SUPPLEMENTAL MATERIAL

$CD11c^+$ DCs accelerate the rejection of older cardiac transplants via IL-17A

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Short Title: Aged DCs augment cardiac allograft rejection

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CV Surgery: [37] transplantation, ventricular assistance, cardiomyopathy
Basic Science Research: [130] animal models of human disease

SUPPLEMENTAL METHODS

Histology, immunohistochemistry and quantification

At day 7 animals were sacrificed and cardiac allografts were procured. Samples were fixed in 10% formalin and embedded in paraffin. Coronary sections were then stained with hematoxylin/eosin (Sigma-Aldrich, St. Louis, MO), and analyzed by light microscopy. The degree of rejection was determined according to a score system based on the degree of inflammatory infiltration and myocyte damage (0=no rejection to 4=severe rejection) according to the International Society of Heart and Lung Transplantation (ISHLT) rejection score [11,12]. An independent pathologist (R.B.) analyzed all sections in a blinded fashion. Immunohistochemistry was performed in paraffin-embedded tissue sections with an automated immunostainer (Nexes; Ventana, Tucson, Arizona, USA), applying the streptavidin–biotin–peroxidase technique with diaminobenzidine as chromogen. Rat anti-mouse CD4 mAb (BD Biosciences, San Diego, CA), as well as rat anti-mouse CD8 mAb (BD Biosciences, San Diego, CA) were used as primary antibodies. For antigen retrieval heat pretreatment in an autoclave at 121°C for 5 minutes with citrate buffer (pH 6.0) was performed. Positive cells in 5 randomly selected high power fields were counted (HPF; 400 magnification). Data are given as mean ± SEM. An independent pathologist analyzed all sections in a blinded fashion. Immunohistochemistry for $CD11c^+$ DCs was performed using OCT-embedded, 4μm acetone-fixed mouse tissue sections. Slides were pre-treated with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity. Monoclonal hamster anti-murine $CD11c^+$ (BD Biosciences, San Diego, CA) was applied in DAKO diluent at 1:200 for 1 hour. After washing, rabbit anti-rat immunoglobulin antibody was applied at 1:750 for 1 hour. Slides were then washed in 50-mM Tris-Cl, pH 7.4 and analyzed with anti-rabbit Envision+ kit (DAKO) per manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DAKO) prior to counterstaining with hematoxylin.

Cell culture, cell proliferation assays

Isolated naïve $CD4^+$ T cells ($0.3x10^6$ cells per well) were co-cultured with isolated $CD11c^+$ DCs ($0.06x10^6$ cells per well; ratio 5:1) in 48-well flat bottom plates in 0.5ml of complete RPMI 1640

media supplemented with 10% FCS, 200mM L-glutamine, 100U/ml penicillin/streptomycin and $5x10^5$ M 2-mercaptoethanol (RP-10) in presence of 10μg/ml plate-bound anti-mouse α-CD3 (17A2) and 2μg/ml soluble α-CD28 (37.51). LPS (Sigma-Aldrich) was diluted in PBS and added at a concentration of 1μg/ml. Where indicated, cells were cultured in Th17-polarizing conditions (10ng/ml of recombinant TGFβ, 100ng/ml of recombinant IL-6, 10mg/ml of anti-IFNγ, and 10mg/ml of anti-IL4). All recombinant cytokines and antibodies were purchased from eBioscience except recombinant TGFβ (R&D Systems, Minneapolis, MN). After 7 days of culture supernatants and cells were collected and analyzed by ELISA and flow cytometry, respectively.

For cell proliferation assays, 0.5×10^6 splenocytes from transplanted DBA/2J mice were co-cultured with irradiated donor-type splenocytes from naïve C57BL/6 mice in 96-well round bottom cell culture plates. After 72 hours, cells were pulsed with ^{3}H thymidine (1μCi/ well) and incubated for another 12 hours. Incorporation of ^{3}H thymidine indicating cell proliferation was then assessed as counts/min, using a Wallac Liquid Scintillation Counter (PerkinElmer Inc., Boston, MA, USA).

To test immunogenic properties of old versus young dendritic cells *in vitro*, 1×10^6 splenocytes from naïve DBA/2J mice were co-cultured with 10^4 splenic CD11c$^+$ DCs from young or old naïve B6 mice with no LPS-stimulation, with low LPS-stimulation (10ng/ml) or with high LPS-stimulation (100ng/ml) in 96-well round bottom cell culture plates and further processed as described above.

Flow Cytometry

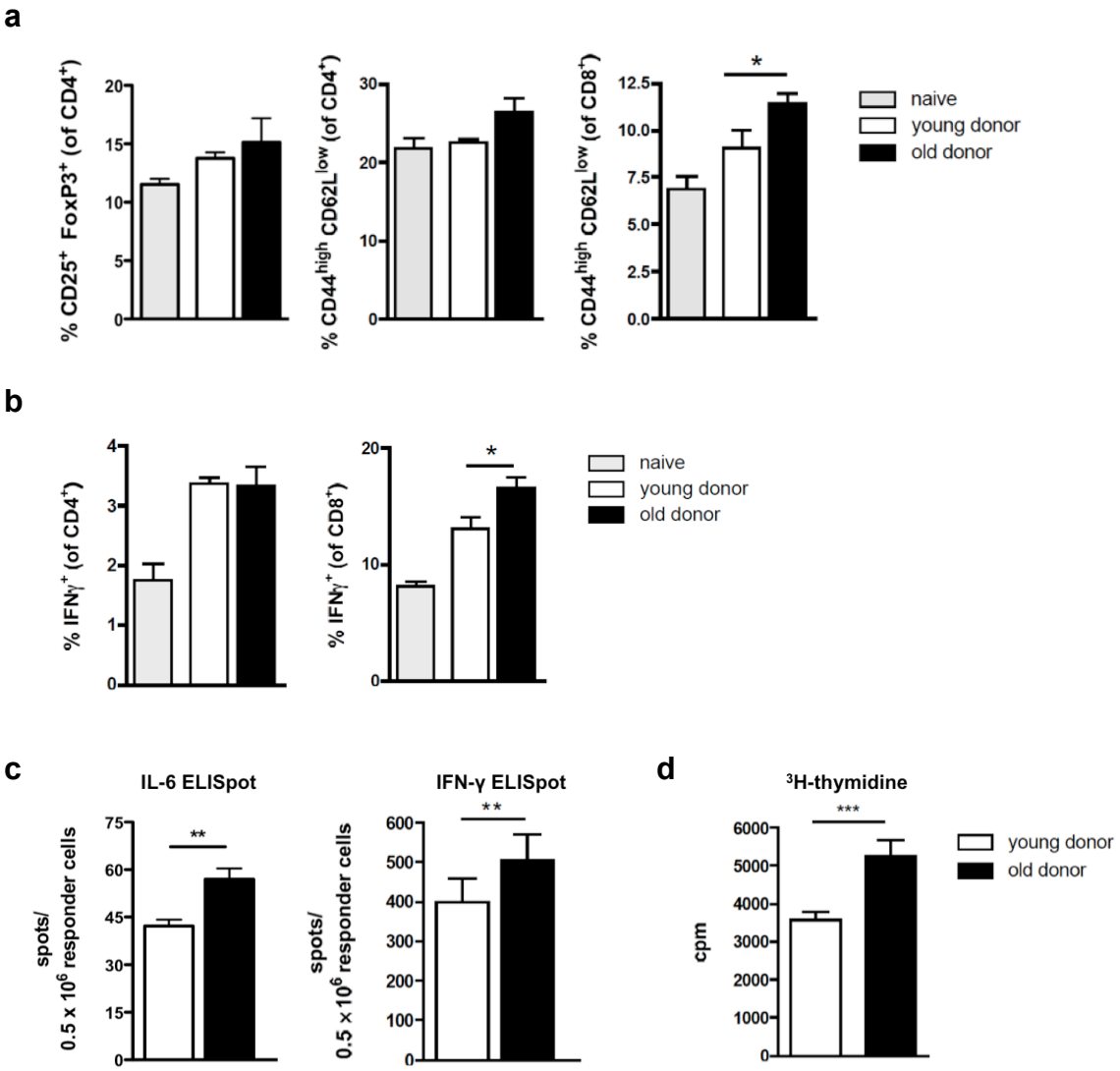
Fluorescence labeled anti-mouse α-CD4 (GK1.5), α-CD8 (53-6.7), α-CD25 (PC61), α-CD11c (HL3), α-CD40 (3/23), α-CD44 (IM7), α-CD62L (MEL-14), α-CD80 (16-10A1), α-CD86 (GL1), α-IA^b (AF6-120.1) antibodies were obtained from BD Biosciences (San Jose, CA, USA). Fluorescence labeled anti-mouse α-Foxp3 (150D/E4), α-IFNγ (XMG1.2) and α-IL-17A (eBio17B7) were purchased from eBioscience (San Diego, CA, USA). For compensation and gate setting, permeabilized and unpermeabilized unstained cells were used. Intracellular staining for Foxp3, IFNγ and IL-17A was performed according to manufacturer's protocols. Cells were re-stimulated in complete media (RPMI media containing 10% FCS, 1% L-Glutamine, 1% Penicillin/Streptomycin; all Bio Whittaker,

Walkersville, MD) for 4 hours at 37°C with ionomycin, phorbol 12-myristate 13-acetate and Brefelding A (eBioscience). Cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences). Flow cytometry measurements were performed using a FACSCalibur system (BD), and data were analyzed using FlowJo (Tree Star, Ashland, OR, USA).

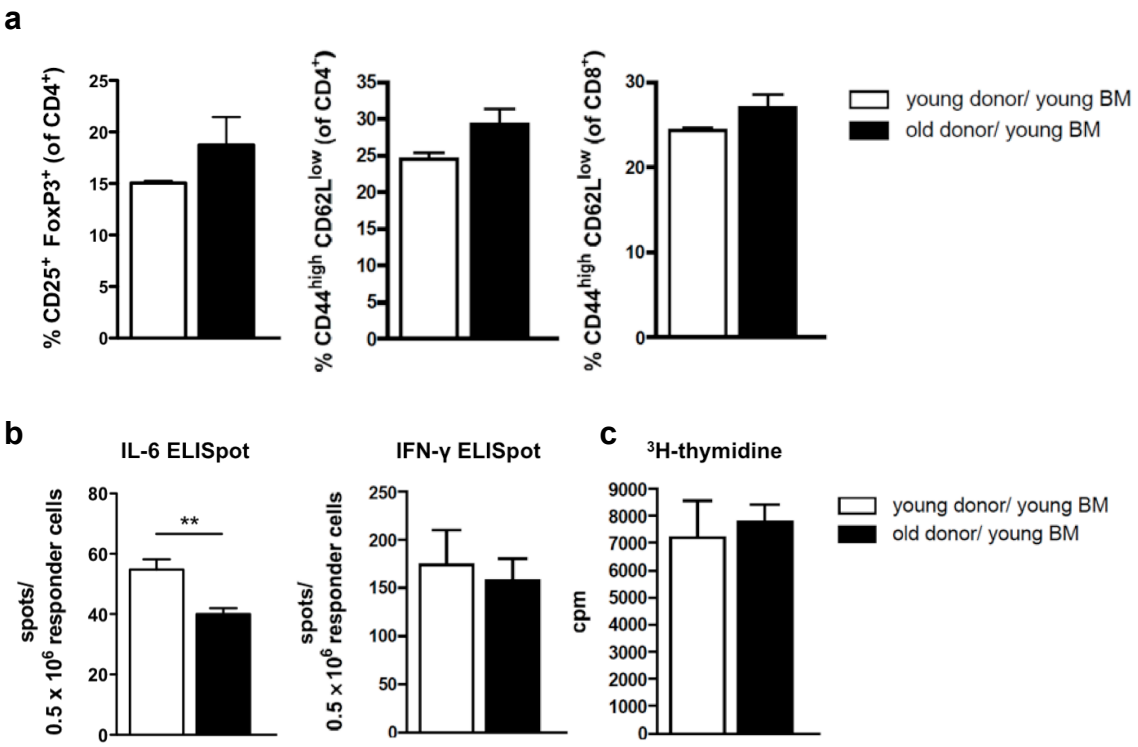
RNA extraction and real-time PCR

RNA extraction from cardiac allografts was performed using RNAqueous extraction kit according to the manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Briefly, 1 mg of heart tissue was homogenized in lysis buffer (total volume of 0.5 ml) and passed through a column. After successive washes, RNA was eluted and reverse transcription was performed using i-Script® cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). For real-time PCR reactions *IL-17A* (MM5419) measurements were performed with Taqman primers and probes from Applied Biosystems. Relative gene expression was determined using the housekeeping gene *GAPDH* (MM99999915_g1) as control.

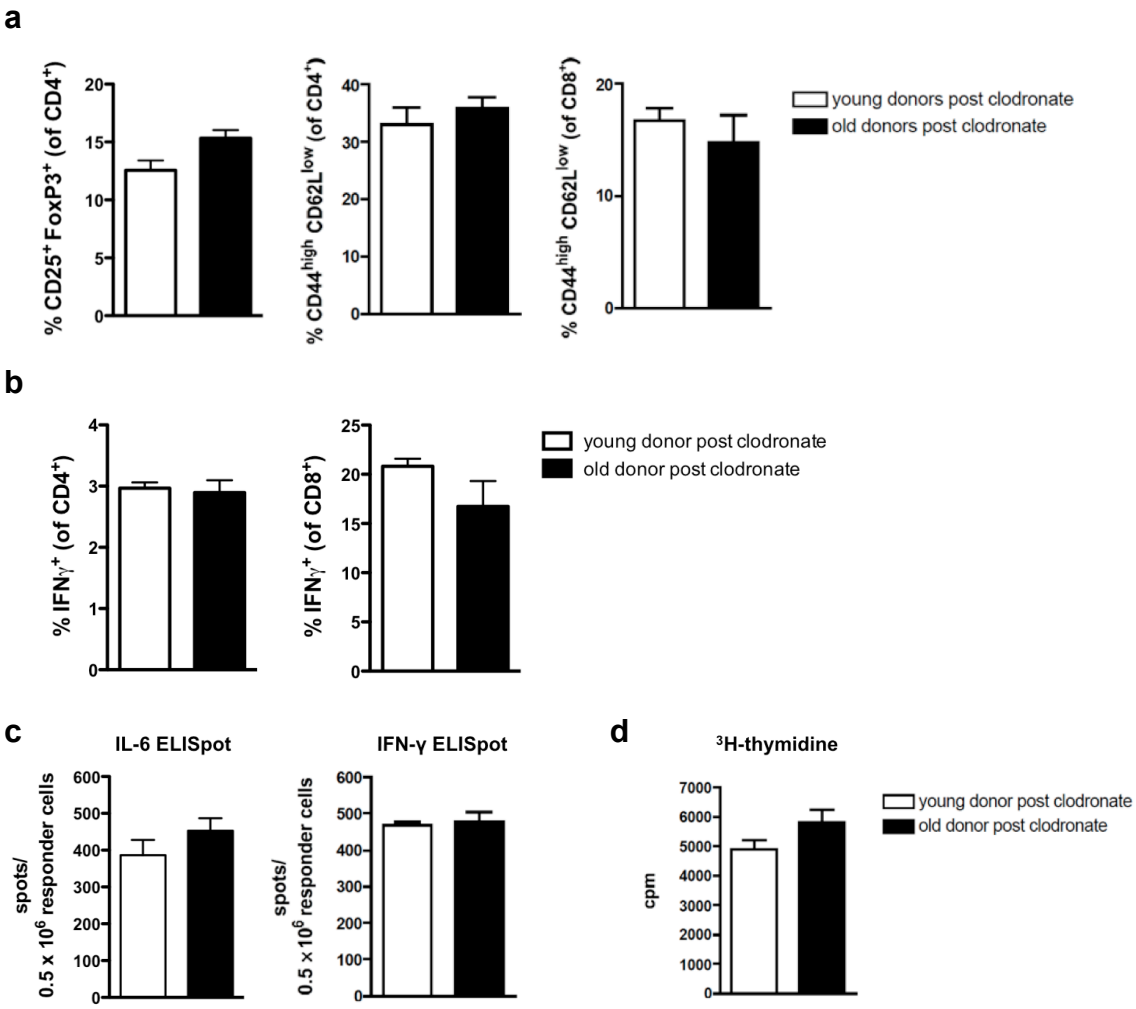
SUPPLEMENTAL FIGURES AND LEGENDS



Supplemental Figure 1. Older cardiac allografts elicit more potent systemic alloimmune responses. Hearts from old and young C57BL/6 mice were transplanted into young DBA/2J mice; 7 days after transplantation, mice were euthanized and splenocytes were analyzed by flow cytometry for ***a***, frequencies of $CD4^+CD25^+FoxP3^+$ Tregs and CD4+/CD8+ effector cells ($CD44^{high}$ $CD62L^{low}$) (n=4/group; **P*<0.05) and ***b***, frequencies of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells (n=4/group; **P*<0.05). ***c-d***, Splenocytes from young DBA/2J mice were co-cultured with irradiated donor-type splenocytes from old or young C57BL/6 mice and (***c***), frequencies of splenocytes producing IL-6 and IFNγ and (***d***) proliferation rates were analyzed by ELISpot and ^{3}H-thymidine incorporation, respectively (n=4/group; ***P*<0.01; ****P*<0.001; data are representative of three independent sets of experiments).



Supplemental Figure 2. Systemic immune responses were comparable after the transplantation of chimeric young and old hearts. 24 hours after irradiation, bone marrow transplantation from young syngeneic C57BL/6 mice was performed to reconstitute passenger leukocytes within donor hearts. 7 days after transplantation, mice were euthanized and splenocytes were analyzed by flow cytometry for ***a***, frequencies of $CD4^+CD25^+Foxp3^+$ Tregs and $CD4^+/CD8^+$ effector T cells ($CD44^{high}$ $CD62L^{low}$) (n=4/group; NS, non-significant). ***b-c***, Splenocytes from young DBA/2J mice were co-cultured with splenocytes from old or young chimeric C57BL/6 mice and (***b***) frequencies of splenocytes producing IL-6 and IFNγ and (***c***) proliferation rates were analyzed by ELISpot and ^3H-thymidine incorporation, respectively (n=4/group; $^{**}P<0.01$; data are representative of three independent sets of experiments).



Supplemental Figure 3. Systemic alloimmune responses to grafts procured from donors pretreated with clodronate were age-independent. DC-depleted hearts from old and young C57BL/6 mice were transplanted into young DBA/2J mice; 7 days after transplantation, mice were euthanized and splenocytes were analyzed by flow cytometry for (***a***) frequencies of $CD4^+CD25^+FoxP3^+$ Tregs and $CD4^+/CD8^+$ effector T cells ($CD44^{high}$ $CD62L^{low}$) (n=4/group; NS, non-significant) and (***b***) for frequencies of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells (n=4/group; NS, non-significant). ***c-d***, Splenocytes from young DBA/2J mice where co-cultured with splenocytes from DC-depleted old or young C57BL/6 mice and (***c***), frequencies of splenocytes producing IL-6 and IFNγ and (***d***) proliferation rates were analyzed by ELISpot and ^{3}H-thymidine incorporation, respectively (n=4/group; NS, non-significant, data are representative of three independent sets of experiments).