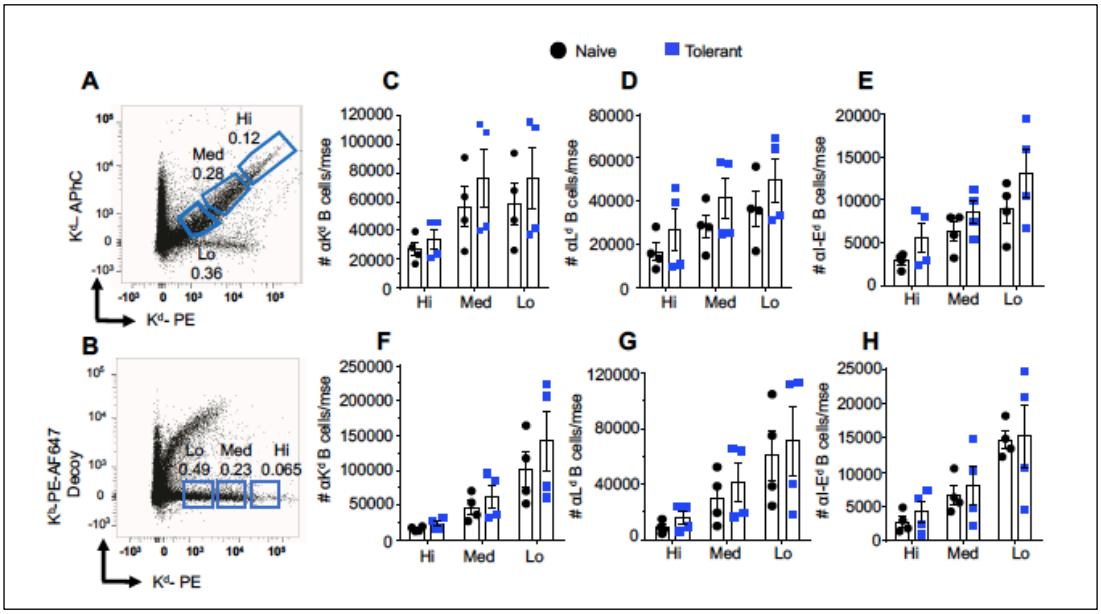
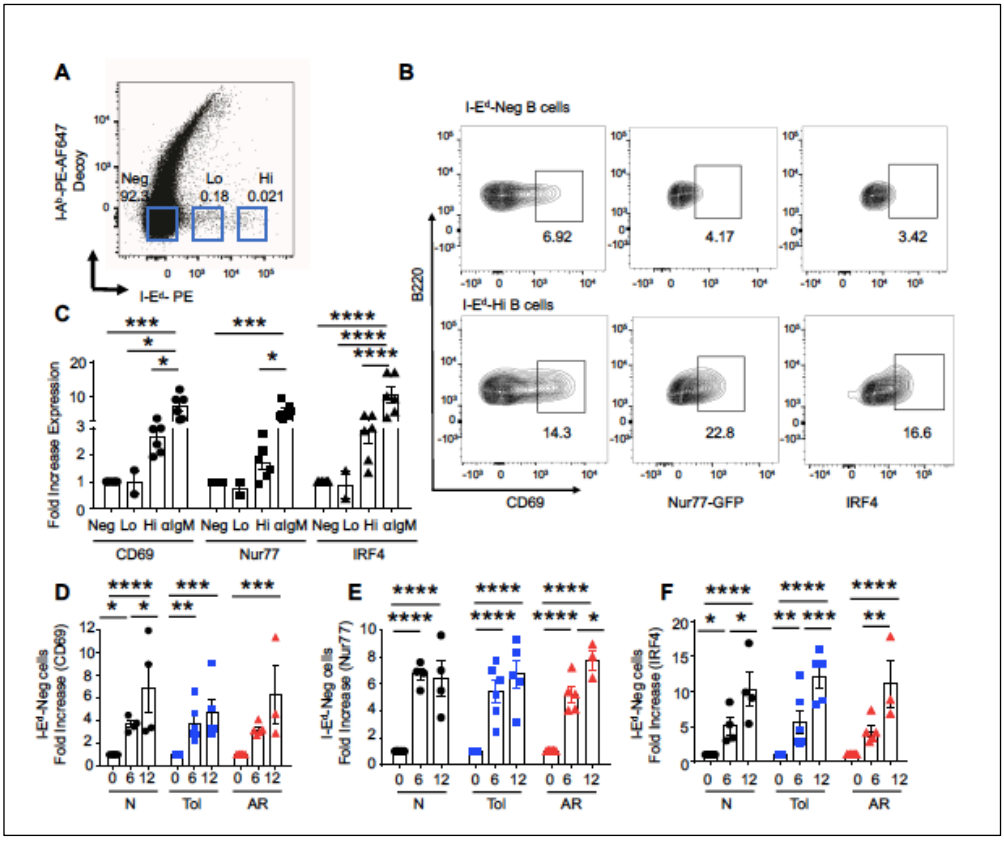
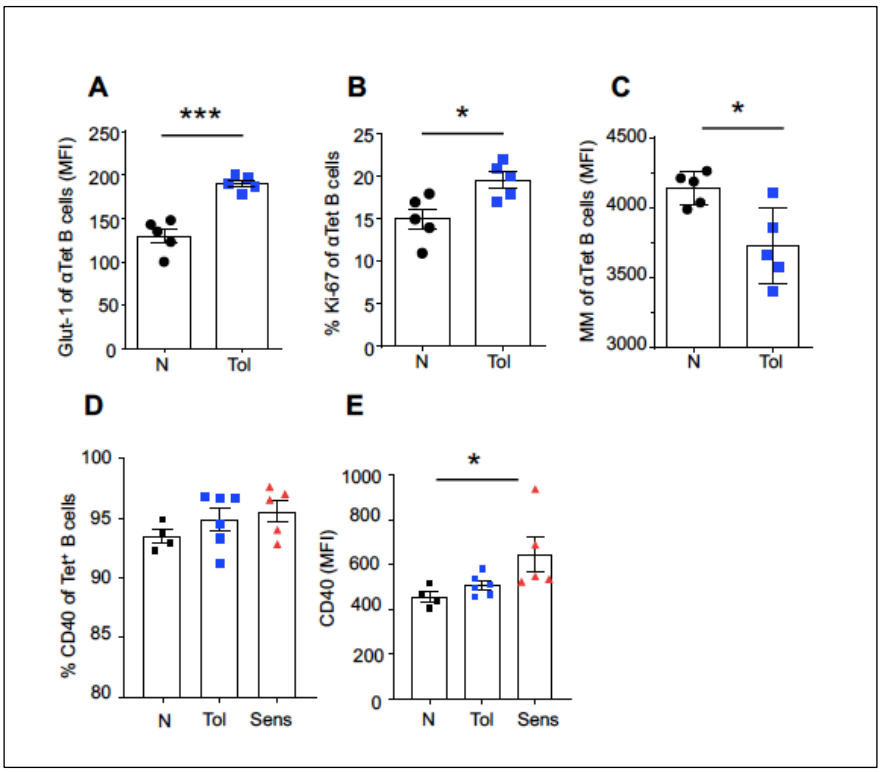
Supplemental Figures



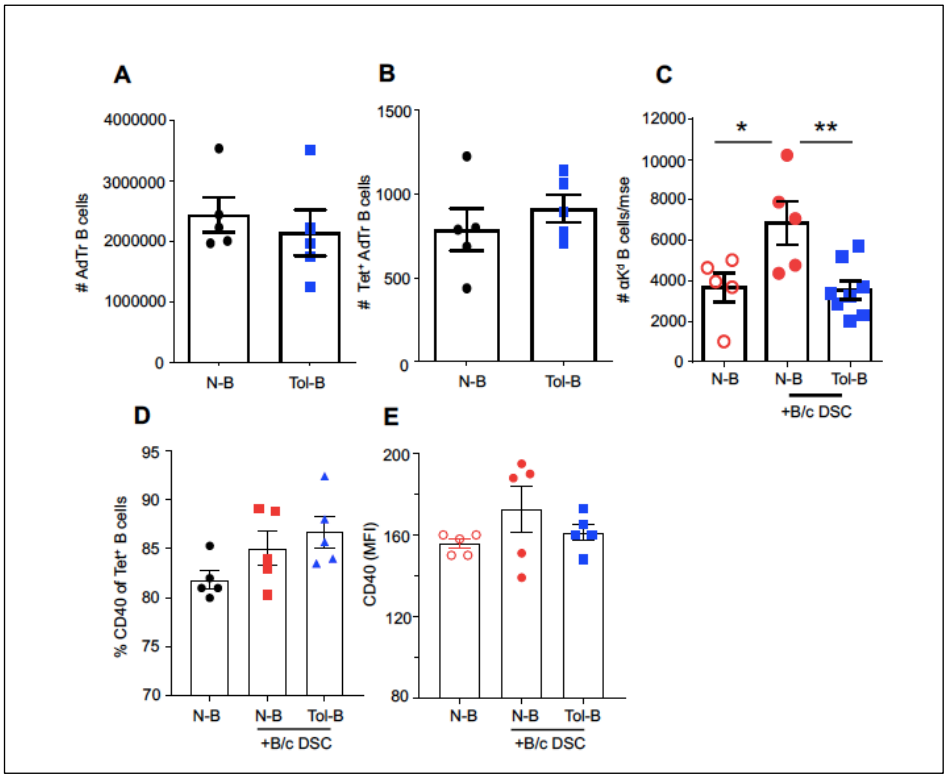
Supplemental Figure 1. Comparable numbers of B cells from B/6 naïve or tolerant mice bind to donor MHC tetramers with high (Hi), medium (Med) and low (Lo) mean fluorescence intensity (MFI). Representative flow plots of H-2K^d tetramer-binding B cells from naïve B/6 mice were divided into tetramer Hi, Med and Lo subsets, using (**A**) double-positive donor MHC Class I (K^d) tetramer conjugated to PE or APhC fluorochromes, and alternative (**B**) decoy K^b (recipient MHC)-tetramer conjugated to PE and AF647 and incubated with K^d-PE tetramers. Total number of (**C**) αK^d, (**D**) αL^d, (**E**) αI-E^d-tetramer binding B cells with Hi, Med and Lo (MFI) from naïve (black circle) or Tol mice (blue square) using dual fluorochrome single tetramer approach, *n=4/group*. Total number of (**F**) αK^d, (**G**) αL^d, (**H**) αI-E^d specific B cells with Hi, Med and Lo (MFI) from naïve (black circle) vs Tol mice (blue square) using decoy tetramer approach, *n=4/group*. Data represent mean ± SEM. Statistical significance by unpaired two-tailed Student's t test.



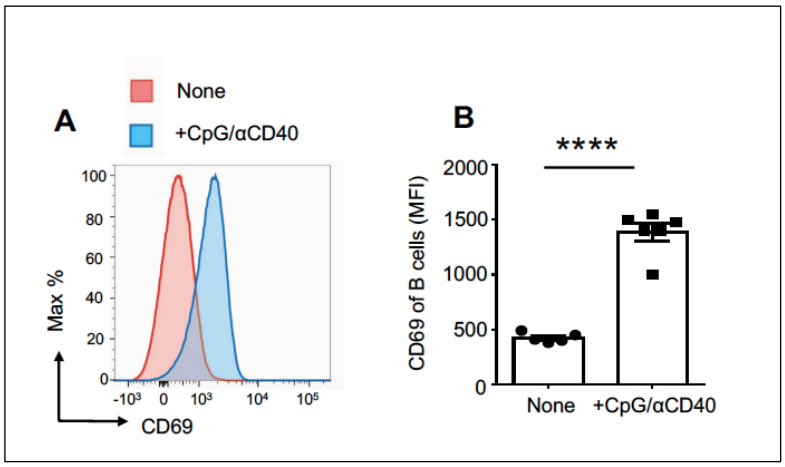
Supplemental Figure 2. Early BCR signaling by alloreactive B cells. (**A**) Representative flow plot showing flow sort I-E^d-Neg, I-E^d-Lo, and I-E^d-Hi B cells populations. (**B**) Representative flow plots depicting CD69, Nur77, and IRF4 expression in I-E^d-Neg B cells and I-E^d-Hi B cells. (**C**) Fold increase in the percentage of αI-E^d B cells expressing CD69, Nur77 and IRF4, after stimulation with immobilized I-E^d for 12 hours*, n=2-6 mice/group*. Controls were unstimulated or αIgM stimulated I-E^d-Neg B cells. Time-dependent expression of (**D**) CD69, (**E**) Nur77 and (**F**) IRF4 by αIgM stimulated I-E^d-Neg B cells from naïve, tolerant (≥day 30 post-transplant) and AR (day 7-10 post-transplant) mice, *n=4-6/ group*. Data were normalized to unstimulated I-E^d-Neg B cells cultured for 6 or 12 hours. Each dot represents an individual mouse, pooled from ≥ 2 independent experiments. Data are presented as mean ± SEM. **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001* by two-way ANOVA with Tukey's post test.



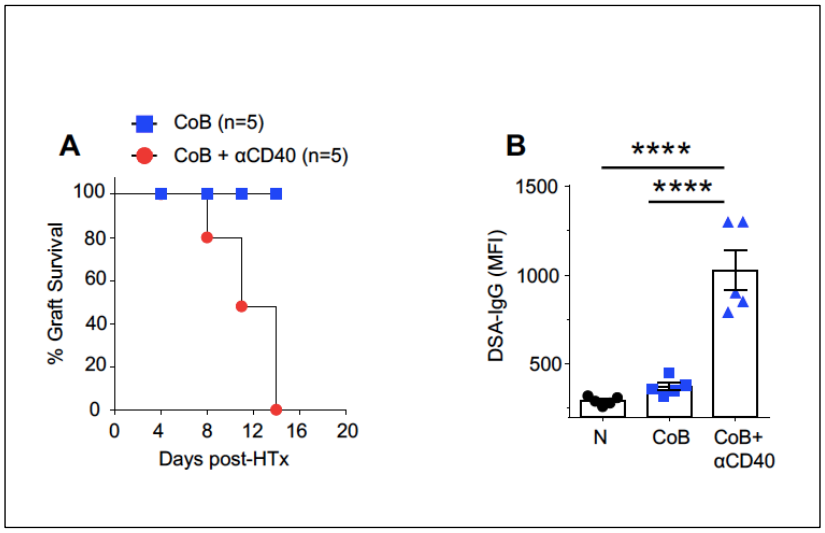
Supplemental Figure 3. Alloreactive B cells from tolerant recipients have upregulated Glut-1 and Ki-67 expression compared to alloreactive B cells from naïve B/6. (**A**) Glut-1, (**B**) Ki-67, and (**C**) Mitochondrial mass (MM) of αTet B cells (MFI) analyzed from the spleen and LNs of naïve and Tol mice (≥D30 post-transplant), *n=5/group*. (**D**) Percentage of CD40 positive and (**E**) MFI of CD40 expression by tetramer-binding B cells in naïve, tolerant (Tol) or sensitized (Sens) mice that had been immunized with $2x10^7$ donor B/c splenocytes in the flank, 12 days before analysis, *n=4-6/group*. Data represent mean ± SEM. **p<0.05, ***p<0.001* by unpaired student's t-test.



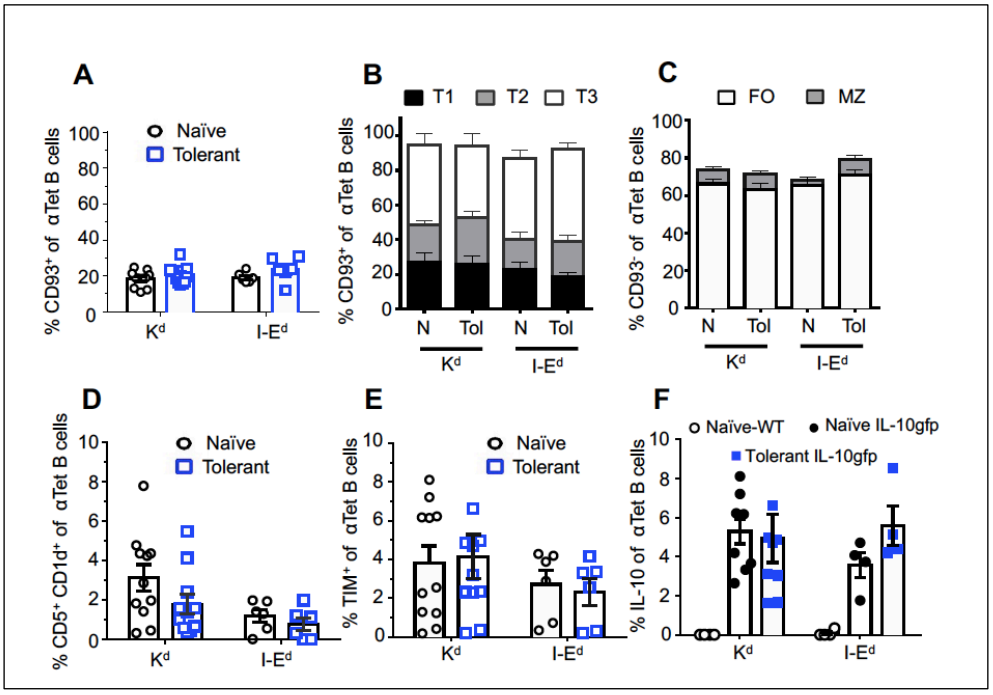
Supplemental Figure 4. Comparable numbers of anti-K^d B cells recovered from MD4 host receiving naïve or tolerant B cells. Spleen and LNs (inguinal, axillary, branchial) were harvested from MD4 recipients receiving naïve B cells with or without B/c DSC immunization, or tolerant B cells with B/c immunization, and analyzed on day 14 post-AdTr. (**A**) Total number of recovered all AdTr naïve and Tol B cells, (**B**) and Tet$^+$ AdTr naïve B and Tol B cells, recovered on day 14 post-AdTr into MD4 mice (no DSC immunization), *n=5/group.* (**C**) Total number of αK^d binding B cells, *n=5-8/group*. (**D**) Percentage of CD40-positive and (**E**) MFI of CD40 expression by naïve B and Tol B cells on day 14 post-AdTr into MD4 hosts, *n=5/group.* Data represent mean ± SEM. **p<0.05, **p<0.01* by one-way ANOVA with Bonferroni post test.



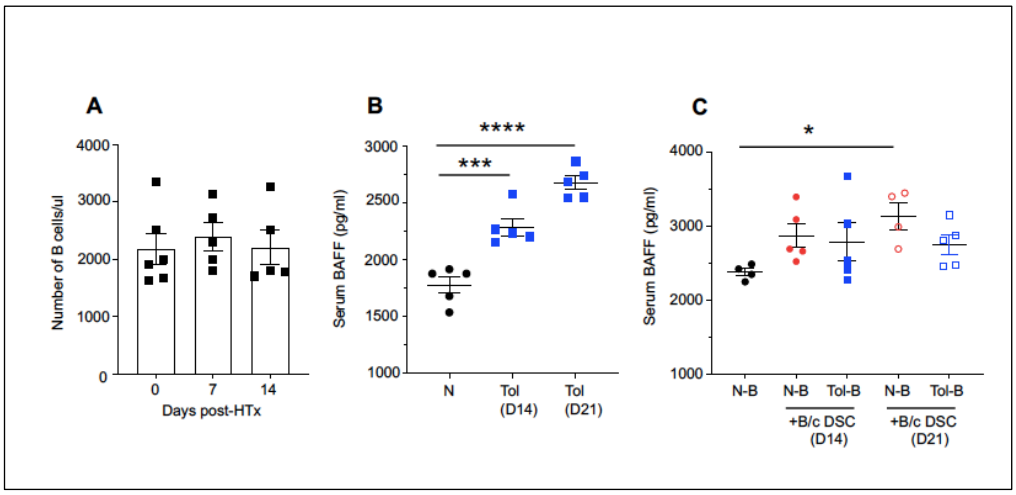
Supplemental Figure 5. Agonistic αCD40+CpG stimulates polyclonal B cell activation. (**A**) Representative histogram and (**B**) quantification of CD69 (MFI) expression on B cells from naïve mice receiving CpG (100μg/mse, i.v. given on day 0 and 50μg/mse, i.p. at day 1, 2) + αCD40 (100μg/mse, i.v. at day 0). Mice were sacrificed on day 3 post-CpG+αCD40, *n=5-6/group*. Data were pooled from 2 independent experiments. Data represent mean ± SEM. ****$p<0.0001$ by unpaired student's t-test.



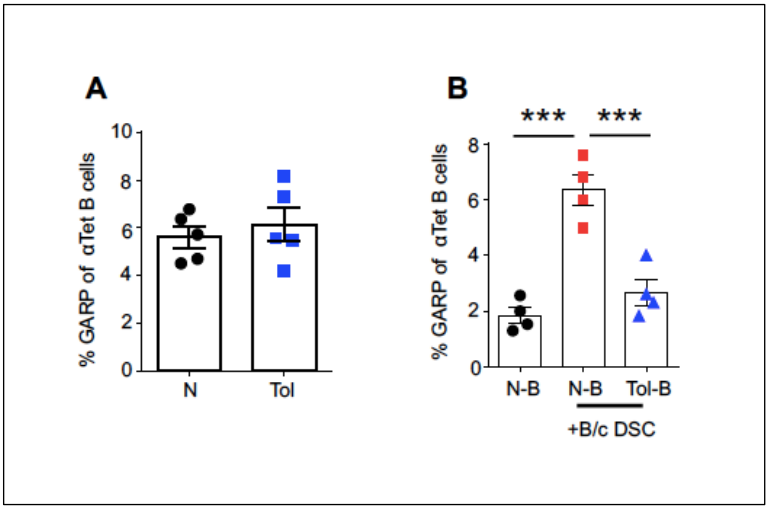
Supplemental Figure 6. Agonistic anti-CD40 induces allograft rejection and DSA production. Agonistic αCD40 (100μg/mse, i.v.) administered on D0 and D7 post-heart transplantation and tolerance treatment with anti-CD154+DSC (CoB). (**A**) Percent graft survival in tolerant mice and tolerant mice ± agonistic αCD40. (**B**) DSA-IgG (MFI) measured on D14 post-transplant, *n=5/group*. Data were pooled from 2 independent experiments. Data represent mean ± SEM. ****$p<0.0001$ by one-way ANOVA with Bonferroni post test and percent graft survival of the heart allografts by log-rank test.



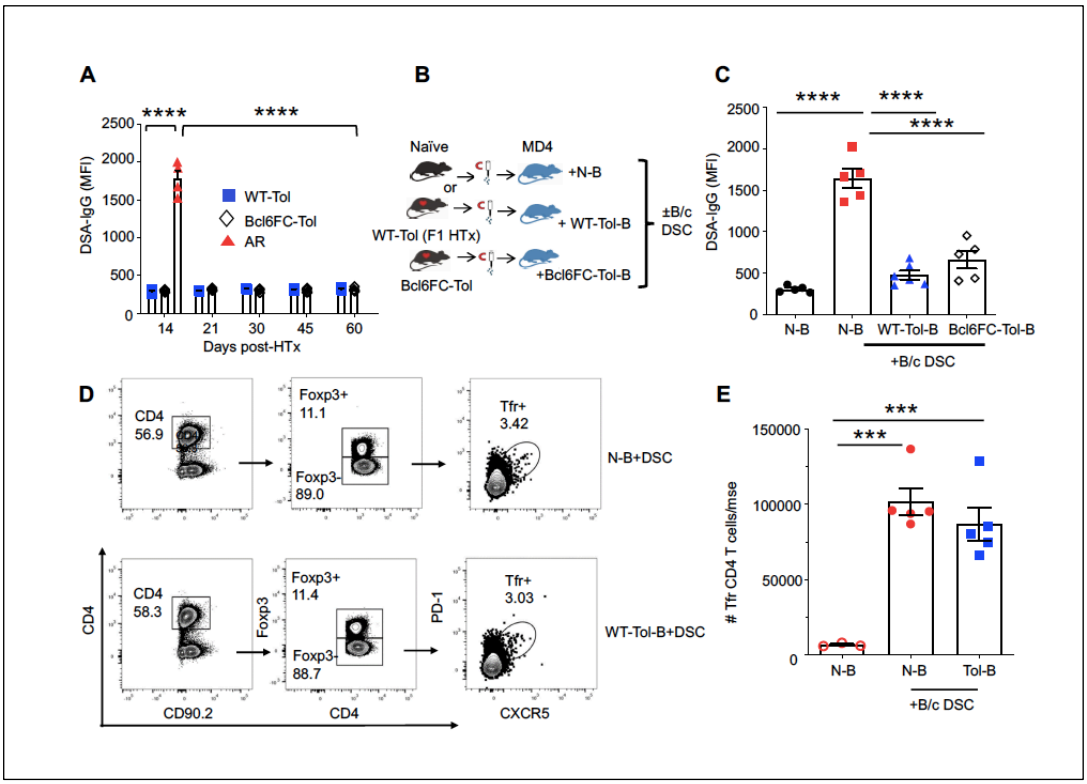
Supplemental Figure 7. Donor-specific B cells from tolerant recipients do not exhibit enrichment of markers associated with transitional B cells and Bregs. Analyses of B220, CD93, IgM and CD23 expression by alloreactive B cells from naive and Tol mice. Following gating on $B220^{+}Dump^{-}$ cells, quantification of (**A**) % CD93 of αK^{d} or αI-E^{d} specific B cells, *n=6-11/group*. (**B**) T1 ($IgM^{+}CD23^{-}$), T2 ($IgM^{+}CD23^{+}$), T3 (IgM^{-} $CD23^{+}$) within the $CD93^{+}$ αK^{d} or αI-E^{d} B cell populations, *n=6-9/group*. (**C**) % follicular ($CD21^{+}CD23^{hi}$) and marginal zone ($CD21^{hi}CD23^{-}$) within $CD93^{-}$ αK^{d} or αI-E^{d} B cells, *n=6-11/group*. (**D**) % $CD5^{+}CD1d^{+}$ of αK^{d} or αI-E^{d}-specific B cells, *n=6-11/group*. (**E**) % TIM-1^{+} of αK^{d} or αI-E^{d} B cells in tolerant vs naïve mice, *n=6-11/group*. (**F**) % of IL-10 of αK^{d} or αI-E^{d} B cells from naïve B/6, or naïve and tolerant IL-10 gfp-reporter mice (B6.129S6-*Il10*^{tm1Flv}/J). Spleen and lymph node cells were stimulated with LPS, PMA + Ionomycin for 8-10 hours, and then incubated with MHC tetramers and other antibodies. GFP expression was used to assess IL-10, *n=4-9/group*. Data represent mean ± SEM. Statistical significance by two-way ANOVA with Tukey's post test and Kruskal-Wallis or Holm-Sidak's multiple comparison tests.



Supplemental Figure 8. No B cells deletion and no reduction of circulating BAFF in tolerant recipients. (**A**) Tolerant recipients have comparable total number of circulating B cells on day 7 and 14 post-HTx. Heparinized peripheral blood from Tol mice were collected on days 0, 7 and 14 post-HTx and anti-154+DSC treatment. Absolute counts (cells per microliter) of B cells in the peripheral blood of Tol mice were analyzed on day 0, 7 and 14. *n=5-6/group*. Data were pooled from 2 independent experiment. (**B**) Circulating serum BAFF levels measured by ELISA in naïve or tolerant mice on day 14 and 21 post-HTx, *n=5/group*. (**C**) Serum BAFF levels from MD4 mice that received naïve or tolerant B cells immunized with DSC. *n=4-5/group*. Data were pooled from 2 independent experiments and are presented as mean ± SEM. **p<0.05, ***p<0.001*, *****p<0.0001* by one-way ANOVA with Bonferroni post-test.



Supplemental Figure 9. GARP expression is not upregulated in tolerant B cells. (**A**) % GARP^{+} of αTet B cells in naïve and Tol mice, *n=5/group*. (**B**) % GARP of αTet B cells in MD4 hosts of naïve B cells or Tol B cells ± immunization with B/c DSC, and analyzed on day 14 post-AdTr, *n=5/group*. Data were pooled from 2 independent experiments. Data represent mean ± SEM. ****p<0.001* by one-way ANOVA with Bonferroni post test.



Supplemental Figure 10. Lack of alloantibody production and induction of B cell tolerance in F1 HTx Bcl6FC recipients treated with CoB. (**A**) Tolerant (Tol) C57BL/6 or Bcl6FC mice received F1 HTx and treated with anti-CD154 (D0, 7, 14) + DSC (D0), while AR mice received F1 HTx without treatment. DSA-IgG from WT and Bcl6FC-Tol recipients on day 14, 21, 30, 45, 60, and AR recipients on day 14 post-HTx, *n=4/group*. *****p<0.0001* by two-way ANOVA with Tukey's post-test for multiple comparisons. (**B**) Experimental design. $2x10^7$ enriched B cells from naive, and WT or Bcl6FC-Tol mice on day 60 post-HTx, together with $1x10^3$ purified TCR75 T cells and $5x10^6$ purified B/6 T cells were adoptive transferred (AdTr) into MD4 recipients, which were then immunized with B/c DSC the following day. (**C**) DSA-IgG (MFI) from naïve B, WT-Tol-B or Bcl6FC-Tol-B cells recipients were measured on day 14 post-AdTr, *n=5-6/group.* (**D**) Gating strategy used to identify follicular regulatory T cells (Tfr). Spleens and LNs (inguinal, axillary, branchial lymph nodes) were harvested from MD4 hosts that received naïve B cells or Tol B cells on day 14 post-AdTr. $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells were gated as in the flow plot. Tfr cells are defined as $Foxp3^+CXCR5^{hi}PD\text{-}1^{hi}$. (**E**) Total number of Tfr ($Foxp3^+CXCR5^+PD\text{-}1^+$) CD4 T cells/mse from the spleen and LNs harvested on day 14 post-AdTr, from MD4 mice receiving naïve B or Tol B cells, then immunized with $2x10^7$ B/c DSC, *n=3-5/group.* Data are presented as mean ± SEM. ****p<0.001, ****p<0.0001* by one-way ANOVA with Bonferroni post-test.