An Age-Specific CD8+ T Cell Pathway That Impairs the Effectiveness of Strategies To Prolong Allograft Survival

Wei Du, Hua Shen, Anjela Galan and Daniel R. Goldstein

J Immunol 2011; 187:3631-3640; Prepublished online 26 August 2011;
doi: 10.4049/jimmunol.1100441
http://www.jimmunol.org/content/187/7/3631

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/08/26/jimmunol.1100441.DC1

References
This article cites 38 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/187/7/3631.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
An Age-Specific CD8+ T Cell Pathway That Impairs the Effectiveness of Strategies To Prolong Allograft Survival

Wei Du,* Hua Shen,* Anjela Galan,†‡ and Daniel R. Goldstein*

Age-related decline in immunity can impair cell-mediated responses during an infection, malignancy, and acute allograft rejection. Although much research has been allocated to understand the immune responses that impact the former two conditions, the cellular mechanisms by which aging impacts the immune acceptance of organ allografts are not completely clear. In this study, we examined how recipient age impacts the efficacy of therapies that modulate immune recognition of allografts using an immunogenic murine skin transplant model. We found that costimulatory blockade-based treatment failed to extend allograft survival in older recipients to the same extent as that observed in younger recipients. CD8+ T cells were critical for the inability of aged recipients to achieve maximal allograft survival. Although aged mice displayed a larger number of effector memory T cells prior to transplantation, these cells did not exhibit enhanced alloreactivity compared with young memory T cells. In contrast, naive aged CD8+ T cells exhibited enhanced IFN-γ production to allostimulation compared with young naive T cells. Our results provide evidence that aging enhances CD8+ T cell alloreactivity. This could impair the ability of costimulatory blockade-based therapies to prolong allograft survival. Thus, targeting CD8+ T cells in humans may be a way to improve outcomes in older patients requiring immune modulatory therapy. The Journal of Immunology, 2011, 187: 3631–3640.

Older people often exhibit increased incidences of malignancy and infection that are associated with age-related decline in immunity (1, 2). To date, most studies that examine this decline in immunity have focused on adaptive immunity, and findings consistently indicate that aging leads to reduced adaptive T cell function, often in the form of a decline in CD8+ T cell responses of both naive and memory CD8+ T cells (3, 4). Moreover, aging leads to restriction of the naive T cell repertoire and an accumulation of memory T cells, further decreasing the body’s ability to respond against new infections and vaccinations (5, 6).

Studies on aging and its relation to immunological tolerance point to a dichotomy for which the mechanism is not well understood. On the one hand, aging leads to an accumulation of CD4+ regulatory T cells (Treg) (7–9), which could promote immunological tolerance (10). On the other hand, aging can also lead to an accumulation of alloreactive memory T cells (11), most likely the result of prior environmental exposures such as viral infections (11). Prior experimental studies performed exclusively in young recipients have found that memory CD8+ T cells pose a barrier to transplant tolerance. As older hosts acquire memory T cells, it has been assumed that older transplant recipients would resist immune modulatory therapy, including costimulatory blockade, due to the presence of these memory T cells.

In this study, we examined how recipient age impacts the effectiveness of immune modulatory therapies for organ allografts. Using an experimental murine skin allograft model, we found that the combination of either anti-CD45RB and anti-CD154 or anti-CD154 and donor-specific spleen cell transfusion (DST) does not extend allograft survival in aged recipients to the same degree as it does in young recipients. Enhanced CD8+ T cell responses were necessary for the observed effect in aged recipients. In contrast, CD8+ T cells played no role in the efficacy of costimulatory blockade to extend allograft survival in young recipients. Although aged recipients exhibited slightly higher numbers of effector memory T cells, these cells did not exhibit allosensitization prior to transplantation or compared with young memory T cells. However, aging augmented alloreactivity of naive CD8+ T cells. Although other studies have shown that a decline in CD8+ T cell function is a major cause for age-related immune phenotypes, our study has revealed that enhanced CD8+ T cell function impairs immune regulation in aged mice.

Materials and Methods

Mice

Young (2–4 mo) and aging recipient mice (14–20 mo) on either the C57BL/6 (H2b) or CBA (H2d) background were obtained from the National Institute of Aging rodent facility. Mice used as donors (BALB/c [H2b] or C57BL/6 [H2b]) were obtained from The Jackson Laboratory. Yale University Institutional Animal Care and Use Committee approved the use of animals in this study. All mice were kept in pathogen-free conditions. No animals were used in the study if they had evidence of skin lesions, weight loss, or lymphadenopathy.

Skin transplantation

Full-thickness trunk skin was transplanted from donor mice and stapled on recipients, as previously described (12). Rejection was defined as graft necrosis >90% of the graft area.

Reagents

Anti-CD154 (10 mg/kg body weight, administered i.p. on days 0, 2, 4, and 6 relative to transplantation) and anti-CD45RB (4 mg/kg body weight administered i.p. on days −1, 0, 1, 2, 5, and 8 relative to transplantation) were administered i.p. on days 0, 2, 4, and 6 relative to transplantation; MPO, myeloperoxidase; Treg, regulatory T cell.

*Department of Internal Medicine and Immunobiology, Yale University School of Medicine, New Haven, CT 06525; †Department of Dermatology, Yale University School of Medicine, New Haven, CT 06525; and ‡Department of Pathology, Yale University School of Medicine, New Haven, CT 06525

Received for publication February 10, 2011. Accepted for publication July 29, 2011.

This work was supported by National Institutes of Health Grants AI064660 and AG028082 (to D.R.G.). D.R.G. is supported by an established investigator award from the American Heart Association (0940006N) and an independent career development grant from the National Institute on Aging (AG030349).

Address correspondence and reprint requests to Dr. Daniel R. Goldstein, 333 Cedar Street, 3FMP, New Haven, CT 06525. E-mail address: daniel.goldstein@yale.edu

The online version of this article contains supplemental material.

Copyright © 2011 by The American Association of Immunologists, Inc.

0022-1767/11/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100441
purchased from BioXcell (Lebannon, NH) and were administered, as previously described (13). For the regimen of donor-specific transfusion and anti-CD154, 1 × 10^7 donor spleen cells were i.v. injected on day 0 relative to transplantation. PC61 (250 μg i.p. on days −1 and 0), GK1.5 (200 μg i.p. on days 0, 1, 2, and 3), and 2.43 (150 μg i.p. on days −1, 2, 9, 16, and 23) mAbs were also purchased from BioXcell.

**Histology and graft myeloperoxidase assay**

Lysates of skin grafts were prepared by homogenizing tissue and myeloperoxidase (MPO) measured by ELISA, as previously described (14). Skin allografts were collected, embedded in paraffin, sliced into 5-μm-thick sections, and stained with H&E or with the rat anti-mouse Ly-6B.2 Ab (Abd Serotec, Raleigh, NC) for immune histochemical analysis. Samples were evaluated by investigators blinded to the experimental group. Images were obtained using an Olympus BX61 microscope with a Spot RT color 2.2.1 camera. Images were acquired with Spot Diagnostic Instruments version 4.5.9.9 software.

**Flow cytometry, cell sorting, and ELISA**

Stainings for CD4, CD25, CD27, CD28, CD45RB, CD44, CD62L, granzyme B, T-bet, and Foxp3 were achieved using fluorescently labeled mAbs and isotype controls purchased from eBiosciences (San Diego, CA). Foxp3, T-bet, and granzyme B stainings were achieved using an intracellular staining protocol, according to manufacturer’s instructions. All analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software. T cells were purified via negative magnetic selection using EasySep reagents (StemCell Technologies, Vancouver, Canada). In certain experiments, CD8+ T cells were subsequently sorted into naive (CD44low; CD62Lhigh), central memory (CD44high; CD62Lhigh), and effector memory (CD44high; CD62Llow) subpopulations via FACS. IFN-γ and IL-2 concentrations were measured using ELISA kits, as previously described (14).

**MLR and generation of bone marrow-derived dendritic cells**

Magnetically enriched CD8+ T cells or magnetically enriched and FACS-sorted CD8+ T cells (2 × 10^6/ml) were cultured with irradiated BALB/c bone marrow-derived dendritic cells (BMDCs; 6 × 10^6 cells/ml) in RPMI 1640 supplemented with 10% FBS. BMDCs were generated in the presence of GM-CSF, as previously described (15). Supernatants were obtained at indicated time points, and the amount of cytokines was quantified by ELISA. At day 4 of culture, CD8+ T cells were stained with indicated fluorescently tagged mAb, and expression was assessed by flow cytometry. Cells that were not stimulated with BALB/c dendritic cells served as negative controls.

**ELISPOT**

T cells purified, as described above, from transplant recipients were stimulated with irradiated donor cells overnight or indicated time points on ELISPOT plates that were coated with an IFN-γ capture Ab. Subsequent spot development and analysis were performed, as described in our past publication (16). T cells and donor cells cultured alone were used as controls.

**Treg suppression assay and anti-CD3 stimulation**

Treg suppression assays were performed, as described previously (15). FACS-sorted 1 × 10^5 CD4+CD25- T effector cells were stimulated with soluble anti-CD3 (1 μg/ml) in the presence of 5 × 10^4 irradiated syngeneic splenic cells. In certain experiments, titrated numbers of CD4+CD25+ Treg cells were added for 72 h. Tritium thymidine was then added to the wells, and cellular proliferation was measured. Results are expressed as cpm or as

---

**FIGURE 1.** Costimulatory blockade-based regimens exhibit an impaired ability to extend allograft survival in aged transplant recipients compared with those in young recipients. A and B. Aged (18–20 mo) and young (aged 2–4 mo) mice received a skin allograft and perioperative anti-CD45RB plus anti-CD154 injection. A, BALB/c donor, C57BL/6 recipient; B, C57BL/6 donor, CBA recipient. Allograft survival was recorded. Differences between aged and young were significant, p < 0.01 (log rank). Mice that received a transplant, but did not receive immune modulation (denoted as no treatment), are shown. C. Anti-CD45RB and anti-CD154 induced inferior allograft survival in 14-mo-old recipients. C57BL/6 mice aged 14 mo and young recipients aged 2–4 mo each received a BALB/c skin transplant and anti-CD45RB and anti-CD154 injections. Difference between aged and young allograft survival was significant, p < 0.01 (log rank). D. Increased recipient age impaired the ability of DST and anti-CD154 to extend allograft survival. C57BL/6 mice, aged 14 mo, and young mice, aged 2–4 mo, all received a BALB/c skin allograft. They were administered a regimen consisting of anti-CD154 and DST, and graft survival was recorded. p < 0.01 between groups (log rank).
the percentage suppression of T effector cell proliferation mediated by the presence of Treg.

Statistical analyses

Survival analysis between groups was calculated using the log rank method. Comparison of means was performed using a two-tailed t test and repeated measures using ANOVA. All results were generated using GraphPad prism software (San Diego, CA). A p value < 0.05 was considered statistically significant. Error bars = SEM.

Results

Aged mice resist the allograft-prolonging properties of costimulatory blockade-based treatment

To empirically test how aging impacts the effectiveness of immune modulatory therapy, we employed a skin transplant model in which graft recipients were administered the following costimulatory blockade reagents: anti-CD45RB and anti-CD154 mAbs (13). This experimental model was chosen because these Abs have been shown to be effective in preventing or delaying skin graft rejection (13, 17–19). Moreover, the median allograft survival time for this regimen in young murine transplant recipients is ~50 d (13), long enough to allow for the assessment of Ab effects on allograft survival. Furthermore, because aging leads to an accumulation of CD4+CD25+ Tregs (7, 9), we employed a regimen, which we determined to be Treg dependent (Supplemental Fig. 1A).

Prior to administering the protocol, we confirmed that aged and young mice expressed similar amounts of CD45RB on both CD4+ and CD8+ T cells (Supplemental Fig. 1B). We did not detect CD154 expression on CD4+ and CD8+ T cells of young or aged mice prior to transplantation (data not shown). Young (2–4 mo of age) and aged (18–20 mo of age) C57BL/6 (H2b) mice were transplanted with a BALB/c (H2d) skin allograft and treated perioperatively with anti-CD45RB and anti-CD154. Additionally, CBA (H2k) strain young and aged mice were transplanted with a C57BL/6 skin graft and treated with the same regimen. Without immune modulation via the costimulatory blockade Abs, we observed that aged C57BL/6 mice exhibited a similar time course of allograft rejection as young mice (Fig. 1A), consistent with previously reported observations (20). However, we found that aged mice displayed significantly reduced allograft survival time compared with their younger counterparts when treated with perioperative anti-CD45RB and anti-CD154 (Fig. 1A, 1B). Similar results were noted in mouse recipients 14 mo of age (Fig. 1C). (Note, for the rest of the study, aged mice were 14 mo of age.)

To evaluate whether the age-associated response was limited to the use of the anti-CD45RB and anti-CD154 Ab protocol, we repeated the above transplant experiments using an alternative regimen that induces delayed allograft survival via depletion of graft-reactive T cells. In this case, skin allograft survival is modulated by perioperative D5T and administration of anti-CD154 (21, 22). In this study, too, we found that older transplant recipients exhibited a shorter allograft survival time than young recipients (Fig. 1D). Our results suggest that the effectiveness of immune modulatory therapies to allografts may be age dependent. Moreover, such an age-dependent response can cut across a number of different costimulatory blockade-based mechanisms, such as those involving anti-CD45RB/anti-CD154.

Aged transplant recipients treated with anti-CD45RB and anti-CD154 exhibit increased allograft inflammation compared with young recipients

Histological examination of allografts at 3 wk posttransplantation revealed that aged recipients treated with anti-CD45RB and anti-CD154 exhibited increased cellular infiltration compared with young recipients. These infiltrations consisted not only of lymphocytes and histiocytes, but largely of polymorphonuclear cells (Fig. 2A–C). Young recipients manifested little intragraft inflammation; when inflammation was observed, it involved mostly lymphocytes and histiocytes (Fig. 2A–C). In contrast to those of their young counterparts, allografts from aged recipients manifested elevated granulocyte activity, as measured by MPO activity (Fig. 2D). These results demonstrate that allografts from aged recipients treated with costimulatory blockade-based regimens led to more inflammation than allografts from similarly treated young recipients.

Aged recipients exhibit elevated T cell alloreactivity compared with young recipients

Cytokines derived from T cells, such as IFN-γ, have been shown to be important markers of allograft rejection (23). To examine the

![FIGURE 2. Aged transplant recipients treated with anti-CD45RB and anti-CD154 exhibit increased graft inflammation relative to young recipients. Skin allografts were harvested from transplanted aged and young mice for histological examination. Results in A–C are representative of three independent experiments with n = 3/group/experiment. A–C. Aged recipients exhibited increased inflammation after transplantation and treatment with anti-CD45RB and anti-CD154. BALB/c skin grafts were harvested at day 21 posttransplantation from C57BL/6 mice that received anti-CD45RB and anti-CD154 (A, original magnification ×4; B, original magnification ×40; Scale bars, A = 0.25 mm; B and C = 0.06 mm. In aged recipient mice, the inflammatory infiltrate was predominantly neutrophilic, as indicated with arrows (B). Immunohistochemical staining with rat anti-mouse Ly-6B.2 Ab identifies neutrophils, as indicated with arrows (C). D. BALB/c skin grafts harvested at day 21 posttransplantation from C57BL/6 mice that received anti-CD45RB and anti-CD154. Grafts were homogenized, and MPO activity was measured by ELISA. Differences in MPO activity between the aged and young reached significance (*p < 0.05, t test). Pooled data from two independent experiments with consistent results; n = 6/group. Error bars = SEM.](http://www.jimmunol.org/)

Downloaded from http://www.jimmunol.org/ by guest on July 29, 2017
role of T cell responses in allograft transplantation in young and aged mice, we measured the cytokine levels of mice treated with or without anti-CD45RB and anti-CD154. Prior to transplantation, we noted that T cells from aged mice exhibited similar antidonor IFN-γ responses as T cells of young mice (Fig. 3). Three weeks after skin transplantation, IFN-γ T cell responses were equally enhanced in young and aged recipients (Fig. 3).

In addition, we found that treatment with anti-CD45RB and anti-CD154 reduced the antidonor IFN-γ T cell responses in both aged groups, although the response of the aged T cells remained significantly higher than that of the young group (Fig. 3). These results demonstrate that aged transplant recipients treated with anti-CD45RB and anti-CD154 exhibited elevated IFN-γ T cell responses, an important readout of alloreactivity, relative to young transplant recipients.

CD4+CD25+ Treg function is similar in both the aged and the young transplant recipients treated with anti-CD45RB and anti-CD154

The results above indicate that aged recipients are not responsive to costimulatory-based regimens that prolong allograft survival in young transplant recipients. This phenotype is associated with increased T cell alloreactivity and enhanced allograft inflammation. Because the anti-CD45RB/anti-CD154 protocol used in these studies is costimulatory-based regimens that prolong allograft survival, aged mice were expected to be less responsive to treatment. However, this was not the case, as aged recipients exhibited an increased proportion of splenic Foxp3+ CD4 T cells before transplantation, which was maintained after transplantation regardless of costimulatory blockade treatment (Fig. 4A). Absolute numbers of splenic Tregs were not significantly different between the young and aged groups before or after transplantation (Fig. 4B). Furthermore, the suppressive function of splenic Tregs was unaltered with aging either before or after transplantation and treatment with anti-CD45RB and anti-CD154 (Fig. 4C, 4D). These results imply that neither alterations in splenic Treg numbers nor function appear to explain the impaired efficacy of anti-CD45RB and anti-CD154 to extend allograft survival with aging.

Aged mice resist the allograft-prolonging effects of anti-CD45RB and anti-CD154 via a CD8+ T cell-dependent mechanism

We next examined whether the resistance to the allograft prolonging effects of anti-CD45RB and anti-CD154 in aged recipients required T cells. We first assessed the role of CD4+ T cells by employing a CD4+ T cell-depleting Ab. CD4+ T cell depletion did not extend allograft survival mediated by this regimen in aged recipients relative to aged recipients treated with isotype control (Fig. 5A). However, we noted that CD4+ T cell depletion also abrogated allograft prolongation mediated by CD45RB and anti-CD154 in young recipients (Fig. 5A), which may be due to removal of CD4+CD25+ Tregs, critical for allograft-prolonging effects of the regimen (Supplemental Fig. 1A).

When we administered a CD8+ T cell-depleting mAb weekly for up to 3 wk posttransplantation to aged recipients treated with anti-CD45RB and anti-CD154, we found that allograft survival was significantly enhanced compared with that for aged recipients treated with anti-CD45RB, anti-CD154, and isotype control for the CD8+ T cell-depleting mAb (Fig. 5B). The CD8-depleting mAb did not impact the tempo of allograft rejection without immune modulatory treatment (Supplemental Fig. 2A) and was effective in depleting CD8+ T cells in both young and aged mice (Supplemental Fig. 2B). Unlike the response of the aged
recipients, CD8+ T cell depletion did not significantly extend the allograft-prolonging effects of anti-CD45RB and anti-CD154 in young mice (Fig. 5B). Indeed, aged transplant recipients that were CD8+ T cell depleted exhibited an increased allograft survival time than young recipients that were similarly CD8+ T cell depleted (Fig. 5B). In contrast, aged mice that received anti-CD45RB and anti-CD154 in addition to the isotype control for the CD8-depleting Ab manifested a shorter allograft survival period than similarly treated young recipients (Fig. 5B). Five weeks post-CD8+ T cell depletion, CD8+ T cells started to emerge in aged CD8+-depleted mice (Fig. 5C). The emergence of these CD8+ T cells was accompanied by allograft rejection (compare Fig. 5B, 5C). Aged recipients that were CD8+ T cell depleted also exhibited reduced allograft MPO levels compared with aged recipients that were treated with isotype control (Fig. 5D). In sum, aged mice resist the allograft-prolonging effects of anti-CD45RB and anti-CD154 via a CD8+ T cell-dependent mechanism.

Aged naive CD8+ T cells, but not memory subpopulations, exhibit more IFN-γ alloreactivity than young CD8+ T cells

Given that aged transplant recipients resisted the allograft-prolonging effects of anti-CD45RB and anti-CD154 via a CD8+ T cell-dependent mechanism, we examined the in vitro alloimmune responses of young and aged CD8+ T cells. We FACS sorted CD8+ T cells from young and aged C57BL/6 mice into naive (CD44low, CD62Lhigh), central memory (CD44high, CD62Lhigh), and effector memory (CD44high, CD62Llow) subpopulations (Fig. 6A). We then cultured these cells with BALB/c BMDCs and measured IFN-γ in the culture supernatants. Aged naive CD8+ T cells exhibited higher IFN-γ responses to alloactivation than young naive CD8+ T cells (after 48-h stimulation) (Fig. 6B). Central memory and effector memory CD8+ T cells from young and aged mice produced similar quantities of IFN-γ during allostimulation, although effector memory T cells produced lower levels of IFN-γ in this assay (Fig. 6B). Other measures of CD8+ T cell function, including IL-2 production and the upregulation of granzyme B in response to allostimulation, did not differ between the young and aged groups (Supplemental Fig. 3). As these responses were similar, it is unlikely that cell viability was different between young and aged CD8+ T cells in this assay. In support of this, we found the proapoptotic molecule, BIM, which has recently been reported to be similarly expressed on aged and young naive CD8+ T cells (24), to be similarly expressed on aged and young naive CD8+ T cells (data not shown). Finally, we could not detect either IL-4 or IL-17 in the culture supernatants of young or aged CD8+ naive T cells activated with allogeneic dendritic cells. In summary, aging enhances naive, but not memory alloreactive CD8+ T cell IFN-γ responses.

Aged naive CD8+ T cells from nontransplanted mice do not exhibit memory T cell characteristics

Aging is known to lead to an accumulation of memory T cells and a restriction of the naive T cell repertoire. We found that before transplantation, aged mice exhibited a small increase in the numbers of effector memory cells, but not central memory splenic CD8+ T cells (with less naive CD8+ T cells) when compared with young mice (Fig. 7A). One possible explanation as to why naive CD8+ T cells from aged mice exhibited increased IFN-γ alloreactivity is that aged naive CD8+ T cells exhibited more memory T cell features at baseline than young naive CD8+ T cells.
To examine the antidonor memory responses within the CD8+ T cell pool from aged mice, we first enriched CD8+ T cells, containing both memory and naive populations from C57BL/6 young and aged, nontransplanted mice. In addition, as a positive control, we obtained CD8+ T cells from C57BL/6 young mice that had rejected a BALB/c skin graft >6 wk prior, and thus contained antidonor memory CD8+ T cells. CD8+ T cells were then stimulated with irradiated BALB/c spleen cells, and IFN-γ was measured after 3-h stimulation as a readout of an effector memory T cell response (25). We noted that bulk-purified CD8+ T cells from young mice that had previously rejected an allograft exhibited a rapid and robust IFN-γ response (Fig. 7B). In contrast, CD8+ T cells from nontransplanted young or aged cells exhibited a similar low level and inferior response to that observed from the young transplanted mice (Fig. 7B). These data indicate that within the total CD8+ T cell pool, aged nontransplanted mice do not display increased antidonor memory responses when compared with young mice.

Next, we assessed rapid antidonor IFN-γ responses of young and aged naive (i.e., CD44low, CD62Lhigh) CD8+ T cells to specifically determine whether aged naive CD8+ T cells exhibited functional evidence of memory T cell behavior. Naive T cells were purified from the spleens of young and aged nontransplanted mice in addition to those purified from the spleens of young mice that had previously rejected a BALB/c skin allograft >6 wk prior. We also included bulk-purified CD8+ T cells from young transplanted mice, which contained both naive and memory T cells, to serve as a positive control. CD8+ T cells were stimulated with irradiated BALB/c spleen cells, and IFN-γ responses were measured after 12 h of stimulation. This time point was chosen due to practical considerations in purifying and culturing CD8+ T cells (see Materials and Methods). Within this 12-h time frame, memory, but not naive T cells are likely to produce an alloimmune response. Indeed, bulk-purified CD8+ T cells from transplanted young mice that had rejected their allografts >6 wk prior exhibited a strong IFN-γ antidonor response after 12-h stimulation (Fig. 7C). CD44low, CD62Lhigh CD8+ T cells from young transplanted mice also exhibited an anti-BALB/c IFN-γ response, although this was significantly weaker than the response of bulk-purified CD8+ T cells (Fig. 7C). Importantly, CD44low, CD62Lhigh CD8+ T cells from nontransplanted young or aged mice failed to elicit an antidonor IFN-γ response (Fig. 7C). These results indicate that naive (i.e., CD44low, CD62Lhigh) CD8+ T cells from nontransplanted aged mice do not exhibit functional features consistent with memory T cells.

**Aged naive CD8+ T cells do not exhibit enhanced IFN-γ responses when compared with young naive CD8+ T cells in response to anti-CD3 activation**

We next examined whether aged naive CD8+ T cells exhibit altered IFN-γ responses to anti-CD3 stimulation. We found that young and aged naive T cells exhibited similar IFN-γ production in response to CD3 cross-linking (Fig. 8A), indicating that the enhanced IFN-γ by aged naive T cells occurs with Ag allostimulation (Fig. 6B), but not with nonspecific T cell activation.

We next examined the expression of several activation markers on aged and young naive (CD44low, CD62Lhigh) CD8+ T cells in response to either anti-CD3 cross-linking or stimulation with allogeneic BMDCs. With alloactivation, we found that CD25 upregulation was similar between young and aged naive CD8+ T cells (Supplemental Fig. 4). There was also no difference in the upregulation of either CD40L or CD28 between young and aged cells, although the expression above baseline was minimal or not evident, respectively (Supplemental Fig. 4). We noted that aged naive CD8+ T cells exhibited a small impairment in the upregulation of CD27 (Supplemental Fig. 4). We found similar results using anti-CD3 activation (Supplemental Fig. 4), except that with anti-CD3 stimulation there was more obvious upregulation of both

![FIGURE 6. Aging enhances naive CD8+ T cell IFN-γ alloreactivity. A, Representative flow cytometry plot to illustrate the gates used to sort CD8+ T cells into naive, central memory, and effector subpopulations. B, Naive aged CD8+ T cells, but not memory CD8+ T cells, exhibited enhanced IFN-γ alloreactivity compared with young CD8+ T cells. Naive, central memory, and effector memory CD8+ T cells from C57BL/6 mice were stimulated with allogeneic BMDCs, and IFN-γ responses were recorded/time by ELISA. With naive CD8+ T cells, the difference in IFN-γ response at day 2 or 3 of culture was significant, *p < 0.05 (t test). These results are representative of four independent experiments. In each experiment, six to eight mice were used from young and aged mice as a source of CD8+ T cells. CD8+ T cells that were not stimulated with donor cells did not produce IFN-γ (data not shown).](http://www.jimmunol.org/252773323)
CD40L and CD28 on naive CD8+ T cells, and that CD28 upregulation was impaired with aging, consistent with prior reports (26–28). We also found that the expression of the T-bet transcription factor, which has been implicated in the production of IFN-γ by CD8+ T cells, was similarly upregulated in young and aged naive CD8+ T cells upon allostimulation or anti-CD3 activation (Supplemental Fig. 4). Finally, we assessed proliferation of naive aged and young CD8+ T cells using activation with either anti-CD3 or allogeneic BMDCs and found no differences in the proliferative response with aging (Fig. 8B). In sum, aged naive CD8+ T cells exhibit some defects in the upregulation of activation markers in response to anti-CD3 stimulation, but the enhanced IFN-γ that occurs in response to allostimulation is not evident in response to anti-CD3 polyclonal activation.

**Discussion**

Many studies have reported that aging impairs CD8+ T cell function (6, 26, 29, 30), which may explain the decline in cellular immunity often associated with aging (6, 26, 30, 31). CD8+ T cell alterations associated with aging include defective upregulation of activation markers, impaired proliferation in response to mitogens or pathogens, defective IFN-γ responses to viral infection, and an accumulation of memory CD8+ T cells (26, 30, 32). Furthermore, there is a loss of TCR naive T cell repertoire with increasing age, which may be linked to thymic involution and chronic viral infections (33). These prior reports generally employed activating stimuli, for example, mAbs to activate CD3 or CD28 (28) and infection with viruses (29) to assess CD8+ T cell responses. In contrast, we employed agents that induce immune regulation. Using this methodology, we demonstrated that enhanced CD8+ T cell alloreactivity after transplantation and treatment with anti-CD45RB and anti-CD154 reduced the antidonor IFN-γ response in both aged and young transplanted mice, aged CD8+ T cells exhibited greater antidonor IFN-γ responses than young CD8+ T cells (Fig. 9). These results demonstrate that aging leads to enhanced CD8+ antidonor IFN-γ responses after transplantation and treatment with anti-CD45RB and anti-CD154.

**Aged mice exhibit enhanced IFN-γ CD8+ T cell alloreactivity after transplantation and treatment with anti-CD45RB and anti-CD154**

We next assessed CD8+ T cell antidonor IFN-γ responses in vivo, specifically after transplantation and treatment with anti-CD45RB and anti-CD154 in young and aged transplant recipients. After transplantation, but without treatment, aged CD8+ T cells exhibited similar antidonor IFN-γ responses than young aged CD8+ T cells (Fig. 9). Although treatment with anti-CD45RB and anti-CD154 reduced the antidonor IFN-γ response in both aged and young transplanted mice, aged CD8+ T cells exhibited greater antidonor IFN-γ responses than young CD8+ T cells (Fig. 9). These results demonstrate that aging leads to enhanced CD8+ antidonor IFN-γ responses after transplantation and treatment with anti-CD45RB and anti-CD154.

**FIGURE 7.** Aged or young CD44low, CD62Lhigh CD8+ T cells from nontransplanted mice do not exhibit rapid antidonor IFN-γ secretion. A, Prior to transplantation, aged mice exhibited reduced numbers of naive CD8+ T cells with slightly increased effector memory T cell number than young mice. Spleen cells were harvested from nontransplanted young and aged C57BL/6 mice, and absolute numbers of CD8+ T cells were calculated after flow cytometry analysis. n = 6–10 mice per group, pooled data from three independent experiments with consistent results between experiments. *p < 0.01 (t test). B, CD8+ T cells were magnetically enriched from the spleens of nontransplanted young and aged mice and from young mice that had rejected a BALB/c skin transplant >6 wk prior. The cells then were stimulated with irradiated BALB/c spleen cells for 3 h, and IFN-γ was measured by ELISPOT. *p < 0.01 (t test). These results are representative of two independent experiments. In each experiment, three to four mice were used from young and aged mice as a source of CD8+ T cells. CD8+ T cells that were not stimulated with donor cells did not produce IFN-γ (data not shown). C, CD44low, CD62Lhigh CD8+ T cells from nontransplanted aged and young mice or from young mice that had rejected a BALB/c skin graft >6 wk previously. IFN-γ was measured by ELISPOT. *p < 0.01 (t test). These results are representative of two independent experiments. In each experiment, six to eight mice were used from young and aged mice as a source of CD8+ T cells. CD8+ T cells that were not stimulated with donor cells did not produce IFN-γ (data not shown).
T cell responses with aging impair the ability of these agents to prolong allograft survival in an experimental transplant model. We showed that naive, but not memory, CD8+ T cells display elevated IFN-\(\gamma\) responses during allostimulation compared with young naive CD8+ T cells, in vitro. Thus, our results contrast with the current immunosenscent paradigm, which indicates that declining immunity explains age-related phenotypes.

Several studies have demonstrated that memory T cells pose a barrier to transplant tolerance induction (11). Environmental exposures to pathogens, which induce cross-reactive, heterologous memory T cells, may be one mechanism (34). Heterologous CD8+ T cells have been implicated in mediating resistance to costimulatory blockade-induced allograft survival in young recipients (35, 36). Given that aging leads to an accumulation of memory T cells (1), one may predict that these cells may be responsible for the impaired ability of aged recipients for prolonged allograft survival mediated by costimulatory blockade-based therapies.

However, our study found no evidence that aged recipients exhibited enhanced memory CD8+ T cell responses toward allografts. First, CD8+ T cells from aged mice did not exhibit elevated rapid IFN-\(\gamma\) responses prior to transplantation, a characteristic of effector memory T cells (25) (Fig. 7B, 7C). Second, neither aged central memory CD8+ T cells nor effector memory CD8+ T cells exhibited enhanced alloreactivity in vitro compared with young memory CD8+ T cells (Fig. 6B). The weak effector memory response in our assay may reflect that these cells were purified from nontransplanted mice in pathogen-free conditions, and, thus, did not contain clones of donor-reactive T cells. Rather, our results demonstrate that naive CD8+ T cells exhibit enhanced antidonor IFN-\(\gamma\) responses in aged mice. The molecular mechanism as to why naive aged CD8+ T cells exhibit enhanced antidonor IFN-\(\gamma\) responses will require further study. Regardless, current strategies to target memory T cells to improve allograft survival in young recipients may not succeed in older organ transplant recipients.

Our study, which predominantly employed a signal 1 inhibitor between the T cell and APC (anti-CD45RB), with signal 2 blockade (anti-CD154), showed that CD8+ T cells did not impair the ability of this regimen to extend allograft survival in young recipients (35, 36). Given that aging leads to an accumulation of memory T cells (1), one may predict that these cells may be responsible for the impaired ability of aged recipients for prolonged allograft survival mediated by costimulatory blockade-based therapies.

However, our study found no evidence that aged recipients exhibited enhanced memory CD8+ T cell responses toward allografts. First, CD8+ T cells from aged mice did not exhibit elevated rapid IFN-\(\gamma\) responses prior to transplantation, a characteristic of effector memory T cells (25) (Fig. 7B, 7C). Second, neither aged central memory CD8+ T cells nor effector memory CD8+ T cells exhibited enhanced alloreactivity in vitro compared with young memory CD8+ T cells (Fig. 6B). The weak effector memory response in our assay may reflect that these cells were purified from nontransplanted mice in pathogen-free conditions, and, thus, did not contain clones of donor-reactive T cells. Rather, our results demonstrate that naive CD8+ T cells exhibit enhanced alloreactive responses in aged mice. The molecular mechanism as to why naive aged CD8+ T cells exhibit enhanced antidonor IFN-\(\gamma\) responses will require further study. Regardless, current strategies to target memory T cells to improve allograft survival in young recipients may not succeed in older organ transplant recipients.

Our study, which predominantly employed a signal 1 inhibitor between the T cell and APC (anti-CD45RB), with signal 2 blockade (anti-CD154), showed that CD8+ T cells did not impair the ability of this regimen to extend allograft survival in young recipients (Fig. 5B). We administered CD8 depletion up to the first 3 wk posttransplantation. In this time frame, CD8+ T cells only posed a barrier to the efficacy of anti-CD45RB and anti-CD154 in extending allograft survival in aged recipients. Indeed, CD8+ T cell depletion in aged recipients who received costimulatory blockade allowed these recipients to exhibit superior allograft survival compared with young CD8+ T cell-depleted recipient (Fig. 5B). It is possible that extending the duration of CD8 depletion in young recipients would lead to enhancement of the protocol. Other studies in young recipients have indicated that

**FIGURE 8.** Naive T cell IFN-\(\gamma\) responses to nonspecific stimulation via CD3 cross-linking are preserved with aging. A. Aged and young C57BL/6 FAC-sorted naive splenic CD8+ T cells were stimulated with anti-CD3 in the presence of syngeneic APCs, and IFN-\(\gamma\) was measured by ELISA. B. Aged and young C57BL/6 FAC-sorted naive splenic CD8+ T cells were stimulated by either BALB/c BMDCs or anti-CD3 cross-linking. Cell proliferation was measured by thymidine incorporation at indicated time points. Results in A and B are representative of four to five independent experiments. In each experiment, six to eight mice were used from young and aged mice as a source of CD8+ T cells. Nonstimulated cells did not produce IFN-\(\gamma\) or proliferate (data not shown).

**FIGURE 9.** Aged transplant recipients treated with anti-CD45RB and anti-CD154 exhibit elevated CD8+ T cell alloreactivity compared with young recipients. CD8+ T cells were negatively enriched from C57BL/6 mice transplanted with a BALB/c skin allograft, and mice transplanted with a BALB/c skin graft and treated with anti-CD45RB and anti-CD154 mice. CD8+ T cells were then cultured with irradiated donor cells overnight, and IFN-\(\gamma\) was measured by ELISPOT. *p < 0.01, t test. Pooled data from three independent experiments with consistent results.
memory CD8+ T cells mediate the resistance to costimulatory blockade using CTLA4 Ig and anti-CD154, a regimen that inhibits signal 2 between APCs and T cells (35, 36). Differences between our study and earlier reports (36) may be due to either the different regimens employed, differences in basal immune status of young mice, or lack of previous examination in aged recipients. Currently, more people >55 y need organ replacement therapy than ever before. This population is expected to grow as the baby boom generation approaches retirement age (37, 38). Yet, the vast majority of experimental approaches to either induce transplant tolerance or develop adjuvant therapies to improve allograft outcomes and patient morbidities are being tested in young recipients. Our study provides evidence that there are clear fundamental differences in immunological barriers in young compared with aged recipients. Another recent study has also documented that recipient age impairs the ability of anti-CD45RB monotherapy to induce transplant tolerance to cardiac allografts, transplants that are less immunogeneic to skin allografts (39). Our study found that the impaired ability of aged recipients to exhibit extended allograft survival was not limited to anti-CD45RB and anti-CD154 therapy, as we found that aged recipients also resisted the allograft survival was not limited to anti-CD45RB and anti-CD154 stringency allograft models. Proc. Natl. Acad. Sci. USA 102: 13230–13235.


References