Efficacy and limitations of combined A2.CAR Treg and anti-CD154 therapy in a mouse model of haplo-mismatched heart transplantation

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Abbreviations:

APC: Antigen presenting cells

Acpt: Accept

CAR: Chimeric antigen receptor

FoxP3: Forkhead box protein 3

GC: Germinal center

HTx: Heart transplantation

MFI: Median fluorescence intensity

Rej: Reject

SLOs: Secondary lymphoid organs

Tregs: Regulatory T cells

Abstract:

The ability to induce allograft specific tolerance would reduce the need for daily pharmacological immunosuppression, improve post-transplant quality of life and transplant outcomes. Adoptive cell therapy with regulatory T cells expressing donor-specific Chimeric Antigen Receptors (CAR-Tregs) is a promising strategy, but monotherapy has not resulted in prolonged survival of grafts with multiple MHC mismatches. We used a clinically-relevant, haplo-mismatched model of heart transplantation in immune-competent C57BL/6 recipients to test the ability of HLA-A2-specific (A2) CAR Tregs to synergize with CD154 blockade to enhance graft survival. We found that in combination with a single low dose of anti-CD154, A2.CAR Tregs significantly prolonged heart allograft survival. Through use of grafts expressing the 2W-OVA transgene, tetramer tracking of 2W- and OVA-specific cells revealed that in mice with accepted grafts, the effects of A2.CAR Tregs included inhibition of endogenous donor-specific $CD4^+$ and $CD8^+$ T cell expansion, and B cell and antibody responses. Moreover, within the 2W-specific $CD4^+$ T cell population, there was a significant increase in the proportion of $FoxP3^{pos}$ cells, suggestive of infectious tolerance. In mice where CD154 blockade and A2.CAR Tregs failed to prolong graft survival, there was preferential expansion of $FoxP3^{neg}$ A2.CAR T cells within the rejecting allograft. Thus, this study provides the first evidence for a synergistic effect between CAR Tregs and CD40-pathway blockade and supports the further refinement of this strategy as a promising future direction towards the goal of transplantation tolerance.

1. Introduction

Finding ways to induce allograft specific tolerance would reduce the need for daily pharmacological immunosuppression and its associated side effects, as well as improving long-term allograft survival. One way to induce tolerance is by combined transplantation of allogeneic organs with hematopoietic stem cells, which has resulted in some success in the clinic, but limitations of the conditioning regimen intensity and risk of graft versus host disease limit its broad applicability[1-3]. Another strategy to induce transplantation tolerance is to harness the natural immunosuppressive properties of forkhead box protein P3 (FoxP3)-expressing regulatory T cells (Tregs) through adoptive cell therapy [2,3]. Building upon evidence in mice that infusion of polyclonal, expanded Tregs can induce transplantation tolerance [4 5 6], several Phase 1 trials have tested the effects of infusing polyclonal Tregs in solid organ or stem cell transplantation. These studies have uniformly shown that the approach is feasible, safe, and may allow a reduction of pharmacological immunosuppression[6-9].

One limitation of cell therapy with polyclonal Tregs is that only a small proportion are alloantigen specific. Work in pre-clinical models of transplantation has shown that antigen-specific Tregs are significantly more effective than are polyclonal cells [10-22]. Alloantigen-specific Tregs can be generated by repetitive stimulation with donor-derived antigen-presenting cells (APCs) [11-14] or donor-antigen specificity can be introduced into Tregs by the expression of Chimeric Antigen Receptors (CARs) [7,15]. Several groups expressed HLA-A2 specific CARs (A2.CARs) in human Tregs and showed that they have superior therapeutic effects over polyclonal Tregs in humanized mouse models of graft versus host disease or skin transplantation [16,17], leading to rapid clinical translation of this approach (NCT05234190, NCT04817774) .

In order to dissect mechanistic effects and iteratively improve efficacy, A2.CAR-encoding mouse Tregs have been studied in mouse transplant models. A2.CAR Tregs were reported to

delay rejection of A2-mismatched skin grafts and A2-specific antibody formation[18,19]. Similarly, in a model of A2-mismatched heart transplantation (HTx), A2.CAR Tregs significantly promoted graft survival over polyclonal Tregs, although graft palpation scores started to decline by day 20 post-HTx. However, A2.CAR Treg therapy alone was ineffective in a haplo-mismatched A2.C57BL/6 X BALB/c (A2.F1) model of HTx, and significant extension of allograft survival was only observed when combined with 9-days of rapamycin treatment[20]. Notably, even in combination with rapamycin, A2.CAR Treg therapy did not induce long-term graft acceptance, highlighting the need for further work to identify combination therapies that support CAR Treg-mediated tolerance induction.

We previously reported that co-stimulation blockade with CTLA-4Ig and rapamycin prevented the expansion of endogenous donor-specific $CD4^+$ Tconvs and Tregs, whereas anti-CD154 preferentially inhibited Tconv expansion but not Tregs[21]. These observations are consistent with the fact that Tregs express lower amounts of CD154 compared to Tconvs [22]. Together with the resurgence of interest in clinical use of modified versions of anti-CD154 without pro-thrombotic properties [23] (NCT04046549, NCT05983770), there was a strong rationale to investigate whether anti-CD154 blockade could enhance the pro-tolerogenic properties of CAR Tregs. Here we used a clinically relevant haplo-mismatched (A2.2W-OVA.F1) HTx model in immune-competent C57BL/6 (B6) recipients to show that A2.CAR Tregs synergized with anti-CD154 to enhance graft survival, and inhibit the expansion of endogenous donor-reactive $CD4^+FoxP3^{neg}$ Tconvs and $CD8^+$ T cells, which were identified with 2W:I-A^b and OVA:K^b tetramers. Endogenous donor-reactive IgG responses that were specific for A2 as well as donor MHC I (K^d, L^d) and MHC II (I-A^d, I-E^d) was also inhibited. Remarkably, endogenous donor-reactive (2W:I-A^b) $CD4^+FoxP3^{pos}$ Tregs expanded in these recipients, thus providing evidence that A2.CAR Tregs synergized with anti-CD154 to promote infectious tolerance, defined as the ability of cells inducing tolerance to transfer this ability to another cell population and to spread tolerance to third-party antigens [24].

2. Materials and Methods

2.1 Mice.

Heart transplant recipients were eight- to twelve-week-old female C57BL/6 (B6, H-2^b) mice purchased from Harlan Sprague Dawley (Indianapolis, IN). Heart donor (A2.2W-OVA.F1) mice were generated by crossing 2W-OVA.BALB/c males [25] (gift from James J Moon, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, USA) with B6.HLA-A*0201 females (Taconic Biosciences, Germantown, NY) to generate 2W-OVA.BALB/c mice. For Treg donor mice, C57BL/6-Foxp3^{gfp} Thy1.1 mice were bred in-house [16,19]. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago or University of British Columbia and adhered to the standards of the NIH Guide for the Care and Use of Laboratory Animals or Canadian Council of Animal Care.

2.2 Generation of A2.CAR Tregs.

A2.CAR Tregs were generated as described previously [7,16]. Briefly, lymph nodes and spleen from female C57BL/6-Foxp3^{gfp} Thy1.1 mice were collected and $CD4^+$ T cells were isolated by negative selection (STEMCELL Technologies). Tregs were sorted as live $CD4^+CD8^-Thy1.1^+Foxp3^{gfp+}$ or $CD4^+CD8^-Thy1.1^+CD25+CD62L^+$ cells using a MoFlo Astrios cell sorter (Beckman Coulter), stimulated with anti-CD3/CD28 Dynabeads (Thermo Fisher Scientific), expanded with recombinant human IL-2 (1,000 U/mL; Proleukin) in the presence of rapamycin (50 nmol/L; Sigma-Aldrich). After 2 days, cells were transduced with a retrovirus encoding an A2-specific, second generation A2.28.z CAR (Supplementary Figure 1A). Dynabeads were removed on day 7 and CAR expression and Treg purity were determined (Supplementary Figure 1B-C). Cells were cryopreserved prior to injection as a cell therapy.

2.3 Adoptive transfer of A2.CAR Tregs and heterotopic heart transplantation.

A2.CAR Tregs were administered on the day prior to HTx at the indicated dose via the intravenous (i.v) route. Heterotopic HTx were performed as described [26], by anastomosing female donor A2.2W-OVA F1 hearts to the inferior vena cava and aorta in the peritoneal cavity of female C57BL/6 recipients. Anti-CD154 (MR1, BioXCell) was injected intra-venously on the day of HTx at the indicated doses. Allograft survival was monitored twice weekly starting at post-operative day (POD) 7 by transabdominal graft palpation and was scored from a scale of zero to four. Allograft rejection was defined as the last day of palpable heartbeat.

2.4 Harvesting of allograft, secondary lymphoid organs, and tissue processing for flow cytometry.

Mice were euthanized at the indicated time post-HTx. Allografts were harvested after perfusing with cold PBS, disintegrated and digested by incubating for 20 min at 37ºC with 2mg/ml collagenase type IV (Sigma Aldrich). Secondary lymphoid organs (SLO) comprising spleens and pooled lymph nodes (brachial, inguinal, axillary and mediastinal) were harvested, and single cell suspensions were obtained from passing through a 40µm cell strainer (Corning Inc., USA). Red blood cells were lysed via 2–3-minute incubation with ammonium chloride-potassium (ACK) lysis buffer (Quality Biological). T cells were enriched with a Pan-T cell isolation kit II (Miltenyi Biotech) and passed through LS columns on a QuadroMACS separator (Miltenyi Biotech), followed by elution with MACS buffer (2%FBS + 2mM EDTA).

2.5 Tetramer, fluorescent antibodies and reagents.

After T cell enrichment, cells were stained with Fixable Aqua Live/Dead staining (Thermo Fisher Scientific OR Invitrogen), then Fc block (anti-CD16/32, S17011E /Biolegend). Tetramer staining was carried out for 40-45 min at 4º C; APC and PE conjugated tetramers (NIH tetramer core

facility). APC- and PE-conjugated 2W (EAWGALANWAVDSA):I-A^b tetramers were used to track allo-reactive $CD4^+$ and OVA (SIINFEKL):H-$2K^b$ for $CD8^+$ T cells. pCons-CDR1 (FIEWNKLRFRQGLEW):I-E^d tetramers (PE-conjugated) and H2-K^b (SIINFEKL) decoy (AF647-PE conjugated) tetramers were used to identify donor MHC-II reactive B cells as previously described [27,28].

Antibodies used to define the phenotype of T cells included: Invitrogen: CD44-BUV737 (IM7), CD8a-PE (53-6.7).; Abcam: c-Myc- AF488 (Y69); Biolegend: IgG2a- PeCy7 (RMG2a-62), IgD-BV605 (11-26c.2a), T and B cell activation marker-FITC (GL7), CD73-BV605 (TY/11.8), PD1-APC-Cy7 (RMP1-30), TIGIT-PECy7 (1G9), LAG3-BV785 (C9B7W), Helios-PE-Dazzle594 (22F6); BD Biosciences: CD19-BV421 (1D3), CD95- BUV737 (Jo2), CD90.2-BUV395 (53-2.1), CD4-BV510/BV786 (GK1.5), CD4-BUV496 (RMA.5), Thy1.1- BUV496 (HIS51), CD8-BUV805 (53-6.7), FR4- BV421 (12A5), FoxP3-AlexaFluor532/PECy7 (FJK-16s); and Ablab UBC: c-Myc-AF647/AF488 (9E10). Antibodies used to exclude non-T or B cells included were CD11c-BUV661 (N418), F4/80-BUV661 1 (T45-2342), NK1.1-2 BUV661 (PK136), TER119-BUV661 (TER-119) (Biolegend).

2.6 Multiplex bead assay to measure donor specific antibodies.

Multiplex streptavidin (SA) beads (PAK-5067-10K; Spherotech) were coated with biotinylated MHC-I (K^d, L^d), MHC-II (I-E^d, I-A^d) and HLA-A*0201 monomers (NIH Tetramer facility). Sera were incubated with pooled MHC I, MHC II, and HLA-A2 coated beads at 4ºC for 1 hour. Beads were washed and then stained with FITC-conjugated anti-mouse IgG (1070-09; Southern Biotech). The mean fluorescence intensity (MFI) of DSA binding to the multiplex beads was determined by flow cytometry (Novocyte Quanteon, Agilent) [27].

2.8 Statistical analysis.

Statistical significance analyses were performed using GraphPad Prism, version 10. Graft-survival significance was assessed using Kaplan-Meier/Mantel-Cox log-rank tests. Statistical differences between experimental groups were determined by 1-way-ANOVA or Mann-Whitney unpaired t test. P values ≤ 0.05 were considered statistically significant.

3. Results:

3.1: A2.CAR Tregs synergize with low dose anti-CD154 to prolong the survival of haplo-mismatched heart grafts.

Previous reports indicated that A2.CAR Tregs failed to promote the survival of haplo-mismatched HTx, and that adjunct immunosuppression is necessary [20]. Our previous observations that anti-CD154 was superior to CTLA-4Ig or rapamycin in permitting expansion of endogenous donor reactive Tregs expansion[21] prompted us to test if a suboptimal dose (250 µg/mouse) of anti-CD154 could synergize and enhance the efficacy of A2.CAR Tregs in B/6 recipients of haplo-mismatched HTx (Figure 1A). An anti-CD154 dose of 250µg was chosen as it delayed rejection to approximately 30 days post HTx (Figure 1B) whereas the high dose of 350µg induced graft acceptance in 100% of recipients.

$1x10^6$ A2.CAR Tregs were administered intra venous (i.v.) one day prior to HTx and a single dose of 250µg anti-CD154 was administered on the day of HTx. Untreated recipients acutely rejected their heart allografts with a median graft survival of 12 days, while those receiving anti-CD154 monotherapy had a median survival of 28 days. Recipients receiving $1.0X10^6$ A2.CAR Tregs + anti-CD154 had significantly prolonged median survival of 56 days. However, a higher dose of $1.4X10^6$ A2.CAR Tregs (henceforth referred to as + A2.CAR Treg) did not further extend graft survival (Figure 1C). Notably, there was considerable heterogeneity in graft survival (Figure 1C-1E), with 23% (n=3/13) rapidly rejecting their grafts by day 14, and an additional 23% (n=3/13) by day 35 post-HTx. The remaining recipients (n=7) maintained a graft palpation score of >3+ until the

day of sacrifice (day 45-63 post-HTx). Collectively, these recipients were grouped into rejecting (Rej) and accepting (Acpt) groups.

3.2: Rejection in A2.CAR Treg recipients is associated with reduced FoxP3^{pos}: FoxP3^{neg} A2.CAR T cell ratios in the heart allograft.

Heterogeneity in graft survival in A2.CAR Treg recipients prompted us to track circulating CD4^{+}Thy1.1^{+} A2.CAR Tregs at weeks 2-6 post-HTx (supplementary Figure 1D). At all timepoints, a ~10-fold higher number of A2.CAR Tregs was detected in the blood from Rej compared to Acpt recipients (Figure 2A). However, the percentage of CD4^{+}Thy1.1^{+} A2.CAR T cells expressing FoxP3 in Rej versus Acpt recipients was not significantly different across all three time points (Figure 2B). Consequently, both FoxP3^{pos} and FoxP3^{neg} circulating A2.CAR T cells were present in higher numbers in Rej compared to Acpt recipients (Figure 2C, D). CAR (Myc) expression was also significantly elevated in CD4^{+}Thy1.1^{+} T cells from Rej versus Acpt recipients (supplementary Figure 2A-B). This suggests that an early expansion of circulating A2.CAR Tregs is unexpectedly associated with poor allograft survival.

To verify the association between expanded numbers of A2.CAR Tregs and poor allograft survival, we quantified FoxP3^{pos} and FoxP3^{neg} of CD4^{+}Thy.1^{+} cells from the SLO (spleen + lymph nodes) or allografts at the time of sacrifice (day 45-63 post-HTx). Similar to blood, significantly more (6-7-fold) CD4^{+}Thy1.1^{+} cells were recovered from the SLOs (Figure 2E) of Rej versus Acpt recipients, although the percentages of FoxP3^{pos} of CD4^{+}Thy1.1^{+} cells were comparable (Figure 2F; 87-93%); thus, more FoxP3^{pos} and FoxP3^{neg} A2.CAR T cells were recovered from Rej vs Acpt recipients (Figure 2G-H). CAR

(Myc) expression was also significantly elevated on A2.CAR Tregs from the SLO of Rej versus Acpt recipients (supplementary Figure 2C-D).

The number of total $CD4^{+}Thy1.1^{+}$ cells infiltrating Rej allografts was also higher (27±28-fold) compared to Acpt recipients (Fig 2I). Notably, only 52 ± 30% of $Thy1.1^{+}$ cells were $FoxP3^{pos}$ in Rej compared to 85 ± 5% in Acpt allografts (Figure 2J). As a result, the recovered $Foxp3^{pos}$ cells (Figure 2K) were only a mean 8-fold (1880±2492) higher. In contrast, $Foxp3^{neg}$ cells (Figure 2L) were ~130-fold higher (3562±4368) in Rej allografts compared to Acpt. The overall CAR (Myc) expression by graft infiltrating $CD4^{+}Thy1.1^{+}$ T cells was lower compared to those in blood or SLO, nevertheless, CAR expression was significantly higher on cells from Rej versus Acpt allografts (supplementary Figure 2E-F). These data demonstrate a preferential accumulation of $FoxP3^{neg}$ A2.CAR T cells that was apparent only in the Rej allograft and not in the blood or SLO.

3.3: $FoxP3^{pos}$ and $FoxP3^{neg}$ A2.CAR T cells acquire a distinct activation phenotype in rejection and acceptance.

The differential expansion of Foxp3 positive versus negative A2.CAR T cells in Rej versus Acpt recipients prompted a phenotypic analysis of expression markers associated with Treg function or T cell dysfunction, namely FR4, CD73, PD1, SLAMF6, LAG3 and TIGIT. Uniform Manifold Approximation and Projection (UMAP) was used to visualize the phenotype at single cell resolution (Figure 3A-E) and revealed that $Foxp3^{pos}$ and $FoxP3^{neg}$ A2.CAR T cells from Acpt versus Rej recipients were phenotypically distinct. Specifically, $Foxp3^{pos}$ A2.CAR Tregs (Figure 3G-K) from Acpt recipients had higher expression of CD44, FR4, and CD73, but comparable levels of PD-1 and SLAMF6. Similarly, $FoxP3^{neg}$

A2.CAR T cells from Acpt recipients expressed higher FR4 and lower PD-1 and SLAMF6 (Figure 3L-P). Collectively, these observations suggest that in Rej recipients, A2.CAR T cells not only exhibited significantly greater expansion but also acquired a distinct phenotype, suggesting that these phenotypes characterize CAR-Tregs with reduced regulatory function.

3.4: A2.CAR Tregs promote endogenous Treg expansion and suppress donor-reactive Tconv and CD8$^+$ T cells.

In our model, donor grafts not only expressed A2 and BALB/c alloantigens, they also expressed the 2W-OVA transgene thus enabling tracking of endogenous polyclonal 2W- and OVA-specific CD4$^+$ and CD8$^+$ T cells by utilizing 2W:I-A^b and OVA:K^b tetramers, respectively (Supplementary Figure 3). We tracked these T cells from HTx-recipients receiving low anti-CD154 in combination with A2.CAR Tregs, and from control groups of naïve B6, B6 HTx recipients with no treatment (acute rejecting, AR), or receiving low or high anti-CD154 monotherapy (Figure 4A). Overall, the total number of CD4$^+$ 2W:I-A^b CD44^{hi} T cells recovered from Acpt recipients was significantly lower compared to Rej recipients (Figure 4B). Remarkably, the total number of Foxp3^{pos} 2W:I-A^b Tregs was significantly higher in Acpt versus Rej recipients and the total number of Foxp3^{neg} 2W:I-A^b Tconvs was lower in Acpt (Figure 4C-E). Consequently, the percentage of Foxp3^{pos} of 2W:I-A^b cells was higher in Acpt versus Rej recipients (35-56% versus 5-15%). The total number of OVA:K^b CD8$^+$ T cells was also significantly lower in Acpt recipients compared to Rej recipients (Figure 4G).

Flow cytometric analysis of 2W:I-A^b Tregs and Tconvs from Acpt versus Rej recipients revealed that they were phenotypically comparable, in contrast to A2.CAR-Tregs that were phenotypically distinct. Notably, this panel of markers was able to distinguish 2W:I-A^b Tregs and Tconvs, and OVA:K^b $CD8^+$ T cells from control rejection (untreated or low Anti-CD154) versus control acceptance (high anti-CD154; Supplementary Figures 4-6). Thus, while endogenous donor-specific $CD4^+$ and $CD8^+$ T cells expanded in Rej recipients, their shared phenotype prompted us to determine whether they participated in graft rejection.

Histology of transplanted hearts (Figure 5A-E) confirmed that A2.CAR Tregs prevented lymphocyte infiltration in Acpt but failed to do so in Rej grafts. Furthermore, flow cytometry to quantify the endogenous (Thy1.1^{neg}) T cells and B cells accumulating allografts confirmed that endogenous $CD4^+$ and $CD8^+$ T cells, as well as B cell, infiltration, was significantly reduced in Acpt versus Rej grafts, but there was no significant difference in the percentage of $FoxP3^{pos}$ of $CD4^+$ T cells (Fig 5F-I). Taken together, these data illustrate the ability of A2.CAR Tregs to synergize with anti-CD154 to mediate infectious tolerance that resulted in the control endogenous donor-specific $CD4^+$ and $CD8^+$ T cell responses leading to improved allograft survival. In contrast, in A2.CAR Tregs recipients that rejected their grafts, no infectious tolerance was observed.

3.5: A2.CAR Tregs inhibit anti-A2 and anti-donor MHC B cell responses.

Prior studies reported that A2.CAR Tregs suppressed anti-A2 IgG responses in single antigen mismatched transplant models [19]. Thus we next tested if A2.CAR Tregs can suppress antibody responses to HLA-A2 and BALB/c MHC-I (K^d, L^d) and MHC-II (I-

E^d, I-A^d) by utilizing multiplex bead based assay. As shown in Figure 6B-6D, the donor specific antibody (DSA) levels were highest in the acute rejecting group on POD-21. Anti-CD154 only treatment and the Rej group also developed significantly higher levels of DSA by day 45 post-HTx compared to naïve mice. In Acpt recipients, IgG responses to all three specificities were significantly suppressed and comparable to recipients treated with high anti-CD154 and naive mice.

Furthermore, by utilizing donor MHCII I-E^d tetramers, we tracked endogenous I-E^d specific B cells and characterized their phenotype (Figure 6E-H). Recipients with Acpt allografts had decreased germinal center B (GC: Fas^+GL7^+) cell responses compared to recipients with Rej allografts. Thus, these data show that A2.CAR Treg therapy controls endogenous DSA responses specific for A2 and donor MHC.

Discussion

CAR Tregs are already being tested clinically, despite limited experimental evidence that they can promote the stable acceptance of multiple antigen-mismatched allografts in immunocompetent recipients [20]. The type of immunosuppression capable of synergizing with CAR-Tregs is also under investigation. Our study provides evidence that in combination with CD154 blockade, A2.CAR Tregs extended the survival of a haploidentical heart allografts by suppressing endogenous Tconv, $CD8^+$ T cell and B cell responses that were donor (A2 and non-A2) -specific. Remarkably, A2.CAR Tregs promoted the expansion of $FoxP3^+$ cells within the endogenous donor-specific (2W:I-A^b) $CD4^+$ cell population. To the best of our knowledge, these are the first data showing the ability of A2.CAR Treg therapy to suppress non-A2 donor-reactive responses, while also promoting non-A2 donor-reactive Treg accumulation. The general consensus is that long-term allograft tolerance is mediated by endogenous Tregs with indirect specificity [29,30], so evidence that infused A2.CAR Tregs can promote the expansion of endogenous Treg with indirect specificity is a key advance. A caveat is that the A2.CAR Tregs also express endogenous TCR, so we cannot rule out the possibility that some of the effects may be mediated by endogenous TCRs with indirect alloreactivity.

Tregs are known to control antigen-presenting cells (APCs) and alloreactive T and B cells through a variety of mechanisms [31,32]. Some of the pathways mediating these effects include expression of coinhibitory molecules, trogocytosis of stimulatory molecules on APCs, secretion of immunoregulatory cytokines, and depletion of IL-2 [33]. That HTx acceptance was associated with $FoxP3^+$ A2.CAR Tregs upregulating FR4 and CD73 suggest a more regulatory phenotype. Indeed, CD73 together with CD39 function

as ectonucleotidases that catabolize ATP into adenosine, a molecule with immunosuppressive properties [34,35]. FR4 is surface receptor for folic acid (Vitamin B9) that is highly expressed on potent Tregs and has been implicated in mediating Treg function in models of auto, allo and tumor immunity [36]. Interestingly, in mice with accepted grafts, FoxP3^{-} A2.CAR T cells upregulated FR4 and downregulated activation markers, PD-1 and SLAMF6, indicative of T cell quiescence.

It was previously reported that A2.CAR Tregs can suppress A2-specific B cells and antibody production [19], but the ability to control other alloantibodies was unknown. Here, we showed that both A2-specific and IgG responses specific for donor MHCI and II were successfully controlled, and that the differentiation of donor MHC-specific B cells into GC B cells was inhibited. Whether this control of donor-specific humoral immunity could have been mediated by A2.CAR Tregs preventing the generation of T follicular helper (Tfh) cells or promoting the differentiation of regulatory T follicular regulatory (Tfrs) or both, will require further investigation [37].

It is notable that the beneficial effects of anti-CD154 and A2.CAR Treg therapy only occurred in approximately half of the recipients. In mice where allograft survival was not prolonged, there was no evidence for infectious tolerance. Rather, as early as 2 weeks post-adoptive transfer, there was a significant expansion in A2.CAR-Tregs in the blood, and SLOs of Rej compared to Acpt recipients, although the percentage of cells that were FoxP3^{pos} remained comparable. We observed higher ratios of FoxP3^{neg}:FoxP3^{pos} A2.CAR T cells in Rej versus Acpt grafts at the time of sacrifice (45-60 days post-HTx) that was not apparent in the blood or SLO, therefore emphasizing the need to investigate events within the allograft. Finally, the origin of the FoxP3^{neg} cells could either be preferential

expansion of FoxP3^{neg} A2.CAR T cells in the infused product, which contained ~1% Foxp3^{neg} cells, and/or destabilization of the FoxP3^{+} cells [38-40]. Investigations into the origin of FoxP3^{neg} A2.CAR Tregs, and why FoxP3^{neg} failed to re-shape host immunity in some mice will be important future directions for improving CAR Treg products for consistent success.

In conclusion, our study supports the clinical testing of CAR Treg therapy in combination of CD40 pathway blockade as a promising future direction. However, heterogeneity in the efficacy of this combination therapy highlights the need for continual improvement of the CAR Treg product to maximize its potency and consistency.

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Data availability statement

The data are available from the corresponding author upon request.

Disclosures:

MKL is an inventor on pending patents related to HLA-A2-specific CARs (PCT/CA2028/051167 and PCT/CA2018/051174). The other authors have no conflict of interest to disclose.

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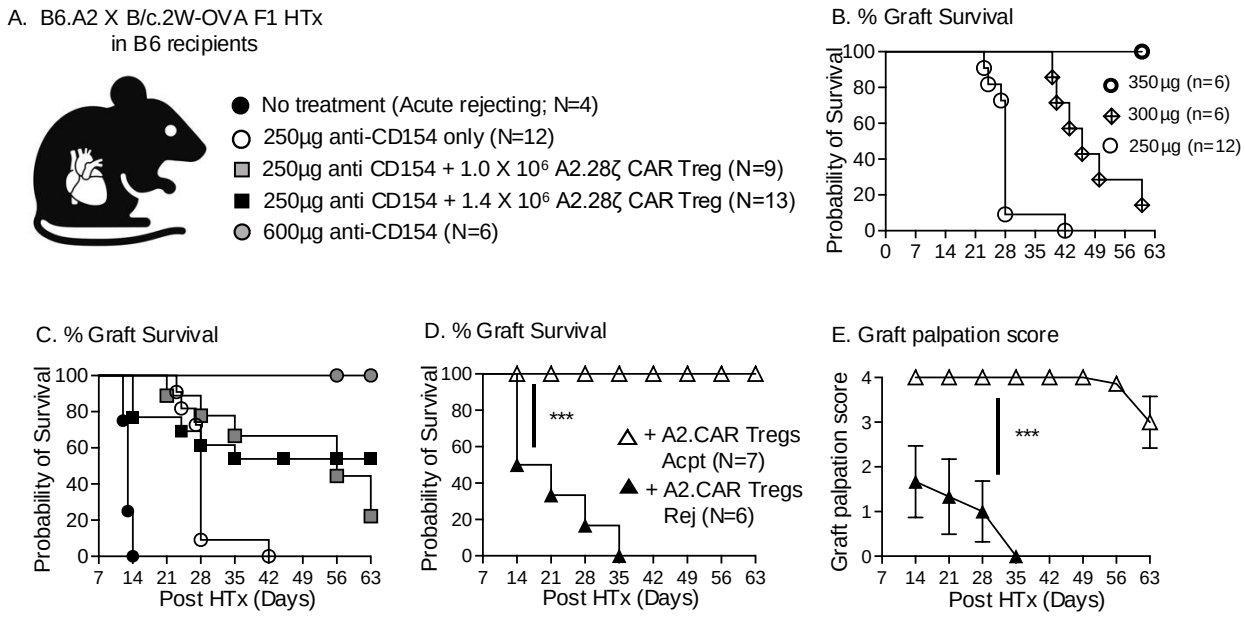

Figure 1: A2.CAR Tregs synergize with low dose anti-CD154 to prolong the survival of haplo-mismatched heart grafts. (A) Experimental design. (B) Graft survival curve in recipients of A2.2W-OVA.F1 heart transplant (HTx) with three different doses of anti-CD154 on day 0 of heart transplant. (C) Graft survival of B6 recipients of A2.2W-OVA.F1 HTx treated with low anti-CD154 (250µg), low anti-CD154 + $1x10^6$ or $1.4x10^6$ A2.CAR Tregs and high anti-CD154 (600µg) (D) Graft survival and (E) palpation scores in recipients of $1.4x10^6$ A2.CAR Tregs + low anti-CD154 with rejected (Rej) or accepted (Acpt) grafts. Log-rank (Mantel-Cox) test for statistical significance. Each symbol represents one mouse. Data are presented as mean $\pm$ SEM and statistical significance assessed by Mann-Whitney Test. *P <0.05; **P <0.01; ***P <0.001.

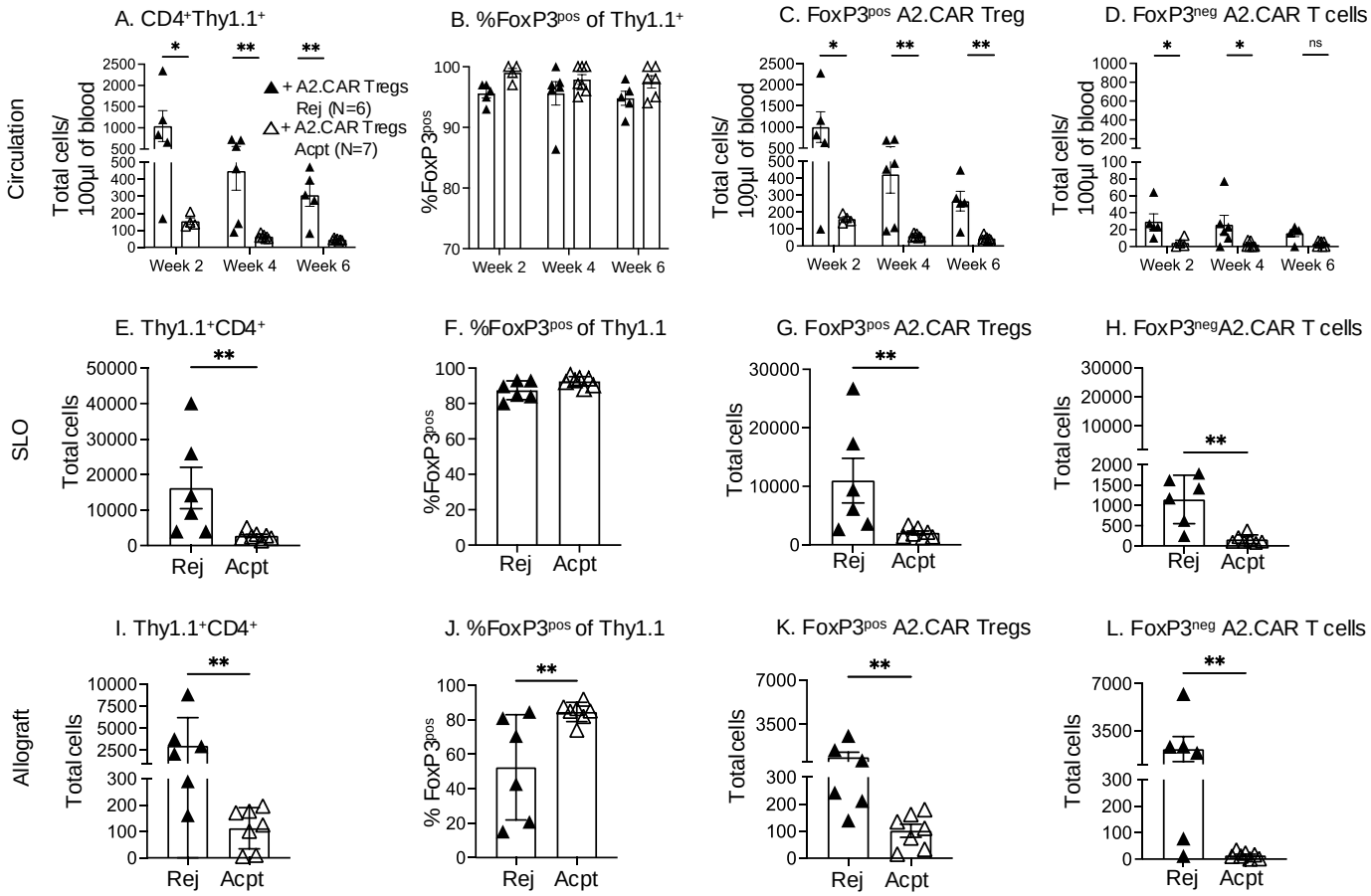

Figure 2: Mice with rejecting allografts have an increased accumulation of CD4⁺Thy1.1⁺ cells and reduced ratio of FoxP3^{pos}: FoxP3^{neg} A2.CAR T cells. (A-D) Quantification of circulating Thy1.1+ A2.CAR Tregs in low anti-CD154 recipients with Acpt or Rej allografts at the indicated week. (A) The number of CD4⁺Thy1.1⁺ cells per 100µl of blood. (B) %FoxP3^{pos} of Thy1.1⁺ cells. (C, D) Number of FoxP3^{pos} or FoxP3^{neg} A2.CAR T cells per 100µl blood. (E, I) Total number or CD4⁺Thy1.1⁺ T cells from SLOs and allografts from Rej (n=6) or Acpt (n=7) recipients. (F, J) %FoxP3^{pos} of Thy1.1⁺ cells. Number of FoxP3^{pos} A2.CAR Tregs (G, K) or FoxP3^{neg} A2.CAR T cells (H, L) recovered from SLO and allograft. Each symbol represents each mouse. Data are presented as mean ± SEM, and statistical significance assessed by Mann-Whitney Test. **P <0.01. SLO: Pooled lymph nodes and spleen. Total number of CD4⁺Thy1.1⁺ cells from SLO normalized to $3x10^6$ events. Total number of CD4⁺Thy1.1⁺ cells from allograft normalized to $1x10^6$ events.

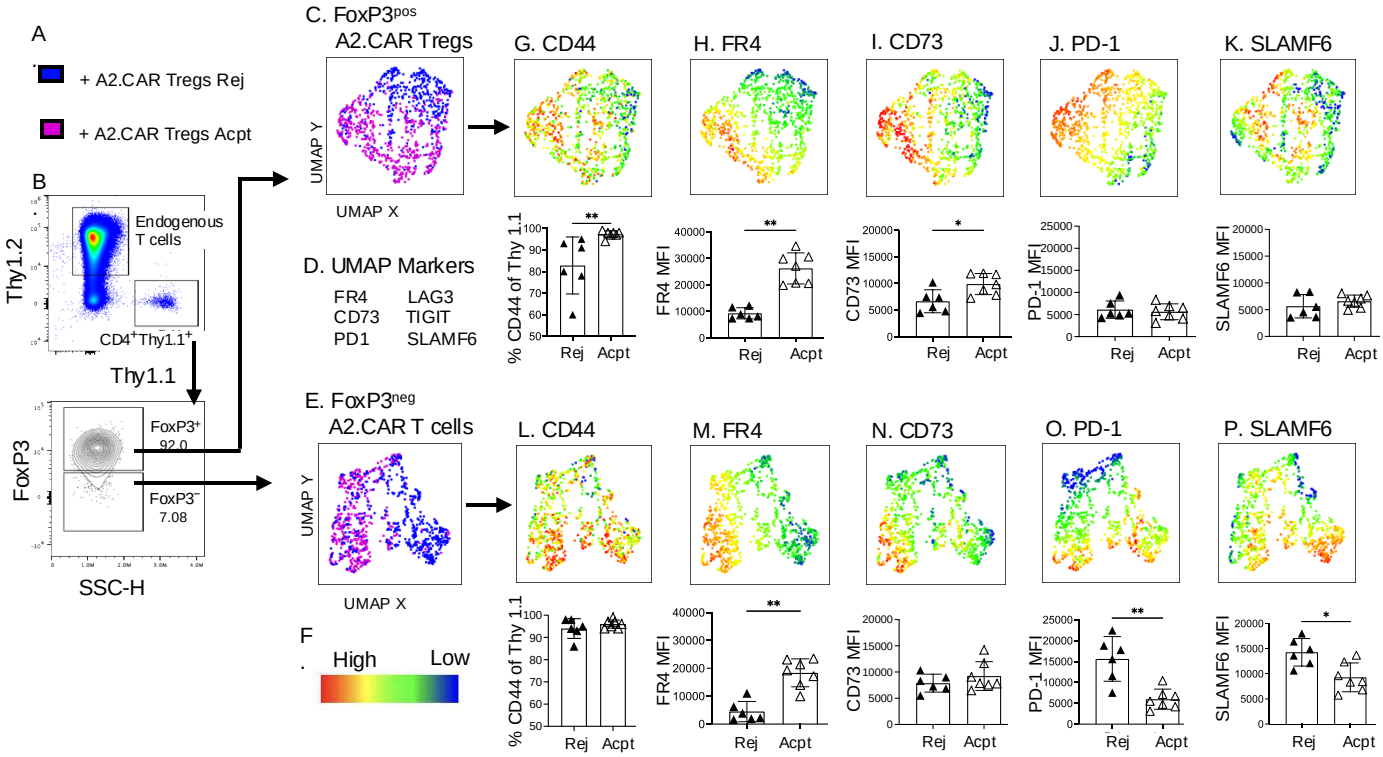

Figure 3: FoxP3^{pos} and FoxP3^{neg} A2.CAR T cells are phenotypically distinct in A2.CAR Treg recipients at rejection (Rej) and acceptance (Acpt). (B) Gating strategy showing FoxP3^{pos} and FoxP3^{neg} of transferred CD4^{+}Thy1.1^{+} A2.CAR T cells from SLO. (C-E) UMAP demonstrating phenotypic differences of (G-K) FoxP3^{pos} and (L-P) FoxP3^{neg}A2.CAR T cells from Rej and recipients, based on expression of FR4, CD73, PD1, SLAMF6, LAG3, TIGIT. UMAP with heatmap showing relative expression of indicated markers based on normalized median fluorescence intensity (MFI). Each symbol in the bar plots represents one mouse. Data are presented as mean ± SEM and statistical significance determined by Mann Whitney Test (*). *P <0.05; **P <0.01.

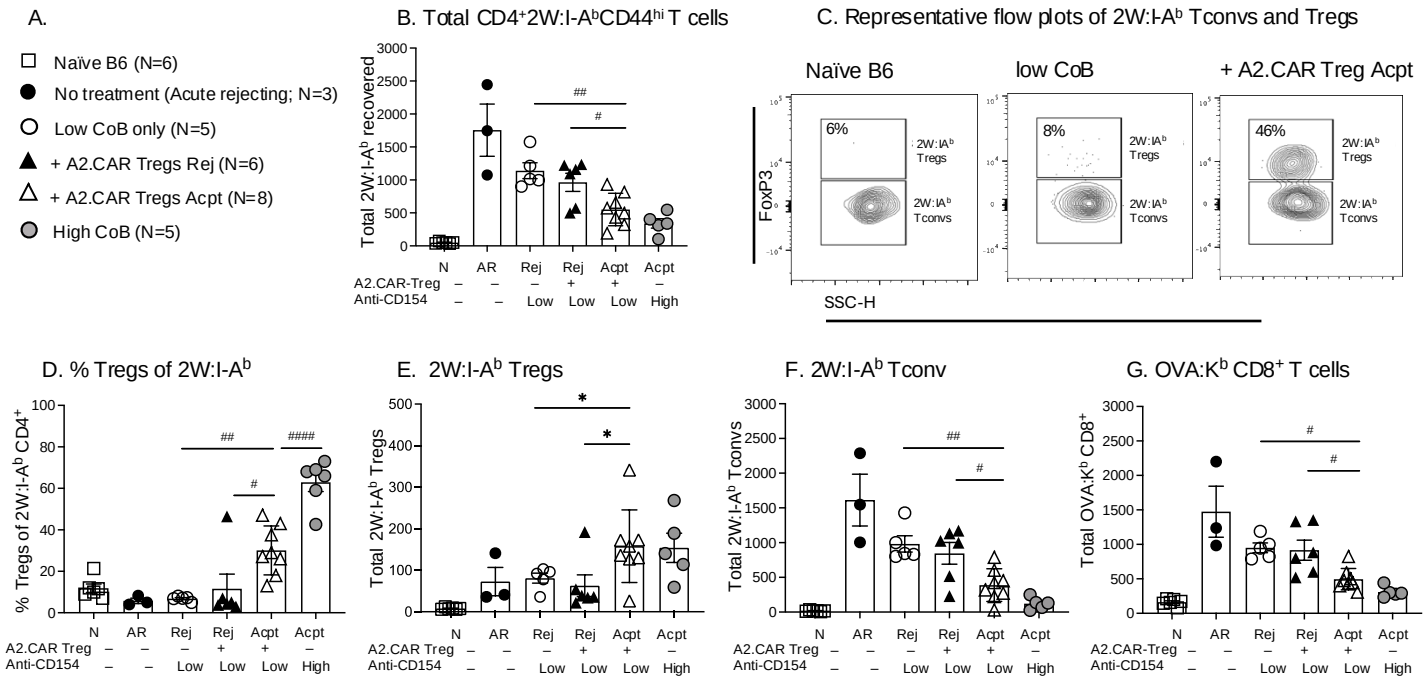

Figure 4: Graft acceptance in A2.CAR Treg treated recipients is associated with reduced endogenous donor-specific T cell responses and expanded Tregs. (A) Experimental groups. (B) Total number of $CD4^+2W{:}I\text{-}A^bCD44^{hi}$ T cells recovered from SLOs. (C) Representative flow plots of FoxP3 expression within gated $CD4^+2W{:}I\text{-}A^b$ $CD44^{hi}$ T cells from SLOs harvested between day 45-60 post-HTx. (D, E) Frequency and normalized total number of $2W{:}I\text{-}A^bCD44^{hi}$ Tregs. Total number of (F) $2W{:}I\text{-}A^bCD44^{hi}$ T Tconvs and (G) $OVA{:}K^b$ $CD8^+$ T cells. Each symbol represents one mouse. (One mouse received $1x10^6$ A2.CAR Tregs (from Fig 1) with accepted allograft at day 100 post-HTx was included in the Acpt group). Total $2W{:}I\text{-}A^b$ $CD4^+$ T cells and $OVA{:}K^b$ $CD8^+$ T cells from SLOs normalized to $3x10^6$ events. Data are presented as mean $\pm$ SEM and statistical significance determined by 1 way ANOVA (#) and Mann Whitney Test (*). *P or $^{\#}$P <0.05; $^{\#\#}$P <0.01; $^{\#\#\#\#}$P <0.0001.

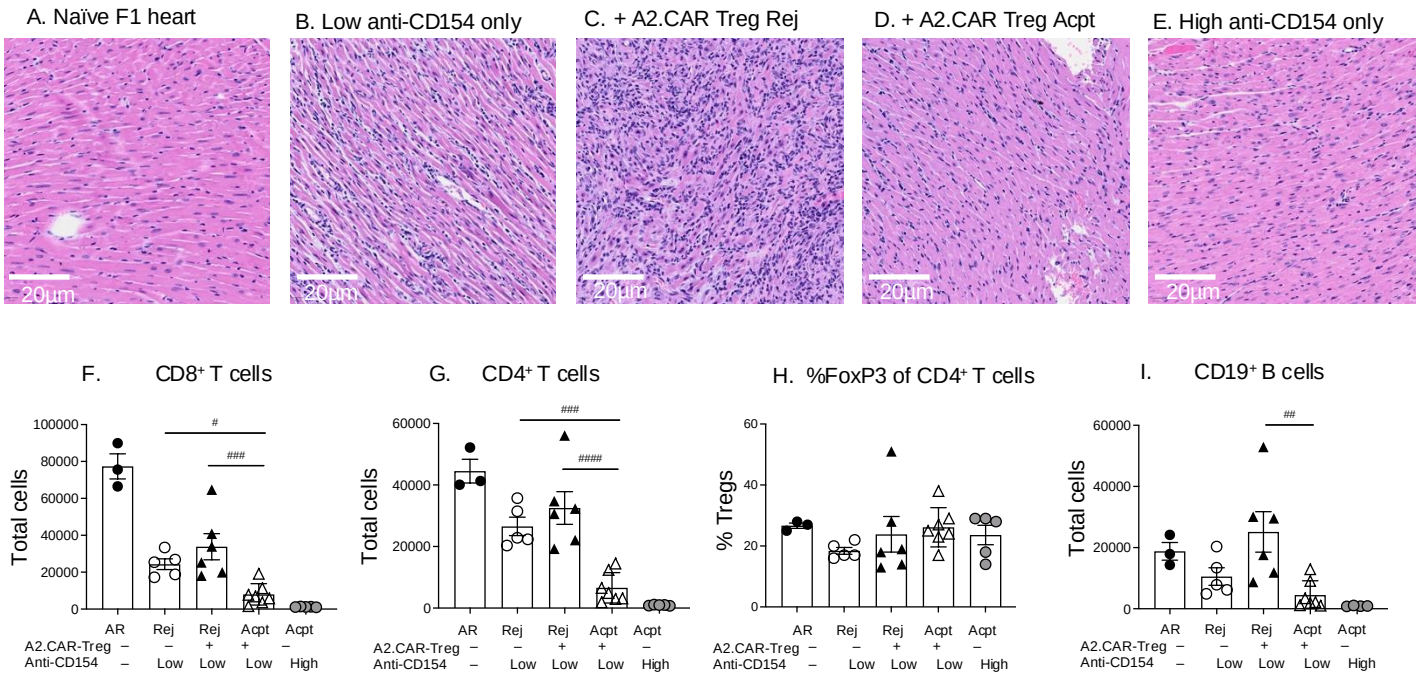

Figure 5: Graft acceptance in A2.CAR Treg recipients is associated with reduced accumulation of endogenous T and B cells in heart allografts. (A-E) Representative H&E-stained histological sections of naïve or transplanted heart allografts from the indicated groups sacrificed on days 45-55 post-HTx. A portion of the graft was analyzed by flow cytometry. Normalized total graft infiltrating (F) $CD8^+$ T cells, (G) $CD4^+$ T cells, (H) % Tregs of $CD4^+$ T cells, and (I) $CD19^+$ B cells. Recipients without treatment, low anti-CD154 only, A2.CAR Treg Rej, A2.CAR Treg Acpt (one mouse received 10^6 A2.CAR Tregs (from Fig 1) with accepted allograft at day 100 post-HTx was included), high anti-CD154 only Acpt between day 45-55 post-HTx. Total number of $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells from allografts normalized to $1x10^6$ events. Each symbol represents each mouse. Data are presented as mean ± SEM, and statistical significance assessed by one-way-ANOVA. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

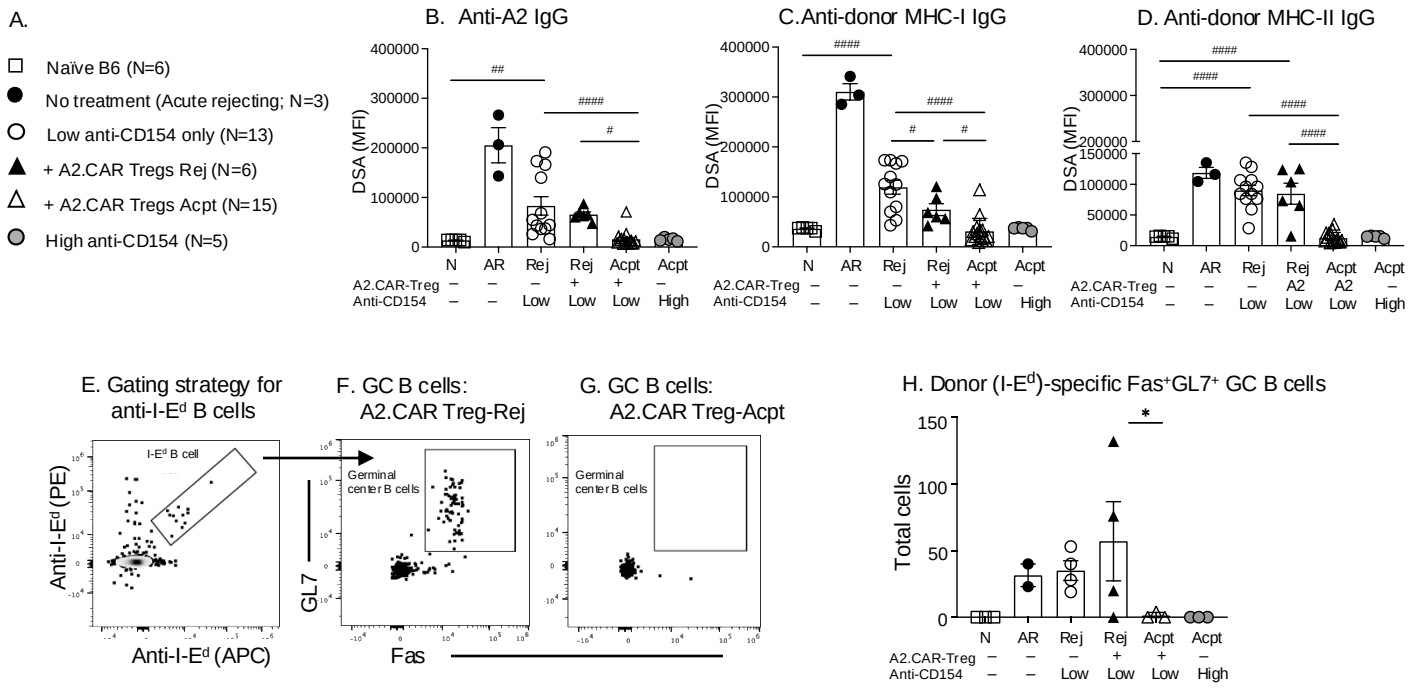

Figure 6: A2.CAR Tregs synergize with low anti-CD154 to suppress non-A2 donor-specific B cell responses. (A) Experimental groups. (B) Quantification of anti-A2 IgG, and (C) donor MHC Class I IgG or (D) Class II IgG at day 45 post HTx. (E-G) Gating strategy to track MHC-II I-E^d and GC B cells (Fas$^+$GL7$^+$) from (F) +A2.CAR Treg Rej or (G) +A2.CAR Treg Acpt. (H) Total GC B cells (Fas$^+$GL7$^+$ of I-E^d) recovered from draining lymph nodes (normalized to 2x10^6 events). Each symbol represents one mouse. Data are presented as mean $\pm$ SEM and statistical significance determined by 1 way ANOVA (#) and Mann Whitney Test (*). *P or $^{\#}$P <0.05; $^{\#\#}$P <0.01; $^{\#\#\#\#}$P <0.0001. MFI: Median fluorescence intensity. DSA: Donor specific antibody.