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CTLA-4Ig inhibits memory B cell responses and promotes allograft survival in sensitized recipients

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Abstract

Sensitized recipients with pre-transplant donor-specific antibodies (DSA) are at higher risk for antibody-mediated rejection (AMR) than non-sensitized recipients, yet little is known about the properties of memory B cells that are central to the recall alloantibody responses. Using cell enrichment and MHC Class I tetramers, C57BL/6 mice sensitized with BALB/c splenocytes were shown to harbor H-2K^d-specific IgG⁺ memory B cells with a post-GC phenotype (CD73⁺CD273⁺CD38^{hi}CD138⁻GL7⁻). These memory B cells adoptively transferred in naïve mice without memory T cells recapitulated class-switched recall alloantibody responses. During recall, memory H-2K^d-specific B cells preferentially differentiated into antibody-secreting cells (ASCs), whereas in primary response, H-2K^d-specific B cells differentiated into germinal center (GC) cells. Finally, our studies revealed that despite fundamental differences in alloreactive B cell fates in sensitized versus naïve recipients, CTLA-4Ig was unexpectedly effective at constraining B cell responses and heart allograft rejection in sensitized recipients.

Introduction

Desensitization protocols with intravenous immunoglobulin (IVIG) in combination with plasmapheresis, rituximab, bortezomib, rabbit antithymocyte globulin and edulizumab are being used in sensitized recipients to reduce pre-transplant DSA and inhibit the increase in DSA post-transplantation (1, 2) (3). Despite these efforts, the rates of antibody-mediated rejection in sensitized patients remain significantly higher compared to non-sensitized recipients (4) (5), and there continues to be a need for better desensitization and immunosuppressive strategies.

Long-lived plasma cells and quiescent memory B cells confer memory in sensitized individuals (6). Plasma cells reside in specialized niches in the bone marrow, secondary lymphoid organs and inflammatory sites, and are responsible for maintaining elevated DSA levels. In contrast, memory B cells remain quiescent in the absence of antigen but are responsible for the faster, more vigorous and class-switched antibody response upon antigen

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re-exposure. Plasma cells have been extensively investigated (7, 8), while there is limited information on memory B cells in the setting of allograft transplantation. Recent breakthroughs in tracking rare endogenous B cells to model antigens have identified new features of memory B cells including their heterogeneity (9–17). In this study, we used Class I MHC tetramers (18) to identify endogenous memory alloreactive B cells, track their fate after heart allograft transplantation, and define their susceptibility to continuous CTLA-4Ig therapy.

Materials and Methods

Animals and Tetramers

4–6 weeks old C57BL/6 and BALB/c mice were purchased from Harlan Sprague Dawley. Congenic Igha mice, B6.Cg-Gpi1a Thy1a Igha/J, were purchased from Jackson Laboratory (Bar Harbor, ME). Mice sensitized with BALB/c spleen or hearts, were injected 500 µg of CTLA4-Ig (Nulojix; Bristol-Myers Squibb) per mouse, intraperitoneally, on day –2, 0 and 2 and then twice per week until the end of the experiment. H2K^d-biotin monomers, H2K^d tetramers, loaded with the SYIPSAEKI peptide from malaria *Plasmodium berghei*, were conjugated with PE or APC (NIH Tetramer Core Facility, Atlanta, GA).

Statistics

Data are presented as mean ± SEM. Student's t test (unpaired) was used when two groups were compared or one-way ANOVA when there were 2 groups. Two-way ANOVA was used when there were 2 variables, followed by Turkey test to compare pairs of means. Survival curves were compared using Mantel-Cox test. All analyses were performed using GraphPad Prism. P values < 0.05 were considered statistically significant.

Study approval

All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of University of Chicago.

Results and Discussion

Recall antibody response is B cell intrinsic

Specific sensitization to HLA antigens can be induced by blood transfusion, pregnancies and previous allografts, as well as through cross-reactivity to environmental antigens or microbes. For our studies, we sensitized C57BL/6 mice with a single s.c. injection of BALB/c splenocytes, and elicited a recall response with a second injection 10 weeks later (Fig 1A). The primary donor-specific and anti-H2K^d IgM response peaked on day 7, while the IgG response peaked at 4 weeks post-sensitization and gradually returned towards baseline by 8–10 weeks post-immunization (Fig 1A). The recall donor-specific and anti-H2K^d antibody response was predominantly IgG, detected by day 7 post-immunization and peaked at titers that were significantly higher than the primary response (Fig 1A).

To investigate whether the features of the vigorous class-switched recall response were due to memory B cells that did not require the presence of memory T cells, we transferred

purified B cells from sensitized or naïve C57BL/6 mice (IgHb) into naïve syngeneic recipients (IgHa). This model avoids homeostatic proliferation, yet allows antibodies secreted by transferred or recipient B cells to be identified by their IgHb and IgHa allotype, respectively. One day after transfer we sensitized with BALB/c splenocytes, and 7 days later we observed a significant increase in BALB/c-specific IgG1a that was absent in recipients receiving B cells from naïve mice (Fig 1B). These observations confirmed that memory B cells in the presence of naïve T cells were capable of generating a rapid class-switched alloantibody response. An endogenous B cell IgMa response was observed in mice receiving memory B cells, suggesting a lack of interference by the BALB/c-specific IgG. In mice receiving naïve B cells, no IgG1a responses were observed, and the observed BALB/c-specific IgMa response confirmed the efficacy of the immunization.

Phenotype and fate of endogenous memory alloreactive B cells

Low numbers and lack of specific cell surface markers have made the study of endogenous antigen-specific memory B cells in mice challenging, however, recently described enrichment protocols have allowed these low-frequency B cells to be identified (10, 15). Memory B cells can express IgM or IgG, but because of higher affinity for antigen, memory IgG⁺ B cells were shown to dominate the recall response when circulating antigen-specific IgG was present (10). Because low levels circulating IgG alloantibodies are present in our sensitized mice, so we focused our efforts on characterizing the IgG⁺ memory alloreactive B cells. Memory IgG⁺ B cells from mice sensitized with BALB/c splenocytes 10 weeks prior were enriched using FITC-conjugated anti-IgG followed by anti-FITC magnetic bead capture. Very few IgG⁺ cells were detected in the flow through (Supplemental Fig 1), indicating efficient enrichment with this approach. Alloreactive B cells within the enriched IgG⁺ population were then identified as double-positive for PE- or APC-conjugated H-2K^d tetramers (Fig 1C). Approximately 100–200 quiescent (GL7⁻CD138⁻) H-2K^d-reactive IgG⁺ B cells were recovered from each sensitized mouse, representing approximately 0.5% of total IgG⁺ expressing B cells (Fig 1D).

Shlomchik and colleagues (9, 12, 13) reported that memory B cells can be subdivided into at least five phenotypic subsets based on the expression of CD273 (PD-L2), CD80 and CD73. Of these, the CD80⁺CD273⁺ subset was most “memory-like” and dominated the recall response by preferentially differentiating into antibody secreting cells (ASCs), whereas the CD273⁻CD80⁻ subset was most “naïve-like” and differentiated into GC cells. We determined the phenotype of the H-2K^d-reactive IgG⁺ B cells as quiescent CD38⁺, CD73⁺ and CD273⁺ (Fig 1C) and CD80^{low} (data not shown). In contrast, the IgG⁻ (predominantly IgM) population was CD273^{low/-} and CD73⁻, while there were too few H-2K^d-reactive IgG⁺ B cells in naïve mice for reliable assessment (Fig 1C). The expression of CD73 marks post-GC memory cells (12, 13, 15), while the single-positive expression of CD273 is consistent with an “intermediate” memory phenotype (13) (9). We speculate that differences in the long-lived memory B cell phenotypes following allo-sensitization compared to immunization with NP-CGG in alum, arose from different innate inflammatory signals during the primary antigen encounter. Supporting this is the observation by Katsuri et al. (19) that innate signals triggered by Toll-like receptors affected the persistence of GC and plasma cell responses, and the programming towards B cell memory. Whether this

“intermediate” memory B cell phenotype induced by donor splenocytes (DSC) is preserved following other different clinically-relevant routes of sensitization, such as pregnancy or organ transplantation, is not known.

Differentiation of endogenous memory alloreactive B cells after allogeneic heart transplantation

Recent studies on memory B cells using model antigens and adjuvants have identified two possible fate trajectories of memory B cells following antigen reencounter. Most studies in mice and humans show that class-switched memory B cells preferentially differentiate into plasma cells whereas unswitched (IgM⁺) memory B cells preferentially differentiate into GC cells (9–11, 20). However others suggest that IgG⁺ memory B cells retain the potential to differentiate into GCs and undergo further BCR-diversification (21). We previously reported that BALB/c heart transplantation into naïve C57BL/6 recipients induced a preferential differentiation of K^d-specific B cells into GC cells (18). We here report that when BALB/c hearts were transplanted into sensitized C57BL/6 mice, minimal differentiation of K^d-specific memory B cells into GL7⁺Fas⁺ GC B cells was observed at both early (7 days) or late (14–15 days) times post-transplant (Fig 2A–B).

We next investigated whether K^d-specific memory B cells differentiated preferentially into antibody secreting cells (ASCs) using a K^d-specific enzyme-linked immunospot (ELISPOT) assay. An increase in the frequency of ASCs from pooled spleen and lymph node cells was observed on day 7 post-transplant in sensitized recipients, and this response was significantly more vigorous than in naïve recipients (Fig 2C). Thus similar to memory B cells generated to model antigens adjuvanted in alum or complete Freund’s adjuvant (9, 10, 13, 17), and in contrast to those generated with monophosphoryl lipid A adjuvant (21), memory alloreactive B cells generated by DSC immunization almost exclusively differentiated into ASC following allogeneic heart transplantation. Interestingly the generation of ASCs to the allograft was unexpectedly short-lived and no longer detectable by day 14 post-transplant. While it is possible that a subset of ASC may have migrated into the BM, we were unable to reliably detect them (data not shown). Similar transient ASC responses have been described for recall B cell responses in humans (22, 23), and rapid clearance of antigen by IgG produced during the recall response as well as the short-lived lifespan of ASC have been proposed to explain the rapid decline of the ASC response (23). In our studies, allografts are not removed after rejection so it is unlikely that clearance of alloantigen causes the rapid abrogation of ASC response, and the signals controlling the kinetics of recall ASC response warrants further investigation.

CTLA-4Ig inhibits memory alloreactive B cell responses to heart allografts

The correlation between pre-transplant or *de novo* alloantibody responses, AMR and poor graft outcomes suggests a need for therapies that inhibit the B cell recall response. Belatacept, a high affinity CTLA-4Ig, as continuous dosing regimen has been approved for the prophylaxis of rejection in kidney transplant recipients (24, 25). We tested whether CTLA-4Ig in a continuous regimen (500 µg/mouse; day –2 and 0 and then 2 times/week till sacrifice), could modulate the recall B cell response to BALB/c hearts in DSC-sensitized recipients (Fig 3). CTLA-4Ig was unexpectedly efficacious, inhibiting both recall ASC and

DSA responses in sensitized recipients, whereas control Fc-Ig1 did not (Fig 3A–B; Supplemental Fig 2). Immunohistochemistry confirmed an absence of C4d deposition in the graft at day 7 post-transplantation (Fig 3C). Coincident with this control of recall antibody responses, continuous CTLA-4Ig treatment prolonged the survival of heart allografts in sensitized recipients; with the majority (7 of 11) of the allografts still beating on day 30 post-transplantation (Fig 3D). Thus recall alloantibody responses are unexpectedly dependent on CD28-CD80/CD86 interactions, and can be controlled by CTLA-4Ig. However, we cannot exclude the possibility that sensitization through routes that elicit more vigorous alloreactive responses may result in memory B cells that are less sensitive to CTLA-4Ig.

The efficacy of CTLA-4Ig in sensitized mice contrasts with reports that CTLA-4Ig is only effective at inhibiting naïve but not memory T cell responses because of redundancy of co-stimulatory molecules on memory T cells (26, 27). However, those studies were based on the transient administration of CTLA-4Ig to induce tolerance, whereas our treatment protocol involves a continuous administration of CTLA-4Ig. The inhibition of memory as well as ongoing (18) B cell responses by CTLA-4Ig may explain the clinical observation of DSA being significantly lower in transplant patients on Belatacept compared to those on calcineurin-inhibitors, despite higher rates of acute rejection (28). Recently, CTLA-4Ig has been shown to be expressed on both Tfh and regulatory Tfh cells and to be dominantly critical for constraining B cell responses (29, 30). In light of these observations, it is reassuring that the outcome of CTLA-4Ig treatment is the inhibition of B cell responses. Finally, our results together with the observation that the majority of memory CD4 cells in humans retain CD28 expression (31) provide a rationale for testing whether Belatacept can inhibit recall antibody responses in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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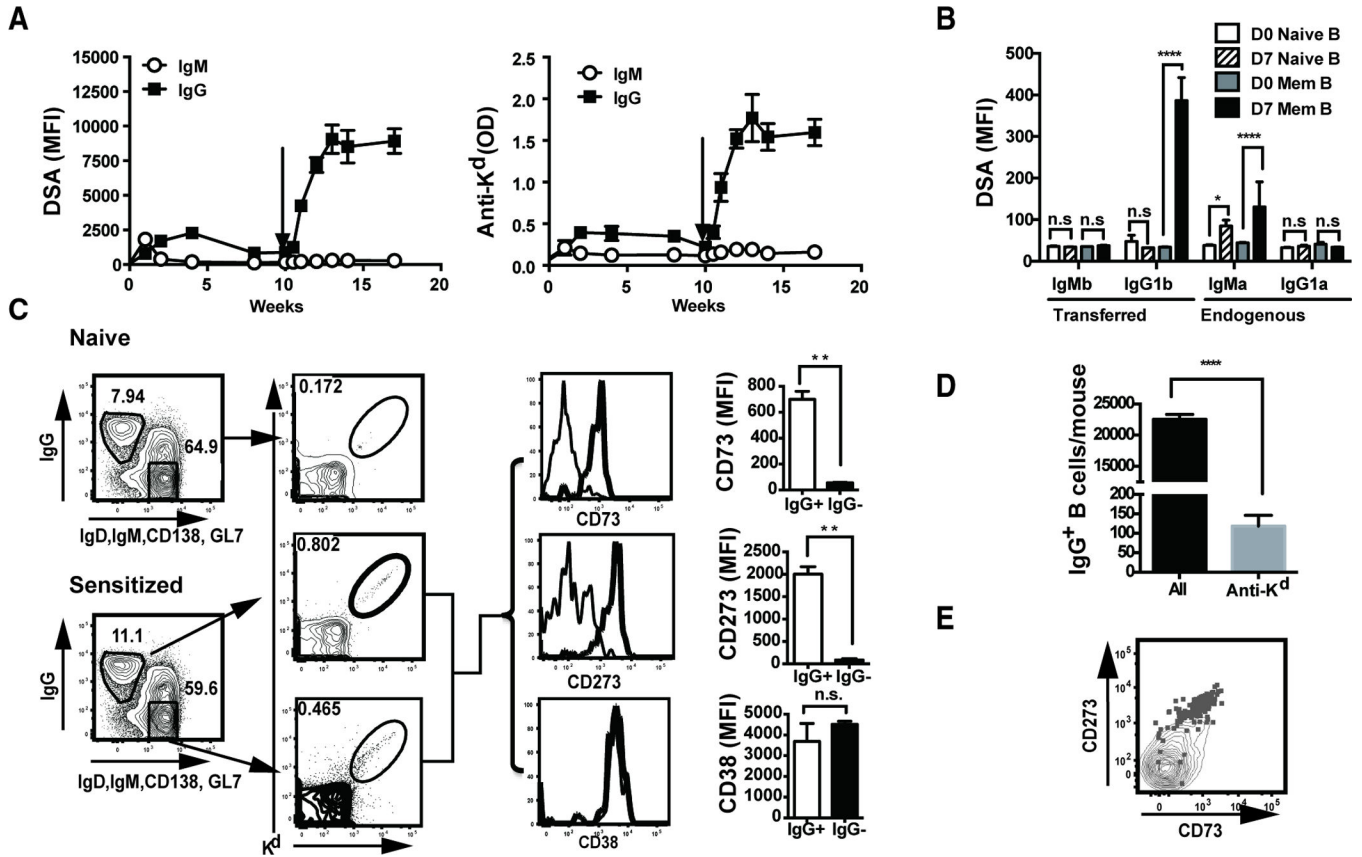


Fig 1. The recall alloantibody response is B cell intrinsic. (A) Antibody responses following primary and secondary immunization with BALB/c spleen cells (s.c.). Donor specific antibodies (DSA) were measured by flow cytometry (mean fluorescence intensity (MFI)) and anti-K^d antibodies by ELISA. N=5 mice per experiment, repeated twice. (B) Adoptive transfer to demonstrate memory B cell responses. C57BL/6 mice (IgHb) were immunized with BALB/c splenocytes (s.c.), and 10 weeks later, mice were sacrificed and B cells were purified and adoptively transferred ((20 × 10⁶) into congenic IgHa recipients. One day after transfer, IgHa recipient mice were immunized with BALB/c splenocytes, and 7 days later, donor specific IgMb and IgG1b produced by transferred B cells, and IgMa and IgG1a produced by recipient B cells, were quantified by flow cytometry. N=2–3/group, repeated 2 times. (C) Gating strategy and percentage of IgG⁺ B cells and H-2K^d-binding IgG⁺ B cells from naïve or sensitized mice (left two panels), and phenotype of memory H-2K^d-binding IgG⁺ versus IgG⁻ B cells. Representative histograms and MFI for CD73, CD38 and CD273 expression are presented (right two panels). N=3–4/group, repeated 3 times. (D) Total number of IgG memory and H-2K^d-binding IgG⁺ cells per sensitized or naïve mouse (pooled spleen plus 6 lymph nodes). (E) Co-expression of CD273 and CD73 on memory H-2K^d-binding IgG⁺ B cells.

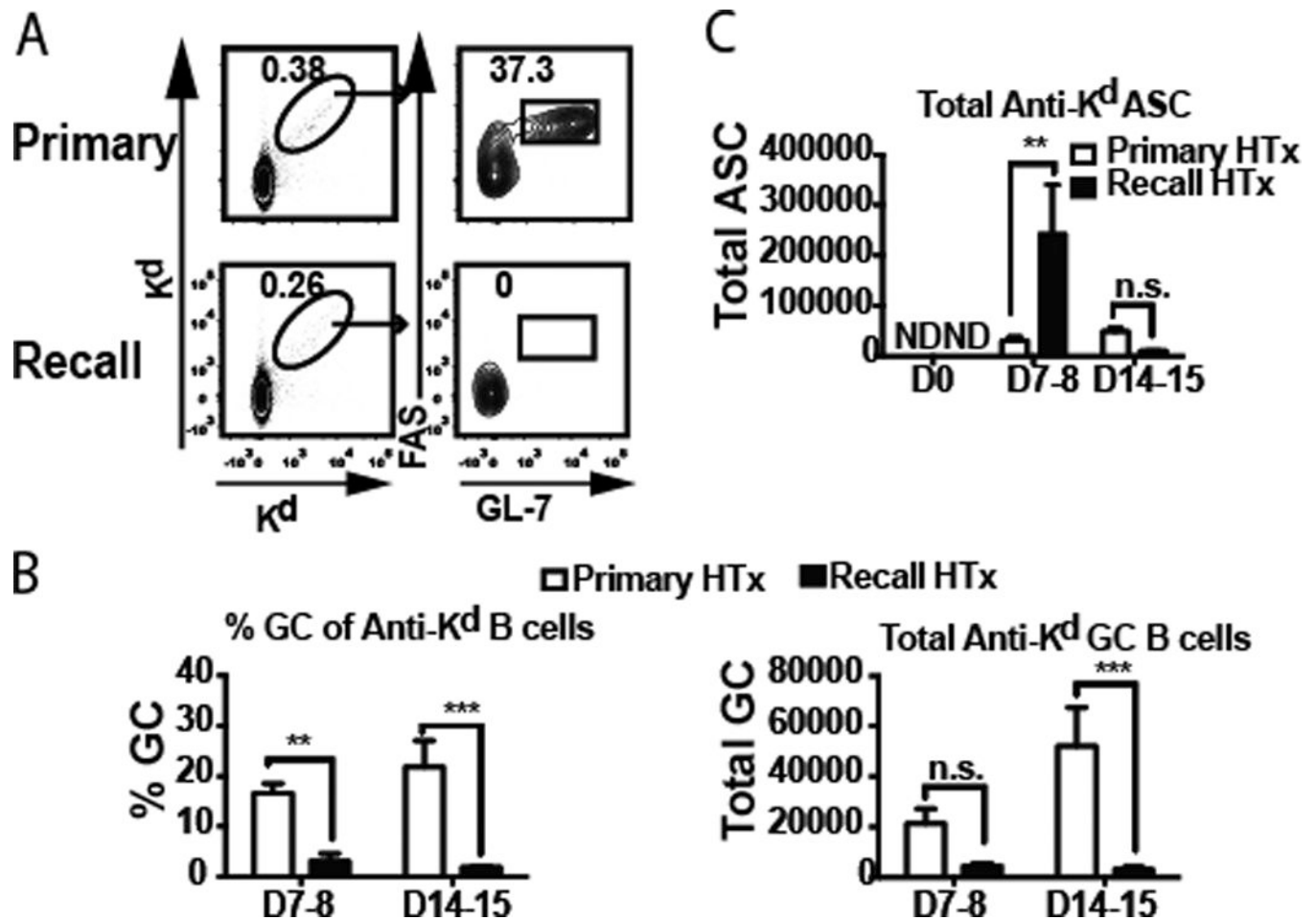


Fig 2. Germinal center (GC) responses predominate during a primary response whereas ASC responses predominate during secondary response. (A) Flow cytometry analysis of pre-gated B220⁺IgD^{low} B cells binding to APC- and PE-conjugated H2K^d tetramers, and expressing a GC phenotype (Fas+GL7+). (B) Percentage and total numbers of H-2K^d-binding GC B cells, and (C) total number of H-2K^d-specific IgG secreting ASCs per mouse (one spleen plus 6 lymph nodes) from naïve or sensitized recipients, and analyzed on the indicated days post-heart transplant. N=3–5/group, repeated twice; N.D. not detected.

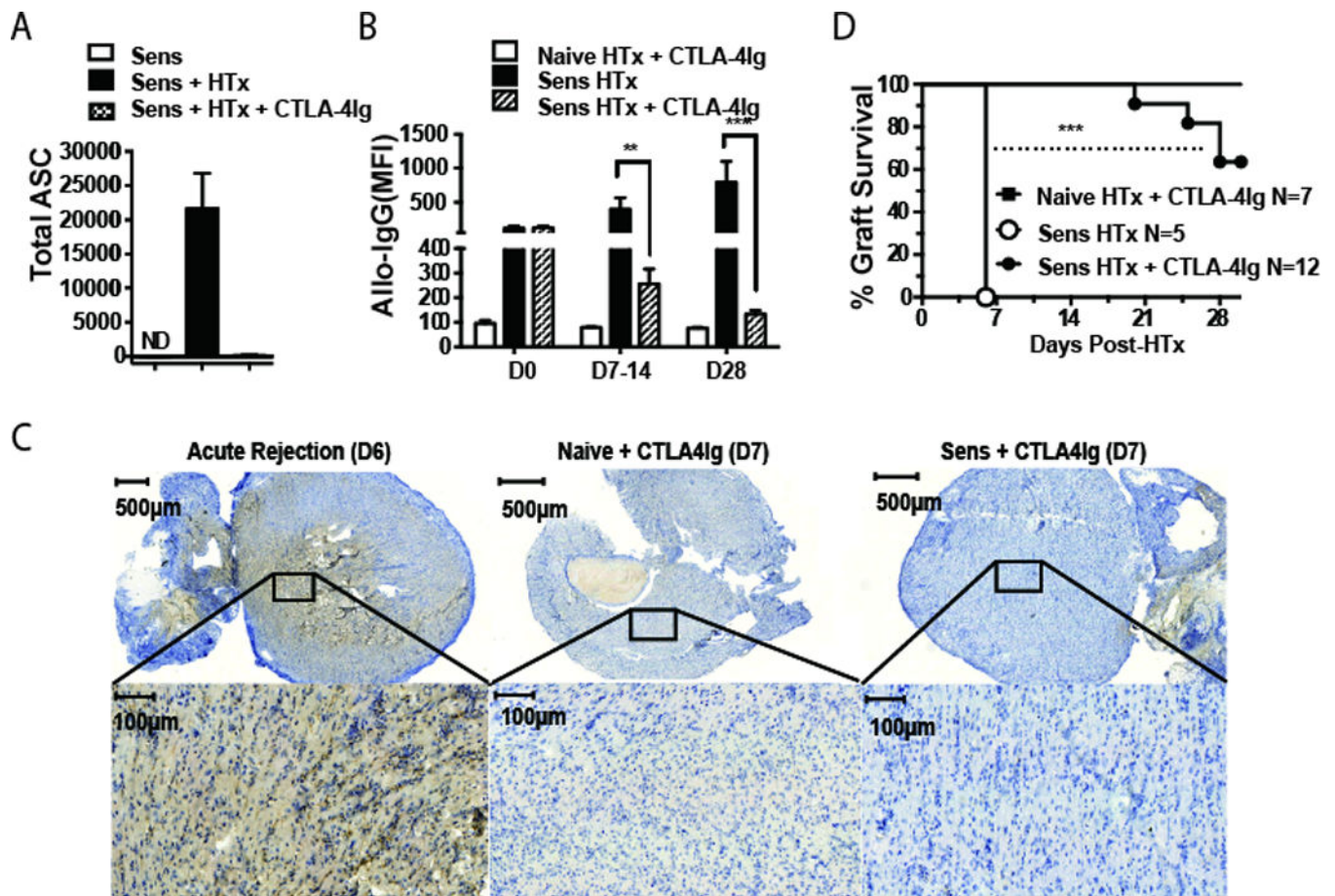


Fig 3. Continuous CTLA-4Ig inhibits the recall antibody response and prolongs allograft survival in sensitized recipients. (A) Spleen and lymph nodes were harvested from sensitized mice, prior to or on day 7 post-transplantation, and the total number of H-2K^d-specific ASC per mouse was quantified by H-2K^d-IgG ELISPOT. (B) Donor-specific IgG production was quantified by flow cytometry, and (C) C4d deposition in allografts on day 7 post-transplant was assessed by immunohistochemistry. N=2–3/group, repeated twice. (D) BALB/c allograft survival in sensitized C57BL/6 recipients.