Recipient Sex and Estradiol levels affect transplant outcomes in an age-specific fashion

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Abbreviations

CD, cluster of differentiation

CI, confidence interval

FCM, flow cytometry

IFN, interferon

IL, interleukin

LC-MS/MS, Liquid Chromatography – Tandem Mass Spectrometry

Treg, regulatory T cell

OVX, ovariectomy

PBS, phosphate buffered saline

Abstract

Sex-specific influences have been shown for a variety of diseases. Whether donor or recipient sex and sex-hormone levels impact alloimmune responses remains unclear.

In uni- and multifactorial analyses of more than 400.000 SRTR listed kidney transplant patients, we found that younger female recipients had an inferior death-censored graft survival that was independent of donor sex. In contrast, graft survival was superior in older female recipients, suggesting the impact of recipient sex-hormones over chromosomal sex mismatches.

Those clinical changes were delineated in experimental skin and heart transplant models showing a prolongation of graft survival in ovariectomized young female recipients. In contrast, graft survival was comparable in ovariectomized and naïve old female recipients. Young ovariectomized mice showed reduced amounts and a compromised T cell proliferation. Deprivation of female hormones dampened the production of IFN-ɣ and IL-17^{+} by CD4^{+} T cells while augmenting systemic counts of T regs. Increasing estradiol concentrations in-vitro promoted the switch of naïve CD4^{+} T-cells into Th1 cells; high physiological estradiol concentrations dampening Th1 responses, promoted T regs, and prolonged graft survival.

Thus, clinical observations demonstrate age-specific graft survival patterns in female recipients. Estrogen levels, in turn, impact the fate of T-cell subsets, providing relevant and novel information on age and sex-specific alloimmunity.

Introduction

Biological sex has been shown to distinctively impact diseases with females being more susceptible to autoimmune diseases (1), some cardiovascular disorders (2) and neurodegenerative conditions (3). Male sex, on the other hand has been shown to be a risk factor for infections including COVID (4, 5), obstructive coronary artery disease (6) or Parkinson's disease (7).

Although of similar chemical structure, the divergent expression of sex hormones and their receptors throughout various stages of adulthood contributes significantly to molecular and physical diversity (8). Female hormones change dramatically with age and during pregnancy. Menopause is characterized by lower estrogen levels as a consequence of age dependent ovarian insufficiency (9, 10).

The hormonal milieu plays a cardinal role in regulating immunity. Estrogen, for instance, modulates T helper 1 cells (11, 12) through the interaction of the estrogen receptor with the promoter region of the IFN-γ gene and through the induction of the transcription factor T-bet (13). Moreover, elevated estrogen levels have been shown to promote an augmented T-helper 2 cell response (14). Immune effects of estrogen appear to be dose-dependent as highly elevated estrogen levels during pregnancy induce regulatory T-cells (T-reg) contributing to an intact pregnancy of the semi-allogenic fetus (15).

Although, effects of sex-hormones on immunity have been recognized, their impact on alloimmune responses over a lifetime remains unclear.

In organ transplantation, donor/recipient sex-mismatches may include aspects beyond age-dependent levels of sex-hormones (16). Kidney size mismatches are of relevance when transplanting female kidneys into male recipients (17); nephron counts have been shown to contribute to an inferior graft function and compromised outcomes (18). Likewise, female hearts and livers transplanted into male recipients displayed inferior survival rates when compared to male to male donor/recipient combinations (18, 19). Interestingly, female kidneys also exhibited compromised graft survival rates when transplanted into female recipients(18, 20), suggesting that hormonal aspects in addition to graft size mismatches are of relevance.

Sex-chromosome mismatches may also play a role: female (xx) recipients receiving sex-chromosome incompatible (xy) transplants have shown higher rates of graft loss (21, 22). Furthermore, hematopoietic stem cells from female donors transplanted into male recipients

demonstrated higher rates of graft versus host disease (GVHD) regardless of HLA-matching, suggesting a significant role of sex-chromosome incompatibility (23).

It is well known that aging impacts immune responses. We and other have previously detailed the consequences of aging on alloimmunity and transplant outcomes (24, 25). Hormonal changes in aging may provide an additional aspect explaining age-specific alloimmune response.

Material and Methods

Study Population and Design of Human Clinical data

Clinical transplant data were extracted from the Scientific Registry of Transplant Recipients (SRTR October 1,1987 - December 31, 2017). Recipients (15 – 74 years) were included; recipients of a renal plus non-renal transplant were excluded; patients with incomplete data were excluded in the analysis. For the clinical study, we assumed sexual reproduction starting between 10 to 13 years linked to an increase in female sex-hormones. Menopause was assumed to begin between 42 to 58 years with a dramatic drop in female hormones (16).

Animals

Young C57BL/6 (H2b; 2-3 months, both sex) mice were purchased from Charles River Laboratory (Wilmington, MA); wild-type DBA/2 (male, 2-3 months) from Jackson Laboratory (Bar Harbor, ME); old male and female C57BL/6 (18 months) from the National Institute of Aging (NIA, Bethesda, MD, USA).

Skin transplantation and ovariectomy

Recipient mice included naïve young and old animals (male and female, 2-3 and 18 months, respectively); additional groups consisted of young and old ovariectomized female mice in addition to sham operated animals. For surgical procedures, mice were anesthetized with intraperitoneal (i.p.) injections of Ketamine/Xylazine (100 and 10 mg/kg, respectively); ovariectomies were performed through flank incisions.

Fully mismatched skin transplants were performed two weeks after ovariectomies or sham surgeries as described previously (26-29); graft rejection was monitored daily and defined as necrosis exceeding 90%.

Hormone treatment

17β-estradiol (E2, Sigma-Aldrich, St Louis, MO) was dissolved in ethanol. For in-vitro experiments, varying doses of E2 (10^{-12} M to 10^{-8} M) were diluted in PBS and added into a hormone-deficient cell culture.

For exogenous estradiol replacement experiments, mice were randomly assigned to four treatment groups at the time of ovariectomy and implanted subcutaneously with a silastic capsule

(28, 29) (Silastic Laboratory Tubing, Dow Corning Co, Midland, MI) containing either a sesame oil (sham treatment) or various E2 concentrations in oil (E2(0)); 50 μg/mL (E2(50)) and 500 μg/mL (E2(500)). Serum estradiol concentrations were measured 14 days after surgical implantation.

Estradiol assay

Circulating estradiol was assayed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Shimadzu, Columbia, MD and Agilent Technologies, Santa Clara, CA). Intra-assay coefficients of variation (c.v.) were 8.21 %, 2.41 %, 2.94 % and 2.30 % at 8.7, 57.1, 157.0 and 540.0 pg/mL, respectively. Inter-assay c.v. values were 10.07 %, 8.49 %, 6.25 % and 2.77 %, at 8.7, 57.1, 157.0 and 540.0 pg/mL, respectively.

Cell Isolation and T-Cell Differentiation

$CD4^+$ T cells were isolated from splenocytes by negative selection (Stemcell technologies, Vancouver, BC, Canada); cells were used at a purity greater than 95%.

$CD4^+$ T cells were cultured in 96-well plates (2.0×10^5 cells per well) suspended with 0.2 mL of hormone-deficient medium at 37°C under 5% CO_2 and 95% air atmosphere with saturated humidity.

Cells were cultured in polarizing T helper type 1 (Th1) (50 ng/mL recombinant IL-2, 100 ng/mL recombinant IL-12 and 100 μg/mL anti-IL-4), T helper type 17 (Th17) (50 ng/mL recombinant IL-2, 100 μg/mL anti-IL-4, 50 ng/mL recombinant TGF-β and 100 μg/mL anti-IFN-γ) and T-reg (100 μg/mL anti-IL-4, 100 ng/mL recombinant IL-6, 50 ng/mL recombinant TGF-β and 100 μg/mL anti-IFN-γ) conditions. For Th0 conditions, $CD4^+$ T cells were cultured with recombinant mouse IL-2 (50 ng/mL) in the presence of α-CD3 and α-CD28 maintaining T-cell survival. E2 diluted in PBS was added to wells as indicated. All recombinant cytokines and antibodies were from Invitrogen (Waltham, MA).

Flow Cytometry

Splenocytes were labeled for surface and intracellular antigens with fluorescence α-CD4, α-CD8, α-CD25, α-FOXP3, α-IFN-γ, α-IL17 and α-IL10 anti-mouse antibodies (Invitrogen). Intracellular cytokine staining for FOXP3 was performed with a commercially available staining kit

(Invitrogen). Flow cytometry measurements were performed on a FACS Canto II (BD Bioscience); data were analyzed using FlowJo (FlowJo Software, OR).

Mixed Lymphoid Reaction

To determine the proliferation of $CD4^+$ T cells, splenocytes (collected by day 8) were stained with CFSE dilution (Invitrogen). Thereafter, total splenocytes were incubated with stimulator DBA/2 splenocytes treated with mitomycin (Sigma-Aldrich). After 5 days of culture, cells were analyzed by flow cytometry.

ELISA

IFN-γ was measured in supernatants after stimulation with donor-type splenocytes for 5 days. A commercial ELISA kit (Invitrogen) was used according to the manufacture's instruction.

Statistics

Statistical analyses of SRTR data was performed with JMP Software 13 Pro version (SAS Institute Inc., Cary, NC, USA); two-sided *P*-values were assessed. Demographics were measured by frequency counts and expressed as mean ± standard deviation (SD), median and interquartile range (IQR). Our primary hypothesis was assessed by dissecting recipient sex and age in a multivariate Cox proportional hazards regression model using the Wald test for interaction. Death-censored graft survival rates were initially estimated by Kaplan-Meier. A univariate Log-rank test was used to examine prognostic factors for the final multivariate Cox regression analysis model; factors that reached significance in the univariate analysis were subsequently included in the multivariate Cox model to determine independent effects of each factor. Covariates included: Donor cold ischemia time, Donor BMI, Donor hypertension, Non-heart-beating donor (donors after cardiac death), Donor diabetes, HLA mismatch, Donor ethnic category, Donor age group, Donor living vs deceased, Donor sex, Recipient age, recipient gender, recipient time on dialysis, Prior transfusion, recipient PRA, Recipient diabetes, recipient prior transplantation, and Transplant era. We also examined acute allograft rejection episodes in recipients as a function of sex and age in the study population. We also analyzed the data by re-coding the sex and age into mutually exclusive "dummy" categorical variables of sex combined with age group, and determined the contribution of each sex/age group to death censored graft

survival by estimating the exponent parameters using the Cox model. Experimental animal data were compared using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). Differences between groups were analyzed using unpaired Student t test and 1-way ANOVA. Graft survival was compared by Log-rank test; a *P*-value less than 0.05 was considered significant (**P*<0.05; ***P*<0.01; ****P*<0.0001).

Results

SRTR Recipient Clinical Characteristics

We evaluated 407,963 recipients, dividing them into three age categories (15-34, 35-54 and 55-74 years, **Fig. 1**).

Clinical characteristics of donor/recipients assigned by age category are summarized in **Table 1;** time on dialysis, ABO-compatibility, HLA mismatches, ratios of either female or male donors, recipient/donor BMI, donor age, deceased/living donor rates and ethnicities were comparable per recipient age group; type II diabetes was more frequent in male vs. female recipients in the age groups 35-54 and 55-74. Recipients aged 15-54 years had more frequently received a previous transplant or had previous blood transfusions compared to transplant recipients of 55-74 years, however, those differences were not sex-specific. Of note, panel reactive antibodies were consistently higher in female recipients regardless of age (**Table 1**).

Recipient sex and age affect allograft survival outcome

Five-year death censored graft survival for both, kidneys from male ($P < .0001$) and female donors *(*$P = 0.0002$) had been inferior in young female recipients (15-34 years) (**Fig. 2A**). When comparing recipients by age and sex, we observed inferior graft survival rates in young female recipients (15-34 years) and improved survival rates in both, female and male recipients 35-54 years. Of note, graft survival was prolonged in older female recipients (> 55 years), exceeding that of male recipients of comparable age (male donor, Log-rank, $P = 0.0294$; female donor, Log-rank, $P = 0.0032$) (**Fig. 2A, B**).

Notably, inferior outcomes in young compared to old female recipients were independent of donor age and sex (**Supp. Fig. 1**).

Next, we assessed the impact of recipient age and sex by adjusted hazard ratios (HR) in a multivariable-adjusted cox regression analysis. Recipient sex by itself, did not impact graft survival (HR 1.002, 95% CI 0.960-1.045 from male donor; HR 0.930, 95% CI 0.888-0.974 from female donor). The combinatorial analysis of recipient age and sex, however had a significant impact on graft survival. Remarkably, young female recipients demonstrated significantly inferior 5-year graft survival rates (HR 1.128, 95% CI 1.061-1.198). Interestingly, graft survival was also inferior in young female recipients receiving male kidneys (HR 1.175, 95% CI 1.083-1.273).

To delineate the relationship between recipient age and sex in more detail, we calculated the interaction of sex and age in a multivariate cox regression model and observed a statistically significant interaction between recipient sex and age on graft survival that had been present independently of transplanting male or female kidneys (male and female donor, $P < .0001$ and $P = 0.0031$, respectively **Table 2**).

Figure 2 and **Supp. Figure 2** illustrate multivariate hazard ratios for death-censored graft survival including covariates noted in the methods/statistical sections above.

In addition, we calculated the odds ratio for treated rejections within 1 year by recipient age gender, and found a two-fold increase of acute rejections in young female recipients (15-35 years old) compared to old female recipients (55-75 years old) (**Supp. Figure 3**).

As the cause of ESRD may impact graft loss in an age and sex specific way, we also calculated multi-factorial estimates including ESRD (**Suppl. Fig. 4).** This analysis demonstrated that graft loss was highest in young females while improving in females 35-54 and 55-74 years old.

Taken together, graft survival rates had been inferior in young compared to older female recipients. This effect was even more pronounced in a multi-variate analysis when male kidneys had been utilized, suggesting that both recipient age-dependent hormonal effects in addition to sex-chromosome incompatibilities may be playing a role.

Estrogen levels change with Aging

We next delineated our clinical findings in an experimental model dissecting the effects of estrogen on alloimmune responses and graft outcomes. As shown in **Fig. 3A**, estradiol serum levels were significantly different in female young and old mice (2-3 months: 14.2±7.9 pg/mL, 18 months: 2.5±0.5 pg/mL); estrogen levels in male young and old mice were not significantly different (2-3 mths: 1.9±0.7 pg/mL; 18 mths: 1.6±0.6 pg/mL). To simulate conditions of menopause, we also measured estradiol serum levels following bilateral ovariectomies (30, 31) (2.1±1.1 pg/mL and 1.6±0.6 pg/mL in 2-3 and 18 mths old animals, respectively).

In support of our clinical observations, graft survival in young female recipients had been significantly shorter (median survival time, 9 days vs. 12 days, $P = 0.0020$ in female vs male recipients, respectively) (**Fig. 3B**). Notably, graft survival was not only prolonged in older female but also in older male recipients (old male vs old female recipients, 14 vs 12 days, $P =$

0.0231). Graft survival in young and old ovariectomized female recipients, old naïve or sham-control animals was comparable (p=n.s.) (**Fig. 3C**).

Next, we tested whether the observed effects of estradiol levels on graft survival will also be present when utilizing immunosuppression. Thus, we treated recipient mice with CTLA4-Ig, a fusion protein blocking costimulatory signaling pathways. CTLA4-Ig treatment prolonged allograft survival in all groups. Differences in graft survival based on donor/recipient sex mismatches or hormonal changes, however remained (**Fig.3D and E**). These results suggest that differences in graft survival are also present under immunosuppression, data that are also supported by others who have shown prolonged murine cardiac allograft survival in ovariectomized, female recipients under cyclosporine treatment(32).

Next, we probed if sex and estrogen level specific differences in graft survival observed in our skin transplant model will also be present in a vascularized cardiac transplant model. As in the skin transplant model, young male heart recipients demonstrated prolonged graft survival times compared to young female recipients. Moreover, ovariectomies of young, female recipient animals prolonged cardiac allograft survival comparable to those observed in young, male recipients (**Fig.3F)**. In addition, ovariectomies in old cardiac transplant recipients did not impact graft survival (**Fig. 3G**).

Thus, our experimental data in a skin transplant model were confirmed in a vascularized heart transplant model and in line with our clinical studies showing the impact of sex, hormonal levels and aging on graft survival.

Hormonal deprivation via ovariectomies dampens alloimmune responses

Our previous findings have shown that ovariectomies promoted allograft survival in young but not old female recipient mice. Moreover, our clinical analysis demonstrated that younger female recipients (15-34 years) had an inferior graft survival when compared to any other age group (**Fig.2A**). Thus, we next investigated the impact of sex-hormones on alloimmunity and tested whether female hormones were the driver of an augmented alloimmunity in younger female recipients.

To dissect the effects of female hormones on alloimmune response, we took advantage of our major HLA mismatched skin transplant model using young female recipients that underwent ovariectomies or sham surgeries as recipients.

One day prior to graft rejection, we isolated $CD4^+$ and $CD8^+$ splenocytes to assess their activation and cytokine profile. We found that ovariectomies in young animals had been linked to reduced numbers of $CD4^+$ and $CD8^+$ T-cells ($p< 0.01$ and $p< 0.001$; **Fig.4**).

In more detail, our findings indicated that ovariectomies dampened pro-inflammatory Th1 ($CD4^+IFN\text{-}\gamma^+$, $p< 0.001$) and Th17 ($CD4^+IL\text{-}17^+$, $p< 0.05$) responses, while promoting $CD4^+CD25^+FOXP3^+$ T regs ($p< 0.01$). Of note, no significant differences between mice undergoing ovariectomies or sham procedures were observed on either Th2 ($CD4^+IL\text{-}4^+$) or $CD8^+IFN\text{-}\gamma^+$ T-cell responses (**Fig. 5**).

Estrogen deprivation dampens $CD4^+$ T-cell responses in Mixed Lymphocyte Reactions:
To further characterize the impact of ovariectomies on antigen specific immune responses, splenocytes of ovariectomized and sham surgery mice were isolated and tested in Mixed Lymphocyte Reactions (MLR). Our analysis demonstrated a compromised proliferative capacity of $CD4^+$ T cell subsets isolated from ovariectomized (ovx) mice when compared to mice subjected to sham surgeries (ovx vs sham, 22.0 ± 0.6 vs 27.1 ± 0.8 %, $p = 0.0024$) (**Fig. 6A)** that was associated with an increased cell death rate (**Fig. 6B**), and reduced IFN-γ production (**Fig. 6C**).

Taken together, our experimental data supported our clinical observations of inferior graft survivals in younger female recipients linked to estrogen levels as estrogen deprivation diminished the robustness of $CD4^+$ T-cell alloimmunity.

17-β-Estradiol levels impact functional changes of T-cell subsets

While ovariectomies prolonged allograft survival associated with reduced pro-inflammatory Th1 and Th17 responses and the promotion for Tregs, underlying mechanisms remained unclear.

To dissect the role of estradiol on $CD4^+$ T-cell development and cytokine profiles, naïve $CD4^+$ T-cell were isolated from splenocytes of young female C57BL/6 and cultured in Th1, Th17 and iTreg polarizing conditions. In addition, naïve $CD4^+$ T-cells were cultured in presence of placebo solution (PBS) or increasing estradiol concentration that reflected physiological estrus cycles (10^{-10} M), pregnancy (10^{-8} M) and menopause (10^{-12} M) (33-35).

Under Th1 polarizing condition, estradiol promoted $CD4^+IFN\text{-}\gamma^+$ cell frequencies in a dose-dependent manner (PBS, 10^{-12} M and 10^{-10} M). In contrast, at much higher estradiol levels

reflective of physiological pregnancy levels frequencies of $CD4^+IFN\text{-}\gamma^+$ cells decreased significantly (**Fig. 7A)**.

Moreover, under Th17 polarizing conditions, estradiol did not alter $CD4^+IL\text{-}17^+$ cell differentiation (**Fig. 7B)**. With iTreg polarizing conditions, however, we observed declining numbers of $CD4^+CD25^+FOXP3^+$ Tregs in a dose-dependent fashion (PBS, 10^{-12} M and 10^{-10} M). Furthermore, at estradiol levels comparable to those observed during pregnancy, $CD4^+CD25^+FOXP3^+$ cell frequencies increased significantly (**Fig. 7C)**.

These results indicate that estradiol plays an important role in regulating $CD4^+$ T-cell fate suggesting estradiol levels as critically important for age-dependent alloimmunity and graft survival rates.

Hormone Replacement

Next, we investigated the impact of estrogen replacement on alloimmunity and graft rejection.

As shown in **Fig. 8A**, mice that underwent sham surgery exhibited estradiol concentrations of 14.1±7.9 pg/ml. Ovariectomies, in contrast, resulted into significantly decreased estradiol concentrations (1.8±0.3 µg/ml, **Fig.8A**; E2(0)). Estrogen replacement (50 µg/mL; E2(50)) or 500 µg/ml; E2(500)) increased systemic levels (23.5±33.4 pg/mL 361.5±50.6 pg/ml, respectively) comparable to those observed during the estrus cycle (2-20 pg/mL(34, 35)) or pregnancy phase (200-2,000 pg/mL.(33, 36, 37).

Of note, estrogen replacement prolonged graft survival with significant differences between E2(0) and E2(500) levels ($P = 0.0035$; **Fig. 8A**). Notably, young ovariectomized mice demonstrated prolonged graft survival times (9 vs 12 days, $P = 0.0031$ for sham vs. ovariectomized young recipients, respectively)

To assess T cell responses under hormone replacement, we next isolated $CD4^+$ and $CD8^+$ T cells just prior to rejection and assessed their activation and cytokine profile. Notably, high estrogen doses decreased both, relative and absolute numbers of CD4 and CD8 T cells. Moreover, both, ovariectomies and high estrogen replacement doses E2(500) reduced the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells ($P < 0.001$; **Fig. 8B**); in addition, ovariectomies and high estrogen doses increased absolute and relative amounts of T-regs significantly (**Fig. 8C**).

Taken together, our in vivo findings are in line with our in vitro findings showing that estradiol levels impact graft survival while playing an important role in T cell activation and alloimmune responses.

Discussion

We have analyzed more than 408,000 kidney transplant recipients, classified graft survival rates and categorized recipient groups in an age-dependent manner. Although panel reactive antibodies were higher in all females at any age, graft outcomes differed in an age-dependent and sex-specific fashion. Younger female recipients (15-34 years) exhibited worse graft survival rates independently of kidneys originating from male or female donors. Inferior graft survival rates in young female recipients were even more pronounced when donors were male, suggesting that both sex-mismatch and age-specific hormonal changes are playing a role. In contrast, outcomes in recipients 35-54 years were comparable for male or female recipients. For older recipients, outcomes were improved in females compared to males, albeit, differences were not as pronounced as in younger recipients. Notably is also that differences did not depend on donor sex, although male donor kidneys are usually associated with improved outcomes based on a superior functioning kidney mass (38), suggesting a protective impact of lower estrogen levels in aging.

Aging is associated with changes of sex hormone levels. Estrogen is a key female sex hormone that is playing a central role in reproduction; its impact on T and B cell immunity has been recognized (39). Both progesterone and estradiol are major female hormones that have been shown to impact immunity. With the complexity of donor/recipient mismatches impacted by organ/recipient size and sex mismatches, we focused our mechanistic analysis on alloimmune responses impacted by estradiol levels, known to regulate $CD4^+$ T-cell response and IFN-γ production (40). While a previous experimental study has recognized the impact of estrogen levels on transplant survival (32), a detailed analysis on aging, hormonal levels and alloimmunity has not been performed.

Observations in our preclinical skin transplant model demonstrated a prolonged graft survival in ovariectomized recipients supporting the concept that estrogen deprivation dampens alloimmunity. In line with our previous reports (25, 26, 41, 42) aging extended graft survival in both, male and female recipients. Of note, ovariectomies extended graft survivals only in young but not old recipients, emphasizing on a fine-tuned link between estrogen levels and alloimmunity.

Previous studies have shown that ovariectomies decrease TLR4 expression and cytokine production in murine macrophages. The addition of estradiol, on the other hand, increased the

expression of TLR4 and enhanced cytokine production following lipopolysaccharide challenge (43). Moreover, estradiol administration has been shown to drive the switch of murine bone marrow precursor cells towards mature $CD11c^+CD11b^+$ DCs (35, 44) that have the ability to promote $CD4^+$ T cell proliferation (45). In contrast, ovariectomies have been linked to declining numbers of murine $CD11c^+CD11b^+$ DCs (46) and compromised T-cell proliferation based on changes of estrogen levels (47). $CD4^+$ T cells in females may also exhibit an increased propensity towards Th1 cytokine production (48, 49). This observation is in line with our in vitro findings showing that increased estradiol doses promoted the differentiation of naïve $CD4^+$ T cells towards Th1 pro-inflammatory cell subsets. Moreover, mouse models suggest that estrogen administration at pregnancy levels increases Treg numbers (50), an observation that is consistent with our in-vitro results showing a decreased Th1 differentiation and an increased differentiation of naïve $CD4^+$ T cells towards Tregs. Although increasing evidences including our in-vitro findings are suggestive of a significant role of female hormones in regulating immune responses, hormonal effects on alloimmunity in aging remain to be determined.

While the impact of male hormones on immunity has been well studied (51, 52) and most experimental models remain to be performed using male animals, little research has been done on the impact of female hormones on alloimmunity.

Previously, ovariectomies and/or the administration of tamoxifen, an inhibitor of the estrogen signaling machinery have shown to prolong cardiac allograft survival (32, 53). Notably, more detailed clinical reports linking aging as a surrogate of estrogen levels with transplant outcomes and a more detailed analysis of estrogen levels affecting T-cell alloimmune responses have been missing. Our data are filling the knowledge gap that aging and levels of female hormones impact alloimmune responses and graft loss. Moreover, our clinical observations are supported by a detailed experimental analysis in ovariectomized young mice showing not only a decreased $CD4^+$ and $CD8^+$ T cell population, but also changing T-cell subsets. In detail, ovariectomies reduced systemic levels of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ T cells and their activation markers while augmenting the T reg population.

Estrogen exerts its biological effect through estrogen receptors (ERs) that manifest in two different phenotypes ER-α (54, 55) and β(56); ER-α is also expressed on $CD4^+$ T cells (57) regulating gene transcription. Estrogen treatment, for instance, has been shown to induce a strong

inhibition of autoantigen-specific Th1 and Th17 cell responses communicated through ER-α causing a long-lasting protection towards experimental autoimmune encephalomyelitis (EAE) (58-61). Those observations are consistent with our findings that administration of high estradiol levels, reflective of physiological pregnancy levels significantly decreased frequencies of $CD4^+IFN\text{-}\gamma^+$ cells under TH1 polarizing conditions. Moreover, estradiol treatment has been shown to modulate IFN-γ secretion by enhancing IFN-γ gene expression in $CD8^+$ T cells through a direct interaction of ER-α with an estrogen response element in the promoter region of the IFN-γ gene (11). It is thus likely that the augmented Th1 and Th17 immune responses in young, female recipients receiving an allogeneic, male transplant are communicated through ERs activation. However, further studies dissecting the molecular mechanism of estradiol on T cell signaling pathways in depth are necessary.

Moreover, estrogen signaling, via ERs down-regulates Fas-ligand expression, thus impeding apoptosis (62). In support, our results have shown that splenocytes of ovariectomized females demonstrated not only a compromised proliferation but also an accumulation of apoptotic T cells.

Consistent with those reports, our results have shown that that both, very low and very high estradiol levels reduced amounts of $CD4^+IFN\text{-}\gamma^+$ T cells while higher estrogen levels, reflective of those during pregnancy increased amounts of iTregs. Th17 cells and their products had not been significantly affected by changes of estrogen levels. Indeed, published data appear difficult to interpret. While estradiol exerted inhibitory effects on Th17 cells in one study (63), estradiol promoted IL-17 in another experimental model (64). In our own in vivo experiments, we observed a prolongation of skin graft survival with increased estrogen levels linked to reduced $CD4^+IFN\text{-}\gamma^+$ and augmented $CD4^+CD25^+FOXP3^+$ T-cell amounts.

Our study demonstrates the complex interplay of estrogens, aging and alloimmunity while emphasizing on the necessity of including female recipients and hormonal analysis to experimental designs. Nevertheless, interactions with other relevant sex-hormones need to be evaluated and additional mechanistic studies dissecting the role of hormone receptors on immune cells are in need.

In summary, our clinical analysis demonstrated an impact of donor and recipient sex on transplant outcomes in an age-specific fashion. Experimentally, we were able to reproduce our

clinical observations with inferior skin and heart graft survival rates in young and prolonged survival rates in old recipients. Notably, age and sex-specific rejection kinetics were also observed under immunosuppression. On a cellular level, we observed reduced Th1 responses under hormonal deprivation and increased $CD4^{+}CD25^{+}FOXP3^{+}$ counts with higher estrogen levels.

Authorship

RM, YN, JI and YL performed experiments, analyzed data and wrote the manuscript. KM performed and analyzed experiments. EM analyzed data and helped with the preparation of the manuscript; TR, RA, HZ, FM and HA helped with preparation of the manuscript.
SGT and AE designed, supervised experiments, wrote and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author (SGT).

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Figures

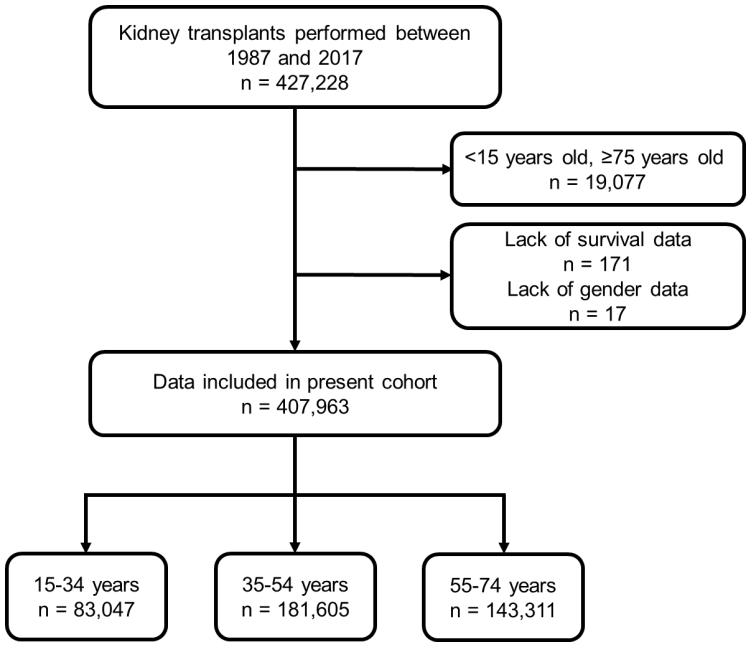


Figure 1. Out of a total of 427,228 recipients transplanted between 1987 – 2017, we excluded 19,077 (<15 years or ≥75 years); 188 patients were excluded with insufficient data. The remaining 407,963 cases were separated into three groups according to younger (15-34 years, n = 83,047), middle age (35-54 years, n = 181,605) or older (55-75 years, n = 143,311).

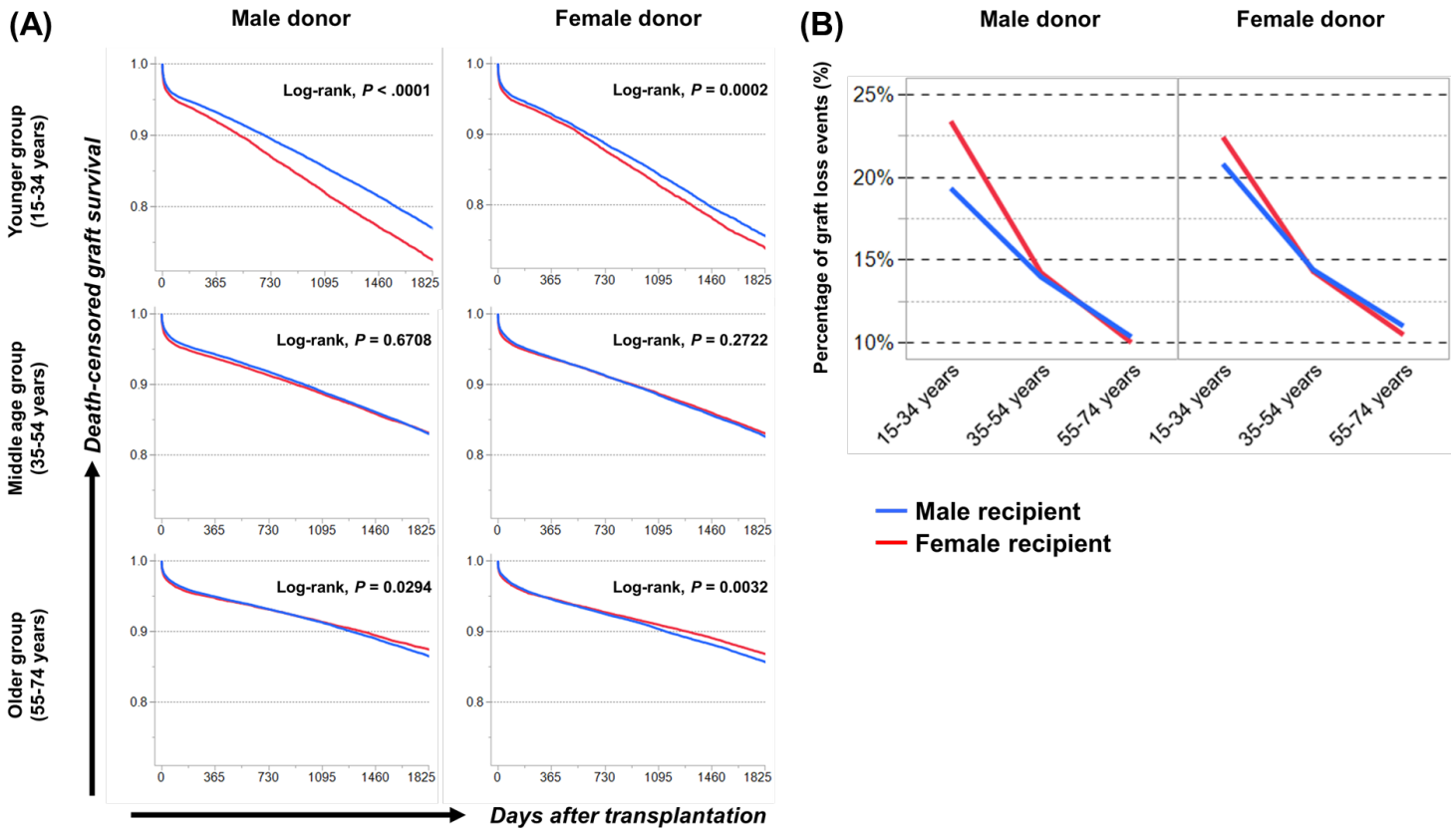


Figure 2. Both, donor sex and recipients age impacted death-censored graft survivals outcome. (A) Kaplan-Meier curves showing the impact of recipients age and donor sex on 5 years death-censored graft survival outcome. (B) Percentage of graft loss/recipient age (15-34, 35-54 and 55-74 years) and donor sex at 5 years after transplantation; *P* values were calculated using a two-sided log rank test. Blue lines represent male and red line female recipients; **P* < 0.05; ***P* < 0.01; ****P* < 0.0001).

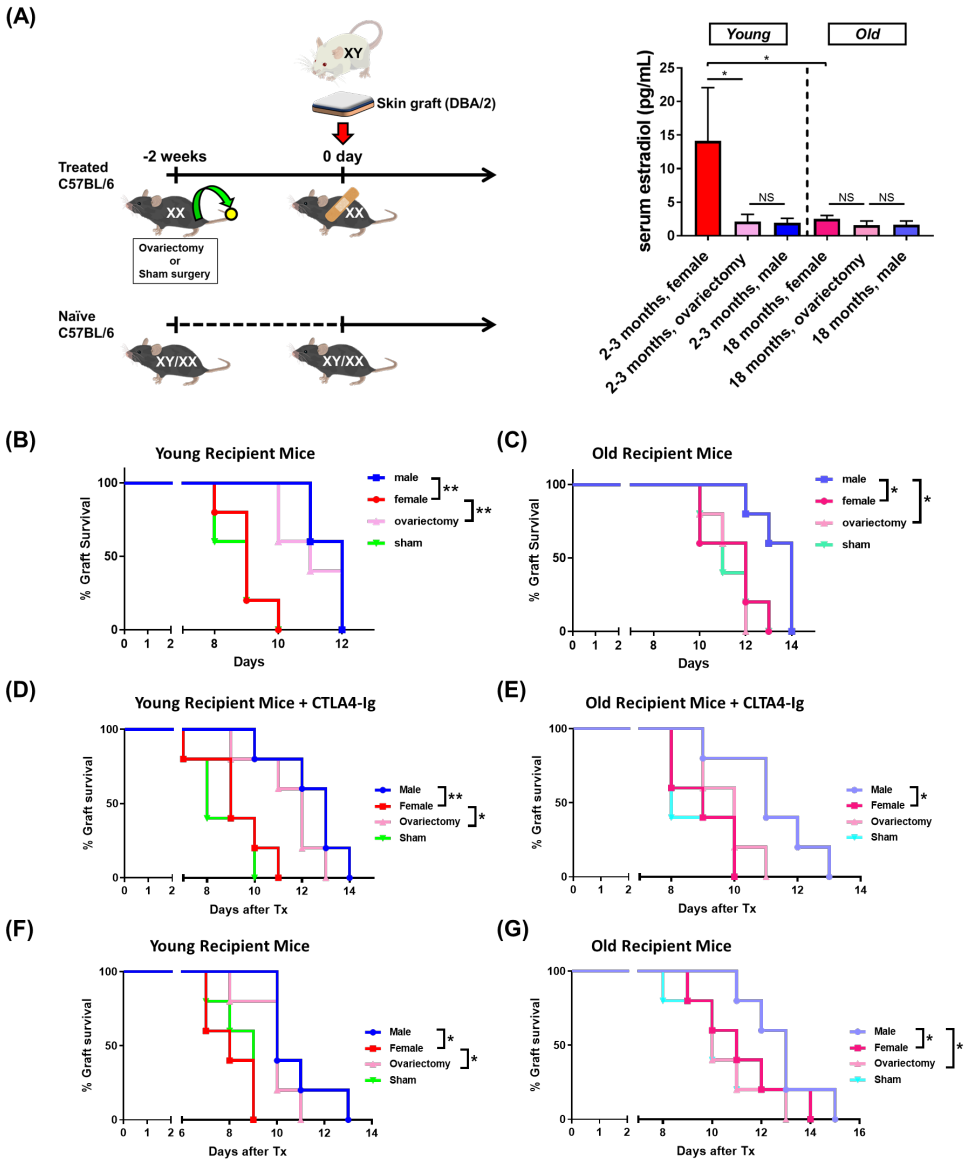


Figure 3. Survival of fully mismatched skin allografts after ovariectomies are shown. (A) Model: C57BL/6 underwent ovariectomies or sham surgeries prior to skin transplantation; after two weeks, mice received a fully mismatch skin transplant. Skin allografts from young male DBA/2 mice were transplanted onto young (2-3 months) or old C57BL/6 mice (18 months). (B) Skin survival of naïve young male and female recipients in addition to female animals that underwent ovariectomies or sham surgeries are shown (n = 5 per group). (C) Skin survival of old naïve male and female recipients, old females that underwent ovariectomies or sham surgeries are shown (n = 5/group). (D) (E) Survival after skin transplants onto young and old naïve male, naïve female, ovariectomized female and sham operated recipients treated with either CTLA4-Ig or PBS are shown. (F) and (G) cardiac allograft survival in young and old naïve male, naïve female, ovariectomized female and sham operated recipients are shown. Log-rank test was used to compare graft survival; *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$

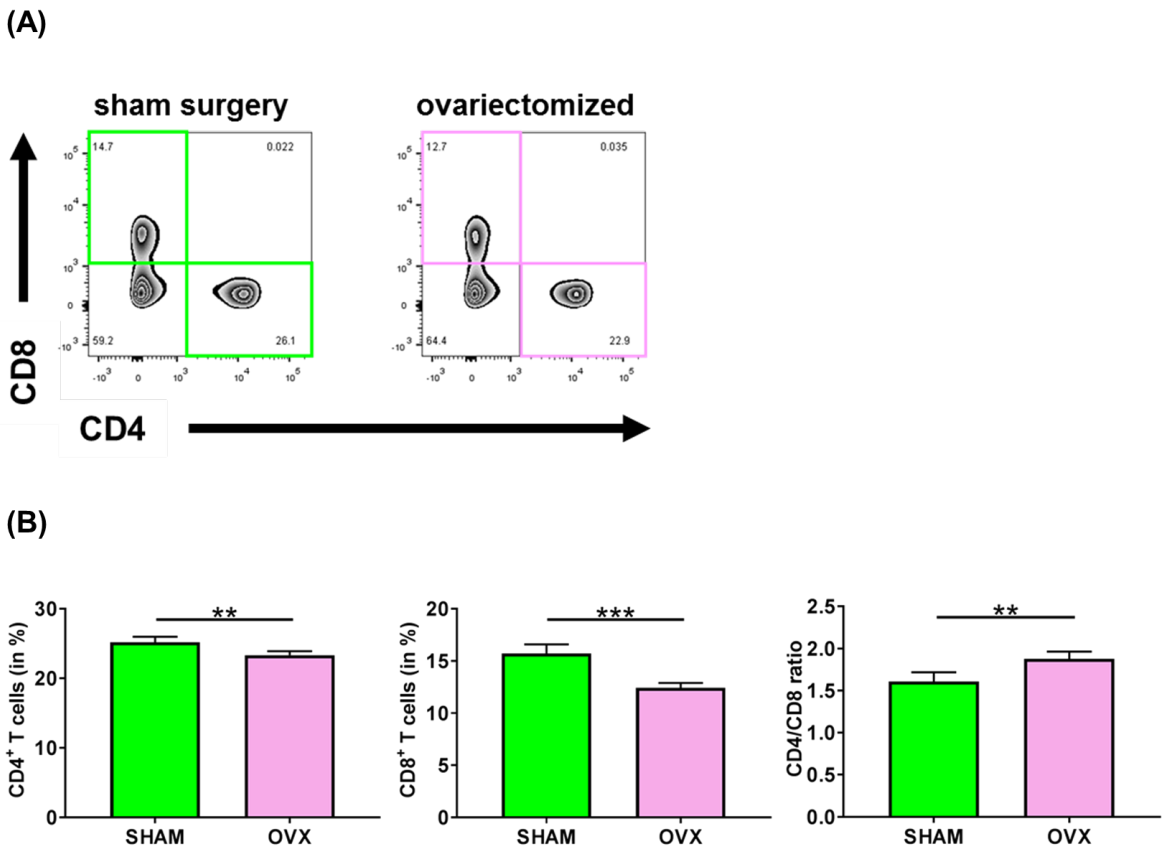


Figure 4. $CD4^+$ and $CD8^+$ T cell frequencies of young skin transplanted animals after ovariectomies or sham surgeries are shown. Eight days after skin transplantation and prior to rejection, spleens were collected, and single leukocytes suspensions were obtained. Frequencies of $CD4^+$ and $CD8^+$ T cells were assessed using flow cytometry (n = 5 per group; Flow cytometric data are displayed as representative estimated density plots. Column plots represent mean ± SD; data were compared by applying Student *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$).

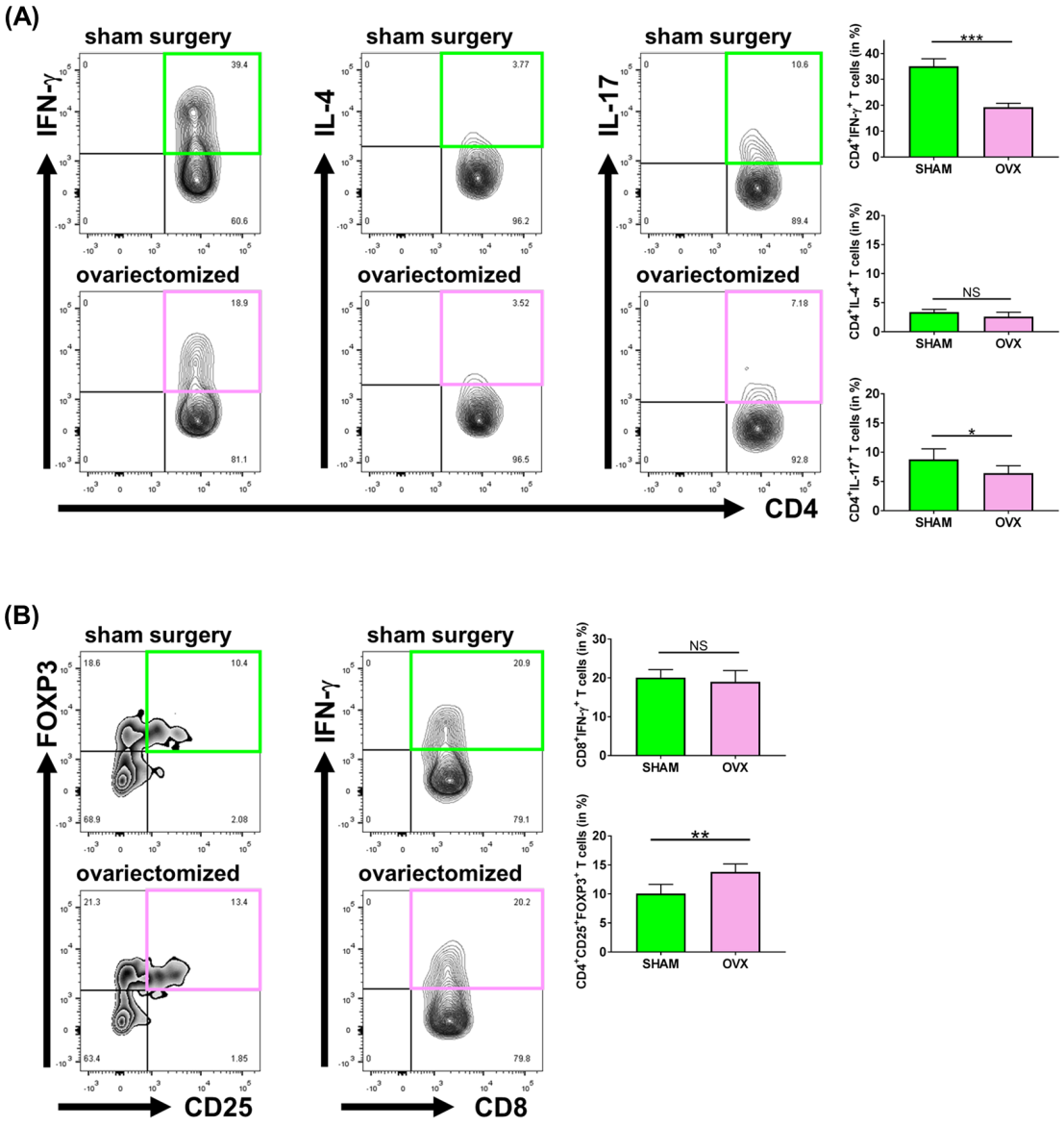


Figure 5. Ovariectomies modified alloimmune responses in young female recipients. Young (2-3 months) C57BL/6 female mice underwent ovariectomies or sham surgeries and received fully mismatch skin transplants from male donors. By day 8, prior to skin transplantation, spleens were collected, and single leukocytes suspensions were obtained. Frequencies of $CD4^+IFN\text{-}\gamma^+$ (Th1), $CD4^+IL\text{-}4^+$ (Th2), $CD4^+IL\text{-}17^+$ (Th17), $CD4^+CD25^+FOXP3^+$ (Treg) and $CD8^+IFN\text{-}\gamma^+$ cells were assessed by flow cytometry (n = 5 per group; Flow cytometric data are displayed as representative estimated density plots. Column plots represent mean ± SD; The Student *t* test was used to compare groups; $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$).

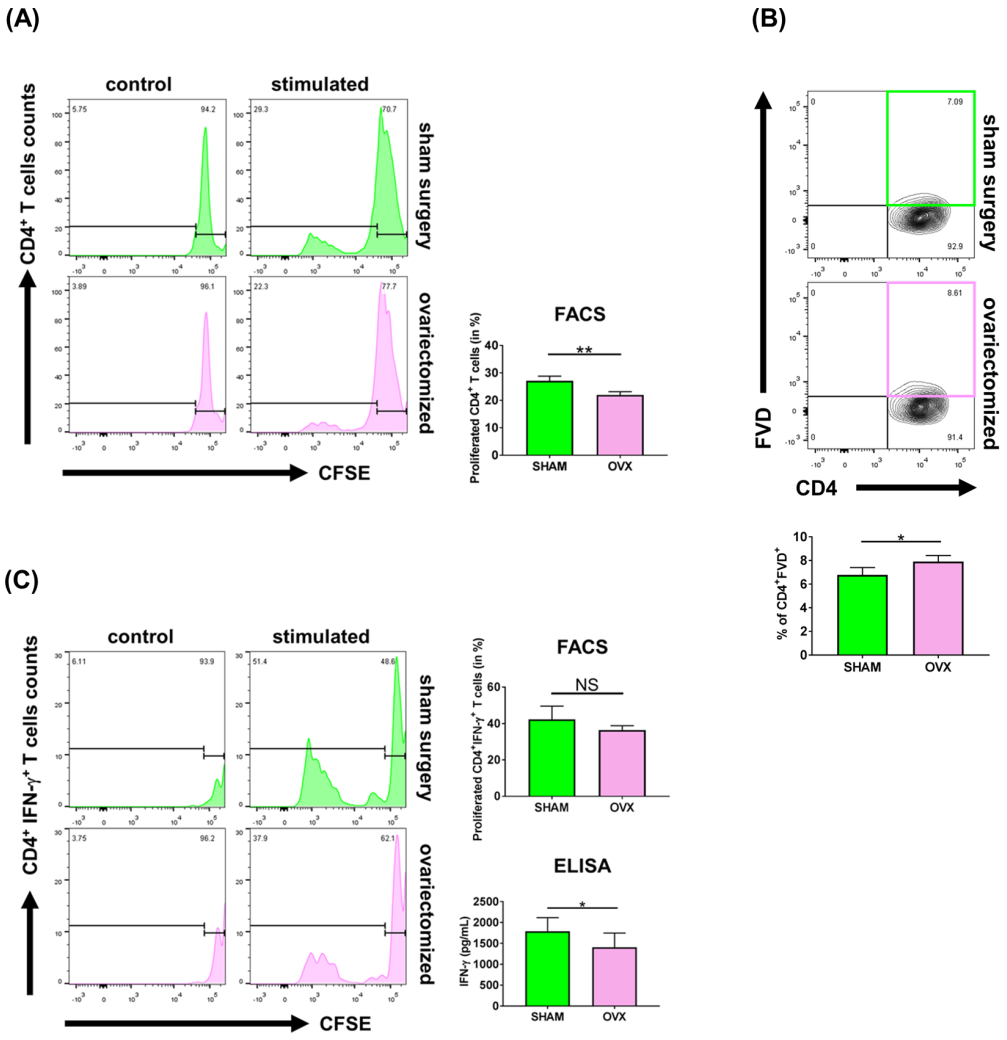


Figure 6. Ovariectomies reduced T cell proliferation and promoted T cell death in young female recipients. Young (2-3 months) C57BL/6 female mice underwent ovariectomies or sham surgeries and received fully mismatch skin transplant (DBA/2 young (2-3 month) male donors). Eight days after skin transplantation (a day prior full rejection), spleens were collected, and single leukocytes suspensions were obtained. To determine the proliferation of $CD4^+$ T and $CD4^+IFN\text{-}\gamma^+$ cells, splenocytes were stained with CFSE dilution and cultured with stimulator DBA/2 splenocytes (MLR) treated with mitomycin. After 5 days of culture, cells were collected and analyzed by flow cytometry (n = 5 per group; Flow cytometric data are displayed as representative estimated density plots. Column plots represent mean ± SD; Student *t* test was used to compare groups; $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$).

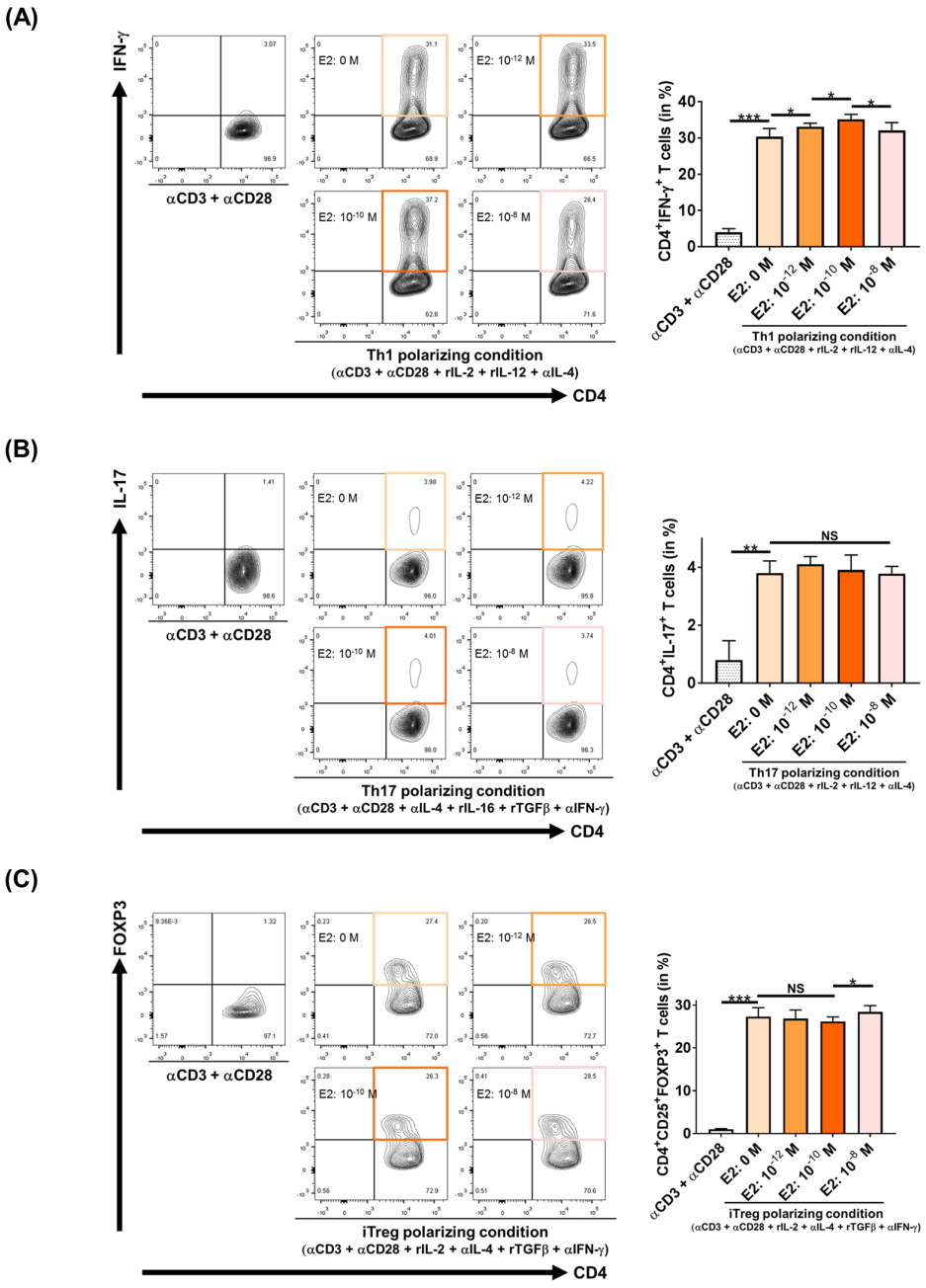


Figure 7. 17β-estradiol modifies IFN-γ$^+$ production and CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells under Th1 and iTreg polarizing conditions. Single cell suspensions were obtained from spleens of young C57BL/6 mice; CD4$^+$ T cells were isolated by negative selection (purity > 95%). CD4$^+$ T cells were then cultured under (A) Th1, (B) Th17 or (C) iTreg polarizing conditions with differing concentrations of 17β-estradiol. By 5 days, cells were stained for double stained for CD4, CD25 and the intracellular expression of IFN-γ, IL-17 and FOXP3 (n = 5 per group; Flow cytometric data are displayed as representative estimated density plots. Column plots represent mean ± SD; Student *t* test and ANOVA were used to compare groups; **P* < 0.05; ***P* < 0.01; ****P* < 0.0001).

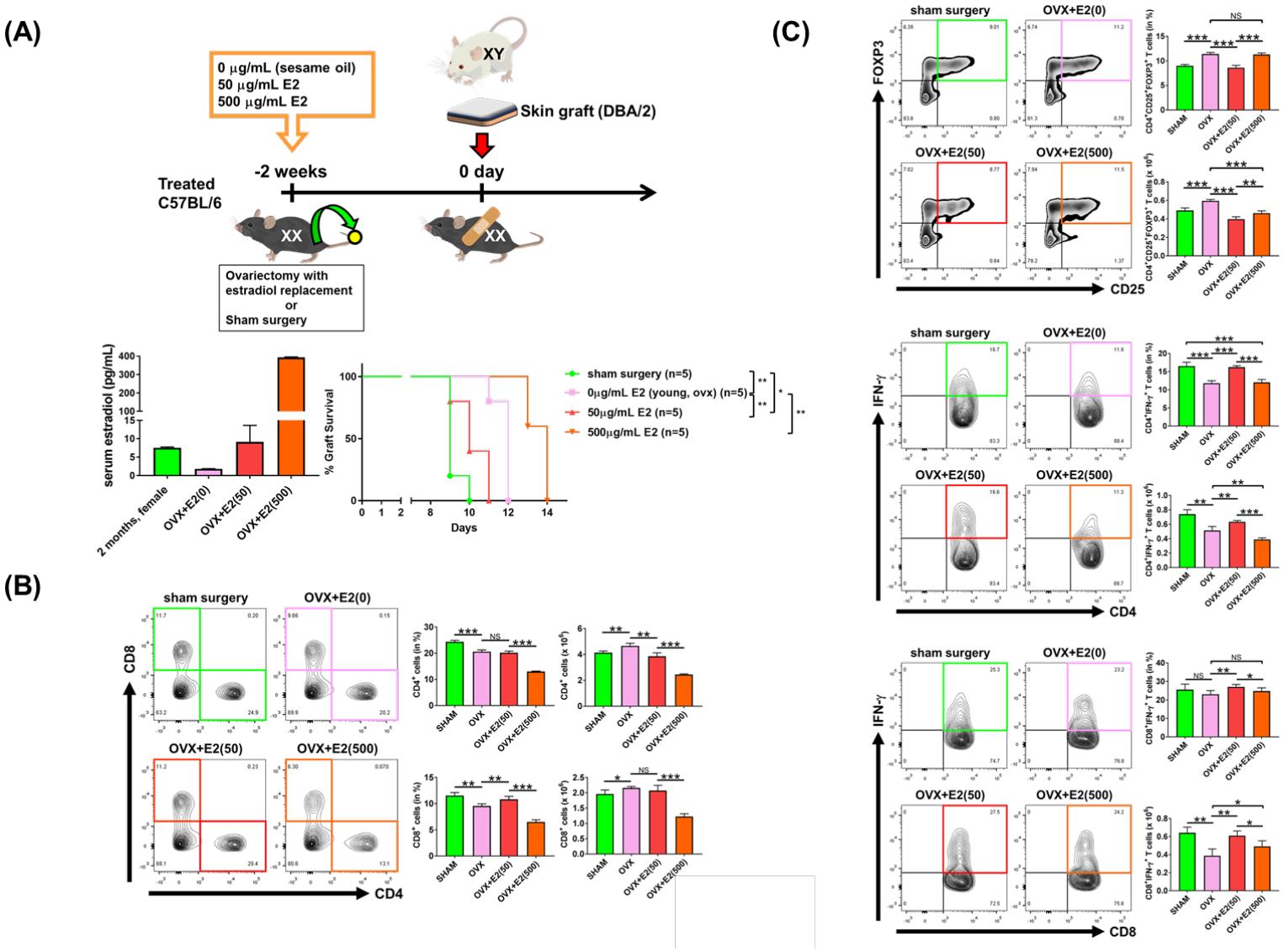
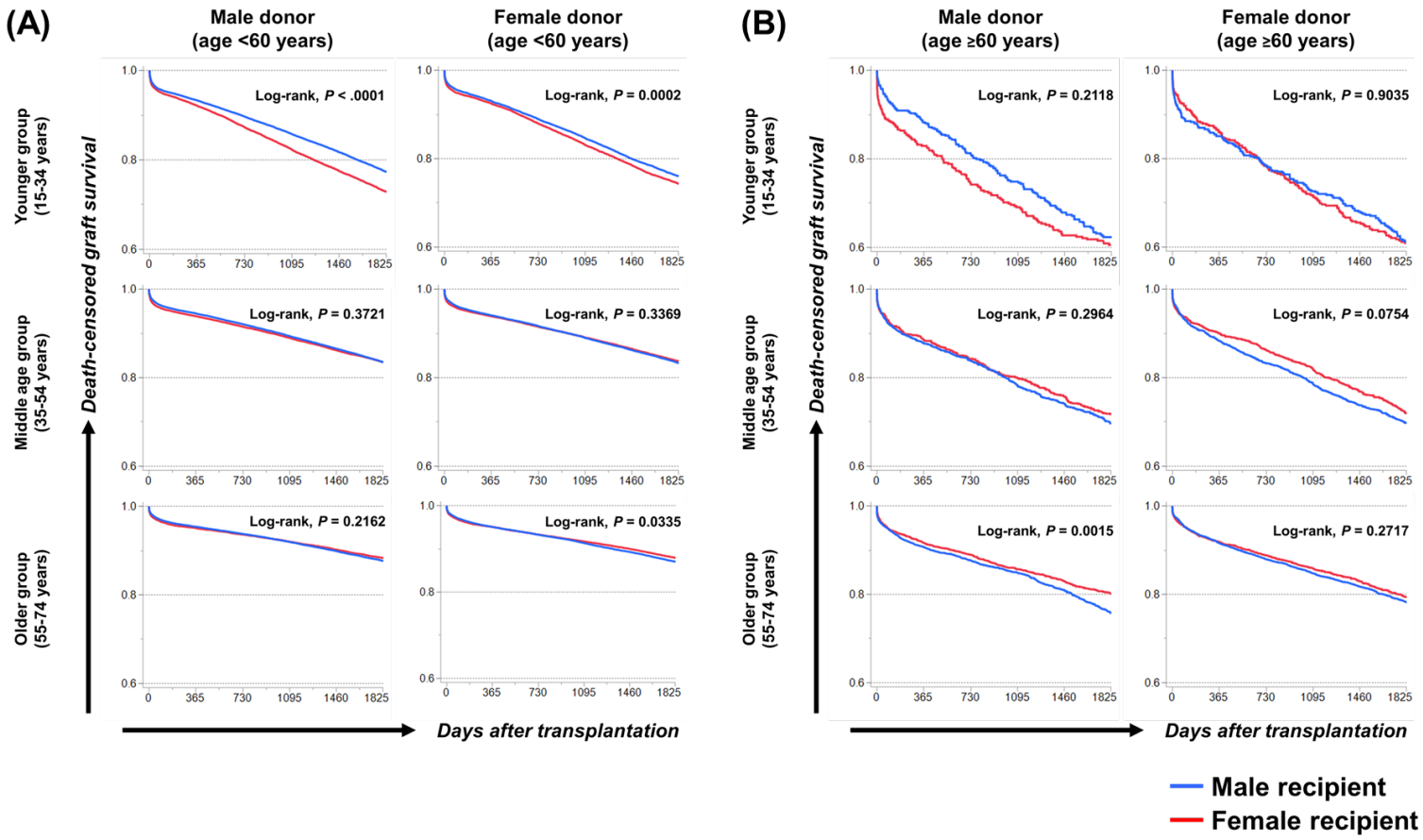


Figure 8. Hormone replacement reverses the effects of ovariectomies on alloimmune responses and transplant survival. (A) Young female C57BL/6 mice (2-3 months) underwent sham or estradiol treatment prior to skin transplantation. Treatments changed mice serum estradiol level. Two weeks later, mice received fully mismatched skin transplants. Skin allografts from young male DBA/2 mice were transplanted onto young female recipient mice (2-3 months). (B) Prior to rejection, spleens were collected, and single leukocytes suspensions were obtained. Frequencies of young $CD4^+$ and $CD8^+$ T cells (2-3 months) were assessed by flow cytometry (n = 5 animals/group). (C) Frequencies of $CD4^+CD25^+FOXP3^+$ Tregs, $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ T cells were assessed by flow cytometry (n = 5 per group; Flow cytometric data are displayed as representative estimated density plots. Column plots represent mean ± SD; Student *t* test and ANOVA were used to compare groups; $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$).



Supplementary Figure 1. Impact of donor age (<60 and ≥60 years) on death-censored graft survivals). (A) Impact of recipients age and donor sex in an analysis of 5 year death-censored graft survival by Kaplan-Meier curves (male donors <60 years – young, recipients, 15-34 year old, n = 44074; recipients, 35-54 years, n = 94855; recipients, 55-74 years, n = 67817; recipients 15-34 years receiving kidneys from female donors, n = 37012; recipients, 35-54 years, n = 79015; recipients, 55-74 years, n = 57464). (B) Impact of recipients age and donor sex in a 5 years death-censored graft survival (donors ≥60 years; recipients, 15-34 years receiving male donor kidneys, n = 950; recipients, 35-54 years, n = 3741; recipients, 55-74 years, n = 8127; 15-34 years recipients receiving female kidneys, n = 1011; 35-54 years recipients, n = 4264; recipients 55-74 years, n = 9903). *P* values were calculated using (two-sided) log rank analysis; Blue lines represent male recipients, red lines, female recipients' survival curves. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$).

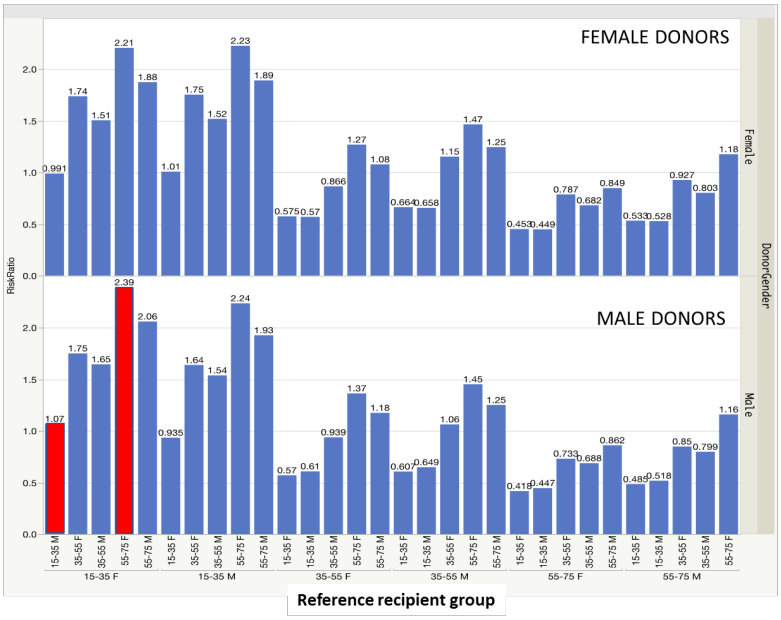
(A)

Hazard Ratios of Covariates Used in Proportional Hazards Analysis

Covariate	Comparison	Risk Ratio	Lower 95%	Upper 95%	
Donor Cold Ischemia Time	Per hour CIT	1.0060	1.0045	1.0074	<0.0001
Donor BMI	Per unit BMI	0.9974	0.9951	0.9998	NS
Donor Non Heart-Beating	Yes vs No	0.9271	0.8730	0.9847	0.0100
Donor Diabetes	Yes vs No	1.2923	1.2172	1.3720	<0.0001
Donor HLA Mismatch	6 vs 0	1.4378	1.3529	1.5280	<0.0001
Donor Ethnic Category	Black vs White	1.2020	1.5528	1.2506	<0.0001
Donor Age Group	>57 vs 17-22	2.2251	2.0992	2.3586	<0.0001
	>57 vs 22-27	2.0784	1.9512	2.2138	<0.0001
Donor Hypertension	Yes vs No	1.0848	1.0468	1.1242	<0.0001
Recipient BMI	Per unit BMI	1.0135	1.0109	1.0160	<0.0001
Recipient Peak PRA	Per unit PRA	1.0027	1.0022	1.0031	<0.0001
Recipient Diabetes	Yes vs No	1.0200	0.9700	1.0900	NS
Recipient Ethnic Category	Black vs White	1.6466	1.5972	1.6976	<0.0001

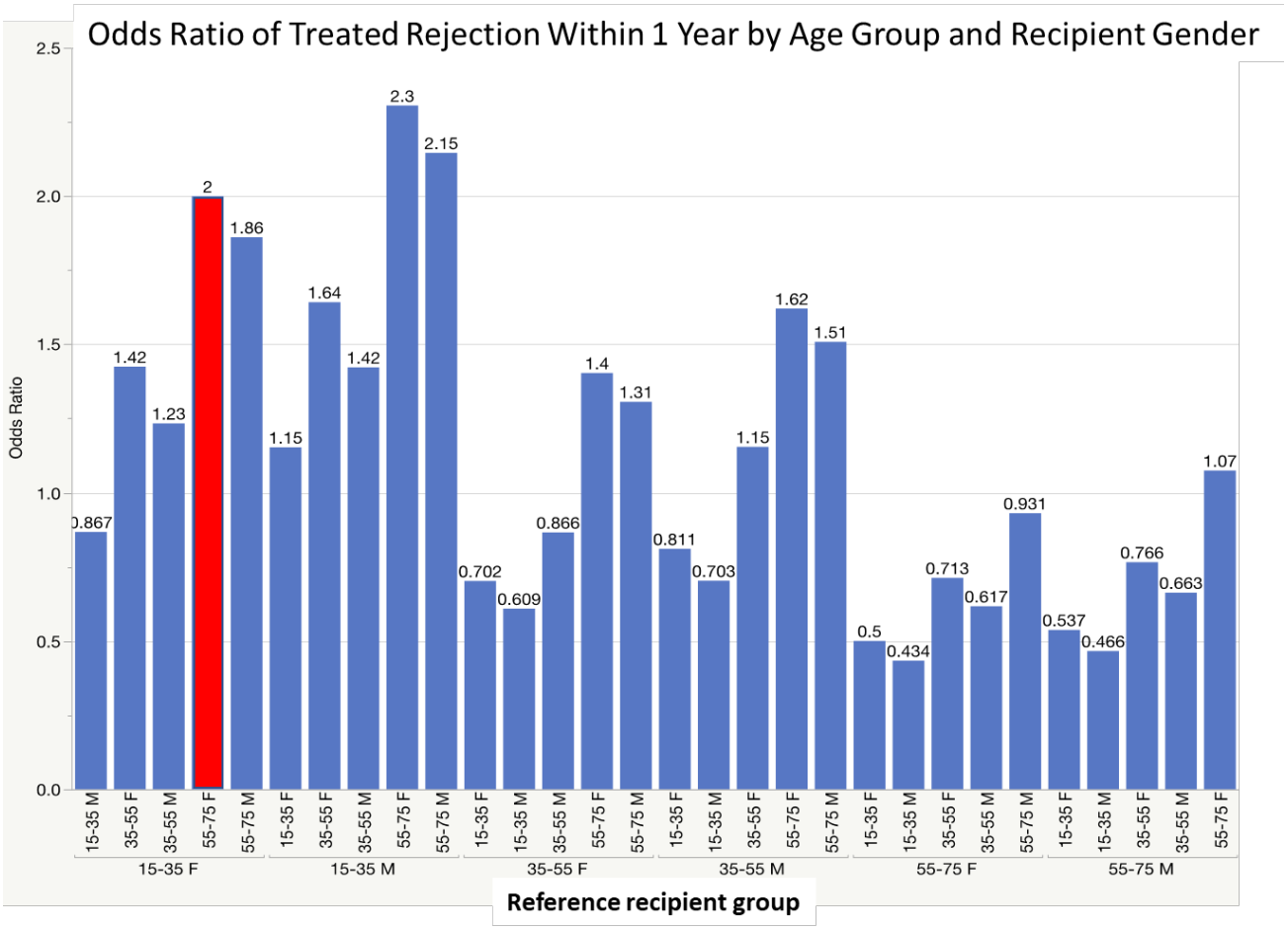
(B)

Detail of Hazard Ratio Graft Loss by Recipient Age and Gender vs Other Age/Gender Groups



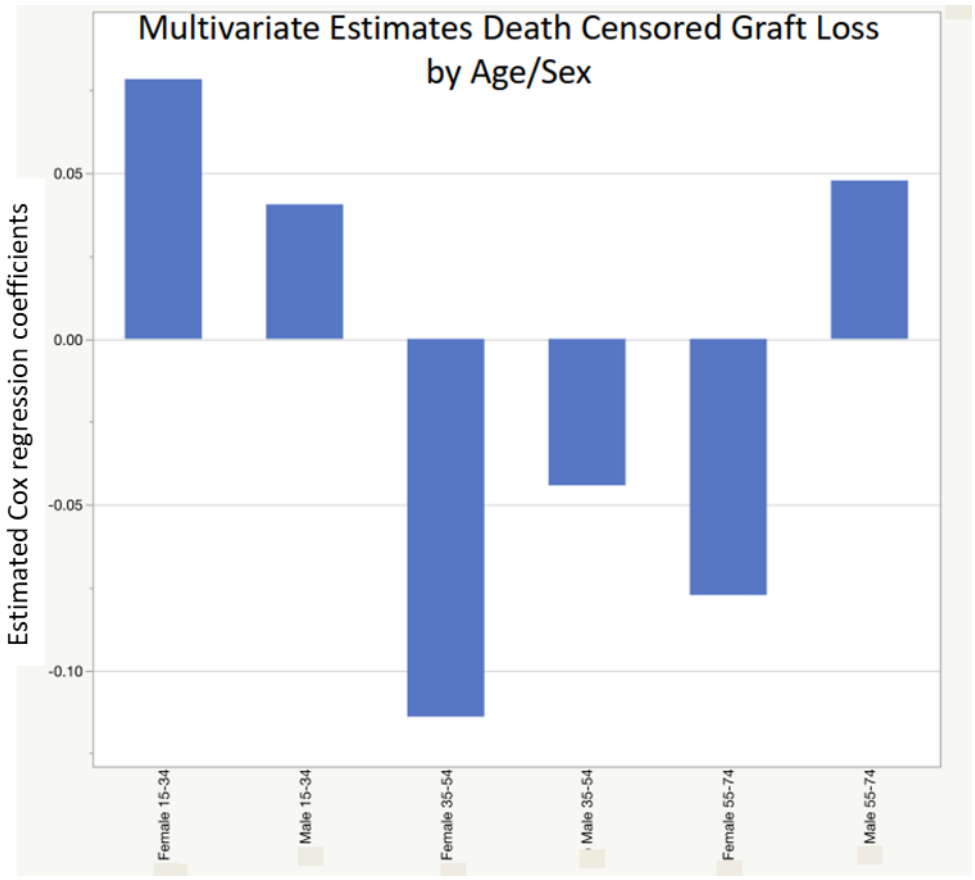
Supplementary Figure 2.

(**A**) Hazard ratios of covariates used in proportional hazard analysis (**B**) Hazard ratios in a multivariate analysis comparing donor/recipient sex and age mismatches are shown.



Supplementary Figure 3.

Odds ratios in a multivariate analysis of acute rejection within 1 year by age group and recipient gender are shown.



Supplementary Figure 4:

Multivariate Cox parameter estimates of death censored graft survival including "dummy" categorical variables for mutually exclusive categories of sex combined with age group as noted on the ordinate axis. Y-axis displays the estimated Cox regression coefficients for each group. Positive values of the coefficient estimates are associated with an increased hazard ratio. Negative values are associated with decreased hazard ratio for death-censored graft survival.

Table 1. Clinical Characteristics of Kidney Transplant Recipients by Age Groups.

	All patients (N = 407,963)		15-34 years		35-54 years		55-74 years	
	Male	Female	Male	Female	Male	Female	Male	Female
Characteristics*	(N = 245820)	(N = 162143)	(N = 47200)	(N = 35847)	(N = 109851)	(N = 71754)	(N = 88769)	(N = 54542)
Recipient age, median (IQR)	49 (38, 59)	48 (36, 58)	28 (23, 31)	27 (23, 31)	46 (41, 50)	45 (40, 50)	62 (58, 66)	62 (58, 66)
Transplantation era, %								
1987-1996	21.4%	21.7%	32.7%	33.1%	24.1%	23.4%	12.1%	11.8%
1997-2006	33.6%	34.3%	34.1%	34.2%	35.3%	36.3%	31.3%	31.8%
2007-2017	45.0%	44.0%	33.3%	32.7%	40.7%	40.3%	56.4%	56.4%
Prior transplantation, %	13.8%	14.4%	18.7%	19.9%	15.3%	16.0%	9.4%	8.7%
Blood transfusion, %	30.2%	37.4%	34.7%	42.5%	30.4%	38.1%	27.4%	32.8%
Diabetes, %								
No	67.6%	72.5%	92.7%	89.3%	71.3%	75.2%	52.7%	60.5%
Type I	3.1%	3.5%	1.6%	2.9%	4.0%	4.4%	2.7%	2.7%
Type II	17.3%	12.8%	1.2%	1.5%	11.9%	8.5%	30.1%	23.8%
Dialysis history, %	85.4%	83.4%	86.6%	86.0%	85.9%	83.4%	84.3%	81.7%
Ethnic, %								
White	56.6%	54.8%	55.6%	52.9%	54.8%	53.7%	59.5%	57.3%
Black	24.0%	24.8%	22.6%	23.3%	26.4%	26.1%	21.7%	24.0%
Hispanic	13.3%	13.1%	16.2%	17.3%	13.0%	12.6%	12.2%	11.1%
Asian	4.4%	5.3%	4.0%	4.4%	4.1%	5.5%	4.9%	5.6%
Recipient BMI, mean ± SD	27.1 ± 5.1	26.7 ± 6.0	24.8 ± 5.2	24.5 ± 5.9	27.3 ± 5.2	27.0 ± 6.0	27.9 ± 4.7	27.7 ± 5.7
ABO mismatch, %								
Identical	88.8%	88.8%	87.6%	87.6%	88.6%	88.5%	89.6%	90.1%
Compatible	10.5%	10.5%	11.8%	11.8%	10.7%	10.8%	9.6%	9.2%
Incompatible	0.7%	0.7%	0.6%	0.6%	0.7%	0.7%	0.8%	0.7%
HLA mismatch, median (IQR)	4 (3, 5)	4 (2, 5)	3 (2, 5)	3 (2, 5)	4 (3, 5)	4 (2, 5)	4 (3, 5)	4 (3, 5)
PRA, mean ± SD	0 (0, 7)	3 (0, 33)	0 (0, 11)	3 (0, 30)	0 (0, 8)	4 (0, 38)	0 (0, 5)	2 (0, 29)
CIT (hours), mean ± SD	14.3 ± 11.6	14.2 ± 11.5	12.6 ± 11.9	12.6 ± 12.0	14.5 ± 11.7	14.4 ± 11.6	14.8 ± 11.2	15.0 ± 11.0
Donor sex, %								
Male	53.8%	53.6%	54.8%	53.5%	54.0%	54.4%	53.2%	52.7%
Female	46.2%	46.4%	45.2%	46.5%	46.0%	45.6%	46.8%	47.3%
Donor age, median (IQR)	39 (26, 50)	38 (25, 49)	32 (23, 45)	32 (23, 45)	38 (26, 48)	38 (25, 48)	43 (30, 54)	42 (29, 53)
Donor BMI, mean ± SD	26.6 ± 6.0	26.4 ± 6.0	25.9 ± 5.6	25.8 ± 5.7	26.5 ± 5.9	26.3 ± 6.0	27.1 ± 6.1	26.9 ± 6.2
Donor, Type								
Living	33.4%	34.0%	44.4%	44.3%	33.3%	34.3%	27.7%	26.9%
Deceased	66.6%	66.0%	55.6%	55.7%	66.7%	65.7%	72.3%	73.1%

Abbreviations: BMI, body mass index; CIT, cold ischemia time; DM, diabetes mellitus; HLA, human leukocyte antigen; IQR, interquartile range; PRA, panel reactive antibody; SD, standard deviation.

*Percentage indicates the proportion of patients with specific clinical characteristics among all patients or in strata of age and recipient's sex.

Table 2. Age categories and Recipient Sex (Male or Female) in Relation to 5 years Graft Survival.

	Male donor				Female donor			
	No. of Patients	No. of Events	Univariate HR (95% CI)	Multivariate HR† (95% CI)	No. of Patients	No. of Events	Univariate HR (95% CI)	Multivariate HR† (95% CI)
Recipient, Sex								
Male	132372	18137	1 (reference)	1 (reference)	113448	16273	1 (reference)	1 (reference)
Female	86922	12891	1.072 (1.048 to 1.097)	1.002 (0.960 to 1.045)	75221	11107	1.015 (0.991 to 1.040)	0.930 (0.888 to 0.974)
Recipient, Age category								
15-34 years	45024	9470	1 (reference)	1 (reference)	38023	8173	1 (reference)	1 (reference)
35-54 years	98326	13812	0.665 (0.647 to 0.682)	0.606 (0.576 to 0.638)	83279	11947	0.668 (0.650 to 0.687)	0.622 (0.587 to 0.658)
55-74 years	75944	7746	0.514 (0.499 to 0.529)	0.496 (0.467 to 0.526)	67367	7260	0.537 (0.520 to 0.554)	0.515 (0.482 to 0.549)
Recipient, 15-34 years								
Male (Recipient)	25852	4991	1 (reference)	1 (reference)	21348	4437	1 (reference)	1 (reference)
Female	19172	4479	1.232 (1.183 to 1.283)	1.175 (1.083 to 1.273)	16675	3736	1.086 (1.039 to 1.134)	1.068 (0.974 to 1.171)
Recipient, 35-54 years								
Male (Recipient)	59292	8268	1 (reference)	1 (reference)	50559	7275	1 (reference)	1 (reference)
Female	39034	5544	1.007 (0.974 to 1.042)	0.944 (0.882 to 1.010)	32720	4672	0.980 (0.944 to 1.016)	0.900 (0.833 to 0.972)
Recipient, 55-74 years								
Male (Recipient)	47228	4878	1 (reference)	1 (reference)	41541	4561	1 (reference)	1 (reference)
Female	28716	2868	0.950 (0.907 to 0.995)	0.902 (0.830 to 0.981)	25826	2699	0.931 (0.888 to 0.976)	0.836 (0.764 to 0.915)
$P_{interaction}$‡			<.0001	<.0001			<.0001	0.0031

Abbreviations: CI, confidence interval; CIT, cold ischemia time; DM, diabetes mellitus; HLA, human leukocyte antigen; HR, hazard ratio; PRA, panel reactive antibody.

†The multivariate Cox regression model initially included Prior transplant, Blood transfusion, Recipient BMI, Peak PRA, HLA mismatch, ABO mismatch, Ethnic, Diabetes, Dialysis history, Donor age (age≥60 / age<60), Donor BMI, Donor type and Cold ischemia time.

‡$P_{\text{interaction}}$ (two-sided) was calculated using the cross-product of recipient sex (female vs male) and recipient age (younger, middle and older) in the Cox regression model.