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Supporting Information

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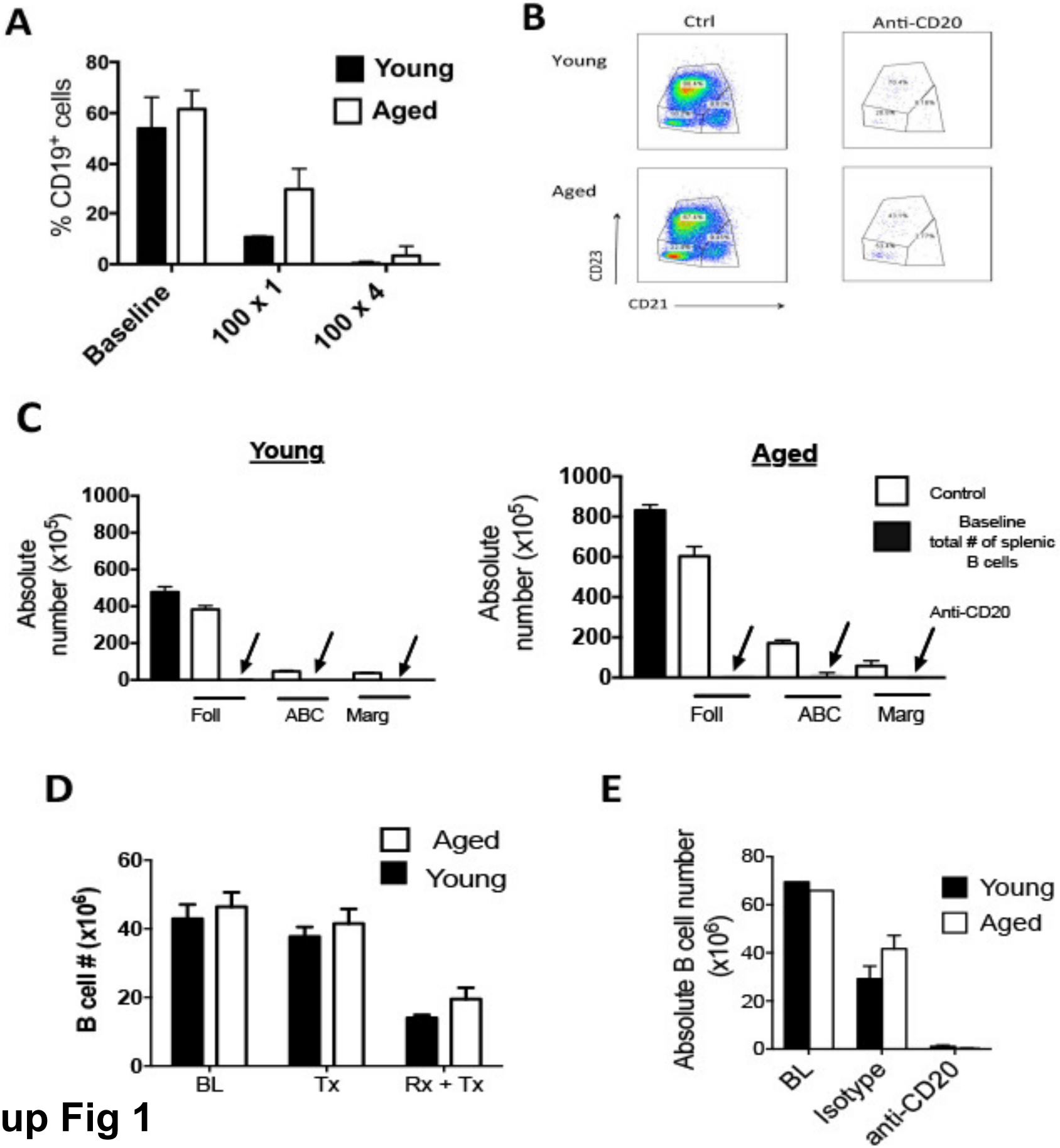
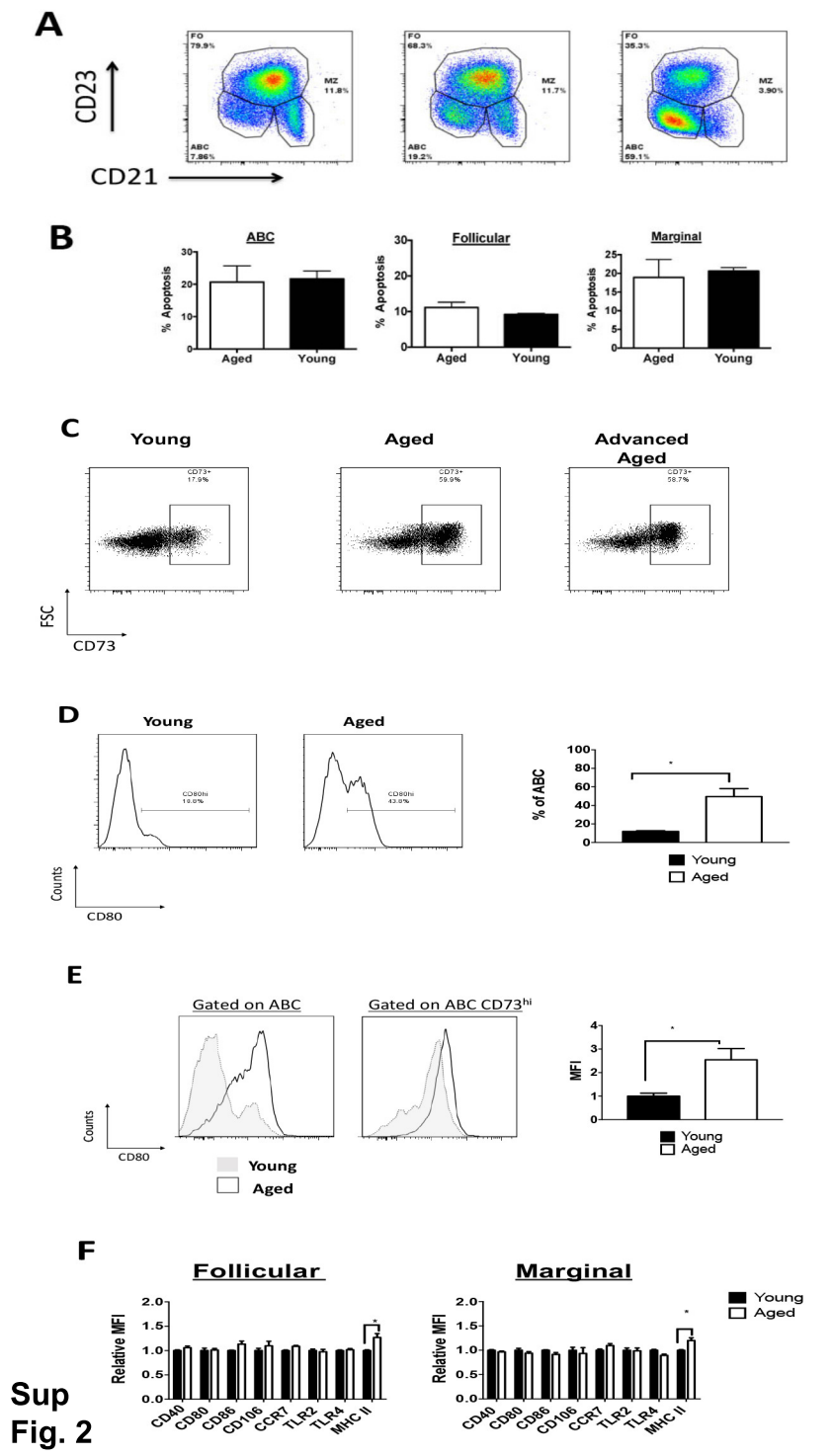
Daniel N. Mori, Hua Shen, Anjela Galan and Daniel R. Goldstein

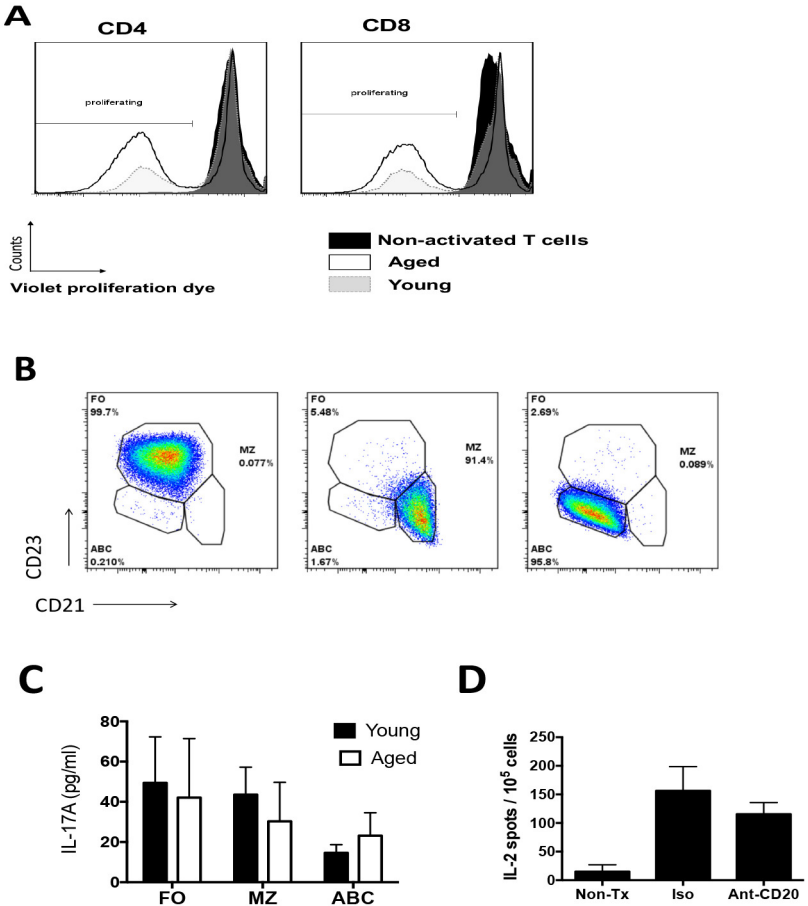
Aged B cells alter immune regulation of allografts in mice


A
% CD19+ cells
Young
Aged
Baseline
100 x 1
100 x 4
B
Ctrl
Anti-CD20
Young
Aged
CD23
CD21
C
Young
Absolute number (x10^5)
Foll
ABC
Marg
Aged
Control
Baseline total # of splenic B cells
Anti-CD20
D
B cell # (x10^6)
Aged
Young
BL
Tx
Rx + Tx
E
Absolute B cell number (x10^6)
Young
Aged
BL
Isotype
anti-CD20

Sup Fig 1


A
CD23
CD21
FO
79.9%
MZ
11.8%
ABC
7.86%
FO
68.3%
MZ
11.7%
ABC
19.2%
FO
36.3%
MZ
3.90%
ABC
59.1%
B
ABC
Follicular
Marginal
% Apoptosis
Aged
Young
C
Young
Aged
Advanced Aged
CD73+
17.9%
CD73+
59.9%
CD73+
58.7%
FSC
CD73
D
Young
Aged
CD80hi
10.0%
CD80hi
43.0%
Counts
CD80
% of ABC
Young
Aged
E
Gated on ABC
Gated on ABC CD73hi
Counts
CD80
Young
Aged
MFI
Young
Aged
F
Follicular
Marginal
Young
Aged
Relative MFI
CD40
CD80
CD86
CD106
CCR7
TLR2
TLR4
MHC II

Sup Fig. 2


A
CD4
CD8
proliferating
proliferating
Counts
Violet proliferation dye
Non-activated T cells
Aged
Young
B
FO
99.7%
MZ
0.077%
ABC
0.210%
FO
5.48%
MZ
91.4%
ABC
1.67%
FO
2.69%
MZ
0.089%
ABC
95.8%
CD23
CD21
C
IL-17A (pg/ml)
Young
Aged
FO
MZ
ABC
D
IL-2 spots / 10^5 cells
Non-Tx
Iso
Ant-CD20

Sup Fig 3

Supporting Information Figure 1. Efficacy of anti-CD20 mAb to deplete B-cells in young and aged mice.

A: Aged and young non-transplanted mice were either treated with 100 µg anti-CD20 mAb every 10 days in four serial doses or with one dose of 100 µg anti-CD20 mAb. Ten days after the last dose, spleens were obtained, and the proportion of $CD19^+$ cells was assessed via flow cytometry (n = 4 mice/group). The representative experiment shown was repeated once with similar results. Four serial doses led to >97% depletion of B cells in both age groups.

B: B-cell subpopulations of the spleens of young or aged mice that were treated as per A. Ctrl = isotype control antibody for anti-CD20 mAb. The percent of total B cells is shown within each B-cell subpopulation.

C: Quantification of sub populations of B cells of the spleens of young or aged mice that were treated as per A. Control = isotype control antibody for anti-CD20 mAb (n = 3 mice/group). Representative of a single experiment.

D: Young and aged C57BL/6 mice either received BALB/c skin transplants (Tx) or Tx and treatments (Rx) with anti-CD45RB and anti-CD154, and the number of $CD19^+$ cells were enumerated following flow cytometric staining. Pooled data from 2 independent experiments with n = 6-9 / group).

E: Young and aged C57BL/6 mice received BALB/c skin transplants, anti-CD45RB, anti-CD154, and anti-CD20 mAb (four serial doses as described in A) or an isotype control to anti-CD20. Three weeks post transplantation, spleens were obtained, and the number of B cells was enumerated following flow cytometric staining (n = 4-6 mice/group). BL = non-transplanted, non-treated baseline, control.

Supporting Information Figure 2. Representative flow cytometric plots of different B-cell subpopulations in aged and young mice.

A: Spleens were obtained from young (left panel), aged (middle panel), and advanced aged (right panel) C57BL/6 mice and stained with the relevant fluorescently tagged monoclonal antibodies. Representative flow cytometry plot gated on $CD19^+$ cells. The percentage of cells with ABC, follicular (FO), or marginal zone (MZ) B cells are shown.

B: Within each of the B-cell subpopulations shown in A, cells were assessed for Annexin V expression (an apoptosis marker). The proportion of apoptotic cells for each subpopulation is plotted (n = 3 / group). Error bars = SEM. Representative a single experiment repeated once with consistent results.

C: Spleens were obtained from young, aged, and advanced aged C57BL/6 mice and stained with the relevant fluorescently tagged monoclonal antibodies. Representative flow cytometry plot gated on ABC ($CD19^+$, $CD21^-$, $CD23^-$). N = 4 mice group. Representative a single experiment repeated once with consistent results.

D: Enriched splenic B-cells from non-transplanted and non-treated young and aged mice were stained with the indicated fluorescent antibody. The cells were gated on the ABC subpopulation and the proportion of $CD80^{hi}$ cells are shown

E: Enriched splenic B-cells from non-transplanted and non-treated young and aged mice were stained with the indicated fluorescent antibody. The cells were gated on the ABC subpopulation or the ABC $CD73^{hi}$ population ($CD73^{hi}$ gate shown in C above) and CD80 expression measured. Within the ABC $CD73^{hi}$ population, aged cells exhibit a higher CD80 expression than young cells quantified in adjacent bar graph. **D-E**: Relative

median fluorescence intensity (MFI) is normalized to average MFI of young mice. Pooled data from 2 independent experiments with n = 5-6/group. * <0.01 (t-test)

F: Enriched splenic B cells from non-transplanted young and aged mice were stained with the indicated fluorescent antibodies and gated on either the follicular cell subpopulation or the marginal zone subpopulation . Samples were analyzed at baseline. Relative median fluorescence intensity (MFI) is normalized to average MFI of young mice. (n = 3-5 mice/group). *, P < 0.01 (t-test). Similar results were noted with a repeat experiment.

Supporting Information Figure 3. Additional information regarding effects of aged B cells and T-cell alloimmunity.

A: The total B-cell pool ($CD19^+$ cells) was enriched by negative magnetic separation from young and aged mice per Figure 3A. Cells were irradiated and cultured with allogeneic, (BALB/c), enriched T cells that were stained with violet proliferation dye. A representative flow cytometric plot is shown for $CD8^+$ and $CD4^+$ T cells respectively, (n = 3 biological replicates/age group/experiment). Consistent results were observed in four independent experiments.

B: Representative flow plots show the purity of sorting into follicular cells, marginal zone cells, and ABCs.

C: MLC culture supernatants from were harvested from cultures of T cells cultured with the indicated B-cell subpopulation, and IL-17 production was measured by ELISA. Error bars = SEM. Data are pooled from two independent experiments with three biological replicates / experiment.

D: Young C57LB/6 mice were transplanted with BALB/c skin allografts, treated with CD45RB, anti-CD154, and anti-CD20 mAb or an isotype control. At three weeks post transplantation, spleens were obtained, and T cells were enriched by magnetic negative selection and cultured with irradiated donor spleen cells. The number of IL-2-producing T-cells was measured via ELISPOT (n = 6 mice/group). Pooled data from 2 independent experiments.