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Aging Augments IL-17 T cell Alloimmune Responses

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Abstract

As increasing numbers of elderly patients require solid organ transplantation, the need to better understand how aging modifies alloimmune responses increases. Here, we examined whether aged mice exhibit augmented, donor-specific memory responses prior to transplantation. We found that elevated donor-specific IL-17, but not IFN- γ , responses were observed in aged mice compared to young mice prior to transplantation. Further characterization of the heightened IL-17 alloimmune response with aging demonstrated that memory CD4⁺ T cells were required. Reduced IL-2 alloimmune responses with age contributed to the elevated IL-17 phenotype *in vitro*, and treatment with an anti-IL-17 antibody delayed the onset of acute allograft rejection. In conclusion, aging leads to augmented, donor-specific IL-17 immune responses that are important for the timing of acute allograft rejection in aged recipients. IL-17 targeting therapies may be useful for averting transplant rejection responses in older transplant recipients.

Introduction

The number of patients over 65 years of age waiting for kidney transplants has increased considerably within the last decade to more than 9% of all patients awaiting donor organs (1, 2). As the elderly subpopulation is increasing in Western countries and many endstage organ disorders are associated with increased age, it is likely that a growing number of older patients will be considered as transplant recipients in coming years (2,3).

Older patients suffer from increased morbidity and mortality resulting from infections and malignancy, but also exhibit an impaired ability to reject organ allografts (4-11). Some reports have indicated that reduced alloimmune responses are due to declining cellular immunity (12,13). An experimental study has shown that the reduced ability of aged recipients to reject skin allografts is associated with declining CD4⁺ T cell responses (14). While several clinical studies have indicated that aging is associated with lower frequencies of acute allograft rejection, other reports have suggested that when rejection occurs in older recipients the episodes are more severe, reviewed in (8). Furthermore, there is evidence indicating that aging is associated with increased chronic rejection (15).

Studies using non-allogeneic models have indicated that aging impairs adaptive T cell function. Specifically, TCR activated aged T cells exhibit impairments in CD28 signaling, IL-2 receptor activation, and downstream TCR signaling pathways (16-18). Additionally, immune senescence may be influenced by the accumulation of memory T cells (4,19). This is potentially significant because cross-reactive effector memory T cells participate in increased rates of acute allograft rejection and can impair the induction of transplantation tolerance (20). Cross-

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reactive memory T cells can be detected through rapid production of IFN- γ . However, it is not clear whether aged hosts exhibit augmented donor-specific memory responses prior to transplantation, and if this is the case, the characteristics of the memory response are not well elucidated. Finally, the impact of any aged-altered, pre—transplant, donor specific memory responses on transplantation outcomes has not been clearly defined.

We examined whether aged mice exhibit increased donor specific IFN- γ responses prior to transplantation. We found that aged hosts showed increased pre-transplant, donor-specific IL-17 responses, but not IFN γ responses, compared to young hosts. CD4⁺ memory T cells were responsible for the heightened IL-17 alloimmune response with aging. Aged mice exhibited a slightly delay to time of allograft rejection as compared to young mice, and inhibiting IL-17 in aged mice significantly extended allograft survival. Our results indicate that augmented IL-17 alloimmune responses influence the tempo of allograft rejection in aged transplant recipients.

Materials and Methods

Mice

Aged (18-23 months) and young (2-4 months) CBA (H2^k) and C57BL/6 (H2^b) mice were purchased from the NIA rodent facility, and animal use was approved by the Yale University IACUC. All mice were housed under pathogen free conditions, and animals were not used if they had skin lesions, or exhibited weight loss or lymphadenopathy. Additionally, sentinel mice were regularly tested for common murine pathogens: mouse hepatitis virus, parvovirus, mycoplasma pneumonia, sendai virus, lymphocytic choriomeningitis virus, ectromelia virus, pneumonia virus, and epizootic diarrhea.

Skin transplantation

Full-thickness trunk skin was removed from donor mice and transplanted (stapled) onto recipients as previously described (21). Rejection was defined as graft necrosis for >90% of the graft area.

Generation of bone marrow derived DCs (BMDCs) and mixed lymphocyte reaction (MLR)

The generation of BMDCs has been described previously (22). To perform the MLR, 1×10^5 purified T cells/well were cultured with 1×10^5 irradiated (28Gy) allogeneic BMDCs and incubated in 96-well plates at 37°C in a 5% CO₂ incubator for 4 days.

Reagents

An anti-IL-2 monoclonal antibody (5 μ g/ml, clone S4B6), an isotype control, and recombinant IL-2 (2000 pg/ml) were obtained from BD Biosciences (San Diego, CA). An anti-IL-17 monoclonal antibody (clone M210, IgG2a) was generously provided by Amgen (Seattle, Washington) (23). For the *in vivo* experiments, anti-IL-17 was administered at a dose of 100 μ g on days -1, +1, +3, +5, and +7 relative to transplantation. Isotype control antibody was obtained from BioXcell (New Lebanon, NH).

ELISPOT

ELISPOT analysis was performed as described previously (22).

Immunization with allogeneic spleen cells

Mice were immunized with 1×10^7 allogeneic spleen cells via i.p. injection.

Cell sorting and flow cytometry

T cells were purified via negative magnetic selection using EasySep reagents (StemCell Technologies, Vancouver, Canada). CD4, CD8, CD44, CD62L, IL-2, and IL-17 fluorescently labeled monoclonal antibodies and isotype controls were purchased from eBiosciences (San Diego, CA). Memory (CD44^{hi}/CD62L^{lo}) / naïve (CD44^{lo}/CD62L^{hi}) cells were further sorted by FACS on a FACSAria cell sorter (BD Bioscience, San Diego, CA). Intracellular cytokine staining was achieved by harvesting spleen cells post-transplantation and stimulating the cells ex-vivo with PMA/ionomycin in the presence of cell permeability agents and golgi stop (eBiosciences). All analysis was performed on a FACS CALIBUR flow cytometer (BD Bioscience) and analyzed with Flow Jo software (Treestar, San Carlos, CA).

Statistical analysis

Survival analysis between groups was calculated using the Logrank method. Comparison of means was performed using a two-tailed *t*-test, and repeated measures using analysis of variance (ANOVA). All results were generated using GraphPad prism software (San Diego, CA). Statistical significance was considered at a *p* value < 0.05.

Results

Aged mice exhibit increased numbers of anti-donor IL-17 producing spleen cells

Cross-reactive effector memory T cells increased the rates of acute allograft rejection, impaired the induction of transplantation tolerance (20), and could be detected based on rapid production of IFN γ (i.e., within 20 h of stimulation) (24). Accordingly, we examined whether aged or young non-transplanted CBA mice produced, rapid anti-donor (i.e. C57BL/6) IFN γ responses upon exposure to donor spleen cells. We found that aged (20-22 months of age) and young spleen cells (2-4 months of age) obtained from non-transplanted mice exhibited comparable numbers of IFN γ producing cells upon ex vivo exposure to irradiated donor spleen cells (Figure 1A). Young CBA mice that were immunized with C57BL/6 spleen cells for one month demonstrated increased IFN γ producing cells compared to other groups, and this effect was donor-specific (Figure 1A). Based on these results, aged non-transplanted mice do not exhibit significant anti-donor memory T cell responses. However, spleen cells harvested from non-transplanted aged CBA mice exhibited significantly increased numbers of anti-donor IL-17 producing spleen cells as compared to young spleen cells (Figure 1B). IL-17 is another cytokine produced by memory T cells (25). Similar results were observed in non-transplanted C57BL/6 recipients stimulated ex vivo with irradiated BALB/c spleen cells (data not shown). In summary, these results demonstrate that aged hosts exhibit increased anti-donor IL-17 responses, but not anti-donor IFN γ responses, prior to transplantation.

Aged T cells manifest augmented IL-17 responses, but impaired IL-2 responses after in vitro activation by young allogeneic DCs

To further examine the altered IL-17 alloimmune response with aging, we cultured CBA T cells with irradiated, *young* C57BL/6 BMDCs. The results demonstrated that aged T cells manifested increased IL-17 responses both over time and in dose response, compared to young T cells (Figure 2A). Similar results were obtained when using a different stimulator and responder strain combination (Supplemental Figure 1A). Furthermore, we found that increased IL-17 alloimmune response occurred with advanced aged (17 months of age), but not in intermediate aged T cells (8 months of age, Supplemental Figure 1B). Further analysis demonstrated that the augmented IL-17 T cell alloimmune response with aging was a consequence associated with CD4⁺ T cells rather than CD8⁺ T cells (Supplemental Figure 2). In contrast to the IL-17 response, aged T cells exhibited an impaired ability to produce IL-2 over time and in response to a dose range of stimulators (Figure 2B), which was in agreement

with a prior report (13). Our results indicate that increased age augments IL-17 *in vitro* T cell alloimmune response, but impairs IL-2 alloimmunity.

IL-2 responses contribute to heightened IL-17 alloimmune response

IL-2 is a negative regulator of the Th17 pathway (26), and results from *in vitro* assays showed that aged T cells produce less IL-2 than young T cells. It is possible that the reduced IL-2 environment contributed to the augmented IL-17 response with aging. We examined whether IL-2 influences the IL-17 alloimmune response in aged CD4⁺ T cells. Inhibiting IL-2 in young CD4⁺ T cells resulted in a greater than double proportion of IL-17 producing cells (Figure 2C). Furthermore, the addition of recombinant IL-2 reduced the IL-17 response of aged CD4⁺ T cells (Figure 2C). These data provide evidence that reduced IL-2 production may facilitate increased IL-17 response by aged CD4⁺ T cells during allostimulation and that heightened IL-2 response in young T cells may suppress the IL-17 response.

Aged mice manifest increased numbers of memory CD4⁺ T cells, which produce more IL-17 during alloactivation

In agreement with prior work (6), we found that aging led to an increased proportion of memory CD4⁺ T cells and a reduced proportion of naïve CD4⁺ T cells (Figure 3A). We next FACs purified memory (i.e., CD44^{hi}, CD62L^{lo}) or naïve (i.e., CD44^{lo}, CD62L^{hi}) populations from CD4⁺ T cells to determine if aged memory or naïve CD4⁺ T cells produced elevated IL-17 responses on a per cell basis compared to young T cells during allostimulation. In the MLR, naïve CD4⁺ T cells did not produce IL-17, regardless of age (Figure 3B). However, we found that aged memory CD4⁺ T cells exhibited a higher proportion of IL-17 secreting cells than young memory CD4⁺ T cells (Figure 3C). Furthermore, we noted that aged memory CD4⁺ T cells that secreted IL-17 did not co-secrete IL-2, but we observed a small proportion of double cytokine-producing memory T cells in this assay (Figure 3C). Aged memory CD4⁺ T cells produce augmented IL-17 alloimmune responses on a per cell basis, but aged mice also generate higher numbers of memory CD4⁺ T cells that could contribute to the elevated IL-17 alloimmune response of the greater T cell pool with age.

Generation of allo-specific, memory T cells in young hosts does not replicate the aged IL-17 T cell phenotype

It is possible that the augmented IL-17 phenotype with aging is due to the accumulation of memory T cells, which characteristically produce IL-17 (25). If this is the case, it might be possible to replicate the aged phenotype in young hosts by immunizing them and allowing them to generate memory T cells. To test this hypothesis, we immunized young CBA mice with C57BL/6 spleen cells to generate memory T cells reactive to alloantigens. One-month post immunization, T cells were purified and stimulated with allogeneic C57BL/6 BMDCs, and IL-17 production was measured. Our results indicate that T cells from young, immunized mice produced a similar amount of IL-17 to T cells from young, non-immunized mice. Both of these groups demonstrated an inferior IL-17 response compared to that of T cells from aged non-immunized mice (Figure 4A). However, T cells from young, immunized mice produced more IL-2 than the other groups (Figure 4B).

We then transplanted young CBA mice with C57BL/6 skin allografts, representing a more chronic exposure to alloantigens than injection with allogeneic spleen cells. One month after transplantation, T cells from young mice were compared by MLR to T cells from non-transplanted aged and young mice, respectively. However, T cells from the young transplanted mice exhibited an inferior IL-17 response during the MLR as compared to T cells from aged, non-transplanted mice (Figure 4C). (Although, at 72h of culture, T cells from the transplanted young mice exhibited slightly increased IL-17 levels compared to T cells from young non-

transplanted mice). These results indicate that the generation of donor specific memory T cells in a young host with these immunization protocols does not replicate the aged T cell phenotype.

Aged individuals exhibit impaired Th1 alloimmune response post transplantation

We investigated the impact of recipient age on alloimmunity using *in vivo* T cell polyclonal systems. Aged or young CBA recipients received a skin transplant from young C57BL/6 donors. ELISPOT examination of harvested recipient splenocytes at days seven and fourteen post transplantation demonstrated that aged recipients exhibit a reduced ability to produce IL-2 and IFN- γ after *ex vivo* stimulation by young, irradiated donor spleen cells (Figure 5A-B). Additionally, aged transplant recipients expressed lower serum Th1 (IgG2a) alloantibodies than young transplant recipients (data not shown). However, Th2 immune responses, quantified by the number of IL-4 producing spleen cells, were preserved in aged recipients at 14 days post transplantation (Figure 5B). In contrast to the above data, at 14 days post transplantation, aged recipients exhibited augmented IL-17 alloimmune responses (Figure 5C). These data indicate that age impairs recipient Th1 T cell responses, but elevates IL-17 responses after skin allotransplantation.

Aged recipients reject skin allografts more slowly than young recipients and inhibiting IL-17 significantly delays the onset of acute allograft rejection

We repeated the experiment where aged or young CBA recipients received a young C57BL/6 skin allograft, and this time we measured the tempo of allograft rejection. We observed a slightly delayed time to allograft rejection in the aged recipients in this model (Figure 6A), although a majority of aged mice were able to reject their allografts at a similar rate to that observed for young transplant recipients.

To examine the role of heightened IL-17 alloimmune responses with age, we transplanted CBA aged recipients with C57BL/6 skin allografts and treated the recipients with an anti-IL-17 mAb or isotype control. Previously, anti-IL-17 was observed to impair IL-17-dependent collagen induced arthritis (23). Both aged and young recipients that were treated with an anti-IL-17 antibody exhibited significantly increased time to acute allograft survival as compared to isotype control treated recipients. The median survival time to acute allograft rejection in aged recipients (20 days) treated with anti-IL-17 was significantly longer than that for young recipients treated with the identical therapy (17 days, $p = 0.03$). These data indicate that heightened IL-17 alloimmune responses that are associated with aging are an important pathway for acute allograft rejection.

Discussion

Aging has a complex effect on immune responses (6). One consistent effect is that aging leads to an accumulation of memory T cells (5,6), even though a prior study indicated that the function of memory T cells declines with aging (27). Based on these studies, we predicted that aged hosts would exhibit increased numbers of donor-specific effector memory responses, characterized by rapid IFN- γ secretion (24), as compared to young hosts, prior to transplantation. However, our study found that that aged hosts exhibited augmented donor-specific, effector memory IL-17, but not IFN- γ , responses prior to transplantation compared to young mice. We showed that CD4⁺ memory T cells are responsible for the elevated alloimmune response with aging, a phenotype that may be exacerbated by weaker IL-2 alloimmune responses. Importantly, we demonstrated that the heightened IL-17 alloimmune responses contributed to acute allograft rejection in aged transplant recipients. Our results indicate that aging elevates memory IL-17 alloimmune responses.

IFN- γ producing donor-specific memory T cells, predominantly of the effector memory phenotype, may be an indicator of an increased tendency toward acute allograft rejection (28). Based on our results, IFN γ measurement may not be sufficient for indicating the presence of donor-specific memory responses with aging. Our work suggests that IL-17 may be a more reliable indicator of the presence of cross-reactive effector memory T cells in aged recipients. This is particularly relevant to solid organ transplantation, for which the fastest growing cohort of patients awaiting transplantation is greater than 65 years of age. Our results await validation using human cells.

Prior studies have associated increased systemic IL-6 levels, a Th17 promoting factor, with aging (29). Furthermore, aging is associated with an increased number of regulatory T cells (19), which can act as a source of TGF- β , another Th17 promoting factor (30). In contrast, it is well known that aging leads to reduced IL-2 responses, a negative regulator of the Th17 pathway (4,31). Apparently, with aging there are increases in Th17 promoting factors and a reduction in Th17 regulatory factors. Thus, it is plausible that, with aging, CD4⁺ T cells may transition to a Th17 phenotype upon exposure to environmental antigens. Some of these CD4⁺ T cell effectors may then differentiate into IL-17 producing memory T cells, which may cross-react with alloantigens. This may explain why the IL-17 T cell alloimmune response in young mice was lower than that in aged mice after immunization with alloantigens. Young hosts may not be prone to IL-17 immune responses. Future studies will be required to mechanistically examine how aging leads to accumulated numbers of IL-17 producing memory CD4⁺ T cells.

Our study shows that aged hosts exhibit increased numbers of memory CD4⁺ T cells that produce IL-17 when stimulated with donor alloantigens. These memory cells could present a barrier to transplantation. However, since aged hosts have increased numbers regulatory T cells (19), which may aid in immune tolerance induction, it is not clear how age will affect the application of protocols that induce transplantation tolerance. This will clearly be an important topic for future investigation.

In contrast, a previous report found that aged recipients exhibit increased IFN γ and IL-2 responses after transplantation (32). However, a major difference between that study and ours is that acute rejection in the prior study was abrogated by the use of cyclosporine. Clinically, this distinction may be relevant, because several clinical studies have documented an association of increasing recipient age with reduced rates of acute allograft rejection (9-11). In contrast, aging reportedly leads to increased chronic rejection (33). Given the recent findings that IL-17 may contribute to chronic lung rejection (34), it will be important for future studies to determine whether heightened IL-17 alloimmune responses with aging impact the development of chronic rejection.

Our study provides evidence that aging leads to elevated IL-17 alloimmune responses that can influence the rate of acute allograft rejection in individuals. When translated to the clinic, this information may be important for tailoring therapies to treat acute allograft rejection in older transplant recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Aging leads to increased donor specific IL-17 responses prior to transplantation

(A): Spleen cells from the indicated groups of mice were stimulated with irradiated donor spleen cells. Aged spleen cells did not develop into a significant number of IFN- γ producing spleen cells compared to non-stimulated controls. Similar findings were noted for young spleen cells. Spleen cells from previously immunized young mice developed into a significant number of IFN- γ producing spleen cells compared to non-ex vivo stimulated cells from the same group (*, $p = 0.01$, T test). This response was donor specific. Allo SPC = allogeneic spleen cells. 3rd party = BALB/c

(B): A similar experiment to that described in A is presented here, except that IL-17 was measured by ELISPOT. Aged spleen cells produced increased numbers of IL-17 producing

spleen cells compared to non-stimulated spleen cells (*, $p < 0.0001$, T test). Spleen cells from young mice exhibit an augmented response during allostimulation in comparison to non-stimulated responders (**, $p = 0.04$, T test). However, this stimulated young response was inferior to the stimulated aged response (***, $p = 0.0003$, T test). Allo SPC = allogeneic spleen cells

Figure 2. Aged T cells manifest elevated IL-17 alloimmune responses

(A): Aged and young CBA T cells were stimulated with irradiated C57BL/6 BMDCs. Aged T cells manifested increased IL-17 responses over time ($p = 0.002$, ANOVA) and in response to a dose range of stimulators ($p = 0.0006$, ANOVA) vs. young T cells. Non-stimulated T cells did not produce IL-17 (data not shown). Data shown is a representative of five independent experiments ($n = 3$ / experiment).

(B): Aged and young T cells were stimulated with irradiated C57BL/6 BMDCs. Aged T cells manifested impaired IL-2 responses over time (ELISA) ($p < 0.0001$, ANOVA) and in response to a dose range of stimulators (ELISPOT) ($p = 0.009$, ANOVA) vs. young T cells. Non-stimulated T cells did not produce IL-2 (data not shown).

(C): Purified CD4⁺ T cells were stimulated with irradiated, allogeneic BMDCs. In the absence of recombinant IL-2, approximately 30% of aged CD4⁺ T cells produced IL-17. This number was reduced by the addition of recombinant IL-2 (2000 pg/ml) to the culture. Young allostimulated CD4⁺ T cells manifest a log fold lower proportion of IL-17 producing T cells vs. aged T cells, and this was doubled by the addition of an anti-IL-2 monoclonal antibody to the culture. Flow plots are gated on the CD4⁺ population.

Figure 3 **Figure 3. The CD4⁺ T cell pool of aged mice contains higher proportion of memory T cells**

(A): Representative flow cytometric plots demonstrating naïve (CD62L^{hi}, CD44^{lo}) and memory (CD62L^{lo}, CD44^{hi}) T cell gates of magnetically enriched CD4⁺ T cells, harvested from spleen and lymph nodes from young and aged mice. The percentages of naïve and memory cells are shown. The histogram shows pooled results from three experiments consisting of four mice / group / experiment. (* p<0.01, T test)

(B): Aged memory CD4⁺ T cells produce more IL-17 during the course of the MLR compared to young memory CD4⁺ T cells (* p<0.01, T test). Naïve T cells (young or aged) do not produce IL-17 in this assay.

(C): Intracellular flow cytometric analysis of aged and young CD4⁺ memory T cells that were stimulated for 96 h with allogeneic BMDCs. N= 4-6 / group. Flow plots are gated on CD4⁺ T cell population, and % positive are shown within each quadrant.

Figure 4. Immunization of young mice with alloantigens does not recapitulate the aged IL-17 T cell alloimmune phenotype

(A): T cells harvested from young mice immunized with allogeneic spleen cells one month earlier did not exhibit an augmented IL-17 response compared to T cells harvested from young non-immunized mice or an inferior response compared to aged T cells harvested from non-immunized aged mice.

(B): T cells from young mice immunized with allogeneic spleen cells exhibited augmented IL-2 responses compared to T cells from young, non-immunized mice. Aged non-immunized mice manifested an inferior IL-2 response as a function of time in this assay.

(C): T cells were isolated from young CBA mice one month after the mice received a C57BL/6 skin allograft, and were then stimulated with irradiated C57BL/6 BMDCs. These T cells exhibited an inferior IL-17 response during the course of the MLR as compared to non-transplanted aged T cells, although the response was elevated compared to young-non-transplanted mice at 72 h of culture.

Figure 5. Recipient age impairs Th1 alloimmune responses after skin transplantation

(A): Aged or young CBA recipients received a young C57BL/6 skin allograft. Seven days post transplantation, recipient spleens were harvested and stimulated ex vivo for <20 hours in the presence of irradiated donor spleen cells. The results showed that aged recipients exhibited inferior IFN γ (*, $p=0.03$; T test) and IL-2 (*, $p=0.02$; T test) responses vs. young counterparts. Non-restimulated spleens from either aged or young mice did not elicit a response in this assay (data not shown). Results are representative of two independent experiments. N = 3-5 / experiment.

(B): Data from a similar experiment to (A) above is presented, except spleen cells harvested at 14 days post transplantation. The results show that aged recipients exhibit inferior IFN γ (*, $p=0.02$; T test) and IL-2 (*, $p=0.02$; T test) responses, but retained IL-4 responses (***, $p=0.04$; T test) vs. young counterparts. Note, stimulation with either irradiated third party or syngeneic spleen cells did not elicit a response in this assay (data not shown). Results are representative of two independent experiments. N = 3-5 / experiment.

(D): Spleen cells harvested at 14 days post transplantation demonstrate that aged recipients exhibited augmented donor specific IL-17 responses vs. young recipients (* $p=0.01$). N = 3-6 / group.

Figure 6. Recipient age delays the onset to acute allograft rejection. Inhibiting IL-17 delays the onset of acute allograft rejection

(A): Aged and young CBA recipients received young C57BL/6 skin allografts. Aged recipients exhibited a slightly, but significantly, extended time to allograft rejection. (*, $p = 0.01$; Log rank).

(B): Aged and young CBA mice that received C57BL/6 skin allografts were treated with an anti IL-17 mAb or isotype control. Groups that received the anti-IL-17 treatment exhibited a significant delay time to acute allograft rejection as compared to isotype control groups. For aged group, isotype control: median survival time (MST) = 12 days vs. anti-IL-17 treated group, MST = 20 days ($p = 0.005$, Log Rank). For young isotype treated group (MST) = 12 days vs. anti-IL-17 treated group, MST = 17 days ($p = 0.001$, Log Rank). The aged anti-IL-17 treated group exhibited a significantly delayed time to acute allograft rejection vs. the young anti-IL-17 treated group ($p = 0.03$, Log Rank).