Supplemental Material

B cells mediate chronic allograft rejection independently of antibody production

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Index

1. Supplemental Methods

Mice: C57BL/6 (H-2^b; hereafter WT, B6.129S2-Igh-6^{tm1Cgn}/J (H-2^b; hereafter μMT) (1), B6(C)-*H2^{bm12}*/KhEgJ (H-2^b-$Ab1^{bm12}$; hereafter Bm12) (2, 3), C57BL/6-Tg(IghelMD4)4Ccg/J (H-2^b; hereafter Ig^{HEL}) (4) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory. B6.129-H2Ab1t^{m1Gru}B2m^{tm1Jae} (B2m/Abb; H-2^b; hereafter MHC-KO) mice were purchased from Taconic Farms. Secretory IgM (*Ighm*) deficient (μS KO) and Activation-Induced Deaminase (AID; *Aicda*) deficient (AID KO), double-knockout mice (H-2^b; hereafter AID/μS KO) were obtained from Dr. Frances E. Lund (5, 6). All animals were maintained under SPF conditions and prophylactic antibiotics to prevent opportunistic infections. All procedures were performed as per IACUC guidelines.

Bone marrow chimeras: μMT mice were lethally irradiated and transplanted with a combination of syngeneic (H-2^b) T and B cell depleted bone marrow cells (1x 10^7) from μMT and WT (80% + 20%) or μMT and MHC-KO (80% + 20%) or μMT (100%) donors. All recipients were treated with anti-NK1.1 (200μg on day -1 and day -2) (PK136, Bioexpress Inc) to deplete NK cells and limit rejection of donor bone marrow cells lacking MHC I expression (7). Restricting non-μMT bone marrow cells to 20% of transferred donor bone marrow allowed reconstitution of all B cells from these cells while limiting reconstitution of non-B cell hematopoietic lineages such as T cells and DCs from these donors to ≤ 20% of the specific population as confirmed by FACS analyses at ≥ 8 weeks after reconstitution (Supplemental Figure 3A).

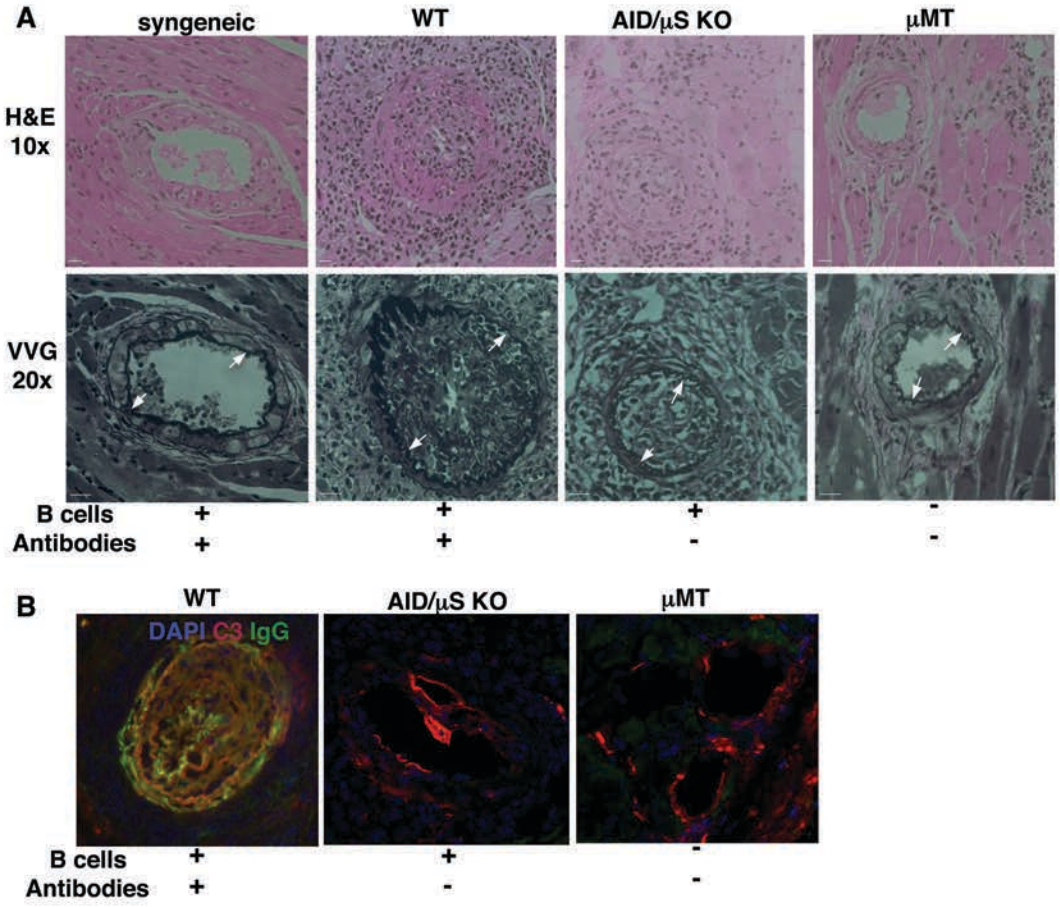
Heterotopic heart transplantation and costimulation blockade: Heart grafts were transplanted heterotopically in the abdomen of the recipient as previously described (8). Recipients of BALB/c heart allografts were treated with CTLA4Ig and anti-CD40L (MR1) (Bioexpress Inc), 0.25mg each, on days 2, 4, 6, and 8 after transplantation to prevent T cell activation by blocking costimulation and inhibit acute rejection (9). As established previously, recipients treated with costimulation blockade maintained palpable allograft heartbeat and contraction for >100 days of monitoring (9). Untreated recipients of Bm12 heart allografts develop chronic allograft rejection (2, 10) and were harvested between 90 – 100 days after transplantation (MST > 90 days in all groups, $P > 0.05$, NS). Logrank test (Graphpad Prism 5.0, Graphpad Software, Inc) was used to assess differences in allograft survival and P value of < 0.05 was considered significant.

Histological analyses and immunofluorescence staining: Transplanted hearts were harvested between 90 – 110 days. Grafts were sectioned transversely. Fixed, paraffin-embedded tissue sections of the base of the heart were stained with H&E and Verhoeff Van Gieson (VVG) for elastin fibers. Images of coronary arteries stained with VVG were analyzed by morphometry using ImageJ software for quantitation of obliterative vasculopathy. Intima was identified as lined by Internal Elastic Lamina (IEL). IEL and lumen were traced in each vessel and luminal occlusion (%) was calculated as (IEL area – luminal area/IEL area) x 100 (8). Vessels with any luminal occlusion were included to quantify percentage of vessels involved in each recipient and percentage of luminal occlusion in each vessel. Immunofluorescence studies were done on 6-8μm frozen sections of heart allograft and spleen tissues harvested from recipients between 90 – 110 days after transplantation. All antibodies were purchased from ebioscience and BD pharmingen except where indicated. Spleen cryosections were stained with biotinylated anti-MOMA-1 (ab51814, Abcam) for marginal zone metallophilic macrophages, anti-B220 (RA3-6B2) for B cells, anti-CD4 (RM4-5) for CD4 T cells and anti-CR1/2 (7E9) for follicular dendritic cells. Streptavidin conjugated Alexafluor-488, Alexafluor-555 and/or Alexafluor-647 (Invitrogen) were used as secondary antibodies. Heart allograft cryosections were stained with biotinylated anti-CD4 and anti-CD8α (53-6.7) or anti-mouse IgG and anti-mouse C3 (11H9; Abcam) followed by streptavidin conjugated Alexafluor-488 and Alexafluor-555 secondary antibodies. 11H9 antibody used for detecting C3 reacts with mouse C3, C3b, iC3b and C3d. All sections were stained with DAPI, sealed under vectashield mounting media (Vector Labs) and imaged using Nikon Eclipse 800 microscope.

B cell transfer, flow cytometry, intracellular cytokine staining and detection of alloantibodies: B cells were isolated from spleens of naïve AID/μS KO mice by labeling with biotinylated antibodies against CD3, CD11b, CD11c and NK1.1 (BD Pharmingen and eBioscience) followed by streptavidin MACS beads (Miltenyi Biotec) and magnetic depletion of non-B cells (11). 2×10^7 B cells (90 ± 3% purity) were adoptively transferred into μMT recipients where indicated 2 - 4 days prior to heart transplantation. Cells were harvested from spleen (SP), lymph node (LN), and bone marrow (BM) of transplant recipients at the time of graft harvest between 90 – 110 days after transplantation. BM cells were obtained from femurs and tibia, and multiplied by a factor of 5.4 to estimate total body BM cells (12, 13). Harvested cells were stained with fluorochrome-tagged antibodies to CD8α (53-6.7), CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2 F3), IFNγ (XMG1.2) and TNFα (MP6-XT22) from BD Pharmingen and eBioscience for FACS. Intracellular IFNγ and TNFα in T cells were measured after stimulation with BALB/c splenocytes ($H\text{-}2^d$) (1:1) for 6hr or 24hr as indicated. Naïve WT, AID/μS KO and μMT mice showed intracellular IFNγ and TNFα in CD4 T cells (0.13%, 0.11%, 0.14% and 0.14%, 0.16%, 0.18%, respectively) and CD8 T cells (0.08%, 0.06%, 0.07% and 0.09, 0.11 and 0.13%, respectively) that were not significantly different, and are not depicted separately. Flow cytometry acquisition was performed on LSRII analyzer (BD Biosciences), and data were analyzed using Flowjo software (Treestar). BALB/c-reactive alloantibodies were detected in the serum of allograft recipients at the time of graft harvest by flow cytometry using BALB/c thymocytes as target cells (14).

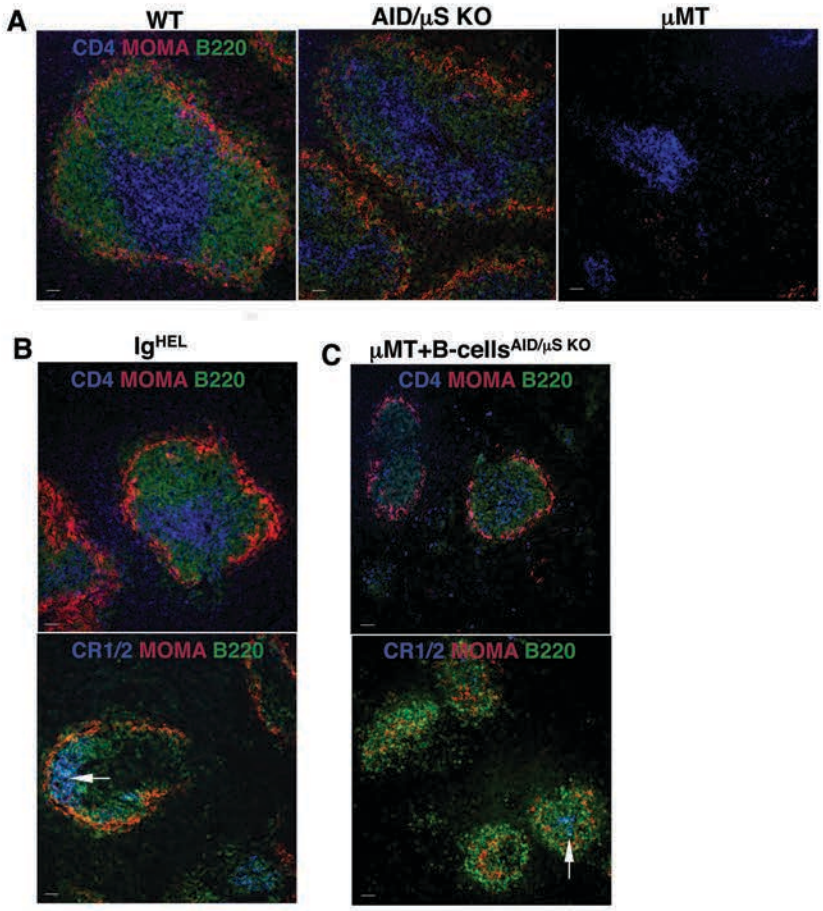
2. Supplemental Figures

Supplemental Figure 1. Histological analyses of heart allografts.



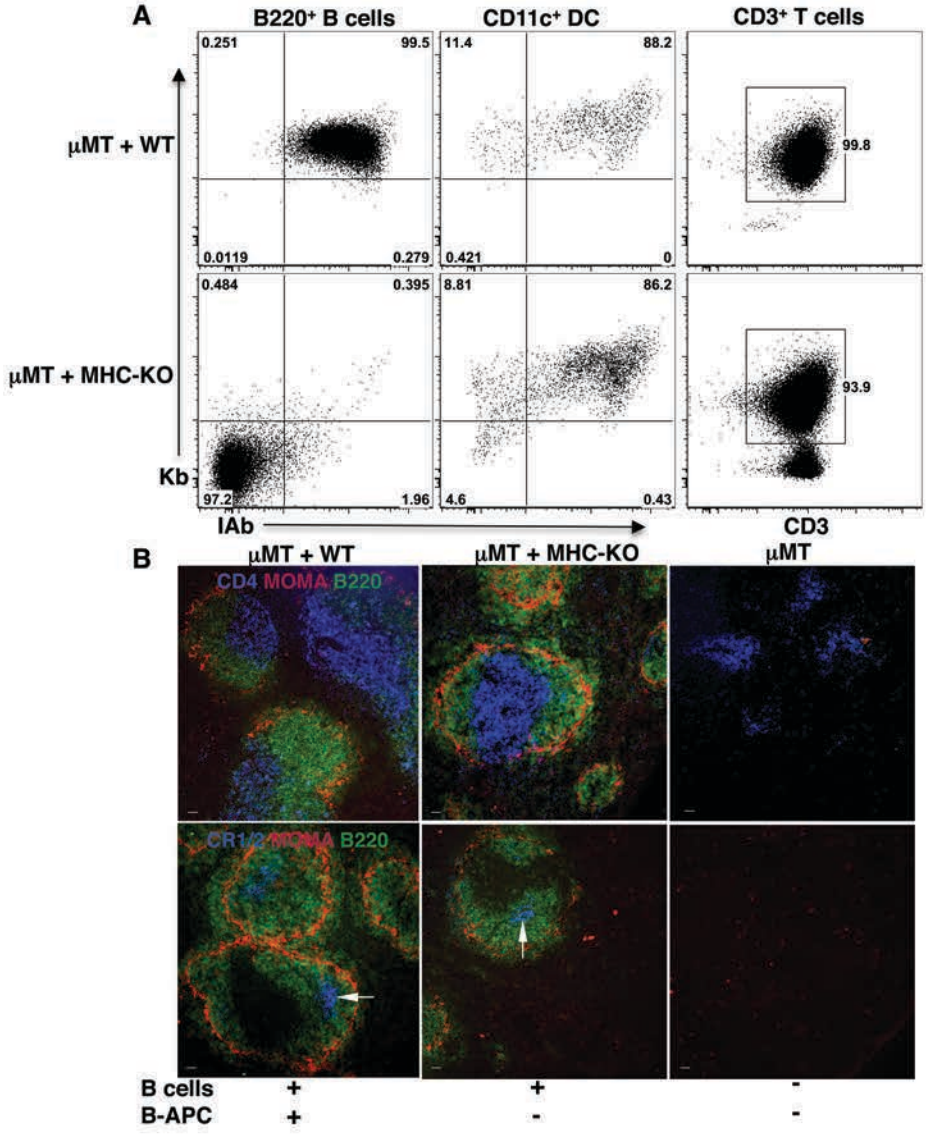
WT, AID/μS KO and μMT recipients were transplanted with BALB/c hearts and treated with costimulation blockade (CTLA4Ig and anti-CD40L, 0.25mg each, on days 2, 4, 6, and 8 after transplantation). Allografts were harvested at 100 – 110 days after transplantation. (**A**) Representative images of heart allograft sections (arrows point to internal elastic lamina, scale bar: 150μm). (**B**) Representative images of IgG and C3 staining in heart allograft cryosections (20x, blue for DAPI, red for C3 and green for IgG).

Supplemental Figure 2. Splenic lymphoid architecture in allograft recipients.



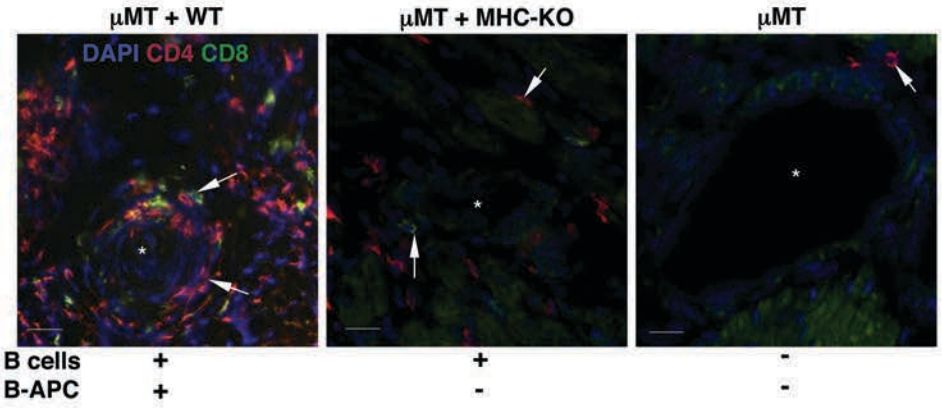
WT, AID/μS KO, μMT, Ig^{HEL} and $\mu MT+B^{AID_{/\mu}S\ KO}$ recipients were transplanted with BALB/c hearts and treated with costimulation blockade (CTLA4Ig and anti-CD40L, 0.25mg each, on days 2, 4, 6 and 8 after transplantation). $\mu MT+B^{AID_{/\mu}S\ KO}$ recipients were μMT mice that received naïve AID/μS KO B cells (2×10^7, 2 - 4 days before transplantation). Mice were harvested between 90 – 110 days after transplantation. (**A**) Representative images of spleen cryosections from recipients stained for CD4 (blue), MOMA-1 (red) and B220 (green) are shown (10x, scale bar 150μm). (**B - C**) Representative images of spleen cryosections from Ig^{HEL} (B) and $\mu MT+B\text{-cell}^{AID_{/\mu}S\ KO}$ (C) recipients showing CD4 (blue), MOMA-1 (red), B220 (green) in top panels and CR1/2 (blue), MOMA-1 (red), B220 (green) in bottom panels (arrow points to $CR1/2^+$ follicular dendritic cell areas) (10x, scale bar 150μm).

Supplemental Figure 3. MHC expression and splenic lymphoid architecture in bone marrow chimeras.



μMT mice were lethally irradiated and reconstituted with bone marrow cells from μMT and WT (10^7, 80:20, μMT+WT→μMT, μMT+WT) or μMT and MHC I & II deficient, *H2-Ab1*$^{-/-}$x*b2m*$^{-/-}$ (10^7, 80:20, μMT+MHC-KO→μMT, μMT+MHC-KO) bone marrow cells. At 8 – 12 weeks after reconstitution, chimeras were transplanted with BALB/c heart allografts, treated with costimulation blockade (CTLA4Ig and anti-CD40L) and harvested between 90 – 100 days after transplantation. (**A**) Representative FACS plots of spleen cells from μMT+WT and μMT+MHC-KO chimeras are shown for Kb (MHC I) and IAb (MHC II) expression on B220$^+$ B cells (99 ± 0.5% and 99 ± 0.3% vs. 0.5 ± 0.6% and 1.5 ± 0.5%, respectively) and CD11c$^+$ DCs (99 ± 0.5% and 88 ± 2% vs. 95 ± 4% and 85 ± 3%, respectively) cells, and Kb expression on CD3$^+$ T cells (99 ± 0.8% vs. 90 ± 4%, respectively). (**B**) Representative images of spleen cryosections from chimeras showing CD4 (blue), MOMA-1 (red), B220 (green) in top panels and CR1/2 (blue), MOMA-1 (red), B220 (green) in bottom panels (arrow points to CR1/2$^+$ follicular dendritic cell areas) (10x, scale bar 150μm).

Supplemental Figure 4. Heart allograft infitrates in chimera recipients.



μMT mice were lethally irradiated and reconstituted with bone marrow cells from μMT and WT (10^7, 80:20, μMT+WT➔μMT, μMT+WT) or μMT and MHC I & II deficient, *H2-Ab1*$^{-/-}$x*b2m*$^{-/-}$ (10^7, 80:20, μMT+MHC-KO➔μMT, μMT+MHC-KO) bone marrow cells. At 8 – 12 weeks after reconstitution, chimeras were transplanted with BALB/c heart allografts, treated with costimulation blockade (CTLA4Ig and anti-CD40L) and harvested between 90 – 100 days after transplantation. Representative images of CD4 (red), CD8 (green) and DAPI (blue) in heart allograft cryosections from chimeras (20x, scale bar: 150μm, arrows point to $CD4^+$ or $CD8^+$ T cells).

3. Supplemental References

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