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Inhibition of CD8⁺ T cell derived CD40 signals is necessary but not sufficient for Foxp3⁺ iTreg induction in vivo

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Abstract

Current models of CD4⁺ T cell help suggest a major role for CD154 binding to CD40 expressed on DC, with a lesser role for direct T: T interactions via CD40 expressed on CD8⁺ T cells. However, the contribution of CD8⁺ T cell-derived CD40 signals during the donor-reactive T cell response to a transplant has never been studied. Here, we examined the graft rejection kinetics and CD4⁺ and CD8⁺ donor-reactive T cell responses under conditions where CD40 was genetically ablated only on antigen presenting cells, and under conditions where CD40 was genetically ablated only on donor-reactive CD8⁺ T cells. Our results revealed a significant role for CD8⁺ T cell-expressed CD40 in the augmentation of donor-reactive CD8⁺ T cell responses following transplantation, and showed that CD40 expressed on CD8⁺ T cells must be inhibited to allow conversion of CD4⁺ T cells into iTreg. Thus, this study identifies a major role of CD8⁺ T cell-derived CD40 signals as a critical switch factor which both promotes optimal differentiation of cytokine-producing CD8⁺ effector T cell responses, and inhibits the differentiation of antigen-specific Foxp3⁺ iTreg *in vivo*.

Introduction

The licensing model of CD4⁺ T cell help suggests that the major role of CD154/CD40 interactions in the immune system involves the binding of CD154 expressed on activated CD4⁺ T cells to CD40 expressed on DC (1-3). CD40 signaling results in maturation of DC and resultant upregulation of costimulatory molecules and cytokines, which provide signals 2 and 3, respectively, for the optimal priming and differentiation of antigen-specific CD8⁺ T cell responses (4). Direct T:T interactions are thought to play a lesser role in the provision of CD4⁺ T cell help to CD8⁺ T cells (5). However, it is well known that CD40 can be expressed on a considerable fraction of activated CD8⁺ T cells during the T cell response to antigen (6-8). Previous studies have examined the role of CD40 expressed by CD8⁺ T cells following activation with nominal antigens and during the course of pathogen infection. While a 2002 report examining H-Y specific CD8⁺ T cell responses concluded that CD40 expressed on CD8⁺ T cells was fundamental for CD8⁺ T cell memory generation and thus that CD8⁺ T cells receive CD4 help directly through CD40 ligation (9), subsequent studies failed to detect a role for intrinsic CD40 signals in the effector or memory phases of the CD8⁺ T cell response (10-12). However, the requirement of CD8⁺ T cell-intrinsic CD40 signals during the donor-reactive T cell response to a transplant have never been studied.

The role of T cell intrinsic CD40 signals during transplantation are particularly relevant because blockade of the CD40/CD154 pathway is a potential therapeutic target for the prophylaxis of immune-mediated transplant rejection (13). Indeed, blockade of this pathway

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remains one of the most effective means of inducing long-term graft survival and in some cases even tolerance (4). However, translation of this approach to clinical transplantation has been stymied by the observation that administration of CD154-specific monoclonal antibodies resulted in thromboembolism in an Fc γ RI-dependent manner (14). Thus, understanding the cellular and molecular pathways at play in the observed alloimmune hypo-responsiveness following CD154 antagonism are critical in the future development of therapeutic blockade of this pathway. A 2003 report suggested that the ability of anti-CD154 mAbs to prolong graft survival was dependent on Fc γ RI and complement component C3, invoking the idea of complement-mediated depletion of CD154-expressing antigen-reactive T cells (15). A subsequent study instead demonstrated that anti-CD40 blocking mAbs functions similarly to anti-CD154 mAbs, suggesting that the major mechanism of action of these reagents involves blocking the CD40/CD154 interaction, and not antibody-mediated depletion of activated antigen-specific CD4⁺ T cells (16). However, the relative importance of CD40 blockade on APC vs. blockade on T cells is unknown.

We and others have demonstrated that one of the key processes underlying the efficacy of CD154/CD40 blockade *in vivo* is the generation of induced CD4⁺ Foxp3⁺ Treg (iTreg) (17, 18). Under conditions of CD154 antagonism, CD4⁺ Foxp3⁺ iTreg differentiate from Foxp3⁻ precursors, a process which is mediated by plasmacytoid dendritic cells (18). Foxp3⁺ iTreg are known to be potent suppressors of cell-mediated immunity *in vivo*, and may be involved in the maintenance of long-term allograft survival in the face of inflammatory stimuli such as injury and infection (19). The factors governing differentiation of naïve CD4⁺ T cells into iTreg have been the subject of many studies revealing that high ambient concentrations of TGF- β in the absence of IL-6 results in opening of the Foxp3 locus and conversion into a regulatory T cell (20-22). Thus, while recent work has shown pharmacologic CD154 antagonism results in differentiation of Foxp3⁺ iTreg (17, 18), the precise CD40/CD154 interactions that must be blocked in order to generate cells of this phenotype are completely unknown. Here, we sought to determine whether blockade of DC-derived CD40 signals or CD8⁺ T cell-derived CD40 signals are necessary and/or sufficient for the peripheral induction of CD4⁺ Foxp3⁺ iTreg.

Using a surrogate minor antigen mismatch skin transplant model (23, 24), we examined the graft rejection kinetics and CD4⁺ and CD8⁺ donor-reactive T cell responses under conditions where CD40 was genetically ablated only on antigen presenting cells (of both donor and host origin), and under conditions where CD40 was genetically ablated only on donor-reactive CD8⁺ T cells. Our results revealed a significant role for CD8⁺ T cell intrinsic CD40 in the augmentation of donor-reactive CD8⁺ T cell responses following transplantation, and showed that CD40 expressed on CD8⁺ T cells must be inhibited to allow conversion of CD4⁺ T cells into iTreg. Thus, this study identifies a major role of CD8⁺ T cell-derived CD40 signals as a critical switch factor which both promotes optimal differentiation of cytokine-producing CD8⁺ effector T cell responses, and inhibits the differentiation of antigen-specific Foxp3⁺ iTreg *in vivo*.

Materials and Methods

Mice

C57BL/6 (H-2^b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (25) and OT-II (26) transgenic mice, purchased from Taconic Farms (Germantown, NY), were bred to Thy1.1⁺ background at Emory University. mOVA mice (C57BL/6 background, H-2^b) (23) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). CD40^{-/-} animals on a B6 background were obtained from Jackson Labs (Bar Harbor, ME), and were bred onto mOVA and OT-IxThy1.1 backgrounds at Emory University. All animals were maintained in accordance with Emory University Institutional

Animal Case and Use Committee guidelines (Atlanta, GA). All animals were housed in pathogen-free animal facilities at Emory University.

Donor-Reactive T Cell Adoptive Transfers and DST Administration

For adoptive transfers of donor-reactive T cells, spleen and mesenteric LNs isolated from Thy1.1⁺ OT-I and Thy1.1⁺ OT-II mice were processed and stained with monoclonal antibodies for CD4 and CD8 (both from Invitrogen), Thy1.1, and Vα2 (BD Pharmingen) for flow cytometry analysis. Cells were resuspended in PBS and 1.5×10^6 of each Thy1.1⁺ OT-I and OT-II were injected i.v. 24–48 h prior to skin transplantation. For DST administration, mOVA spleens were processed into single cell suspensions, and 10^7 splenocytes were injected i.v. on the day of skin transplantation.

Skin Transplantation and In Vivo CD154 Blockade

Full thickness tail and ear skins were transplanted onto dorsal thorax of recipient mice and secured with adhesive bandages as previously described (27). Where indicated, mice were treated with 500 μg hamster monoclonal anti-mouse CD154 (MR-1, BioExpress, West Lebanon, NJ) on days 0, 2, 4, and 6 post transplantation.

Flow Cytometry and Intracellular Cytokine Staining

Spleens or draining axillary and brachial LNs were stained for CD4 and CD8 (both from Invitrogen) and Thy1.1 (BD Pharmingen). Samples were analyzed on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (Treestar, San Carlos, CA). For ICCS, responder splenocytes were stimulated with 10nM OVA₂₅₇₋₂₆₄ (SIINFEKL) (Genscript Inc), in the presence of 10 μg/mL Brefeldin A for 4 hours. Intracellular staining kit was used to detect TNF and IFN-γ (all from BD Pharmingen), according to manufacturer's instructions.

T_{reg} Staining

Splenocytes and draining lymph nodes were harvested at day 10 post transplantation, processed and stained with antibodies to CD4 and CD8 (both from Invitrogen), Thy1.1, Helios, and CD25 (all from BD Pharmingen). Intracellular staining with anti-Foxp3 was performed using intra-nuclear staining kit (eBiosciences), according to manufacturer's instructions.

In Vitro T cell Stimulation Assay

Splenocytes from OT-I × Thy1.1 or OT-II × Thy1.1 animals (1×10^6 /well in a 96 well plate) were incubated in vitro in complete media with cognate peptide antigen (OVA 257–264 and OVA323–339, respectively (Genscript, Inc.) for 4 d. Culture media consisted of RPMI medium 1640 supplemented with 10% FBS (Mediatech, Herndon, VA), 2mM L-glutamine, 0.01 M Hepes buffer, 100 μg/ml gentamycin (Mediatech), and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Cultures without peptide antigen served as negative controls. Cells were then harvested and CD11c⁺ DC, CD4⁺ Thy1.1⁺ T cells, and CD8⁺ Thy1.1⁺ T cells were then stained for surface expression of CD40.

LM-OVA infection

Listeria monocytogenes containing full-length OVA insert with streptomycin-resistance (LM-OVA) was made by H. Shen (28). 5 mls BHI broth (Teknova) supplemented with 50 μg/ml streptomycin was inoculated with LM-OVA and incubated overnight at 37° C. Mice were infected with 10^4 colony-forming units (CFU) of LM-OVA intraperitoneally in 500 μl of sterile PBS on day 0.

Statistical Analysis

Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. For analysis of T cell responses, one-way ANOVA tests were performed, followed by Bonferroni post-test on significant results. All analyses were done using GraphPad Prism software (GraphPad Software Inc).

Results

Genetic deficiency of CD40 on both donor and recipient derived APCs fails to recapitulate the effects of CD154 blockade

Current dogma suggests that engagement of the CD154/CD40 pathway contributes to enhancement of cellular immune responses via interaction of CD154 expressed on activated antigen-specific CD4⁺ T cells with CD40 expressed on DC. CD40 signaling into DC results in licensing of the APC, resulting in upregulation of CD80, CD86, and other costimulatory molecules for the optimal stimulation of CD8⁺ antigen-specific T cell responses (4, 29). To ascertain whether this mechanism is at play in the generation of donor-reactive T cell responses during transplantation, and is thus an important pathway that is blocked following CD154 antagonism, we asked whether genetic deficiency of CD40 on both donor- and recipient-derived APCs recapitulates the effects of CD154. We utilized a surrogate minor antigen mismatch model in which the donor and recipient differed by a single protein antigen, membrane-bound chicken ovalbumin ubiquitously expressed under the β -actin promoter. In this model, OVA-specific CD4⁺ and CD8⁺ T cells are adoptively transferred into naïve B6 hosts, which then receive an OVA-expressing skin graft under the cover of donor-specific transfusion (DST) and CD154 antagonism (Figure 1A). While recipients treated with DST alone reject their grafts with a median survival time (MST) of 14 days, those treated with CD154 blockade exhibit long-term graft survival with an MST of > 100 days (Figure 1B)(17). To interrogate the impact of loss of CD40 on antigen presenting cells, wild-type (WT) donor-reactive CD4⁺ and CD8⁺ T cells were adoptively transferred into CD40^{-/-} hosts that subsequently received a CD40^{-/-} OVA-expressing skin graft and CD40^{-/-} OVA-expressing DST (Figure 1A). Strikingly, these experiments revealed that CD40 deficiency on antigen presenting cells alone resulted in a significant, but not complete, prolongation in graft survival (MST 20 days, Figure 1B). Importantly, CD40 deficiency on all APCs (donor and host) failed to recapitulate the effects of treatment with anti-CD154 mAb.

We further assessed the donor-reactive T cell response in these recipients at day 10 post-transplantation, which is the peak of the response (24). We found that genetic deficiency in CD40 only on APC resulted in a profound diminution in the donor-reactive CD4⁺ T cell response (Figure 1C, D), but failed to attenuate the antigen-specific CD8⁺ T cell response both in terms of frequency and absolute numbers (Figure 1E, F). In contrast, pharmacologic CD154 antagonism significantly attenuated both CD4⁺ and CD8⁺ donor-reactive T cell responses. Furthermore, while CD154 antagonism resulted in a significant increase in both the frequency and absolute number of antigen-specific CD4⁺ CD25⁺ Foxp3⁺ iTreg, CD40 deficiency on APCs alone failed to recapitulate this increase in Foxp3⁺ iTreg (Figure 1G, H). Taken together, these data suggested two possibilities: 1) that complete loss of CD40 signaling does not mimic the effect of CD154 antagonism, or 2) that CD40 expressed on some other cell type was contributing to the increased rejection observed in these animals as compared to those treated with CD154 blockade. We proceeded to test these two possibilities in turn.

CD40 is upregulated on donor-reactive CD8⁺ T cells activated following activation

A 2003 report suggested that the mechanism by which anti-CD154 mAbs mediate attenuation of graft-reactive T cells and result in prolongation of graft survival is through complement and FcR-dependent antibody-mediated depletion of CD154⁺ T cells (15). Thus, one explanation of the results presented in Figure 1 might be that complete loss of CD40 signaling would not mimic the results of anti-CD154 due to the absence of antibody-mediated depletion of CD154-expressing cells. Thus, to determine whether complete loss of CD40 would mimic the effect of anti-CD154 monoclonal antibody treatment, we performed skin graft experiments in which CD40^{-/-} OVA-expressing skin grafts were transplanted onto CD40^{-/-} recipients (Figure 2A). In these experiments no exogenous T cells were adoptively transferred, thus the both the CD4⁺ and CD8⁺ donor-reactive T cell responses were also CD40^{-/-}. Results indicated that equivalent rejection kinetics were observed in CD40^{-/-} recipients of CD40^{-/-} grafts and wild-type animals treated with anti-CD154 (Figure 2B), again suggesting that the primary effect of anti-CD154 mAbs is to block CD154/CD40 interaction, not to mediate antibody-dependent depletion of CD154-expressing T cells. These results, coupled with the findings from Figure 1 showing that complete CD40 deficiency mimicked the effect of anti-CD54 mAbs but that CD40 deficiency only on APC failed to do so, suggested that in these recipients another cell type that expresses CD40 may be contributing to the increased graft rejection. Evidence in models of pathogen infection and autoimmunity has suggested that CD40 may be expressed on activated T cells (6-8). In order to determine whether CD40 was expressed on activated CD4⁺ or CD8⁺ T cells in our model, we stimulated donor-reactive CD4⁺ or CD8⁺ T cells in vitro for 4 days in the presence or absence of cognate antigen before assessing the surface expression of CD40. Results indicated that positive control CD11c⁺ DC exhibited a high degree of CD40 expression at baseline which was upregulated on activated DCs, and that antigen-specific CD4⁺ T cells failed to upregulate CD40 following activation (Figure 2C, 2D). Importantly, while unstimulated CD8⁺ T cells exhibited a low degree of background CD40 expression, activation resulted in the upregulation of CD40 on about 50% of antigen-reactive T cells (Figure 2C, 2D). This result was validated by the absence of positive staining in activated CD40^{-/-} CD8⁺ T cells (data not shown).

In order to confirm these findings in an in vivo model, we adoptively transferred WT Thy1.1⁺ OVA-specific CD4⁺ and CD8⁺ T cells into naïve WT recipients that then received an OVA-expressing skin graft and no further treatment (Figure 3A). On day 10 post-transplant graft draining LN were harvested and CD40 expression was assessed on activated graft-specific Thy1.1⁺ CD8⁺ T cells (Figure 3B). Results revealed that in contrast to naïve CD8⁺ T cells, activated antigen-specific CD8⁺ T cells exhibited an upregulation in CD40 expression (Figure 3C). Approximately 40% of activated donor-reactive CD8⁺ T cells were CD40⁺, as compared to ~8% of endogenous CD8⁺ T cells in grafted animals and <1% of CD8⁺ T cells in naïve animals (Figure 3D). These data demonstrate that within the context of transplantation, a large population of donor-reactive CD8⁺ T cells expresses CD40 in vivo.

CD40 functions in a T cell intrinsic manner to augment donor-reactive CD8⁺ T cell responses and inhibit Foxp3⁺ iTreg conversion in CD4⁺ T cells

Based on the findings that loss of CD40 on APC alone failed to recapitulate the effect of CD154 antagonism (Figure 1), and that CD40 is expressed on activated donor-reactive T cells (Figures 2 and 3), we hypothesized that CD40 expressed on activated donor-reactive T cells could contribute to the enhancement of the donor-reactive CD8⁺ T cell response. To test this, we established a system in which CD40 was deficient only on the donor-reactive CD8⁺ T cells responding to the graft (Figure 4A). Remarkably, we found that graft survival was significantly prolonged in animals that received CD40^{-/-} donor-reactive CD8⁺ T cells

as compared to WT donor-reactive CD8⁺ T cells (MST of 26 d vs. 15.5 d, respectively, Figure 3B). Furthermore, while the donor-reactive CD4⁺ T cell responses were not inhibited in mice that received CD40^{-/-} CD8⁺ T cells as compared to WT CD8⁺ T cells (Figure 4C, 4D), the donor-reactive CD8⁺ T cell response was significantly attenuated in terms of both frequency and absolute number in recipients of CD40^{-/-} CD8⁺ T cells relative to recipients of WT CD8⁺ T cells (Figure 4E, 4F). These results suggesting that in the setting of transplantation, CD40 functions in a cell intrinsic manner to augment donor-reactive CD8⁺ T cell responses.

Functional analysis of donor-reactive CD8⁺ T cell responses revealed that loss of CD40 signaling on CD8⁺ T cells functioned in a cell-intrinsic manner to reduce the frequency of IFN- γ ⁺ TNF⁺ double cytokine producers to a degree comparable to that observed following CD154 blockade (Figure 4G, 4H). However, while antigen-specific CD4⁺ CD25⁺ Foxp3⁺ iTreg were increased in recipients treated with CD154 blockade, our analysis showed that loss of CD40 signaling on donor-reactive CD8⁺ T cells failed to result in an increase in these cells. Taken together, these results suggest that CD40 on CD8⁺ T cells functions in a cell-intrinsic manner to augment both the magnitude and functionality of the donor-reactive CD8⁺ T cell response, but that loss of CD40 on CD8⁺ T cells alone is insufficient to promote Foxp3⁺ iTreg conversion in donor-reactive CD4⁺ T cells.

OVA-specific CD8⁺ T cells can dispense with T cell-intrinsic CD40 signals in the context of pathogen infection

The above results demonstrating that T cell intrinsic CD40 signals are required for optimal donor-reactive CD8⁺ T cell responses are in contrast to what has been previously observed in models of pathogen-specific CD8⁺ T cell responses (10). Thus, in order to determine whether these discrepancies are due to fundamental differences pathogen- vs. graft-specific CD8⁺ T cell responses, or to other differences in the systems (i.e. T cell specificity, affinity, etc.), we assessed the ability of CD40^{-/-} OT-I T cells to respond in the setting of infection with *Listeria monocytogenes*. WT or CD40^{-/-} CD8⁺ OVA-specific OT-I T cells were adoptively transferred into naïve animals that were then infected with an OVA-expressing *Listeria* (Figure 5A). Results showed that in contrast to the diminished response we observed when CD40^{-/-} OT-I responded in the context of a transplant, the magnitude of CD40^{-/-} OT-I response in the context of a pathogen infection was indistinguishable from the WT OT-I response (Figure 5B, 5C). Similarly, the ability of CD40^{-/-} OT-I to produce IFN- γ and TNF following ex vivo restimulation was identical to that of WT OT-I (Figure 5D, 5E). Thus, these data confirm previous findings in other infection models demonstrating that in the context of infection (10), CD40 expression on CD8⁺ T cells is dispensable for generation of optimal CD8⁺ T cell responses. This is in stark contrast to our results in a model of transplantation (Figure 4), in which CD8⁺ T cell-intrinsic CD40 signals are required for optimal expansion and effector function of donor-reactive CD8⁺ T cell responses.

Loss of CD40 signaling on both donor-reactive CD8⁺ T cells and APC synergize to promote Foxp3⁺ iTreg induction

Results from Figure 1 demonstrated that Foxp3⁺ iTreg failed to be induced when CD40 was absent from all sources of APCs, and results from Figure 4 revealed that Foxp3⁺ iTreg similarly failed to be induced when CD40 was absent from antigen-specific CD8⁺ T cell responses. Thus, we next asked if iTreg are induced when CD40 is ablated both on APC and CD8⁺ T cells. In order to accomplish this, we conducted similar experiments in which CD40 was genetically ablated on all sources of APCs (the recipient, the graft, and the DST), and also on the donor-reactive CD8⁺ T cells (Figure 6A). Donor-reactive CD4⁺ T cells in these experiments were WT. Loss of CD40 on both donor-reactive CD8⁺ T cells and APC resulted

in significant attenuation of both CD4⁺ (Figure 6B) and CD8⁺ (Figure 6C) donor-reactive T cell responses, to a level similar to that observed following treatment with anti-CD154 mAbs. Importantly, we found that loss of CD40 signaling on both APC and CD8⁺ T cells synergized in increasing the frequency of Foxp3⁺ iTreg induced following transplantation (Figure 6D, 6E). These Foxp3⁺ iTreg were also uniformly Helios⁺ (Figure 6D), likely signifying recent encounter with cognate antigen (30). These data suggest that CD8⁺ T cell-derived CD40 is sufficient to inhibit the peripheral generation of graft-specific Foxp3⁺ iTreg, and thus, that blockade of CD8⁺ T cell-intrinsic CD40 signals is a critical switch factor in determining the programmed differentiation of naïve CD4⁺ T cells into effector T cells vs. Treg.

Discussion

The observations presented here define the role of CD8⁺ T cell-expressed CD40 in the augmentation of effector alloimmune responses and the attenuation of regulatory T cell responses following transplantation. Previous studies showed that CD8⁺ T cell-derived CD40 signals were critical for the generation of optimal memory T cell responses when male cells were injected into female recipients (9), however three additional studies in models of pathogen infection and our data presented here (Figure 5) revealed no role for CD8⁺ T cell derived CD40 signals in either the generation or maintenance of effector and memory CD8⁺ T cell responses (10-12). Our results reveal that in the setting of transplantation, CD8⁺ T cell derived CD40 signals are critical to drive optimal expansion and differentiation of donor-reactive CD8⁺ T cell responses. These results are consistent with the recent work of Tanchot and colleagues, who found that CD40 expressed on CD8⁺ T cells played a critical role in the differentiation of CD8⁺ memory T cell responses in a GVHD-like model (31). We speculate that the discrepancy between our findings and those of the studies of pathogen-specific CD40^{-/-} CD8⁺ T cell responses is a result of the fact that CD8⁺ T cell derived CD40 signals may be dispensable when there are sufficient pathogen-associated molecular pattern-mediated signals to independently drive CD8⁺ T cell responses in the absence of CD40 signals. The fact that graft-specific CD8⁺ T cell responses appear to require intrinsic CD40 signals for optimal differentiation while pathogen-specific CD8⁺ T cells obviate the requirement for intrinsic CD40 signals suggests that there may be a therapeutic window in which blockade of CD40 only on CD8⁺ T cells may significantly attenuate graft-specific T cell responses while leaving pathogen-specific protective immune responses intact.

A significant component of the long term graft survival/ donor-specific tolerance that is observed following antagonism of the CD154 pathway *in vivo* stems from the generation of donor-reactive CD4⁺ Foxp3⁺ Treg under these conditions. Studies from Bromberg and colleagues have shown that the DC generated in the absence of CD154/CD40 signals were responsible for transmitting this tolerogenic phenotype and programming iTreg induction (18). Our results showed increased antigen-specific iTreg in the setting of CD40 deficient APC only under conditions in which the activated CD8⁺ T cell response was also deficient for CD40. These suggest that a concomitant activated CD8⁺ T cell response can prevent the tolerogenic status of DC in which CD40 signaling is absent. Instead, our results support a model in which CD40 signals from either the CD8⁺ T cell or the APC can result in the programmed differentiation of naïve Th cells into effectors instead of regulators.

As shown in Figure 6, the peripherally induced iTreg generated following CD40 deletion or CD154 antagonism also express high levels of the Ikaros- family transcription factor Helios (32). Expression of Helios in Foxp3⁺ Treg was first purported to represent thymically-derived natural Treg (nTreg) as opposed to peripherally-induced iTreg that were generated following administration of oral antigen (33). We have previously confirmed, using OT-II ×

RAG^{-/-} naïve T cells that contain absolutely no pre-existing nTreg, that the emergence of antigen-specific Thy1.1⁺ Foxp3⁺ T cells in this system represents the peripheral conversion of Foxp3⁻ naïve T cells into Foxp3⁺ regulatory T cells, and not the preferential expansion of thymically-derived nTreg (17). Thus, the fact that these antigen-specific iTreg observed following CD154/CD40 blockade express high levels of Helios fails to support the contention that Helios is uniformly a marker of nTreg. These findings are corroborated by more recent studies examining iTreg induced in a variety of experimental models (30, 34). These studies have suggested that the observation of Helios expression in iTreg may signify more recent encounter with antigen, as continued antigen-dependent stimulation was found to be required for maintenance of Helios expression in vivo. Our results therefore demonstrate that iTreg that arise under conditions in which CD40 signaling is absent may differ in their stimulation history as compared to those generated via other mechanisms such as oral tolerance. Elucidation of the mechanisms of regulation of Helios expression and functionality of Helios⁺ vs. Helios-iTreg therefore remains of high interest.

The nature of the CD8⁺ T cell-derived signals that function to inhibit iTreg induction is currently unknown. However, data from our recently published studies suggest that the primary function of anti-CD154 monoclonal antibodies on DC is to inhibit inflammatory cytokine release, as treatment with these antibodies has no effect on the CD40-driven maturation associated increase in class I, class II, or costimulatory molecule expression on DC (29). Thus, we speculate that it is the decreased secretion of inflammatory cytokines from both DC and antigen-specific CD8⁺ T cells that is responsible for the programmed differentiation of naïve antigen-specific CD4⁺ T cells into iTreg. Given the pivotal role for inflammatory cytokines in preventing the differentiation of naïve CD4⁺ T cells into Foxp3⁺ Tregs (20), we speculate that CD8⁺ T cell-derived cytokines may be involved in the programmed differentiation of Teff vs. Treg; this remains an important area of investigation.

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Figure 1. Genetic deficiency of CD40 on both donor and recipient derived APCs fails to recapitulate the effects of CD154 blockade

A, 1.5×10^6 WT OVA-specific CD4⁺ and CD8⁺ T cells were adoptively transferred into naïve WT or CD40^{-/-} hosts, which then received a WT or CD40^{-/-} OVA-expressing skin graft under the cover of WT or CD40^{-/-} DST. WT recipients received WT graft and DST, while CD40^{-/-} recipients received CD40^{-/-} DST. Additional controls included WT recipients that were treated with anti-CD154. B, Skin graft survival data. Median survival times (MSTs) were 14, 100, and 20 days in untreated, anti-CD154-treated, and CD40^{-/-} recipients, respectively. No Rx = WT animals that have received a skin graft and DST but no other treatment or manipulation of the CD40 pathway. C-D, Representative examples and summary data of frequency and absolute numbers (per spleen) of donor-reactive CD4⁺ T cells at day 10 post-transplant. E-F, Representative examples and summary data of frequency and absolute numbers (per spleen) of donor-reactive CD8⁺ T cells at day 10 post-transplant. G-H, Representative examples and summary data of frequency and absolute numbers (per spleen) of donor-reactive CD4⁺ Foxp3⁺ iTreg at day 10 post-transplant. Data shown are cumulative from four independent experiments, with a total of 12 animals per group. *p<0.05.

Figure 2. CD40 is upregulated on donor-reactive CD8⁺ T cells activated following activation
A, B. CD40^{-/-} hosts received CD40^{-/-} OVA-expressing skin grafts in the absence of any T cell adoptive transfer. Untreated WT animals receiving WT OVA-expressing skin grafts served as negative controls and anti-CD154 treated WT recipients of WT OVA-expressing skin grafts served as positive controls (A). While untreated recipients rejected their grafts with an MST of 16 d, CD40^{-/-} recipients and those treated with anti-CD154 experienced increased graft survival that was comparable between the two groups (B). C, D. Splenocytes from OT-I × Thy1.1 or OT-II × Thy1.1 animals were incubated in vitro with cognate peptide antigen (OVA 257-264 and OVA323-339, respectively) for 4 d in vitro. Cultures without peptide antigen served as negative controls. Cells were then harvested and CD11c⁺ DC, CD4⁺ Thy1.1⁺ T cells, and CD8⁺ Thy1.1⁺ T cells were then stained for surface expression of CD40 or were stained with an isotype control. C, Representative histograms of CD40 staining (black histograms) or isotype control (grey shaded histograms) on DCs, CD4⁺ T cells, and CD8⁺ T cells from both unstimulated and peptide stimulated cultures. D, Summary data from three independent experiments. *p<0.05.

Figure 3. CD40 is upregulated on donor-reactive CD8⁺ T cells in vivo

1.5×10^6 WT Thy1.1⁺ OVA-specific CD4⁺ and CD8⁺ T cells were adoptively transferred into naïve WT recipients that then received an OVA-expressing skin graft and no further treatment (A). DLN were harvested on day 10 post-transplant (B) and CD40 expression was assessed on activated graft-specific Thy1.1⁺ CD8⁺ T cells. C, Representative histograms of CD40 staining (black histograms) or isotype control (grey shaded histograms) on naïve endogenous CD8⁺ T cells isolated from naïve B6 animals, endogenous Thy1.1⁺ CD8⁺ T cells isolated from grafted animals, and Thy1.1⁺ CD8⁺ donor-reactive T cells isolated from grafted animals. D, Summary data of frequencies of CD40⁺ cells among CD8⁺ T cells within the three groups. Data are cumulative of two independent experiments (n=8/group).

Figure 4. CD40 functions in a T cell intrinsic manner to enhance donor-reactive CD8⁺ T cell responses and inhibit Foxp3⁺ iTreg conversion

A, 1.5×10^6 WT OVA-specific CD4⁺ and 1.5×10^6 WT OVA-specific CD8⁺ or 1.5×10^6 CD40^{-/-} OVA-specific CD8⁺ T cells were adoptively transferred into naïve WT hosts, which then received a WT OVA-expressing skin graft under the cover of WT DST. Additional controls included WT recipients that were treated with anti-CD154. B, Skin graft survival data. MSTs were 15.5 days in WT controls vs. 26 days in recipients of CD40^{-/-} T cells. No Rx = WT animals that have received a skin graft and DST but no other treatment or manipulation of the CD40 pathway. C-D, Representative examples and summary data of frequency and absolute numbers (per spleen) of donor-reactive WT CD4⁺ T cells at day 10 post-transplant in recipients containing either WT or CD40^{-/-} T cells. E-F, Representative examples and summary data of frequency and absolute numbers (per spleen) of donor-reactive CD8⁺ T cells at day 10 post-transplant. G-H, Representative examples and summary data of frequency of donor-reactive CD8⁺ cytokine producing cells at day 10 post-transplant. I-J, Representative examples and summary data of frequency of donor-reactive CD4⁺ Foxp3⁺ iTreg at day 10 post-transplant. Data shown are cumulative from three independent experiments, with a total of 9 animals per group. *p<0.05.

Figure 5. OVA-specific CD8⁺ T cells can dispense with T cell-intrinsic CD40 signals in the context of pathogen infection

WT or CD40^{-/-} Thy1.1⁺ CD8⁺ OVA-specific OT-I T cells (10⁶) were adoptively transferred into naïve animals that were then infected with an OVA-expressing *Listeria* (A). Animals were sacrificed on day 10 and OVA-specific Thy1.1⁺ CD8⁺ T cell responses were analyzed in the spleen. Representative flow plots (B) and summary data (C) of frequencies of WT vs. CD40^{-/-} OVA-specific Thy1.1⁺ T cells. Representative flow plots (D) and summary data (E) of frequencies of IFN-γ⁺ TNF⁺ producing cells in either WT or CD40^{-/-} OT-I populations following ex vivo restimulation. Data shown are cumulative from two independent experiments (n=10/group).

Figure 6. Loss of CD40 signaling on both donor-reactive CD8⁺ T cells and APC synergize to promote Foxp3⁺ iTreg induction

A, 1.5×10^6 WT OVA-specific CD4⁺ and 1.5×10^6 CD40^{-/-} CD8⁺ T cells were adoptively transferred into naïve CD40^{-/-} hosts, which then received a CD40^{-/-} OVA-expressing skin graft under the cover of CD40^{-/-} DST. Negative controls were WT recipients that received WT graft and DST. Positive controls were WT recipients that were treated with anti-CD154. No Rx = WT animals that have received a skin graft and DST but no other treatment or manipulation of the CD40 pathway. B, Analysis of frequency and absolute numbers (per spleen) of donor-reactive CD4⁺ T cells at day 10 post-transplant. C, Analysis of frequency and absolute numbers (per spleen) of donor-reactive CD8⁺ T cells at day 10 post-transplant. D-E, Representative examples and summary data of frequency and absolute numbers (per spleen) of donor-reactive CD4⁺ Foxp3⁺ iTreg at day 10 post-transplant. Data shown are cumulative from two independent experiments, with a total of 6 animals per group. *p<0.05.