs conditioned with dexamethasone in a rat model of kidney transplantation. In other study, using the same approach to generate tolerogenic DCs, it has been shown that the infusion of donor DCs, although induces T cell hypo-responsiveness, fail to prolong graft survival in a similar model of transplantation [306]. Nonetheless, dexamethasone-treated DCs co-expressing both self and donor MHC molecules in combination with CTLA4-Ig and cyclosporine A, but not in the absence of immunosuppression, promote tolerance [159]. Regardless of the origin of DCs, these studies highlight the need to combine cell therapy with immunosuppression to induce tolerance in transplantation. In fact, it has been observed in different rodent models that recipient DCs infusion in combination with suboptimal doses or short post-operative course of immunosuppressive drugs induces stronger tolerogenic responses in transplantation. Among these concomitant immunosuppressive therapies are LF15-095 and FK506 (which inhibit DC maturation), depletion of lymphocytes by administration of anti-lymphocyte serum or the classical immunosuppressive drugs rapamycin and cyclosporine A [145–151,166]. Rapamycin could be a good candidate since prevents the

up-regulation of co-stimulatory molecules during maturation of LPS-treated BMDCs [345]. This situation could be beneficial in transplantation by blocking the maturation of donor APCs and thereby, inhibiting direct presentation. Furthermore, rapamycin induces the expansion of CD4+ Foxp3+ regulatory T cells in vitro, what could be favourable in the induction of tolerance in transplantation [346–348]. Therefore, it should be important to include rapamycin in combination with tolerogenic DCs to induce a desirable effect on graft outcome.

Tolerogenic DCs modulate B cell responses

The absence of additional immunosuppression in transplanted animals allowed us to study the regulatory effect of tolerogenic DCs in vivo without any interference. We observed a clear modulation in the percentage of peripheral blood NK and B lymphocytes in recipient rats treated with tolerogenic DCs. Because our model is defined by an acute humoral rejection, we decided to further investigate the role of tolerogenic DCs in the regulation of B lymphocytes. DCs modulate B cell activation indirectly through the stimulation of T cells. DCs present antigens via MHC molecules to helper T cells that in turn influence B cell responses through cytokine secretion or cell contact-dependent mechanisms. Alternatively, lymphoid tissue-resident and circulating antigen-bearing DCs are able to reprocess and present native antigen to B cells directly, participating in T-cell independent [60,62,64,349] and T-cell dependent humoral responses [350–352]. Finally, DCs can modulate B cell responses through different mechanisms that include soluble factors such as cytokines, BAFF, APRIL and TNF-family receptors and cell contact-dependent pathways such as CD40L-CD40 interaction, thus regulating their differentiation, proliferation and class switching [65,67,69]. Besides their role in initiation/modulation of B cell responses, DCs have been also involved in the inhibition of B cell functions, even in the context of transplantation [120].

Recently, two studies reported that immature BMDCs suppress TLR and BCR-mediated B cell proliferation [315,316]. Furthermore, immature BMDCs inhibit B cell differentiation to plasma cells and IgM production. Additionally, immature DCs induce apoptosis of TLR-4- and BCR-stimulated splenic and bone marrow B cells [316]. Finally, this study shows the induction of bone marrow B cell apoptosis by immature BMDCs through an antigen-

dependent mechanism. The inhibition of B cell proliferation was dependent on CD22 expression by B cells [315,316]. CD22 is a B-cell inhibitor co-receptor associated to BCR that belongs to the sialic acid-binding immunoglobulin-like lectins (Siglecs), also known as Siglec-2. CD22 is expressed on the surface of B cells at different stages, including activated B cells, memory B cells and short lived-plasma cells. The expression of CD22 is lost when plasma cells migrate to bone marrow (or inflamed tissues such as rejecting allografts) where they become long-lived plasma cells [114,353]. CD22 ligands are glycans containing α2,6-linked sialic acids that can be present on B cells (cis-ligand interaction of CD22) or other cell types (trans-ligand interaction of CD22) such as bone marrow endothelial cells [353]. Importantly, the expression of α2,6-sialic acids on immature (tolerogenic) DCs has been reported [354].

Therefore, we asked whether the reduction of peripheral blood B lymphocytes observed in vivo could be related with the immunomodulatory properties of tolerogenic DCs. In order to test our hypothesis, we performed in vitro co-cultures of immature, mature and tolerogenic DCs with B cells activated by different stimuli. Our in vitro experiments showed that both immature and tolerogenic BMDC were able to inhibit LPS-mediated B cell proliferation. Moreover, the suppressor ability of immature BMDC seemed to be mediated through the induction of B cells apoptosis, measured as percentage of B cells in the cultures at the end of the experiment. Previous works have demonstrated that immature DC-mediated inhibition of B cell activation is regulated through the induction of apoptosis and the inhibition of B cell differentiation [316]. Conversely, tolerogenic DCs did not reduce the numbers of B cells despite inhibiting the proliferation. The mechanism by which these cells mediate B cell inhibition may be dependent on the secretion of regulatory soluble mediators such as IL-10 [355]. Interestingly, B cell viability was increased in non-stimulated B cell co-cultured with the three types of DCs. It has been described that DCs provide signals for B cell survival [66].

Unfortunately, we did not observe any inhibitory effect on BCR-activated B cells by immature or tolerogenic BMDCs as previously reported [315,316]. The lack of inhibitory effect could depend on the high concentration of anti-IgM used, because B cell

proliferation mediated by a very strong crosslinking of BCR could not be suppressed by immature DCs [315].

Our in vivo experiments of tolerogenic DCs migration showed that, from the first day after injection, DCs (and also exosomes) were localized in bone marrow. This lymphoid organ not only serves as a hematopoietic hub, but as a site where mechanisms of antigen presentation take place. Since antigen-bearing DCs activate bone-marrow resident memory T cell [343], it could be possible that injected tolerogenic DCs migrated to bone marrow and regulated B cell functions in rats. However, because long-lived plasma cells residing in the bone marrow do not express CD22, it seems unlikely that tolerogenic DCs inhibit B cell by this mechanism. Another possibility could be the interaction of injected DCs with B cells in secondary lymphoid organs such as spleen [60,64], where the activation of B cells takes place. Interestingly, among the actual therapies that are being used for tolerance induction in transplantation, the monoclonal antibody targeting CD22 appears to be a good candidate, given the effect in the abolishment of B cells [356]. In patients with systemic lupus erythematosus, treatment with the humanized anti-CD22 antibody reduces by 35% the total number of B cells and inhibits B cell activation and proliferation [357]. Alternatively to DC-mediated inhibition of B cells through CD22, it is possible that other inhibitor receptors, such as CD72 or Siglec-G, [353,358] mediate this process.

The regulation of T cells responses by tolerogenic DCs have been widely reported, however the modulation of B cell by tolerogenic DCs was not investigated so extensively. Recently, it has been described that tolerogenic DCs conditioned with dexamethasone and vitamin D3 induces IL-10-secreting regulatory B cells in vitro [359]. Nevertheless, the mechanism that we propose here is more related to B cell anergy or apoptosis induction. Significantly, it has been reported recently a decrease of total B lymphocytes in ultra-long renal transplant recipients [360].

Although further experiments are required, our results suggest that one of the mechanisms by which tolerogenic DCs could have a biological effect in transplantation is through modulation of B cell responses.

CONCLUSIONS

The most remarkable conclusions from experiments conducted during this thesis can be summarized as follows:

1. Human plasma contains exosomes. However, their use as source of alloantigens for immunotherapeutic purposes is not feasible due to the following reasons:
    - Under healthy conditions (as found in living healthy donors) exosomes content in plasma MV is low, and not sufficient to endeavour an immunotherapeutic procedure.
    - Proteomic analysis of plasma-derived microvesicles detected an elevated number of immunoglobulins and proteins involved in the complement and coagulation processes, which may have important implications in transplantation.
2. Human peripheral blood dendritic cells are able to capture allogeneic exosomes.
    - Confocal microscopy and flow cytometry experiments confirm that plasmacytoid and, more efficiently, conventional dendritic cells capture exosomes.
    - Capture of exosomes capture by conventional dendritic cells is blockade by endocytosis inhibitors in a dose-dependent manner.
    - In conventional dendritic cells, exosomes are partially localized in lisosomal compartments.
    - Capture of exosomes derived from non-activated T cells does not modify the state of activation of plasmacytoid dendritic cells and do not prevent their ulterior activation.
    - Exosomes-loaded plasmacytoid dendritic cells induce the proliferation of autologous T cells.
3. The conditioning of rat bone marrow-derived dendritic cells with dexamethasone generates tolerogenic dendritic cells with immunomodulatory properties.

- Dexamethasone-treated dendritic cells display a semi-mature phenotype, an anti-inflammatory cytokine profile and a reduced ability to stimulate allogeneic splenocytes.
- Dexamethasone-treated dendritic cells are able to capture allogeneic exosomes. These exosomes-loaded tolerogenic dendritic cells show reduced ability to stimulate syngeneic T cells.
- After intravenous infusion in healthy rats, tolerogenic dendritic cells and allogeneic exosomes are localized in primary (bone marrow) and secondary lymphoid organs (spleen) among others.
- In a fully mismatched rat model of kidney transplantation, in the absence of any other co-treatment, the intravenous injection of tolerogenic dendritic cells, loaded or not with allogeneic exosomes, is not sufficient to improve the survival rate or organ function of recipient animals.
- However, treatment with tolerogenic dendritic cells causes a reduction in the number of peripheral blood B cells in recipient animals.
- In vitro experiments reveal that tolerogenic dendritic cells are able to inhibit LPS-mediated proliferation of syngeneic B cells.

BIBLIOGRAPHY

[1] Cornell LD, Smith RN, Colvin RB. Kidney transplantation: mechanisms of rejection and acceptance. Annual Review of Pathology 2008;3:189–220.

[2] Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. Nature Reviews Immunology 2005;5:807–17.

[3] Rocha PN, Plumb TJ, Crowley SD, Coffman TM. Effector mechanisms in transplant rejection. Immunological Reviews 2003;196:51–64.

[4] Burne MJ, Daniels F, El Ghandour A, Mauiyyedi S, Colvin RB, O'Donnell MP, et al. Identification of the CD4+ T cell as a major pathogenic factor in ischemic acute renal failure. Journal of Clinical Investigation 2001;108:1283–90.

[5] Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell a R, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. Journal of Immunology 2001;166:3789–96.

[6] Rosenberg A, Mizuochi T, Singer A. Analysis of T-cell subsets in rejection of Kb mutant skin allografts differing at class I MHC. Nature 1986;322:829–31.

[7] Rogers NJ, Lechler RI. Allorecognition. American Journal of Transplantation 2001;1:97–102.

[8] Lechler RI, Garden O a, Turka L a. The complementary roles of deletion and regulation in transplantation tolerance. Nature Reviews Immunology 2003;3:147–58.

[9] Bolton EM, Bradley JA, Pettigrew GJ. Indirect allorecognition: not simple but effective. Transplantation 2008;85:667–9.

[10] Gökmen MR, Lombardi G, Lechler RI. The importance of the indirect pathway of allorecognition in clinical transplantation. Current Opinion in Immunology 2008;20:568–74.

[11] Herrera OB, Golshayan D, Tibbott R, Salcido Ochoa F, James MJ, Marelli-Berg FM, et al. A novel pathway of alloantigen presentation by dendritic cells. Journal of Immunology 2004;173:4828–37.

[12] Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation 2012;93:1–10.

[13] Turka L a, Lechler RI. Towards the identification of biomarkers of transplantation tolerance. Nature Reviews Immunology 2009;9:521–6.

[14] Moine AL, Goldman M, Abramowicz D. Multiple pathways to allograft rejection. Transplantation 2002;73:1373–81.

[15] Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells a D, Turka L a. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. Journal of Immunology 2001;166:973–81.

[16] Chong AS, Alegre M-L. The impact of infection and tissue damage in solid-organ transplantation. Nature Reviews Immunology 2012;12:459–71.

[17] Terasaki PI, Cai J. Humoral theory of transplantation: further evidence. Current Opinion in Immunology 2005;17:541–5.

[18] Li X. The significance of non-T cell pathways in graft rejection--implications for transplant tolerance. Transplantation 2010;90:1043–7.

[19] Benichou G, Tonsho M, Tocco G, Nadazdin O, Madsen JC. Innate immunity and resistance to tolerogenesis in allotransplantation. Frontiers in Immunology 2012;3:73.

[20] Sacks SH, Zhou W. The role of complement in the early immune response to transplantation. Nature Reviews Immunology 2012;12:431–42.

[21] Murphy SP, Porrett PM, Turka L a. Innate immunity in transplant tolerance and rejection. Immunological Reviews 2011;241:39–48.

[22] LaRosa DF, Rahman AH, Turka L a. The innate immune system in allograft rejection and tolerance. Journal of Immunology 2007;178:7503–9.

[23] Kirk AD, Hale D a, Mannon RB, Kleiner DE, Hoffmann SC, Kampen RL, et al. Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (CAMPATH-1H). Transplantation 2003;76:120–9.

[24] He H, Stone JR, Perkins DL. Analysis of robust innate immune response after transplantation in the absence of adaptive immunity. Transplantation 2002;73:853–61.

[25] Boros P, Bromberg JS. New cellular and molecular immune pathways in ischemia/reperfusion injury. American Journal of Transplantation 2006;6:652–8.

[26] Leventhal JS, Schröppel B. Toll-like receptors in transplantation: sensing and reacting to injury. Kidney International 2012;81:826–32.

[27] Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature 2006;442:39–44.

[28] Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010;140:805–20.

[29] Alegre M-L, Florquin S, Goldman M. Cellular mechanisms underlying acute graft rejection: time for reassessment. Current Opinion in Immunology 2007;19:563–8.

[30] Gill R. NK cells: elusive participants in transplantation immunity and tolerance. Current Opinion in Immunology 2010;22:649–54.

[31] Villard J. The role of natural killer cells in human solid organ and tissue transplantation. Journal of Innate Immunity 2011;3:395–402.

[32] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.

[33] Pulendran B, Tang H, Denning T. Division of labor, plasticity, and crosstalk between dendritic cell subsets. Current Opinion in Immunology 2008;20:61–7.

[34] Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nature Reviews Immunology 2007;7:19–30.

[35] Crozat K, Guiton R, Guilliams M, Henri S, Baranek T, Schwartz-Cornil I, et al. Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. Immunological Reviews 2010;234:177–98.

[36] Shortman K, Heath WR. The CD8+ dendritic cell subset. Immunological Reviews 2010;234:18–31.

[37] Belz GT, Nutt SL. Transcriptional programming of the dendritic cell network. Nature Reviews Immunology 2012;12:101–13.

[38] Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. Immunology 2013.

[39] Mittag D, Proietto AI, Loudovaris T, Mannering SI, Vremec D, Shortman K, et al. Human dendritic cell subsets from spleen and blood are similar in phenotype and function but modified by donor health status. Journal of Immunology 2011;186:6207–17.

[40] Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, et al. Dendritic cell subsets in health and disease. Immunological Reviews 2007;219:118–42.

[41] Piccioli D, Tavarini S, Borgogni E, Steri V, Nuti S, Sammicheli C, et al. Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. Blood 2007;109:5371–9.

[42] Swiecki M, Colonna M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. Immunological Reviews 2010;234:142–62.

[43] Lande R, Gilliet M. Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. Annals of the New York Academy of Sciences 2010;1183:89–103.

[44] Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood 2010;116:e74–80.

[45] Voisine C, Hubert F-X, Trinité B, Heslan M, Josien R. Two phenotypically distinct subsets of spleen dendritic cells in rats exhibit different cytokine production and T cell stimulatory activity. Journal of Immunology 2002;169:2284–91.

[46] Hubert F. Rat plasmacytoid dendritic cells are an abundant subset of MHCII CD4 CD11b OX62 and type I IFN producing cells that exhibit selective expression of toll like receptors 7 and 9 and strong responsivennes to CpG. The Journal of Immunology 2004;172:7485–94.

[47] Liu L, Zhang M, Jenkins C, MacPherson GG. Dendritic cell heterogeneity in vivo: two functionally different dendritic cell populations in rat intestinal lymph can be distinguished by CD4 expression. Journal of Immunology 1998;161:1146–55.

[48] Powell T, Jenkins C. Rat bone marrow derived dendritic cells, but not ex vivo dendritic cells, secrete nitric oxide and can inhibit T cell proliferation. Immunology 2003;109:197–208.

[49] Chen-Woan M. In vitro characterization of rat bone marrow-derived dendritic cells and their precursors. Journal of Leukocyte Biology 1996;59:196–207.

[50] Talmor M, Mirza A, Turley S. Generation or large numbers of immature and mature dendritic cells from rat bone marrow cultures. European Journal of Immunology 1998;28:811–7.

[51] Grauer O, Wohlleben G, Seubert S, Weishaupt A, Kämpgen E, Gold R. Analysis of maturation states of rat bone marrow-derived dendritic cells using an improved culture technique. Histochemistry and Cell Biology 2002;117:351–62.

[52] Pêche H, Trinité B, Martinet B, Cuturi MC. Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. American Journal of Transplantation 2005;5:255–67.

[53] Taieb A, Breitinger JJ, Unadkat JV, Shufesky WJ, Morelli AE, Thomson AW, et al. Intrinsic ability of GM+IL-4 but not Flt3L-induced rat dendritic cells to promote allogeneic T cell hyporesponsiveness. Clinical Immunology 2007;123:176–89.

[54] Brissette-Storkus CS, Kettel JC, Whitham TF, Giezeman-Smits KM, Villa L a, Potter DM, et al. Flt-3 ligand (FL) drives differentiation of rat bone marrow-derived dendritic cells expressing OX62 and/or CD161 (NKR-P1). Journal of Leukocyte Biology 2002;71:941–9.

[55] Bowers BWE, Berkowitz MR. Differentiation of dendritic cells in cultures of rat bone marrow cells. Journal of Experimental Medicine 1986;163:872–83.

[56] Villadangos J a, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nature Reviews Immunology 2007;7:543–55.

[57] Shortman K, Liu Y-J. Mouse and human dendritic cell subtypes. Nature Reviews Immunology 2002;2:151–61.

[58] Lin M-L, Zhan Y, Villadangos J a, Lew AM. The cell biology of cross-presentation and the role of dendritic cell subsets. Immunology and Cell Biology 2008;86:353–62.

[59] Randolph GJ, Ochando J, Partida-Sánchez S. Migration of dendritic cell subsets and their precursors. Annual Review of Immunology 2008;26:293–316.

[60] Balázs M, Martin F, Zhou T, Kearney J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. Immunity 2002;17:341–52.

[61] Sornasse T, Flamand V. Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. The Journal of Experimental Medicine 1992;175:15–21.

[62] Colino J, Snapper C. Exosomes from Bone Marrow Dendritic Cells Pulsed with Diphtheria Toxoid Preferentially Induce Type 1 Antigen-Specific IgG Responses in Naive Recipients in the Absence of Free Antigen. The Journal of Immunology 2006;177:3757–62.

[63] Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. Nature Reviews Immunology 2009;9:15–27.

[64] Bergtold A, Desai DD, Gavhane A, Clynes R. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. Immunity 2005;23:503–14.

[65] Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nature Immunology 2002;3:822–9.

[66] Sapoznikov A, Pewzner-Jung Y, Kalchenko V, Krauthgamer R, Shachar I, Jung S. Perivascular clusters of dendritic cells provide critical survival signals to B cells in bone marrow niches. Nature Immunology 2008;9:388–95.

[67] Dubois B, Massacrier C. Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. The Journal of Immunology 1998;161:2223–31.

[68] Jego G, Palucka A, Blanck J, Chalouni C. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity 2003;19:225–34.

[69] Wykes M, MacPherson G. Dendritic cell-B-cell interaction: dendritic cells provide B cells with CD40-independent proliferation signals and CD40-dependent survival signals. Immunology 2000;100:1–3.

[70] Shaw J, Wang Y-H, Ito T, Arima K, Liu Y-J. Plasmacytoid dendritic cells regulate B-cell growth and differentiation via CD70. Blood 2010;115:3051–7.

[71] King C. New insights into the differentiation and function of T follicular helper cells. Nature Reviews Immunology 2009;9:757–66.

[72] Lechler R, Batchelor J. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. The Journal of Experimental Medicine 1982;155:31–41.

[73] O'Larsen C, Morris P, Austyn J. Migration of dendritic leukocytes from cardiac allografts into host spleens. Journal of Experimental Medicine 1990;171.

[74] Coquerelle C, Moser M. DC subsets in positive and negative regulation of immunity. Immunological Reviews 2010;234:317–34.

[75] Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annual Review of Immunology 2003;21:685–711.

[76] Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. The Journal of Experimental Medicine 2001;194:769–79.

[77] Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, Inaba K, et al. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. Journal of Immunology 2008;181:6923–33.

[78] Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, et al. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. The Journal of Experimental Medicine 2003;198:235–47.

[79] Jiang A, Bloom O, Ono S, Cui W. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. Immunity 2007;27:610–24.

[80] Coombes JL, Siddiqui KRR, Arancibia-Cárcamo CV, Hall J, Sun C-M, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. The Journal of Experimental Medicine 2007;204:1757–64.

[81] Sun C-M, Hall J a, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. The Journal of Experimental Medicine 2007;204:1775–85.

[82] Guilliams M, Crozat K, Henri S, Tamoutounour S, Grenot P, Devilard E, et al. Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. Blood 2010;115:1958–68.

[83] Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nature Immunology 2001;2:725–31.

[84] Belz GT, Behrens GMN, Smith CM, Miller JF a. P, Jones C, Lejon K, et al. The CD8 + Dendritic Cell Is Responsible for Inducing Peripheral Self-Tolerance to Tissue-associated Antigens. Journal of Experimental Medicine 2002;196:1099–104.

[85] Matta BM, Castellaneta A, Thomson AW. Tolerogenic plasmacytoid DC. European Journal of Immunology 2010;40:2667–76.

[86] Goubier A, Dubois B, Gheit H, Joubert G, Villard-Truc F, Asselin-Paturel C, et al. Plasmacytoid dendritic cells mediate oral tolerance. Immunity 2008;29:464–75.

[87] de Heer HJ, Hammad H, Soullié T, Hijdra D, Vos N, Willart M a M, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. The Journal of Experimental Medicine 2004;200:89–98.

[88] Jonuleit H, Schmitt E, Steinbrink K, Enk a H. Dendritic cells as a tool to induce anergic and regulatory T cells. Trends in Immunology 2001;22:394–400.

[89] Schwartz RH. T cell anergy. Annual Review of Immunology 2003;21:305–34.

[90] Süss G, Shortman K. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. The Journal of Experimental Medicine 1996;183.

[91] Lu L, Qian S, Hershberger P. Fas ligand (CD95L) and B7 expression on dendritic cells provide counter-regulatory signals for T cell survival and proliferation. The Journal of Immonlogy 1997;158:5676–84.

[92] Maldonado R, Andrian U von. How tolerogenic dendritic cells induce regulatory T cells. Advances in Immunology 2010;108.

[93] Banchereau J, Pascual V, O'Garra A. From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. Nature Immunology 2012;13:925–31.

[94] Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. Blood 2010;116:935–44.

[95] Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. Blood 2005;105:1162–9.

[96] Mayer CT, Berod L, Sparwasser T. Layers of dendritic cell-mediated T cell tolerance, their regulation and the prevention of autoimmunity. Frontiers in Immunology 2012;3:183.

[97] Belladonna ML, Volpi C, Bianchi R, Vacca C, Orabona C, Pallotta MT, et al. Cutting edge: Autocrine TGF-beta sustains default tolerogenesis by IDO-competent dendritic cells. Journal of Immunology 2008;181:5194–8.

[98] Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, et al. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. European Journal of Immunology 2007;37:3054–62.

[99] Matteoli G, Mazzini E, Iliev ID, Mileti E, Fallarino F, Puccetti P, et al. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut 2010;59:595–604.

[100] Mellor A, Baban B. Cutting edge: induced indoleamine 2, 3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. The Journal of Immunology 2003;171:1652–6.

[101] Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002;297:1867–70.

[102] Tuettenberg A, Huter E, Hubo M, Horn J, Knop J, Grimbacher B, et al. The role of ICOS in directing T cell responses: ICOS-dependent induction of T cell anergy by tolerogenic dendritic cells. Journal of Immunology 2009;182:3349–56.

[103] Ito T, Yang M, Wang Y-H, Lande R, Gregorio J, Perng O a, et al. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. The Journal of Experimental Medicine 2007;204:105–15.

[104] Peña-Cruz V, McDonough SM, Diaz-Griffero F, Crum CP, Carrasco RD, Freeman GJ. PD-1 on immature and PD-1 ligands on migratory human Langerhans cells regulate antigen-presenting cell activity. The Journal of Investigative Dermatology 2010;130:2222–30.

[105] Fukaya T, Takagi H, Sato Y, Sato K, Eizumi K, Taya H, et al. Crucial roles of B7-H1 and B7-DC expressed on mesenteric lymph node dendritic cells in the generation of antigen-specific CD4+Foxp3+ regulatory T cells in the establishment of oral tolerance. Blood 2010;116:2266–76.

[106] Manavalan JS, Rossi PC, Vlad G, Piazza F, Yarilina A, Cortesini R, et al. High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. Transplant Immunology 2003;11:245–58.

[107] Brenk M, Scheler M, Koch S, Neumann J, Takikawa O, Häcker G, et al. Tryptophan deprivation induces inhibitory receptors ILT3 and ILT4 on dendritic cells favoring the induction of human CD4+CD25+ Foxp3+ T regulatory cells. Journal of Immunology 2009;183:145–54.

[108] Chang CC, Ciubotariu R, Manavalan JS, Yuan J, Colovai a I, Piazza F, et al. Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nature Immunology 2002;3:237–43.

[109] Hawksworth JS, Leeser D, Jindal RM, Falta E, Tadaki D, Elster E a. New directions for induction immunosuppression strategy in solid organ transplantation. American Journal of Surgery 2009;197:515–24.

[110] Webber A, Hirose R, Vincenti F. Novel strategies in immunosuppression: issues in perspective. Transplantation 2011;91:1057–64.

[111] Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi a B. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. Nature Medicine 2000;6:114.

[112] Pearl JP, Parris J, Hale D a, Hoffmann SC, Bernstein WB, McCoy KL, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2005;5:465–74.

[113] Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. The Journal of Clinical Investigation 2003;111:1887–95.

[114] Clatworthy MR. Targeting B cells and antibody in transplantation. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2011;11:1359–67.

[115] Moreau A, Varey E, Bériou G, Hill M, Bouchet-Delbos L, Segovia M, et al. Tolerogenic dendritic cells and negative vaccination in transplantation: from rodents to clinical trials. Frontiers in Immunology 2012;3:218.

[116] Riquelme P, Geissler EK, Hutchinson J a. Alternative approaches to myeloid suppressor cell therapy in transplantation: comparing regulatory macrophages to tolerogenic DCs and MDSCs. Transplantation Research 2012;1:1–17.

[117] Wood KJ, Bushell A, Hester J. Regulatory immune cells in transplantation. Nature Reviews Immunology 2012;12:417–30.

[118] Franquesa M, Herrero E, Torras J, Ripoll E, Flaquer M, Gomà M, et al. Mesenchymal Stem Cell Therapy Prevents Interstitial Fibrosis and Tubular Atrophy in a Rat Kidney Allograft Model. Stem Cells and Development 2012;00.

[119] Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. Nature Reviews Immunology 2007;7:610–21.

[120] Ezzelarab M, Thomson AW. Tolerogenic dendritic cells and their role in transplantation. Seminars in Immunology 2011;23:252–63.

[121] Valujskikh a. The challenge of inhibiting alloreactive T-cell memory. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2006;6:647–51.

[122] Lu L, McCaslin D, Starzl T, Thomson A. Bone marrow-derived dendritic cell progenitors (NLDC 145+. MHC class II+, B7-1dim, B7-2-) induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. Transplantation 1995;60:1539–45.

[123] Fu F, Li Y, Qian S, Lu L, Chambers F. Costimulatory molecule-deficient dendritic cell progenitors (MHC class II+, CD80dim, CD86-) prolong cardiac allograft survival in nonimmunosuppressed. Transplantation 1996;62:659–65.

[124] Lutz MB, Suri RM, Niimi M, Ogilvie a L, Kukutsch N a, Rössner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. European Journal of Immunology 2000;30:1813–22.

[125] Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nature Reviews Immunology 2004;4:24–34.

[126] Thomson A, Turnquist H, Raimondi G. Immunoregulatory functions of mTOR inhibition. Nature Reviews Immunology 2009;9:324–37.

[127] Nelson HS, Leung DYM, Bloom JW. Update on glucocorticoid action and resistance. Journal of Allergy and Clinical Immunology 2003;111:3–22.

[128] van Kooten C, Stax AS, Woltman AM, Gelderman K a. Handbook of experimental pharmacology “dendritic cells”: the use of dexamethasone in the induction of tolerogenic DCs. Handbook of Experimental Pharmacology 2009:233–49.

[129] Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. Blood 2006;108:1435–40.

[130] Di Rosa M, Malaguarnera M, Nicoletti F, Malaguarnera L. Vitamin D3: a helpful immuno-modulator. Immunology 2011;134:123–39.

[131] Pedersen AW, Claesson MH, Zocca M-B. Dendritic cells modified by vitamin D: future immunotherapy for autoimmune diseases. 1st ed. Elsevier Inc.; 2011.

[132] Bonham CA, Peng L, Liang X, Wang L, Ma L, Hackstein H, et al. Marked Prolongation of Cardiac Allograft Survival by Dendritic Cells Genetically Engineered with NF- B Oligodeoxyribonucleotide Decoys and Adenoviral Vectors Encoding CTLA4-Ig. Journal of Immunology 2002;169:3382–91.

[133] Abe M, Wang Z, de Creus A, Thomson AW. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2005;5:1808–19.

[134] Yamano T, Watanabe S, Hasegawa H, Suzuki T, Abe R, Tahara H, et al. Ex vivo-expanded DCs induce donor-specific central and peripheral tolerance and prolong the acceptance of donor skin grafts. Blood 2011;117:2640–8.

[135] DePaz HA, Oluwole OO, Adeyeri AO, Witkowski P, Jin M-X, Hardy MA, et al. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. Transplantation 2003;75:521–8.

[136] Giannoukakis N, Bonham C a, Qian S, Chen Z, Peng L, Harnaha J, et al. Prolongation of cardiac allograft survival using dendritic cells treated with NF-kB decoy oligodeoxyribonucleotides. Molecular Therapy : the Journal of the American Society of Gene Therapy 2000;1:430–7.

[137] Zhang Z, Ge W, Yang J, Vladau C, Suzuki M, Chen D, et al. Immune Modulation and Tolerance Induction by RelB-Silenced Dendritic Cells through RNA Interference. The Journal of Immonlogy 2007;178:5480–7.

[138] Lan Y, Wang Z, Raimondi G. “Alternatively Activated” Dendritic Cells Preferentially Secrete IL-10, Expand Foxp3+CD4+ T Cells, and Induce Long-Term Organ Allograft Survival in Combination with CTLA4-Ig. The Journal of Immonlogy 2006;177:5868–77.

[139] Hattori T, Saban DR, Emami-Naeini P, Chauhan SK, Funaki T, Ueno H, et al. Donor-derived, tolerogenic dendritic cells suppress immune rejection in the indirect allosensitization-dominant setting of corneal transplantation. Journal of Leukocyte Biology 2012;91:621–7.

[140] Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R. Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America 2001;98:6800–5.

[141] Emmer PM, van der Vlag J, Adema GJ, Hilbrands LB. Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hyporesponsiveness. Transplantation 2006;81:1451–9.

[142] Smyth L a, Ratnasothy K, Moreau A, Alcock S, Sagoo P, Meader L, et al. Tolerogenic Donor-Derived Dendritic Cells Risk Sensitization In Vivo owing to Processing and Presentation by Recipient APCs. Journal of Immunology 2013;190:4848–60.

[143] Divito SJ, Wang Z, Shufesky WJ, Liu Q, Tkacheva O a, Montecalvo A, et al. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. Blood 2010;116:2694–705.

[144] Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. The Journal of Experimental Medicine 2006;203:1851–8.

[145] Garrovillo M, Ali a, Depaz H a, Gopinathan R, Oluwole OO, Hardy M a, et al. Induction of transplant tolerance with immunodominant allopeptide-pulsed host lymphoid and myeloid dendritic cells. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2001;1:129–37.

[146] Bériou G, Pêche H, Guillonneau C, Merieau E, Cuturi M-C. Donor-Specific Allograft Tolerance by Administration of Recipient-Derived Immature Dendritic Cells and Suboptimal Immunosuppression. Transplantation 2005;79:969–72.

[147] Hill M, Thebault P, Segovia M, Louvet C, Bériou G, Tilly G, et al. Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and epstein-barr virus-induced gene 3. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2011;11:2036–45.

[148] Turnquist H, Raimondi G. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ. The Journal of Immonlogy 2007;178:7018–31.

[149] Ikeguchi R, Sacks JM, Unadkat JV, Solari M, Horibe EK, Thomson AW, et al. Long-term survival of limb allografts induced by pharmacologically conditioned, donor alloantigen-pulsed dendritic cells without maintenance immunosuppression. Transplantation 2008;85:237–46.

[150] Horibe EK, Sacks J, Unadkat J, Raimondi G, Wang Z, Ikeguchi R, et al. Rapamycin-conditioned, alloantigen-pulsed dendritic cells promote indefinite survival of vascularized skin allografts in association with T regulatory cell expansion. Transplant Immunology 2008;18:307–18.

[151] Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2005;5:228–36.

[152] Liu X, Mishra P, Yu S, Beckmann J, Wendland M, Kocks J, et al. Tolerance induction towards cardiac allografts under costimulation blockade is impaired in CCR7-deficient animals but can be restored by adoptive transfer of syngeneic plasmacytoid dendritic cells. European Journal of Immunology 2011;41:611–23.

[153] Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. Nature Immunology 2006;7:652–62.

[154] Li XL, Ménoret S, Bezie S, Caron L, Chabannes D, Hill M, et al. Mechanism and localization of CD8 regulatory T cells in a heart transplant model of tolerance. Journal of Immunology 2010;185:823–33.

[155] Tanriver Y, Ratnasothy K, Bucy RP, Lombardi G, Lechler R. Targeting MHC class I monomers to dendritic cells inhibits the indirect pathway of allorecognition and the production of IgG alloantibodies leading to long-term allograft survival. Journal of Immunology 2010;184:1757–64.

[156] Wang Z, Shufesky WJ, Montecalvo A, Divito SJ, Larregina AT, Morelli AE. In situ-targeting of dendritic cells with donor-derived apoptotic cells restrains indirect allorecognition and ameliorates allograft vasculopathy. PloS One 2009;4:e4940.

[157] Pêche H, Heslan M, Usal C. Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection 1. Transplantation 2003;76:1503–10.

[158] Pêche H, Renaudin K, Beriou G, Merieau E, Amigorena S, Cuturi MC. Induction of tolerance by exosomes and short-term immunosuppression in a fully MHC-mismatched rat cardiac allograft model. American Journal of Transplantation : Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2006;6:1541–50.

[159] Mirenda V. Modified Dendritic Cells Coexpressing Self and Allogeneic Major Histocompatability Complex Molecules: An Efficient Way to Induce Indirect Pathway Regulation. Journal of the American Society of Nephrology 2004;15:987–97.

[160] Garrod K, Chang C. Targeted lymphoid homing of dendritic cells is required for prolongation of allograft survival. The Journal of Immunology 2006;177:863–8.

[161] Wang Q. Anti-ICAM-1 antibody and CTLA-4Ig synergistically enhance immature dendritic cells to induce donor-specific immune tolerance in vivo. Immunology Letters 2003;90:33–42.

[162] Sordi V, Bianchi G, Buracchi C, Mercalli A, Marchesi F, D'Amico G, et al. Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. Transplantation 2006;82:826–34.

[163] Barten MJ, Garbade J, Bittner HB, Fiedler M, Dhein S, Thiery J, et al. Affects of immunosuppression on circulating dendritic cells: an adjunct to therapeutic drug monitoring after heart transplantation. International Immunopharmacology 2006;6:2011–7.

[164] Chorny A, Gonzalez-Rey E, Fernandez-Martin A, Ganea D, Delgado M. Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response. Blood 2006;107:3787–94.

[165] Sato K, Yamashita N, Yamashita N, Baba M, Matsuyama T. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. Immunity 2003;18:367–79.

[166] Sacks JM, Kuo Y-R, Taieb A, Breitinger J, Nguyen VT, Thomson AW, et al. Prolongation of composite tissue allograft survival by immature recipient dendritic cells pulsed with donor antigen and transient low-dose immunosuppression. Plastic and Reconstructive Surgery 2008;121:37–49.

[167] Tiao M-M, Lu L, Tao R, Wang L, Fung JJ, Qian S. Prolongation of Cardiac Allograft Survival by Systemic Administration of Immature Recipient Dendritic Cells Deficient in NF-kB Activity. Annals of Surgery 2005;241:497–505.

[168] Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nature Reviews Immunology 2002;2:569–79.

[169] Lamparski HG, Metha-Damani A, Yao J-Y, Patel S, Hsu D-H, Ruegg C, et al. Production and characterization of clinical grade exosomes derived from dendritic cells. Journal of Immunological Methods 2002;270:211–26.

[170] Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nature Reviews Immunology 2009.

[171] Harding C, Heuser J, Stahl P. Receptor-mediated Endocytosis of Transferrin and Recycling of the Transferrin Receptor in Rat Reticulocytes Biochemical Approaches to Transferrin. The Journal of Cell Biology 1983;97:329–39.

[172] Pan BT, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. The Journal of Cell Biology 1985;101:942–8.

[173] Johnstone RM, Adam M, Hammonds JR, Turbide C. Vesicle Formation during Reticulocyte Maturation. The Journal of Biological Chemistry 1987;262:9412–20.

[174] Raposo G, Nijman H. B lymphocytes secrete antigen-presenting vesicles. The Journal of Experimental Medicine 1996;183:1161–72.

[175] Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G. The biogenesis and functions of exosomes. Traffic 2002;3:321–30.

[176] Bobrie A, Colombo M, Raposo G, Théry C. Exosome secretion: molecular mechanisms and roles in immune responses. Traffic 2011;12:1659–68.

[177] Michelet X, Djeddi A, Legouis R. Developmental and cellular functions of the ESCRT machinery in pluricellular organisms. Biology of the Cell / Under the Auspices of the European Cell Biology Organization 2010;102:191–202.

[178] Théry C, Boussac M. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. The Journal of Immunology 2001;166:7309–18.

[179] Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. Journal of Immunology 2002;168:3235–41.

[180] Buschow SI, Liefhebber JMP, Wubbolts R, Stoorvogel W. Exosomes contain ubiquitinated proteins. Blood Cells, Molecules & Diseases 2005;35:398–403.

[181] Geminard C, Gassart A de, Blanc L, Vidal M. Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. Traffic 2004;5:181–93.

[182] Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 2008;319:1244–7.

[183] Gassart A de, Géminard C. Lipid raft-associated protein sorting in exosomes. Blood 2003;102:4336–44.

[184] Wubbolts R, Leckie RS, Veenhuizen PTM, Schwarzmann G, Möbius W, Hoernschemeyer J, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. The Journal of Biological Chemistry 2003;278:10963–72.

[185] Vidal M, Mangeat P, Hoekstra D. Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. Journal of Cell Science 1997;110:1867–77.

[186] Rabesandratana H, Toutant JP, Reggio H, Vidal M. Decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) are released within exosomes during In vitro maturation of reticulocytes. Blood 1998;91:2573–80.

[187] Fang Y, Wu N, Gan X, Yan W, Morrell JC, Gould SJ. Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. PLoS Biology 2007;5:e158.

[188] Muntasell A, Berger AC, Roche P a. T cell-induced secretion of MHC class II-peptide complexes on B cell exosomes. The EMBO Journal 2007;26:4263–72.

[189] Buschow SI, Nolte-'t Hoen ENM, van Niel G, Pols MS, ten Broeke T, Lauwen M, et al. MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. Traffic 2009;10:1528–42.

[190] Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, et al. Syndecan – syntenin – ALIX regulates the biogenesis of exosomes. Nature Cell Biology 2012;14:677–85.

[191] Savina A, Vidal M, Colombo MI. The exosome pathway in K562 cells is regulated by Rab11. Journal of Cell Science 2002;115:2505–15.

[192] Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nature Cell Biology 2010;12:19–30; sup pp 1–13.

[193] Bobrie A, Krumeich S, Reyal F, Recchi C, Moita LF, Seabra MC, et al. Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. Cancer Research 2012;72:4920–30.

[194] Hsu C, Morohashi Y, Yoshimura S-I, Manrique-Hoyos N, Jung S, Lauterbach M a, et al. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. The Journal of Cell Biology 2010;189:223–32.

[195] Booth AM, Fang Y, Fallon JK, Yang J-M, Hildreth JEK, Gould SJ. Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. The Journal of Cell Biology 2006;172:923–35.

[196] Mears R, Craven R a, Hanrahan S, Totty N, Upton C, Young SL, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Proteomics 2004;4:4019–31.

[197] Pisitkun T, Shen R-F, Knepper M a. Identification and proteomic profiling of exosomes in human urine. Proceedings of the National Academy of Sciences of the United States of America 2004;101:13368–73.

[198] Conde-Vancells J. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. Journal of Proteome Research 2008;7:5157–66.

[199] Kalra H, Simpson RJ, Ji H, Aikawa E, Altevogt P, Askenase P, et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biology 2012;10:e1001450.

[200] Mathivanan S, Simpson RJ. ExoCarta: A compendium of exosomal proteins and RNA. Proteomics 2009;9:4997–5000.

[201] Zitvogel L, Regnault A, Lozier A, Wolfers J. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. Nature Medicine 1998;4:594–600.

[202] Théry C, Regnault a, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, et al. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. The Journal of Cell Biology 1999;147:599–610.

[203] Van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, et al. Intestinal Epithelial Cells Secrete Exosome-like Vesicles. Gastroenterology 2001;121:337–49.

[204] Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Molecular Biology of the Cell 1997;8:2631–45.

[205] Admyre C, Bohle B, Johansson SM, Focke-Tejkl M, Valenta R, Scheynius A, et al. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. The Journal of Allergy and Clinical Immunology 2007;120:1418–24.

[206] Skokos D, Le Panse S, Villa I, Rousselle JC, Peronet R, David B, et al. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. Journal of Immunology 2001;166:868–76.

[207] Admyre C, Grunewald J, Thyberg J, Gripenback S, Tornling G, Eklund a., et al. Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. European Respiratory Journal 2003;22:578–83.

[208] Morelli AE, Larregina AT, Shufesky WJ, Sullivan MLG, Stolz DB, Papworth GD, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood 2004;104:3257–66.

[209] Kim S, Bianco N. MHC class II+ exosomes in plasma suppress inflammation in an antigen-specific and Fas ligand/Fas-dependent manner. The Journal of Immunology 2007;179:2235–41.

[210] Segura E, Nicco C, Lombard B, Véron P, Raposo G, Batteux F, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. Blood 2005;106:216–23.

[211] Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. The Journal of Biological Chemistry 1998;273:20121–7.

[212] Coppieters K, Barral AM, Juedes A, Wolfe T, Rodrigo E, Théry C, et al. No significant CTL cross-priming by dendritic cell-derived exosomes during murine lymphocytic choriomeningitis virus infection. Journal of Immunology 2009;182:2213–20.

[213] Saunderson S, Schuberth P. Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor. The Journal of Immunology 2008;4:1–7.

[214] Schorey JS, Bhatnagar S. Exosome function: from tumor immunology to pathogen biology. Traffic 2008;9:871–81.

[215] Clayton A, Turkes A, Dewitt S, Steadman R, Mason MD, Hallett MB. Adhesion and signaling by B cell-derived exosomes: the role of integrins. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 2004;18:977–9.

[216] Véron P, Segura E, Sugano G, Amigorena S, Théry C. Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells. Blood Cells, Molecules & Diseases 2005;35:81–8.

[217] Prado N, Marazuela EG, Segura E, Fernández-García H, Villalba M, Théry C, et al. Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. Journal of Immunology 2008;181:1519–25.

[218] Caby M-P, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. International Immunology 2005;17:879–87.

[219] Clayton A, Mitchell JP, Court J, Linnane S, Mason MD, Tabi Z. Human tumor-derived exosomes down-modulate NKG2D expression. Journal of Immunology 2008;180:7249–58.

[220] Conde-Vancells J, Rodriguez-Suarez E, Gonzalez E, Berisa A, Gil D, Embade N, et al. Candidate biomarkers in exosome-like vesicles purified from rat and mouse urine samples. Proteomics Clinical Applications 2010;4:416–25.

[221] Mallegol J, Van Niel G, Lebreton C, Lepelletier Y, Candalh C, Dugave C, et al. T84-intestinal epithelial exosomes bear MHC class II/peptide complexes potentiating antigen presentation by dendritic cells. Gastroenterology 2007;132:1866–76.

[222] Hegmans JPJJ, Bard MPL, Hemmes A, Luider TM, Kleijmeer MJ, Prins J-B, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. The American Journal of Pathology 2004;164:1807–15.

[223] Pisitkun T, Shen R-F, Knepper M a. Identification and proteomic profiling of exosomes in human urine. Proceedings of the National Academy of Sciences of the United States of America 2004;101:13368–73.

[224] Taylor D, Akyol S, Gercel-Taylor C. Pregnancy-associated exosomes and their modulation of T cell signaling. The Journal of Immunology 2006;176:1534–42.

[225] Luketic L, Delanghe J, Sobol PT, Yang P, Frotten E, Mossman KL, et al. Antigen presentation by exosomes released from peptide-pulsed dendritic cells is not suppressed by the presence of active CTL. Journal of Immunology 2007;179:5024–32.

[226] Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen L-A a, et al. Proteomic analysis of exosomes isolated from human malignant pleural effusions. American Journal of Respiratory Cell and Molecular Biology 2004;31:114–21.

[227] Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology 2007;9:654–9.

[228] Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González MÁ, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nature Communications 2011;2:282.

[229] Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 2012;119:756–66.

[230] Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 2012;119:756–66.

[231] Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecologic Oncology 2008;110:13–21.

[232] Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clinical Lung Cancer 2009;10:42–6.

[233] Denzer K, van Eijk M, Kleijmeer MJ, Jakobson E, de Groot C, Geuze HJ. Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. Journal of Immunology 2000;165:1259–65.

[234] Escrevente C, Keller S, Altevogt P, Costa J. Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer 2011;11:108.

[235] Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, et al. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. Journal of Cell Science 2011;124:447–58.

[236] Barrès C, Blanc L, Bette-Bobillo P, André S, Mamoun R, Gabius H-J, et al. Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. Blood 2010;115:696–705.

[237] Segura E, Guérin C. CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo. The Journal of Immunology 2007;179:1489–96.

[238] Xie Y, Zhang H, Li W, Deng Y, Munegowda MA, Chibbar R, et al. Dendritic cells recruit T cell exosomes via exosomal LFA-1 leading to inhibition of CD8+ CTL responses through downregulation of peptide/MHC class I and Fas ligand-mediated cytotoxicity. Journal of Immunology 2010;185:5268–78.

[239] Feng D, Zhao W-L, Ye Y-Y, Bai X-C, Liu R-Q, Chang L-F, et al. Cellular internalization of exosomes occurs through phagocytosis. Traffic 2010;11:675–87.

[240] Théry C, Duban L, Segura E, Véron P, Lantz O, Amigorena S. Indirect activation of naïve CD4+ T cells by dendritic cell-derived exosomes. Nature Immunology 2002;3:1156–62.

[241] Vallhov H, Gutzeit C, Johansson SM, Nagy N, Paul M, Li Q, et al. Exosomes containing glycoprotein 350 released by EBV-transformed B cells selectively target B cells through CD21 and block EBV infection in vitro. Journal of Immunology 2011;186:73–82.

[242] Nolte-'t Hoen ENM, Buschow SI, Anderton SM, Stoorvogel W, Wauben MHM. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. Blood 2009;113:1977–81.

[243] Izquierdo-Useros N, Lorizate M, Puertas MC, Rodriguez-Plata MT, Zangger N, Erikson E, et al. Siglec-1 is a novel dendritic cell receptor that mediates HIV-1 trans-infection through recognition of viral membrane gangliosides. PLoS Biology 2012;10:e1001448.

[244] Montecalvo A, Shufesky WJ, Stolz DB, Sullivan MG, Wang Z, Divito SJ, et al. Exosomes as a short-range mechanism to spread alloantigen between dendritic cells during T cell allorecognition. Journal of Immunology 2008;180:3081–90.

[245] Admyre C, Johansson SM, Paulie S, Gabrielsson S. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. European Journal of Immunology 2006;36:1772–81.

[246] Qazi KR, Gehrmann U, Domange Jordö E, Karlsson MCI, Gabrielsson S. Antigen-loaded exosomes alone induce Th1-type memory through a B-cell-dependent mechanism. Blood 2009;113:2673–83.

[247] André F, Chaput N, Schartz NEC, Flament C, Aubert N, Bernard J, et al. Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. Journal of Immunology 2004;172:2126–36.

[248] Wolfers J, Lozier a, Raposo G, Regnault a, Théry C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nature Medicine 2001;7:297–303.

[249] Andre F, Schartz NEC, Movassagh M, Flament C, Pautier P, Morice P, et al. Mechanisms of disease Malignant effusions and immunogenic tumour-derived exosomes. The Lancet 2002;360:295–305.

[250] Chaput N, Théry C. Exosomes: immune properties and potential clinical implementations. Seminars in Immunopathology 2011;33:419–40.

[251] Van Niel G, Mallegol J, Bevilacqua C, Candalh C, Brugière S, Tomaskovic-Crook E, et al. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. Gut 2003;52:1690–7.

[252] Aline F, Bout D. Infections-Toxoplasma gondii Antigen-Pulsed-Dendritic Cell-Derived Exosomes Induce a Protective Immune Response against T. gondii Infection. Infection and Immunity 2004;72:4127–37.

[253] Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Cancer Research 2007;67:7458–66.

[254] Wieckowski EU, Visus C, Szajnik M, Szczepanski MJ, Storkus WJ, Whiteside TL. Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T lymphocytes. Journal of Immunology 2009;183:3720–30.

[255] Abusamra AJ, Zhong Z, Zheng X, Li M, Ichim TE, Chin JL, et al. Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. Blood Cells, Molecules & Diseases 2005;35:169–73.

[256] Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nature Medicine 2012;18:883–91.

[257] Hedlund M, Stenqvist A-C, Nagaeva O, Kjellberg L, Wulff M, Baranov V, et al. Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function. Journal of Immunology 2009;183:340–51.

[258] Sabapatha A, Gercel-Taylor C, Taylor DD. Specific isolation of placenta-derived exosomes from the circulation of pregnant women and their immunoregulatory consequences. American Journal of Reproductive Immunology 2006;56:345–55.

[259] Chaput N, Schartz NEC, André F, Taïeb J, Novault S, Bonnaventure P, et al. Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. Journal of Immunology 2004;172:2137–46.

[260] Morse M a, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. Journal of Translational Medicine 2005;3:9.

[261] Escudier B, Dorval T, Chaput N, André F, Caby M-P, Novault S, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. Journal of Translational Medicine 2005;3:10.

[262] Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, et al. Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. Molecular Therapy : the Journal of the American Society of Gene Therapy 2006;13:289–300.

[263] Kim SH, Bianco NR, Shufesky WJ, Morelli AE, Robbins PD. Effective treatment of inflammatory disease models with exosomes derived from dendritic cells genetically modified to express IL-4. Journal of Immunology 2007;179:2242–9.

[264] Kim S, Lechman E. Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. The Journal of Immunology 2005;174:6440–8.

[265] Almqvist N, Lönnqvist A, Hultkrantz S, Rask C, Telemo E. Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma. Immunology 2008;125:21–7.

[266] El Andaloussi S, Mäger I, Breakefield XO, Wood MJ a. Extracellular vesicles: biology and emerging therapeutic opportunities. Nature Reviews Drug Discovery 2013;12:347–57.

[267] Yang X, Meng S, Jiang H, Zhu C, Wu W. Exosomes derived from immature bone marrow dendritic cells induce tolerogenicity of intestinal transplantation in rats. The Journal of Surgical Research 2011;171:826–32.

[268] Li X, Li J-J, Yang J-Y, Wang D-S, Zhao W, Song W-J, et al. Tolerance induction by exosomes from immature dendritic cells and rapamycin in a mouse cardiac allograft model. PloS One 2012;7:e44045.

[269] Admyre C, Johansson SM, Qazi KR, Filén J-J, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. Journal of Immunology 2007;179:1969–78.

[270] Pisitkun T, Gandolfo MT, Das S, Knepper M a, Bagnasco SM. Application of systems biology principles to protein biomarker discovery: urinary exosomal proteome in renal transplantation. Proteomics Clinical Applications 2012;6:268–78.

[271] Falcón-Pérez JM, Nazarian R, Sabatti C, Dell'Angelica EC. Distribution and dynamics of Lamp1-containing endocytic organelles in fibroblasts deficient in BLOC-3. Journal of Cell Science 2005;118:5243–55.

[272] Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Current Protocols in Cell Biology 2006;Chapter 3:Unit 3.22.

[273] Silva JC, Denny R, Dorschel C, Gorenstein MV, Li G-Z, Richardson K, et al. Simultaneous qualitative and quantitative analysis of the Escherichia coli proteome: a sweet tale. Molecular & Cellular Proteomics : MCP 2006;5:589–607.

[274] Pérez-Cabezas B, Naranjo-Gómez M, Bastos-Amador P, Requena-Fernández G, Pujol-Borrell R, Borràs FE. Ligation of Notch receptors in human conventional and plasmacytoid dendritic cells differentially regulates cytokine and chemokine secretion and modulates Th cell polarization. Journal of Immunology 2011;186:7006–15.

[275] Marin-Gallen S, Clemente-Casares X, Planas R, Pujol-Autonell I, Carrascal J, Carrillo J, et al. Dendritic cells pulsed with antigen-specific apoptotic bodies prevent experimental type 1 diabetes. Clinical & Experimental Immunology 2009;160:207–14.

[276] Chen-Woan M, Delaney C. A new protocol for the propagation of dendritic cells from rat bone marrow using recombinant GM-CSF, and their quantification using the mAb OX-62. Journal of Immunological Methods 1995;178:157–71.

[277] Herrero-Fresneda I, Torras J, Cruzado JM, Condom E, Vidal A, Riera M, et al. Do alloreactivity and prolonged cold ischemia cause different elementary lesions in chronic allograft nephropathy? The American Journal of Pathology 2003;162:127–37.

[278] Mignot G, Roux S, Thery C, Ségura E, Zitvogel L. Prospects for exosomes in immunotherapy of cancer. Journal of Cellular and Molecular Medicine 2006;10:376–88.

[279] Viaud S, Théry C, Ploix S, Tursz T, Lapierre V, Lantz O, et al. Dendritic cell-derived exosomes for cancer immunotherapy: what's next? Cancer Research 2010;70:1281–5.

[280] Bianco NR, Kim SH, Ruffner M a, Robbins PD. Therapeutic effect of exosomes from indoleamine 2,3-dioxygenase-positive dendritic cells in collagen-induced arthritis and delayed-type hypersensitivity disease models. Arthritis and Rheumatism 2009;60:380–9.

[281] MacKay P a, Leibundgut-Landmann S, Koch N, Dunn AC, Reith W, Jack RW, et al. Circulating, soluble forms of major histocompatibility complex antigens are not exosome-associated. European Journal of Immunology 2006;36:2875–84.

[282] Batista B, Eng W. Identification of a conserved glycan signature for microvesicles. Journal of Proteome Research 2011;10:4624–33.

[283] Simons M, Raposo G. Exosomes--vesicular carriers for intercellular communication. Current Opinion in Cell Biology 2009;21:575–81.

[284] György B, Módos K, Pállinger E, Pálóczi K, Pásztói M, Misják P, et al. Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. Blood 2011;117:e39–48.

[285] Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Review of Proteomics 2009;6:267–83.

[286] Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Reviews 2007;21:157–71.

[287] Izquierdo-Useros N, Naranjo-Gómez M, Archer J, Hatch SC, Erkizia I, Blanco J, et al. Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway. Blood 2009;113:2732–41.

[288] Fonteneau J-F, Gilliet M, Larsson M, Dasilva I, Münz C, Liu Y-J, et al. Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. Blood 2003;101:3520–6.

[289] Krug A, Veeraswamy R, Pekosz A, Kanagawa O, Unanue ER, Colonna M, et al. Interferon-producing cells fail to induce proliferation of naive T cells but can promote expansion and T helper 1 differentiation of antigen-experienced unpolarized T cells. The Journal of Experimental Medicine 2003;197:899–906.

[290] Salio M, Palmowski MJ, Atzberger A, Hermans IF, Cerundolo V. CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. The Journal of Experimental Medicine 2004;199:567–79.

[291] Schlecht G, Garcia S. Murine plasmacytoid dendritic cells induce effector/memory CD8+ T-cell responses in vivo after viral stimulation. Immunobiology 2004;104:1808–15.

[292] Young LJ, Wilson NS, Schnorrer P, Proietto A, ten Broeke T, Matsuki Y, et al. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. Nature Immunology 2008;9:1244–52.

[293] Ito T, Inaba M, Inaba K, Toki J, Sogo S, Iguchi T, et al. A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. Journal of Immunology 1999;163:1409–19.

[294] Robinson SP, Patterson S, English N, Davies D, Knight SC, Reid CD. Human peripheral blood contains two distinct lineages of dendritic cells. European Journal of Immunology 1999;29:2769–78.

[295] Sapoznikov A, Fischer J a a, Zaft T, Krauthgamer R, Dzionek A, Jung S. Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells. The Journal of Experimental Medicine 2007;204:1923–33.

[296] Dalgaard J, Beckstrøm K. Differential capability for phagocytosis of apoptotic and necrotic leukemia cells by human peripheral blood dendritic cell subsets. Journal of Leukocyte Biology 2005;77:689–98.

[297] Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. The Journal of Experimental Medicine 1997;185:1101–11.

[298] Stent G, Reece JC, Baylis DC, Ivinson K, Paukovics G, Thomson M, et al. Heterogeneity of freshly isolated human tonsil dendritic cells demonstrated by intracellular markers, phagocytosis, and membrane dye transfer. Cytometry 2002;48:167–76.

[299] Angelot F, Seillès E, Biichlé S, Berda Y, Gaugler B, Plumas J, et al. Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases. Haematologica 2009;94:1502–12.

[300] Tel J, Lambeck AJ a, Cruz LJ, Tacken PJ, de Vries IJM, Figdor CG. Human plasmacytoid dendritic cells phagocytose, process, and present exogenous particulate antigen. Journal of Immunology 2010;184:4276–83.

[301] Hoeffel G, Ripoche A-C, Matheoud D, Nascimbeni M, Escriou N, Lebon P, et al. Antigen crosspresentation by human plasmacytoid dendritic cells. Immunity 2007;27:481–92.

[302] Morelli AE, Thomson AW. Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction. Immunological Reviews 2003;196:125–46.

[303] Stuart LM, Lucas M, Simpson C, Lamb J, Savill J, Lacy-Hulbert A. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. Journal of Immunology 2002;168:1627–35.

[304] Verbovetski I, Bychkov H, Trahtemberg U, Shapira I, Hareuveni M, Ben-Tal O, et al. Opsonization of Apoptotic Cells by Autologous iC3b Facilitates Clearance by Immature Dendritic Cells, Down-regulates DR and CD86, and Up-regulates CC Chemokine Receptor 7. Journal of Experimental Medicine 2002;196:1553–61.

[305] van Kooten C, Lombardi G, Gelderman K a., Sagoo P, Buckland M, Lechler R, et al. Dendritic Cells as a Tool to Induce Transplantation Tolerance: Obstacles and Opportunities. Transplantation 2011;91:2–7.

[306] Stax AM, Gelderman KA, Schlagwein N, Essers MC, Kamerling SWA, Woltman AM, et al. Induction of donor-specific T-cell hyporesponsiveness using dexamethasone-

treated dendritic cells in two fully mismatched rat kidney transplantation models. Transplantation 2008;86:1275–82.

[307] Muthana M, Fairburn B, Mirza S. Systematic evaluation of the conditions required for the generation of immature rat bone marrow-derived dendritic cells and their phenotypic and functional characterization. Journal of Immunological Methods 2004;294:165–79.

[308] Stax AM, Crul C, Kamerling SW a, Schlagwein N, van der Geest RN, Woltman AM, et al. CD40L stimulation of rat dendritic cells specifically favors the IL-12/IL-10 ratio resulting in a strong T cell stimulatory capacity. Molecular Immunology 2008;45:2641–50.

[309] Brenan M, Puklavec M. The MRC OX-62 antigen: a useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. The Journal of Experimental Medicine 1992;175:1457–65.

[310] Cabezón R, Ricart E, España C, Panés J, Benitez-Ribas D. Gram-Negative Enterobacteria Induce Tolerogenic Maturation in Dexamethasone Conditioned Dendritic Cells. PLoS ONE 2012;7:e52456.

[311] Piemonti L, Monti P, Allavena P, Sironi M, Soldini L, Leone BE, et al. Glucocorticoids affect human dendritic cell differentiation and maturation. Journal of Immunology 1999;162:6473–81.

[312] Woltman a M, de Fijter JW, Kamerling SW, Paul LC, Daha MR, van Kooten C. The effect of calcineurin inhibitors and corticosteroids on the differentiation of human dendritic cells. European Journal of Immunology 2000;30:1807–12.

[313] Josien R, Heslan M. Rat spleen dendritic cells express natural killer cell receptor protein 1 (NKR-P1) and have cytotoxic activity to select targets via a Ca2+-dependent mechanism. The Journal of Experimental Medicine 1997;186:467–72.

[314] Herrero-Fresneda I, Franquesa M, Torras J, Vidal a, Aran J, Pluvinet R, et al. Role of cold ischemia in acute rejection: characterization of a humoral-like acute rejection in experimental renal transplantation. Transplantation Proceedings 2005;37:3712–5.

[315] Santos L, Draves KE, Boton M, Grewal PK, Marth JD, Clark E a. Dendritic cell-dependent inhibition of B cell proliferation requires CD22. Journal of Immunology 2008;180:4561–9.

[316] Sindhava VJ, Tuna H, Gachuki BW, DiLillo DJ, Avdiushko MG, Onami TM, et al. Bone marrow dendritic cell-mediated regulation of TLR and B cell receptor signaling in B cells. Journal of Immunology 2012;189:3355–67.

[317] Matic G, Schütt W, Winkler RE, Tiess M, Ramlow W. Extracorporeal removal of circulating immune complexes: from non-selective to patient-specific. Blood Purification 2000;18:156–60.

[318] Nishimura M, Ishikawa Y, Satake M. Activation of polymorphonuclear neutrophils by immune complex: possible involvement in development of transfusion-related acute lung injury. Transfusion Medicine 2004;14:359–67.

[319] Burnier L, Fontana P, Kwak BR, Angelillo-Scherrer A. Cell-derived microparticles in haemostasis and vascular medicine. Thrombosis and Haemostasis 2009;101:439–51.

[320] Ramacciotti E, Hawley A, Wrobleski S. Proteomics of microparticles after deep venous thrombosis. Thrombosis Research 2010;125:1–16.

[321] Wakabayashi S, Koide T. Histidine-rich glycoprotein: a possible modulator of coagulation and fibrinolysis. Seminars in Thrombosis and Hemostasis 2011;37:389–94.

[322] Poon IKH, Patel KK, Davis DS, Parish CR, Hulett MD. Histidine-rich glycoprotein: the Swiss Army knife of mammalian plasma. Blood 2011;117:2093–101.

[323] Salge-Bartels U, Breitner-Ruddock S, Hunfeld A, Seitz R, Heiden M. Are quality differences responsible for different adverse reactions reported for SD-plasma from USA and Europe? Transfusion Medicine 2006;16:266–75.

[324] Dasgupta SK, Abdel-Monem H, Niravath P, Le A, Bellera RV, Langlois K, et al. Lactadherin and clearance of platelet-derived microvesicles. Blood 2009;113:1332–9.

[325] Terrisse a D, Puech N, Allart S, Gourdy P, Xuereb JM, Payrastre B, et al. Internalization of microparticles by endothelial cells promotes platelet/endothelial cell interaction under flow. Journal of Thrombosis and Haemostasis : JTH 2010;8:2810–9.

[326] Perez-Hernandez D, Gutiérrez-Vázquez C, Jorge I, López-Martín S, Ursa A, Sánchez-Madrid F, et al. The Intracellular Interactome of Tetraspanin-enriched Microdomains Reveals Their Function as Sorting Machineries toward Exosomes. The Journal of Biological Chemistry 2013;288:11649–61.

[327] van Balkom BWM, Pisitkun T, Verhaar MC, Knepper MA. Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases. Kidney International 2011;80:1138–45.

[328] Alvarez ML, Khosroheidari M, Kanchi Ravi R, Distefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney International 2012;82:1024–32.

[329] Pant S, Hilton H, Burczynski ME. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. Biochemical Pharmacology 2012;83:1484–94.

[330] Fleissner F, Goerzig Y, Haverich A, Thum T. Microvesicles as novel biomarkers and therapeutic targets in transplantation medicine. American Journal of Transplantation 2012;12:289–97.

[331] Segura E. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. Blood 2005;106:216–23.

[332] Tel J, Schreibelt G, Sittig SP, Mathan TSM, Buschow SI, Cruz LJ, et al. Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8+ T cells despite lower Ag uptake than myeloid dendritic cell subsets. Blood 2013;121:459–67.

[333] Wilson NS, Villadangos J a. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. Advances in Immunology 2005;86:241–305.

[334] Tel J, van der Leun AM, Figdor CG, Torensma R, de Vries IJM. Harnessing human plasmacytoid dendritic cells as professional APCs. Cancer Immunology, Immunotherapy : CII 2012;61:1279–88.

[335] Janelidze S, Enell K, Visse E, Darabi A, Salford LG, Siesjö P. Activation of purified allogeneic CD4(+) T cells by rat bone marrow-derived dendritic cells induces concurrent secretion of IFN-gamma, IL-4, and IL-10. Immunology Letters 2005;101:193–201.

[336] Fazekasova H, Golshayan D, Read J. Regulation of rat and human T-cell immune response by pharmacologically modified dendritic cells. Transplantation 2009;87:1617–28.

[337] Matyszak MK, Citterio S, Rescigno M, Ricciardi-Castagnoli P. Differential effects of corticosteroids during different stages of dendritic cell maturation. European Journal of Immunology 2000;30:1233–42.

[338] Rea D, Kooten CV, Meijgaarden KEV, Ottenhoff THM, Melief CJM, Offringa R. Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. Blood 2000;95:3162–7.

[339] Ouabed A, Hubert F. Differential control of T regulatory cell proliferation and suppressive activity by mature plasmacytoid versus conventional spleen dendritic cells. The Journal of Immunology 2008;180:5862–70.

[340] Cohen N, Mouly E, Hamdi H, Maillot M-C, Pallardy M, Godot V, et al. GILZ expression in human dendritic cells redirects their maturation and prevents antigen-specific T lymphocyte response. Blood 2006;107:2037–44.

[341] Unger WWJ, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. European Journal of Immunology 2009;39:3147–59.

[342] Bonasio R, von Andrian UH. Generation, migration and function of circulating dendritic cells. Current Opinion in Immunology 2006;18:503–11.

[343] Cavanagh L, Bonasio R, Mazo I. Activation of bone marrow–resident memory T cells by circulating, antigen-bearing dendritic cells. Nature Immunology 2005;6:1029–37.

[344] Naranjo-Gómez M, Raïch-Regué D, Oñate C, Grau-López L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. Journal of Translational Medicine 2011;9:89.

[345] Wang G-Y, Chen G-H, Li H, Huang Y, Wang G-S, Jiang N, et al. Rapamycin-treated mature dendritic cells have a unique cytokine secretion profile and impaired allostimulatory capacity. Transplant International : Official Journal of the European Society for Organ Transplantation 2009;22:1005–16.

[346] Battaglia M, Stabilini A, Roncarolo M-G. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood 2005;105:4743–8.

[347] Stallone G, Infante B, Schena A, Battaglia M, Ditonno P, Loverre A, et al. Rapamycin for treatment of chronic allograft nephropathy in renal transplant patients. Journal of the American Society of Nephrology : JASN 2005;16:3755–62.

[348] Coenen JJ a, Koenen HJPM, van Rijssen E, Hilbrands LB, Joosten I. Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+CD25+ regulatory T cells. Blood 2006;107:1018–23.

[349] Dubois B, Caux C. Critical role of ITIM-bearing FcgammaR on DCs in the capture and presentation of native antigen to B cells. Immunity 2005;23:463–4.

[350] Chappell CP, Draves KE, Giltiay NV, Clark E a. Extrafollicular B cell activation by marginal zone dendritic cells drives T cell-dependent antibody responses. The Journal of Experimental Medicine 2012;209:1825–40.

[351] Wykes M, Pombo A, Jenkins C, MacPherson GG. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. Journal of Immunology 1998;161:1313–9.

[352] Qi H, Egen JG, Huang AYC, Germain RN. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. Science 2006;312:1672–6.

[353] Nitschke L. CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions. Immunological Reviews 2009;230:128–43.

[354] Jenner J, Kerst G, Handgretinger R, Müller I. Increased alpha2,6-sialylation of surface proteins on tolerogenic, immature dendritic cells and regulatory T cells. Experimental Hematology 2006;34:1212–8.

[355] Sindhava V, Woodman ME, Stevenson B, Bondada S. Interleukin-10 mediated autoregulation of murine B-1 B-cells and its role in Borrelia hermsii infection. PloS One 2010;5:e11445.

[356] Townsend MJ, Monroe JG, Chan AC. B-cell targeted therapies in human autoimmune diseases: an updated perspective. Immunological Reviews 2010;237:264–83.

[357] Dörner T, Kaufmann J, Wegener W a, Teoh N, Goldenberg DM, Burmester GR. Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. Arthritis Research & Therapy 2006;8:R74.

[358] Nitschke L, Tsubata T. Molecular interactions regulate BCR signal inhibition by CD22 and CD72. Trends in Immunology 2004;25:543–50.

[359] Volchenkov R, Karlsen M, Jonsson R, Appel S. Type 1 regulatory T cells and regulatory B cells induced by tolerogenic dendritic cells. Scandinavian Journal of Immunology 2013;77:246–54.

[360] Braun WE, Protiva D a, Schold JD. Decreased Total CD19(+) B Lymphocytes and the Occurrence of Monoclonal Proteins Are Frequent in Ultra-Long (30 to 44-Year) Renal Transplant Recipients: Implications for Allograft and Patient Survival. Transplantation Proceedings 2013;45:1466–8.

PUBLICATIONS

1. Bastos-Amador P, Pérez-Cabezas B, Izquierdo-Useros N, Puertas MC, Martinez-Picado J, Pujol-Borrell R, et al. **Capture of cell-derived microvesicles (exosomes and apoptotic bodies) by human plasmacytoid dendritic cells**. Journal of Leukocyte Biology 2012;91:751–8.

2. Bastos-Amador P, Royo F, Gonzalez E, Conde-Vancells J, Palomo-Diez L, Borras FE, et al. **Proteomic analysis of microvesicles from plasma of healthy donors reveals high individual variability**. Journal of Proteomics 2012;75:3574–84.

3. Pérez-Cabezas B, Naranjo-Gómez M, Bastos-Amador P, Requena-Fernández G, Pujol-Borrell R, Borràs FE. **Ligation of Notch receptors in human conventional and plasmacytoid dendritic cells differentially regulates cytokine and chemokine secretion and modulates Th cell polarization**. Journal of Immunology 2011;186:7006–15.

4. Pérez-Cabezas B, Naranjo-Gómez M, Ruiz-Riol M, Bastos-Amador P, Fernández MA, Carmona F, et al. **TLR-activated conventional DCs promote γ-secretase-mediated conditioning of plasmacytoid DCs**. Journal of Leukocyte Biology 2012;92:133–43.

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