Supplemental Material

Control of humoral response in renal transplantation by Belatacept depends on a direct effect on B cells and impaired T follicular helper (Tfh)- B cells crosstalk.

Authors

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Supplemental figures

Supplemental Figure 1: Characteristics of stimulated B cells in the presence or absence of Belatacept after activation.

B cells were cultured with CD40L and IL-21 stimulation for 5 days in the presence or absence of Belatacept. **(A)** Representative dot plot showing gating strategy of CD19+ B and CD3+ T cells (left panel) and the purity of sorted B cells (right panel), showing that CD3+ contamination represented less than 0.3% of the total lymphocytes in the culture at day 5 **(B)** Mortality of B cells, assessed by a cell viability dye, was not modified by Belatacept adjunction (n=20). **(C)** Similar proportion of memory B cells (CD19+CD27+CD38-) (n=25) in presence or absence of Belatacept. **(D)** Representative dot plot of cell proliferation dye (CPD) dilution in B cells and plasmablasts cultured with or without stimulation. Stimulation with CPG (1µG/ml) was included as a positive control. **(E)** Equal proportion of proliferative plasmablasts ($CD19^{lo}CD27^{hi}CD38^{hi}CPD^{lo}$) (n=10) were observed in the presence or absence of Belatacept.

Supplemental Figure S2: Induction of CD80 and CD86 expression on stimulated B cells, according to effector B cells subsets.

B cells were cultured with or without CD40L and IL-21 stimulation for 5 days in the absence of Belatacept. **(A-B)** Increased proportion of CD80+ (A) (n=7) and CD86+ (B) (n=7) B cells in stimulated B cells. **(C-D)** Proportion (C) and MFI (D) of CD80 and CD86 B cells (n=7) varied according to effector B cells subsets (*i.e.* plasmablasts or memory B cells) described in Figure 1A. **(E-F)** Representative dot plot from a HBD showing gating strategy of CD86 and CD80 on (E) unstimulated or stimulated B cells with or without Belatacept or (F) according to B cells subsets. **(G)** Representative dot plot from a HBD showing gating strategy of CD28 on stimulated B cells and their subsets. $^{\#}p<0.05$ *vs.* unstimulated, $^{*}p<0.05$ memory B cells *vs.* plasmablasts, Wilcoxon matched-pairs signed rank test (A and B) or Mann Whitney (C and D).

Supplemental Figure S3: Gating strategies and purity of sorted Tfh and memory B cells.

(A) Dot plot from a representative HBD showing gating strategies to sort (i) memory B cells ($CD19^{+}CD38^{low}IgD^{-}CD27^{+/-}$) (upper panel) and (ii) Tfh cells (CD4+CD45RA-CXCR5+PD1+) (bottom panels). **(B)** Dot plot from the same representative HBD showing purity of the sorted cells.

Figure S1

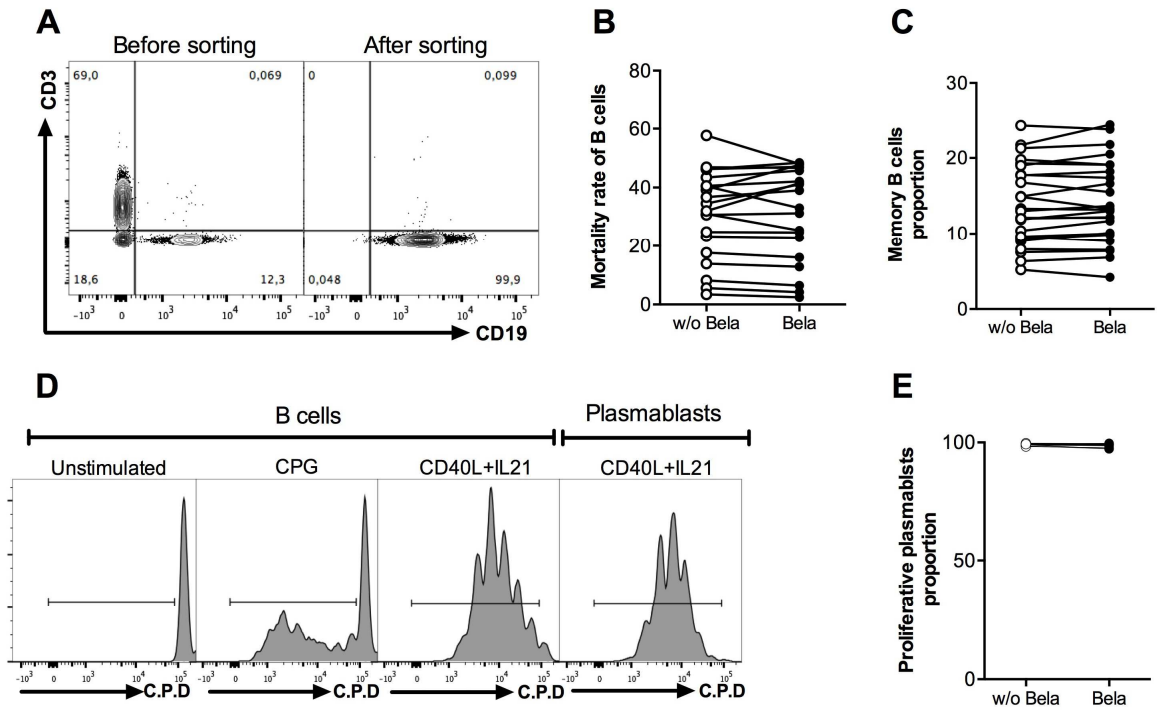

A
Before sorting
After sorting
CD3
CD19
69,0
0,069
18,6
12,3
0
0,099
0,048
99,9
B
Mortality rate of B cells
w/o Bela
Bela
C
Memory B cells proportion
w/o Bela
Bela
D
B cells
Plasmablasts
Unstimulated
CPG
CD40L+IL21
CD40L+IL21
C.P.D
C.P.D
C.P.D
C.P.D
E
Proliferative plasmablsts proportion
w/o Bela
Bela

Figure S2

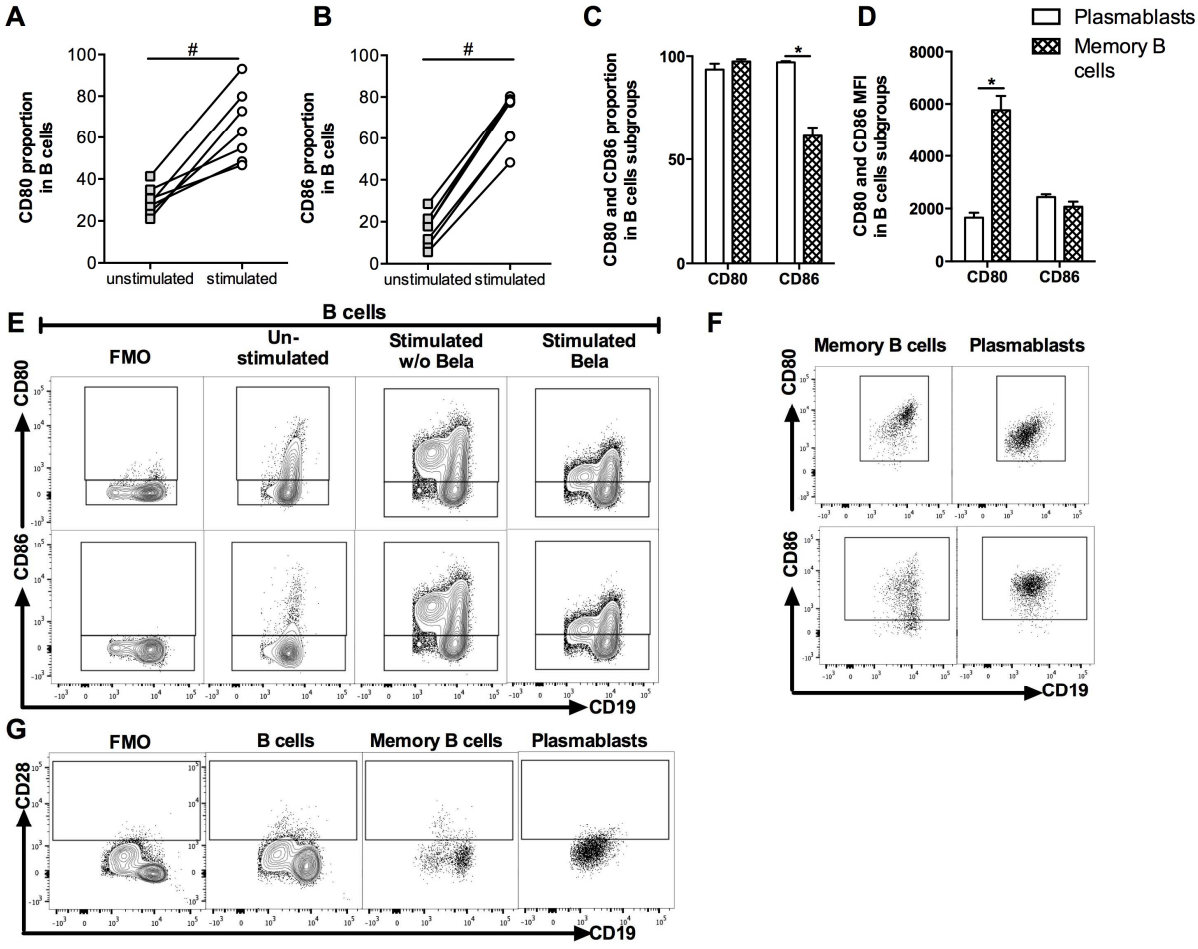
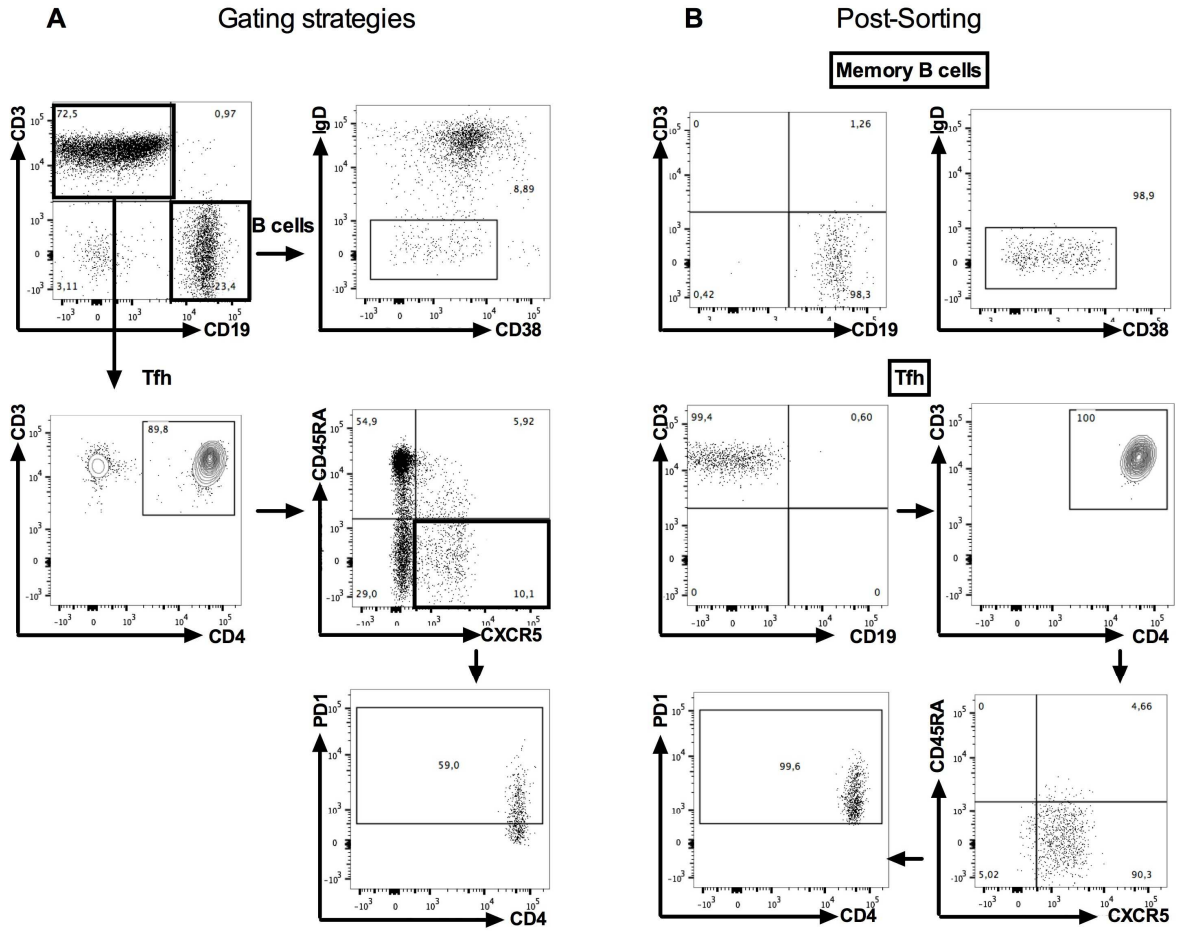

A
CD80 proportion in B cells
100
80
60
40
20
0
#
unstimulated
stimulated
B
CD86 proportion in B cells
100
80
60
40
20
0
#
unstimulated
stimulated
C
CD80 and CD86 proportion in B cells subgroups
100
50
0
*
CD80
CD86
D
Plasmablasts
Memory B cells
CD80 and CD86 MFI in B cells subgroups
8000
6000
4000
2000
0
*
CD80
CD86
E
B cells
FMO
Un-stimulated
Stimulated w/o Bela
Stimulated Bela
CD80
CD86
CD19
F
Memory B cells
Plasmablasts
CD80
CD86
CD19
G
FMO
B cells
Memory B cells
Plasmablasts
CD28
CD19

Figure S3


A
Gating strategies
B
Post-Sorting
Memory B cells
CD3
CD19
72,5
0,97
3,11
23,4
B cells
IgD
CD38
8,89
Tfh
CD4
89,8
CD45RA
CXCR5
54,9
5,92
29,0
10,1
PD1
59,0
99,4
0,60
1,26
0,42
98,3
98,9
100
99,6
4,66
5,02
90,3

Complete methods

Culture of B cells

Flow cytometry analysis of isolated B cells showed a mean (± SEM) purity of 98.2% (±0.45, N=16). B cells were cultured in 96-well plates ($1x10^5$ per well/200µl medium) at 37°C in a humidified 5% CO_2 incubator, in a culture medium consisting of RPMI 1640+ Glutamax supplemented with 10% fetal bovine serum (FCS), 100 IU/ml penicillin, 100mg/ml streptomycin (Life Technologies, Carlsbad, CA), 5mM HEPES (Life Technologies; Thermo Fisher Scientific), ± 50µM B-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). They were stimulated in the presence of 100ng/ml soluble recombinant Mega CD40L (Enzo Life Sciences, Farmingdale, NY) and 50ng/ml IL-21 (Affymetrix eBioscience). The choice of Belatacept concentrations was based on the range previously reported in similar *in vitro* culture, albeit on T or DC cell responses[17].

Flow cytometry

For the analysis of B cell subpopulations and activation markers, the monoclonal antibodies used were CD19 (clone HIB19), CD24 (ML5), CD38 (HB-7), CD80 (L307-4), CD152 (BNI3), CD274 (MIH1), CD28 (L293), CD268 (11C1), IL10 (JES3-19F1), IFNγ (B27) from BD biosciences, CD27 (1A4CD27) from Beckman and Coulter, IgM (SA-DA4), IL6 (MQ2-13A5), IL21 (Ebio 3A-N2) from Ebiosciences, CD86 (FM95), CD275 (MIH12), TNFα (CA-2) from Miltenyi, CD279 (clone EH12) from Biolegend.

Cell viability was assessed with 7AAD (BD biosciences) or a viability dye (E-bioscience ref 65-0866) staining. It is worth mentioning that CD3+ contamination represented less than 0.3% of the total lymphocytes in the culture at day 5.

For intracellular staining, cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA), ionomycin (Sigma-Aldrich, Saint Louis, CA, USA) and Brefeldin A (BD Pharmingen, San Diego, CA, USA). For surface staining, cells were incubated with for 30 min then permeabilized using Fixation/Permeabilization solution and stained with IFNγ, TNF, Il-6, IL-21 or IL-10.

Western Blot Analysis of purified B cells.

Protein concentration of whole-cell extracts was evaluated using the Bradford assay. Lysates were mixed with reducing sample buffer (Laemmli) for electrophoresis and subsequently transferred on nitrocellulose membranes (Amersham, GE Healthcare Life Sciences). Equal loading (20µg) was verified using Ponceau red solution. After blocking with 5% non-fat milk TBS/T (0.05M Tris, 150mM NaCl, 0.05% Tween-20), membranes were probed with anti-CD86 (Abcam), anti-Blimp-1 (ebioscience), anti-XBP1s (Cell signaling), anti-CD80 (BioLegends), anti-phospho-Akt(Ser473) (Cell Signaling), anti-Akt (Cell Signaling), anti-phospho-STAT3(Tyr705) (Cell Signaling), anti-STAT3 (Cell Signaling) and anti-tubulin (Abcam) antibodies. After appropriate secondary antibody incubation (anti-rat HRP, anti-rabbit HRP, or anti-mouse HRP, 1:2000, Cell Signaling), immunodetection was performed using an ECL kit (Diagomics) and bands were revealed with Fusion-SL Imaging system (Vilber Lourmat) and Fusion software. Densitometry analysis with ImageJ software (NIH) was used for quantification.

***In vitro* Tfh-B cells co-culture assays**

For sorting, cells were stained with anti-CD4 (Biolegend), PD1, IgD, CD3, CD45RA, CXCR5, CD19, CD27, CD38 (BD Biosciences) and Aqua Live Dead (Life Technologies). Blood Tfh (CD3+CD4+CD45RA-CXCR5+PD1+) and memory B cells (CD3-CD19+IgD-$CD38^{dim}$CD27+/-) were sorted using a BD FACSAria III (BD Biosciences) with a purity > 95%.

Memory B cells (1-3x10^4) were co-cultured with blood Tfh at a 1:1 ratio in complete RPMI (supplemented with 5% fetal bovine serum (FCS), 100 IU/ml penicillin, 100mg/ml streptomycin (Life Technologies, Carlsbad, CA), 5mM HEPES (Life Technologies; Thermo Fisher Scientific), 1mM sodium pyruvate (Gibco; Thermo Fisher Scientific) and MEM NEAA 1X (Gibco; Thermo Fisher Scientific) with endotoxin-reduced staphylococcal enterotoxin B (SEB) (100 ng/ml; Sigma)).

After co-culture, cells were stained at day 5 with anti-CXCR5 (RF8B2), anti-CD210 (3F9), anti-CD3 (UCHT1), anti-CD19 (HIB19), anti-IgD (IA6-2), anti-CD278 (DX29), anti-360 (17A12) (BD Biosciences), anti-CD4 (RPA-T4), anti-PD1 (EH12), anti-CD275 (MIH12), anti-CD274 (MIH1) (Biolegend), anti-CD38 (HIT2) (E-bioscience), and Aqua Live Dead (Life technologies).

Immunophenotyping of blood B cells and Tfh.

B cells and Tfh subsets were characterized from freshly isolated PBMCs by flow cytometry (BD Canto II) with the following anti-human antibodies: anti-CD19 (clone HIB19), anti-CD24 (ML5), anti-CD38 (HB-7), anti-CD80 (L307-4), anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD45RA (T6D11), anti-CXCR5 (RF8B2), anti-CD274 (MIH1), and anti-CD278 (ISA3), anti-CD279 (EH12) from BD biosciences, anti-CD27 (1A4CD27) from Beckman and Coulter, anti-IgD (IA-62), anti-IgM (SA-DA4) from Ebiosciences, anti-CD86 (FM95), anti-CD197 (REA 108), anti-CXCR5 (REA103), anti-CCR6 (REA190), and anti-CXCR3 (REA 232) from Miltenyi.