Effect of Different Immunosuppressive Drugs on Immune Cells from Young and Old Healthy Persons

Kathrin Welzl^{a,b} Georg Kern^b Gert Mayer^b Birgit Weinberger^a
Marcus D. Säemann^d Gisela Sturm^c Beatrix Grubeck-Loebenstien^a
Christian Koppelstaetter^b

^aImmunology Division, Research Institute of Biomedical Aging Research, University of Innsbruck, and Departments of ^bInternal Medicine IV Nephrology and Hypertension and ^cDermatology and Venerology, Innsbruck Medical University, Innsbruck, and ^dInternal Medicine III/Clinical Division of Nephrology and Dialysis, Vienna Medical University, Vienna, Austria

Key Words

Aging · Immunosuppressive drugs · Peripheral mononuclear cells

Abstract

Background: Life expectancy, as well as the average age of patients undergoing solid organ transplantation, increases constantly. Consequently, immunosuppressive therapy is no longer limited to young organ recipients. **Objective:** Here, we investigate how different types of immunosuppressive therapy, namely the calcineurin inhibitors cyclosporin A and tacrolimus, as well as the mTOR inhibitor rapamycin, affect the function of immune cells in young and elderly persons. **Methods:** Proliferation, cell viability, cytokine production (IL-2, IFN- γ), H₂O₂ production and telomere length of phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMCs) of young (n = 13; median age 27 years) and old (n = 19; median age 71 years) healthy donors were analyzed. **Results:** The inhibition of proliferation was dampened in PBMCs from elderly donors, especially after incubation with rapamycin. All three immunosuppressive drugs inhibited the production of IL-2 equally well, whereas

the production of IFN- γ was less well inhibited by rapamycin. Both calcineurin inhibitors increased H₂O₂ concentrations after stimulation with PHA and led to a shortening of telomeres in PBMCs from young and old individuals. Rapamycin had only minor effects on H₂O₂ production and telomere length. **Conclusion:** Our results demonstrate that the effects of immunosuppressive drugs on PBMCs differ between young and elderly persons. Calcineurin inhibitors compared to rapamycin have a more pronounced prosenescence effect. These data indicate that specific treatment regimens for the elderly might therefore be considered.

© 2014 S. Karger AG, Basel

Introduction

Due to demographic changes the number of elderly persons undergoing solid organ transplantation has increased substantially in the last years [1–4]. In the past,

Beatrix Grubeck-Loebenstien and Christian Koppelstaetter contributed equally to this work.

advanced age was a contraindication, for example, to kidney transplantation. However, data from more recent studies indicate that kidney transplantation doubles the remaining life span of patients older than 65 years [5, 6]. The outcome for elderly patients, as for their younger counterparts, is better when transplantation is performed after short periods of dialysis [7], and even those who are transplanted after a longer waiting time still benefit from transplantation [8]. In Europe the specially designed 'old for old' program resulted in a shortened waiting time for recipients of older age [9].

A higher infection rate and susceptibility to cardiovascular diseases as well as cancer in old age may be due to the fact that the overall function of the immune system declines with age, with hematopoietic stem cells and innate as well as adaptive immune cells being affected [10, 11]. T cells play a crucial role in transplant tolerance and rejection, and their age-related changes are therefore of special interest in this context. A decline in the production of naïve T cells combined with the accumulation of memory and of highly differentiated effector T cells leads to a decreased diversity of the T cell repertoire. This is associated with an impaired response upon challenge [12–15]. These changes can be exacerbated by persistent viral infections, in particular with cytomegalovirus [16–18]. Thus, immunosuppression protocols for older transplant recipients should minimize the risk of acute rejection, but at the same time consider the potentially higher risk of infections in older adults.

Additionally, immunosuppression protocols should consider age-related physiological changes that alter the pharmacokinetics, pharmacodynamics and potential toxicity of immunosuppressive drugs [19]. Studies assessing immunosuppressant pharmacokinetic disposition among older kidney, liver and heart recipients have yielded diverse results [4, 20–23]. Despite age-related immunological and pharmacological changes, current immunosuppression protocols are similar in young and elderly persons [24]. The most commonly used immunosuppressive protocol is the combination of mycophenolate mofetil (a calcineurin inhibitor) and cortisone. In a lower percentage, particularly after renal transplantation, the mTOR inhibitors sirolimus or everolimus are used [25, 26]. It is unclear whether currently employed immunosuppressants differently impact immune cells from elderly versus from young persons. Hence, we investigated the effect of cyclosporin A (CsA) and tacrolimus on isolated peripheral mononuclear cells from young and old healthy donors *in vitro*. In addition, we evaluated the mTOR inhibitor rapamycin in this context and demonstrated that

immunosuppressive drugs affect peripheral blood mononuclear cells (PBMCs) from young and elderly persons to a different extent. Establishing specific treatment regimens for the elderly may therefore be considered and the implications of these findings are discussed.

Methods

Blood Donors

Peripheral blood was obtained from healthy young ($n = 13$; age range 24–30 years, median 27; 9 female) and old ($n = 19$; age range 65–86 years, median 71; 14 female) healthy donors. Persons under immunosuppressive or chemotherapy were excluded from the study. All participants gave their written informed consent, and the study was approved by the Ethics Committee of the Medical University of Innsbruck, Austria. PBMCs were isolated from blood samples by density gradient centrifugation (Ficoll-Hypac; Amersham Biosciences).

ELISpot Assay

IFN- γ and IL-2 secretion was evaluated by ELISpot assay: 96-well MultiScreen™ hydrophobic polyvinylidene fluoride membrane-bottomed plates (Millipore) were coated at 4°C overnight with high-affinity monoclonal antibodies against IFN- γ (5 $\mu\text{g/ml}$) or IL-2 (5 $\mu\text{g/ml}$; Mabtech) in 1 M sodium carbonate buffer (pH 9.6) in PBS. After blocking with 10% FCS, PBMCs were seeded in triplicates at 0.8×10^5 (IL-2, young donors), 1.6×10^5 (IL-2, old donors) or at 4×10^4 (IFN- γ) per well. Cells were incubated for 24 h at 37°C and 5% CO₂ in the presence of phytohemagglutinin (PHA; 2.5 $\mu\text{g/ml}$) only, or in combination with CsA (1, 5 or 10 μM), tacrolimus (1, 5 or 10 μM) or rapamycin (0.1, 0.5 or 1 μM). The plates were washed with 0.05% TWEEN 20, an IFN- γ or IL-2 biotinylated antibody was added and cells were incubated for 1.5 h at 37°C. After further washing, alkaline phosphatase-conjugated streptavidin (dilution 1:1,000; Mabtech) was added and incubated for 1.5 h at room temperature before plates were washed again. Plates were then incubated with alkaline phosphatase substrate BCIP/NBT (Moss Inc., Pasadena, Md., USA) until distinct spots emerged. The spot number was determined using an automated KS-ELISpot reader version 4.13 (Carl Zeiss). The mean number of blank control wells (no PHA, no treatment) was subtracted from the mean number of spots in the experimental wells. Data are expressed as spot-forming cells (SFC)/ 1.6×10^5 per well for the IL-2 ELISpot and as SFC/ 4×10^4 per well for the IFN- γ ELISpot.

Cell Culture

PBMCs were cultivated in RPMI 1640 supplemented with 10% FCS and 1% penicillin-streptomycin at 37°C and 5% CO₂. Cells were stimulated for 5 days with PHA (2 $\mu\text{g/ml}$). The immunosuppressive agents were added to the culture medium. Concentrations were 1, 5 and 10 μM for CsA (Calbiochem) and tacrolimus (Fujisawa Pharmaceutical Co.) and 0.1, 0.5 and 1 μM for rapamycin (LC Laboratories).

CFSE Assay

The proliferation rate of T cells was determined by assessing the reduction of the intensity of the fluorescent cell-permeable dye

CFSE. CFSE fluorescence is equally distributed into both daughter cells during mitosis; 5.7×10^6 PBMCs were incubated for 15 min at 37°C in 1 ml PBS containing 1 μ M CFSE. After 5 days in culture, CFSE-labeled cells were costained with α -CD3-mAb (BD Biosciences) and analyzed by flow cytometry. Proliferation was expressed as an index: $PI = [\sum_{n \geq 1} (P_n/2^n)] / [\sum_{n \geq 0} (P_n/2^n)]$, n = cell doubling, P = % of cells in n .

Cell Viability

The cell viability was determined by performing an annexin V/7AAD costaining assay. After 5 days in culture, PBMCs were stained for annexin V and 7AAD and early and late apoptosis was measured by flow cytometry. Annexin V⁺/7AAD⁻ cells represent early apoptotic cells and annexin V⁺/7AAD⁺ cells late apoptotic cells. 7AAD single positive cells were considered as necrotic. Viable cells are negative for both annexin V and 7AAD. Cell viability was expressed as percentage of viable cells.

Flow Fluorescence in situ Hybridization

Fluorescence in situ hybridization (FISH) with labeled peptide nucleic acid (Panagen) probes specific for telomere repeats combined with FLOW cytometry was used to determine the average telomere length of PBMCs. Using TCL 1301, a tetraploid human T cell leukemia cell line with known telomere length, as a standard for each telomere length measurement, it is possible to calculate the telomere length of a certain cell population. A peptide nucleic acid probe was added to hybridization buffer containing 70% formamide. PBMCs were incubated in hybridization buffer at 85°C for 12 min. After 2 min on ice, cells were incubated at room temperature for 90 min. After washing twice with posthybridization buffer and twice with PBS, cells were analyzed by flow cytometry.

H₂O₂ Production

H₂O₂ production was measured by Amplex Red. PBMCs were exposed to CsA, tacrolimus or rapamycin and stimulated with PHA for 5 days. Freshly collected supernatants (30 μ l) were added to Amplex Red Ultra (30 μ l of 0.16 mM; Molecular Probes) and horseradish peroxidase (1 U/ml; Sigma) in PBS. The plates were incubated at 37°C for 1 h. The H₂O₂ production was determined by determination of fluorescent resorufin, which was formed from Amplex Red in the presence of H₂O₂ and horseradish peroxidase. Fluorescence was measured at 540 nm excitation and 595 nm emission.

Statistical Analysis

Normal distribution was checked for all variables using the Kolmogorov-Smirnov test. Data were analyzed by two-way analyses of variance (ANOVAs) for repeated measures to evaluate the effects of age and immunosuppressive drug (between-subject factor) and of different concentrations (within-subject factor) on cytokine production, proliferation, H₂O₂ concentrations and telomere length. Mauchly's test was applied to check the sphericity assumption of the repeated-measures ANOVA. When sphericity was violated, the Greenhouse-Geisser correction was used. Significant ANOVA models were evaluated by post hoc tests using the Bonferroni correction. Differences between young and old were calculated using *t* tests. For all tests *p* values <0.05 were considered significant. Statistical analyses were performed using IBM SPSS Statistics version 20 (IBM Corp., Armonk, N.Y., USA).

Results

Potent Inhibition of IL-2 and IFN- γ Production by Immunosuppressants

Cytokine production of PBMC was induced with PHA and the impact of CsA, tacrolimus and rapamycin on the production of IL-2 and IFN- γ was analyzed (fig. 1a, b). IL-2 and IFN- γ production was similar in young and old donors after PHA stimulation and treatment with CsA and tacrolimus almost completely abolished cytokine production in both age groups. Rapamycin also inhibited IL-2 secretion very efficiently, whereas IFN- γ production was not fully abolished in both age groups. We could not detect age-dependent differences in the effect of immunosuppressants on cytokine production and there were no differences between the different concentrations of immunosuppressive substances used.

Inhibition of T Cell Proliferation by Immunosuppressive Drugs Is Decreased in Elderly Persons

Next, we analyzed the impact of calcineurin and mTOR inhibition on T cell proliferation in response to PHA. No age-related differences were observed in the proliferation index of T cells stimulated in the absence of immunosuppressive drugs (fig. 2). The inhibitory effect of CsA, and even more pronounced of rapamycin, was reduced in the elderly compared to young adults (fig. 2).

Whereas the effects of both calcineurin inhibitors and rapamycin were similar for the young age group with slightly less inhibition by tacrolimus, proliferation of T cells from elderly donors was higher in the presence of mTOR inhibition by rapamycin compared to CsA and tacrolimus.

One possible reason for impaired cytokine production and cell proliferation could be an increased rate of cell death. We therefore analyzed cell viability in stimulated PBMCs from young and elderly donors in the presence or absence of immunosuppressive drugs. Tacrolimus and rapamycin had only moderate, but still significant, effects on cell viability in all concentrations without differences between PBMCs from young compared to elderly adults (fig. 3). CsA also only moderately reduced cell viability in the low concentrations, whereas the highest concentration of CsA led to substantial cell death. No age-related differences in cell viability were observed for treatment with CsA. We therefore conclude that impaired cytokine production and cell proliferation in the presence of immunosuppressive drugs are not due to increased rates of cell death.

Fig. 1. In vitro treatment of PHA-stimulated (2.5 µg/ml) PBMCs from young and elderly donors with immunosuppressives inhibits IL-2 (a) and IFN-γ (b) production during 24 h of treatment. IL-2 and IFN-γ production were analyzed by ELISpot technique. Mean values ± SEM are shown (n = 4 experiments with cells from differ-

ent donors). **a** Repeated-measures ANOVA showed a significant difference in cytokine production between concentrations ($p < 0.001$). Inhibition of cytokine production was significant in all samples treated with immunosuppressive drugs ($p < 0.001$) compared to untreated controls.

Concentration of H_2O_2 in Activated PBMCs Is Increased in the Presence of CsA and Tacrolimus

Next we investigated whether immunosuppressive drugs influence H_2O_2 concentrations in PBMCs from young and elderly donors. The production of H_2O_2 was firstly determined in PHA-treated PBMC in the absence of immunosuppressants. There was no significant difference in the production of H_2O_2 by PBMCs from young and old donors (fig. 4a). Treatment with immunosuppressive drugs significantly increased H_2O_2 concentrations. We found a sharp increase in H_2O_2 concentrations after treatment with CsA and tacrolimus, but only a minor effect of rapamycin. Differences between rapamycin treatment and calcineurin inhibitors were highly significant ($p \leq 0.005$). For both calcineurin inhibitors the magnitude of the increase was slightly higher in PBMCs from young than from elderly persons, but these differences did not reach statistical significance.

Short-Term Treatment with CsA and Tacrolimus Leads to a Reduction of Telomere Length

We have previously shown that CsA reduces telomere length in renal tubular epithelial cells, even after short-term in vitro treatment [27], and we therefore aimed to determine the effect of short-term immunosuppressive treatment with CsA, tacrolimus or rapamycin on telomere erosion of lymphocytes. We cultured and stimulated PBMCs in the presence or absence of immunosuppressants and measured telomere length by flow-FISH. In the absence of immunosuppressive drugs PBMCs from elderly persons had significantly shorter telomeres than PBMCs from the young group (fig. 5a, b). Taking the results in both age groups as baseline for calculating the effect of immunosuppressive drugs on changes in telomere length during culture we detected that treatment with calcineurin inhibitors induced a pronounced reduction of telomere length in both age groups. In contrast, telomere

Fig. 2. Effect of immunosuppressive drugs on T cell proliferation. **a** Flow cytometry histograms of CFSE-labeled CD3+ T cells from young and old healthy persons after treatment with immunosuppressive drugs and stimulation with PHA for 5 days. **b** Proliferation was assessed by CFSE staining and the proliferation index was calculated. Values represent means $\pm$ SEM of 4 independent ex-

periments. Repeated-measures ANOVA showed a significant difference in proliferation inhibition between concentrations ($p < 0.001$). Proliferation was significantly inhibited in all samples treated with immunosuppressive drugs ($p < 0.001$) compared to untreated controls. Significant differences between age groups are indicated. * $p < 0.05$; ** $p < 0.01$.

shortening was less severe after treatment with rapamycin. Differences between rapamycin treatment and calcineurin inhibitors were significant ($p = 0.032$ for CsA vs. rapamycin and $p = 0.006$ for tacrolimus vs. rapamycin). No age-related differences were observed (fig. 5c).

Discussion

In solid organ transplantation, a variety of immunosuppressive drugs is in use to avoid rejection of the graft and therapy should be carefully tailored to each patient

since effects and side effects are manifold and dependent on several factors [28, 29]. Parameters for this decision are comorbidities, the immune status of the recipient, earlier transplantations, or the infectological background. However, in most cases the chronological age of the patient does not influence the choice of treatment. Even though specific transplant programs have been developed for elderly patients ('old for old') only a limited amount of information for specific immunosuppressive regimens are available [30–32].

One major impact on the choice of therapy, particularly after renal transplantation, is the potency of nephro-

Fig. 3. a Flow cytometry dot plots for PBMC of 1 representative donor are shown. PBMCs were cultured for 5 days in the presence of PHA and viable cells were defined as annexin V⁻/7AAD⁺. Shown are examples for untreated PBMC as well as PBMC cultured in the presence of 10 μM CsA, 10 μM tacrolimus or 1 μM rapamycin. **b** The bar graphs show mean values $\pm$ SEM for 4 young and 4 elderly donors. Repeated-measures ANOVA showed a significant mean cell

viability difference between concentrations ($p < 0.001$). Cell viability was significantly decreased in all samples treated with immunosuppressive drugs ($p < 0.001$) compared to untreated controls. Significant differences between CsA and tacrolimus ($p < 0.001$), CsA and rapamycin ($p = 0.005$) and tacrolimus and rapamycin ($p = 0.019$) were found (post hoc test; Bonferroni correction). Differences between age groups were not significant.

Fig. 4. a H₂O₂ production by PBMCs incubated with PHA for 5 days. H₂O₂ concentrations in supernatants from PBMC of young and elderly donors were determined by Amplex Red. Shown are means $\pm$ SEM ($n = 5$ for each age group). **b** The effect of immunosuppressive drugs on H₂O₂ production of PHA-stimulated PBMCs. After 5 days in culture, H₂O₂ concentrations in supernatants from young and elderly donors were determined by Amplex Red. Values represent means $\pm$ SEM ($n = 5$). Repeated-measures ANOVA

showed a significant difference in H₂O₂ concentrations between concentrations ($p < 0.001$). H₂O₂ concentrations were significantly increased in all samples treated with immunosuppressive drugs ($p < 0.001$) compared to untreated controls. Significant differences between CsA and rapamycin ($p = 0.005$) as well as between tacrolimus and rapamycin ($p < 0.001$) were found (post hoc test; Bonferroni correction). Differences between age groups were not significant.

Fig. 5. **a** Representative example for telomere length assessment by flow-FISH technique after 5 days of culture in the presence of PHA. Shown are FACS histograms for 1 young (black) and 1 old (grey) donor as well as for the cell line TCL 1031 (white), which was used as a standard for calculation of telomere length. **b** Relative telomere length of PBMCs from young and elderly donors incubated for 5 days with PHA. Values represent means $\pm$ SEM ($n = 4$).

** $p < 0.01$, young versus old. **c** The effect of immunosuppressive drugs on the reduction of telomere length. Telomere length was assessed after 5 days in cell culture by flow-FISH. Values represent means $\pm$ SEM ($n = 4$). Telomere shortening was significant in all samples treated with immunosuppressive drugs ($p < 0.001$) compared to untreated controls.

toxicity of each substance. Even though calcineurin inhibitors are well established in this field, their grade of deleterious effects on the transplanted kidney may lead to application of other substances. For the last 10 years mTOR inhibitors have been available for immunosuppression in renal transplantation [33, 34]. However, the exclusion criteria in the use of these agents are manifold, and only a small cohort of patients may benefit from treatment with mTOR inhibitors [35]. Despite the fact that the renal side effects are well researched, the influence of these substances on the immune system of patients with different ages is not yet investigated.

In this in vitro study on the effect of CsA, tacrolimus and rapamycin on stimulated PBMCs from healthy sub-

jects of different ages, we could show the expected suppression of IL-2 and IFN- γ according to the well-known mode of action of these substances [28], with no significant differences between the age groups. Stimulation of PBMC with alloantigen instead of mitogen would have mimicked the in vivo situation even closer. However, differences in the level of mismatch would presumably result in higher variation between the individual donors.

Whereas inhibition of T cell proliferation was similar for all substances in cells from young adults, rapamycin did not inhibit T cell proliferation to the same extent in cells from older individuals, suggesting a less pronounced alteration of the immune state of older patients

by rapamycin. One could suspect a higher rate of rejection. However, no studies describing such an effect have yet been published to our knowledge, which may be due to the fact that only a limited number of studies are available using mTOR inhibitors in transplant recipients of older age. Only a reduced rate of nephrotoxicity in this age group has been described by Pascual et al. [31] in a study analyzing the use of mTOR inhibitors in elderly patients.

To determine a possible impact of toxicity on the results discussed above, we determined the viability of exposed cells. Cell viability after rapamycin treatment was only slightly reduced and did not differ between PBMCs from young and old individuals. Therefore, toxic/pro-apoptotic effects influencing proliferation could be excluded. Similar results were found after exposure to tacrolimus, whereas a clear toxic effect is triggered by high doses of CsA, which is also independent of age. Comparable results have been previously described in renal cells where the generation of reactive oxygen species (ROS) was discussed as a possible mode of action [34].

In PBMCs of young as well as elderly individuals the calcineurin inhibitors enhanced H_2O_2 concentrations after stimulation with PHA to a greater extent than rapamycin [36–38]. In addition, shortening of telomeres was only observable to a small extent in the rapamycin treatment group, whereas after exposure to CsA and tacrolimus reduction of telomere length was more pronounced. Such an effect has been shown in different cell types exposed to ROS as well as in a variety of publications interpreting reduction of leukocyte telomere length as a marker for senescence in certain kinds of diseases and even after exposure to lifestyle predictors [37, 39–43]. Nevertheless, no studies have been published showing a direct effect of immunosuppressive substances on telomere length, suggesting the lowest degree of induction of cellular senescence by mTOR inhibition.

Further studies are needed to dissect the role of different cell types in these processes. It has also been taken into consideration that ROS concentration in the supernatant might not fully reflect ROS levels in the cells. Several factors such as the rate of proliferation and telomerase expression under in vitro culture conditions might also influence telomere shortening. Further studies with lymphocytes isolated directly from patients under immunosuppressive therapy should be conducted to clarify the impact of immunosuppressive drugs on telomere length in vivo.

As a limitation of this study the exclusion of steroids and mycophenolate mofetil in our treatments groups can be discussed. It draws a picture which will not be seen in the in vivo situation since most of the transplant patients worldwide are treated with a triple combination of these two substances with calcineurin or mTOR inhibitors. Due to the complexity which is evoked by such a triple combination, we decided to exclude these substances and to investigate the specific impact of the primary immunosuppressives on PBMCs.

The dosage of immunosuppressives in cell culture experiments is still a matter of discussion since the direct effect on cellular mechanisms cannot be fully elucidated due to differences in target through and peak levels. However, we decided to use equivalent doses compared to other publications to guarantee comparability.

As the total population of PBMC was analyzed, it was not possible to determine whether the age-related differences observed were due to changes at the cellular level or at the cell population level. The composition of the T cell pool, particularly within the CD8+ compartment, is altered with age as the number of naïve T cells decreases whereas highly differentiated effector T cells accumulate. It has been demonstrated that mTOR is a major regulator of murine CD8+ T cell differentiation and that mTORC1 inhibition by rapamycin has immunostimulatory effects on the generation of memory CD8+ T cells [44]. One could speculate that different T cell subsets are differentially susceptible to the effects of rapamycin. Nevertheless, the relevance of our findings for clinical considerations is independent of these mechanistic details, which could be clarified in further studies. It is currently unclear why the effect of immunosuppression shows age-related differences for some functional T cell parameters (e.g. proliferation) but not for others (e.g. cytokine production).

It would also be of interest to further investigate whether other cell types such as myeloid cells or B cells might influence T cell function under immunosuppression.

In summary, the effect of calcineurin inhibitors or rapamycin treatment on human PBMCs shows several differences. Age-related differences were more pronounced after rapamycin treatment. Increased H_2O_2 concentrations and telomere shortening were observed after calcineurin treatment, but only to a lesser extent in the presence of rapamycin. As a consequence of these findings, studies would be needed to explore the age-dependent role of immunosuppressive drugs on the immune system after solid organ transplantation.

Acknowledgments

We thank Prof. Peter Landsdorp from Terry Fox Laboratory, Vancouver, for the permission and teaching of telomere length determination by flow-FISH. This work was partly supported by an unrestricted grant from Pfizer Pharmaceutical Company.

Disclosure Statement

Dr. Koppelstaetter received industrial grants from Novartis, Wyeth and Astellas.

References

- Cohen DJ, St Martin L, Christensen LL, Bloom RD, Sung RS: Kidney and pancreas transplantation in the United States, 1995–2004. *Am J Transplant* 2006;6:1153–1169.
- Danovitch GM, Cohen DJ, Weir MR, Stock PG, Bennett WM, Christensen LL, Sung RS: Current status of kidney and pancreas transplantation in the United States, 1994–2003. *Am J Transplant* 2005;5:904–915.
- Singhal A, Sezginsoy B, Ghuloom AE, Hutchinson IV, Cho YW, Jabbour N: Orthotopic liver transplant using allografts from geriatric population in the United States: is there any age limit? *Exp Clin Transplant* 2010;8:196–201.
- Weiss ES, Nwakanma LU, Patel ND, Yuh DD: Outcomes in patients older than 60 years of age undergoing orthotopic heart transplantation: an analysis of the UNOS database. *J Heart Lung Transplant* 2008;27:184–191.
- Oniscu GC, Brown H, Forsythe JL: Impact of cadaveric renal transplantation on survival in patients listed for transplantation. *J Am Soc Nephrol* 2005;16:1859–1865.
- Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, Held PJ, Port FK: Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *New Engl J Med* 1999;341:1725–1730.
- Meier-Kriesche HU, Port FK, Ojo AO, Rudich SM, Hanson JA, Cibrik DM, Leichtman AB, Kaplan B: Effect of waiting time on renal transplant outcome. *Kidney Int* 2000;58:1311–1317.
- Gill JS, Tonelli M, Johnson N, Kiberd B, Landsberg D, Pereira BJ: The impact of waiting time and comorbid conditions on the survival benefit of kidney transplantation. *Kidney Int* 2005;68:2345–2351.
- Frei U, Noeldeke J, Machold-Fabrizii V, Arbogast H, Margreiter R, Fricke L, Voiculescu A, Kliem V, Ebel H, Albert U, Lopau K, Schnuelle P, Nonnast-Daniel B, Pietruck F, Offermann R, Persijn G, Bernasconi C: Prospective age-matching in elderly kidney transplant recipients – a 5-year analysis of the Eurotransplant Senior Program. *Am J Transplant* 2008;8:50–57.
- Weinberger B, Grubeck-Loebenstein B: Vaccines for the elderly. *Clin Microbiol Infect* 2012;18(suppl 5):100–108.
- Weinberger B, Weiskopf D, Grubeck-Loebenstein B: Immunology and aging; in Hazard's Geriatric Medicine and Gerontology. New York, McGraw-Hill, 2009.
- Arnold CR, Wolf J, Brunner S, Herndler-Brandstetter D, Grubeck-Loebenstein B: Gain and loss of T cell subsets in old age – age-related reshaping of the T cell repertoire. *J Clin Immunol* 2011;31:137–146.
- Cicin-Sain L, Smyk-Pearson S, Currier N, Byrd L, Koudelka C, Robinson T, Swarbrick G, Tackitt S, Legasse A, Fischer M, Nikolich-Zugich D, Park B, Hobbs T, Doane CJ, Mori M, Axthelm MK, Lewinsohn DA, Nikolich-Zugich J: Loss of naive T cells and repertoire constriction predict poor response to vaccination in old primates. *J Immunol* 2010;184:6739–6745.
- Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E, Witkowski J, Fulbright J, Weyand CM, Goronzy JJ: The influence of age on T cell generation and TCR diversity. *J Immunol* 2005;174:7446–7452.
- Yager EJ, Ahmed M, Lanzer K, Randall TD, Woodland DL, Blackman MA: Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J Exp Med* 2008;205:711–723.
- Almanzar G, Schwaiger S, Jenewein B, Keller M, Herndler-Brandstetter D, Wurzner R, Schonitzer D, Grubeck-Loebenstein B: Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *J Virol* 2005;79:3675–3683.
- Brunner S, Herndler-Brandstetter D, Weinberger B, Grubeck-Loebenstein B: Persistent viral infections and immune aging. *Ageing Res Rev* 2011;10:362–369.
- Pawelec G, Koch S, Gouttefangeas C, Wikby A: Immunorejuvenation in the elderly. *Rejuvenation Res* 2006;9:111–116.
- Danovitch GM, Gill J, Bunnapradist S: Immunosuppression of the elderly kidney transplant recipient. *Transplantation* 2007;84:285–291.
- Miura M, Satoh S, Kagaya H, Saito M, Inoue T, Tsuchiya N, Suzuki T, Habuchi T: No impact of age on dose-adjusted pharmacokinetics of tacrolimus, mycophenolic acid and prednisolone 1 month after renal transplantation. *Eur J Clin Pharmacol* 2009;65:1047–1053.
- Staatz CE, Tett SE: Pharmacokinetic considerations relating to tacrolimus dosing in the elderly. *Drugs Aging* 2005;22:541–557.
- Wang CX, Meng FH, Chen LZ, Ren B, Li SX, Fei JG, Qiu J, Deng SX, Li J, Chen SY: Population pharmacokinetics of mycophenolic acid in senile Chinese kidney transplant recipients. *Transplant Proc* 2007;39:1392–1395.
- Waki K: UNOS Liver Registry: ten year survivals. *Clin Transpl* 2006:29–39.
- Gill J, Sampaio M, Gill JS, Dong J, Kuo HT, Danovitch GM, Bunnapradist S: Induction immunosuppressive therapy in the elderly kidney transplant recipient in the United States. *Clin J Am Soc Nephrol* 2011;6:1168–1178.
- Meier-Kriesche HU, Li S, Gruessner RW, Fung JJ, Bustami RT, Barr ML, Leichtman AB: Immunosuppression: evolution in practice and trends, 1994–2004. *Am J Transplant* 2006;6:1111–1131.
- Veroux M, Tallarita T, Corona D, Sinagra N, Giaquinta A, Zerbo D, Guerrieri C, D'Assoro A, Cimino S, Veroux P: Conversion to sirolimus therapy in kidney transplant recipients with new onset diabetes mellitus after transplantation. *Clin Dev Immunol* 2013;2013:496974.
- Jennings P, Koppelstaetter C, Aydin S, Abberger T, Wolf AM, Mayer G, Pfaller W: Cyclosporine A induces senescence in renal tubular epithelial cells. *Am J Physiol Renal Physiol* 2007;293:F831–F838.
- Halloran PF: Immunosuppressive drugs for kidney transplantation. *New Engl J Med* 2004;351:2715–2729.
- Schiffer M: Renal co-morbidity after solid organ transplantation. *Dtsch Med Wochenschr* 2012;137:2332–2335.
- Pascher A, Pratschke J, Neuhaus P, Tullius SG: Modifications of immune regulations with increasing donor and recipient age. *Ann Transplant* 2004;9:72–73.
- Pascual J, Marcen R, Ortuno J: Clinical experience with everolimus (Certican) in elderly recipients: the 'old-for-old' concept. *Transplantation* 2005;79:S85–S88.

- 32 Ponticelli C: Present and future of immuno-suppressive therapy in kidney transplantation. *Transplant Proc* 2011;43:2439–2440.
- 33 Halloran PF: Immunosuppression in the post-adaptation period. *Transplantation* 2000;70:3–5.
- 34 Kahan BD: Sirolimus: a comprehensive review. *Expert Opin Pharmacother* 2001;2: 1903–1917.
- 35 Lebranchu Y: Can we eliminate both calcineurin inhibitors and steroids? *Transplant Proc* 2010;42:S25–S28.
- 36 Babizhayev MA, Yegorov YE: Smoking and health: association between telomere length and factors impacting on human disease, quality of life and life span in a large population-based cohort under the effect of smoking duration. *Fundam Clin Pharmacol* 2011;25: 425–442.
- 37 Batty GD, Wang Y, Brouillette SW, Shiels P, Packard C, Moore J, Samani NJ, Ford I: Socio-economic status and telomere length: the West of Scotland Coronary Prevention Study. *J Epidemiol Community Health* 2009;63: 839–841.
- 38 Richter T, Proctor C: The role of intracellular peroxide levels on the development and maintenance of telomere-dependent senescence. *Exp Gerontol* 2007;42:1043–1052.
- 39 Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A, Erusalimsky JD: Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *J Cell Sci* 2004;117:2417–2426.
- 40 Scalera F, Borlak J, Beckmann B, Martens-Loebenhoffer J, Thum T, Tager M, Bode-Boger SM: Endogenous nitric oxide synthesis inhibitor asymmetric dimethyl L-arginine accelerates endothelial cell senescence. *Arterioscler Thromb Vasc Biol* 2004;24:1816–1822.
- 41 Brouillette SW, Moore JS, McMahon AD, Thompson JR, Ford I, Shepherd J, Packard CJ, Samani NJ: Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study. *Lancet* 2007;369:107–114.
- 42 Benetos A, Gardner JP, Zureik M, Labat C, Xiaobin L, Adamopoulos C, Temmar M, Bean KE, Thomas F, Aviv A: Short telomeres are associated with increased carotid atherosclerosis in hypertensive subjects. *Hypertension* 2004;43:182–185.
- 43 Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber RA: Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* 2003; 361:393–395.
- 44 Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R: mTOR regulates memory CD8 T-cell differentiation. *Nature* 2009;460:108–112.