Supplementary Material

IRF4 ablation in B cells abrogates allogeneic B cell responses and prevents chronic transplant rejection

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Supplementary Material and Methods

Flow cytometric analysis

Fluorochrome-conjugated antibodies specific for B220 (clone RA3-6B2), CD43 (1B11), CD45 (30-F11), IgD (11-26c.2a), IgM (RMM-1), CD24 (M1/69), CD93 (AA4.1), CD23 (B3B4), CD21 (7E9), CD138 (281-2), GL-7 (GL7), Fas (SA367H8), CD4 (GK1.5), CD8 (53-6.7), IgG1 (RMG1-1), CD38 (90), CD44 (IM7), CD62L (MEL-14), IFN-γ (XMG1.2), IL-17A (TC11-18H10.1), Blimp-1 (5E7), CD3 (145-2C11), CD11b (M1/70), CD11c (N418), and Gr-1 (RB6-8C5) were purchased from BioLegend. Fluorochrome-conjugated antibodies specific for BP-1 (6C3), Foxp3 (FJK-16s), CD5 (53-7.3), CD19 (eBio1D3), and PD-1 (J43) were purchased from Thermo Fisher Scientific. Fluorochrome-conjugated antibodies specific for Bcl6 (K112-91) and NK1.1 (Pk136) were purchased from BD Biosciences. Phycoerythrin-conjugated NP (Cat: sc-396483) was purchased from Santa Cruz Biotechnology. In brief, splenocytes and bone marrow cells were stained with fluorochrome-conjugated antibodies and analyzed on an LSR II cytometer. For detecting CXCR5 expression, cells were stained with biotin-conjugated antibody for CXCR5 (2G8; BD Biosciences) and BV421 streptavidin (BioLegend). Dead cells were excluded from analysis by using the Zombie Aqua Fixable Viability Kit (BioLegend). Expression of transcription factors was determined by using the Foxp3 staining Buffer Set (Thermo Fisher Scientific). For intracellular staining of cytokines, cells were re-stimulated for 4 hours with 50 ng/ml phorbol 12-myristate 13-acetate and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences), fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences), followed by staining with fluorochrome-conjugated antibodies against cytokines. The data were processed by using the FlowJo v10 software (Tree Star, Inc.).

NP-KLH immunization

Eight-week-old male *Irf4*$^{fl/fl}$ control and *Irf4*$^{fl/fl}$*Cd19-Cre* mice were i.p. immunized with 100 μg of 4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin (NP-KLH) (Biosearch Technologies) precipitated in alum (Thermo Fisher Scientific). Host immune response to NP-KLH was determined by ELISA and flow cytometry analysis.

ELISA

Diluted serum samples (1:8100) were added into 96-well ELISA plates that were coated with 5 μg/ml NP5-BSA or NP25-BSA (Biosearch Technologies) and incubated overnight at 4 ^{o}C, followed by incubation with HRP-conjugated goat anti-mouse IgM (SouthernBiotech) and IgG1 (SouthernBiotech) for 1 hour at room temperature. The reactions were detected with TMB peroxidase EIA substrate Kit (Bio-Rad Laboratories), and measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments) at 450 nm. The average blank OD was subtracted from OD values of each sample.

Heart and skin transplantation

Balb/c hearts were transplanted into 8- to 10-week-old male *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$*Cd19-Cre* mice as previously described.[16] Recipients were administered i.p. with 250 μg human recombinant CTLA4-Ig (BristolMyers-Squibb) on days 0 and 2 post-transplant, or left untreated. Graft survival was monitored daily by transabdominal palpation.

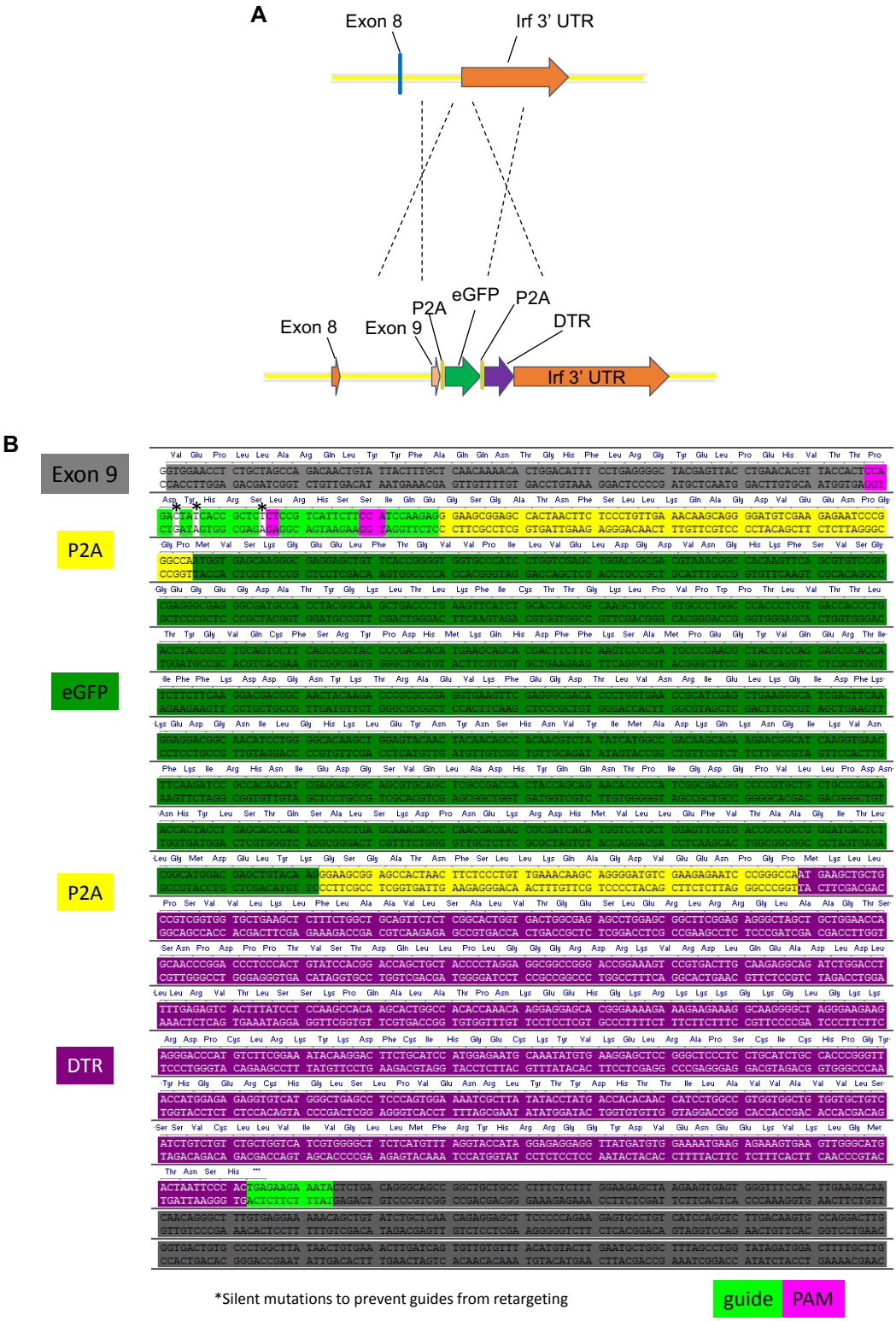
For the donor-antigen sensitization model, *Irf4*$^{fl/fl}$ and *Irf4*$^{fl/fl}$*Cd19-Cre* mice were transplanted with Balb/c skins as previously described.[19] Thirty days later, recipient mice were

transplanted again with Balb/c hearts and treated with 250 µg CTLA4-Ig on days 0, 2, 4, and 6 post-heart transplantation.

Detection of DSA in serum

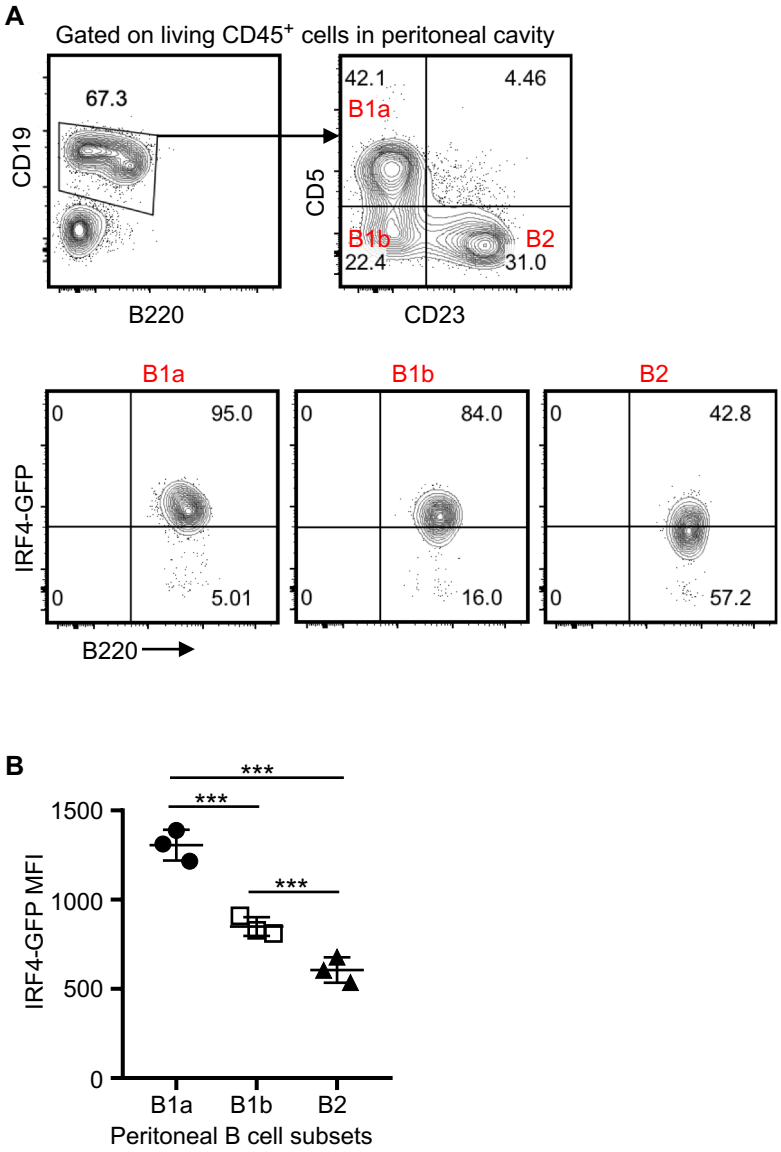
Serum samples were collected from the tail vein of transplant recipients at different days post-transplant as indicated, and were stored in a -80 ℃ freezer until analysis. In brief, Balb/c thymocyte suspensions were incubated with TruStain FcX (anti-mouse CD16/32; BioLegend) for 20 minutes on ice. After washing twice with PBS (2% FBS), Balb/c cells (0.5×10^6 cells in 50 µl) were incubated with 25 µl of serum from transplant recipients for 1 hour at 4 ℃. Cells were washed and incubated with fluorochrome-conjugated anti-mouse IgG1 (RMG1-1, BioLegend) and CD45 (clone 30-F11, BioLegend) for 30 minutes at 4 ℃. IgG1 mean fluorescence intensity (MFI) of $CD45^+$ Balb/c cells were measured by flow cytometry.

Figure S1



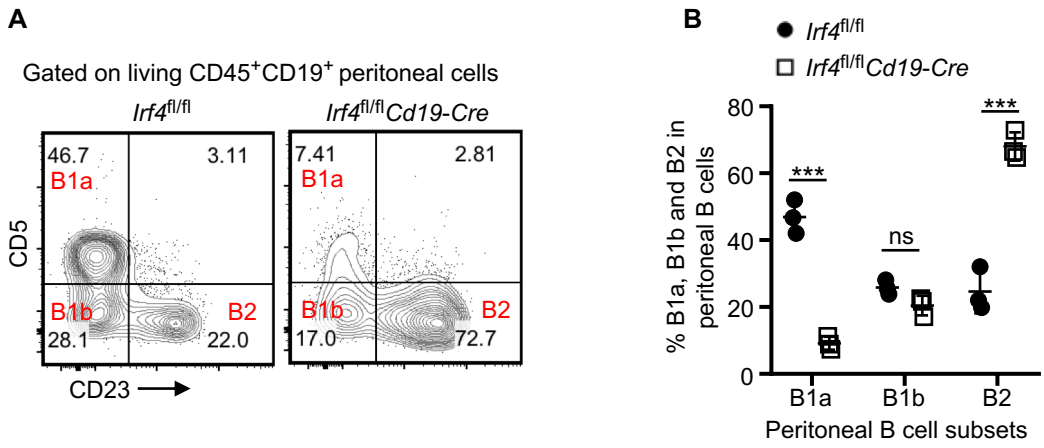
Supplemental Figure 1. The generation of *Irf4*^{gfp} reporter mice. To generate the *Irf4*^{gfp} reporter mice (B6 background), a P2A.eGFP_P2A.DTR_stop cassette was inserted immediately after the last exon of WT B6 mouse *Irf4* by using the CRISPR/Cas9 technique. (**A**) Schematic diagram of knock-in strategy. (**B**) The image shows the knock-in allele.

Figure S2



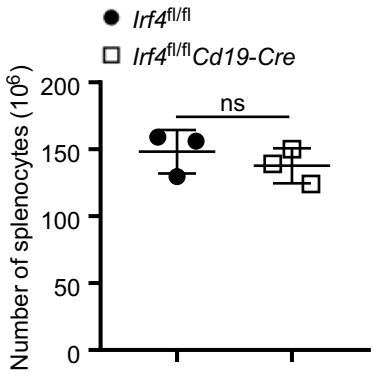
Supplemental Figure 2. Expression of IRF4 in peritoneal B cells. Cells were harvested from the peritoneal cavity of *Irf4*^{gfp} reporter mice at 8 weeks of age, followed by flow cytometry analysis**.** (**A**) Representative contour plots show the gating strategy for detecting peritoneal B cell subsets (upper panels), and % IRF4-GFP^{+} cells in each indicated B cell subset (bottom panels). (**B**) The bar graph displays IRF4-GFP MFI of each peritoneal B cell subset. ***P < 0.001 by unpaired Student's t test. Data in **B** are shown as mean ± SD (n = 3) and are from one experiment that is representative of two independent experiments.

Figure S3



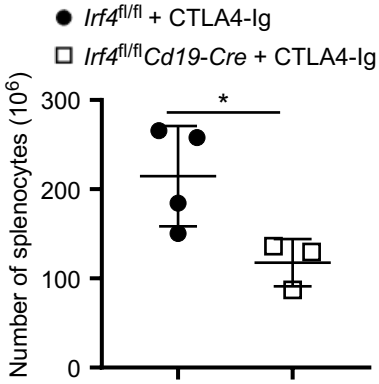
Supplemental Figure 3. Ablation of IRF4 in B cells affects the frequencies of peritoneal B cell subsets. Peritoneal cells were harvested from *Irf4*$^{fl/fl}$ control and *Irf4*$^{fl/fl}$*Cd19-Cre* mice at 8 weeks of age, followed by flow cytometry analysis. (**A** and **B**) Representative contour plots and the bar graph display the frequencies of B1a, B1B and B2 cells in peritoneal B cells. ns, $P > 0.05$; $^{***}P < 0.001$ by unpaired Student's *t* test. Data in **B** are shown mean ± SD (n = 3) and are from one experiment that is representative of two independent experiments.

Figure S4



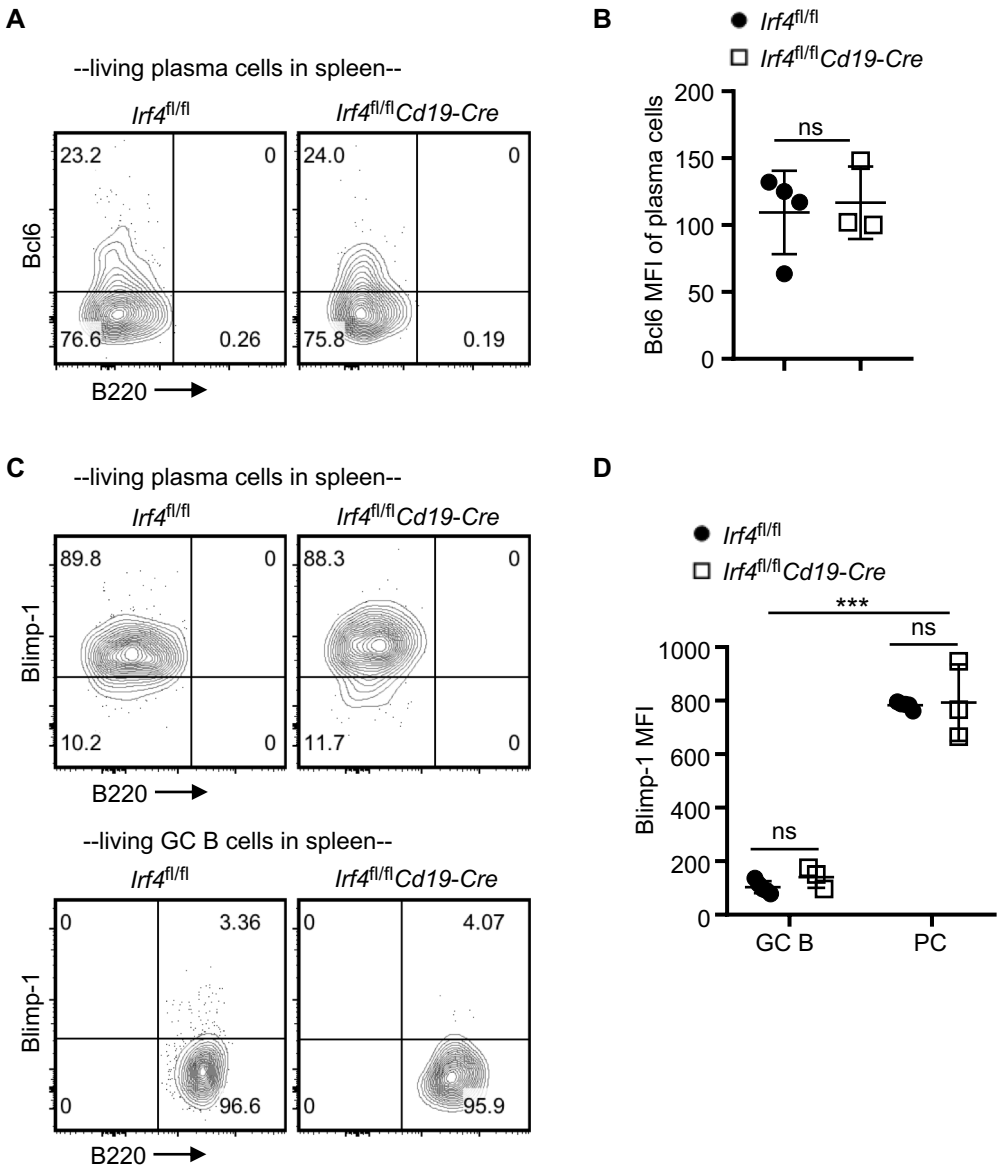
Supplemental Figure 4. Ablation of IRF4 in B cells alone does not affect the number of splenocytes in transplant recipients. *Irf4*$^{fl/fl}$ control and *Irf4*$^{fl/fl}$*Cd19-Cre* mice were transplanted with Balb/c hearts. The graph shows the number of splenocytes in recipients at day 14 post-transplant. ns, $P > 0.05$ by unpaired Student's *t* test. Data are shown as mean ± SD (n = 3) and are from one experiment that is representative of two independent experiments.

Figure S5



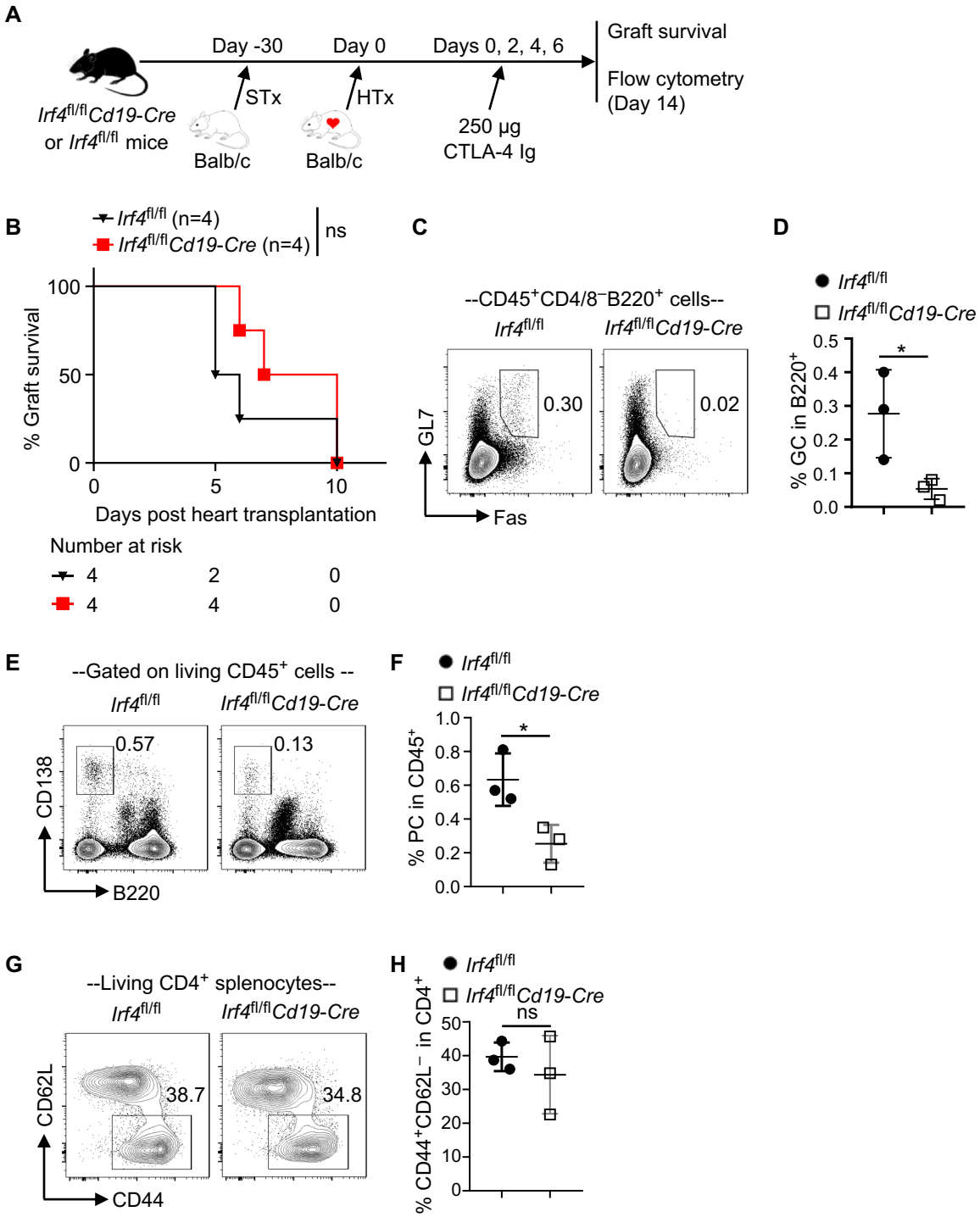
Supplemental Figure 5. Ablation of IRF4 in B cells decreases the number of splenocytes in CTLA4-Ig–treated transplant recipients. *Irf4*$^{fl/fl}$ control (n = 4) and *Irf4*$^{fl/fl}$*Cd19-Cre* (n = 3) mice were transplanted with Balb/c hearts on day 0, and treated with CTLA4-Ig on days 0 and 2. The graph shows the number of splenocytes in recipients at day 35 post-transplant. $^{*}P < 0.05$ by unpaired Student's *t* test. Data are shown as mean ± SD and are from one experiment that is representative of two independent experiments.

Figure S6



Supplemental Figure 6. The expression of Bcl6 and Blimp-1 in GC B cells and plasma cells. *Irf4*$^{fl/fl}$ control (n = 4) and *Irf4*$^{fl/fl}$*Cd19-Cre* (n = 3) mice were transplanted with Balb/c hearts on day 0, and treated with CTLA4-Ig on days 0 and 2. Splenocytes were obtained at day 35 post-transplant for flow cytometry analysis. (**A**) Representative contour plots display % Bcl6^{+} cells in splenic plasma cells. (**B**) The bar graph displays Bcl6 MFI of plasma cells. (**C**) Representative plots display % Blimp-1^{+} cells in plasma cells and GC B cells. (**D**) The bar graph displays Blimp-1 MFI of GC B cells and plasma cells (PC). ns, $P > 0.05$; ***$P < 0.001$ by unpaired Student's *t* test. Data in **B** and **D** are shown as mean ± SD and are from one experiment that is representative of two independent experiments.

Figure S7



Supplemental Figure 7. Ablation of IRF4 in B cells fails to prevent heart allograft rejection in mice that were pre-sensitized with donor skins. *Irf4*$^{fl/fl}$ control and *Irf4*$^{fl/fl}$*Cd19-Cre* mice were transplanted with Balb/c skins (on day -30). Thirty days later, recipients were transplanted again with Balb/c hearts (on day 0) and treated with CTLA4-Ig on days 0, 2, 4 and 6 post-heart transplantation. Splenocytes were obtained at day 14 post-heart transplantation for flow cytometry analysis. (**A**) Schematic of the experimental design. STx, skin transplantation; HTx, heart transplantation. (**B**) % allograft survival after heart transplantation (n = 4). ns, $P > 0.05$ by log-rank test. (**C** and **D**) Representative contour plots and the bar graph show % GC B cells in splenic B cells. (**E** and **F**) % PC in splenic CD45$^+$ cells. (**G** and **H**) % CD62L$^-$CD44$^+$ T cells in CD4$^+$ splenocytes. Data in **D**, **F**, and **H** are shown as mean ± SD (n = 3). ns, $P > 0.05$; *$P < 0.05$ by unpaired Student's *t* test.

Supplementary Table: abbreviations used throughout the manuscript

Abbreviation	Definition
αNP-5 IgG1	anti-NP-5 IgG1 (high-affinity anti-NP-KLH IgG1 antibodies)
αNP-25 IgG1	anti-NP-25 IgG1 (total anti-NP-KLH IgG1 antibodies)
αNP-25 IgM	anti-NP-25 IgM
B220	B cell isoform of 220 kDa (B cell-specific CD45 isoform)
Bcl6	B cell lymphoma 6
Blimp-1	B lymphocyte-induced maturation protein 1
BM	bone marrow
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)
CTLA4-Ig	cytotoxic T lymphocyte-associated antigen 4–immunoglobulin fusion protein
CXCR5	C-X-C chemokine receptor type 5
DSA	donor-specific antibodies
ELISA	enzyme-linked immunosorbent assay
Fol B	follicular B (follicular B cell)
GC	germinal center
GC B	germinal center B (germinal center B cell)
GFP	green fluorescent protein
H&E	hematoxylin & eosin
HTx	heart transplantation
IB	immature B (immature B cell)
IFN-γ	interferon gamma
IL-17A	interleukin-17A
IRF4	interferon regulatory factor 4
mB	mature B (mature B cell)
MFI	mean fluorescence intensity
MST	mean survival time
MZ B	marginal zone B (marginal zone B cell)
NP	(4-hydroxy-3-nitrophenyl)acetyl
NP-KLH	(4-hydroxy-3-nitrophenyl)acetyl (NP)-keyhole limpet hemocyanin (KLH)
OD	optical density
PC	plasma cell
PD-1	programmed cell death protein-1
SD	standard deviation
STx	skin transplantation
Tfh	follicular helper T
Th1	T helper 1
Th17	T helper 17
VVG	Verhoeff-Van Gieson