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ORIGINAL ARTICLE

Modified CD4$^+$ T-cell response in recipients of old cardiac allografts

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Summary

With an increasing demand, organs from elderly donors are more frequently utilized for transplantation. Herein, we analyzed the impact of donor age on CD4$^+$ T-cell responses with regard to regulatory and effector mechanisms. Young (3 months) BM12 recipients were engrafted with young or old (18 months) B6 cardiac allografts. Systemic CD4$^+$ T-cell responses and intragraft changes were monitored and compared to age-matched syngenic transplant controls. While elderly, nonmanipulated hearts contained significantly elevated frequencies of donor-derived leukocytes prior to transplantation, allograft survival was age-independent. T-cell activation, however, was delayed and associated with a compromised immune response in mixed lymphocyte cultures (MLR; $P = 0.0002$) early after transplantation (day 14). During the time course after transplantation, recipients of old grafts demonstrated an augmented immune response as shown by significantly higher frequencies of activated CD4$^+$ T-cells and a stronger *in vitro* alloreactivity (MLR; ELISPOT; $P < 0.01$). In parallel, frequencies of regulatory T-cells had increased systemically and overall fewer CD4$^+$ T-cells were detected intragraft. Interestingly, changes in the CD4$^+$ T-cell response were not reflected by graft morphology. Of note, transplantation of young and old syngenic hearts did not show age-related differences of the CD4$^+$ T-cells response suggesting that old grafts can recover from a period of short cold ischemia time. Our data suggest that donor age is associated with an augmented CD4$^+$ T-cells response which did not affect graft survival in our model. These findings contribute to a better understanding of the immune response following the engraftment of older donor organs.

Introduction

As numbers of patients awaiting transplantation are steadily increasing, organs from older donors are more frequently utilized for transplantation. At the same time, advanced donor age is known as a major risk factor for primary non and delayed graft function as well as long-term allograft dysfunction [1–3].

Studies dissecting the impact of advanced donor age on the recipient's immune response are limited. In a retrospective clinical analysis, de Fijter *et al.* found evidence of an increased immunogenicity of older donor kidneys in parallel to increased frequencies of acute rejections [4]. Several mechanisms have been implied to play a role which include an increased expression of pro-inflammatory cytokines, MHC antigens, and increased recruitment

and activation of APCs [5,6]. Although a recent experimental study had been unable to show an age-related increased immune response [7], our own group has reported on an augmented T- and B-cell response early after transplantation of aged grafts in a MHC class I disparate rat kidney transplant model [8]. In contrast, the development of chronic arterial vasculopathy in old allografts and the alloreactive T-cell response to old grafts had been comparable or weaker compared to young allografts, implying a time-dependent effect of donor age on the recipients T-cell response. We have previously shown that recipient age profoundly impacts $CD4^+$ T-cell responses [9]. In our current study, we aimed to investigate the impact of donor age on systemic and intragraft responses of $CD4^+$ T-cells in a chronic cardiac allograft model. To our knowledge, effects of donor age on the biology of $CD4^+$ T-cell responses have not been studied before. We focused on dissecting the response of $CD4^+$ T-cells in response to organ age using a MHC class II mismatched transplant model which allowed a specific emphasis on chronic immune changes [10]. By using a chronic model, the subtle immunostimulatory effects of donor age became more apparent than in a full MHC-mismatch setting allowing us to investigate $CD4^+$ T-cell mediated immune responses in this model. Although acute rejection does not resemble a major hurdle after transplantation anymore, advanced chronic graft deterioration represents an unsolved clinical issue. Thus, using a chronic model provided clinically relevant information on the long-term immune response to older donor organs.

We found that the long-term $CD4^+$ response to older donor organs was more vigorous compared to young donor organs although graft survival was unaffected by donor age in this model. Interestingly, syngenic heart transplants did not reveal an age-dependent effect on the recipients' immune response, suggesting sufficient and age-independent repair capacities to recover from a short cold ischemia time.

Material and methods

Mice

Mice 3 months old C57Bl/6 and B6.C-$H2^{bm12}$ (bm12: weight: 18–25 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Twelve months and 18 months old C57Bl/6 mice were obtained from the National Institute of Aging (NIA, Bethesda, MD; weight: 40 ± 5g).

This strain combination is mismatched for MHC class II developing histomorphological signs of chronic transplant vasculopathy (CTV) [11,12]. We used this model to specifically analyze CD4+ T-cell responses following transplantation of old donor organs.

Heterotopic heart transplantation

Heterotopic vascularized cardiac transplants from B6 donors into bm12 recipients were performed with standard microvascular techniques [13]. Graft function was monitored by daily palpation. Rejection was defined as complete cessation of cardiac contractility and confirmed by direct visualization. Graft survival is shown as the median survival time in days. All heart grafts were routinely procured on day 100 for analysis ($n = 6$ each).

Flow cytometry

Cells were washed in PBS containing 2% Rat-Serum (Bio Whittaker, Walkersville, MD) to block unspecific FcR binding. Lymphocytes were stained with directly conjugated anti-CD8, anti-CD4, anti-CD69, anti-CD25, anti-CD44, anti-CD62L or anti-$I\text{-}A^b$. For FoxP3-staining, a commercially available kit (eBioscience, San Diego, CA) was used. All mAbs were purchased from BD Biosciences (San Diego, CA).

For analysis of graft infiltrating cells, hearts were flushed, minced and incubated for 1 h at 37 °C in RPMI with Type II collagenase (Sigma, St. Louis, MO). After incubation, samples were passed through 40 micron filters, washed twice in RPMI, counted using a hemocytometer, and stained for phenotypic surface markers using commercially available antibodies (BD Bioscience, San Jose CA; eBioscience). For detection of dead cells, 7AAD staining was performed.

ELISPOT assay

Splenocytes from naive or transplanted bm12 mice were harvested and subsequently stimulated with naive wild-type B6 splenocytes. The ELISPOT assay was employed to measure the frequency of alloreactive T-cells producing IFNγ (Th1) as previously described [14]. The resulting spots were counted on a computer-assisted enzyme-linked immunospot image analyzer (Cellular Technology, Shaker Heights, OH, USA) and frequencies were expressed as the number of cytokine-producing spots per 0.5×10^6 splenocytes.

Proliferation assay (mixed lymphocyte culture)

The bm12 splenocytes were isolated and 0.5×10^6 cells/well was co-cultured with 0.5×10^6 bm12 splenocytes in round-bottomed 96-well plates (BD Biosciences). After 72 h, plates were pulsed for 12 h with 1 μCi [^{3}H]thymidine per well. Proliferation was measured as counts per minute using a Wallac Liquid Scintillation Counter (Perkin Elmer, San Jose, CA, USA).

Combined TUNEL and surface immunofluorescence staining

The OCT-embedded, 5 μm thick paraformaldehyde-fixed mouse tissue sections were permeabilized (1% Triton X 100 sodium citrate; Fisher Scientific, Fair Lawn, NJ, USA), endogenous biotin and endogenous peroxidase were blocked by AKO X0590, USA, Carpinteria, CA and 3% H_2O_2 in methanol, respectively, and TUNEL staining was performed using the TACS 2 TdT-Fluor *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA).

For surface staining, monoclonal rat anti-murine CD4 (clone 4B12; Vector Labs, Burlingame, CA, Cat# VP-C319), CD8b (BD Pharmingen, San Diego, CA, #550797), were applied in DAKO diluent for 1 h, followed by staining with a biotinylated polyclonal rabbit anti-rat Ig (DAKO, Carpinteria, CA, USA) for 1 h. For visualization Streptavidin-PE (BD Pharmingen) was applied for 1 h.

For evaluation, 10 high power fields (HPF; × 400 magnification) were randomly selected and TUNEL/$CD4^+$ and TUNEL/$CD8^+$ double positive T-cells were counted and stated as mean ± SD. Evaluation was performed in a blinded fashion.

Immunohistochemistry

Immunohistochemistry was performed using OCT-embedded 4 μm thick acetone fixed mouse tissue sections. Slides were pretreated with Peroxidase Block (DAKO) for 5 min to quench endogenous peroxidase activity. Samples were incubated with monoclonal rat anti-murine CD4 (clone 4B12, Vector Labs, Cat# VP-C319), CD8b (BD Pharmingen, #550797), CD11c (BD Parmingen, #550292) diluted in DAKO buffer at 1:200 for 1 h, washed, and then stained with rabbit anti-rat immunoglobulin antibody diluted at 1:750. Slides were washed in 50-mM Tris-Cl, pH 7.4, and detected with anti-rabbit Envision+ kit (DAKO) as per the manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin. T-cells in >10 HPF were counted and stated as mean ± SD.

H&E staining

Cardiac graft samples fixed in 10% Formalin were embedded in paraffin, sectioned, and stained with H&E for evaluation of chronic graft lesions (chronic arterial vasculopathy, fibrosis and cellular infiltration) and graded semi-quantitatively with 0 = none, 1 = mild, 2 = moderate, 3 = severe based, 4 = luminal occlusion/complete morphological destruction on the overall extent/severity of the vasculopathy). The evaluation was conducted in a blinded fashion by an independent pathologist (RFP).

Statistics

Comparisons between experimental groups were performed using ANOVA one way tests and presented as Mean ± SEM. All results were generated using GraphPad Prism software (San Diego, CA). A P-value < 0.05 was considered as statistically significant.

Ethics statement

All animal work has been conducted according to the relevant national and local guidelines (Harvard Medical Area, Standing Committee on Animals, Protocol Number 04077). Approval Date: 06/25/2008. The 'Principles of Laboratory animal care' NIH publication Vol 25, No. 28 revised 1996; were followed.

Results

Donor age is associated with increased passenger leukocyte counts

As priming of recipients with donor specific DC's was shown to enhance early alloreactive IFNγ production in recipient's spleens [10], un-manipulated donor hearts were analyzed prior to transplantation for contents of passenger leukocytes. As shown in Fig. 1, old hearts contained significantly higher frequencies of $CD4^+$ and $CD8^+$ T-cells and DCs ($CD11c^+$) ($P < 0.05$) as assessed by FACS analysis. In particular, expression of MHC class II and co-stimulatory molecules (CD40, CD80, CD86) were increased on DC's in old hearts suggesting a higher maturity and activation levels of intragraft DCs ($P < 0.002$).

Graft survival was not influenced by donor age

Survival of young and old cardiac grafts was unaffected by donor age in this MHC II mismatched model (Fig. 2a). Although differences in donor age may not be reflected in this model, differences in immune responses may become apparent *in vitro* and on a cellular and histological level. Thus, we sought to investigate how donor age will affect the recipient's $CD4^+$ T-cell response.

Early increased frequencies of effector/memory T-cells following transplantation of old grafts

The FACS analysis of recipient's spleens revealed an altered T-cell response after engraftment of old hearts. Donor age evoked an expansion of effector/memory T-cells while $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T-cells were

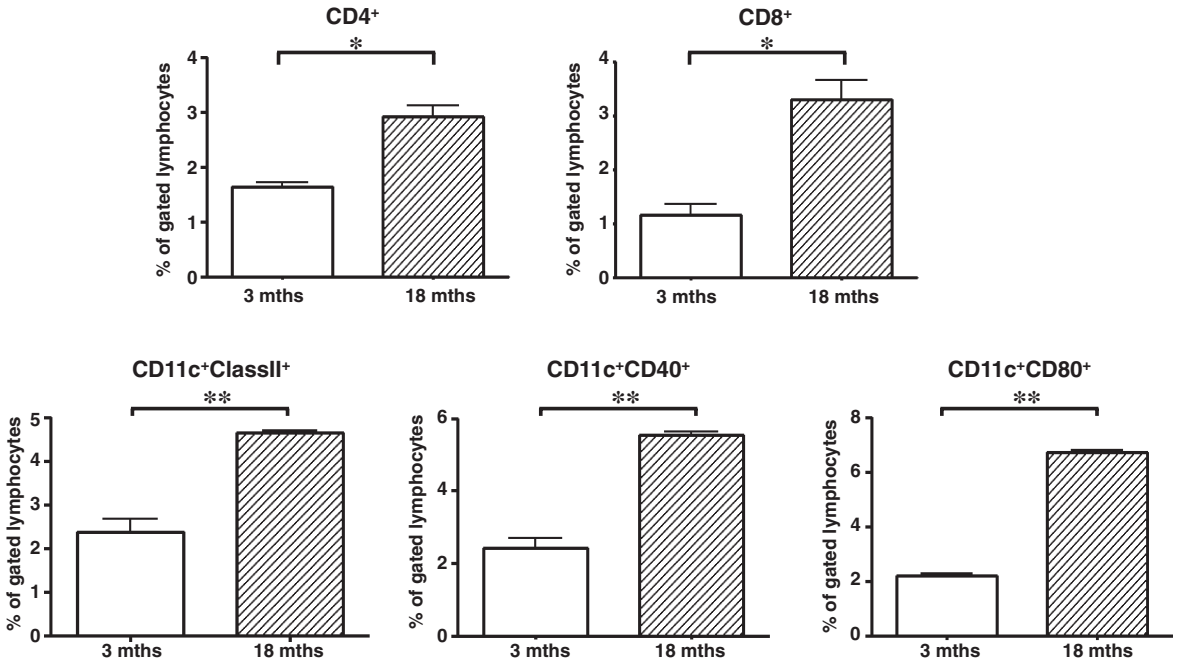

Figure 1 Increased passenger leukocyte count in aged naive hearts 3 and 18 months naive B6 mice were sacrificed, and naive hearts were stained for dendritic cells (DC) and T-cells ($n = 5$). Donor-derived DCs expressed increased levels of MHC class II and co-stimulatory molecules, suggesting a mature phenotype. * $P < 0.05$; ** $P < 0.01$.

reduced by day 14 after transplantation (Fig. 3a). In contrast, CD25 expression on $CD4^+$ T-cells was less intense after the engraftment of older hearts (Fig. 3a; $P < 0.05$). Thus, despite higher percentages of effector/memory T-cells, overall T-cell alloreactivity as measured by IFNγ release in ELISPOT analysis was not different between groups (Fig. 2b). In accordance with a decreased expression of early activation markers and intracellular IL-2 expression by $CD4^+$ T-cells (data not shown), proliferation of splenocytes was significantly impaired after engraftment of old hearts [mixed lymphocyte cultures (MLR)]. Interestingly, transplantation of syngenic hearts did not reveal age-dependent differences indicating that age-dependent modifications of graft immunogenicity were linked to the alloimmune response.

Taken together, old grafts carried higher frequencies of passenger leukocytes leading to an expansion of effector/memory $CD4^+$ T-cells and reduction of regulatory T-cells whereas activation and proliferation of naive T-cells was impaired at 2 weeks after transplantation.

Enhanced systemic T-cell response following transplantation of old grafts

Next, we analyzed consequences of T-cell activation after transplantation of older organs. By 8 weeks, frequencies of activated $CD4^+$ T-cells were significantly elevated following engraftment of old hearts (Fig. 3b). In accordance, *in vitro* alloreactivity as measured by ELISPOT analysis and splenocyte proliferation (3H TdR (Thymidine) uptake in MLR) had been more pronounced when older organs were transplanted (Fig. 2c). The augmented alloreactive T-cell response had been linked to a significant expansion of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T-cells in recipient's spleens. Memory T-cell responses, at the same time, were comparable and donor age-independent (Fig. 3b). Interestingly, donor age did not affect syngenic controls indicating an allo-antigen dependent effect of older donor organs on the immune response.

These data suggest that donor age leads to a stronger activation of naïve T-cells and a systemic expansion of regulatory T-cells while frequencies of effector/memory T-cell were unaltered in recipients of older hearts.

Systemic immune responses do not translate into an accelerated activation intragraft

First, we procured heart grafts by 8 weeks to assess the immune response intragraft. Surprisingly, fewer infiltrating $CD4^+$ T-cells were observed in older grafts (Fig. 4a; $P < 0.05$). Next, we dissected graft infiltrating $CD4^+$ T-cells in more detail. Although comparable frequencies of T-cells with an effector/memory phenotype were found

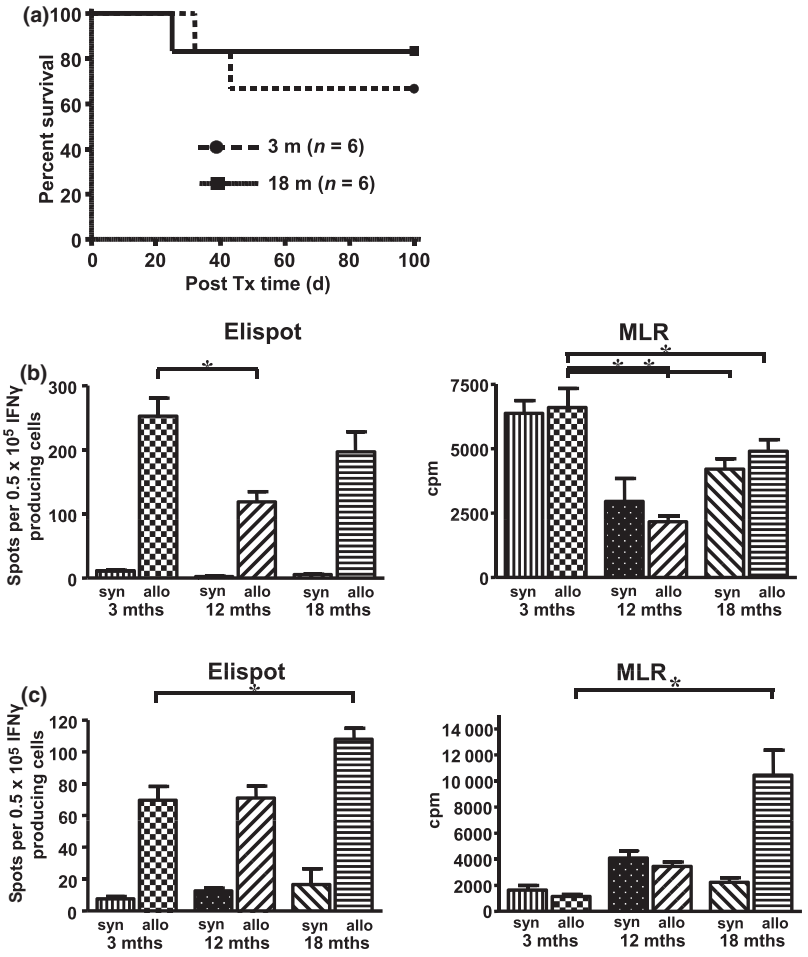

Figure 2 Donor age alters the immune response without affecting long-term heart allograft survival. Despite higher passenger leukocyte counts with increasing donor age, survival of 3 months and 18 months old hearts was comparable (a). However, recipients of old hearts demonstrate a delayed but enhanced functional response to allografts. Early after transplantation (day 14), T-cell proliferation was decreased following the transplantation of (18 months) old hearts (b) while long-term (by week 8) both T-cell proliferation and alloreactive IFNγ - production had significantly (n = 6) (c).

in young and old heart grafts, percentages of $CD4^+$ T-cells expressing FoxP3 were significantly increased (Fig. 4 b). These observations are in line with our systemic phenotypical findings which demonstrated a comparable effector T-cell but expanded regulatory T-cell population in recipients of old hearts.

Next, we explored by immunofluorescent TUNEL staining of intragraft T-cells whether apoptosis mediated suppression by regulatory T-cells may play a role in the $CD4^+$ T-cell response toward older grafts in this model. Interestingly, numbers of apoptotic $CD4^+$ T-cells were comparable in young and old grafts by 8 weeks after transplantation suggesting that regulatory T-cells did not induce apoptosis of alloreactive $CD4^+$ T-cells in this setting (Fig. 5).

Finally, we sacrificed long-term surviving recipients by day 100 after transplantation and examined grafts for structural changes. H&E staining demonstrated signs of CTV assessed by insignificantly different grades of cellular infiltrates, interstitial fibrosis and graft arteriosclerosis suggesting that CTV was not significantly aggravated when older cardiac allografts were transplanted in this model (Fig. 6).

Taken together, aging *per se* did not seem to affect graft immunogenicity in our model as the recipient's immune response to syngenic heart grafts had been comparable to both young and old cardiac isografts. Donor age, however, modified the $CD4^+$ response following allotransplantation. A systemic activation of $CD4^+$ T-cells went along with an increased *in vitro* alloreactivity whereas structural

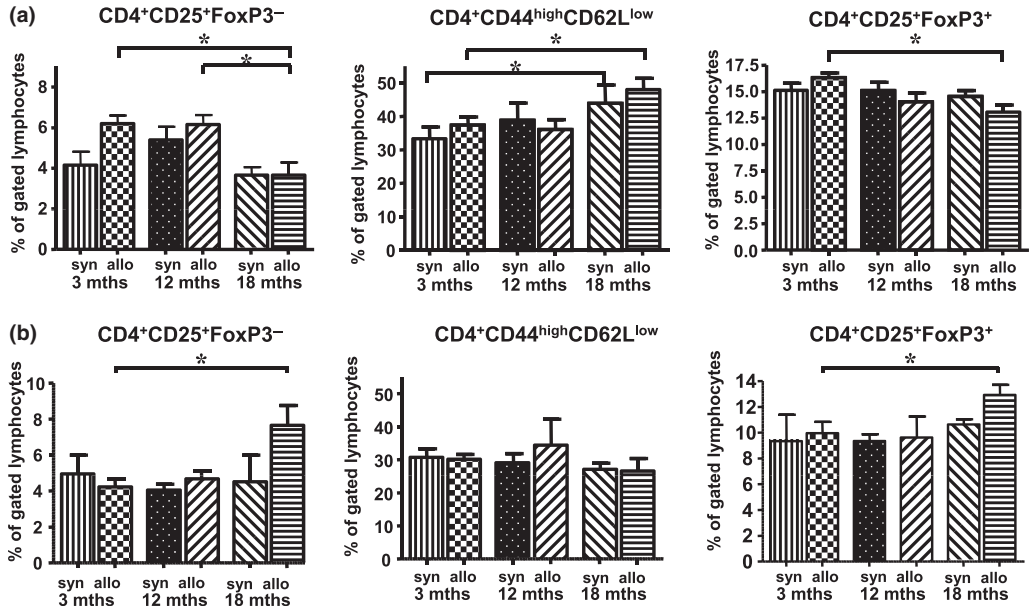

Figure 3 Time course of T-cell activation and proliferation after transplantation of old heart grafts. Recipients of 3, 12, and 18 months old B6 heart grafts were sacrificed by day 14 and recipients spleens were analyzed for the T-cell response ($n = 6$). Donor age was associated with an accumulation of effector/memory CD4+ T-cells, while frequencies of activated T-cells were diminished at this early timepoint after transplantation (a). In line with the augmented *in vitro* response of recipients of old grafts, frequencies of activated T-cells and regulatory T-cells had significantly increased by week 8 after transplantation (b). Of note, amounts of T-cells with an effector/memory phenotype were comparable suggesting an altered ratio of regulatory and effector mechanisms with advanced donor age. $^*P < 0.05$.

changes were overall comparable in young and old cardiac allografts.

Discussion

As a consequence of the increasing organ shortage, old organs are becoming an increasingly valuable source to expand the donor pool. It is recognized that expanded criteria donor organs have a compromised transplant outcome compared with more optimal or standard criteria donor organs. However, studies dissecting the effects of organ aging on the immune response after transplantation remain scarce. At the same time, effects of donor age on the recipients CD4+ T-cell responses have not been investigated. Based on our previous findings of a modified, time-dependent T-cell response with the transplantation of older donor organs we sought to further delineate the impact of advanced donor age on the biology of chronic CD4+ T-cell responses. For this purpose, we made use of a MHC class II mismatched murine cardiac transplant model which would allow us to study the isolated role of CD4+ T-cells in chronic rejection of old allografts as this feature has not been evaluated yet. Although this approach may not reflect the entire clinical situation it allows us to further analyze specifics of the T-cell response on the background of a more tailored immunosuppression in the clinical setting. Older cardiac grafts contained higher frequencies of passenger leukocytes associated with an early memory T-cell expansion. However, recruitment of the naïve T-cell pool was delayed at an early time point. In keeping with previous findings of our group [8] we did not observe an enhanced immune response early after transplantation in our model when engrafting older organs, however, the recipient's immune response to an older organ had become more vigorous by 8 weeks.

In parallel, a systemic activation of CD4+ T-cells was associated with an expansion of regulatory T-cells which was also reflected by an influx of Tregs into old heart grafts. The altered intragraft ratio of effector to regulatory T-cells, however, did not contribute to an accelerated morphological deterioration of older organs as histological features of chronic graft deterioration were age-independent.

The complex role of intragraft T-cell subsets has not been fully elucidated yet. It has recently been suggested that effector T-cells generated during an immune response may actively potentiate suppression by regulatory T-cells, implying a role of regulatory proteins and IL-10 and IFNγ, both of which are found increasingly in aged tissues [15–19].

Yet, regulatory T-cells may have dichotomous effects on graft outcome as they have been implied in both graft

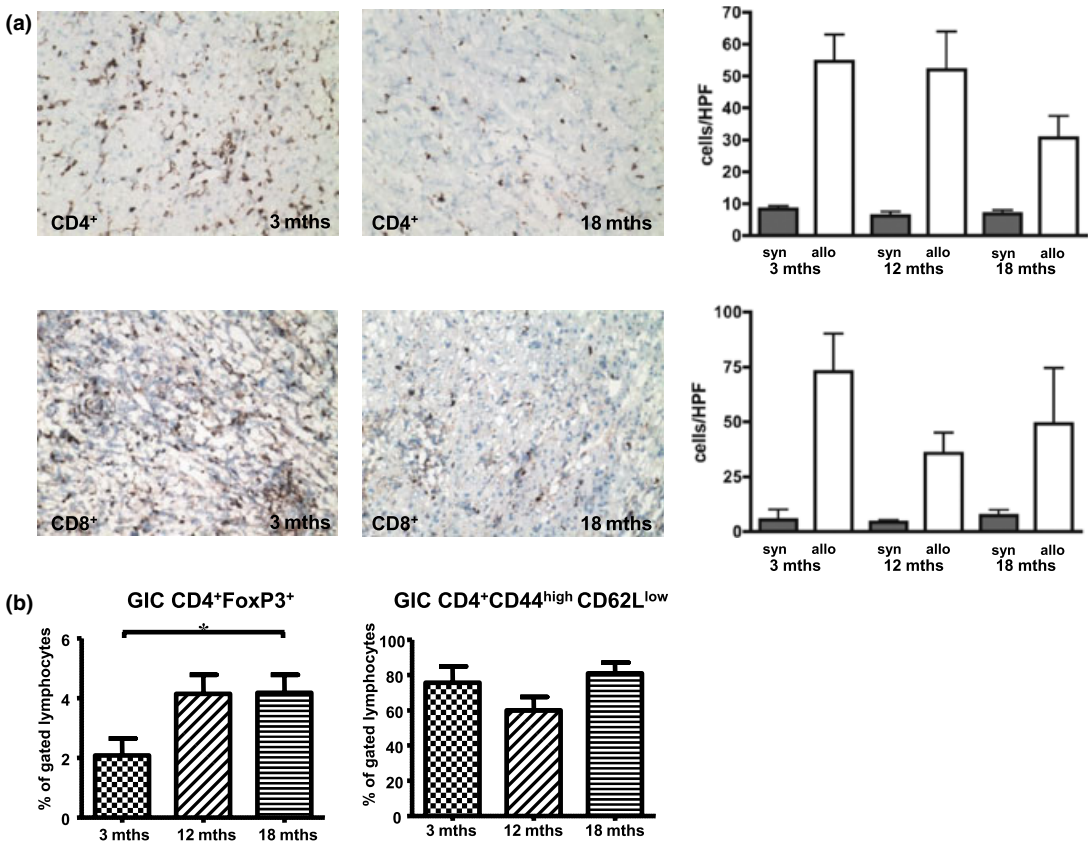

Figure 4 Altered graft infiltration pattern in old hearts. Grafts were procured after 8 weeks and snap frozen sections were stained for graft infiltrating $CD4^+$ T-cells ($n = 6$) (a). Fewer T-cell infiltrates were observed in old compared with young heart grafts (3 months vs. 18 months: CD4: 54.6 vs. 30.5 cells/HPF, $P < 0.05$). As shown in graph (b), a higher proportion of FoxP3 expressing $CD4^+$ T-cells was detected using FACS analysis in aged allografts ($n = 6$). $^*P < 0.05$.

protection and graft deterioration. Grimbert *et al.* reported on a ''tolerogenic'' higher intragraft ratio of FoxP3/cytotoxic T-cells in kidney transplant recipients with borderline changes compared with patients acutely rejecting their grafts [20]. Besides, intragraft regulatory T-cells have also been associated with reduced alloresponsiveness and stable function in human kidney transplant recipients [21–23]. Chronically rejecting patients, on the other hand, exhibited significantly less FoxP3+ regulatory T-cells [24,25]. In contrast, intragraft FoxP3 expression was found to correlate with interstitial fibrosis, acute rejection, and poor graft outcome in kidney transplant recipients [26]. Similar to our findings, FoxP3 expressing T-cell counts were significantly increased during acute rejection in clinical heart transplant recipients suggesting that they parallel the anti[-donor T-cell response rather than preventing acute rejection [27]. In this regard, it has been shown that the suppressive capacity of intragraft regulatory T-cells rather than their absolute numbers may be important for amelioration of acute rejection responses in heart transplant recipients [28].

Taken together, recipients of old heart grafts in our study demonstrated a stronger $CD4^+$ T-cell activation associated with an expansion of regulatory T-cells both in the spleen and in the graft. Yet, cardiac structure and graft outcome of young and old allografts was not significantly different. Although some studies suggested an increased immunogenicity and enhanced graft deterioration of older organs [8,29], others found that elderly murine kidney grafts do not exhibit an increased immunogenicity and show comparable structural changes compared with young grafts [7]. The authors of the latter study concluded that cell senescence, accelerated by peritransplant stress, rather than an age-dependent immunogenicity may account for differences in long-term graft outcome. Of note, syngenic heart grafts in our study did not reveal an age-dependent immunogenicity. In contrast, older heart allografts stimulated an augmented alloreactivity *in vitro*. Interestingly, most *in vitro* results in our study did not show significant differences between recipients of 3 months and 12 months old heart grafts, whereas recipients of 18 months demonstrated significantly

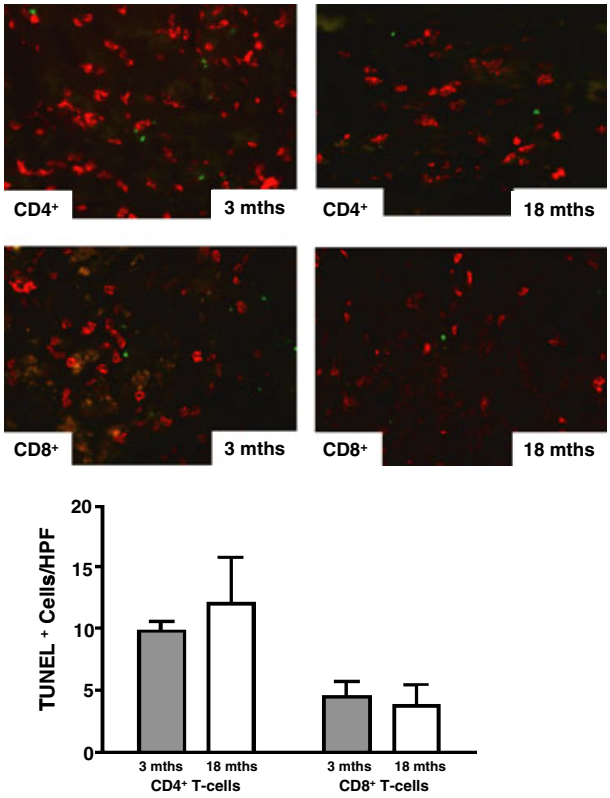

Figure 5 Intragraft T-cell apoptosis. 8 weeks after transplantation, heart allografts were retrieved and tissue sections were stained for apoptotic T-cells using an *in situ* Apoptosis Detection Kit. Following surface staining for CD4+ T-cells, 10 random pictures were taken per slide and counted for double positive cells at 400 × magnification in a blinded fashion (a). Percentages of both apoptotic CD4+ T-cells were not different in old compared with young grafts.

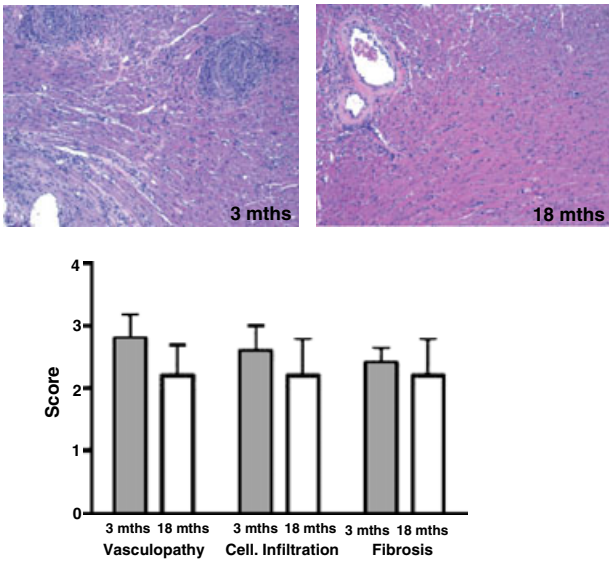

Figure 6 Graft Morphology. To depict consequences of a mitigated intragraft immune responses, grafts from long-term surviving animals (day 100) were procured and chronic lesions were examined by H&E staining (b). Overall structure was comparable in young and old hearts and vasculopathy was similarly pronounced with increasing donor age. (Representative slides of $n = 4$ per group are shown; evaluated by a blinded pathologist).

different elevated *in vitro* parameters. Those results suggest that consequences of aging become apparent only in very old donors. This may be explained by the terminally decreased function of "key cells" such as stem cells, epithelial and endothelial cells, and resident macrophages causing an impaired recovery and repair which ultimately leads to a chronic state of inflammation (so called "inflamm-aging"). These findings are in line with previous *in vitro* results by our group as well as recent data of kidney transplant recipients proving increased rates of acute rejection with advanced donor age [8,30]. Although our model is based on a MHC class II mismatch and thus does not parallel the complex clinical reality, it allowed us to study the isolated role of chronic graft rejection by CD4+ T-cells in the context of donor age. Our experimental data demonstrate an aggravated CD4+ T-cell response toward old heart grafts which may have important clinical implications regarding a modified immunosuppression when transplanting older organs. Further studies in a full MHC-mismatch setting are needed to evaluate the impact of CD4+ T-cell responses on long-term outcome of aged allografts.

Authorship

CD: wrote the article, conducted *in vitro* and *in vivo* experiments and designed the study. XG: conducted experiments (*in vivo*), provided scientific advice. AJ: conducted experiments (*in vitro*). SK: conducted experiments (*in vitro*). IK: conducted experiments (*in vitro*). RFP: conducted histological analysis. AN: conducted experiments (*in vitro*). PF: conducted experiments (*in vitro*). JP: provided scientific advice. SGT, PI: designed the study, provided animals and lab equipment, wrote the manuscript.

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