Aus dem Institut für Medizinische Immunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Impact of immunosenescence and donor age on alloimmunity and transplant outcome

Zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

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Datum der Promotion:
30.05.2015

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1 ABSTRACT

Continuous and complex modifications of the immune system in parallel to aging are major factors impacting transplant outcome and organ quality. Increasing numbers of elderly transplant recipients and a growing utilization of organs from older donors thus pose pressing challenges for transplantation medicine. In clinical practice, transplantation of old donor organs has been associated with reduced patient and graft survival, more frequent episodes of acute rejection and delayed graft function. To elucidate the mechanisms underlying these clinical findings, a fully MHC-mismatched murine model of vascularized heterotopic cardiac transplantation with young (8 to 12 weeks) DBA/2 recipients and either young or old (18 months) C57BL/6 donors was used.

Cardiac allografts procured from old donor mice were subject to significantly accelerated graft rejection compared to young allografts. This difference in graft survival was associated with higher ISHLT rejection scores and increased intragraft infiltration by $CD4^+$ and $CD8^+$ T cells. Futhermore, organ age impacted characteristics of systemic alloimmune responses of the recipients, with increased frequencies of splenic $CD8^+$ effector T cells and $CD8^+$IFN-γ^+ T cells in recipients of old allografts, in addition to higher frequencies of alloreactive IFN-γ and IL-6-producing splenocytes and more potent proliferative responses upon restimulation with donor-type antigen.

To discriminate between differential effects of parenchymal tissue and intragraft passenger leukocytes in this context, chimeric donor animals were generated by transplanting young syngeneic bone marrow into old and young prospective donor mice six weeks prior to organ procurement. When transplanting these chimeric allografts (young tissue/young leukocytes or old tissue/young leukocytes) comparable graft survival, rejection scores and systemic immune responses were observed, thus revealing a crucial role of intragraft passenger leukocytes in mediating augmented immune responses when transplanting old allografts.

Focusing on dendritic cells as one subset of passenger leukocytes, prospective cardiac allografts were then depleted of intragraft DCs by donor pretreatment with liposomal clodronate. Differences in graft survival and rejection scores where abolished when using hearts depleted of intragraft DCs, and systemic alloimmune responses remained independent of donor age. Furthermore, *in-vitro* characterization of allostimulatory priming capabilities of DCs showed significantly higher frequencies of IFN-γ-producing cells and higher proliferative responses among allogeneic splenocytes when stimulated by old DCs, as well as increased expression of MHC-II and costimulatory molecules on old DCs. Dendritic cells as a cellular substrate mediating donor-age-dependent acceleration and exacerbation of allograft rejection may thus represent a potential target for donor pretreatment strategies.

Kontinuierliche und komplexe Veränderungen des Immunsystems mit zunehmendem Alter haben entscheidenen Einfluss auf den klinischen Erfolg von Organtransplantationen und die Qualität von Spenderorganen. Da die Zahl der älteren Transplantatempfänger steigt und zunehmend Organe von älteren Spendern transplantiert werden, ergeben sich drängende Herausforderungen für die Transplantationsmedizin. Klinisch konnte für die Transplantation von älteren Spenderorganen eine Assoziation mit schlechterem Patienten- und Transplantatüberleben sowie häufigeren akuten Abstoßungsreaktionen und verzögerter Transplantatfunktion gezeigt werden. Um diesen klinischen Phänomenen zugrundeliegende Mechanismen zu ergründen, wurde in der vorliegenden Arbeit ein Mausmodell heterotoper, komplett vaskularisierter Herztransplantation mit MHC-disparaten jungen (acht bis zwölf Wochen) DBA/2-Empfängern und jungen oder alten (18 Monate) C57BL/6 Spendern etabliert.

Von alten Spendermäusen gewonnene Herzen zeigten eine signifikant beschleunigte Transplantatabstoßung im Vergleich zu jungen Spenderherzen. Dieser Unterschied im Transplantatüberleben war begleitet von höheren Indizes in der ISHLT Abstoßungsklassifikation und verstärkter Transplantatinfiltration durch $CD4^+$ und $CD8^+$ T-Zellen. Das Alter des transplantierten Organs hatte weiterhin Einfluss auf Charakteristiken der systemischen Alloimmunreaktion des Empfängers, dargestellt durch erhöhte Häufigkeit von milzständigen $CD8^+$ Effektor-T-Zellen und $CD8^+$IFN-γ^+ T-Zellen bei Transplantation von älteren Spenderorganen, zusätzlich zu erhöhten Häufigkeiten von alloreaktiven IFN-γ- und IL-6-produzierenden Splenozyten sowie ausgeprägterer alloreaktiver Proliferation von Splenozyten bei Restimulation mit donorspezifischen Antigenen.

Um Effekte des Parenchyms der transplantierten Organe von denen der mitübertragenen Passagierleukozyten zu unterscheiden, wurden chimäre Spendertiere generiert durch Transplantation von Knochenmark junger syngener Tiere in alte und junge zukünftige Spendertiere sechs Wochen vor Organentnahme. Nach Transplantation dieser chimären Spenderherzen (junges Parenchym mit jungen Passagierleukozyten oder altes Parenchym mit jungen Passagierleukozyten) waren Transplantatüberleben, Abstoßungsindizes und systemische Empfängerantworten vergleichbar, was auf eine entscheidende Rolle von organständigen Passagierleukozyten bei der Vermittlung von verstärkten Immunreaktionen nach Transplantation alter Spenderorgane hindeutete.

Mit Fokus auf dendritische Zellen als eine Untergruppe der Passagierleukozyten wurden nun organständige dendritische Zellen durch Vorbehandlung der Spendertiere mit liposomalen Clodronat aus den Spenderherzen depletiert. Unterschiede in Transplantatüberleben und Abstoßungsindizes waren aufgehoben nach Transplantation von Spenderherzen vorbehandelter Tiere, ebenso waren die systemischen Immunantworten

unabhängig vom Alter der Spendertiere. Weiterhin konnte eine detailliertere Charakterisierung der allostimulatorischen Priming-Fähigkeiten dendritischer Zellen *in-vitro* zeigen, dass Stimulation mit alten dendritischen Zellen zu erhöhten Häufigkeiten von IFN-γ-produzierenden Zellen sowie deutlicheren alloreaktiven Proliferationen unter allogenen Splenozyten führt und alte dendritische Zellen eine erhöhte Expression von MHC-II und kostimulatorischen Molekülen aufweisen. Dendritsche Zellen als zelluläres Substrat der beschleunigten und verstärkten Transplantatabstoßung alter Spenderorgane könnten somit ein Ziel möglicher Strategien der Spendervorbehandlung darstellen.

Die vorliegende Arbeit ist unter Betreuung durch

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das Ergebnis experimenteller Arbeiten am

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2 ABBREVIATIONS

ADCC, antibody-dependent cell-mediated cytotoxicity
AEC, 3-amino-9-ethyl-carbazole
AP-1, activator protein 1
APC, antigen presenting cell
BAFF, B cell activating factor
Bcl-2, B cell lymphoma 2
BCR, B cell receptor
BLyS, B lymphocyte stimulator
CCR, c-c motif receptor
CD, cluster of differentiation
CLP, common lymphoid progenitor
CMV, cytomegalovirus
ConA, Concanavalin A
DAB, Diaminobenzidine
DAMP, damage-associated molecular patterns
DC, dendritic cell
DGF, delayed graft function
DNA, Deoxyribonucleic acid
DTR, diphtheria toxin receptor
ECD, extended criteria donors
ELISpot, enzyme-linked immunosorbent spot
ESP, Eurotransplant Senior Program
ESRD, end-stage renal disease
FACS, fluorescence-activated cell sorting
FasL, Fas ligand
Fc, fragment crystallizable
FCS, fetal calf serum
FDC, follicular dendritic cell
fMLP, N-Formylmethionine leucyl-phenylalanine
FMO, fluorescence minus one
FoxP3, forkhead box P3
GC, germinal center
GH, growth hormone
GM-CSF, granulocyte-monocyte colony-stimulating factor
GMP, granulocyte-macrophage progenitor
GnRH, gonadotropin-releasing hormone
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA, human leukocyte antigen
HPF, high power field
HRP, horse radish peroxidase
HSC, hematopoietic stem cell
ICAM-1, intercellular adhesion molecule 1
IFN, interferon
Ig, immunoglobulin
IL, interleukin
iNOS, inducible nitric oxide synthase
IRI, ischemia reperfusion injury
ISHLT, International Society for Heart and Lung Transplantation
KIR, killer-cell immmunoglobulin-like receptor
LFA-1, lymphocyte-function associated antigen 1
LPS, lipopolysaccharide
MACS, magnetic-activated cell-sorting
MAPK, mitogen-activated protein kinase
mDCs, myeloid dendritic cells
MDDC, monocyte-derived dendritic cell

MHC, major histocompatibility complex
MLR, mixed lymphocyte reaction
mRNA, messenger ribonucleic acid
MST, median survival time
NF-AT, nuclear factor of activated T cells
NK cell, natural killer cell
NKT cell, natural killer T cell
OCT, optimal cutting temperature
PAMP, pathogen-associated molecular pattern
PBS, phosphate-buffered saline
pDCs, plasmacytoid dendritic cell
PDGF, platelet-derived growth factor
PGE_2, Prostaglandin E_2
PMA, Phorbol 12-myristate 13-acetate
PRR, pathogen recognition receptor
RAG, recombination activating gene
ROS, reactive oxygen species
RPMI, Roswell Park Memorial Institute
SEM, standard error of the mean
sjTREC, signal joint T cell receptor excision circle
STAT5, signal transducer and activator of transcription 5
TCR, T cell receptor
Th cell, T helper cell
TLR, toll-like receptor
TNF, tumor necrosis factor
UW, University of Wisconsin
VCAM-1, vascular cell adhesion molecule
VEGF, vascular endothelial growth factor

3 INTRODUCTION AND REVIEW OF THE LITERATURE

The number of patients suffering from end-stage organ disease is continually increasing, with the elderly being the fastest growing segment in this population: for end-stage renal disease (ESRD), in 1980 only 7.6 percent of new patients with ESRD were aged 75 or older; in 2004, however, this population accounted for more than one in four patients beginning kidney replacement therapy [1]. Since organ transplantation is the validated treatment of choice for suitable candidates in this population [2], waiting list demographics are reflecting these changes and almost half of the currently listed patients for renal transplantation are over 50 years of age [3]. In an attempt to meet this rapidly increasing demand, organs from so-called expanded criteria donors (ECD) – including organs from old donors – have been used more frequently. In fact, more than half of all currently transplanted kidneys are from donors older than 50 years [3]. An ongoing increase in age and number of old recipients and organ donors should be expected as changes in demographics will continue to impact age distribution.

3.1 BASIC CONCEPTS OF TRANSPLANT IMMUNOLOGY

3.1.1 Allorecognition

Direct and indirect allorecognition are the two main mechanisms of mounting immune responses against allogeneic tissue. In *direct* allorecognition, recipient T cells recognize determinants on intact donor MHC molecules displayed on the surface of transplanted passenger antigen-presenting cells (APC) [4]. In the high determinant density model it has been proposed that T cells directly recognize residues on allogeneic MHC molecules themselves [5], while in the multiple binary complex model the recognition of peptide bound by allogeneic MHC molecules is of primary importance [6].

Indirect allorecognition on the other hand involves processing of alloantigens and presentation as peptides by self-MHC molecules on the surface of recipient APCs, similar to conventional self-restricted responses [7]. Recipient APCs can obtain alloantigens through various mechanisms: donor DCs can be endocytosed after migration to secondary lymphoid tissue [8], soluble alloantigens shed from the graft can drain to secondary lymphoid organs [9] and, finally, recipient APCs can enter the graft and endocytose alloantigens and subsequently migrate to secondary lymphoid organs [10].

The relative contribution of the different pathways is still a matter of ongoing debate. The direct response is most potent in the early period and should diminish with the destruction of donor APCs over time. The indirect pathway requires antigen capture, processing and presentation and should be less rapid than the direct pathway, but could theoretically continue throughout the life of the graft. In line with this, direct alloreactivity was not detected in renal allograft recipients with chronic allograft dysfunction several years after transplantation [11]. Priming through the direct pathway on the other hand is likely to result in a population of residual donor-reactive memory T cells [12].

More recently, a third mechanism (termed *semi-direct*) linking direct and indirect allorecognition through a single APC has been described [13]. In this pathway, recipient dendritic cells (DC) acquire intact peptide-presenting donor MHC molecules from cells of the graft, either through cell-to-cell contact [14] or release and uptake of small exosomes [15].

It has been established that natural killer (NK) cells express killer cell immunoglobulin-like receptors (KIR) displaying an extensive diversity with more than 20 different haplotypes and at least 40-50 genotypes [16]. They are a group of inhibitory receptors that regulate NK cell function through ligation with self MHC class I molecules and possibly mediate allorecognition through mismatch with MHC class I molecules [17] on cells of the donor organ ("missing self-recognition"), albeit the exact contribution of NK cell allorecognition to allograft rejection remains to be elucidated [18].

3.1.2 Alloimmune response

3.1.2.1 T cells

The T cell compartment plays a major orchestrating role in several non-exclusive pathways leading to allograft rejection. After priming through the direct pathway, T cells exert direct cytotoxicity including bystander killing upon reencountering their alloantigen on intragraft parenchymal cells, donor vascular endothelial cells or donor APCs [19]. The same holds true when indirectly primed T cells recognize their specific ligands on graft-infiltrating recipient APCs [20] or on recipient-derived vascular endothelial cells feeding the graft that present donor-derived peptides on recipient MHC molecules [21].

Directly and indirectly primed $CD4^+$ T cells are able to differentiate into proinflammatory

type-1 cytokine secreting phenotypes that mediate delayed-type hypersensitivity responses. These cells produce cytokines such as IFN-γ and tumor necrosis factor (TNF) upon triggering by antigen, which activates macrophages, granulocytes and NK cells. This causes a further amplification of cytokine and chemokine production, along with other effector molecules such as proteolytic enzymes and nitric oxide that perpetuate the local inflammation in an antigen-independent fashion [22]. $CD4^+$ T-helper 1 (Th1) cells also provide help for induction of $CD8^+$ cytotoxic T cells [23].

Crosstalk between B cells and $CD4^+$ T cells through the CD40-CD40L pathway and production of cytokines such as IL-4, IL-5, IL-10 and IL-13 by Th2-type T cells provides B cells with help for alloantibody production and class switching [24]. IL-4 and IL-5 also seem to promote eosinophil-mediated rejection [25].

3.1.2.2 B cells

The significance of antibody-mediated rejection is illustrated in hyperacute rejection, when an entire organ can be destroyed by preformed HLA antibodies within minutes [26]. Antibodies detected with staining for the product of complement activation C4d have been associated with acute and chronic rejection as well as graft survival [27–29], and antibody-mediated rejection has been shown to be the major cause of late renal allograft failure [30].

After crosstalk between B cells and T cells and subsequent alloantibody production and class switching, complement activation is initiated by interaction of the globular domains of C1q with IgG or IgM bound to antigen epitopes on the graft [31]. Antibodies also lyse target cells through the low-affinity Fc receptor CD16 on NK cells and macrophages. Ligation of alloantibodies on endothelial cells in the absence of complement or inflammatory cells can also cause a cycle of injury and repair or further detrimental changes in cell signaling and gene expression that eventually lead to intimal thickening and allograft vasculopathy characteristic of chronic rejection [32,33]. Besides HLA-antibodies, alloantibodies against epithelia, endothelial lines and monocytes were found in patients rejecting allografts [34].

3.1.2.3 Innate immune system

Based on the observation that for complete allograft rejection T cells are both needed and sufficient [35], transplant immunology has traditionally focused on the involvement of the adaptive immune system in allograft rejection. In fact, most immunosuppressive drugs used in

transplant recipients are aimed at T cells [36]. To control cellular immune responses, however, innate immune cells express a variety of pattern recognition receptors (PRR) that recognize conserved pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMPs) [37]. They also express additional receptors for complement, antibodies as well as receptors that can sense “self” and “missing self” [38]. Although in general they do not mediate direct graft injury in most cases, this wide array allows them to critically determine transplant outcome by influencing the initiation, duration and the overall character of the alloresponse [39].

The surgical procedure with tissue trauma, graft ischemia, vascular dysfunctions, graft conservation and eventual reperfusion results in inevitable graft damage collectively termed ischemia reperfusion injury (IRI). In response to inflammatory cytokines and chemokines as well as complement products, IRI mobilizes intragraft interstitial DCs and causes a rapid and massive cellular infiltration of recipient monocytes/macrophages, neutrophils, NK cells and DCs besides T and B cells [40]. Stimulation of PRRs by DAMPs on damaged graft cells then leads to production of additional inflammatory cytokines and chemokines as well as induction of cytotoxic responses. APCs mature in response to these signals to induce adaptive responses [41] and graft cells expressing markers of cellular stress become the target of NK-mediated killing [42].

Innate immune cells are also significant contributors to graft damage caused by acute rejection responses once they acquire effector functions. In chronic rejection, innate immune cells, alloantibodies and complement depositions dominate the infiltrates in lesions; the effector responses initiated by alloantibodies involve primarily innate pathways and innate immune cells [31]. Especially NK cells might be of, so far, underestimated importance for chronic allograft vasculopathy [43].

3.1.2.3.1 Dendritic cells

Depletion of interstitial kidney DCs in CD11c-DTR reporter mice with diphtheria toxin reduced tubular cell necrosis and renal dysfunction after IRI [44]. Mice lacking specific TLRs or intracellular proteins required for subsequent signaling showed significantly reduced tissue damage after IRI [45] and prolonged allograft survival [46], further illustrating the importance of innate receptors in APC maturation and alloimmune responses. The pivotal role DCs play in allorecoginition mechanisms has been discussed above.

3.1.2.3.2 Monocytes/Macrophages

Upon inflammatory triggering, circulating monocytes rapidly traffick to the site of inflammation and mature to macrophages [47]. Both cell types consist of multiple functionally different subsets that express different characteristic phenotypic markers [48]. At inflammatory sites and in allograft rejection, macrophages phagocytose foreign entities and present antigenic peptides to T cells and produce, and respond to, numerous inflammatory cytokines [49]. They present the predominant cell type in delayed-type hypersensitivity responses [40] and mediate antibody-dependent cell-mediated cytotoxicity through their Fc receptors.

Depletion of macrophages or inhibiting their trafficking led to less important tissue damage after IRI [44]. The importance of macrophage effector functions for graft injury in allograft rejection was highlighted in similar studies [50,51]. Renal transplant recipients that experience acute rejection episodes despite T cell depletion with alemtuzumab show graft infiltrations predominantly with monocytes [52]. In models of chronic allograft rejection, macrophages contributed to allograft vasculopathy [53] and were found to accelerate chronic allograft injury [54]. A recent report suggested distinct capabilities of macrophages to recognize and reject adoptively transferred allogeneic cells [55].

3.1.2.3.3 Natural killer (NK) cells

Recognition of target cells by NK cells leads to rapid release of granules packed with primarily perforin and granzymes, causing target cell apoptosis [56]. Non-secretory mechanisms for lysis of target cells involve the binding of tumor necrosis factor (TNF) and receptor-like molecules such as FAS/CD95 with their respective ligands (FasL) [57]. NK cells are also able to impact the nature of immune responses by producing large amounts of pro- and anti-inflammatory cytokines. Besides promoting Th1-differentiation through production of IFN-γ [58], they can also interact with T cells through contact-dependent costimulatory signals [59]. Recently, a number of reports about reciprocal influences between NK cells and DCs have been published [60].

A variety of activating and inhibitory cell surface receptors control development, maturation and effector functions of NK cells [61]. Besides “missing self-recognition” as a mechanism of allorecognition mentioned earlier, a process called “induced self-recognition” is also of importance in the setting of allograft rejection, especially IRI: damaged and stressed cells

often express ligands for NK-activating receptors that override the inhibitory signals by self MHC class I molecules and lead to NK cell induced cell death [62].

In CD28$^{-/-}$ mice, blockade of NKG2D – an activating receptor – prolonged cardiac allograft survival, suggesting that NK cells have at least a facilitating role in rejection [63]. In one study, NK cells activated by IL-15 were even capable of mediating acute rejection in the absence of adaptive immune cells [64]. A recent study identified NK cells as effector cells in a complement-independent pathway of antibody-mediated transplant vasculopathy [43]. A role for NK cells in chronic graft loss is also suggested by the clinical observation that KIR-mismatched kidney transplants are associated with worse graft survival [65].

3.1.2.3.4 Neutrophil granulocytes

In the setting of acute humoral rejection, chemotactic response to complement factors and ligation of intragraft antigen-antibody complexes to Fc receptors are important effector pathways involving neutrophils [66]. Experimental evidence that neutrophils play a role in acute rejection was provided by the observation that prevention of neutrophil activation led to less potent acute allograft rejections in rats [67]. A recent study identified neutrophils as an important link between innate and adaptive immunity in IRI and acute rejection: neutrophils stimulated donor DCs in a contact-dependent fashion to augment production of IL-12 and expand alloantigen-specific T cells, thus exacerbating IRI and acute rejection [68].

3.1.2.3.5 Mast cells and basophil granulocytes

Mast cells have long been characterized as effector cells in immunoglobulin E (IgE)-associated responses such as allergy or immunity to parasites. More recent reports have demonstrated a role for mast cells in shaping the adaptive immune response [69] and through studies on tolerance induction there is indirect evidence that mast cells might be of importance in the rejection process: in a model of tolerance induction through costimulatory blockade, tolerance could not be established in mast cell-deficient mice but was achieved after reconstitution with mast cells [70]. This study also reported on a functional link of Treg cells with mast cells through the production of the mast cell growth factor IL-9. Others have reported on a loss of Treg mediated skin graft tolerance through mast cell degranulation [71]. Recently, a role for basophils in shaping the adaptive immune response has been described: they are able to enhance humoral immune responses through cytokine secretion [72] or in a CD40L-dependent manner [73] and can drive the differentiation of CD4^{+} Th2 T cells [74].

Implications of these findings for the field of transplant immunology are so far not understood.

3.1.2.3.6 Eosinophil granulocytes

A number of studies suggest an important role for eosinophils in models with rejection mediated by a predominant Th2-type response. Th2-type cells produce a number of cytokines, among them IL-4, IL-5 and IL-13. These cytokines have been shown to recruit eosinophils to the graft and upon activation of these eosinophils, graft damage can be caused through release of leukotrienes, superoxides, major basic protein, eosinophilic cationic protein and eosinophil peroxidase [25].

In models of Th2-dominant allograft rejection, histological examination revealed intense infiltration by eosinophils [25]. When neutralizing antibodies to IL-4 were given, vasculopathy and eosinophil infiltration were abrogated in a model of chronic skin graft rejection. In addition, there are some reports on important eosinophil infiltrates in allograft rejection in humans [75,76] and more recently, eosinophil-driven acute rejection has been described in T cell-depleted patients after intestinal transplantation [77].

3.1.2.3.7 Complement

The complement system consists of numerous serum proteins that are primarily produced in the liver. The classical pathway of complement activation is triggered by antigen-antibody complexes, for example on graft cells, and ends – like the alternative and the lectin pathway – with the formation of membrane-attacking complexes that mediate lysis of targets cells by disruption of the cell membrane [78]. It also elicits signals for the proliferation of endothelial cells, such as platelet-derived growth factor (PDGF) [79].

All three pathways also lead to the generation of complement fragments C3a and C5a that function as strong chemoattractants and opsonins for innate immune cells. Activation of the C3a or C5a receptor at the surface of endothelial cells causes cytoskeletal changes and cytokine release [80] and exposure of endothelial cells to sub-lytic concentrations of C5b-C9 increases the expression of adhesion molecules and pro-inflammatory cytokines [81].

The classical pathway is a primary effector mechanism in antibody-mediated vascular injury [31]. Besides the effector mechanisms mentioned above, the capability to function as

costimulation in T cell activation and to induce maturation of DCs have recently been described: both cells can produce complement components [82,83] and express receptors for C3a and C5a [82,84].

In hyperacute rejection, large quantities of preformed antibodies bind antigens on endothelial cells and activate the classical pathway, which leads to the generation of procoagulant, chemoattractive and adhesive interfaces and rapid graft loss through widespread intravascular thrombosis, hemorrhage and tissue injury [85]. Complement also provides an effector pathway for the prominent role already mentioned above that antibody-mediated alloresponses have in chronic rejection.

Many studies have shown the importance of various complement factors like C1 esterase, C3 and C5 in different animal models of IRI [86]. When transplanted with C3-deficient kidneys, transplant recipients showed prolonged graft survival [87] and transplantation of such grafts prevented IRI in another murine model [88]. Both studies also illustrate the importance of locally produced complement over circulating complement.

3.2 IMMUNOSENESCENCE AND ALLOIMMUNE RESPONSES

Increasing age has been associated with a variety of alterations in the immune system, often summarized as immunosenescence. Changes can affect all components in all compartments of the immune system and represent not necessarily a uniform deterioration of functions but rather individual shifts in function, regulation and balance of a complex system, depending on numerous intrinsic and extrinsic factors. Most of these aspects are of great significance in the setting of solid organ transplantation, as age-dependent altered alloimmune responses impact overall transplant outcome as well as patient and graft survival.

It should be emphasized that in many instances, the reports in the literature on immunosenescence are inconsistent and even contradictory. This may be attributed to the varying sources of cells and the methods used. In addition, comparing results from different human studies in this field is difficult because of comorbidities, medication and varying criteria for the selection of subjects, notably the inconsistent use of the admission criteria specified in the SENIEUR protocol which attempts to exclude unhealthy individuals [89].

Most detailed data is often published in the field of kidney transplantation due to the relatively high numbers of patients and the wide age spectrum.

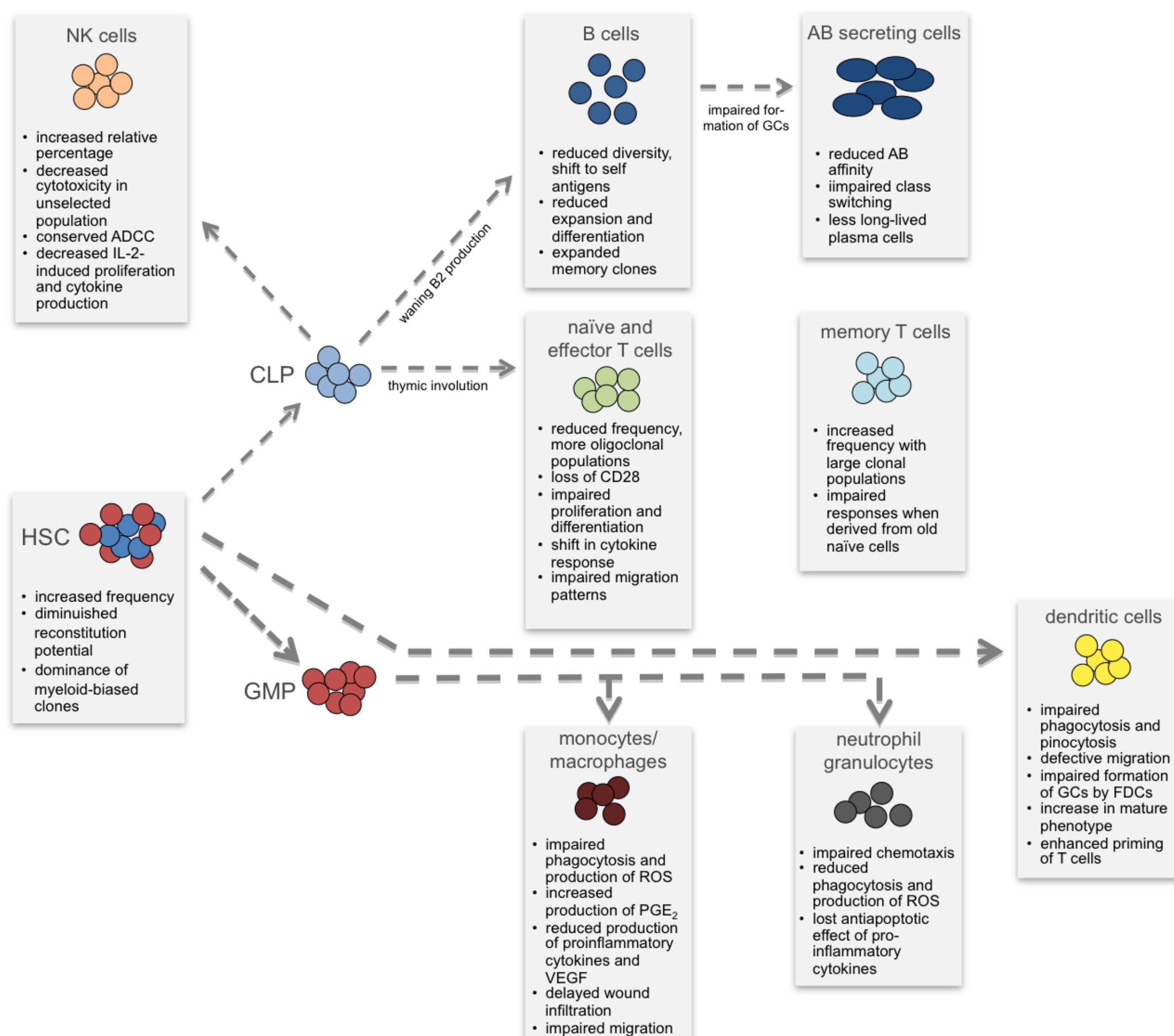

Figure 1: Immunosenescence impacts all cellular compartments of the immune system in a multifaceted and complex way. HSC=hematopoietic stem cell, CLP=common lymphoid progenitor, ADCC=antibody-dependent cell-mediated cytotoxicity, GC= germinal center, AB=antibody, FDC=follicular dendritic cell, GMP=granulocyte-macrophage progenitor, ROS=reactive oxygen species, PGE2=Prostaglandin E2, VEGF=vascular endothelial growth factor. Dendritic cells comprise several distinct subtypes deriving from different precursors. Although most DCs are thought to be of myeloid origin, some subtypes share early steps of development with B and T cells.

3.2.1 Hematopoietic stem cells

The cellular components of the immune system are mostly short-lived and do therefore require continuous replenishment. Hematopoietic stem cells (HSC) comprise only 0.01% of the bone marrow population and have the potential at any given cell division to give rise to all blood cell types of the myeloid and lymphoid lineages or to self-renew to generate more

HSCs. They are long-lived and usually not depleted during a lifetime. Despite their extensive proliferative and regenerative capacity, a growing body of evidence suggests that these cells show signs of aging with important functional implications for the whole immune system [90,91].

3.2.1.1 Reconstitution potential

Purified HSCs from old mice show a measurable and successive functional decline in their overall reconstitution potential [92]. Significant differences in the importance of these changes between different strains of common laboratory mice have been reported, but might be due to different timescales for the functional decline [93]. Similar results have been obtained when comparing human HSC cells from fetal liver tissue, umbilical cord blood and adult bone marrow [94].

Since HSCs synthesize telomerase to maintain telomeric length [95], telomeres do not seem to be a plausible explanation for HSC senescence. Given that the HSC compartment predominantly consists of cells in a state of proliferative and metabolic quiescence, exposure to oxidative stress as a natural by-product of cellular metabolism is less likely to be of primary importance, although this presents an important and probably universal mechanism that leads to a variety of intracellular damages [96]. Age-dependent accumulation of damage to genomic and mitochondrial DNA, to which oxidative stress contributes, may in turn be the most important factor driving the functional decline of HSCs [97,98]. This implies very strict requirements of the DNA repair machinery or a reduced ability of HSCs to repair even small DNA damages, with important changes in cell cycle regulation. The expression of the cell-cycle inhibitor p16INK4a appears to increase with age in HSCs [99]. Stromal cells are exposed to similar environmental damage and stress that might alter the support provided by the stromal niches that directly affect the regulation of HSCs [100].

3.2.1.2 Quantitative changes

The functional decline is concomitant with an elevated frequency and an increased expansion potential of old murine HSCs [101,102], raising the possibility of a feedback mechanism compensating for the per-cell loss in function. Despite the prevailing view that HSC frequency diminishes in parallel to a reduced cellularity in the bone marrow of the elderly [103], more recent results suggest an increase in the frequency of human HSCs with aging [91,104].

3.2.1.3 Lineage potentials

Murine HSCs also show changes in lineage potential with aging, resulting in attenuated lymphoid lineage output and preserved or even increased myeloid lineage output [105]. A more robust self-renewal potential [106] and a differential response to the aged cytokine milieu [107] have been proposed for the progressive predominance of myeloid-biased HSC clones. These changes are accompanied by down-regulation of genes mediating lymphoid specification and function and up-regulation of genes involved in myeloid specification and function as well as of proto-oncogenes involved in the development of myeloid leukemia [108].

Interestingly, pediatric leukemias tend to involve lymphoid lineages, whereas leukemias in the adult population – besides an overall elevated incidence in malignant transformations of the hematopoietic system – tend to involve myeloid leukemias [109,110]. How aging impacts the clonal composition of the human HSC compartment remains nevertheless to be addressed.

3.2.2 T cell compartment

3.2.2.1 T cell generation

After entering the thymic microenvironment, thymic progenitors originating from the bone marrow traffic through stromal cells, macrophages and dendritic cells while undergoing a number of proliferative and differentiation events that lead to the emigration of mature and functional T cells into the peripheral T cell pool.

Reduction of thymic mass in humans starts at the age of one year after the task of populating the peripheral immune system is essentially completed and continues gradually throughout later life, with another important reduction in thymic mass at the time of puberty [111]. This atrophy, or involution, has been shown to correlate with an enlargement of the perivascular space (which does not contain any developing thymocytes) possibly due to the loss of thymocytes, thymic stroma and thymic epithelial space (where all thymopoiesis takes place) [112]. Adipose tissue eventually fills up the enlarged perivascular space [113].

Despite significant atrophy, the aged thymus retains the capacity to produce naïve T cells. More reliable measurements of changes in thymic output by means of the signal joint T cell receptor excision circle (sjTREC) assay have recently revealed that T cell output declines

as a function of thymopoietic tissue, but the production of naïve T cells per gram of thymopoietic tissue remains constant [114]. sjTRECs are DNA fragments that are generated during the V(D)J rearrangement of the T cell receptor (TCR) genes and are present in T cells that have not yet undergone cell division.

Several hypotheses – that also reveal potential therapeutic strategies – have been proposed to explain thymic involution, among them a decline in supply of bone marrow progenitors (as mentioned above), alterations in the productive rearrangement of the TCR, loss of cells in the thymic microenvironment and alterations in the levels of several circulating (GH, GnRH) or intrathymic (IL-7, neurotrophins, thymic hormones) hormones, cytokines or growth factors [115]. The infiltrating adipose tissue may also be a contributing factor, either through direct interaction or the release of soluble factors [116].

3.2.2.2 T cell receptor repertoire

The loss in thymic output with age does not result in a significant change in the total number of peripheral T cells [117], as this number seems to be regulated via a peripheral mechanism of thymus-independent homeostatic proliferation of mature T cells following low-affinity interactions with self-peptide/MHC complexes [118,119]. The decreased number of naïve T cells and this peripheral expansion result in a significantly limited TCR repertoire – in one study, young adults had a repertoire diversity of 20 million different TCR-β chains, whereas those participants older than 70 years had only 200,000 [120].

3.2.2.3 $CD28^-$ T cells

By age 80 and above, 10-15% of peripheral blood CD4 T^+ cells and 50-60% of CD8 T^+ cells lack expression of CD28, while at birth, virtually all human T cells express CD28 [121]. Interestingly, these changes are not observed in mice [122]. As these cells are frequently oligoclonally expanded, their T cell receptors display reduced diversity [123]. Their increased frequency in the aged might cause the overall limitation of the TCR repertoire mentioned above [124] and let these cells occupy important immunological niches by consuming stimulatory signals such as cytokines or surface ligands.

$CD28^-$ T cells show an altered expression of further costimulatory receptors [125] and a gain in cytolytic functions [126]. They also acquire expression of NK cell receptors such as KIRs [127], which fundamentally influences signal recognition by these cells, as ligands for these receptors are not limited to APCs.

Loss of CD28 expression in T cells with age has been attributed to repeated antigenic stimulation, a process that can also be observed *in-vitro* [128]. $CD28^-$ T cells have shorter telomeres than their $CD28^+$ counterparts within the same clonal population and might thus already have reached their limit of proliferative potential [129]. In addition, presence of type I interferons during TCR activation increases the proportion of $CD28^-$ T cells in culture [130], pointing to the relevance of a proinflammatory environment.

There is evidence suggesting that generation of $CD28^-$ T cells might be driven or accelerated by chronic viral stimulation, most commonly linked to herpesviridae like CMV [131]. Chronic viral stimulation represents a repeated antigenic stimulation and creates a proinflammatory environment.

Although even with increased age an efficient CMV immunosurveillance can be maintained, in immunocompromised patients such as transplant recipients this balance can be disturbed, accelerating the generation of greater clonality in the $CD8^+$ T cell repertoire and contributing to the higher level of chronic subclinical inflammation [132]. Transfer of these expanded T cells from aged mice into young mice led to reduced resistance to viral challenge [133] and cytomegalovirus infection has interfered with tolerance induction in rats [134].

3.2.2.4 Memory T cells

While naïve T cell numbers decrease with aging, the proportion of memory T cells increases with age, possibly due to cumulative exposure to pathogens and environmental antigens [135]. Selected changes in lymphocyte turnover have been reported, notably a reduced turnover of memory $CD8^+$ T cells in mice. Human and murine $CD8^+$ memory T cells were also found to have a much longer half-life than other T cell subsets with age, and large clonal populations were found among this specific subset [136,137]. Furthermore, memory cells derived from aged naïve cells proliferate less well, produce reduced levels of cytokines and provide less cognate helper function [138].

3.2.2.5 Proliferative response

T cells enter a state of reduced proliferative capacity when telomere lengths are reduced to a critical length after a certain number of cell divisions known as the ‘Hayflick’ limit has been reached [139,140]. The loss of CD28 expression described above has been associated with loss of proliferative capacity of T cells during repeated cycles of replication, a process termed replicative senescence [141]. $CD28^-$ T cells show irreversible cell-cycle arrest, apoptosis

resistance and reduced proliferative responses [142]. Previous studies had already established a reduced proliferative response of aged T cells to antigenic and mitotic stimuli [143,144].

3.2.2.6 Signaling

T cell proliferation is induced by IL-2, but with increasing age, T cells lose their capacity to produce and respond to IL-2: naïve T cells from aged mice produce less IL-2 upon stimulation with antigens presented by APCs [145]. Impaired activation of transcription factors AP-1 and NF-AT have been closely associated with this decreased expression also in humans [146].

Both signals required for T cell activation can be affected by aging. Aged murine $CD4^+$ T cells establish less efficient TCR synapses with APCs [147], and reduced expression of several activation and differentiation markers like CD40L/CD154, CD25, CD28 and CD62L have been reported [148,149].

Further changes in signaling cascades of aged T cells include impairments in calcium metabolism, tyrosine kinase phosphorylation and protein kinase C translocation [150] as well as alterations in cell membrane lipid rafts [151].

3.2.2.7 Cytokine responses

A number of studies suggest an imbalance between Th1 and Th2 responses in aging. Some published data support the notion that human aging is associated with a decreased Th1/Th2 ratio [152], while one recent study reported an increased Th1/Th2 ratio [153]. Studies in mice have generally provided evidence for enhanced Th1 immune responses [154].

The overall frequency of type 1 and type 2 T helper cells seems to increase with age [155,156]. The increased frequency of memory T cells might be of importance for this finding, since memory T cells typically have less strict requirements for stimulation and produce a broader set of cytokines. High levels of lymphocyte function-associated antigen 1 (LFA-1) in $CD28^-$ T cells also lower the activation threshold of these cells [157].

The role of Th17 immune responses in aging is still unclear, although some groups have reported a shift in cytokine expression toward IL-17 in coronary arteries [158] or stressed the importance of augmented IL-17 alloimmune responses in aged animals [159].

3.2.2.8 Migration

Recent observations indicate that expression of selected proinflammatory chemokines and chemokine receptors is increased in aged human and murine T cells, possibly influencing T

cell migration patterns. Changes in the expression of CCR7 and CD62L, for example, may explain the observed defective T cell homing to secondary lymphoid organs.[160]

3.2.2.9 Regulatory T cells

A number of disorders such as autoimmune diseases, chronic inflammation and cancer have been linked with quantitative and qualitative defects of Tregs and since most of these disorders have higher incidences in the aged population, age-related changes in this subset have been of increasing interest [161] and might also be relevant in the context of reduced recipient alloresponsiveness with age.

Thymic output of Tregs would presumably decline, but Tregs can also be generated through a peripheral mechanism [162]. Although most studies report no correlation between numbers of Tregs and aging, few selected studies have shown a positive correlation [163]. These differences might in part be due to different phenotypic definitions such as the use of high expression of FoxP3 in addition to CD25 as molecular markers.

Functional activity of Tregs has been reported to decline with age, perhaps due to the decrease in IL-2 expression and related signaling described above [164]. However, it has recently been shown in a model of murine skin transplantation that Treg functions remained unaltered with age [165]. Gregg et al. have reported similar findings in humans.[166]

3.2.2.10 Apoptosis and T cell survival

Aging affects major signaling pathways of apoptosis in T cells [167]. Studies in humans have shown that naïve and memory T cells have an increased CD95/Fas expression [168] and an decreased expression of Bcl-2 with age, both correlating with enhanced apoptosis [169]. The functional relevance of these findings is still being discussed [170]. Successive shortening of telomeric DNA, as described for T cells with advancing age, is an additional independent factor for increased apoptosis [171].

3.2.3 B cell compartment

3.2.3.1 B cell generation

In line with age-related changes in HSCs mentioned earlier, the production of B cells wanes with increasing age [172]. Early B cell progenitors are reduced with age [173] and expression of essential transcriptional regulators including E2A gene products such as E47 is reduced

[174]. In line with this, the expression of RAG enzymes, crucial for the passage through pro- and pre-B cell stages, is diminished in old individuals [175]. Reduced expression of downstream products of E2A has also been shown to be present in peripheral B cells from old mice [176]. Finally, *in-vivo* labeling has revealed that production rates in pro-, pre- and immature bone marrow B cell pools diminishes with age [177]. One homeostatic mechanism to maintain the number of peripheral B cells despite decreased output seems to be reduction in turnover of mature B cells [178].

As B2 production wanes with age, the proportional contribution of B1 cells might increase [179]. B1 cells are a self-renewing pool that predominates in peritoneal and pleural cavities whose repertoire is characterized by polyspecificity and low-affinity self-reactivity [180].

3.2.3.2 B cell receptor diversity and specificity

Studies have shown a significant loss in diversity of the B cell receptor (BCR) in the peripheral blood with age that was correlated with poor health and survival [181]. Apart from reduced output of naïve B cells and intrinsic repertoire differences generated from aged HSCs, some truncation of the repertoire might reflect expanded clones of memory B cells [182]. In keeping with this, reduced levels of panel reactive antibodies [183] have been found in transplant candidates with increasing age.

This might also lead to the shift observed in antibody specificity to autologous antigens. In most inbred mouse strains, spontaneous appearance of autoreactive antibodies is associated with age. Another proposed explanation for this is less selective stringency when interclonal competition is less intense: the B lymphocyte stimulator (BLyS or BAFF) is a limiting survival resource for which newly formed and mature B cells compete [184], regulating crucial survival pathways via BLyS receptor 3 [185]. Expanded clones of memory B cells might also lead to increased autoantibody titers since some of these were initially expanded and selected by cross-reactive antigens or self-antigens.

3.2.3.3 B cell responses

Germinal center (GC) formation and kinetics in T cell dependent responses of B cells are impaired in aged mice [186]. Correspondingly, B cell expansion, antibody affinity maturation, memory B cell differentiation and long-lived plasma cells in the bone marrow are reduced [187,188].

Since successful interaction between T and B cells is required for GC formation, the reduced capacity for T cell help might be reflected by reduced CD40L/CD154 expression and the altered cytokine environment [148]. B cells, in turn, express less CD40 with increasing age [182]. Furthermore, follicular dendritic cells have been found to be less effective at antigen trapping and dispersal, correlating with fewer and smaller GCs [189]. Intrinsic class switching defects secondary to decreased induction of E47 and activation-induced cytidine deaminase have also been described, further reducing the generation of high affinity antibodies [190].

3.2.4 Innate immune system

3.2.4.1 Dendritic cells

Several studies have reported on unchanged numbers and phenotypes of DCs with age [191–193], whereas others found that the numbers of myeloid DCs (mDCs) in human blood progressively decline with age while there is no change in plasmacytoid DCs (pDC) [194]. The same group observed a higher frequency of mature phenotypes in aged DCs with increased expression of costimulatory molecules CD86 and CD83.

Since various numerical and phenotypic changes with age have been described for skin [195], mucosal immune system [196], thymus [197] and brain [198], changes during aging may probably vary with the subsets of DCs and tissue of their residence.

To present antigens to T cells, DCs must first internalize these antigens by phagocytosis, receptor-mediated endocytosis or pinocytosis [199]. Monocyte-derived DCs (MDDCs) from aged humans showed impaired capacities of phagocytosis and pinocytosis [200]. The same study also found an impaired capacity to phagocytose apoptotic cells. This could lead to an accumulation of necrotic cells which in turn favors activation of DCs and secretion of pro-inflammatory cytokines [201].

There is conflicting evidence on age-dependent changes in antigen sensing and activation of DCs. Several studies have reported comparable levels of TLR-induced activation and cytokine secretion by human MDDCs [192,193], while others found decreased [191] cytokine secretion upon TLR-dependent stimulation.

Impaired migration of DCs from aged mice to draining lymph nodes was observed *in-vivo* in a TCR transgenic mouse model [202] and similar results were obtained with human DCs [203]. Besides intrinsic defects of DCs, the aged microenvironment should be of importance in this setting as well.

There is no consistent data on the capacity of aged DCs to prime and activate T cells. Early studies demonstrated decreased T cell priming capacity of DCs in lymph nodes of aged mice [204]. Comparable stimulatory capacities of MDDCs from young and aged subjects have been reported more recently [205]. However, one should keep in mind that MDDCs represent a particular subset of DCs whose equivalence with naturally occurring human DC subsets is controversial [206]. Implications of age-related changes in DCs for the B cell compartment through impaired formation of GC by FDCs have been mentioned above.

3.2.4.2 Monocytes/Macrophages

While in mice there appears to be an increase in the macrophage population of the bone marrow [207], a significant decrease in macrophage precursors and macrophages was found in the bone marrow of old humans [103]. Both aging human and rodent macrophages seem to have reduced levels of MHC class II expression [208], which may contribute to poorer T cell responses [209].

Several reports using murine models indicate a decline in phagocytosis, opsonization and tumor cell killing by peritoneal macrophages from aged mice [210,211]. Macrophages from aged rats also demonstrated a decrease in the ability to produce superoxide anion upon incubation with IFN-γ or opsonized zymosan [212]. A different group reported an abrogation in the mitogen-activated protein kinase (MAPK) pathway as a functional explanation for this finding [213].

There have been reports about decreased [214] as well as increased [215] inducible nitric oxide synthase (iNOS) mRNA in aged murine macrophages. Recently, different age-specific nitrite-production patterns based on the dose of IFN-γ used for stimulation were demonstrated [216].

The production of prostaglandin E_2 (PGE_2) by macrophages was found to be increased in aged mice [217]. PGE_2 is able to alter DC function in mice by reducing the secretion of IL-12, increasing production of IL-10 and decreasing expression of MHC class II molecules [218].

Furthermore, it is able to decrease IL-2 production and subsequent proliferative responses in T cells [219] and murine splenocytes cocultivated with PGE_2 also up-regulate Th2 cytokines [220]. Both findings present a possible link to the aforementioned age-related changes in the T cell compartment.

Some attention has been paid to the question whether macrophages are the source of the elevated levels of proinflammatory cytokines found in the aged [221]. In fact, several recent reports suggested a decrease in the production of proinflammatory cytokines by human and murine macrophages, associated with alterations in TLR-dependent signaling [222,223]. Instead of an intrinsic aging process, chronic inflammatory disease and poor nutrition may be responsible for this process [224,225].

In wound healing, macrophages keep the wound bed free from infection and promote angiogenesis. Studies on excisional wound healing in humans demonstrated a delay in monocyte and macrophage infiltration with age, associated with a decreased expression of adhesion molecules VCAM-1 and ICAM-1 [226]. Peritoneal macrophages from aged mice also produced less VEGF upon stimulation [227].

3.2.4.3 Natural killer cells

When strictly following the SENIEUR protocol, NK cells of aged individuals had conserved [228] or even enhanced [229] cytotoxicity. In other studies and in animals, age-specific decreases in NK cell cytotoxicity were reported [230,231]. Impaired turnover of inositol triphosphate has been correlated in one study with this decline [232]. Interestingly, antibody-dependent cell-mediated cytotoxicity does seem to be preserved with aging [233] and changes in intracellular signaling were not observed in this pathway [232].

An age-related increase in the relative percentage of human NK cells has been found in several studies [234] and may represent a compensatory mechanism [235]. These changes were also associated with a phenotypic and functional shift, as an increase in the more mature, highly cytotoxic $CD56^{dim}$ population was found [236].

Human and murine NK cells also showed a decreased proliferative response following IL-2 stimulation, associated with a decrease in Ca^{2+} mobilization [236]. IL2-induced production of IFN-γ and other chemokines was decreased in NK cells from aged subjects [236,237], possibly compromising adaptive immune responses driven by NK cells.

3.2.4.4 Natural killer T (NKT) cells

NKT cells are a unique and heterogeneous T cell population characterized by coexpression of TCRs and NK cell-associated receptors [238]. Classical human NKT cells express semi-invariant CD1d-restricted αβ TCRs, show a strong response to α-galctosyl ceramide and rapidly secrete mainly IL-4 and IFN-γ upon activation, thus influencing adaptive immune responses [238,239]. The same lytic mechanisms as those reported for NK cells have been observed in NKT cells, though the FAS/FasL mechanism seems to be the preferred system [240]. Besides these classical or invariant NKT cells, there is a subset of mainly $CD8\alpha^+$ non-classical NKT cells that are not CD1c-restricted and express NK cell-associated receptors such as CD16, CD56, CD57, CD161, CD94 and NKG2A [239].

A critical role for NKT cells in transplant tolerance has been proposed by studies with corneal [241], islet [242] and cardiac [243] allografts. Another study highlighted the importance of chemokines involved in NKT trafficking in this context [244]. Recently, human NKT cells were reported to be able to establish a cross-talk involving KIRs leading to maturation of allogeneic APCs and increased reactivity of classical NKT cells [245].

A decreased percentage of classical NKT cells was found in peripheral blood of old donors that seems to be associated with impaired proliferation, cytotoxicity and cytokine production [246–248]. Impaired IFN-γ production by NKT cells may in turn lead to inefficient NK cell cytotoxicity [249]. Human classical NKT cells also showed a shift toward a Th2 cytokine profile *in-vitro* [250]. Non-classical NKT cells expressing CD56 and being $CD28^-$ seem to be an increasing population among $CD3^+$ cells in the elderly [251]. Cross-linking of CD56 without TCR ligation led to production of proinflammatory cytokines in these cells, presenting a possible mechanism for TCR-independent T cell activation in aged individuals [252].

3.2.4.5 Neutrophil granulocytes

Circulating neutrophil numbers in the blood seem to be unaltered with age [253,254] and migration of neutrophils to the site of inflammation seems to be unimpaired in aged individuals [255,256]. Studies *in-vitro* have confirmed an unimpaired adhesion of neutrophils to vascular endothelial cells [257]; chemotaxis, however, was found to be impaired [258,259].

There seems to be an age-dependent loss of microbiocidal capacity of neutrophils [260]. Impaired phagocytosis of opsonized bacteria or yeast by neutrophils in the elderly has

been observed [261,262] and Fc receptor-mediated production of reactive oxygen species (ROS) was found to be significantly decreased in aged individuals [263,264], although this seems not to apply in response to fMLP [265], a chemoattractant produced by some bacteria. Mechanistically, decreased intracellular Ca^{2+} levels in stimulated neutrophils [266] and diminished actin polymerization [267] have been associated with this.

In the absence of stimulation, no differences in apoptotic cell death have been reported in human aged neutrophils [268]. Proinflammatory signals like IL-2, LPS or GM-CSF, on the other hand, were found to have decreased antiapoptotic effects on neutrophils from aged individuals, associated with alterations in the STAT5 signaling pathway [269] and compromised lipid raft function [270,271].

3.2.4.6 Mast cells and basophil granulocytes

Early studies reported an impaired degranulation of basophils [272], whereas more recently a higher reactivity was observed [273].

3.2.4.7 Eosinophil granulocytes

Eosinophils of old human subjects showed decreased degranulation in one study [274]. The same study also noted a trend toward decreased production of superoxide anions. Although in a different study airway eosinophilia was increased in response to antigen challenge in old mice, airway hyper-responsiveness was reduced, suggesting reduced eosinophil effector function [275]. Partly in contrast, others reported on a failure to accumulate eosinophils in allergic inflammation of the airway [276].

3.2.4.8 Complement

At present, limited data exists on age-related changes in complement functionality, although the majority of the literature report a preserved function in aged humans [277]. Serum levels of components of the classical pathway were increased [278] or unchanged [279] between young and old subjects. Similar results were found for components of the alternative pathway [279,280]. One study found decreased alternative pathway-induced hemolysis [281], whereas Bellavia et al. [279], using the SENIEUR protocol, found no functional changes in either the classical or the alternative pathway to induce hemolysis.

3.3 THE CLINICAL SIGNIFICANCE OF AGE AND IMMUNOSENESCENCE IN TRANSPLANTATION

3.3.1 Recipient age and transplant outcome

Old individuals listed for transplantation with end-stage renal disease that are eventually undergoing transplantation show better long-term survival than matched patients staying on organ replacement therapy: projected life expectancy almost doubled from 6 to 10 years in the group of 60-74 year olds in a longitudinal mortality comparison by Wolfe et al [2]. This is in spite of the fact that older recipients are more likely to receive kidneys from less well matched older donors with risk factors like obesity, diabetes and hypertension [282]. Older recipients, on the other hand, present a highly selected patient population [2,283].

Old recipients are more likely to die with a functioning graft, but graft survival censored for death with a functioning graft was found to be higher in old recipients [284,285]. Almost half of all graft loss in old recipients was a result of death, compared to 15% in young recipients [286]. Over half of the causes of death were attributable to cardiovascular disease, infection or malignancy – complications that are all exacerbated by immunosuppressive therapy [287].

A reduced incidence of acute rejection episodes in old recipients has been confirmed in numerous clinical trials in corneal, kidney, cardiac, liver and lung transplantation [282,288–293]. Renal transplantation offers a wider age spectrum for more detailed analysis showing that only one quarter of graft failures in old recipients resulted from rejection, whereas about 50% of graft failures in recipients under 45 years of age were due to rejection [183].

If old recipients experience an acute rejection episode, however, this rejection is more likely to significantly shorten patient and graft survival [294]. This points to the initial quality of the graft – since old recipients are more likely to receive organs from old donors [282] – and the impact of donor age on transplant outcome. These aspects will be discussed in the context of the aged immune system of the donor, together with the finding that reduced incidence of acute rejection in old recipients may not apply when old recipients receive grafts from old donors. In addition, recipient age seems to be an independent risk factor for chronic allograft failure [284]. Part of this increased risk might be explained by an increased susceptibility to calcineurin inhibitor-related nephrotoxicity.

3.3.2 The aged immune system of the donor

A large retrospective study demonstrated that transplantation of kidneys from old donors is associated with an increased risk of transplant failure: the projected graft half-life was reduced to 5 years if the donor was older than 60 years, compared to 10.2 years with young donors [295]. An adverse effect of donor age was not observed in living donor transplants, which show lower incidences of acute rejection and chronic allograft nephropathy as well as better graft-survival rates [296,297]. This may be attributed to strict selection of donors in terms of renal function and glomerular filtration rate, much shorter ischemic times and avoided systemic consequences of brain death or even cardiac death, all of which might be of importance in determining the impact of immunological changes on donor organs with increasing age.

Part of the reduced projected graft half-life may be due to intrinsic functional deterioration of aged kidneys. In fact, autopsy studies showed a decrease in kidney weight, number of glomeruli and mean glomerular volume with increasing age [298]. Longitudinal studies have shown a diminished renal reserve with aging, accompanied by functional deficits in the ability to respond to challenges of excess or deficits [299], potentially leading to unspecific injury and cellular distress when exposed to the challenges of the transplantation procedure. It is unclear whether this is due to aging itself or an accumulation of injuries by undetected or minimal renal disease.

Poorer graft survival of old kidneys has also been attributed to increased susceptibility to IRI and delayed graft function (DGF), thus leading to a higher risk of acute rejection and graft failure. A retrospective analysis indeed demonstrated an increased need for postoperative dialysis after transplantation of old kidneys [295] and another study identified donor age as an independent risk factor for DGF [294]. DGF in turn did not influence graft survival, but was shown to increase the risk for acute rejection episodes [300].

There are a number of studies that demonstrate increased susceptibility for IRI with increasing age. Early reports focused cardiac allografts [301] and significant attention has been paid to the concept that an increased release of reactive oxygen species from the mitochondria of old hearts is responsible for the observed age-related differences [302]. Other studies showed an age-related increase of IR injury for liver [303], kidney [304,305] as well

as skeletal muscle [306,307] models. Similar early observations have been made clinically [308,309].

Organs from older donors may already have undergone age-related injury and an increased IR injury may lead to an even higher level of damage at the time of transplantation. Tissue injury induces a stereotyped injury response by the immune system that promotes immune recognition and can lead to new immunologic injury [310]. This immunologic injury can then initiate a vicious circle of repeated injury and injury response [311], leading to higher immunogenicity of older grafts [312]. There is experimental evidence that suggests that IRI facilitates immune recognition through increased expression of MHC molecules [313] and subsequent injury may be mediated by a local release of inflammatory cytokines that was found after IRI [314]. The transplantation procedure also leads to increased migration of DCs into the graft irrespective of alloreactivity [315].

An increased immunogenicity of old donor organs independently from IR injury might also be a cell-intrinsic phenomenon mediated by DCs residing in the graft. Enhanced antigen-presenting capacities of DCs were reported previously [316–318], possibly a compensatory mechanism for functional impairments of aged T cells.

An age-related defect of DCs might also be linked to this phenomenon. DCs isolated from aged individuals are impaired in their capacity to phagocytose apoptotic cells [200]. Apoptotic cells may accumulate and become necrotic, lyse and release auto-antigens. In contrast to apoptotic cells, necrotic cells in turn induce maturation of DCs with enhanced antigen presentation and increased secretion of proinflammatory cytokines [201].

The concept of inflamm-aging presents a more integrated idea of how aging may impact donor organ immunogenicity. Aging is associated with reduced competence and integrity of the epithelial barriers of skin, gut and lung [319]. This increasing influence of extrinsic factors with chronic subclinical infections, accumulating antigenic burden and exposure to damaging factors poses a growing challenge to the innate immune system, which – together with the aforementioned deficiencies in adaptive immunity and relatively less lymphoid biased HSCs – gains in importance for preserving immunologic protection [320]. This shift may lead to the reported elevated levels of proinflammatory cytokines in the elderly [321] and may also increase the overall proinflammatory state of procured organs.

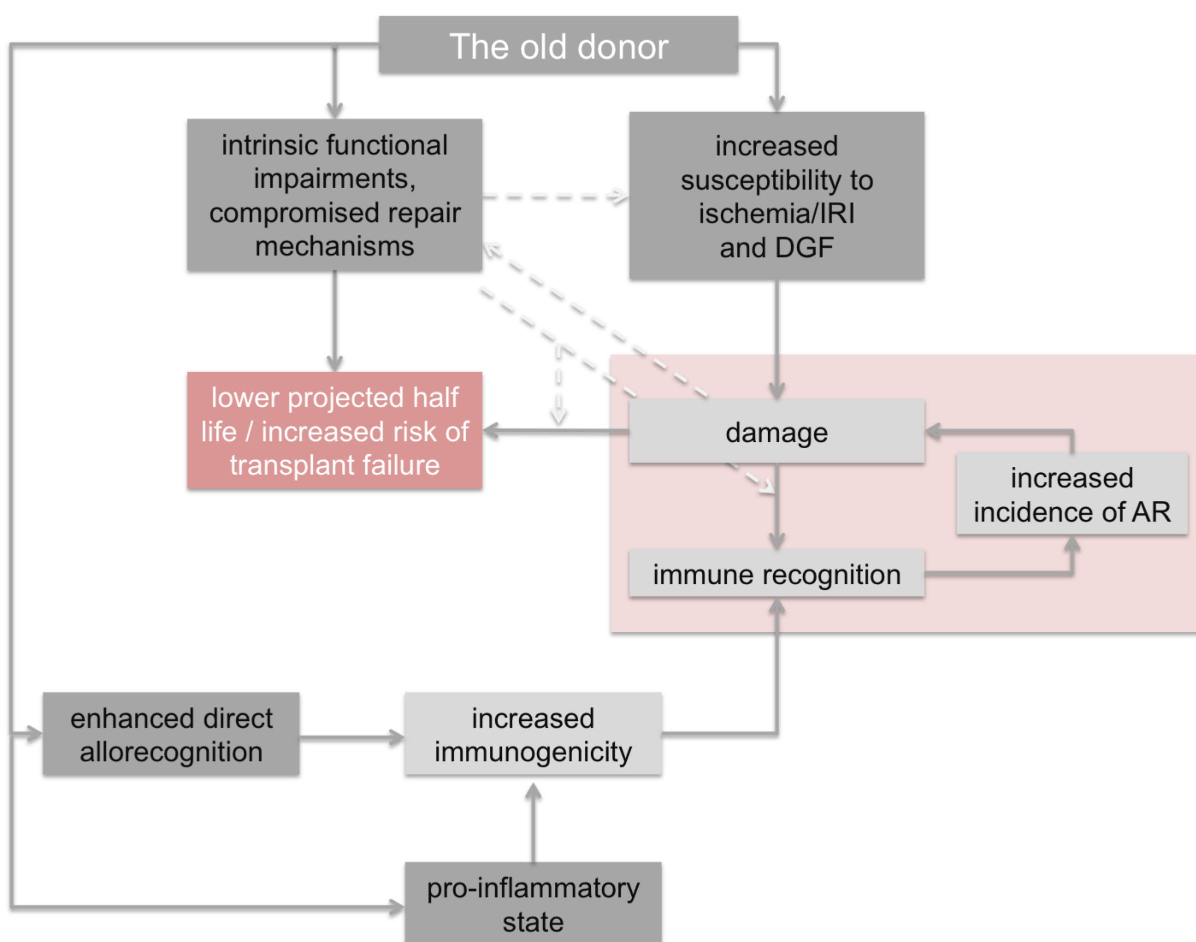

Figure 2: Intrinsic functional impairments, susceptibility to IRI and DGF, enhanced direct allorecognition, and a more pro-inflammatory state of organs from old donors contribute to a cycle of damage, modified immune recognition and compromised repair that ultimately translates into increased risk of transplant failure and inferior transplant outcome. AR, acute rejection.

All these elements discussed above may lead to the finding that a reduced incidence of acute rejection episodes in old recipients may not apply when old recipients receive grafts from old donors. In fact, an increased incidence of acute rejection episodes after transplantation of old kidneys was noted by several investigators, both in young and old recipients [294,322,323]. Evaluation of the Eurotransplant Senior Program reported higher rejection rates, although allocation was done regardless of HLA matching [324]. In a recent large retrospective analysis, increased donor age was associated with higher frequency of acute rejection episodes, while this effect was blunted by increased recipient age [282,293].

When old organs are subject to an acute rejection episode, they are also less capable of mounting a repair response to this injury. In one study, an increased graft loss was observed for kidneys from old donors if these kidneys had experienced acute rejection episodes or DGF [325]. A simple explanation may be that old kidneys have fewer functioning nephrons and

that summation of damage results in an earlier depletion of renal function. It has also been proposed that as a result of multiple injuries and repair, parenchymal cells of old donor organs reach a state of premature senescence earlier than parenchymal cells of younger organs would [326].

3.4 Aims and objectives, experimental outline

As outlined and reviewed in the preceding sections, age and immunosenescence impact the quality and immunogenicity of donor organs as well as the alloimmune response of the recipient in a multifaceted and clinically relevant way. With current demographic changes in mind, these insights gain increasing significance for the field of transplantation.

As a relatively recent approach to the growing gap between numbers of patients waitlisted for transplantation and scarcity of donor organs, the Eurotransplant Senior Program (ESP) was established as an allocation scheme for kidney transplantation that allocates organs from donors >65 years of age regardless of HLA matching to non-immunized local recipients ≥65 years of age undergoing their first transplantation [327]. As reported in a follow-up after 5 years, waiting time for patients listed in the ESP program decreased significantly and allocation to local recipients led to reduced cold ischemic time and lower incidence of DGF [328]. Patient and graft survival were comparable to the standard allocation scheme, although an increased rate of acute rejection episodes was noted [328].

Thus, while clinical evidence seems to be compelling and recent approaches towards using the aged donor pool are promising, the mechanistical aspects underlying graft quality and immunogenicity in relation to aging are still poorly understood. Dendritic cells and other passenger leukocytes seem to be subject to significant age-related modifications in phenotype and function, while mechanisms of injury and repair, premature parenchymal senescence and functional physiological reserve of donor organs seem by no means to be excluded from age-dependent alterations. This work aims to elucidate this complex interplay by means of *in-vitro* and *in-vivo* experiments:

I. Is an experimental animal model of transplantation able to reproduce the clinical findings of more frequent and more severe acute rejection episodes when transplanting old donor organs? What are the characteristics of alloimmune responses elicited in this setting?

For this aim, cardiac allografts procured from young or old mice were transplanted into young fully MHC-mismatched mice in a murine model of heterotopic, fully vascularized heart transplantation. Graft survival was monitored and subsequent alloresponses were characterized locally in the allograft by means of immunohistochemical staining and independent pathohistological grading. Systemically, T cell subsets of the recipient were characterized at day 7 after transplantation (flow cytometry) and cytokine (ELISpot) as well as proliferative response (^{3}H thymidine MLR) of recipient lymphocytes upon reencounter of donor-type antigen were analyzed.

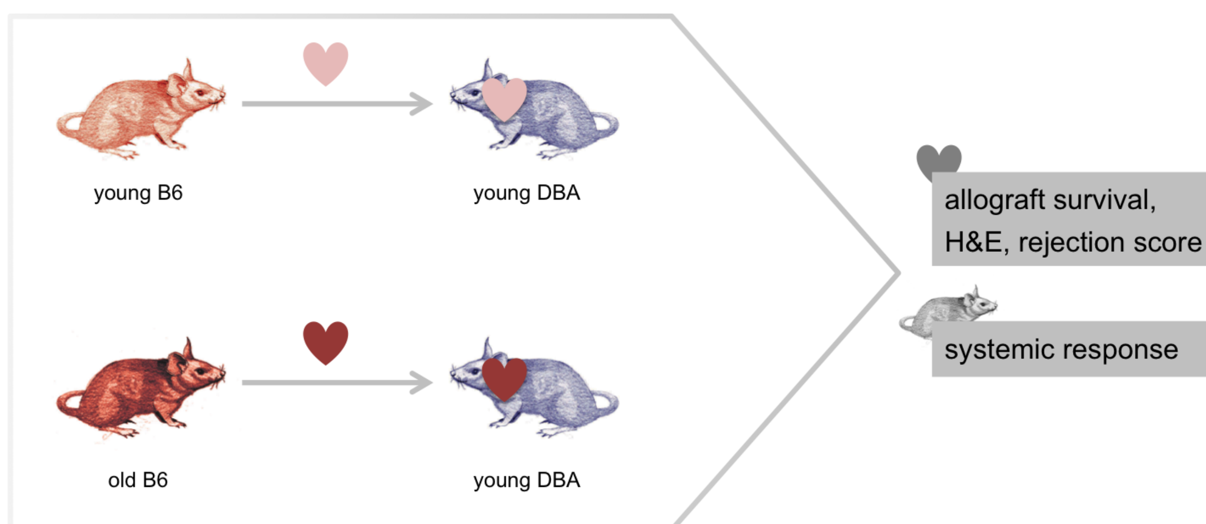

Figure 3: Young or old mismatched cardiac allografts were transplanted into young recipients to monitor graft survival and to characterize alloresponses elicited in this setting. Conventional and immunohistochemical staining was performed on allografts for pathohistological grading. Splenic T cell subsets among recipient splenocytes at day 7 post transplantation as well as cytokine and proliferative responses of recipient splenocytes were characterized.

II. If any differences are detectable between the transplantation of young and old donor organs, can these differences be ascribed to changes related to parenchymal tissue or to passenger leukocytes impacted by immunosenescence?

For this aim, old and young chimeric mice with hearts populated by young passenger leukocytes were generated by total body irradiation and subsequent syngeneic bone marrow transplantation with bone marrow procured from syngeneic young animals. These animals were used as cardiac allografts donors for fully MHC-mismatched young recipient mice. Subsequent alloimmune responses were then monitored as outlined above.

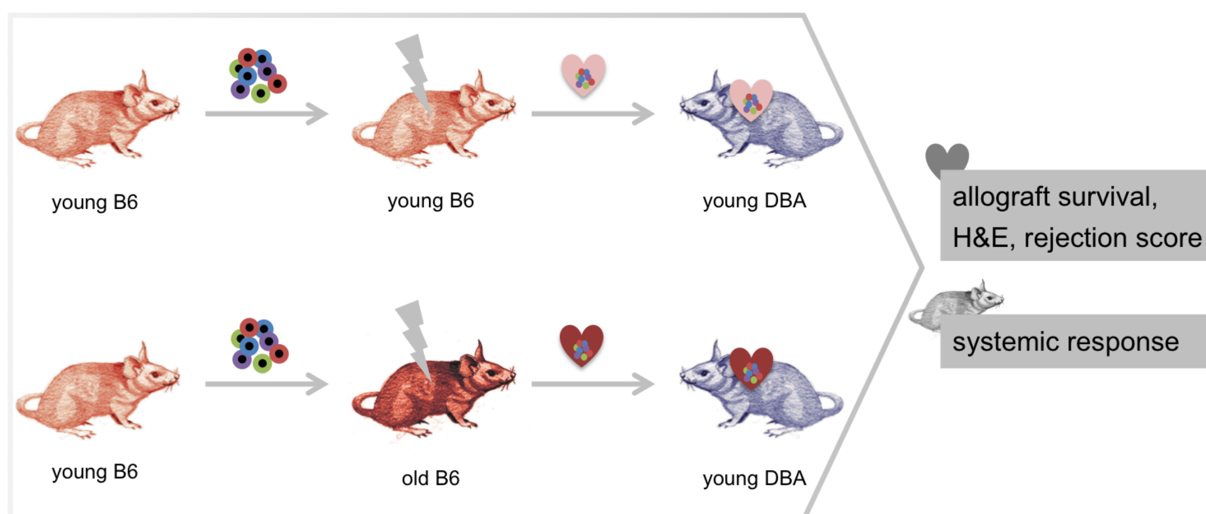

Figure 4: Chimeric donor animals with young or old hearts populated by young passenger leukocytes were generated by total body irradiation and subsequent syngeneic bone marrow transplantation prior to graft procurement. Survival and alloimmune responses were monitored and characterized using pathohistological grading, T cell subsets among recipient splenocytes as well as cytokine and proliferative response of recipient splenocytes.

In a second experimental design, young and old donor animals were pretreated with liposomal clodronate to deplete dendritic cells residing in the cardiac tissue prior to graft procurement. Hearts from pretreated donors were then transplanted into fully MHC-mismatched young recipients and alloimmune responses were monitored as in the previous approaches.

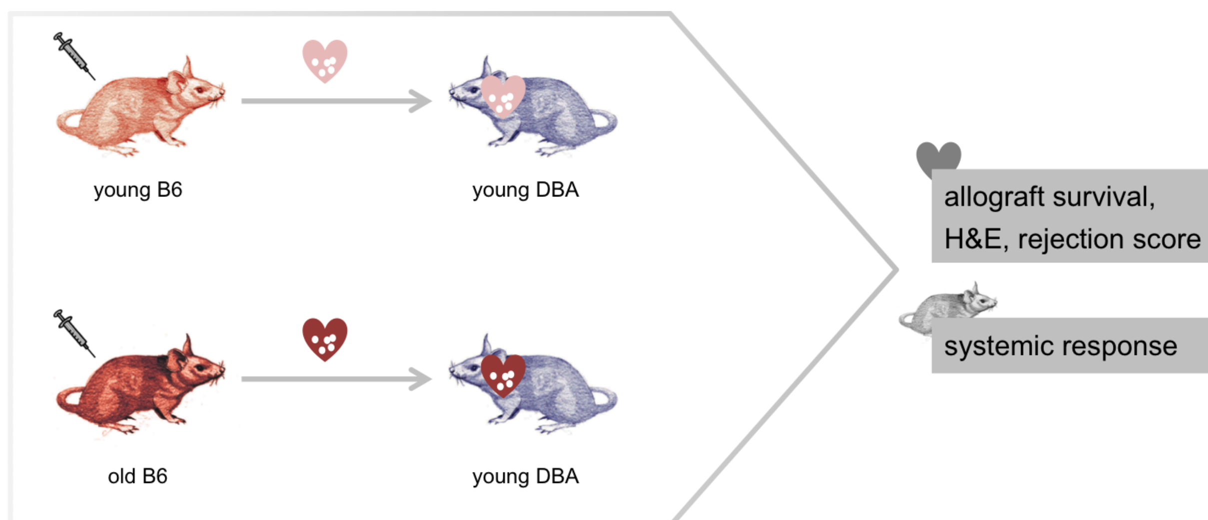

Figure 5: To generate young and old donor animals with hearts depleted of dendritic cells, mice were treated with liposomal clodronate prior to transplantation into young mismatched recipients. Subsequent alloimmune responses were then characterized as in the two previous experimental approaches.

4 MATERIAL AND METHODS

4.1 MATERIAL

4.1.1 Reagents

DNAse	Sigma-Aldrich (St. Louis, MO, USA)
Gentamicin	Sigma-Aldrich (St. Louis, MO, USA)
Fetal calf serum (FCS)	Sigma-Aldrich (St. Louis, MO, USA)
Penicillin/Streptomycin	Sigma-Aldrich (St. Louis, MO, USA)
L-glutamine	Sigma-Aldrich (St. Louis, MO, USA)
Lipopolysaccharide (LPS)	Sigma-Aldrich (St. Louis, MO, USA)
Formalin	Boston BioProducts (Ashland, MA, USA)
Paraffin	Sigma-Aldrich (St. Louis, MO, USA)
Hematoxylin/eosin	Sigma-Aldrich (St. Louis, MO, USA)
Concanavalin A (ConA)	Sigma-Aldrich (St. Louis, MO, USA)
Tween-20	Boston BioProducts (Ashland, MA, USA)
Acetone	Boston BioProducts (Ashland, MA, USA)
Ionomycin	Sigma-Aldrich (St. Louis, MO, USA)
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich (St. Louis, MO, USA)
Golgi-Stop	BD Biosciences (San Diego, CA, USA)
Collagenase D	Sigma-Aldrich (St. Louis, MO, USA)
Percoll	GE LifeSciences (Pittsburgh, PA, USA)

4.1.2 Buffers and solutions

Saline (NaCl) solution	Boston BioProducts (Ashland, MA, USA)
University of Wisconsin solution	PSI (Elkhorn, WI, USA)
Phosphate buffered saline (PBS)	Boston BioProducts (Ashland, MA, USA)
Citrate buffer	Boston BioProducts (Ashland, MA, USA)
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer	Boston BioProducts (Ashland, MA, USA)
RPMI (Roswell Park Memorial Institute) 1640 medium	Corning Cellgro (Manassas, VA, USA)
Tissue-Tek optimal cutting temperature	Thermo Fisher Scientific (Waltham, MA,

(OCT) compound	USA)
Dako diluent	Dako USA (Carpinteria, CA, USA)
Tris-Cl	Boston BioProducts (Ashland, MA, USA)
Diaminobenzidine (DAB)	Dako USA (Carpinteria, CA, USA)
70% Ethanol	Boston BioProducts (Ashland, MA, USA)
Trypan blue	Boston BioProducts (Ashland, MA, USA)
Lysis buffer	Sigma-Aldrich (St. Louis, MO, USA)
flow cytometry buffer	1000 ml PBS, supplemented with 0.02% sodium azide (Boston BioProducts) and 2% rat serum (Sigma-Aldrich)

4.1.3 Cell culture media

Bone marrow (BM) medium	500 ml RPMI 1640 medium, supplemented with 5 ml HEPES buffer, 5 mg DNAse, and 2 mg Gentamicin
Cell culture medium	RPMI 1640 medium supplemented with 10% FCS, 1% Penicillin/Streptomycin and 1% L-glutamine

4.1.4 Anesthetics, analgesics, anticoagulants and other pharmaceuticals

Ketamin	Phoenix Ketaject (St. Joseph, MO, USA)
Xylazin	Lloyd AnaSed (Shenandoah, IA, USA)
Buprenorphin	Bedford Laboratories (Bedford, OH, USA)
Isoflurane	Abbott Laboratories (North Chicago, IL, USA)
Heparin	Sigma-Aldrich (St. Louis, MO, USA)
Liposomal clodronate (dichloromethylene diphosphonate)	Encapsula NanoSciences (Nashville, TN, USA)

4.1.5 Experimental kits

Mouse IFN-γ ELISpot kit	BD Biosciences (San Diego, CA, USA)
Mouse IL-6 ELISpot kit	BD Biosciences (San Diego, CA, USA)
Peroxidase Block kit	Dako USA (Carpinteria, CA, USA)
Rabbit Envision+ kit	Dako USA (Carpinteria, CA, USA)
FoxP3 staining kit	eBioscience (San Diego, CA, USA)
Intracellular staining kit	BD Biosciences (San Diego, CA, USA)

4.1.6 Supplies

Syringes and needles	BD Medical (Franklin Lakes, NJ, USA)
Petri dishes	BD Falcon (Franklin Lakes, NJ, USA)
Centrifuge tubes	BD Falcon (Franklin Lakes, NJ, USA)
Pasteur pipettes	Corning Falcon (Manassas, VA, USA)
Flow cytometry tubes	BD Falcon (Franklin Lakes, NJ, USA)
Small flow cytometry tubes	BD Falcon (Franklin Lakes, NJ, USA)
Pipette tips	Thermo Fisher Scientific (Waltham, MA, USA)
LS columns	Miltenyi Biotec (Bergisch Gladbach, Germany)
Scalpel blades	Thermo Fisher Scientific (Waltham, MA, USA)

4.1.7 Equipment

Operating microscope	Nikon Instruments SMZ800 (Melville, NY, USA)
Vessel cuffs	RiverTech Medical (Chattanooga, TN, USA)
Surgical instruments	Owens & Minor (Richmond, VA, USA) and KLS Martin (Jacksonville, FL, USA)
Isoflurane inhaler	Harvard Apparatus Labvent 783 (Holliston, MA, USA)
Sutures und ligatures	Covidien (Mansfield, MA, USA)

Light microscope	Olympus America (Center Valley, PA, USA)
Automated immunostainer	NexES, Ventana (Tucson, AZ, USA)
Autoclave	Thermo Fisher Scientific (Waltham, MA, USA)
Incubators	Thermo Fisher Scientific (Waltham, MA, USA)
ELISpot plate analyzer	Immunospot, Cellular Technology (Shaker Heights, OH, USA)
Liquid scintillation counter	Perkin Elmer (Waltham, MA, USA)
Cell harvester	Perkin Elmer (Waltham, MA, USA)
Cell and mouse irradiator	^{137}CS self-contained blood irradiator
Pipettes	Eppendorf (Hamburg, Germany)
Flow cytometer	FACSCalibur, BD Biosciences and FlowJo software 9.3.2 for Mac (Tree Star, Ashland, OR)
Flow sorter	FACSAria II (BD Biosciences) and Diva software (BD Biosciences)
MidiMACS separator	Miltenyi Biotec (Bergisch Gladbach, Germany)

4.1.8 Antibodies

4.1.8.1 Immunohistochemistry

CD4	Clone 1F6; Novocastra Laboratories (Newcastle, United Kingdom); Cat# NCL-CD4-1F6
CD8	Clone C8/144B; Dako USA (Carpinteria, CA, USA), Cat# M7103
CD11c	Clone HL3, BD Biosciences (San Diego, CA, USA), Cat# 550283

4.1.8.2 Surface staining

CD4-FITC	Clone GK1.5, BD Biosciences (San Diego,

	CA, USA), Cat# 553729
CD4-PE	Clone GK1.5, BD Biosciences (San Diego, CA, USA), Cat# 553730
CD4-PerCP	Clone RM4-5, BD Biosciences (San Diego, CA, USA), Cat# 553052
CD4-APC	Clone RM4-5, BD Biosciences (San Diego, CA, USA), Cat# 553051
CD8α-FITC	Clone 53-6.7, BD Biosciences (San Diego, CA, USA), Cat# 553031
CD25-PE	Clone PC61, BD Biosciences (San Diego, CA, USA), Cat# 553866
CD44-PE	Clone IM7, BD Biosciences (San Diego, CA, USA), Cat# 553134
CD62L-APC	Clone MEL-14, BD Biosciences (San Diego, CA, USA), Cat# 553152
I-A^b-PE	Clone AF6-120.1, BD Biosciences (San Diego, CA, USA), Cat# 553552
CD80-PE	Clone 16-10A1, BD Biosciences (San Diego, CA, USA), Cat# 553769
CD86-FITC	Clone GL1, BD Biosciences (San Diego, CA, USA), Cat# 553691
CD40-FITC	Clone 3/23, BD Biosciences (San Diego, CA, USA), Cat# 553790
CD11c-APC	Clone HL3, BD Biosciences (San Diego, CA, USA), Cat# 550261

4.1.8.3 Intracellular staining

FoxP3-APC	Clone FJK-16s, eBioscience (San Diego, CA, USA), Cat# 17-5773
IFN-γ-PE	Clone XMG1.2, BD Biosciences (San Diego, CA, USA), Cat# 554412

4.1.8.4 Magnetic cell sorting

B220-PE	Clone RA3-6B2, BD Biosciences (San Diego, CA, USA), Cat# 553090
anti-PE microbeads	Miltenyi Biotec (Bergisch Gladbach, Germany)

4.1.8.5 Flow sorting

CD11c-APC	Clone HL3, BD Biosciences (San Diego, CA, USA), Cat# 550261

4.1.9 Animals

C57BL/6 (B6), $H2^b$, ♂, 8-12 weeks	Charles River Laboratories (Wilmington, MA, USA)
DBA/2, $H2^d$, ♂, 8-12 weeks	Charles River Laboratories (Wilmington, MA, USA)
C57BL/6 (B6), $H2^b$, ♂, 18 months	National Institute of Aging (Bethesda, MD, USA)

All mice were housed in accordance with institutional and National Institutes of Health guidelines. All procedures were approved by the local Animal Welfare Committee.

4.2 METHODS

4.2.1 Murine heterotopic heart transplantation model

All hearts were transplanted using a non-suture cuff technique. This technique was first described for rabbits and rats by Heron et al. in 1971 [329], and was later adapted for mice by Matsuura et al. [330]. More recently, Gu et al. proposed improvements for some aspects of the procedure [331].

4.2.1.1 Preparation and equipment

Procedures were performed in a semi-sterile fashion, using sterile gloves and face masks. Surgical field and instruments were disinfected at the beginning of the procedure. Heating

pads were placed under the surgical field. An operating microscope was used for all procedures.

All cuffs used for arterial and venous anastomoses were made of autoclavable nylon tubing. For the carotid artery, cuffs with an inside diameter of 0.5 mm and an outside diameter of 0.63 mm were used. Cuffs for the external jugular vein had an inside diameter of 0.75 mm and outside diameter of 0.94 mm. The total length of all cuffs was 2 mm, including a handle of 1 mm for the microvascular clamp.

4.2.1.2 Anesthesia

All mice were anesthetized with an i.p. injection of Ketamin/Xylazin (95/22 mg per kg body weight). Postoperative pain management was done using Buprenorphine. Isoflurane was applied in case of intraoperative insufficiency of anesthesia.

4.2.1.3 Graft procurement

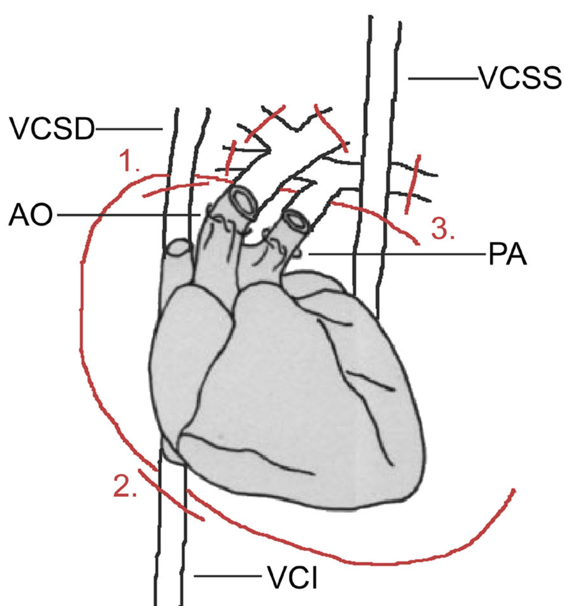

Figure 6: Anatomical hallmarks of the donor procedure [331]**.** AO, aorta; PA, pulmonary artery; VCSS, vena cava superior sinistra; VCSD, vena cava superior dextra; VCI, vena cava inferior. See 4.2.1.3 for a detailed explanation of the graft procurement procedure.

Following anesthesia, shaving of the abdominal wall and fixation of all four limbs, median laparotomy was performed. The small bowel was pulled sidewards for proper access to the inferior caval vein and 0.4 ml of a 1:4 heparin-saline solution was injected. The inferior caval vein was then cut for exsanguination. After opening of the thoracic cage through the diaphragm and two bilateral incisions, the whole anterior thoracic wall was flipped cranially. *Figure 6* shows anatomic landmarks of the donor procedure.

The pericardium was then opened after removal of thymic tissue. Prior to injection of 5 ml of cold (4°C) University of Wisconsin (UW) solution into the aortic arch for retrograde cardioplegia, left and right superior caval veins were incised. For further protection, a gauze swab was placed on the heart that was intermittently rinsed with cold saline solution throughout the remaining donor procedure.

After ligation using a 7/0 silk suture, the right superior caval vein was cut close to the atrium. Following appropriate dissection, aorta (distal to the brachiocephalic trunk) and brachiocephalic trunk were cut. The pulmonary trunk was then cut at the confluence of both pulmonary arteries. The heart was elevated to reveal the inferior caval vein, which was subsequently ligated with a 7/0 suture and cut. Finally, a large 7/0 silk ligature was placed around the pulmonary veins and the remaining left superior caval vein to place the heart in UW solution at 4°C after removal of both lungs.

4.2.1.4 Preparation of the recipient

After anesthesia, shaving and fixation of all four limbs and the head, a skin incision in the right cervical region from the suprasternal notch to the lower mandible was performed. Following opening of the platysma and cauterization of a crossing A. transversa colli, the external jugular vein was dissected and all tributaries were cauterized. The external jugular

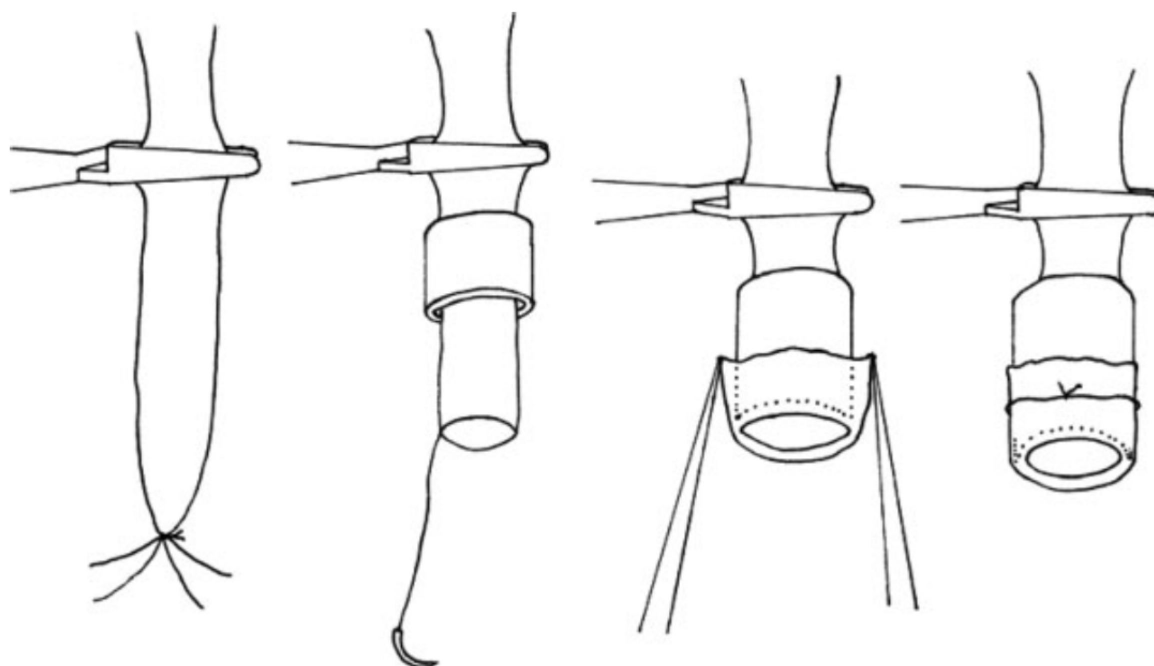

Figure 7: Preparation of the recipient vessels using the cuff technique [331]**.** For the procedure used in this model, the cuff had a semicircular proximal extension for placement of the microvascular clamp and fixation of the cuff during eversion of the vessels. See 4.2.1.4 and 4.2.1.5 for a detailed description of the vascular anastomoses used in this model.

vein was then cut between two 7/0 silk ligatures and the venous cuff was placed, with the microvascular clamp on the 1 mm handle. After removal of the remaining ligature and topical anticoagulation using a 1:4 heparine-saline solution, the vein was pulled over the cuff to evert the intimal layer. A 7/0 silk loop was placed around the vein and the cuff (*Figure 7*).

The right lobe of the submandibular gland and the sternal head of the right sternocleidomastoid muscle were then excised to expose the common carotid artery. While sparing the internal jugular vein and the vagal nerve, the common carotid artery was then dissected and cut between two 7/0 silk ligatures. The arterial cuff was placed with the microvascular clamp on the handle. The artery was passed through the cuff and after removal of the remaining ligature and rinsing with 1:4 heparin-saline solution dilatation of the peripheral end was done using a vascular dilatator. Now the distended end was pulled over the cuff to evert the intimal layer and a 7/0 silk loop was used to secure the artery on the cuff.

4.2.1.5 Engraftment

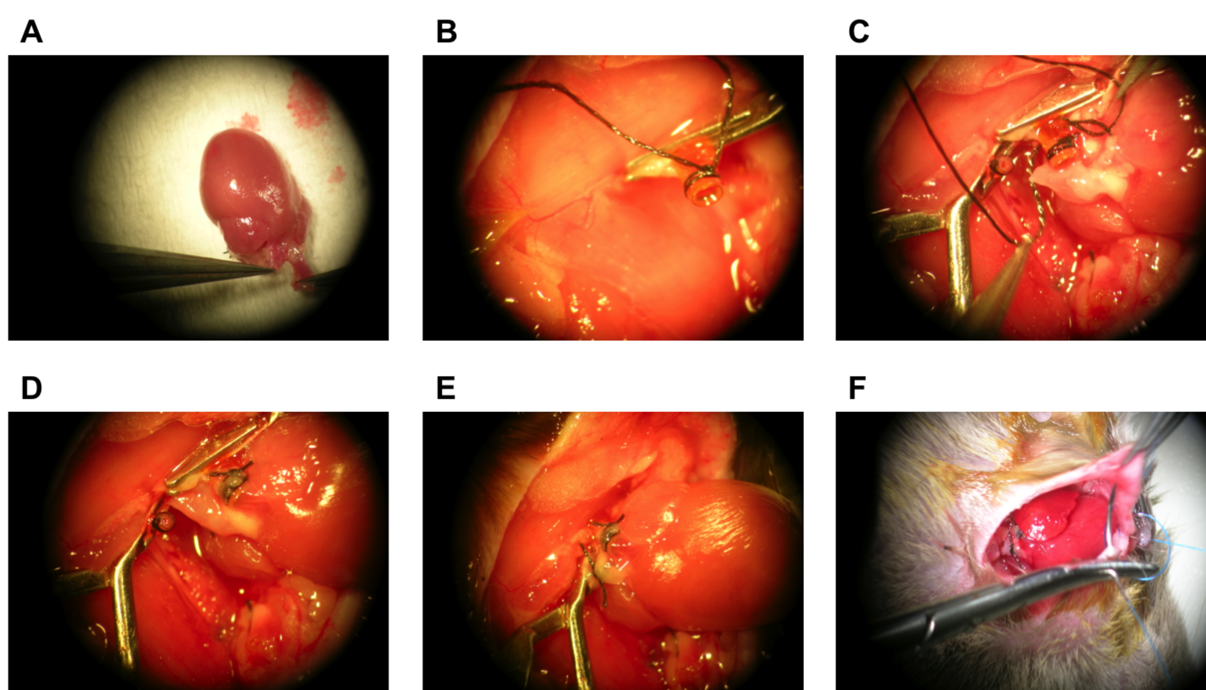

Figure 8: Major steps for the preparation of the recipient and engraftment of the donor organ. A, The donor organ after procurement, with aorta and pulmonary artery. B, The external jugular vein of the recipient everted over the vessel cuff. C, After preparation of both the external jugular vein and carotid artery of the recipient. D, Implantation of the cardiac allograft by preparing the vascular anastomoses. E, Declamping and reperfusion of the graft. F, Skin suture following successful transplantation.

The graft was placed into the right cervical region of the recipient, with the cardiac apex pointing cranial-laterally. After preparation of two circular 7/0 ligatures, donor aorta and recipient carotid artery as well as donor pulmonary trunk and recipient external jugular vein were connected, respectively. The venous clamp followed by the arterial clamp were then removed. The graft was rinsed with warm saline solution and upon successful reperfusion the handle of the venous cuff was removed prior to wound closure using absorbable sutures. The

graft was thus fully vascularized and functional, although it did not contribute to the recipient's circulation.

All transplantations were performed by an experienced microsurgeon and ischemia time upon revascularization was consistently at 40 minutes. *Figure 8* illustrates the major steps of the recipient procedure as well as the engraftment of the donor heart.

4.2.1.6 Postoperative evaluation

Graft function was evaluated in a blinded fashion by an independent reviewer, with allograft rejection being defined as the complete cessation of palpable contractility. Graft survival is shown as the median survival time (MST) in days.

4.2.2 Bone marrow transplantation

Young and old prospective donor B6 mice received a myoablative dose (11 Gy, 0.9 Gy/min) of total body irradiation. Unseparated bone marrow cells were harvested from young syngeneic B6 mice as described earlier [332,333]. Briefly, tibiae, femurs and humeri were procured and remaining muscle tissue was removed. Bones were then placed in a petri dish with 70% ethanol for 30 seconds, washed twice with PBS, and transferred into a new dish with RPMI 1640. Both ends of the bones were cut with scissors, and bone marrow was flushed out with syringes and 25 G needles using 2 ml of RPMI 1640. Cells were suspended, passed through a 100 µm nylon mesh to remove small pieces of bone and debris, and erythrocytes were lysed using lysis buffer. Cells were counted using a Neubauer chamber and trypane blue, diluted in bone marrow media and injected into the tail vene of prospective graft donors ($10x10^6$ cells/animal in 1 ml) 24 hours after irradiation. After six weeks, cardiac tissue of bone marrow transplanted young and old B6 mice was repopulated with bone marrow-derived leukocytes and animals were used as cardiac allograft donors.

4.2.3 Clodronate treatment protocol

Young and old prospective donor B6 mice were treated with liposomal clodronate (dichloromethylene diphosphonate) on days -8, -5, and -1 of cardiac transplantation. 0.5 mg of liposomal clodronate in a total volume of 1 ml was injected into the tail vein. This regimen

insures depletion of circulating $CD11c^+$ dendritic cells and $F4/80^+$ macrophages and reduces the residing DC population within the cardiac tissue prior to transplantation. For control mice, PBS liposomes provided by the manufacturer were administered as described above.

4.2.4 Isolation of leukocytes from hearts

Hearts were procured from B6 mice and perfused with 20 ml of PBS. Tissue was minced in a petri dish with scalpel blades and incubated for 30 minutes in 1 ml of cell culture media per heart with 1 mg/ml Collagenase D at 37°C and 5% CO_2. Tissue was then passed through a 70 µm cell strainer into a centrifuge tube, washed with PBS and spun down at 1800 rpm for 10 min and resuspended in 1.5 ml PBS.

A Percoll gradient was then prepared for density gradient centrifugation. 1.8 ml PBS was added to 3.5 ml Percoll for a total 5.3 ml of 66% Percoll. 3 ml of this solution was pipetted into a 15 ml centrifuge tube. The 1.5 ml cell suspension was then resuspended with 1.5 ml of the remaining 66% Percoll solution and layered gently on the 3 ml of 66% Percoll.

This tube was centrifuged for 20 min at 620 G and room temperature. Leukocytes were then aspirated at the density interface, washed with PBS and counted using a Neubauer chamber and trypan blue. Surface staining for CD11c was performed as described.

4.2.5 Histology

Cardiac allograft samples were harvested 7 days after transplantation. Samples were fixed in 10% formalin, embedded in paraffin, coronally sectioned, stained with hematoxylin/eosin (HE), and analyzed by light microscopy. The degree of rejection was determined according to a score system (see *Table 1*) based on the International Society of Heart and Lung Transplantation (ISHLT) guidelines [334,335]. Evaluation was performed in a blinded fashion by an independent pathologist.

Table 1: ISHLT Standardized Cardiac Biopsy Grading for Acute Cellular Rejection [335]

Grade 0	No rejection
Grade 1, mild	
A – Focal	Focal perivascular and/or interstitial infiltrate without myocyte damage
B – Diffuse	Diffuse infiltrate without myocyte damage
Grade 2, moderate (focal)	One focus of infiltrate with associated myocyte damage
Grade 3, moderate	
A – Focal	Multifocal infiltrate with myocyte damage
B – Diffuse	Diffuse infiltrate with myocyte damage
Grade 4, severe	Diffuse, polymorphous infiltrate with extensive myocyte damage ± edema, ± hemorrhage + vasculitis

4.2.6 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded coronally sectioned cardiac tissue using the streptavidin–biotin–peroxidase technique with diaminobenzidine as chromogen with an automated immunostainer following the manufacturer's protocol. Cross-reactive monoclonal mouse anti-human antibodies (CD4, dilution 1:10; CD8, dilution 1:50) were used. Heat pretreatment at 121°C in an autoclave with citrate buffer (pH 6.0) for five minutes was done for antigen retrieval. Positive T cells in 5 randomly selected high power fields were counted (HPF; 400x magnification) and the data given as mean ± SEM. All slides were analyzed in a blinded fashion by an independent pathologist.

Immunohistochemistry for CD11c was performed using OCT-embedded, 4 µm thick acetone fixed coronally sectioned cardiac tissue. Slides were pre-treated with peroxidase block kit for 5 minutes to quench endogenous peroxidase activity. Monoclonal hamster anti-mouse CD11c was applied in Dako diluent at 1:200 for 1 hour, washed, and rabbit anti-rat immunoglobulin antibody was applied at 1:750 in Dako diluent for 1 hour. Slides were washed in 50-mM Tris-Cl (pH 7.4) and detected with anti-rabbit Envision+ kit following the manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen. Counterstaining was done with hematoxylin.

4.2.7 Isolation of splenocytes

Spleens were removed without fat or connective tissue and passed through 70 µm nylon cell strainers using the back of a syringe plunger, while rinsing with PBS. Cells were resuspended in PBS and centrifuged (1600 rpm, 6 min, 4°C). After lysis of erythrocytes using lysis buffer, cells were passed through a new cell strainer and centrifuged. Cell count was determined using a Neubauer chamber and trypan blue.

4.2.8 Enzyme-linked immunospot (ELISpot) assay

ELISpot assays were performed using mouse IFN-γ and IL-6 ELISpot Kits according to the manufacturer's instructions. Briefly, $0.5x10^6$ unselected splenocytes from DBA/2 recipients were restimulated with $0.5x10^6$ irradiated splenocytes from young naïve donor-type B6 animals within 96-well ELISpot plates for 24 hours in cell culture media at 37°C and 5% CO_2. For *in-vitro* characterization of DCs, $1x10^6$ splenocytes from naïve DBA/2 mice were used as responder cells to $1x10^4$ flow-sorted splenic $CD11c^+$ DCs (untreated or LPS-stimulated) isolated from young or old naïve B6 mice.

Plates were coated overnight at 4°C with 100 µl of capture antibody in a 1:200 dilution with PBS. After washing with PBS (2x) and blocking with cell media for 2 hours, cells were pipetted as indicated above with a final volume of 200 µl per well. For positive controls, ConA with a final concentration of 10 µg/ml was used. After 24 hours, wells were washed with PBS with 0.05% Tween-20 (2x) and PBS (2x). 100 µl of detection antibody in a 1:250 dilution with 0.2 µm filtered PBS containing 10% FCS were then added into the wells and incubated overnight at 4°C. After repeated washing with PBS/Tween (2x) and PBS (2x), 100 µl of the diluted (1:100 in 0.2 µm filtered PBS containing 10% FCS) Streptavidin-HRP enzyme conjugate was added into the wells and incubated for 1 hour at room temperature. Finally, after washing with PBS/Tween (2x) and PBS (2x), 100 µl of the AEC (3-amino-9-ethyl-carbazole) substrate solution were added and formation of spots was monitored. The substrate reaction was stopped with deionized water and the plates were dried overnight in the dark at room temperature. Resulting spots were counted on a computer-assisted ELISpot plate analyzer, and frequencies of alloreactive T cells were expressed as the number of cytokine-producing spots per $0.5x10^6$ or $1x10^6$ responder cells, respectively.

4.2.9 Mixed lymphocyte reaction and ^{3}H-thymidine incorporation assay

$0.5x10^6$ splenocytes from DBA/2 recipient mice were co-cultured with $0.5x10^6$ irradiated donor-type splenocytes from young naïve B6 mice in 96-well U-bottom cell culture plates. The total volume in each well was 200µl and cells were suspended in cell culture media. For positive controls, ConA with a final concentration of 10µg/ml was used. After incubation at 37°C and 5% CO_2 for 72 hours, cells were pulsed with ^{3}H-thymidine (1 µCi/ well) and incubated under the same conditions for another 12 hours. Cells were transferred on filter paper using a cell harvester and incorporation of ^{3}H-thymidine, which indicates cell proliferation, was then assessed as counts per minute using a computer-assisted liquid scintillation counter.

To test the immunogenic properties of old versus young dendritic cells *in-vitro*, $1x10^6$ splenocytes from naïve DBA/2 mice were co-cultured with $1x10^4$ untreated or LPS-stimulated flow-sorted splenic CD11c$^+$ DCs from young or old naïve B6 mice in 96-well U-bottom cell culture plates for 72 hours prior to pulsing with 1 µCi/ well and incubation for another 12 hours. Cells were then processed as described above and counts per minute were quantified.

4.2.10 Flow cytometry

Flow cytometric analyses were performed using a FACSCalibur system and data were analyzed using FlowJo software. Cells were prepared in flow cytometry buffer and stainings were done using 96-well V-bottom plates. Fluorochrome-labeled monoclonal antibodies were prediluted in flow cytometry buffer according to concentrations determined in preliminary titrations. For compensation and controls, unstained, single-stained and quadruple-stained samples were used. For intracellular staining, controls were subject to the same staining and permeabilization buffers. Intracellular cytokine expression was assessed using FMO (fluorescence minus one) controls. For identification of cell populations, size (forward scatter), granularity (sideward scatter), and fluorescence emission were used.

4.2.10.1 Surface staining

100 µl of a cell suspension containing $10x10^6$ cells/ml was pipetted into each well and 10µl of the prepared flow cytometry buffer with prediluted antibodies was added. After incubation for 30 min in the dark at 4°C, cells were centrifuged (2000 rpm, 3 min, 4°C) and washed with

200 µl of flow cytometry buffer. For transfer into flow cytometry tubes, cells were resuspended in 100-200 µl of flow cytometry buffer.

4.2.10.2 Intracellular staining

Intracellular staining for FoxP3 was done using a commercially available staining set, including permeabilization solution and buffer. Following staining for surface antigens as described above, cells were permeabilized overnight at 4°C with 100 µl of permeabilization solution per well. Cells were then washed with 200 µl of permeabilization buffer and incubated for 30 min in the dark at 4°C with 100 µl of permeabilization buffer containing FoxP3 antibody (1:50). After washing with 200 µl of permeabilization buffer, cells were transferred into flow cytometry tubes and samples were read.

For intracellular cytokine staining (IFN-γ), splenocytes were stimulated for 4 hours in 96 well U-bottom plates with Ionomycin and PMA at 37°C and 5% CO_2. Golgi-Stop was added at a final concentration of 0.67 µl/ml. Stimulated cells were then washed twice and staining for surface antigens was done as described above. For permeabilization solution and washing buffer, an intracellular cytokine staining set was used. Following permeabilization overnight at 4°C with 100 µl of permeabilization solution, cells were washed with 200 µl of washing buffer and stained for intracellular cytokine antigens (antibodies diluted in 100 µl of washing buffer) for 30 min at 4°C in the dark. After washing with 200 µl of washing buffer, cells were transferred into flow cytometry tubes using flow cytometry buffer and samples were read.

4.2.11 Isolation of dendritic cells

Splenocytes from young or old naïve B6 mice were prepared as described above. To limit cell death due to extended sorting times, cells were preselected by magnetically depleting the splenic B cell population. This was done using B220-PE antibodies, anti-PE microbeads, LS columns and a MidiMACS separator according to the manufacturer's instructions. Cells were then stained for CD11c as described above. Staining was done in one flow cytometry tube per group, with volumes and concentrations adjusted accordingly. Using a FACSAria II flow sorter, $CD11c^+$ were isolated and collected in cell culture media. Cell count was determined using a Neubauer chamber and trypan blue and cells were cultivated with or without LPS prior to ELISpot assay or ^3H-thymidine incorporation assay.

4.2.12 Prestimulation of dendritic cells

Flow-sorted splenic $CD11c^+$ dendritic cells were cultivated at 37°C and 5% CO_2 for 24 hours in cell culture media without LPS and in presence of 10 or 100 ng/ml of LPS. 96-well U-bottom plates were used, with $0.3x10^6$ cells per well with a total volume of 0.2 ml. After stimulation, dendritic cells were washed twice with PBS and counted using a Neubauer chamber and trypan blue.

4.2.13 Statistics

Kaplan-Meier survival graphs were used for survival analysis. Survival analysis between groups was calculated using the Log-rank method. Unpaired two-tailed Student's t test was used for comparison of means. All results were generated using GraphPad prism software (San Diego, CA). A p value < 0.05 was considered significant. Data were expressed as mean ± standard error of the mean (SEM).

5 RESULTS

5.1 CARDIAC ALLOGRAFTS FROM OLD DONORS ARE SUBJECT TO ACCELERATED GRAFT REJECTION

To test whether the clinically observed impact of donor age on allograft survival was reproducible in an experimental *in-vivo* setting, fully MHC-mismatched cardiac allografts from young (8 to 12 weeks) or old (18 months) B6 mice were transplanted into young DBA/2 recipient mice, using a vascularized model of heterotopic cervical heart transplantation performed with consistent ischemia-reperfusion times.

With all grafts being monitored regularly by an independent reviewer and complete rejection being defined as no palpable contraction, grafts procured from old donors were rejected significantly faster than those from young donors, as illustrated by median survival times of 9 days vs. 11 days (*Figure 9*).

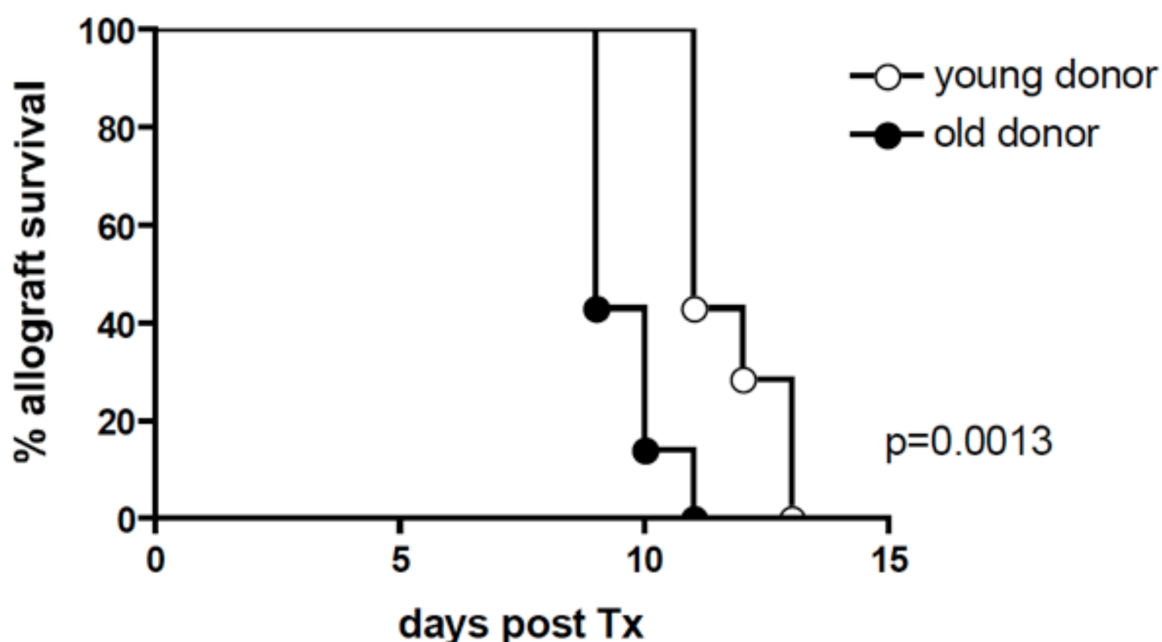

Figure 9: Accelerated rejection of cardiac allografts from old donors in a fully MHC-mismatched murine model of heterotopic cardiac transplantation. Hearts procured from either young (8 to 12 weeks) or old (18 months) B6 mice were transplanted with consistent ischemia-reperfusion times into young (8 to 12 weeks) DBA/2 mice using a model of heterotopic cervical heart transplantation. With complete rejection being defined as no palpable contraction, hearts procured from old donor mice were rejected significantly faster than those procured from young donors. MST=9 vs. 11 days; n=7 per group, p=0.0013.

Old allografts also displayed significantly higher degrees of acute rejection when HE stainings of grafts were graded by an independent pathologist at day 7 post transplantation using the ISHLT score to assess lymphocellular infiltration, thus providing a local histomorphological correlate for the differences in median survival times (*Figure 10*). This finding was also confirmed by immunohistochemical stainings revealing more pronounced infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in old grafts (*Figure 11*).

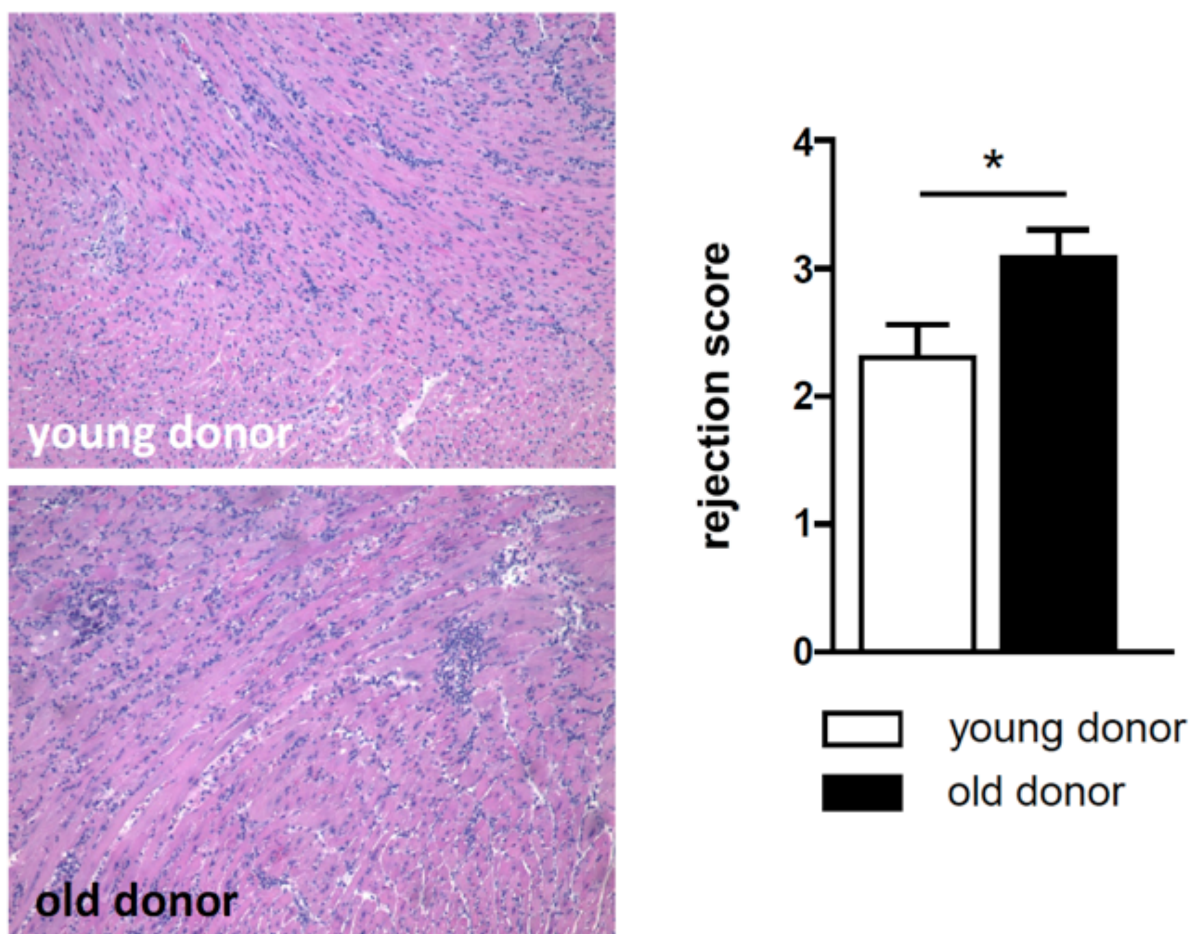

Figure 10: Grafts procured from old donors showed increased lymphocellular infiltration with significantly higher rejection scores after transplantation. Using the same model of heterotopic fully MHC-mismatched murine cardiac transplantation, cardiac allografts from either young or old B6 donor mice transplanted into young DBA/2 recipient mice were harvested at day 7 post transplantation and fixed, embedded, sectioned and stained to allow for histopathological grading of rejection. Analysis revealed increased lymphocellular infiltration and higher rejection scores in cardiac allografts from old donor mice. HE staining, 10x; n=4 per group; *, $p<0.05$.

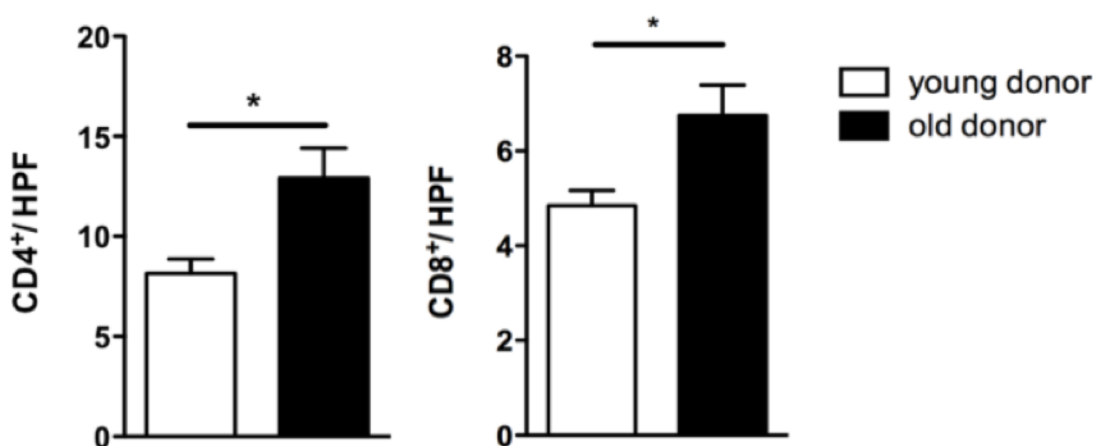

Figure 11: More pronounced infiltration of $CD4^+$/$CD8^+$ T cells in old allografts after transplantation. Allografts procured from either young or old donor mice were transplanted into young recipient mice and harvested at day 7 post transplantation. Lymphocellular infiltration was then further analyzed using immunohistochemical staining for CD4 and CD8, showing increased numbers of $CD4^+$ and $CD8^+$ cells per high power field in old allografts. HPF, high power field; n=4 per group; *, $p<0.05$.

5.2 Accelerated rejection of cardiac allografts from old donors correlates with more pronounced systemic alloimmune responses

To determine donor-age-related effects on the systemic alloimmune response of the recipient, frequencies of splenic T cell populations were assessed by flow cytometry at day 7 post transplantation in spleens procured from recipients. While frequencies of $CD4^+CD25^+FoxP3^+$ regulatory T cells did not differ between the two groups, recipients of old cardiac allografts had significantly increased frequencies of $CD8^+$ effector T cells ($CD44^{high}CD62^{low}$ phenotype) and a marked trend towards higher frequencies of $CD4^+$ effector T cells (*Figure 12*). Transplantation of old grafts also led to higher frequencies of IFN-γ-producing $CD8^+$ T cells, as assessed by intracellular cytokine staining (*Figure 13*).

Recipient splenocytes were further characterized by restimulation with donor-type antigen (splenocytes isolated from young B6 mice) and subsequent evaluation of cytokine production and proliferative response. Using ELISpot assays, recipients of old allografts showed significantly increased frequencies of alloreactive IFN-γ and IL-6-producing responder cells among splenocytes upon restimulation (*Figure 14*). Proliferation of alloreactive splenocytes responding to donor-type antigen in ^{3}H-thymidine assays was also more pronounced in splenocytes of recipients of old allografts (*Figure 14*), further underscoring the age-dependent differences in graft survival and local lymphocellular infiltration.

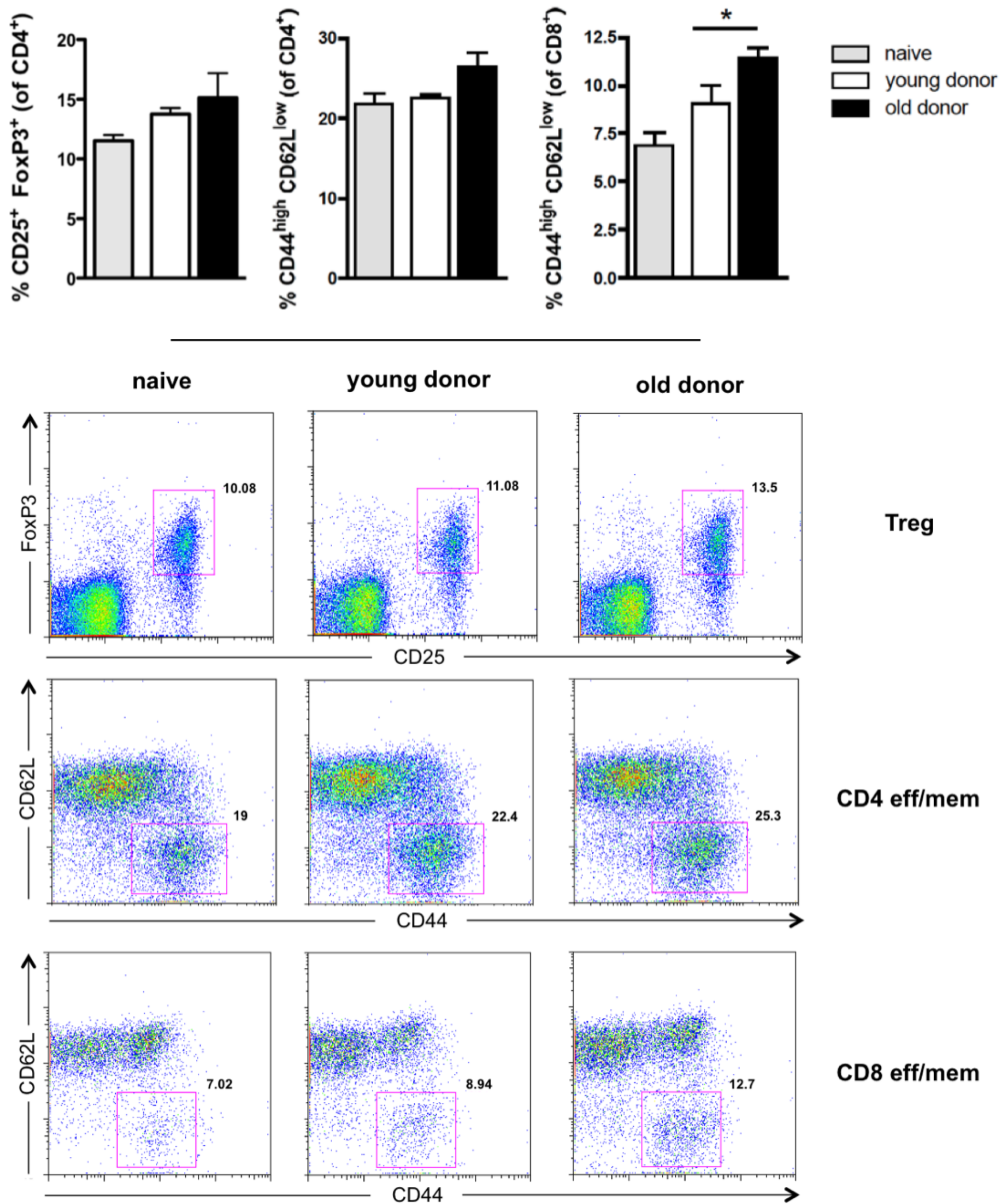

Figure 12: Increased frequencies of splenic $CD8^+$ effector T cells in recipients of old cardiac allografts, with no significant changes in frequencies of regulatory T cells. To determine effects of donor age on systemic alloimmune responses of recipients, spleens of young DBA/2 recipient mice were harvested at day 7 after transplantation of cardiac allografts from either young or old B6 donor mice. Spleens from untransplanted young DBA/2 mice were used as naïve controls. Frequencies of splenic T cell populations among isolated splenocytes were then analyzed using flow cytometry. Recipients of old cardiac allografts showed increased frequencies of $CD8^+$ effector T cells ($CD44^{high}CD62^{low}$ phenotype) and a trend towards higher frequencies of $CD4^+$ effector T cells. Frequencies of regulatory T cells ($CD4^+CD25^+FoxP3^+$ phenotype) did not differ between groups. N=4 per group; *, $p<0.05$; representative plots shown.

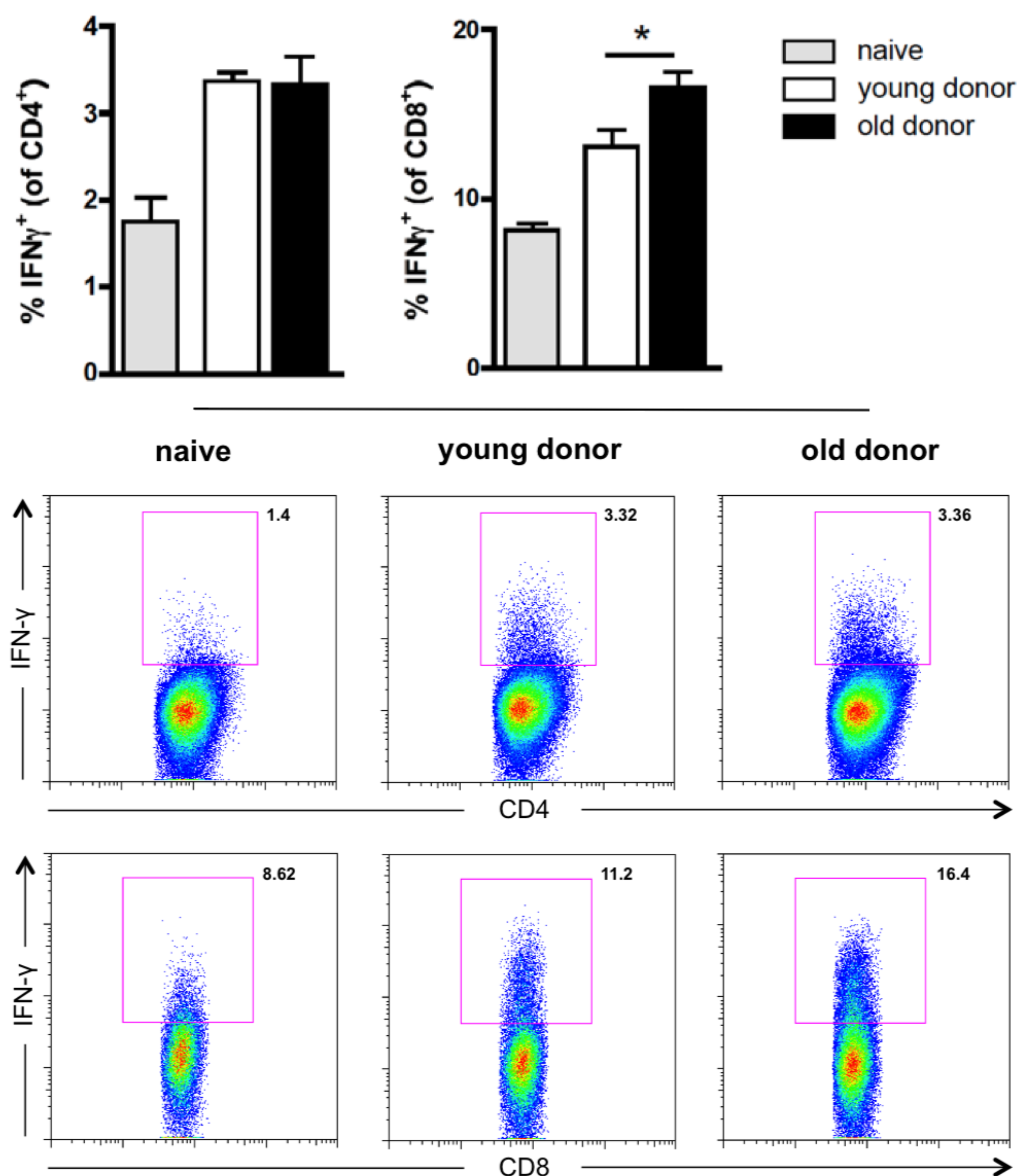

Figure 13: Transplantation of allografts procured from old donors resulted in increased frequencies of IFN-γ-producing $CD8^+$ T cells in recipient splenocytes. Donor-age-related effects on recipient alloimmune responses were analyzed by harvesting spleens of young DBA/2 recipient mice seven days after transplantation of cardiac allografts procured from either young or old B6 donor mice. Untransplanted young DBA/2 mice were used as naïve controls. Isolated splenocytes were restimulated for 4 hours using PMA/Ionomycin in presence of Golgi-Stop prior to surface staining and intracellular cytokine staining for IFN-γ. Splenocytes isolated from recipients of old cardiac allografts showed higher frequencies of IFN-γ-producing $CD8^+$ T cells, while frequencies of $CD4^+$IFN-γ^+ T cells were comparable among all three groups. N=4 per group; *, $p<0.05$; representative plots shown.

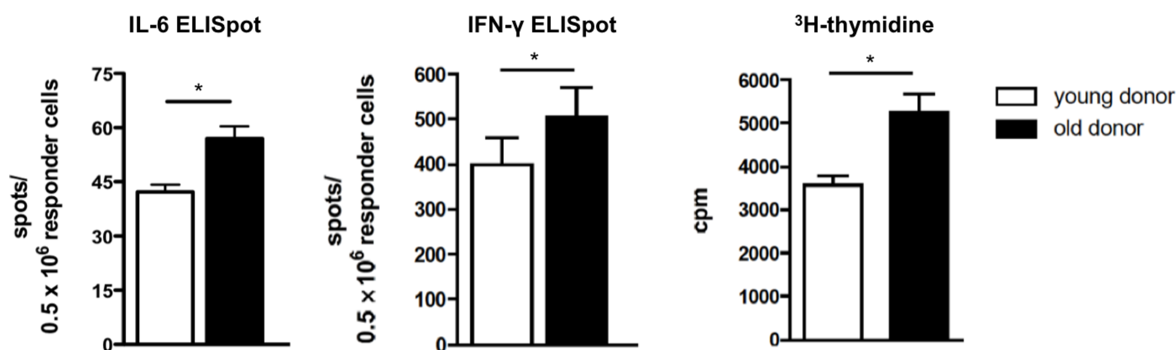

Figure 14: Upon restimulation with donor-type antigen, splenocytes isolated from recipients of old cardiac allografts showed higher frequencies of IL-6 and IFN-γ-producing cells (ELISpot, left and center) as well as more pronounced proliferative responses (^{3}H-thymidine incorporation, right). For further characterization of splenocytes isolated from spleens of young recipient mice at day 7 following transplantation of young or old donor hearts, recipient splenocytes were restimulated with donor-type antigen (splenocytes isolated from young B6 mice). For ELISpot assays (left and center), $0.5x10^6$ unselected splenocytes from DBA/2 recipients were restimulated with $0.5x10^6$ irradiated splenocytes from young naïve donor-type B6 animals for 24 hours. Recipients of old allografts showed increased frequencies of alloreactive IFN-γ and IL-6-producing responder cells upon restimulation. For ^{3}H-thymidine incorporation assays, $0.5x10^6$ splenocytes from DBA/2 recipient mice were co-cultured with $0.5x10^6$ irradiated donor-type splenocytes from young naïve B6 mice. After incubation for 72 hours, cells were pulsed with ^{3}H-thymidine (1 μCi/ well) and incubated for another 12 hours. Following cell harvesting, counts per minute indicating ^{3}H-thymidine incorporation and thus cell proliferation were then assessed. Proliferation of alloreactive splenocytes responding to donor-type antigen was more pronounced in splenocytes of recipients of old allografts. N=4 per group; *, $p<0.05$; experiments were performed in triplicates and means were used for statistical analysis.

5.3 Donor-age-dependent differences in transplant survival and alloimmune responses are mediated by passenger leukocytes

To determine whether these donor-age-related effects on graft survival and alloimmune responses were due to age-dependent changes in parenchymal cells or mediated by age-dependent modifications in passenger leukocytes, chimeric old B6 donor mice were generated by lethal total body irradiation and subsequent hematopoietic reconstitution with bone marrow procured from young naïve syngeneic B6 mice. Thus, old leukocytes residing in the cardiac tissue of old animals were replaced with young leukocytes derived from young transplanted bone marrow. Young B6 donor mice underwent the same treatment, generating animals with young cardiac tissue and young passenger leukocytes. Successful repopulation of cardiac tissue with bone-marrow derived leukocytes was verified after six weeks by immunohistochemistry stainings (*Figure 15*).

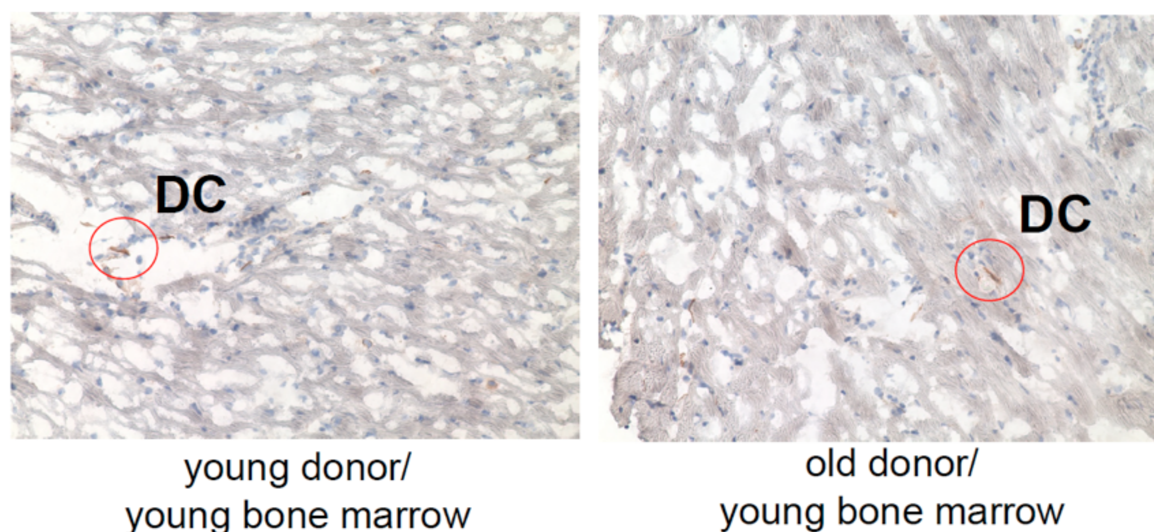

Figure 15: Successful repopulation of cardiac tissue with bone marrow-derived leukocytes after lethal irradiation and syngeneic bone marrow transplantation of donor animals. Chimeric donor animals were generated to delineate whether donor-age-related effects on graft survival and alloimmune responses were due to age-dependent changes in parenchymal cells or mediated by age-dependent modifications in passenger leukocytes. 24 hours following a myoablative dose of total body irradiation, young and old prospective donor B6 mice received $10x10^6$ cells/animal of bone marrow cells procured from young syngeneic B6 mice. After six weeks, these chimeric young and old B6 mice were used as cardiac allograft donors for young DBA/2 mice. To verify successful repopulation of cardiac tissue with bone marrow-derived leukocytes, hearts were harvested and stained for CD11c six weeks after bone marrow transplantation. Representative slides (10x).

Following transplantation into young DBA/2 recipients, an age-dependent effect of parenchymal cells on graft survival was not detectable. Both types of chimeric hearts (young tissue/young leukocytes and old tissue/young leukocytes) showed similar median survival times (*Figure 16*). In line with these findings, grading of acute rejection in HE slides 7 days after transplantation no longer revealed any significant differences in lymphocellular infiltration of the grafts (*Figure 17*).

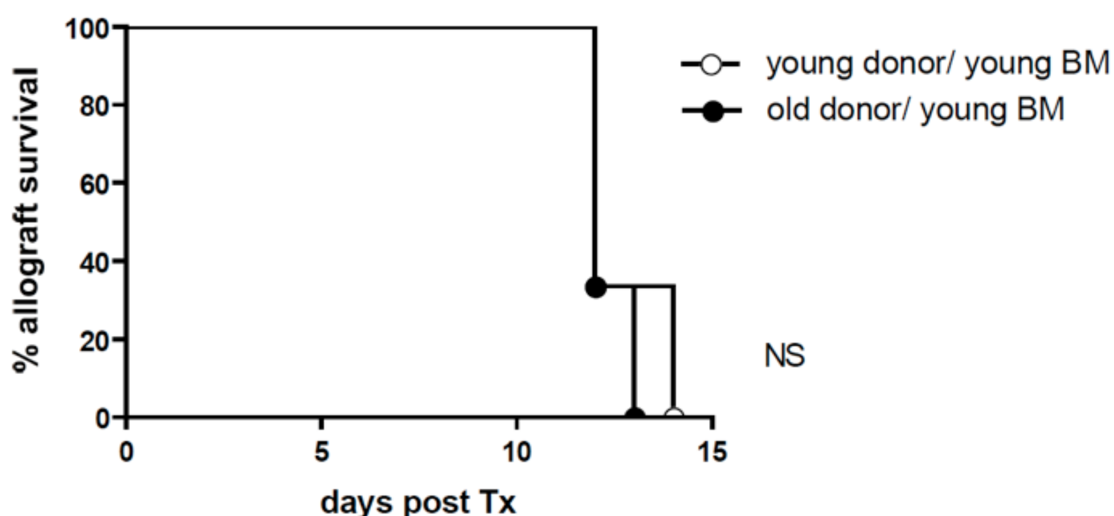

Figure 16: Survival times of chimeric B6 cardiac allografts (young tissue/young leukocytes or old tissue/young leukocytes) in young DBA/2 mice are comparable. When transplanting hearts procured from either young or old chimeric B6 animals, differences in survival observed when transplanting hearts from untreated young or old B6 donor mice were abolished and both groups showed comparable allograft survival. MST=12 days; n=4 per group, p=0.50.

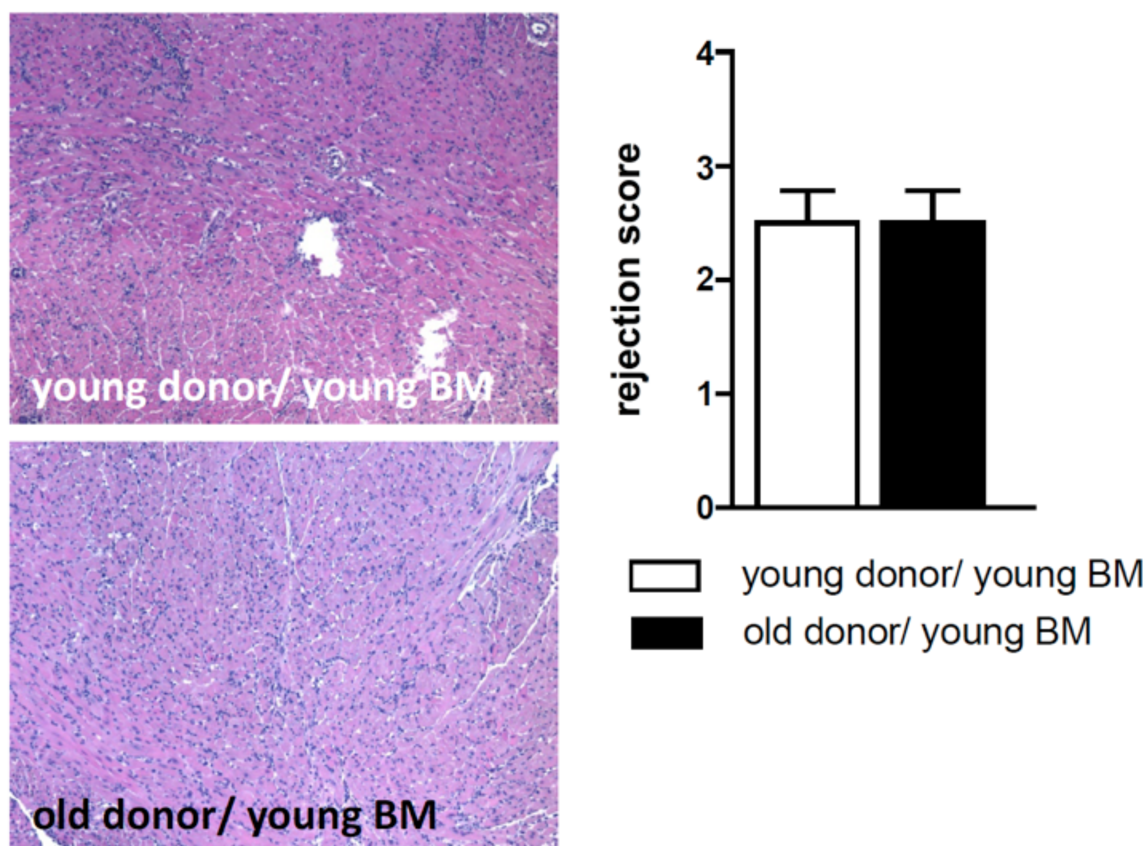

Figure 17: Similar degree of lymphocellular infiltration in allografts procured from young and old chimeric donor mice after lethal total body irradiation and hematopoietic reconstitution with young syngeneic bone marrow. Chimeric cardiac allografts (young tissue/young leukocytes or old tissue/young leukocytes) from either young or old B6 donor mice transplanted into young DBA/2 recipient mice were harvested at day 7 post transplantation and stained for histopathological grading of lymphocellular infiltration and rejection. Contrary to results obtained with cardiac allografts procured from untreated young and old B6 mice, grading of acute rejection no longer revealed any significant differences. HE staining; 10x; n=4 per group.

Moreover, splenic T cell populations at day 7 post transplantation were comparable in recipients of both types of chimeric hearts, indicating that systemic alloimmune responses were not impacted by donor-age-dependent mechanisms when using chimeric animals (*Figure 18).* Most notably, previously detected differences in frequencies of $CD8^+$ effector T cells were abolished. Upon restimulation with donor-type antigen, recipients of old chimeric allografts neither showed increased frequencies of IFN-γ or IL-6-producing alloreactive responder cells among splenocytes, nor did the proliferation rates of alloreactive responder splenocytes differ between recipients of old and young chimeric allografts (*Figure 19*). Surprisingly, frequencies of IL-6-producing responder cells were significantly lower in recipients of old chimeric allografts (*Figure 19).* Taken together, these results suggest that the impact of donor age on alloimmune responses and overall graft survival cannot be ascribed to age-dependent changes in immunogenicity of cardiac tissue, parenchyma or endothelial cells. Conversely, passenger leukocytes seem to play a major role in donor-age-dependent mechanisms of alloimmunity.

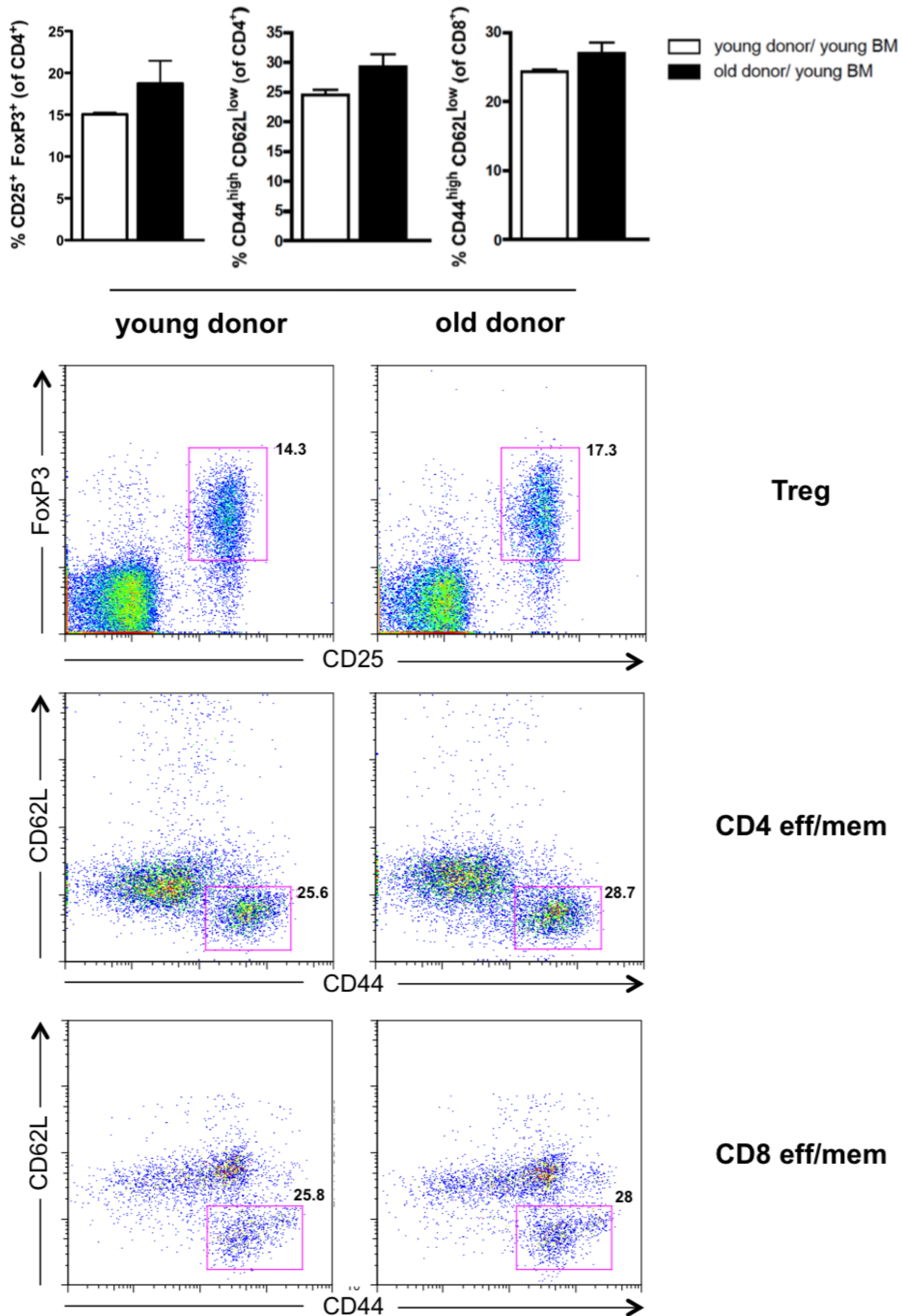

Figure 18: Frequencies of regulatory T cells as well as CD4$^+$ and CD8$^+$ effector T cells did not differ in splenocytes of recipients of young or old chimeric cardiac allografts. Cardiac allografts from either young or old chimeric (young tissue/young leukocytes or old tissue/young leukocytes) B6 donor mice were transplanted into young DBA/2 recipient mice and spleens of recipient mice were harvested at day 7 post transplantation. Frequencies of splenic T cell populations among isolated splenocytes were then analyzed using flow cytometry. While recipients of old untreated cardiac allografts had shown increased frequencies of CD8$^+$ effector T cells (CD44^{high}CD62^{low} phenotype), transplantation of chimeric old donor hearts (old tissue/young leukocytes) did no longer lead to increased frequencies of CD8$^+$ effector T cells. Consistent with previous results, frequencies of regulatory T cells (CD4$^+$CD25$^+$FoxP3$^+$ phenotype) or CD4$^+$ effector T cells did not differ between groups. N=4 per group; representative plots shown.

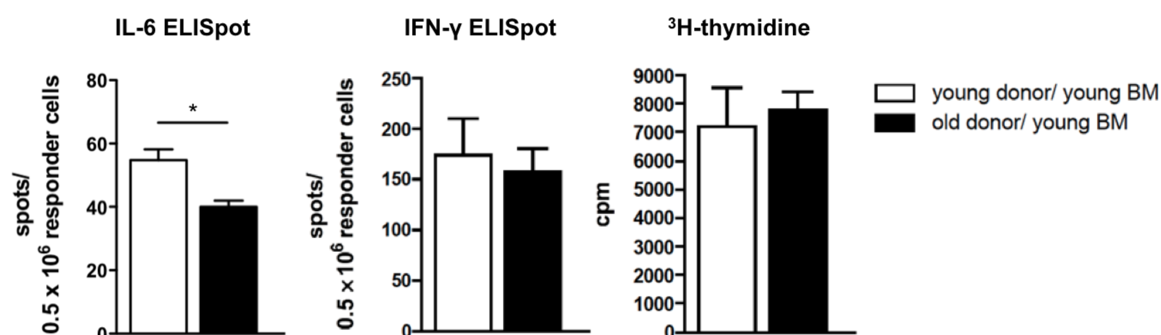

Figure 19: Splenocytes isolated from recipients of young and old chimeric cardiac allografts showed comparable frequencies of IFN-γ-producing cells (ELISpot, center) and comparable proliferation rates (^{3}H-thymidine incorporation, right) upon restimulation with donor-type antigen. Frequencies of IL-6-producing cells (ELISpot, left) were significantly lower in recipients of old chimeric hearts. Splenocytes isolated from spleens of young recipient mice at day 7 following transplantation of young or old chimeric donor hearts were restimulated with donor-type antigen (splenocytes isolated from young B6 mice). For ELISpot assays (left and center), $0.5x10^6$ unselected splenocytes from DBA/2 recipients were restimulated with $0.5x10^6$ irradiated splenocytes from young naïve donor-type B6 animals for 24 hours. Recipients of old chimeric allografts no longer showed increased frequencies of alloreactive IFN-γ and IL-6-producing responder cells upon restimulation. Recipients of old chimeric donor hearts showed significantly lower frequencies of alloreactive IL-6-producing responder cells. For ^{3}H-thymidine incorporation assays, $0.5x10^6$ splenocytes from DBA/2 recipient mice were co-cultured with $0.5x10^6$ irradiated donor-type splenocytes from young naïve B6 mice. After incubation for 72 hours, cells were pulsed with ^{3}H-thymidine (1 µCi/ well) and incubated for another 12 hours. Counts per minute indicating ^{3}H-thymidine incorporation and thus cell proliferation were then quantified. Transplantation of old chimeric donor hearts did not lead to increased proliferation of alloreactive splenocytes responding to donor-type antigen. N=4 per group; *, $p<0.05$; experiments were performed in triplicates and means were used for statistical analysis.

5.4 Dendritic cells are the key mediators of donor-age-related effects of increased alloimmune responses

Passenger dendritic cells have been shown to play a major role in pathways of alloimmunity, in particular during early phases of graft rejection [7]. To further discriminate between the differential roles of aged passenger leukocyte subsets in accelerated graft rejection following transplantation of old allografts, donor B6 mice were pretreated with liposomal clodronate to deplete dendritic cells residing in the cardiac tissue prior to transplantation. Liposomal clodronate reliably depletes circulating dendritic cells and macrophages, but does not penetrate cardiac capillaries [336,337]. Since macrophage turnover in cardiac tissue is much slower than dendritic cell turnover [338], this protocol affects primarily the cardiac DC population. The effect of liposomal clodronate on dendritic cells residing in the cardiac graft was verified prior to transplantation by flow cytometric analysis of leukocytes isolated from donor hearts (*Figure 20*). This strategy thus allowed to assess the effects of aged dendritic cells versus macrophages as well as T, B, and NK lymphocytes.

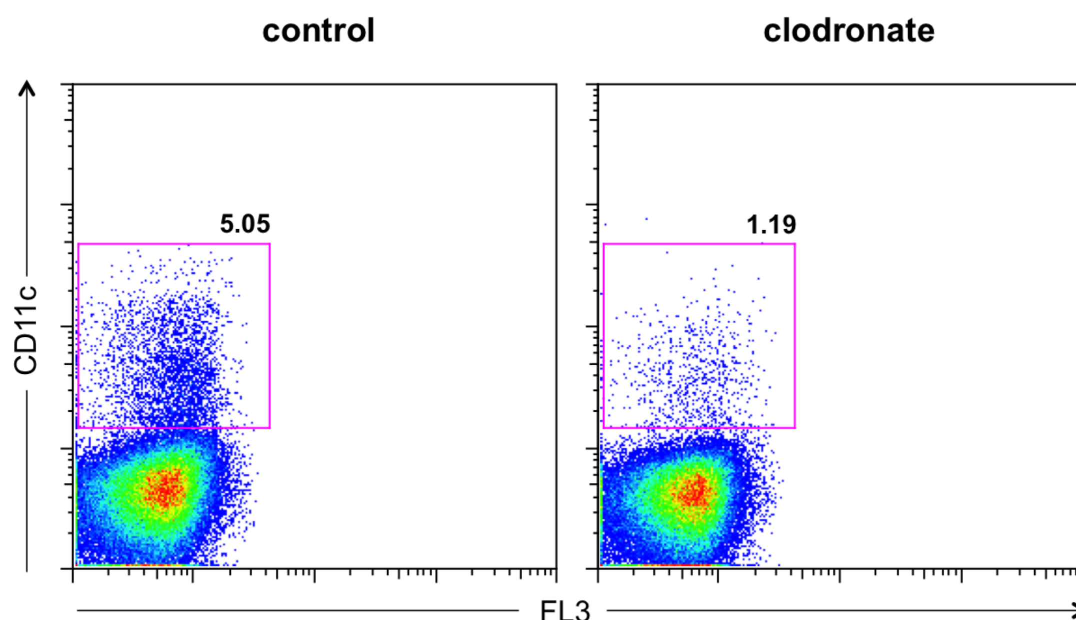

Figure 20: Dendritic cell population among leukocytes isolated from donor hearts is depleted following pretreatment with liposomal clodronate. Hearts were procured from B6 mice pretreated with liposomal clodronate from day -8 and perfused with PBS prior to incubation of minced tissue for 30 minutes in 1 mg/ml Collagenase D. Following straining and washing, leukocytes were isolated using Percoll density gradient centrifugation. Cells were then stained for CD11c and analyzed using flow cytometry. N=3 per group.

Cardiac allografts transplanted from pretreated young and old B6 donor mice into young DBA/2 recipients showed prolonged overall survival times (MST 12 and 14 days) when compared to naïve donors, with differences between the two groups being abolished (*Figure 21*). Histopathological evaluation of the grafts revealed no differences in the degree of acute rejection (*Figure 22*). In contrast to results gained with untreated donors, systemic alloimmune responses of the recipients following transplantation of old donor organs were now comparable to those elicited by young allografts, as assessed by using splenic T cell populations (*Figure 23*), intracellular cytokine staining *(Figure 24*), frequencies of IFN-γ and IL-6-producing alloreactive splenocytes (*Figure 25*), and proliferation of alloreactive splenocytes (*Figure 25*). Taken together, the results gained with donor hearts depleted of dendritic cells point to a crucial role of aged passenger dendritic cells rather than aged lymphocytes and macrophages – which are not targeted by liposomal clodronate – in mediating the impact of donor age on allograft survival and alloimmune responses.

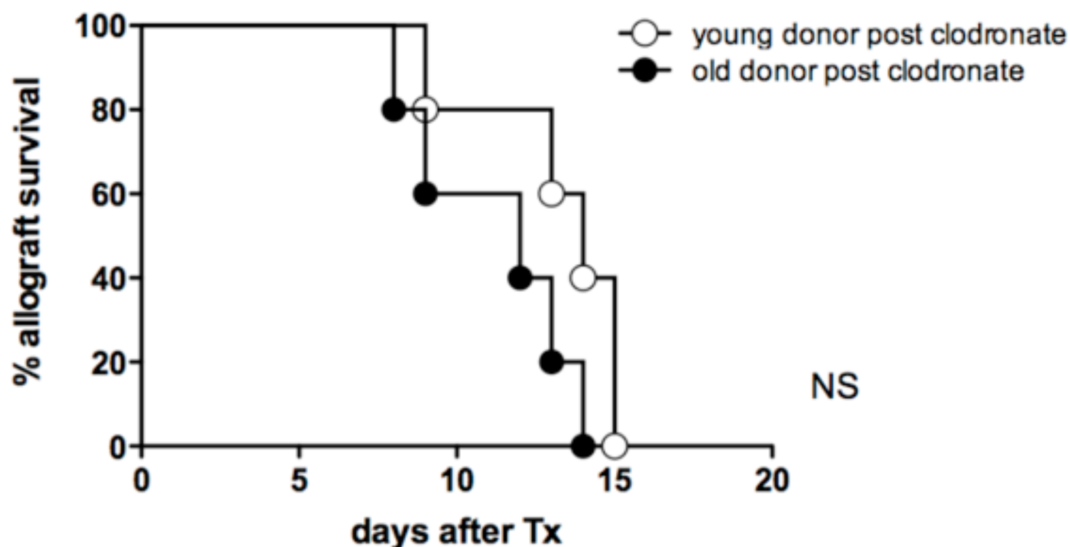

Figure 21: Donor age did not affect allograft survival following donor pretreatment with liposomal clodronate. Contrary to cardiac allografts procured from untreated donor mice, hearts procured from either young or old B6 donor mice pretreated with liposomal clodronate for 8 days did not show differences in allograft survival in young DBA/2 recipients. MST=12 vs. 14 days; n=5 per group; p=0.10.

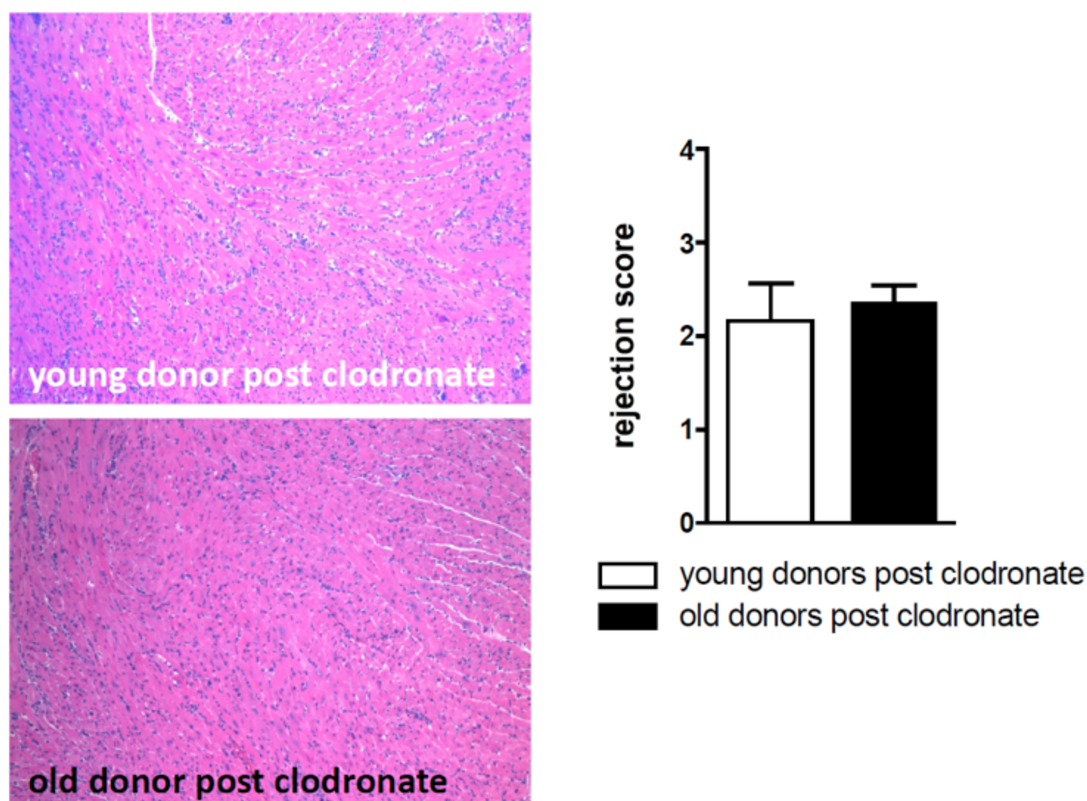

Figure 22: Similar degree of lymphocellular infiltration in allografts from old and young donor mice after donor pre-treatment with liposomal clodronate. Cardiac allografts procured from either young or old B6 donor mice pretreated with liposomal clodronate for 8 days were transplanted into young DBA/2 recipient mice and harvested at day 7 post transplantation to evaluate histopathological degree of lymphocellular infiltration. Allografts procured from old B6 donor mice no longer showed elevated rejection scores when donor mice were pretreated with liposomal clodronate. HE staining; 10x; n=4 per group.

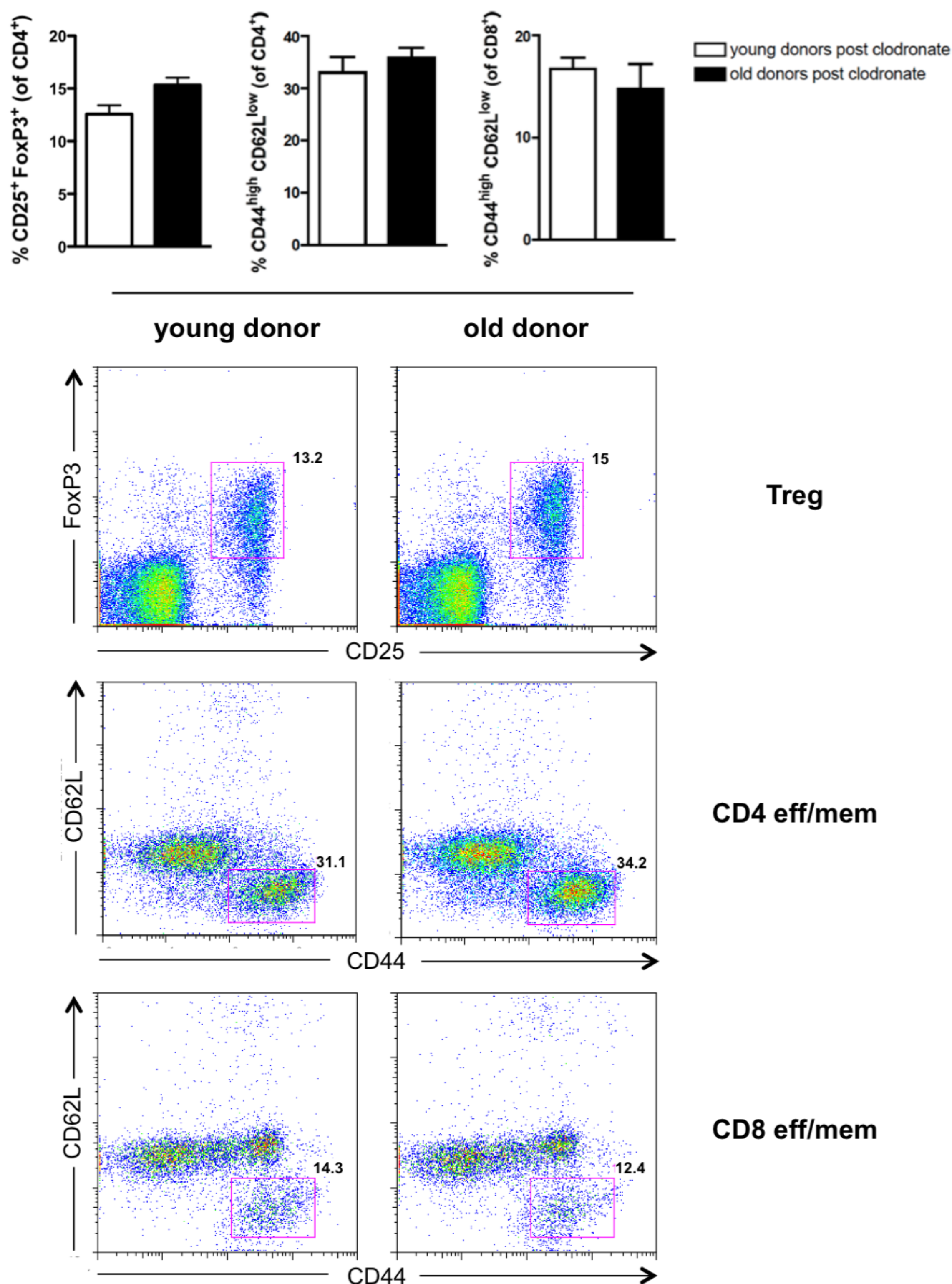

Figure 23: Frequencies of regulatory T cells ($CD4^+CD25^+FoxP3^+$ phenotype) as well as $CD4^+$ and $CD8^+$ effector T cells ($CD44^{high}CD62L^{low}$ phenotype) in recipients of old and young allografts are comparable after donor pre-treatment with liposomal clodronate. B6 donor mice were pretreated with liposomal clodronate for 8 days and used as cardiac allograft donors for young DBA/2 recipient mice. Spleens of recipient mice were then harvested at day 7 post transplantation and frequencies of splenic T cell populations among isolated splenocytes were analyzed. Transplantation of hearts procured from pretreated old donor mice did not lead to increased frequencies of $CD8^+$ effector T cells ($CD44^{high}CD62^{low}$ phenotype), whereas recipients of old untreated cardiac allografts had shown increased frequencies of $CD8^+$ effector T cells. N=4 per group; representative plots shown.

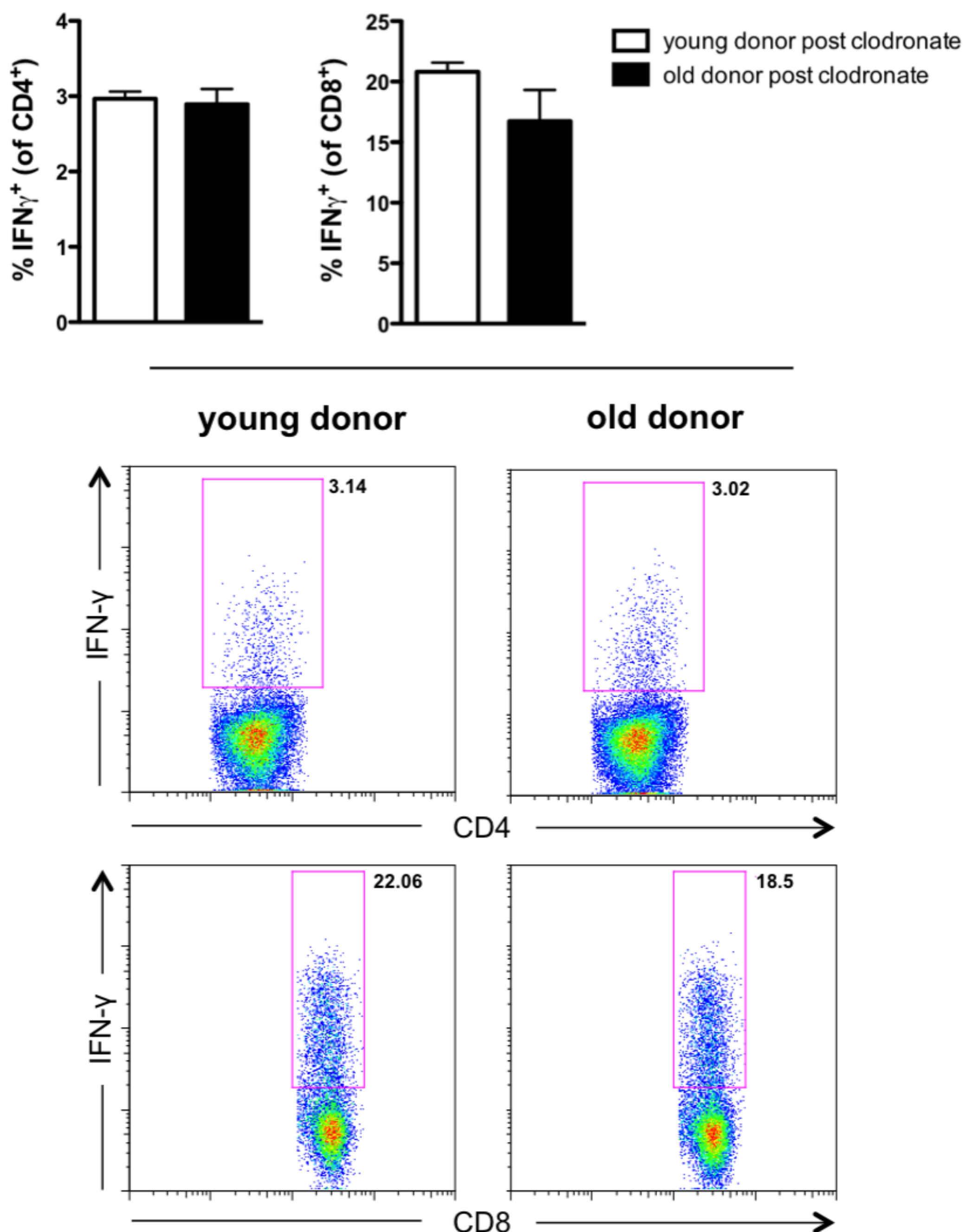

Figure 24: Transplantation of cardiac allografts procured from old donors pretreated with liposomal clodronate does not result in increased frequencies of IFN-γ-producing $CD4^+$ or $CD8^+$ T cells in recipient splenocytes. Spleens of young DBA/2 recipient mice were harvested seven days after transplantation of cardiac allografts procured from either young or old B6 donor mice pretreated with liposomal clodronate for 8 consecutive days. Isolated splenocytes were restimulated for 4 hours using PMA/Ionomycin in the presence of Golgi-Stop prior to surface staining and intracellular cytokine staining for IFN-γ. Splenocytes isolated from recipients showed comparable frequencies of IFN-γ-producing $CD8^+$ and $CD4^+$ T cells, thus no longer revealing increased frequencies of $CD8^+$IFN-γ^+ T cells observed when transplanting untreated old B6 allografts. N=4 per group, representative plots shown.

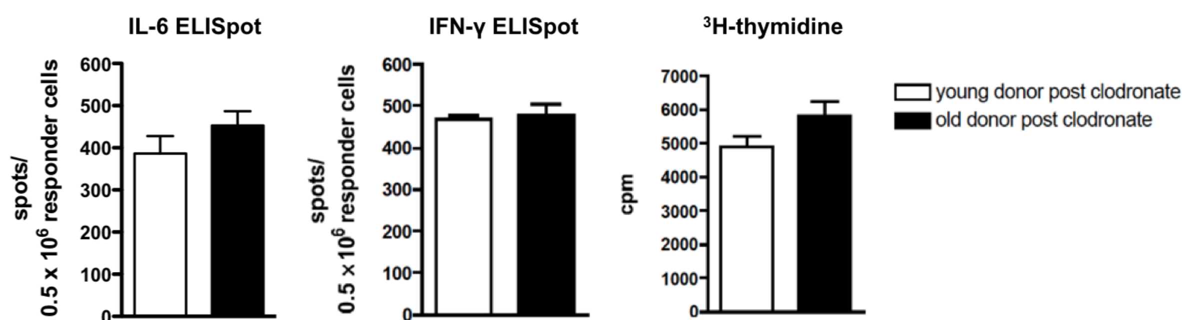

Figure 25: Following restimulation with donor-type antigen, frequencies of IFN-γ (left) and IL-6- (middle) producing splenocytes as well as proliferation rates of responder splenocytes (right) are comparable in recipients of old and young allografts after donor pretreatment with liposomal clodronate. Splenocytes isolated from spleens of young recipient mice at day 7 following transplantation of cardiac allografts from young or old B6 donor mice pretreated with liposomal clodronate were restimulated with donor-type antigen. For ELISpot assays (left and center), $0.5x10^6$ unselected splenocytes from DBA/2 recipients were restimulated with $0.5x10^6$ irradiated splenocytes from naïve donor-type B6 animals for 24 hours. Transplantation of hearts procured from old pretreated B6 donor animals did not lead to increased frequencies of alloreactive IFN-γ or IL-6-producing responder cells upon restimulation, contrary to observations made with allografts from untreated old donors. For ^{3}H-thymidine incorporation assays, $0.5x10^6$ splenocytes from DBA/2 recipient mice were co-cultured with $0.5x10^6$ irradiated donor-type splenocytes from young naïve B6 mice. After incubation for 72 hours, cells were pulsed with ^{3}H-thymidine (1 µCi/ well) and incubated for another 12 hours. Counts per minute indicating ^{3}H-thymidine incorporation and thus cell proliferation were then quantified. Transplantation of old donor hearts following pretreatment with liposomal clodronate did not lead to increased proliferation of alloreactive splenocytes responding to donor-type antigen. N=4 per group; experiments were performed in triplicates and means were used for statistical analysis.

5.5 OLD DENDRITIC CELLS SHOW INCREASED ALLOSTIMULATORY CAPACITIES IN-VITRO

To gain insight into the mechanisms of DC-mediated effects of donor age on alloimmunity, as well as to delineate effects of intrinsic changes in DCs with immunosenescence and effects of increased responses of DCs to danger signals, flow-sorted naïve splenic $CD11c^+$ dendritic cells were further characterized. Dendritic cells isolated from young and old B6 mice were used as allogeneic stimulators to splenocytes isolated from young naïve DBA/2 mice, with or without prior activation by LPS as a strong TLR4 agonist and potent inducer of DC activation and maturation.

Naïve DCs isolated from old B6 mice displayed stronger allostimulatory priming capacities compared with their young counterparts when cocultivated with naïve allogeneic splenocytes, as frequencies of IFN-γ-producing responder cells were significantly elevated (*Figure 26*) and proliferation rates increased (*Figure 27*). Preactivation with LPS increased the overall allostimulatory level of both young and old dendritic cells in a dose-dependent manner, with differences being mainly preserved.

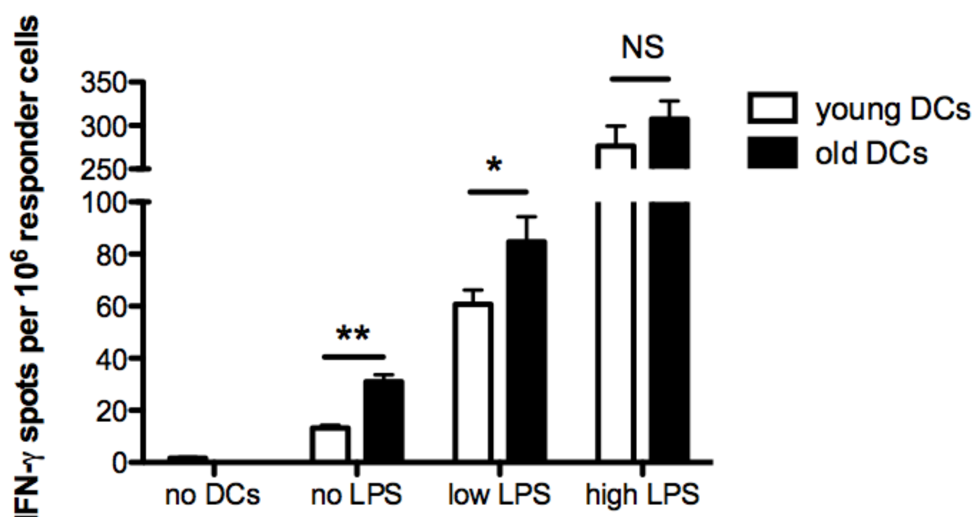

Figure 26: Frequencies of IFN-γ-producing responder splenocytes were significantly increased when stimulated by old flow-sorted splenic dendritic cells, while the overall level of spots increased in a dose-dependent manner with prestimulation of dendritic cells using LPS prior to cocultivation. To test immunogenic properties of old versus young dendritic cells in-vitro and to delineate effects of intrinsic immunosenescence and danger signaling on old DCs, $1x10^6$ splenocytes from naïve DBA/2 mice were co-cultured on ELISpot plates for 24 hours with $1x10^4$ untreated or LPS-stimulated flow-sorted splenic $CD11c^+$ DCs from young or old naïve B6 mice. DCs isolated from old B6 mice displayed stronger allostimulatory priming capacities as frequencies of IFN-γ-producing responder cells were significantly elevated. Age-dependent differences were preserved when prestimulating DCs for 24 hours using 10 ng/ml of LPS, but levelled out when using 100 ng/ml of LPS. N=6 per group; *, $p<0.05$; **, $p<0.05$; experiments were performed in triplicates and means were used for statistical analysis.

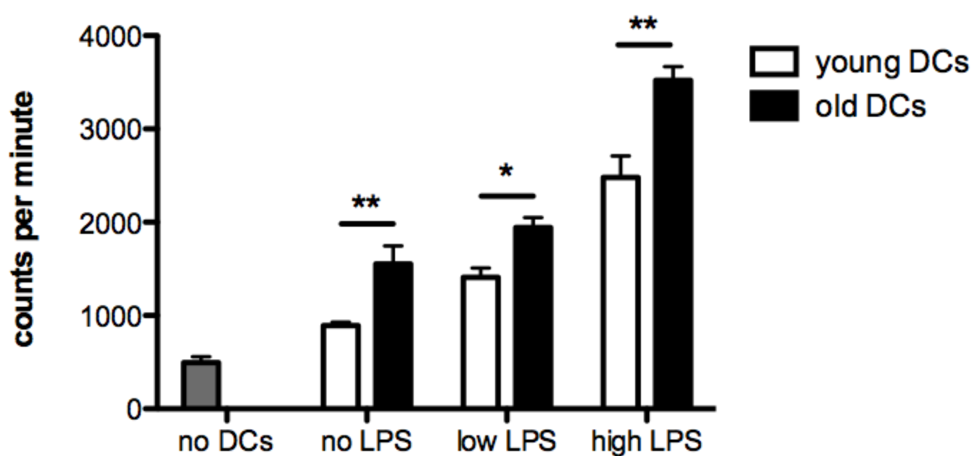

Figure 27: When cocultivated with old flow-sorted splenic dendritic cells, proliferation rates of responder splenocytes were increased. Preactivation of dendritic cells raised proliferation rates of responder cells in both groups in a dose-dependent manner. For further in-vitro characterization of DCs, $1x10^4$ untreated or LPS-stimulated flow-sorted splenic $CD11c^+$ DCs from young or old naïve B6 mice were used as stimulators for $1x10^6$ splenocytes from naïve DBA/2 mice in a mixed leukocyte reaction. Following incubation for 72 hours, cells were pulsed with ^{3}H-thymidine (1 µCi/ well) and incubated for another 12 hours. Counts per minute were then quantified, indicating ^{3}H-thymidine incorporation and cell proliferation. For prestimulation, DCs were cultivated for 24 hours in the presence of 0, 10 or 100 ng/ml of LPS. N=6 per group; *, $p<0.05$; **, $p<0.005$; experiments were performed in triplicates and means were used for statistical analysis.

Since antigen presentation by dendritic cells through MHC-II and expression of costimulatory molecules is a central pathway in triggering allorecognition and alloimmune responses, phenotypic differences between young and old naïve splenic B6 dendritic cells were determined for a more detailed understanding of age-dependent differences in allostimulatory characteristics. Flow cytometric staining for I-A^b, CD40, CD80, and CD86 revealed marked increases on old DCs in surface expression of these markers for maturation and antigen presentation (*Figure 28*).

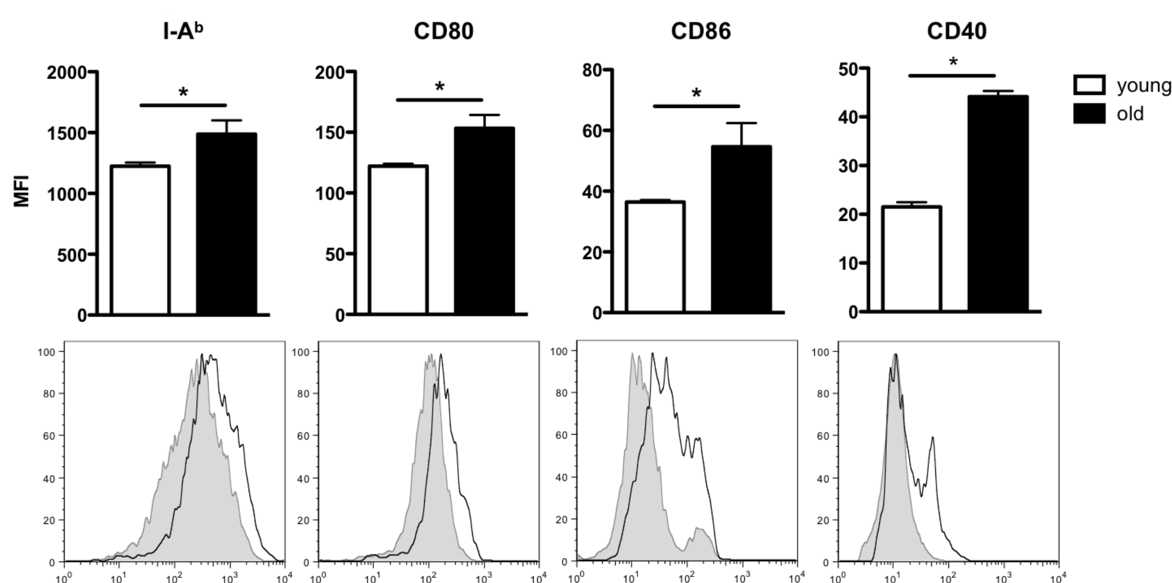

Figure 28: Old splenic sorted DCs show significantly increased expression of I-A^b (MHC-II), CD80, CD86, and CD40 as markers for DC maturation and activation.
Splenocytes from young or old naïve B6 mice were isolated and preselected by magnetically depleting the splenic B cell population using B220-PE antibodies, anti-PE microbeads, LS columns and a MidiMACS separator in order to limit cell death and preactivation due to extended sorting times. Cells were then stained for CD11c and sorted using a FACSAria II flow sorter. Following surface staining for I-A^b and costimulatory molecules, cells were analyzed using flow cytometry. Results were expressed as mean (geometric) fluorescence intensity (MFI). N=3 per group; *, $p<0.05$; representative histogram plots (grey tinted: young, black line: old) shown.

These observations give insight into intrinsic functional modifications of DCs with immunosenescence, in relation to danger signals that occur under conditions such as tissue damage through ischemia. They emphasize the importance of dendritic cells among passenger leukocytes in mediating donor-age-dependent acceleration of allograft rejection.

6 DISCUSSION

Best clinical results in solid organ transplantation have generally been achieved when organs from young donors were transplanted into young recipients, whereas on the other hand the increased use of old organs has been correlated with unfavorable clinical outcomes including increased frequencies of acute rejection in the early post-transplant period, especially in young recipients [324,339]. Clinical outcome, however, was improved when old organs were transplanted into old recipients, emphasizing results gained from allocation strategies such as the Eurotransplant Senior Program [328].

Some authors have attributed this clinical phenomenon to decreased effector, but preserved memory and regulatory T cell functions in older recipients [165,340]. Conversely, these reports could also serve as an explanation for elevated rates of acute rejection episodes in young graft recipients regardless of donor age. In a recent large-scale retrospective database analysis, increased frequencies of acute rejection episodes were found in all age cohorts of recipients when transplanting old donor organs, suggesting an impact independent of donor age on graft survival [282].

Old donor organs per se have been correlated with impaired graft function post transplantation as a result of both limited functional reserve and increased immunological damage [294,311,312]. Physiological alterations related to aging as well as cell senescence have been put forward as non-immunological factors influencing graft survival by also limiting repair mechanisms that are already impaired by transplantation-related stress factors [341,342]. Furthermore, increased susceptibility to tissue damage during the transplantation procedure including cold and warm ischemia in combination with impaired repair mechanisms may provide a pro-inflammatory milieu with increased DAMP signaling that elicits and perpetuates secondary allospecific immune responses [313,314]. Moreover, at least in theory, the magnitude of the alloantibody response as well as the breadth of the specificity of this response may be enlarged to include cryptic self-antigens exposed during necrotic cell death following transplantation of older grafts.

Passenger leukocytes residing in the graft that are transplanted along with the donor organ prime allospecific immune responses within the recipient. A prominent role among these cells has been attributed to antigen-presenting cells, particularly dendritic cells, although there is an ongoing debate on potential tolerogenic capacities of passenger dendritic cells [343]. Increased numbers of intragraft passenger leukocytes, and particularly DCs, with

increased expression of co-stimulatory molecules have been reported [344], along with results suggesting increased immunogenic properties of aged DCs in general [194,316–318].

Whether, especially in terms of early immune responses after transplantation, parenchyma-related or passenger leukocyte-related factors are predominant in mediating donor-age-related clinical outcomes after solid organ transplantation is thus still a matter of ongoing debate. The aim of this work was to discriminate relevant mechanisms of donor-age-dependent alloimmune responses and to shed light on the differential contributions of these concepts to the clinical phenomen. Using a fully MHC-mismatched murine cardiac transplantation model, this work demonstrated (1) that allografts from older donors were rejected significantly faster and displayed higher ISHLT rejection scores, (2) that the more potent recipient immune response was reflected by a higher frequency of $CD8^{+}IFN\text{-}\gamma^{+}$ T cells and $CD8^{+}$ effector T cells, significantly increased frequencies of alloreactive IFN-γ-producing recipient splenocytes as well as a more pronounced proliferative response of recipient splenocytes after re-stimulation with donor type antigen, and (3) that the depletion of old passenger leucocytes, in particular dendritic cells, muted the age-dependent differences in graft survival, histopathological grading and systemic immune responses.

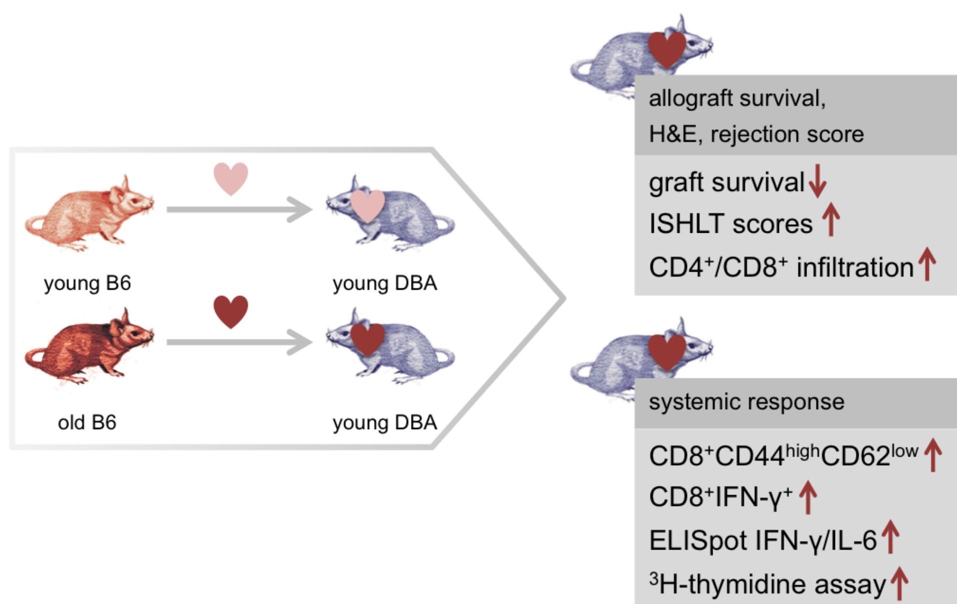

Figure 29: Young or old mismatched cardiac allografts were transplanted into young recipients to monitor graft survival and to characterize alloresponses elicited in this setting. Allografts procured from old donors were rejected significantly faster, displayed higher ISHLT rejection scores and showed increased intragraft infiltration by $CD4^{+}$ and $CD8^{+}$ cells. The more potent alloimmune response mounted by recipients of old allografts was reflected by a higher frequency of $CD8^{+}IFN\text{-}\gamma^{+}$ T cells and $CD8^{+}$ effector T cells among splenocytes, significantly increased frequencies of alloreactive IFN-γ- producing recipient splenocytes as well as a more pronounced proliferative response of recipient splenocytes after re-stimulation with donor type antigen.

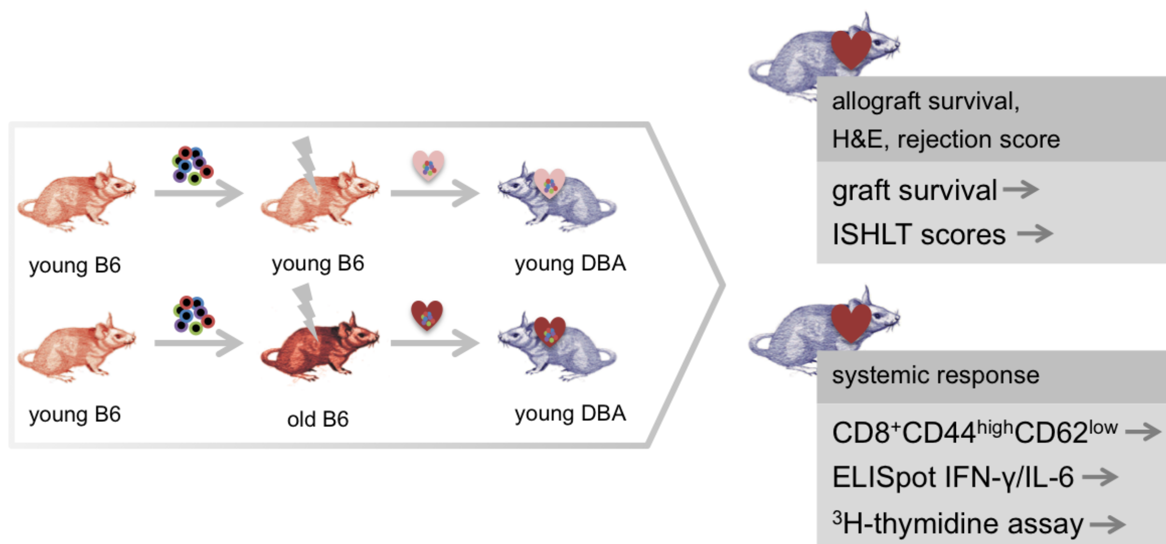

Figure 30: Chimeric donor animals with young or old hearts populated by young passenger leukocytes were generated by total body irradiation and subsequent syngeneic bone marrow transplantation prior to graft procurement and transplantation of chimeric cardiac allografts into young recipients. Age-related differences observed with naïve donor animals in terms of graft survival, local cellular infiltration and systemic alloimmune responses were abolished.

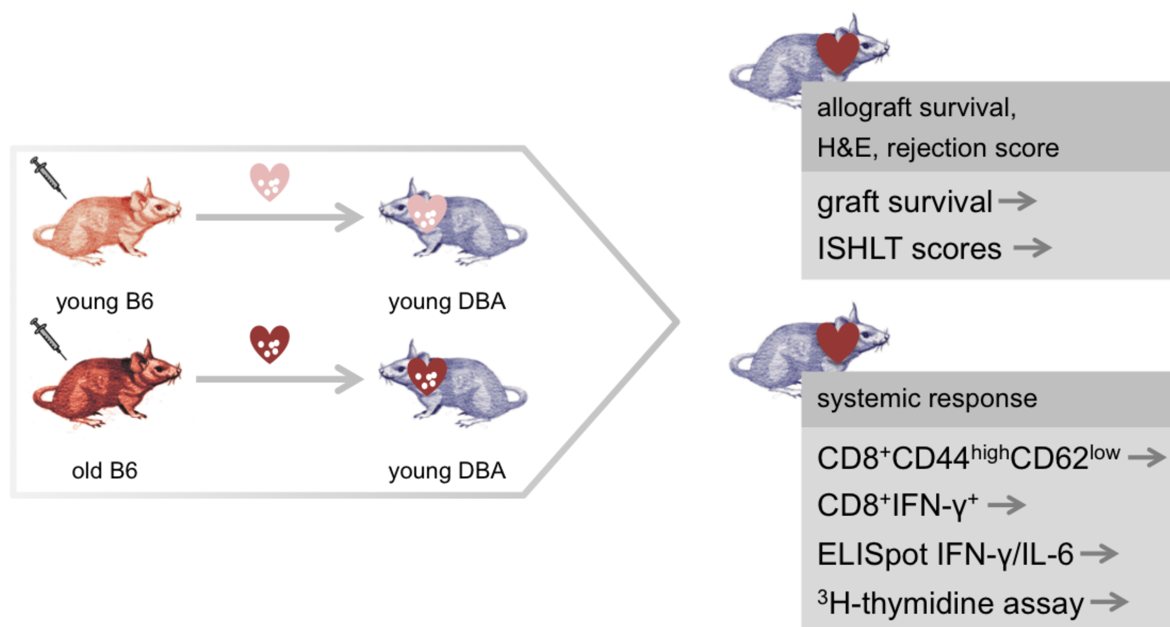

Figure 31: To generate young and old donor animals with hearts depleted of dendritic cells, mice were treated with liposomal clodronate prior to transplantation into young mismatched recipients. Depletion of dendritic cells in old cardiac allografts by pretreatment with liposomal clodronate prolonged graft survival to time periods observed in young grafts. Systemic alloimmune responses and local cellular rejection did not differ between the two groups.

6.1 Accelerated rejection of old allografts in-vivo with more potent alloimmune responses

Following transplantation into young recipients, fully MHC-mismatched cardiac allografts procured from old donor mice were rejected in an accelerated fashion, accompanied by enhanced alloimmune responses correlating with both histomorphological and systemic findings. Local lymphocellular infiltration of grafts was more pronounced and frequencies of $CD8^{+}CD44^{+}CD62^{low}$ effector T cells and $CD8^{+}$ IFN-γ^{+} T cells among recipient splenocytes were increased. Recipient splenocytes showed elevated frequencies of IFN-γ-producing alloreactive splenocytes (ELISpot) and increased proliferative responses (^{3}H thymidine incorporation) upon restimulation with donor-type antigen. For evaluation of histopathological infiltration and systemic alloresponses prior to complete allorejection, day 7 post transplantation was chosen following preliminary experiments.

The results generated with this model were thus able to reproduce the clinical observation that old organs transplanted into young recipients show increased frequencies of acute rejection episodes. Furthermore, these results are consistent with data generated using different experimental transplant models such as a rat model of chronic kidney rejection [312]. Shen et al. [340], however, did not observe a statistically significant difference in survival of skin grafts procured from old versus young donors.

As skin grafts are not vascularized, relevant effects of passenger leukocytes may not be represented adequately in the model used by Shen et al. The technique of heterotopic cervical cardiac transplantation, on the other hand, allows transplantation of fully vascularized grafts that – unlike models of cardiac or renal transplantation into the abdominal cavity – allows regular and direct assessment of graft function and graft survival through palpation. Unspecific injuries related to IRI may be more pronounced in vascularized organs, potentially causing an increased activation and immunogenicity of cardiac DCs and their subsequent migration to recipient secondary lymphoid organs. Furthermore, whereas dermal DCs are equivalent to cardiac DCs, Langerhans cells represent a cell population that is unique to the skin [41]. Interestingly, specific alloimmunogenic properties of skin-derived Langerhans cells comparable to heart-derived DCs have been reported [345].

Differences in procurement procedures should also be taken into account. Whereas skin grafts are procured from living animals, cardiac allografts transplanted in the present model were procured following exsanguination of the donor animal. When translating data

generated with the present model into clinical settings, consideration should be given to the fact that clinical observations have been made with transplantation of organs procured from brain-dead donors. In experimental and clinical studies, complex immunological and physiological consequences have been described following brain death [346].

With transplantation of fully MHC-mismatched grafts without any subsequent immunosuppressive regimen, the technique used in the present work represents a model of severe acute allograft rejection. With a graft survival of less than two weeks, observed differences of two days in allograft survival represent marked differences. Also, initial differences in functional reserve or mismatching in donor/recipient size are likely to be superseded by such vigorous alloimmune responses. The age-related differences in systemic alloimmune responses were mainly based on $CD8^+$ effector T cells, as indicated by increased frequencies of $CD8^+$ effector T cells and increased frequencies of IFN-γ-producing alloreactive splenocytes in ELISpot assays and increased frequencies of $CD8^+IFN\text{-}\gamma^+$ T cells among recipient splenocytes. Subsets of $CD4^+$ T cells, including regulatory T cells, on the other hand, did not differ between the two groups.

These results gained with a model of undampened acute rejection underline the importance of $CD8^+$ T cells in early phases of (direct) allorecognition and allograft rejection. In line with this, $CD4^+$ T cells predominantly mediating chronic allograft vasculopathy through B cells and mechanisms of regulatory T cells seem to be of subordinate relevance in mediating the observed age-dependent differences. Using a single MHC-mismatch model of chronic allograft rejection which allowed to study the isolated role of chronic graft rejection by $CD4^+$ T-cells, it was recently shown that recipients of old cardiac allografts demonstrated a more pronounced $CD4^+$ T-cell activation associated with an expansion of regulatory T cells both in the spleen and in the graft, without significant differences in graft survival or in histopathology [344]. As the procedure-related tissue damage should be similar in both models, the higher degree of MHC-mismatch used in the present work seems to uncover age-related immunogenic mechanisms which result in accelerated graft rejection and enhanced systemic alloimmune responses, pointing to immunological processes as mediators of more severe graft rejection when transplanting old donor organs, at least in the setting of acute rejection.

6.2 THE CONTRIBUTION OF PASSENGER LEUKOCYTES TO DONOR-AGE-DEPENDENT ALLOIMMUNE RESPONSES

To test whether differences in alloimmune responses were mediated by aged passenger leukocytes within the transplanted grafts, prospective young and old donor mice were lethally irradiated and reconstituted with bone marrow procured from young syngeneic mice. This model of complete depletion of intragraft leukocytes through lethal total-body irradiation and subsequent reconstitution with leukocytes derived from transplantated bone marrow has been established and used extensively by other investigators in the analysis of repopulation and residual APCs using flow cytometry and RT-PCR [347,348]. For this work, repopulation of prospective allografts was confirmed by immunohistochemical stainings (*Figure 15*).

This protocol generates mice with chimeric hearts consisting of either young parenchyma and young passenger immune cells or of old parenchyma and young passenger immune cells. As transplantation of these chimeric hearts eliminates the age of passenger leukocytes as an influencing factor, any age-dependent differences in allograft survival or systemic alloimmune response would be mediated by aged parenchyma. Previously observed age-related differences in terms of graft survival, local cellular infiltration and systemic alloimmune responses were abolished in this setting, suggesting that aged parenchyma per se was not responsible for age-dependent differences when transplanting cardiac allografts from untreated young or old donors.

Although successful repopulation of cardiac allografts was confirmed six weeks after bone marrow transplantation by immunohistochemical stainings for CD11c with no differences between the two groups, a potential impact of aged parenchyma on the ability of passenger leukocytes and particularly dendritic cells to migrate into old cardiac tissue may still remain. As less effective repopulation with passenger leukocytes may result in dampened immunogenicity of old allografts, effects of aged parenchyma on migratory capacities may have presented a confounding factor. Increased numbers of dendritic cells, on the other hand, have been reported in untreated old murine hearts [344].

The procedure of bone marrow transplantation may have presented a potential nonspecific stimulus for newly generated bone marrow-derived passenger leukocytes, resulting in an advanced state of activation and maturation and masking any age-dependent differences.

Furthermore, since prospective donor mice were exposed to total-body irradiation, concomitant tissue damage conferred not exclusively to the cardiac tissue may provide a pro-inflammatory milieu with increased DAMP signaling. This may lead to unspecific activation of passenger leukocytes, with any effects of the parenchyma related to age per se being dampened. Conversely, damage caused to potentially more susceptible old parenchyma may have limited stimulatory capacities present in non-irradiated old parenchyma. Both young and old prospective donors, however, received bone marrow procured exclusively from young syngeneic animals, and parameters related to procedure-related damage were kept consistently at comparable levels in both groups.

6.3 The role of dendritic cells among passenger leukocytes

Passenger leukocytes residing in the transplanted allograft are of crucial importance in triggering host alloimmune responses upon encounter with allogeneic tissue. Especially the impact of donor-derived antigen-presenting cells (APCs), particularly dendritic cells, has been studied in this context, as they are able to trigger primary allospecific immune responses via direct alloantigen presentation to recipient responder cells within secondary lymphatic tissue [7,349]. This pathway is of particular importance in early episodes of acute rejection triggered through direct allorecognition. The pivotal immunogenic properties of dendritic cells in this setting have been highlighted in previous studies: the immunogenicity of long-surviving renal allografts retransplanted into secondary recipients was restored by the injection of small numbers of donor strain dendritic cells, whereas neither the passenger volume of donor strain blood nor enriched populations of T or B lymphocytes or macrophages were able to mount comparable episodes of acute rejection [7].

Previous observations regarding the impact of aging on dendritic cells in models of transplantation or allostimulation include enhanced T cell proliferation in syngeneic as well as allogeneic settings upon co-culture of APCs from healthy elderly donors with purified T cells from young donors [317]. More recently, unmanipulated hearts procured form old mice were shown to contain elevated numbers of passenger leucocytes, in particular $CD4^{+}/CD8^{+}$ T cells and dendritic cells [344].

Passenger leukocytes comprise several different cell subsets, such as T and B lymphocytes, macrophages, NK cells and dendritic cells. To further identify the differential effects of these

cell populations in mediating the observed age-dependent effects on allograft survival and alloimmune responses, old and young donor mice were treated with liposomal clodronate prior to organ procurement. Administered in a liposomal formulation, this bisphosphonate is selectively taken up by phagocytic leukocytes, particularly dendritic cells and macrophages [350,351]. Following intracellular disruption of the liposomes through lysosomal phospholipases, accumulating clodronate leads to selective apoptosis of the target cell [352]. Since cardiac capillaries are not permeable to liposomal clodronate [336,337], the primary effect in the present model is thus restricted to a reliable depletion of circulating cells. As resident macrophage turnover is much slower than that of dendritic cells [338], liposomal clodronate thus had no sizable effect on cardiac macrophage populations after one week of treatment, however it subtotally depleted intragraft DCs.

This depletion, as shown by the results of this work and other reports [351,353], however, is not complete. Other techniques targeting passenger dendritic cells might be more specific and result in a more extensive depletion of dendritic cells [354]. In knock-out models, on the other hand, the absence of DCs from birth results in compensatory expansion of other cell types. DTR transgenic mice might not exclusively deplete DCs, while neutrophilia and monocytosis are common confounding factors in this model. Moreover, none of these strains are commercially available at 18 months of age.

Depletion of dendritic cells in old cardiac allografts by pretreatment with liposomal clodronate prolonged graft survival to time periods observed in young grafts, indicating that old DCs were needed to mediate more potent immune responses resulting in a more rapid rejection of older grafts. In addition to abolished differences in graft survival between young and old cardiac allografts, systemic alloimmune responses and local cellular rejection did not differ between the two groups. Taken together with the insights gained from untreated and bone marrow transplanted donor mice, these data indicate that it is the age of passenger dendritic cells rather than lymphocyte and macrophage age that drives donor-age-dependent effects on alloimmunity.

6.4 MECHANISMS OF INCREASED ALLOSTIMULATORY CAPACITY OF DENDRITIC CELLS WITH AGING

To gain further mechanistic insights into DC-mediated donor-age-dependent alloimmune responses, age-specific priming capacities and phenotypic characteristics of DCs were tested *in-vitro*. Flow-sorted splenic CD11c^{+} dendritic cells from young and old C57BL/6 animals were used as allogeneic stimulators of splenocytes from young naïve DBA/2 mice. In ELISpot and ^{3}H thymidine proliferation assays, aged B6 dendritic cells elicited an increased alloimmune response, as assessed by frequencies of alloreactive IFN-γ-producing splenocytes and by responder cell proliferation, respectively. Moreover, this increased allostimulatory capacity of old DCs correlated with an increased expression of costimulatory molecules CD80, CD86, and CD40 and with an increased expression of MHC-II on aged DCs, indicating elevated levels of activation and maturation of splenic dendritic cells in old mice.

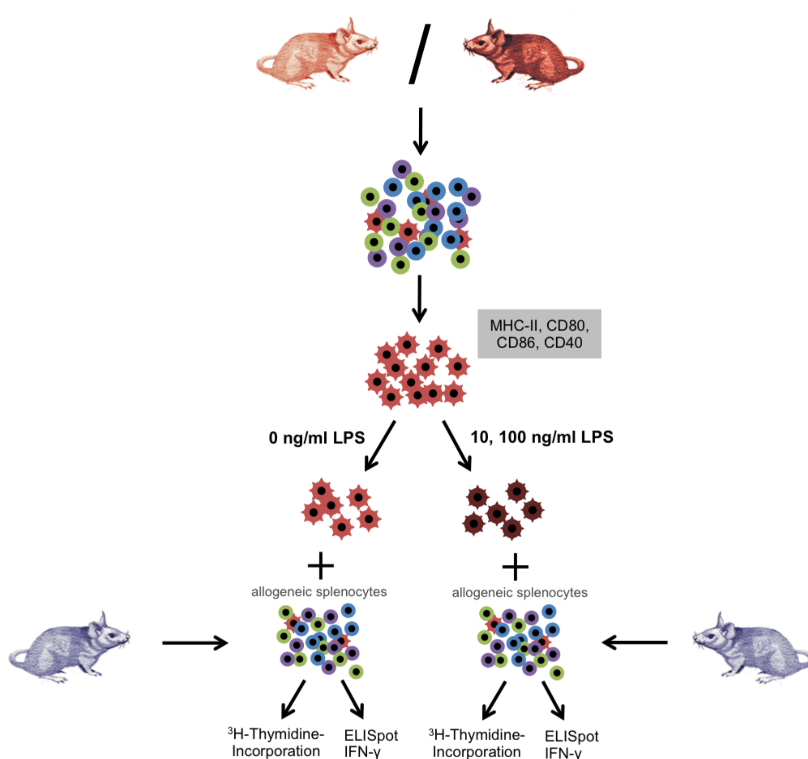

Figure 32: *In-vitro* characterization of allostimulatory priming capabilities of young and old dendritic cells. Stimulation with old DCs lead to significantly higher frequencies of IFN-γ-producing cells and higher proliferative responses among allogeneic splenocytes (*Figures 26 and 27*). Expression of MHC-II and costimulatory molecules was increased on old DCs (*Figure 28*).

These findings are consistent with results by Ordemann et al. showing increased immunogenicity of aged APCs in an experimental model of graft-versus-host-disease [316]. Shen et al., in contrast, detected reduced expression of MHC-II and costimulatory molecules on dendritic cells in aged mice [340]. The same group, however, showed well-preserved priming capabilities of donor APCs with aging. Several other studies reported enhanced antigen presentation of dendritic cells with aging [194,317,318], and comparable stimulatory capacities of MDDCs from young and aged subjects have been reported in humans [205].

Despite providing compelling evidence for increased allostimulatory capacities of aged dendritic cells, these results generated with splenic dendritic cells may not be representative for intragraft DCs residing in the cardiac tissue *in-vivo*. As mentioned above, evidence suggests complex age-dependent changes in dendritic cells depending on phenotypical subsets and tissue of residence. Furthermore, as murine dendritic cells can be further characterized as lymphoid, myeloid and plasmacytoid dendritic cells using surface markers such as CD4, CD8, CD11b, CD205 and mPDCA-1 in addition to CD11c, differential contributions of these subsets to age-dependent differences in allostimulatory capacities could not be discriminated with the present experimental design.

Taken together, this work has found compelling evidence that in mice, age-dependent differences in graft survival as well as local and systemic characteristics of resulting alloimmune responses are driven by phenotypic and functional modifications of dendritic cells with aging. Using the same read-out, differences between recipients of young and old cardiac allografts were no longer detectable when eliminating differences in the age of passenger leukocytes or when depleting intragraft dendritic cells. After ruling out age-dependent changes in parenchymal tissue as mediators of donor-age-dependent alloimmune responses, dendritic cells were identified as the main cellular correlate conferring differences in graft survival and allospecific immune responses. Furthermore, results with isolated splenic DCs demonstrated that these *in-vivo* results correlated with increased antigen presentation and enhanced allostimulatory capacities of old dendritic cells *in-vitro*.

Nevertheless, mechanisms underlying the DC-mediated effects of donor age have not been uncovered completely and interactions between dendritic cells and additional immunological factors represented by the parenchymal tissue remain an intricate network of complex interactions. When studying alloimmune responses, the impact of graft damage caused by the transplant procedure, including ischemia-reperfusion injury with its subsequent major cascades of danger signaling, needs to be taken into account. Thus, it may be conceivable that differences observed in this model were not due to intrinsic age-dependent modifications of dendritic cells in parallel to immunosenescence, but to an increased response of old DCs to DAMP signaling, potentially perpetuated by parenchymal tissue that releases and displays increasing amounts of respective factors with aging. Young and old DCs pretreated with 100 ng/ml of LPS prior to cocultivation with allogeneic responder cells no longer showed significant differences in allostimulatory properties in ELISpot assays (*Figure 26*), thus

displaying characteristics in favor of this concept. LPS as a potent agonist of toll-like receptor 4 causes activation and maturation of dendritic cells.

The majority of differences between young and old dendritic cells in allostimulatory characteristics, however, were preserved regardless of preactivation with various concentrations of LPS. Old dendritic cells also showed increased expression of CD40, CD80, CD86, and MHC-II without prior unspecific activation, a result in favor of the concept of intrinsic effects of aging on dendritic cells that lead to altered functional characteristics including elevated preactivation even in an idle state, resulting in donor-age-dependent alterations of alloimmune responses. On the other hand, cell isolation and cell-sorting may present an unspecific stimulus for dendritic cells regardless of subsequent preactivation with LPS, thus leading to characterization of untreated dendritic cells already in a state of increased DAMP signaling.

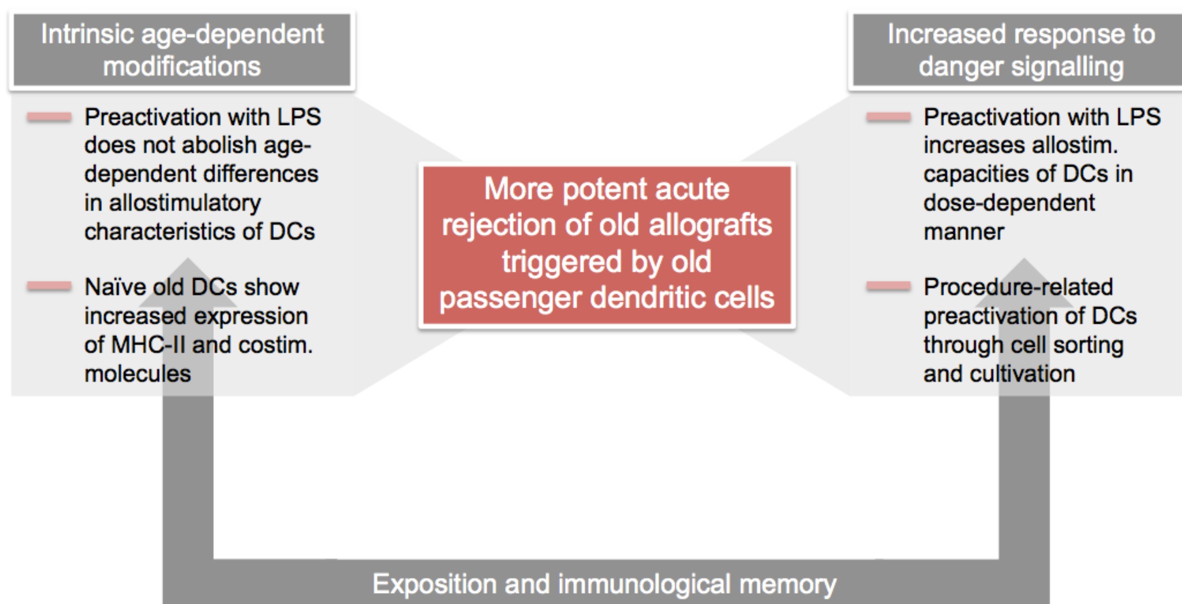

Figure 33: Possible mechanisms underlying DC-mediated effects of donor age on allograft rejection in the present model. Evidence collected in this work suggests that age-dependent differences in graft survival as well as local and systemic characteristics of resulting alloimmune responses were driven by modifications of dendritic cells with aging. These modifications may represent intrinsic age-dependent alterations due to immunosenescence. An increased response of old DCs to danger signaling potentially perpetuated by parenchymal tissue, on the other hand, may lead to increased immunogenicity of old allografts. Furthermore, old animals may have experienced increased exposure to various pathogenic factors, resulting in altered phenotypic and functional characteristics of old DCs in alloimmune responses.

Tightly correlated with aging in terms of immunobiology is the exposure of the immune system to various pathogenic factors over the course of a life span resulting in numerous adaptations not only in terms of generation of memory B and T cells [355]. This may also lead to altered responses of dendritic cells in settings of alloimmunity. It is important to note

that, to minimize these external factors, all aged mice were procured from the National Institutes of Aging (NIA). NIA maintains colonies of barrier-raised, specific pathogen-free (SPF) rodents specifically for use in aging research. For institutional housing, a viral antigen-free facility complying with AAALAC (Association for Accreditation and Assessment of Laboratory Animal Care) standards was used.

6.5 Implications and prospective studies

In sum, the evidence presented in this work suggests that donor-age-related effects on allograft survival and alloimmune response are due to aged passenger dendritic cells within the graft rather than aged parenchyma, and that aged dendritic cells trigger an enhanced primary allospecific immune response, unlike their young counterparts. These observations may have relevant clinical implications, as they point to dendritic cells as potential therapeutic targets for protocols of donor pre-treatment prior to organ procurement, during cold ischemia or even immediately following reperfusion. Also, as these results stress the important independent effect of donor age on transplant outcome, giving more consideration to donor age in allocation schemes may prove beneficial in clinical settings. As effects of donor age have been observed particularly in the early post-transplant period, immunosuppressive regimens may need to be reconsidered with particular attention to the age of the respective donor.

Further experimental studies are needed to characterize intragraft dendritic cells *in-vivo* in their respective biological setting. Analysis of gene expression may be able to reveal functional properties of intragraft, splenic, and circulating dendritic cells prior to and following transplantation. Experiments using syngeneic and allogeneic models in different degrees of mismatch may be able to uncover further interactions of dendritic cells and parenchymal tissue *in-vivo*. Adoptive transfer of young and old donor-type dendritic cells may prove that dendritic cells are both needed and sufficient to confer age-dependent differences in transplant outcome. Analysing DC cocultivation with allogeneic responder cells on a protein level through ELISA and Luminex assays and experiments using transwell plates may allow the focus to be shifted to contact-independent pathways of interaction between old and young dendritic cells and responder cells in allogeneic settings.

7 REFERENCES

1. Collins AJ, Foley RN, Chavers B, et al. United States Renal Data System 2011 Annual Data Report: Atlas of chronic kidney disease & end-stage renal disease in the United States. Am J Kidney Dis 2012;59:A7, e1–420.
2. Wolfe RA, Ashby VB, Milford EL, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med 1999;341:1725–30.
3. Wolfe RA, Roys EC, Merion RM. Trends in organ donation and transplantation in the United States, 1999-2008. Am J Transplant 2010;10:961–72.
4. Warrens AN, Lombardi G, Lechler RI. Presentation and recognition of major and minor histocompatibility antigens. Transpl Immunol 1994;2:103–7.
5. Smith P a, Brunmark A, Jackson MR, Potter T a. Peptide-independent recognition by alloreactive cytotoxic T lymphocytes (CTL). J Exp Med 1997;185:1023–33.
6. Weber DA, Terrell NK, Zhang Y, et al. Requirement for peptide in alloreactive CD4+ T cell recognition of class II MHC molecules. J Immunol 1995;154:5153–64.
7. Lechler RI, Batchelor JR. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. J Exp Med 1982;155:31–41.
8. Benichou G, Takizawa PA, Olson CA, McMillan M, Sercarz EE. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. J Exp Med 1992;175:305–8.
9. Demaria S, Bushkin Y. Soluble HLA proteins with bound peptides are released from the cell surface by the membrane metalloproteinase. Hum Immunol 2000;61:1332–8.
10. Denton MD, Geehan CS, Alexander SI, Sayegh MH, Briscoe DM. Endothelial cells modify the costimulatory capacity of transmigrating leukocytes and promote CD28-mediated CD4(+) T cell alloactivation. J Exp Med 1999;190:555–66.
11. Baker RJ, Hernandez-Fuentes MP, Brookes PA, et al. Loss of direct and maintenance of indirect alloresponses in renal allograft recipients: implications for the pathogenesis of chronic allograft nephropathy. J Immunol 2001;167:7199–206.
12. Zhai Y, Meng L, Gao F, Busuttil RW, Kupiec-Weglinski JW. Allograft rejection by primed/memory CD8+ T cells is CD154 blockade resistant: therapeutic implications for sensitized transplant recipients. J Immunol 2002;169:4667–73.

13. Herrera OB, Golshayan D, Tibbott R, et al. A novel pathway of alloantigen presentation by dendritic cells. J Immunol 2004;173:4828–37.
14. Game DS, Rogers NJ, Lechler RI. Acquisition of HLA-DR and costimulatory molecules by T cells from allogeneic antigen presenting cells. Am J Transplant 2005;5:1614–25.
15. Morelli AE, Larregina AT, Shufesky WJ, et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood 2004;104:3257–66.
16. Hsu KC, Chida S, Geraghty DE, Dupont B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. Immunol Rev 2002;190:40–52.
17. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002;295:2097–100.
18. Vilches C, Parham P. Do NK-cell receptors and alloreactivity affect solid organ transplantation? Transpl Immunol 2006;17:27–30.
19. Pietra BA, Wiseman A, Bolwerk A, Rizeq M, Gill RG. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. J Clin Invest 2000;106:1003–10.
20. Vella JP, Vos L, Carpenter CB, Sayegh MH. Role of indirect allorecognition in experimental late acute rejection. Transplantation 1997;64:1823–8.
21. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. Nat Immunol 2002;3:844–51.
22. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. J Immunol 1999;162:352–8.
23. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allorejection. J Exp Med 1996;184:2013–8.
24. Steele DJ, Laufer TM, Smiley ST, et al. Two levels of help for B cell alloantibody production. J Exp Med 1996;183:699–703.
25. Le Moine A, Goldman M, Abramowicz D. Multiple pathways to allograft rejection. Transplantation 2002;73:1373–81.
26. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med 1969;280:735–9.
27. Crespo M, Pascual M, Tolkoff-Rubin N, et al. Acute humoral rejection in renal allograft recipients: I. Incidence, serology and clinical characteristics. Transplantation 2001;71:652–8.

28. Mauiyyedi S, Pelle PD, Saidman S, et al. Chronic humoral rejection: identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. J Am Soc Nephrol 2001;12:574–82.
29. McKenna RM, Takemoto SK, Terasaki PI. Anti-HLA antibodies after solid organ transplantation. Transplantation 2000;69:319–26.
30. Einecke G, Sis B, Reeve J, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant 2009;9:2520–31.
31. Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. Nat Rev Immunol 2005;5:807–17.
32. Jin Y-P, Singh RP, Du Z-Y, et al. Ligation of HLA class I molecules on endothelial cells induces phosphorylation of Src, paxillin, and focal adhesion kinase in an actin-dependent manner. J Immunol 2002;168:5415–23.
33. Bian H, Harris PE, Reed EF. Ligation of HLA class I molecules on smooth muscle cells with anti-HLA antibodies induces tyrosine phosphorylation, fibroblast growth factor receptor expression and cell proliferation. Int Immunol 1998;10:1315–23.
34. al-Hussein KA, Talbot D, Proud G, Taylor RM, Shenton BK. The clinical significance of post-transplantation non-HLA antibodies in renal transplantation. Transpl Int 1995;8:214–20.
35. Rosenberg AS, 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.
36. Taylor AL, Watson CJE, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol 2005;56:23–46.
37. Palm NW, Medzhitov R. Pattern recognition receptors and control of adaptive immunity. Immunol Rev 2009;227:221–33.
38. Medzhitov R, Janeway C a. Decoding the patterns of self and nonself by the innate immune system. Science 2002;296:298–300.
39. Murphy SP, Porrett PM, Turka L a. Innate immunity in transplant tolerance and rejection. Immunol Rev 2011;241:39–48.
40. Rocha PN, Plumb TJ, Crowley SD, Coffman TM. Effector mechanisms in transplant rejection. Immunol Rev 2003;196:51–64.
41. Shortman K, Liu Y-J. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151–61.

42. Kroemer A, Edtinger K, Li XC. The innate natural killer cells in transplant rejection and tolerance induction. Curr Opin Organ Transplant 2008;13:339–43.
43. Hirohashi T, Chase CM, Della Pelle P, et al. A novel pathway of chronic allograft rejection mediated by NK cells and alloantibody. Am J Transplant 2012;12:313–21.
44. Li L, Okusa MD. Macrophages, dendritic cells, and kidney ischemia-reperfusion injury. Semin Nephrol 2010;30:268–77.
45. Zhai Y, Shen X, O'Connell R, et al. Cutting edge: TLR4 activation mediates liver ischemia/reperfusion inflammatory response via IFN regulatory factor 3-dependent MyD88-independent pathway. J Immunol Am Assoc Immnol; 2004;173:7115–9.
46. McKay D, Shigeoka A, Rubinstein M, Surh C, Sprent J. Simultaneous deletion of MyD88 and Trif delays major histocompatibility and minor antigen mismatch allograft rejection. Eur J Immunol Wiley Online Library; 2006;36:1994–2002.
47. Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 2007;317:666–70.
48. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5:953–64.
49. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011;11:723–37.
50. Gao W, Topham PS, King JA, et al. Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. J Clin Invest 2000;105:35–44.
51. Jose MD, Ikezumi Y, van Rooijen N, Atkins RC, Chadban SJ. Macrophages act as effectors of tissue damage in acute renal allograft rejection. Transplantation 2003;76:1015–22.
52. Kirk AD, Hale D a, Mannon RB, 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.
53. Kitchens WH, Chase CM, Uehara S, et al. Macrophage depletion suppresses cardiac allograft vasculopathy in mice. Am J Transplant 2007;7:2675–82.
54. Yang J, Reutzel-Selke A, Steier C, et al. Targeting of macrophage activity by adenovirus-mediated intragraft overexpression of TNFRp55-Ig, IL-12p40, and vIL-10 ameliorates adenovirus-mediated chronic graft injury, whereas stimulation of macrophages by overexpression of IFN-gamma accelerates chron. J Am Soc Nephrol 2003;14:214–25.

55. Liu W, Xiao X, Demirci G, Madsen J, Li XC. Innate NK cells and macrophages recognize and reject allogeneic nonself in vivo via different mechanisms. J Immunol 2012;188:2703–11.
56. Shresta S, MacIvor DM, Heusel JW, Russell JH, Ley TJ. Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells. Proc Natl Acad Sci U S A 1995;92:5679–83.
57. Trapani JA. Dual mechanisms of apoptosis induction by cytotoxic lymphocytes. Int Rev Cytol 1998;182:111–92.
58. Martín-Fontecha A, Thomsen LL, Brett S, et al. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat Immunol 2004;5:1260–5.
59. Zingoni A, Sornasse T, Cocks BG, et al. Cross-talk between activated human NK cells and CD4+ T cells via OX40-OX40 ligand interactions. J Immunol 2004;173:3716–24.
60. Degli-Esposti MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat Rev Immunol 2005;5:112–24.
61. Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225–74.
62. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495–502.
63. Kim J, Chang CK, Hayden T, et al. The activating immunoreceptor NKG2D and its ligands are involved in allograft transplant rejection. J Immunol 2007;179:6416–20.
64. Kroemer A, Xiao X, Degauque N, et al. The innate NK cells, allograft rejection, and a key role for IL-15. J Immunol 2008;180:7818–26.
65. Van Bergen J, Thompson A, Haasnoot GW, et al. KIR-ligand mismatches are associated with reduced long-term graft survival in HLA-compatible kidney transplantation. Am J Transplant 2011;11:1959–64.
66. Mauiyyedi S, Crespo M, Collins a B, et al. Acute humoral rejection in kidney transplantation: II. Morphology, immunopathology, and pathologic classification. J Am Soc Nephrol 2002;13:779–87.
67. Hirayama S, Shiraishi T, Shirakusa T, Higuchi T, Miller EJ. Prevention of neutrophil migration ameliorates rat lung allograft rejection. Mol Med 2006;12:208–13.
68. Kreisel D, Sugimoto S, Zhu J, et al. Emergency granulopoiesis promotes neutrophil-dendritic cell encounters that prevent mouse lung allograft acceptance. Blood 2011;118:6172–82.
69. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol 2005;6:135–42.

70. Lu L-F, Lind EF, Gondek DC, et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature 2006;442:997–1002.
71. De Vries VC, Wasiuk A, Bennett K a, et al. Mast cell degranulation breaks peripheral tolerance. Am J Transplant 2009;9:2270–80.
72. Denzel A, Maus UA, Rodriguez Gomez M, et al. Basophils enhance immunological memory responses. Nat Immunol 2008;9:733–42.
73. Gauchat JF, Henchoz S, Mazzei G, et al. Induction of human IgE synthesis in B cells by mast cells and basophils. Nature 1993;365:340–3.
74. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. Nat Immunol 2008;9:310–8.
75. Almirall J, Campistol JM, Sole M, Andreu J, Revert L. Blood and graft eosinophilia as a rejection index in kidney transplant. Nephron 1993;65:304–9.
76. Martinez OM, Ascher NL, Ferrell L, et al. Evidence for a nonclassical pathway of graft rejection involving interleukin 5 and eosinophils. Transplantation 1993;55:909–18.
77. Wu T, Bond G, Martin D, et al. Histopathologic characteristics of human intestine allograft acute rejection in patients pretreated with thymoglobulin or alemtuzumab. Am J Gastroenterol 2006;101:1617–24.
78. Nakashima S, Qian Z, Rahimi S, Wasowska BA, Baldwin WM. Membrane attack complex contributes to destruction of vascular integrity in acute lung allograft rejection. J Immunol 2002;169:4620–7.
79. Benzaquen LR, Nicholson-Weller A, Halperin JA. Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. J Exp Med 1994;179:985–92.
80. Monsinjon T, Gasque P, Chan P, et al. Regulation by complement C3a and C5a anaphylatoxins of cytokine production in human umbilical vein endothelial cells. FASEB J 2003;17:1003–14.
81. Brunn GJ, Saadi S, Platt JL. Differential regulation of endothelial cell activation by complement and interleukin 1alpha. Circ Res 2006;98:793–800.
82. Strainic MG, Liu J, Huang D, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. Immunity 2008;28:425–35.
83. Castellano G, Woltman AM, Nauta AJ, et al. Maturation of dendritic cells abrogates C1q production in vivo and in vitro. Blood 2004;103:3813–20.

84. Peng Q, Li K, Wang N, et al. Dendritic cell function in allostimulation is modulated by C5aR signaling. J Immunol 2009;183:6058–68.
85. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. J Am Soc Nephrol 2007;18:1046–56.
86. Diepenhorst GMP, van Gulik TM, Hack CE. Complement-mediated ischemia-reperfusion injury: lessons learned from animal and clinical studies. Ann Surg 2009;249:889–99.
87. Pratt JR, Basheer S a, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. Nat Med 2002;8:582–7.
88. Farrar C a, Zhou W, Lin T, Sacks SH. Local extravascular pool of C3 is a determinant of postischemic acute renal failure. FASEB J FASEB; 2006;20:217–26.
89. Ligthart GJ, Corberand JX, Fournier C, et al. Admission criteria for immunogerontological studies in man: the SENIEUR protocol. Mech Ageing Dev 1984;28:47–55.
90. Geiger H, Van Zant G. The aging of lympho-hematopoietic stem cells. Nat Immunol 2002;3:329–33.
91. Beerman I, Maloney WJ, Weissmann IL, Rossi DJ. Stem cells and the aging hematopoietic system. Curr Opin Immunol Elsevier Ltd; 2010;22:500–6.
92. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. Nat Med 1996;2:1011–6.
93. Chen J, Astle CM, Harrison DE. Genetic regulation of primitive hematopoietic stem cell senescence. Exp Hematol 2000;28:442–50.
94. Lansdorp PM, Dragowska W, Mayani H. Ontogeny-related changes in proliferative potential of human hematopoietic cells. J Exp Med 1993;178:787–91.
95. Morrison SJ, Prowse KR, Ho P, Weissman IL. Telomerase activity in hematopoietic cells is associated with self-renewal potential. Immunity 1996;5:207–16.
96. Hasty P. The impact energy metabolism and genome maintenance have on longevity and senescence: lessons from yeast to mammals. Mech Ageing Dev 2001;122:1651–62.
97. Reese JS, Liu L, Gerson SL. Repopulating defect of mismatch repair-deficient hematopoietic stem cells. Blood 2003;102:1626–33.
98. Nijnik A, Woodbine L, Marchetti C, et al. DNA repair is limiting for haematopoietic stem cells during ageing. Nature 2007;447:686–90.

99. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 2006;443:421–6.
100. Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A 2001;98:12072–7.
101. Pearce DJ, Anjos-Afonso F, Ridler CM, Eddaoudi A, Bonnet D. Age-dependent increase in side population distribution within hematopoiesis: implications for our understanding of the mechanism of aging. Stem Cells 2007;25:828–35.
102. De Haan G, Nijhof W, Van Zant G. Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. Blood 1997;89:1543–50.
103. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. Mech Ageing Dev 2000;117:57–68.
104. Taraldsrud E, Grøgaard HK, Solheim S, et al. Age and stress related phenotypical changes in bone marrow CD34+ cells. Scand J Clin Lab Invest 2009;69:79–84.
105. Cho RH, Sieburg HB, Muller-Sieburg CE. A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood 2008;111:5553–61.
106. Beerman I, Bhattacharya D, Zandi S, et al. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. Proc Natl Acad Sci U S A 2010;107:5465–70.
107. Challen GA, Boles NC, Chambers SM, Goodell MA. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell 2010;6:265–78.
108. Rossi DJ, Bryder D, Zahn JM, et al. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 2005;102:9194–9.
109. Signer RAJ, Montecino-Rodriguez E, Witte ON, McLaughlin J, Dorshkind K. Age-related defects in B lymphopoiesis underlie the myeloid dominance of adult leukemia. Blood 2007;110:1831–9.
110. Edwards BK, Howe HL, Ries LAG, et al. Annual report to the nation on the status of cancer, 1973-1999, featuring implications of age and aging on U.S. cancer burden. Cancer 2002;94:2766–92.
111. Aspinall R, Andrew D. Thymic involution in aging. J Clin Immunol 2000;20:250–6.

112. Mackall CL, Gress RE. Thymic aging and T-cell regeneration. Immunol Rev 1997;160:91–102.
113. Flores KG, Li J, Sempowski GD, Haynes BF, Hale LP. Analysis of the human thymic perivascular space during aging. J Clin Invest 1999;104:1031–9.
114. Jamieson BD, Douek DC, Killian S, et al. Generation of functional thymocytes in the human adult. Immunity 1999;10:569–75.
115. Taub DD, Longo DL. Insights into thymic aging and regeneration. Immunol Rev 2005;205:72–93.
116. Pond CM. Paracrine relationships between adipose and lymphoid tissues: implications for the mechanism of HIV-associated adipose redistribution syndrome. Trends Immunol 2003;24:13–8.
117. Berzins SP, Boyd RL, Miller JF. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J Exp Med 1998;187:1839–48.
118. Mackall CL, Bare C V, Granger LA, et al. Thymic-independent T cell regeneration occurs via antigen-driven expansion of peripheral T cells resulting in a repertoire that is limited in diversity and prone to skewing. J Immunol 1996;156:4609–16.
119. Kieper WC, Jameson SC. Homeostatic expansion and phenotypic conversion of naïve T cells in response to self peptide/MHC ligands. Proc Natl Acad Sci U S A 1999;96:13306–11.
120. Naylor K, Li G, Vallejo AN, et al. The influence of age on T cell generation and TCR diversity. J Immunol 2005;174:7446–52.
121. Fagnoni FF, Vescovini R, Mazzola M, et al. Expansion of cytotoxic CD8+ CD28- T cells in healthy ageing people, including centenarians. Immunology 1996;88:501–7.
122. Ortiz-Suárez A, Miller RA. A subset of CD8 memory T cells from old mice have high levels of CD28 and produce IFN-gamma. Clin Immunol 2002;104:282–92.
123. Batliwalla F, Monteiro J, Serrano D, Gregersen PK. Oligoclonality of CD8+ T cells in health and disease: aging, infection, or immune regulation? Hum Immunol 1996;48:68–76.
124. Weng N-P, Akbar AN, Goronzy J. CD28(-) T cells: their role in the age-associated decline of immune function. Trends Immunol 2009;30:306–12.
125. Watts TH. TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 2005;23:23–68.

126. Azuma M, Phillips JH, Lanier LL. CD28- T lymphocytes. Antigenic and functional properties. J Immunol 1993;150:1147–59.
127. Li G, Weyand CM, Goronzy JJ. Epigenetic mechanisms of age-dependent KIR2DL4 expression in T cells. J Leukoc Biol 2008;84:824–34.
128. Vallejo AN. CD28 extinction in human T cells: altered functions and the program of T-cell senescence. Immunol Rev 2005;205:158–69.
129. Monteiro J, Batliwalla F, Ostrer H, Gregersen PK. Shortened telomeres in clonally expanded CD28-CD8+ T cells imply a replicative history that is distinct from their CD28+CD8+ counterparts. J Immunol 1996;156:3587–90.
130. Borthwick NJ, Lowdell M, Salmon M, Akbar AN. Loss of CD28 expression on CD8(+) T cells is induced by IL-2 receptor gamma chain signalling cytokines and type I IFN, and increases susceptibility to activation-induced apoptosis. Int Immunol 2000;12:1005–13.
131. Fletcher JM, Vukmanovic-Stejic M, Dunne PJ, et al. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. J Immunol 2005;175:8218–25.
132. Khan N, Shariff N, Cobbold M, et al. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol 2002;169:1984–92.
133. Messaoudi I, Lemaoult J, Guevara-Patino JA, Metzner BM, Nikolich-Zugich J. Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. J Exp Med 2004;200:1347–58.
134. Pascher A, Proesch S, Pratschke J, et al. Rat cytomegalovirus infection interferes with anti-CD4 mAb-(RIB 5/2) mediated tolerance and induces chronic allograft damage. Am J Transplant 2006;6:2035–45.
135. Saule P, Trauet J, Dutriez V, et al. Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. Mech Ageing Dev 2006;127:274–81.
136. Zhang X, Fujii H, Kishimoto H, et al. Aging leads to disturbed homeostasis of memory phenotype CD8(+) cells. J Exp Med 2002;195:283–93.
137. Wallace DL, Zhang Y, Ghattas H, et al. Direct measurement of T cell subset kinetics in vivo in elderly men and women. J Immunol 2004;173:1787–94.

138. Haynes L, Eaton SM, Burns EM, Randall TD, Swain SL. CD4 T cell memory derived from young naive cells functions well into old age, but memory generated from aged naive cells functions poorly. Proc Natl Acad Sci U S A 2003;100:15053–8.
139. Effros RB. From Hayflick to Walford: the role of T cell replicative senescence in human aging. Exp Gerontol 2004;39:885–90.
140. Kaszubowska L. Telomere shortening and ageing of the immune system. J Physiol Pharmacol 2008;59 Suppl 9:169–86.
141. Effros RB, Boucher N, Porter V, et al. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: a possible cause for both in vivo and in vitro immunosenescence. Exp Gerontol 1994;29:601–9.
142. Brzezińska A, Magalska A, Szybińska A, Sikora E. Proliferation and apoptosis of human CD8(+)CD28(+) and CD8(+)CD28(-) lymphocytes during aging. Exp Gerontol 2004;39:539–44.
143. Hobbs M V, Ernst DN, Torbett BE, et al. Cell proliferation and cytokine production by CD4+ cells from old mice. J Cell Biochem 1991;46:312–20.
144. Murasko DM, Weiner P, Kaye D. Decline in mitogen induced proliferation of lymphocytes with increasing age. Clin Exp Immunol 1987;70:440–8.
145. Haynes L, Linton PJ, Eaton SM, Tonkonogy SL, Swain SL. Interleukin 2, but not other common gamma chain-binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice. J Exp Med 1999;190:1013–24.
146. Whisler RL, Beiqing L, Chen M. Age-related decreases in IL-2 production by human T cells are associated with impaired activation of nuclear transcriptional factors AP-1 and NF-AT. Cell Immunol 1996;169:185–95.
147. Sadighi Akha A a, Miller R a. Signal transduction in the aging immune system. Curr Opin Immunol 2005;17:486–91.
148. Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. J Exp Med 2004;200:1613–22.
149. Kovaiou RD, Grubeck-Loebenstein B. Age-associated changes within CD4+ T cells. Immunol Lett 2006;107:8–14.
150. Fulop T, Larbi A, Wikby A, et al. Dysregulation of T-cell function in the elderly: scientific basis and clinical implications. Drugs Aging Adis International; 2005;22:589–603.

151. Larbi A, Douziech N, Dupuis G, et al. Age-associated alterations in the recruitment of signal-transduction proteins to lipid rafts in human T lymphocytes. J Leukoc Biol 2004;75:373–81.
152. Shearer GM. Th1/Th2 changes in aging. Mech Ageing Dev 1997;94:1–5.
153. Uciechowski P, Kahmann L, Plümäkers B, et al. TH1 and TH2 cell polarization increases with aging and is modulated by zinc supplementation. Exp Gerontol 2008;43:493–8.
154. Li SP, Miller RA. Age-associated decline in IL-4 production by murine T lymphocytes in extended culture. Cell Immunol 1993;151:187–95.
155. Huang H, Patel DD, Manton KG. The immune system in aging: roles of cytokines, T cells and NK cells. Front Biosci 2005;10:192–215.
156. Zanni F, Vescovini R, Biasini C, et al. Marked increase with age of type 1 cytokines within memory and effector/cytotoxic CD8+ T cells in humans: a contribution to understand the relationship between inflammation and immunosenescence. Exp Gerontol 2003;38:981–7.
157. Yung R, Powers D, Johnson K, et al. Mechanisms of drug-induced lupus. II. T cells overexpressing lymphocyte function-associated antigen 1 become autoreactive and cause a lupuslike disease in syngeneic mice. J Clin Invest 1996;97:2866–71.
158. Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G. Aging-induced proinflammatory shift in cytokine expression profile in coronary arteries. FASEB J 2003;17:1183–5.
159. Tesar BM, Du W, Shirali AC, et al. Aging augments IL-17 T-cell alloimmune responses. Am J Transplant 2009;9:54–63.
160. Mo R, Chen J, Han Y, et al. T cell chemokine receptor expression in aging. J Immunol 2003;170:895–904.
161. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol Nature Publishing Group; 2010;11:7–13.
162. Taams LS, Akbar AN. Peripheral generation and function of CD4+CD25+ regulatory T cells. Curr Top Microbiol Immunol 2005;293:115–31.
163. Dejaco C, Duftner C, Schirmer M. Are regulatory T-cells linked with aging? Exp Gerontol 2006;41:339–45.
164. Tsaknaridis L, Spencer L, Culbertson N, et al. Functional assay for human CD4+CD25+ Treg cells reveals an age-dependent loss of suppressive activity. J Neurosci Res 2003;74:296–308.

165. Denecke C, Bedi DS, Ge X, et al. Prolonged graft survival in older recipient mice is determined by impaired effector T-cell but intact regulatory T-cell responses. PLoS One 2010;5:e9232.
166. Gregg R, Smith CM, Clark FJ, et al. The number of human peripheral blood CD4+ CD25high regulatory T cells increases with age. Clin Exp Immunol 2005;140:540–6.
167. Gupta S. Molecular mechanisms of apoptosis in the cells of the immune system in human aging. Immunol Rev 2005;205:114–29.
168. Potestio M, Pawelec G, Di Lorenzo G, et al. Age-related changes in the expression of CD95 (APO1/FAS) on blood lymphocytes. Exp Gerontol 1999;34:659–73.
169. Aggarwal S, Gupta S. Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. J Immunol 1998;160:1627–37.
170. Gupta S, Gollapudi S. CD95-mediated apoptosis in naïve, central and effector memory subsets of CD4+ and CD8+ T cells in aged humans. Exp Gerontol 2008;43:266–74.
171. Yang Y, An J, Weng N. Telomerase is involved in IL-7-mediated differential survival of naive and memory CD4+ T cells. J Immunol 2008;180:3775–81.
172. Cancro MP, Hao Y, Scholz JL, et al. B cells and aging: molecules and mechanisms. Trends Immunol 2009;30:313–8.
173. Miller JP, Allman D. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. J Immunol 2003;171:2326–30.
174. Riley RL, Blomberg BB, Frasca D. B cells, E2A, and aging. Immunol Rev 2005;205:30–47.
175. Labrie JE, Sah AP, Allman DM, Cancro MP, Gerstein RM. Bone marrow microenvironmental changes underlie reduced RAG-mediated recombination and B cell generation in aged mice. J Exp Med 2004;200:411–23.
176. Frasca D, Van der Put E, Landin AM, et al. RNA stability of the E2A-encoded transcription factor E47 is lower in splenic activated B cells from aged mice. J Immunol 2005;175:6633–44.
177. Johnson KM, Owen K, Witte PL. Aging and developmental transitions in the B cell lineage. Int Immunol 2002;14:1313–23.
178. Kline GH, Hayden TA, Klinman NR. B cell maintenance in aged mice reflects both increased B cell longevity and decreased B cell generation. J Immunol 1999;162:3342–9.
179. Dorshkind K, Montecino-Rodriguez E. Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. Nat Rev Immunol 2007;7:213–9.

180. Chumley MJ, Dal Porto JM, Cambier JC. The unique antigen receptor signaling phenotype of B-1 cells is influenced by locale but induced by antigen. J Immunol 2002;169:1735–43.
181. Gibson KL, Wu Y-C, Barnett Y, et al. B-cell diversity decreases in old age and is correlated with poor health status. Aging Cell 2009;8:18–25.
182. Colonna-Romano G, Bulati M, Aquino A, et al. B cells in the aged: CD27, CD5, and CD40 expression. Mech Ageing Dev 2003;124:389–93.
183. Cecka JM, Terasaki PI. Optimal use for older donor kidneys: older recipients. Transplant Proc 1995;27:801–2.
184. Harless SM, Lentz VM, Sah AP, et al. Competition for BLyS-mediated signaling through Bcmd/BR3 regulates peripheral B lymphocyte numbers. Curr Biol 2001;11:1986–9.
185. Schiemann B, Gommerman JL, Vora K, et al. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. Science 2001;293:2111–4.
186. Zheng B, Han S, Takahashi Y, Kelsoe G. Immunosenescence and germinal center reaction. Immunol Rev 1997;160:63–77.
187. Han S, Marinova E, Zheng B. Rectification of age-related impairment in Ig gene hypermutation during a memory response. Int Immunol 2004;16:525–32.
188. Lazuardi L, Jenewein B, Wolf AM, et al. Age-related loss of naïve T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes. Immunology 2005;114:37–43.
189. Aydar Y, Balogh P, Tew JG, Szakal AK. Altered regulation of Fc gamma RII on aged follicular dendritic cells correlates with immunoreceptor tyrosine-based inhibition motif signaling in B cells and reduced germinal center formation. J Immunol 2003;171:5975–87.
190. Frasca D, Van der Put E, Riley RL, Blomberg BB. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. J Immunol 2004;172:2155–62.
191. Grolleau-Julius A, Garg MR, Mo R, Stoolman LL, Yung RL. Effect of aging on bone marrow-derived murine CD11c+CD4-CD8alpha- dendritic cell function. J Gerontol A Biol Sci Med Sci 2006;61:1039–47.

192. Tesar BM, Walker WE, Unternaehrer J, et al. Murine [corrected] myeloid dendritic cell-dependent toll-like receptor immunity is preserved with aging. Aging Cell 2006;5:473–86.
193. Lung TL, Saurwein-Teissl M, Parson W, Schönitzer D, Grubeck-Loebenstein B. Unimpaired dendritic cells can be derived from monocytes in old age and can mobilize residual function in senescent T cells. Vaccine Elsevier; 2000;18:1606–12.
194. Della Bella S, Bierti L, Presicce P, et al. Peripheral blood dendritic cells and monocytes are differently regulated in the elderly. Clin Immunol 2007;122:220–8.
195. Sprecher E, Becker Y, Kraal G, et al. Effect of aging on epidermal dendritic cell populations in C57BL/6J mice. J Invest Dermatol 1990;94:247–53.
196. Fujihashi K, McGhee JR. Mucosal immunity and tolerance in the elderly. Mech Ageing Dev 2004;125:889–98.
197. Varas A, Sacedón R, Hernandez-López C, et al. Age-dependent changes in thymic macrophages and dendritic cells. Microsc Res Tech 2003;62:501–7.
198. Stichel CC, Luebbert H. Inflammatory processes in the aging mouse brain: participation of dendritic cells and T-cells. Neurobiol Aging 2007;28:1507–21.
199. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 1995;182:389–400.
200. Agrawal A, Agrawal S, Cao J-N, et al. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. J Immunol 2007;178:6912–22.
201. Sauter B, Albert ML, Francisco L, et al. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000;191:423–34.
202. Linton P-J, Li SP, Zhang Y, et al. Intrinsic versus environmental influences on T-cell responses in aging. Immunol Rev 2005;205:207–19.
203. Agrawal A, Agrawal S, Tay J, Gupta S. Biology of dendritic cells in aging. J Clin Immunol 2008;28:14–20.
204. Donnini A, Argentati K, Mancini R, et al. Phenotype, antigen-presenting capacity, and migration of antigen-presenting cells in young and old age. Exp Gerontol 2002;37:1097–112.

205. Grewe M. Chronological ageing and photoageing of dendritic cells. Clin Exp Dermatol 2001;26:608–12.
206. Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 2007;7:19–30.
207. Wang CQ, Udupa KB, Xiao H, Lipschitz DA. Effect of age on marrow macrophage number and function. Aging (Milano) 1995;7:379–84.
208. Herrero C, Sebastián C, Marqués L, et al. Immunosenescence of macrophages: reduced MHC class II gene expression. Exp Gerontol 2002;37:389–94.
209. Plowden J, Renshaw-Hoelscher M, Engleman C, Katz J, Sambhara S. Innate immunity in aging: impact on macrophage function. Aging Cell 2004;3:161–7.
210. De La Fuente M. Changes in the macrophage function with aging. Comp Biochem Physiol A Comp Physiol 1985;81:935–8.
211. Khare V, Sodhi A, Singh SM. Effect of aging on the tumoricidal functions of murine peritoneal macrophages. Nat Immun 1996;15:285–94.
212. Davila DR, Edwards CK, Arkins S, Simon J, Kelley KW. Interferon-gamma-induced priming for secretion of superoxide anion and tumor necrosis factor-alpha declines in macrophages from aged rats. FASEB J 1990;4:2906–11.
213. Ding a, Hwang S, Schwab R. Effect of aging on murine macrophages. Diminished response to IFN-gamma for enhanced oxidative metabolism. J Immunol 1994;153:2146–52.
214. Kissin E, Tomasi M, McCartney-Francis N, Gibbs CL, Smith PD. Age-related decline in murine macrophage production of nitric oxide. J Infect Dis 1997;175:1004–7.
215. Chen LC, Pace JL, Russell SW, Morrison DC. Altered regulation of inducible nitric oxide synthase expression in macrophages from senescent mice. Infect Immun 1996;64:4288–98.
216. Rollo EE, Denhardt DT. Differential effects of osteopontin on the cytotoxic activity of macrophages from young and old mice. Immunology 1996;88:642–7.
217. Wu D, Hayek MG, Meydani S. Vitamin E and macrophage cyclooxygenase regulation in the aged. J Nutr 2001;131:382S–8S.
218. Harizi H, Grosset C, Gualde N. Prostaglandin E2 modulates dendritic cell function via EP2 and EP4 receptor subtypes. J Leukoc Biol 2003;73:756–63.
219. Wood JJ, Grbic JT, Rodrick ML, Jordan A, Mannick JA. Suppression of interleukin 2 production in an animal model of thermal injury is related to prostaglandin synthesis. Arch Surg 1987;122:179–84.

220. Hilkens CM, Snijders A, Snijdewint FG, Wierenga EA, Kapsenberg ML. Modulation of T-cell cytokine secretion by accessory cell-derived products. Eur Respir J Suppl 1996;22:90s–94s.
221. Franceschi C, Bonafè M, Valensin S, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci 2000;908:244–54.
222. Boehmer ED, Goral J, Faunce DE, Kovacs EJ. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. J Leukoc Biol 2004;75:342–9.
223. Renshaw M, Rockwell J, Engleman C, et al. Cutting edge: impaired Toll-like receptor expression and function in aging. J Immunol 2002;169:4697–701.
224. Krause D, Mastro AM, Handte G, et al. Immune function did not decline with aging in apparently healthy, well-nourished women. Mech Ageing Dev 1999;112:43–57.
225. Mazari L, Lesourd BM. Nutritional influences on immune response in healthy aged persons. Mech Ageing Dev 1998;104:25–40.
226. Ashcroft GS, Horan MA, Ferguson MW. Aging alters the inflammatory and endothelial cell adhesion molecule profiles during human cutaneous wound healing. Lab Invest 1998;78:47–58.
227. Swift ME, Kleinman HK, DiPietro LA. Impaired wound repair and delayed angiogenesis in aged mice. Lab Invest 1999;79:1479–87.
228. Kmiec Z, Myśliwska J, Rachón D, et al. Natural killer activity and thyroid hormone levels in young and elderly persons. Gerontology 2001;47:282–8.
229. Myśliwska J, Bryl E, Zorena K, et al. Overactivity of tumor necrosis factor-alpha but not interleukin 6 is associated with low natural killer cytotoxic activity in the elderly. Gerontology 1997;43:158–67.
230. Ravaglia G, Forti P, Maioli F, et al. Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged >/=90 y. Am J Clin Nutr 2000;71:590–8.
231. Hsueh CM, Chen SF, Ghanta VK, Hiramoto RN. Involvement of cytokine gene expression in the age-dependent decline of NK cell response. Cell Immunol 1996;173:221–9.
232. Mariani E, Mariani AR, Meneghetti A, et al. Age-dependent decreases of NK cell phosphoinositide turnover during spontaneous but not Fc-mediated cytolytic activity. Int Immunol 1998;10:981–9.

233. Fernandes G, Gupta S. Natural killing and antibody-dependent cytotoxicity by lymphocyte subpopulations in young and aging humans. J Clin Immunol 1981;1:141–8.
234. Sansoni P, Cossarizza A, Brianti V, et al. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. Blood 1993;82:2767–73.
235. Mocchegiani E, Malavolta M. NK and NKT cell functions in immunosenescence. Aging Cell 2004;3:177–84.
236. Borrego F, Alonso MC, Galiani MD, et al. NK phenotypic markers and IL2 response in NK cells from elderly people. Exp Gerontol 1999;34:253–65.
237. Mariani E, Meneghetti A, Neri S, et al. Chemokine production by natural killer cells from nonagenarians. Eur J Immunol 2002;32:1524–9.
238. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol 2007;25:297–336.
239. Emoto M, Kaufmann SHE. Liver NKT cells: an account of heterogeneity. Trends Immunol 2003;24:364–9.
240. Inui T, Nakagawa R, Ohkura S, et al. Age-associated augmentation of the synthetic ligand- mediated function of mouse NK1.1 ag(+) T cells: their cytokine production and hepatotoxicity in vivo and in vitro. J Immunol 2002;169:6127–32.
241. Sonoda K-H, Taniguchi M, Stein-Streilein J. Long-term survival of corneal allografts is dependent on intact CD1d-reactive NKT cells. J Immunol 2002;168:2028–34.
242. Yang SH, Jin JZ, Lee SH, et al. Role of NKT cells in allogeneic islet graft survival. Clin Immunol 2007;124:258–66.
243. Seino KI, Fukao K, Muramoto K, et al. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. Proc Natl Acad Sci U S A 2001;98:2577–81.
244. Jiang X, Shimaoka T, Kojo S, et al. Cutting edge: critical role of CXCL16/CXCR6 in NKT cell trafficking in allograft tolerance. J Immunol 2005;175:2051–5.
245. Patterson S, Chaidos A, Neville DCA, et al. Human invariant NKT cells display alloreactivity instructed by invariant TCR-CD1d interaction and killer Ig receptors. J Immunol 2008;181:3268–76.
246. DelaRosa O, Tarazona R, Casado JG, et al. Valpha24+ NKT cells are decreased in elderly humans. Exp Gerontol 2002;37:213–7.
247. Peralbo E, DelaRosa O, Gayoso I, et al. Decreased frequency and proliferative response of invariant Valpha24Vbeta11 natural killer T (iNKT) cells in healthy elderly. Biogerontology 2006;7:483–92.

248. Tsukahara A, Seki S, Iiai T, et al. Mouse liver T cells: their change with aging and in comparison with peripheral T cells. Hepatology 1997;26:301–9.
249. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol 2001;13:458–64.
250. Jing Y, Gravenstein S, Chaganty NR, et al. Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood. Exp Gerontol 2007;42:719–32.
251. Peralbo E, Alonso C, Solana R. Invariant NKT and NKT-like lymphocytes: two different T cell subsets that are differentially affected by ageing. Exp Gerontol 2007;42:703–8.
252. Lemster BH, Michel JJ, Montag DT, et al. Induction of CD56 and TCR-independent activation of T cells with aging. J Immunol 2008;180:1979–90.
253. Born J, Uthgenannt D, Dodt C, et al. Cytokine production and lymphocyte subpopulations in aged humans. An assessment during nocturnal sleep. Mech Ageing Dev 1995;84:113–26.
254. Chatta GS, Andrews RG, Rodger E, et al. Hematopoietic progenitors and aging: alterations in granulocytic precursors and responsiveness to recombinant human G-CSF, GM-CSF, and IL-3. J Gerontol 1993;48:M207–12.
255. Biasi D, Carletto A, Dell'Agnola C, et al. Neutrophil migration, oxidative metabolism, and adhesion in elderly and young subjects. Inflammation 1996;20:673–81.
256. Fransson C, Mooney J, Kinane DF, Berglundh T. Differences in the inflammatory response in young and old human subjects during the course of experimental gingivitis. J Clin Periodontol 1999;26:453–60.
257. Damtew B, Spagnuolo PJ, Goldsmith GG, Marino JA. Neutrophil adhesion in the elderly: inhibitory effects of plasma from elderly patients. Clin Immunol Immunopathol 1990;54:247–55.
258. Corberand J, Ngyen F, Laharrague P, et al. Polymorphonuclear functions and aging in humans. J Am Geriatr Soc 1981;29:391–7.
259. Niwa Y, Kasama T, Miyachi Y, Kanoh T. Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross-sectional and longitudinal studies. Life Sci 1989;44:1655–64.
260. Seres I, Csongor J, Mohácsi A, Leövey A, Fülöp T. Age-dependent alterations of human recombinant GM-CSF effects on human granulocytes. Mech Ageing Dev 1993;71:143–54.

261. Emanuelli G, Lanzio M, Anfossi T, et al. Influence of age on polymorphonuclear leukocytes in vitro: phagocytic activity in healthy human subjects. Gerontology 1986;32:308–16.
262. Mege JL, Capo C, Michel B, Gastaut JL, Bongrand P. Phagocytic cell function in aged subjects. Neurobiol Aging 1988;9:217–20.
263. Fu YK, Arkins S, Li YM, Dantzer R, Kelley KW. Reduction in superoxide anion secretion and bactericidal activity of neutrophils from aged rats: reversal by the combination of gamma interferon and growth hormone. Infect Immun 1994;62:1–8.
264. Tortorella C, Polignano A, Piazzolla G, et al. Lipopolysaccharide-, granulocyte-monocyte colony stimulating factor and pentoxifylline-mediated effects on formyl-methionyl-leucine-phenylalanine-stimulated neutrophil respiratory burst in the elderly. Microbios 1996;85:189–98.
265. Esparza B, Sanchez H, Ruiz M, et al. Neutrophil function in elderly persons assessed by flow cytometry. Immunol Invest 1996;25:185–90.
266. Fülöp T, Varga Z s, Jacob MP, Robert L. Effect of lithium on superoxide production and intracellular free calcium mobilization in elastin peptide (kappa-elastin) and FMLP stimulated human PMNS. Effect of age. Life Sci 1997;60:PL 325–32.
267. Rao KM, Currie MS, Padmanabhan J, Cohen HJ. Age-related alterations in actin cytoskeleton and receptor expression in human leukocytes. J Gerontol 1992;47:B37–44.
268. Tortorella C, Piazzolla G, Spaccavento F, et al. Spontaneous and Fas-induced apoptotic cell death in aged neutrophils. J Clin Immunol 1998;18:321–9.
269. Fülöp T, Fouquet C, Allaire P, et al. Changes in apoptosis of human polymorphonuclear granulocytes with aging. Mech Ageing Dev 1997;96:15–34.
270. Alvarez E, Ruiz-Gutiérrez V, Sobrino F, Santa-María C. Age-related changes in membrane lipid composition, fluidity and respiratory burst in rat peritoneal neutrophils. Clin Exp Immunol 2001;124:95–102.
271. Fortin CF, Larbi A, Lesur O, Douziech N, Fulop T. Impairment of SHP-1 down-regulation in the lipid rafts of human neutrophils under GM-CSF stimulation contributes to their age-related, altered functions. J Leukoc Biol 2006;79:1061–72.
272. Schwarzenbach HR, Nakagawa T, Conroy MC, de Weck AL. Skin reactivity, basophil degranulation and IgE levels in ageing. Clin Allergy 1982;12:465–73.

273. Marone G, Giugliano R, Lembo G, Ayala F. Human basophil releasability. II. Changes in basophil releasability in patients with atopic dermatitis. J Invest Dermatol 1986;87:19–23.
274. Mathur SK, Schwantes EA, Jarjour NN, Busse WW. Age-related changes in eosinophil function in human subjects. Chest 2008;133:412–9.
275. Busse PJ, Zhang TF, Srivastava K, Schofield B, Li X-M. Effect of ageing on pulmonary inflammation, airway hyperresponsiveness and T and B cell responses in antigen-sensitized and -challenged mice. Clin Exp Allergy 2007;37:1392–403.
276. Yagi T, Sato A, Hayakawa H, Ide K. Failure of aged rats to accumulate eosinophils in allergic inflammation of the airway. J Allergy Clin Immunol 1997;99:38–47.
277. Plackett TP, Boehmer ED, Faunce DE, Kovacs EJ. Aging and innate immune cells. J Leukoc Biol 2004;76:291–9.
278. Nagaki K, Hiramatsu S, Inai S, Sasaki A. The effect of aging on complement activity (CH50) and complement protein levels. J Clin Lab Immunol 1980;3:45–50.
279. Bellavia D, Fradà G, Di Franco P, et al. C4, BF, C3 allele distribution and complement activity in healthy aged people and centenarians. J Gerontol A Biol Sci Med Sci 1999;54:B150–3.
280. Menzel EJ, Zlabinger GJ, Dunky A, Steffen C. Autoimmunity and T-cell subpopulations in old age. Arch Gerontol Geriatr 1988;7:249–60.
281. Hazlett LD, Masinick-McClellan SA, Barrett RP. Complement defects in aged mice compromise phagocytosis of Pseudomonas aeruginosa. Curr Eye Res 1999;19:26–32.
282. Tullius SG, Tran H, Guleria I, et al. The combination of donor and recipient age is critical in determining host immunoresponsiveness and renal transplant outcome. Ann Surg 2010;252:662–74.
283. Kasiske BL, Cangro CB, Hariharan S, et al. The evaluation of renal transplantation candidates: clinical practice guidelines. Am J Transplant 2001;1 Suppl 2:3–95.
284. Meier-Kriesche HU, Ojo AO, Cibrik DM, et al. Relationship of recipient age and development of chronic allograft failure. Transplantation 2000;70:306–10.
285. Lufft V, Kliem V, Tusch G, Dannenberg B, Brunkhorst R. Renal transplantation in older adults: is graft survival affected by age? A case control study. Transplantation 2000;69:790–4.
286. Takemoto S, Terasaki PI. Donor age and recipient age. Clin Transpl 1988;345–56.
287. Bradley B a. Rejection and recipient age. Transpl Immunol 2002;10:125–32.

288. Vail A, Gore SM, Bradley BA, et al. Conclusions of the corneal transplant follow up study. Br J Ophthalmol 1997;81:631–6.
289. Pirsch JD, Stratta RJ, Armbrust MJ, et al. Cadaveric renal transplantation with cyclosporine in patients more than 60 years of age. Transplantation 1989;47:259–61.
290. Renlund DG, Gilbert EM, O'Connell JB, et al. Age-associated decline in cardiac allograft rejection. Am J Med 1987;83:391–8.
291. Zetterman RK, Belle SH, Hoofnagle JH, et al. Age and liver transplantation: a report of the Liver Transplantation Database. Transplantation 1998;66:500–6.
292. Snell GI, De Hoyos A, Winton T, Maurer JR. Lung transplantation in patients over the age of 50. Transplantation 1993;55:562–6.
293. Tullius SG, Milford E. Kidney allocation and the aging immune response. N Engl J Med Massachusetts Medical Society; 2011;364:1369–70.
294. De Fijter JW, Mallat MJ, Doxiadis II, et al. Increased immunogenicity and cause of graft loss of old donor kidneys. J Am Soc Nephrol 2001;12:1538–46.
295. Terasaki PI, Gjertson DW, Cecka JM, Takemoto S, Cho YW. Significance of the donor age effect on kidney transplants. Clin Transplant 1997;11:366–72.
296. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med 1995;333:333–6.
297. Cecka JM. Living donor transplants. Clin Transpl 1995;11:363–77.
298. Nyengaard JR, Bendtsen TF. Glomerular number and size in relation to age, kidney weight, and body surface in normal man. Anat Rec 1992;232:194–201.
299. Epstein M. Aging and the kidney. J Am Soc Nephrol 1996;7:1106–22.
300. Boom H, Mallat MJ, de Fijter JW, Zwinderman AH, Paul LC. Delayed graft function influences renal function, but not survival. Kidney Int 2000;58:859–66.
301. Lesnefsky EJ, Gallo DS, Ye J, Whittingham TS, Lust WD. Aging increases ischemia-reperfusion injury in the isolated, buffer-perfused heart. J Lab Clin Med 1994;124:843–51.
302. Lesnefsky EJ, Hoppel CL. Ischemia-reperfusion injury in the aged heart: role of mitochondria. Arch Biochem Biophys 2003;420:287–97.
303. Okaya T, Blanchard J, Schuster R, et al. Age-dependent responses to hepatic ischemia/reperfusion injury. Shock 2005;24:421–7.
304. Qiao X, Chen X, Wu D, et al. Mitochondrial pathway is responsible for aging-related increase of tubular cell apoptosis in renal ischemia/reperfusion injury. J Gerontol A Biol Sci Med Sci 2005;60:830–9.

305. Kusaka J, Koga H, Hagiwara S, et al. Age-dependent responses to renal ischemia-reperfusion injury. J Surg Res Elsevier Inc; 2012;172:153–8.
306. Li X, Cooley BC, Gould JS. Effect of age upon ischemia/reperfusion injury in rat muscle free flaps. J Surg Res 1993;55:193–200.
307. Mowlavi A, Reynolds C, Neumeister MW, et al. Age-related differences of neutrophil activation in a skeletal muscle ischemia-reperfusion model. Ann Plast Surg 2003;50:403–11.
308. Liu M, Zhang P, Chen M, et al. Aging might increase myocardial ischemia / reperfusion-induced apoptosis in humans and rats. Age (Omaha) 2012;34:621–32.
309. Devan AE, Umpierre D, Harrison ML, et al. Endothelial ischemia-reperfusion injury in humans: association with age and habitual exercise. Am J Physiol Heart Circ Physiol 2011;300:H813–9.
310. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994;12:991–1045.
311. Halloran PF, Homik J, Goes N, et al. The “injury response”: a concept linking nonspecific injury, acute rejection, and long-term transplant outcomes. Transplant Proc 1997;29:79–81.
312. Reutzel-Selke A, Jurisch A, Denecke C, et al. Donor age intensifies the early immune response after transplantation. Kidney Int 2007;71:629–36.
313. Shoskes DA, Parfrey NA, Halloran PF. Increased major histocompatibility complex antigen expression in unilateral ischemic acute tubular necrosis in the mouse. Transplantation 1990;49:201–7.
314. Goes N, Urmson J, Ramassar V, Halloran PF. Ischemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-gamma, transforming growth factor-beta 1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10. Transplantation 1995;59:565–72.
315. Penfield JG, Dawidson IA, Ar’Rajab A, et al. Syngeneic renal transplantation increases the number of renal dendritic cells in the rat. Transpl Immunol 1999;7:197–200.
316. Ordemann R, Hutchinson R, Friedman J, et al. Enhanced allostimulatory activity of host antigen-presenting cells in old mice intensifies acute graft-versus-host disease. J Clin Invest Am Soc Clin Investig; 2002;109:1249–56.
317. Castle SC, Uyemura K, Crawford W, Wong W, Makinodan T. Antigen presenting cell function is enhanced in healthy elderly. Mech Ageing Dev Elsevier; 1999;107:137–45.

318. Sidman CL, Luther EA, Marshall JD, et al. Increased expression of major histocompatibility complex antigens on lymphocytes from aged mice. Proc Natl Acad Sci U S A 1987;84:7624–8.
319. Gomez CR, Boehmer ED, Kovacs EJ. The aging innate immune system. Curr Opin Immunol 2005;17:457–62.
320. Franceschi C, Capri M, Monti D, et al. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev 2007;128:92–105.
321. Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med 2000;51:245–70.
322. Basar H, Soran A, Shapiro R, et al. Renal transplantation in recipients over the age of 60: the impact of donor age. Transplantation 1999;67:1191–3.
323. Waiser J, Schreiber M, Budde K, et al. Age-matching in renal transplantation. Nephrol Dial Transplant 2000;15:696–700.
324. Pratschke J, Merk V, Reutzel-Selke A, et al. Potent early immune response after kidney transplantation in patients of the European senior transplant program. Transplantation 2009;87:992–1000.
325. Moreso F, Serón D, Gil-Vernet S, et al. Donor age and delayed graft function as predictors of renal allograft survival in rejection-free patients. Nephrol Dial Transplant 1999;14:930–5.
326. Halloran PF, Melk A, Barth C. Rethinking chronic allograft nephropathy: the concept of accelerated senescence. J Am Soc Nephrol 1999;10:167–81.
327. Smits JMA, Persijn GG, van Houwelingen HC, Claas FHJ, Frei U. Evaluation of the Eurotransplant Senior Program. The results of the first year. Am J Transplant 2002;2:664–70.
328. Frei U, Noeldeke J, Machold-Fabrizii V, et al. Prospective age-matching in elderly kidney transplant recipients--a 5-year analysis of the Eurotransplant Senior Program. Am J Transplant 2008;8:50–7.
329. Heron I. A technique for accessory cervical heart transplantation in rabbits and rats. Acta Pathol Microbiol Scand A 1971;79:366–72.
330. Matsuura A, Abe T, Yasuura K. Simplified mouse cervical heart transplantation using a cuff technique. Transplantation 1991;51:896–8.
331. Gu X, Xiang J, Zhou Y, Qian S, Chen Z. Improved cuff technique for cervical heart transplantation in mice. Microsurgery 2007;27:317–9.

332. Blaha P, Bigenzahn S, Koporc Z, et al. The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. Blood 2003;101:2886–93.
333. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992;176:1693–702.
334. Billingham ME, Cary NR, Hammond ME, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant 1990;9:587–93.
335. Stewart S, Winters GL, Fishbein MC, et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transplant 2005;24:1710–20.
336. Danenberg HD. Macrophage Depletion by Clodronate-Containing Liposomes Reduces Neointimal Formation After Balloon Injury in Rats and Rabbits. Circulation 2002;106:599–605.
337. Van Rooijen N, Hendrikx E. Liposomes for specific depletion of macrophages from organs and tissues. Weissig V, editor. Methods Mol Biol Totowa, NJ: Humana Press; 2010;605:189–203.
338. Spencer SC, Fabre JW. Characterization of the tissue macrophage and the interstitial dendritic cell as distinct leukocytes normally resident in the connective tissue of rat heart. J Exp Med 1990;171:1841–51.
339. Heldal K, Hartmann A, Leivestad T, et al. Clinical outcomes in elderly kidney transplant recipients are related to acute rejection episodes rather than pretransplant comorbidity. Transplantation 2009;87:1045–51.
340. Shen H, Tesar BM, Du W, Goldstein DR. Aging impairs recipient T cell intrinsic and extrinsic factors in response to transplantation. PLoS One 2009;4:e4097.
341. Melk a, Schmidt BMW, Braun H, et al. Effects of donor age and cell senescence on kidney allograft survival. Am J Transplant 2009;9:114–23.
342. Melk A, Schmidt BMW, Vongwiwatana A, Rayner DC, Halloran PF. Increased expression of senescence-associated cell cycle inhibitor p16INK4a in deteriorating renal transplants and diseased native kidney. Am J Transplant Wiley Online Library; 2005;5:1375–82.

343. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. Nat Rev Immunol 2007;7:610–21.
344. Denecke C, Ge X, Jurisch A, et al. Modified CD4(+) T-cell response in recipients of old cardiac allografts. Transpl Int 2012;25:328–36.
345. Molinero LL, Zhou P, Wang Y, et al. Epidermal Langerhans cells promote skin allograft rejection in mice with NF-kappa B-impaired T cells. Am J Transplant 2008;8:21–31.
346. Floerchinger B, Oberhuber R, Tullius SG. Effects of brain death on organ quality and transplant outcome. Transplant Rev (Orlando) Elsevier Inc.; 2012;26:54–9.
347. Eiref SD, Zhang W, Popma SH, et al. Creation of chimeric hearts: a tool for testing the “passenger leukocyte” hypothesis. Ann Thorac Surg 1997;64:628–33.
348. Krasinskas a M, Eiref SD, McLean a D, et al. Replacement of graft-resident donor-type antigen presenting cells alters the tempo and pathogenesis of murine cardiac allograft rejection. Transplantation 2000;70:514–21.
349. Lechler RI, Batchelor JR. Immunogenicity of retransplanted rat kidney allografts. Effect of inducing chimerism in the first recipient and quantitative studies on immunosuppression of the second recipient. J Exp Med 1982;156:1835–41.
350. Van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. J Immunol Methods 1996;193:93–9.
351. Rabinovich GA, Riera CM, Iribarren P. Granulocyte-macrophage colony-stimulating factor protects dendritic cells from liposome-encapsulated dichloromethylene diphosphonate-induced apoptosis through a Bcl-2-mediated pathway. Eur J Immunol 1999;29:563–70.
352. Naito M, Nagai H, Kawano S, et al. Liposome-encapsulated dichloromethylene diphosphonate induces macrophage apoptosis in vivo and in vitro. J Leukoc Biol 1996;60:337–44.
353. Cho WY, Choi HM, Lee SY, et al. The role of Tregs and CD11c(+) macrophages/dendritic cells in ischemic preconditioning of the kidney. Kidney Int Nature Publishing Group; 2010;78:981–92.
354. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. Nat Rev Immunol 2011;11:788–98.
355. Shanley DP, Aw D, Manley NR, Palmer DB. An evolutionary perspective on the mechanisms of immunosenescence. Trends Immunol 2009;30:374–81.

8 APPENDIX

8.1 CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

8.2 PUBLICATIONS

Oberhuber R[*], Heinbokel T[*], Biefer HR[*], Boenisch O[*], Hock K, Bronson RT, Wilhelm MJ, Iwakura Y, Edtinger K, Uehara H, Quante M, Voskuil F, Krenzien F, Slegtenhorst B, Abdi R, Pratschke J, Elkhal A, Tullius SG.
CD11c+ DCs accelerate the rejection of older cardiac transplants via IL-17A.
Circulation (accepted for publication).

Heinbokel T, Quante M, Tullius SG.
Author's reply.
Transplantation. 2013 Nov 27;96(10):e72-3.

Heinbokel T, Elkhal A, Liu G, Edtinger K, Tullius SG.
Immunosenescence and organ transplantation.
Transplant Rev (Orlando). 2013 Jul 29;27(3):65–75.

Heinbokel T[*], Floerchinger B[*], Schmiderer A, Edtinger K, Liu G, Elkhal A, Tullius SG.
Obesity and its impact on transplantation and alloimmunity.
Transplantation. 2013 Jul 15;96(1):10–6.

Heinbokel T, Hock K, Liu G, Edtinger K, Elkhal A, Tullius SG.
Impact of immunosenescence on transplant outcome.
Transpl Int. 2013 Mar 29;26(3):242–53.

Linneweber J, Heinbokel T, Christ T, Claus B, Kossagk C, Konertz W.
Clinical experience with the ATS 3F stentless aortic bioprosthesis: five years' follow up.
J Heart Valve Dis. 2010 Nov;19(6):772–7.

Berlin, 21/04/15

8.3 Eidesstattliche Versicherung

Ich, Timm Heinbokel, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema "Impact of immunosenescence and donor age on alloimmunity and transplant outcome" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe Uniform Requirements for Manuscripts (URM) des ICMJE – *www.icmje.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM und werden von mir verantwortet.

Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.

Berlin, den 21. April 2015

8.4 DANKSAGUNG

Mein Dank gilt Stefan Tullius für das große mir entgegengebrachte Vertrauen und die bedingungslose Unterstützung vor, während und lange nach meinem Aufenthalt in seiner Arbeitsgruppe in Boston. Die enge und persönliche Zusammenarbeit mit ihm in einer einzigartigen Umgebung stellt eine große Inspiration und anhaltende Motivation für mich dar. Die kontinuierliche, geduldige persönliche Förderung und die steten Herausforderungen im laborexperimentellen und wissenschaftlichen Denken haben mir eine einzigartige und anhaltende Entwicklung ermöglicht, die sich nicht nur im erfolgreichen Abschluss dieses Projektes ausdrückt sondern weit über meinen Forschungsaufenthalt hinausweist.

Die intensiven Diskussionen mit Hans-Dieter Volk haben mir eine wissenschaftliche Orientierung gegeben, die nicht nur in diesem Projekt Ziel meiner zukünftigen wissenschaftlichen Herausforderungen bleiben wird. Die pragmatische und doch tiefgreifende Unterstützung des Projektes hat die Fertigstellung der vorliegendenen Arbeit entscheidend begleitet. Neben einer großen Begeisterung und Bewunderung für die Immunologie wird auch ein tiefer Eindruck auf mein wissenschaftliches Denken und meine methodischen Herangehensweisen bleiben.

Für die enge Zusammenarbeit an diesem Projekt sowie die vielen Diskussionen und Erläuterungen danke ich Rupert Oberhuber. Olaf Boenisch gilt mein großer Dank für die Teilhabe an seiner umfangreichen methodischen Erfahrung, Abdala Elkhal danke ich für die besonders enge persönliche und wissenschaftliche Unterstützung. Dem ganzen Team des Transplant Surgery Research Laboratory, insbesondere auch Karin Hock, bin ich überaus dankbar für den gemeinsamen Weg in einem ereignisreichem Jahr. Die wertvolle Unterstützung durch Mitarbeiter des Instituts für Medizinische Immunologie insbesondere schon vor Beginn meines Forschungsaufenthaltes hat eine unschätzbare methodische Grundlage gelegt, für die ich besonders Stephan Schlickeiser, Mathias Streitz und Levent Akyüz sehr dankbar bin.

Neben der finanziellen Unterstützung danke ich der International Academy of Life Sciences um Prof. Dr. Hilmar Stolte besonders für die wertvolle direkte persönliche Unterstützung, die ich zusammen mit allen Teilnehmern meines Jahrganges erfahren durfte. Mein Dank gilt ebenso der Stiftung Charité für die Förderung im Rahmen eines Promotionsstipendiums.