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## Belatacept inhibit human B cell germinal center development in immunodeficient mice

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1 Belatacept inhibit Human B cell germinal center development  
2 in immunodeficient mice

3  
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23

24 **Abstract**

25 The humoral response mediated by alloantibodies directed against donor HLA molecules  
26 (DSAs) is one of the main causes of graft loss in kidney transplantation. Understanding the  
27 pathophysiology leading to humoral kidney rejection as the development of therapeutic tools  
28 is therefore a main objective in the field of solid organ transplantation and necessitate adapted  
29 experimental models. Among the immunosuppressive agents used in renal transplantation,  
30 belatacept, a fusion protein targeting T costimulatory molecules has shown its ability to  
31 prevent more efficiently the secretion of DSA by different mechanisms including a direct  
32 action on plasma cells but also on B lymphocytes and follicular helper T lymphocytes (Tfh)  
33 cooperation. This cellular cooperation occurs within germinal centers (GC), the seat of B  
34 lymphocytes differentiation. Here, we aimed to develop a dedicated mouse model in which  
35 human GC would be functional to study the effect of belatacept on GC formation and the  
36 ability of B lymphocytes to secrete immunoglobulin. We next demonstrate that belatacept  
37 inhibits the formation of these GCs, by inhibiting the frequency of Tfh and B lymphocytes.  
38 This alters the B maturation and therefore the generation of plasma cells and consequently,  
39 immunoglobulin secretion.

40

## 41 **Introduction**

42 Kidney transplantation remained the treatment of choice for end-stage chronic kidney disease,  
43 but long-term survival improvement is stagnating (1-3). This observation is mainly related to  
44 the occurrence of antibody-mediated rejection (ABMR) which remains the death-censored  
45 leading cause of transplant loss across all solid organ transplants (4). Anti-HLA donor-  
46 specific antibodies (DSAs) arising after kidney transplantation, also called *de novo* donor-  
47 specific antibodies (*dn*DSAs), have increasingly been recognized as the leading cause of both  
48 acute and chronic ABMR(5). Approximately 15%–30% of kidney transplant recipients  
49 develop *dn*DSAs(6).

50 Belatacept (cytotoxic T lymphocyte-associated antigen 4 [CTLA4]-Ig; LEA29Y; Bristol  
51 Myers Squibb) is a human fusion protein combining the extracellular portion of CTLA-4 that  
52 has been mutated to confer greater binding avidity to CD80 and CD86 and the constant region  
53 fragment of human IgG1. CTLA-4 binds to surface costimulatory ligands of antigen-  
54 presenting cells (APCs) and in a lesser extent on T cells, and thus, prevents their interaction  
55 with CD28, thereby blocking T cell activation(7, 8).

56 Long-term follow-up analysis of phase 3 clinical trial using belatacept showed that recipient  
57 mortality and the graft failure rate at 7 years after transplantation were significantly lower in  
58 the group of recipients treated with belatacept compared with the control recipients treated  
59 with calcineurin inhibitors (CNIs). Interestingly, the incidence of *dn*DSAs at year 7 was  
60 significantly lower in belatacept-treated patients than in CNI-treated patients(9). These  
61 clinical results remain to be explained by experimental studies aiming to analyze the effect of  
62 belatacept, as other costimulatory inhibitors molecules, on different steps of the B cell-  
63 mediated response in humans.

64 Using a preclinical model of ABMR after renal transplantation in macaques, Kim et al  
65 showed that belatacept prevents the maturation of B cells in peripheral blood and memory T  
66 cell populations in secondary lymphoid organs and finally suppresses germinal center  
67 reconstruction after T cell depletion. However, in this non-human model, belatacept was used  
68 as additional immunosuppressive regimen including Steroids, Tacrolimus, monoclonal Anti-  
69 CD3 antibodies and Alefacept (10). Our previous in vitro human studies showed that  
70 belatacept reduces plasmablast differentiation, Ig production, and expression of the major  
71 transcription factor involved in plasma cell function, Blimp-1, independently of T  
72 lymphocytes (11) . When Tfh and B lymphocytes are co-cultured, belatacept blocks CD28-  
73 mediated Tfh activation. Moreover, patients treated with belatacept, exhibited a reduced  
74 proportion of blood effector B lymphocytes, and activated Tfh (PD1<sup>+</sup> ICOS<sup>+</sup>) compared to  
75 control transplant patients treated with CNI(11). Taken all together, our results showed that  
76 belatacept modulates the function of B cells directly and at the level of the B cell-Tfh  
77 interaction.

78 Studying human B and T cells interaction in humanized mice is quite a challenge. B cells do  
79 not survive long time post infusion in classical humanized mice models. Some authors have  
80 developed models like the one of Jangalwe *et al.*(12) but they are technically hardly  
81 achievable: irradiation, human fetal tissues and special NSG mice were used. Here, using an  
82 original, easily reproducible experimental *in vivo* model of human germinal centers  
83 formation in NSG (NOD/scid/IL-2R $\gamma$ <sup>-/-</sup>) mice, we demonstrate for the first time on human  
84 cells the detrimental and specific role of belatacept on human germinal centers formation as  
85 single immunosuppressive regimen.

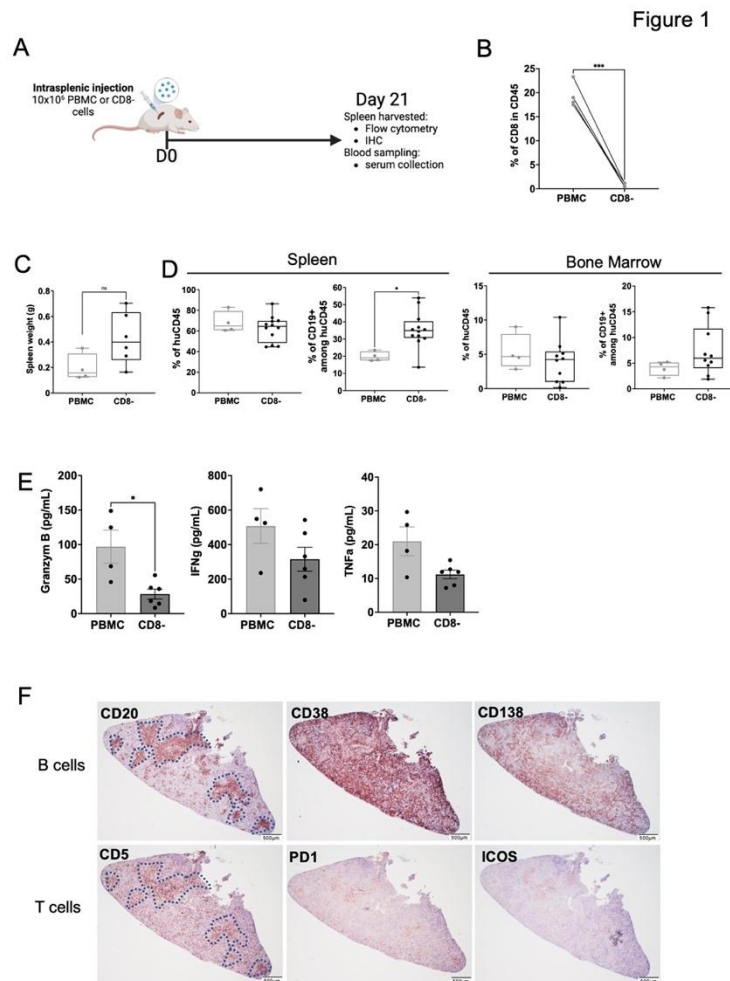
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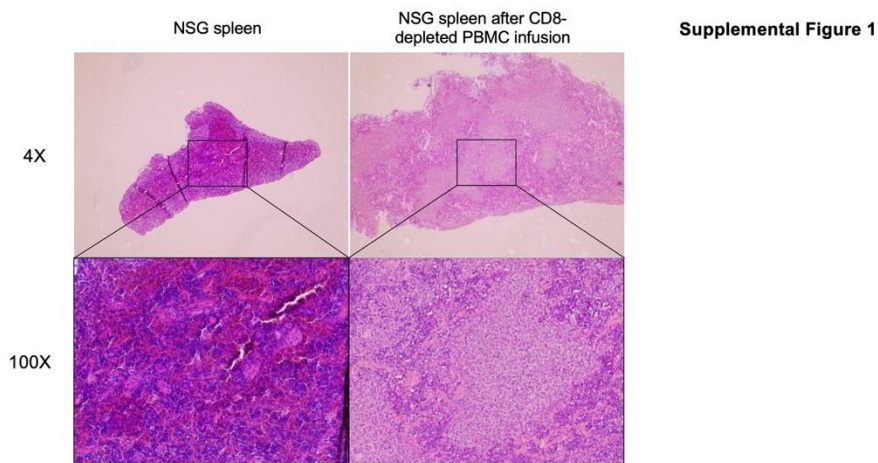
87 **Results**

88 **B cells enrichment in mice spleen using CD8 depleted PBMCs.**

89 Few *in vivo* models allow the study of human germinal centers and the B and T cells  
90 interaction. Then, to facilitate the B cells engraftment, we infuse human PBMC directly in the  
91 spleen of immunodeficient NSG mice by intrasplenic infusion. Since CD8 T cells seem to be  
92 directly implicated in xeno-GVHD clinical symptoms development, we compared infusion of  
93 total PBMC with an infusion of CD8-depleted PBMC (Fig. 1A). CD8 proportion after  
94 depletion was significantly reduced (Fig. 1B). On day 21, spleen weight with CD8-depleted  
95 PBMC were higher than in PBMC mice (Fig. 1C). On day 21, no difference was observed in  
96 total human cells reconstitution evaluated by the percentage of huCD45, between mice  
97 infused with PBMC or CD8- depleted human cells (Fig. 1D). However, CD8 depletion  
98 significantly favored B cells survival. The proportion of CD19+ among huCD45 cells  
99 increases from 19.83% to 35.48% in CD8-depleted injected mice. Human B cells were also  
100 detected in the bone marrow of the grafted mice (Fig. 1D). Cytokines production such as  
101 Granzyme-B, IFN- $\gamma$  and TNF- $\alpha$  known to be implicated in xeno-GVHD was reduced (Fig.  
102 1E). For these reasons, we decided to use CD8-depleted infusion for the next experiments to  
103 be able to follow the cell composition *in vivo* without being limited by potential mortality due  
104 to xeno-GVHD. We then analyzed the germinal center formation in the spleen by  
105 immunohistochemistry at D21. The comparison of hematoxylin and eosin staining of normal  
106 NSG mice with CD8-depleted PBMC infused NSG spleen show difference in size and cells  
107 structure (Fig. S1). Immunohistochemical staining formally confirmed the presence of a B  
108 cell zone (Fig. 1F). Indeed, CD20 expression showed the naive B cells in the middle of the  
109 GC. CD38 expression showed the differentiated cells at the periphery of follicles whereas the  
110 CD138 positive cells outside of GCs showed the terminal stage of B cell differentiation which  
111 corresponds to plasma cells (Fig. 1F). The distribution of the different stages of maturation of

112 B cells was thus hierarchically respected compared to a classic secondary lymphoid organ.  
 113 The presence of the T zone was also evaluated by the CD5+ expression (Fig. 1F). Thus, the  
 114 CD5+ expression in the NSG mice spleen highlights the presence of T cells. The joint  
 115 expression of ICOS and PD1 suggests that these T cells may be Tfh (Fig. 1F). All, those  
 116 results suggest the presence of functional human germinal centers in mice.





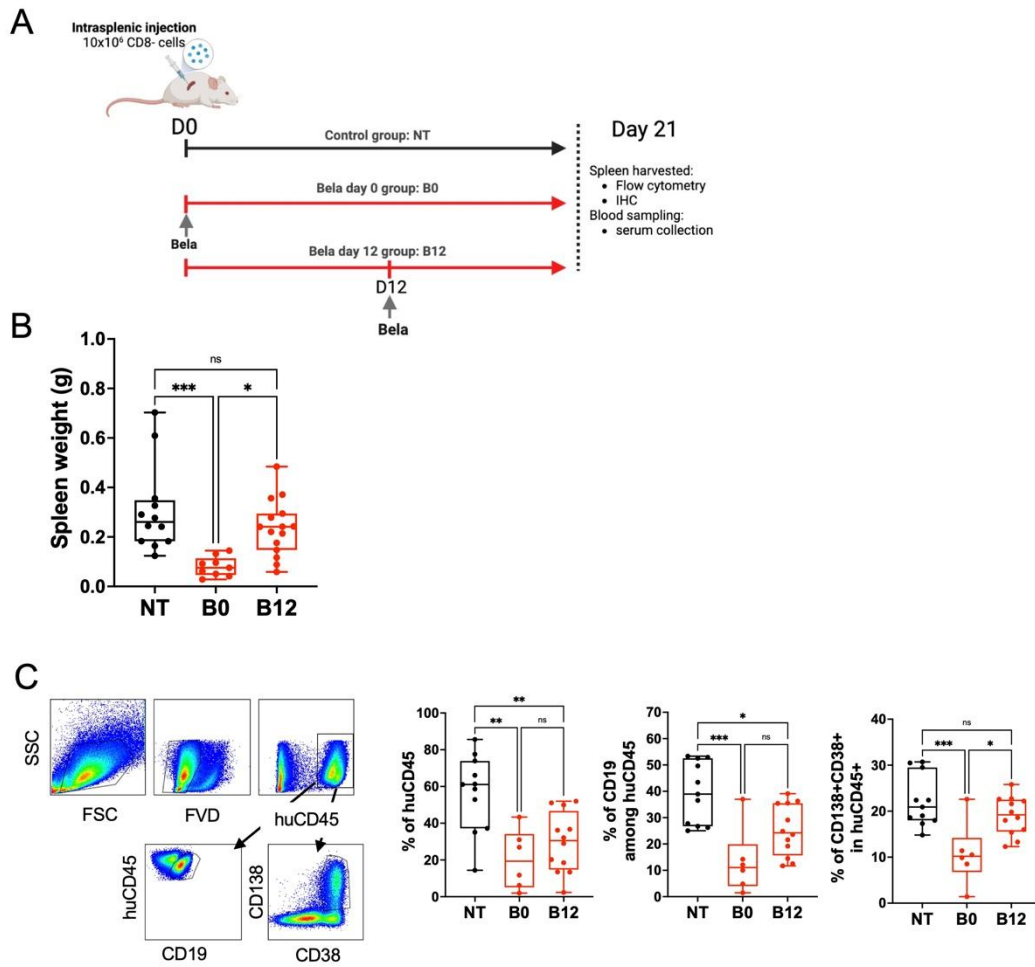
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119 **Belatacept reduces B cells survival and human germinal centers formation**

120 To assess the effect of belatacept on B cells differentiation and on the GC formation in the  
121 spleen of NSG mice, we adopted two different strategies: early treatment on day 0 (B0) to  
122 study the effect on the structural establishment, and at day 12 (B12) to determine the effect of  
123 belatacept on existing functional germinal structures (Fig. 2A). Only day 0 treatment with  
124 belatacept (B0), significantly reduced the spleen weight and size compared to the untreated  
125 mice (Fig. 2B). Also, both belatacept treatments (B0 and B12) significantly decreased the  
126 percentage of huCD45+ cells in the spleen (Fig. 2C). In addition, B cells compartment was  
127 strongly affected by belatacept at day 0, with a decrease in total CD19+ percentage and fewer  
128 plasma cells identified by CD138 and CD38 positive cells (Fig. 2C) only with the treatment at  
129 day 0.



Figure 2

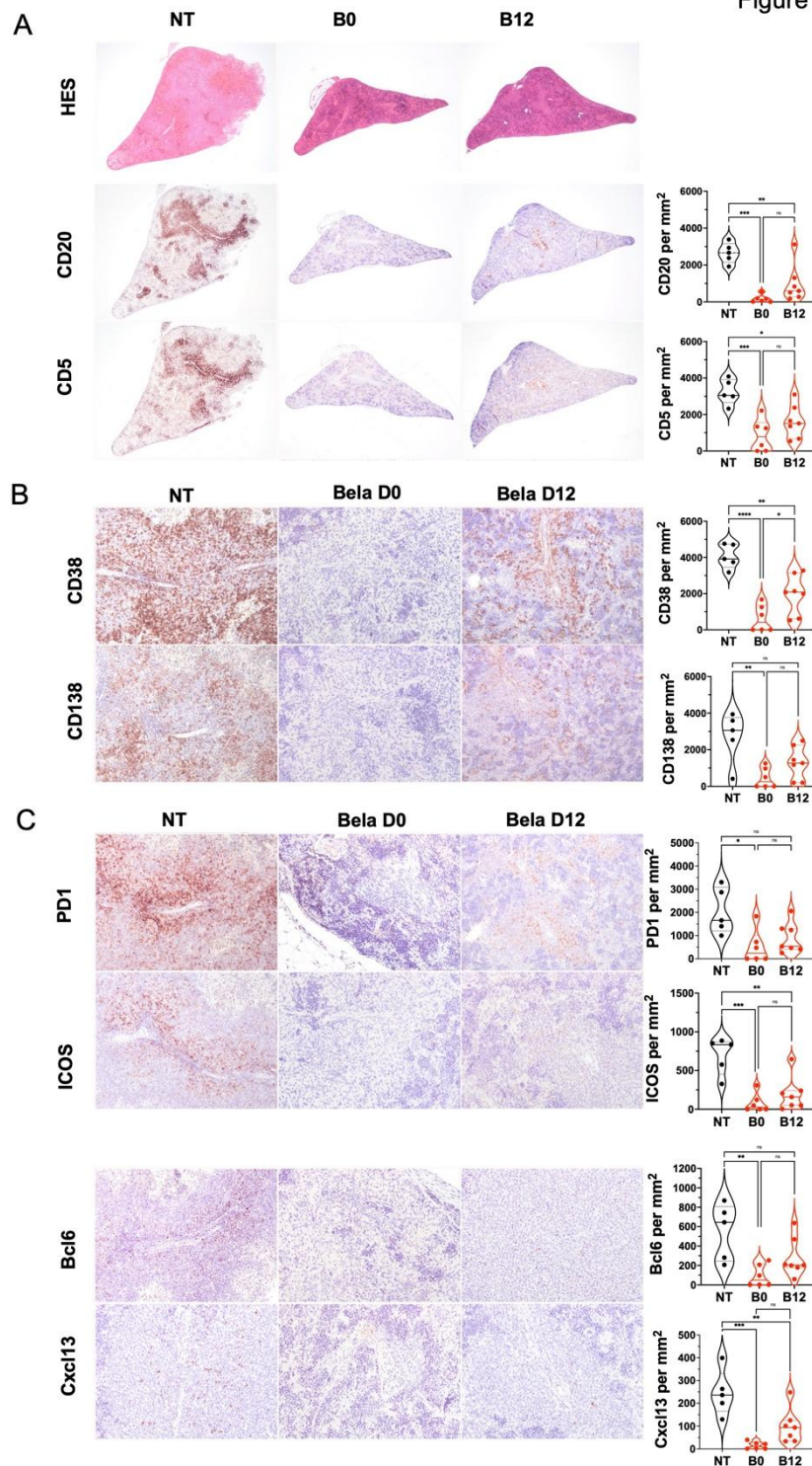


130

131 We next analyzed by histology the human GC formation and structure 21 days after the IS  
 132 infusion of CD8- human PBMCs. In the presence of belatacept, spleens of NSG mice show  
 133 significantly fewer B zones, both in terms of naive cells (CD20+) (Fig. 3A) and differentiated  
 134 cells (CD38+ and CD138+) (Fig. 3B). We observe a significant reduction in CD5+ T cells

135 from the T cells area, only in the belatacept day 0 group (Fig. 3A). Furthermore, a significant  
136 reduction of Tfh markers expression (ICOS, PD1, Bcl6, CXCL13) was observed in the  
137 spleens of mice treated with belatacept (Fig. 3C). Belatacept appears to have a specific effect  
138 on the development and maintenance of secondary lymphoid structures.

Figure 3



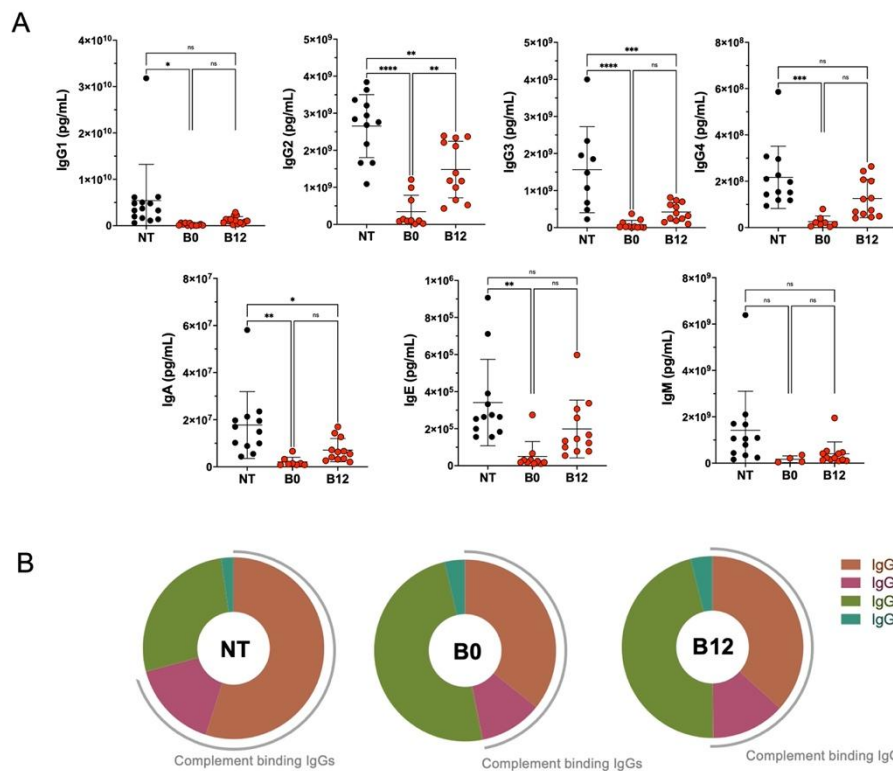
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140 We next investigate the functionality of germinal center B cells by analyzing human

141 immunoglobulin production in the sera of mice. Human Ig (IgG1,2,3,4, IgA, IgE, IgM) were

142 detected in large amounts in untreated mice (Fig. 4A). However, belatacept treatment at day 0

143 and to a lesser extent at day 12 induced a significantly lower level of all IgG subtype tested  
 144 but also IgA, IgE, and IgM suggesting an altered B cell function in the germinal center.  
 145 Complement binding IgGs (IgG1 and IgG2) proportion were decreased with belatacept  
 146 treatment B0 and B12 (Fig. 4B) suggesting a switch in IgG subtype secretion with belatacept.  
 147



148  
 149 **Discussion**

150 Controlling the alloantibody response is a key factor for allograft survival. Although this  
 151 humoral response largely depends on the development of GC in secondary lymphoid organs,  
 152 this step remains poorly understood and studied in human due to the difficulty of access to  
 153 these tissues. To date, experimental models allowing analysis of the allospecific immune  
 154 response within GC response are limited (13, 14). Here we establish an original in vivo model  
 155 of human GC formation in NSG mice and next demonstrate that belatacept specifically  
 156 impairs the formation of these GC.

157 In the field of solid organ transplantation, DSA secretion implies the migration of previously  
158 activated B and T cells to the border of B-cell follicles and T-cell zones, where they undergo  
159 cognate interactions. At this step, Tfh–B-cell crosstalk leads to the development of the GC  
160 and the generation of memory B cells and long-lived plasma cells(15). Although co-  
161 stimulation molecules are fundamental for T-cell priming by dendritic cells they have also  
162 been proven to play an important role in the different steps of Tfh differentiation and function  
163 during CG formation(16, 17). Indeed, it has recently been found that continuous CD28  
164 expression is required for Tfh differentiation(18). Along parallel lines, CTLA-4 expression,  
165 the inhibitory competitor of CD28, restrains Tfh responses and inhibits their B-cell  
166 stimulatory function. Specifically, deletion of CTLA4 on Tfh increased GC B cell numbers  
167 and serum antibody titers(19, 20).

168 Although belatacept has been mainly developed to target DC-priming of T cells, preclinical  
169 models have interestingly shown that the use of belatacept inhibited humoral responses in  
170 transplantation settings. Follicle size, GC proportion, and IL-21 secretion were decreased in  
171 belatacept-treated primate recipients, suggesting a specific role of belatacept on Tfh–B-cell  
172 crosstalk(10). Studies by Chen et al.(21) and Young et al.(22) further showed, in murine  
173 models, that late CTLA4-Ig administration could inhibit ongoing humoral response, even if  
174 priming of allogeneic T cells had already occurred. Indeed, the introduction of CTLA4-Ig  
175 treatment 14 days after sensitization inhibited alloantibody production and collapsed GC  
176 responses. These results suggested that CTLA4-Ig action on B-cell stimulatory capacities  
177 were important, independently of T-cell priming by DCs. Our model offers mechanistic  
178 understanding of both clinical and in vitro data suggesting that CTLA4-mediated  
179 costimulatory blockade impairs specifically several steps of the humoral response. In fact,  
180 belatacept appears to have a specific effect on the development and maintenance of secondary  
181 lymphoid structures. We observed a decrease in the size of the follicle and the amount of GC.

182 We further studied mechanisms underlying belatacept action on humoral response in  
183 humans(11). We have previously show that belatacept inhibited Tfh–B-cell crosstalk in vitro  
184 by decreasing the proportion of activated PD-1<sup>+</sup>ICOS<sup>+</sup> Tfh cells, decreasing Tfh proliferation,  
185 and decreasing the differentiation of B cells into plasmablasts. These *in vitro* data account for  
186 clinical results showing a marked reduction in de novo DSA synthesis in renal transplant  
187 recipients treated with belatacept when compares to CNI (9). Here again, the co-stimulatory  
188 blockade agents have serious consequences on the quality of GC. It implies that targeting co-  
189 stimulatory pathway led to the induction of a new equilibrium of T and B cells population and  
190 functions. A significant reduction in Ig production confirms functional consequences of these  
191 observations.

192 With the development of new molecules aiming to control the humoral response in the field of  
193 transplantation, it is now essential to have adequate models and tools to evaluate their  
194 effectiveness and understand their mechanisms. The model of GC reconstitution described in  
195 this work allows to address these two points. It should also allow to test combined approaches  
196 associating several therapeutic molecules targeting different cell populations at the same time.  
197 Even if this model is not perfect because it does not resume in its integrality what happens in  
198 humans, it is to date the model that seems to us the most successful allowing in vivo the study  
199 of the B response. This model could be used in the field of alloreactivity as well as in that of  
200 autoimmune diseases.

201

202 **Methods**

203 **Mice**

204 NSG (NOD/scid/IL-2R $\gamma$ <sup>-/-</sup>) mice were obtained from Charles River (Miserey, France).  
205 Manipulations were performed according to European Union guidelines and with approval of  
206 the Regional Ethics Committee in Animal Experimentation no. 16, Ile- de- France, France  
207 (authorization no. 11/12/12- 11B). In vivo experiment reported in this study were done in  
208 accordance with ARRIVE guidelines.

209 **Blood sample**

210 Human PBMCs were obtained from healthy donors (Etablissement Francais du Sang, Créteil,  
211 France). Ethical review and approval were not required for the study on human participants in  
212 accordance with the local legislation and institutional requirements. The participants provided  
213 their written informed consent to participate in this study. PBMCs were isolated by density  
214 gradient centrifugation (Lymphocyte Separation Medium; Eurobio®, France). CD8 depletion  
215 was made thanks to a negative depletion with CD8<sup>+</sup> T Cell Isolation Kit from Miltenyi Biotec  
216 (Paris, France).

217 ***In vivo* experiment**

218  $10 \times 10^6$  PBMC (depleted or not in CD8<sup>+</sup>) are injected in recipient mice by an intrasplenic  
219 injection. Before surgery, mice were given an analgesic solution of buprenorphine. Then, they  
220 are kept anesthetized under isoflurane gas. The mice weight and their general state of health  
221 (signs of xenoGVHD) are checked 3 times a week.

222 **Belatacept treatments**

223 Recipient mice were treated by one intraperitoneal injection at day 0 (B0 group) or day 12  
224 (B12 group) of belatacept (Nulojix, Bristol-Myers Squibb Pharma) at 5mg/kg.

225 **Flow cytometry**

226 Human cells suspensions from mice spleen and bone marrow were prepared by mechanical  
227 dilacerations and then stained for the phenotype analysis. APC/Cyanine7 anti-CD19 (clone  
228 HIB19) was purchased from Biolegend (Paris, France). APC anti-CD45 (clone HI30) and PE  
229 anti-CD8 were purchased from BD Biosciences (Le Pont de Claix, France). PE-Vio770 anti-  
230 CD38 (IB6) was purchased from Miltenyi Biotec (Paris, France). Efluor 506 fixable viability  
231 dye was purchased from eBioscience (Paris, France). PE anti-CD138 (clone B-A38) was  
232 purchased from Beckman Coulter (Villepinte, France). Events were acquired on a FACS  
233 Canto II flow cytometer using FACS Diva software (BD Biosciences), and data were  
234 analyzed using FlowJo software (Tree Star, Ashland, OR).

### 235 **Immunohistochemistry**

236 For immunoenzymatic staining, spleens were collected. Formalin fixation, paraffin inclusion,  
237 as well as standard staining (hematein-eosin) were performed on this tissue.  
238 Immunohistochemical staining was performed on 3-  $\mu\text{m}$ -thick tissue sections from the  
239 formalin- fixed paraffin- embedded spleen specimens, by a standardized automated method  
240 (Bond; Leica Menarini) using anti-CD20 M0755 (L26 clone, Dako, anti-CD5, NCL-L-CD5-  
241 4C7, 4C7 clone, Leica Biosystems), anti-CD38 (NCL-L-CD38-290, SPC32 clone, Leica  
242 Biosystems), anti-CD138 (M7228, MI15 clone, Dako, anti-CXCL13, MABB801, clone  
243 53610, R&D systems), anti-BCL6 (PA0204, LN22 clone, Leica Biosystems), anti-ICOS  
244 (ab105227, SP98 clone, Abcam), and anti-PD1 (ab52587, NAT105 clone, Abcam). Slides  
245 were scanned using NanoZoomer Digital Pathology System (Nanozoomer 2.0-HT slide  
246 scanner (Hamamatsu, Hamamatsu City, Japan)). Cell detection was conducted using QuPath's  
247 digital software (<https://qupath.github.io>) built-in 'Positive cell detection'. Cell densities of  
248 immune cells were expressed as the mean number of positive cells per  $\text{mm}^2$ .

### 249 **Luminex**



250 Ig concentrations were measured in the mice serum by Luminex following the manufacturer's  
251 protocol (Affymetrix E-bioscience; Human Isotyping procartaplex). Cytokines were  
252 quantified in the mice serum using the human Premixed Multi-Analyte kit from Bio-Techne  
253 with Luminex-based technology as specified by manufacturer. The following cytokines were  
254 analyzed: IFN- $\gamma$ , TNF- $\alpha$  and Granzyme B (23).

### 255 **Statistical analysis**

256 Statistical analyses of differences between groups were performed using paired t test or one-  
257 way anova follow by Tukey's multiple comparisons test, with the software Prism 9.0 (Graph  
258 Pad Software, Inc., La Jolla, CA). All statistical tests were considered statistically different  
259 when  $p < 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0,0001$ .

260

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267 **Author Contributions**

268 CS, AT, CP, and PG designed the study; CS, AT, AM and CP performed experiments; CS,  
269 AT, AM, CP and PG analyzed the data. CS, AT, JLC, CP and PG wrote the manuscript.

270 **Disclosure**

271 The authors of this manuscript have no conflicts of interest to disclose.

272 **Data availability statement**

273 Data are available on reasonable request, please contact corresponding author.

274

275 **Figure Legend**

276 **Figure 1: B cells engraftment after human CD8 depleted PBMCs infusion**

277 Intraspinal injection of NSG mice were realize with  $10 \times 10^6$  of PBMC or CD8 depleted  
278 PBMCs (CD8-). Mice were sacrificed at day 21 and spleen were harvested for analysis (A).  
279 **B)** Proportion of CD8 in CD45 cells before and after negative selection of CD8. Statistical  
280 significance of CD8 proportion of before and after negative selection was determined using  
281 paired t-test, \*\*\*  $p \leq 0.001$ . **C)** Spleen weight (g) of both group at day 21. **D)** Flow cytometry  
282 detection of human cells at day 21 in the spleen and bone marrow of NSG mice. Histograms  
283 represent percentage of human CD45+ and CD19+ cells are represented. All samples were  
284 initially gated for living lymphocytes by forward and side scatter. The cumulative data of five  
285 independent experiments are shown. Each point represents a single xenograft; data are

286 presented as mean percentage  $\pm$  SEM. **E)** Granzyme-B, IFN- $\gamma$  and TNF- $\alpha$  concentration in the  
287 mice serum at D21. Statistical significance from the controls (PBMC) was determined using  
288 Mann-Whitney tests. \* $p \leq 0.05$ . **F)** Serial sections were stained with anti-human CD20 /  
289 CD38 / CD138 for B cells analysis and anti-human CD5 / ICOS / PD1 for T cells analysis.  
290 Original magnification x20 for CD5 and CD20 staining, x100 for CD38, CD138, PD1 and  
291 ICOS staining. Dotted line in CD20 and CD5 images represents T and B cells colocalisation.

292

293 **Figure 2: Belatacept decreased human B cells persistence**

294 **A)** Protocol design. Intrasplenic injection of NSG mice were realize with  $10 \times 10^6$  of CD8  
295 depleted PBMCs (CD8-). Belatacept (5mg/kg) were injected either at the same day of cells  
296 injection (B0) nor at day 12 (B12). Untreated mice (NT) served as control group. Mice were  
297 sacrificed at day 21 and spleens were harvested for flow cytometry analysis. **B)** Spleen weight  
298 (g) of each group at day 21. The cumulative data of five independent experiments are shown.  
299 Each point represents a single xenograft; data are presented as mean percentage  $\pm$  SEM. **C)**  
300 Gating strategy and percentage of human CD45+ cells, CD19+ B cells, CD38+ CD138+ B  
301 cells in untreated (NT) and treated groups (B0, B12). The cumulative data of five independent  
302 experiments are shown. Each point represents a single xenograft; data are presented as mean  
303 percentage  $\pm$  SEM. Statistical significance from the controls (NT) was determined using one-  
304 way anova and Tukey's multiple comparisons test.  $p \leq *0.05$ ,  $**0.01$ ,  $***0.001$ .

305

306 **Figure 3: Belatacept inhibited human B and Tfh cells engraftment.**

307 Intrasplenic injection of NSG mice were realize with  $10 \times 10^6$  of CD8 depleted PBMCs (CD8-  
308 ). Belatacept (5mg/kg) were injected either at the same day of cells injection (B0) nor at day  
309 12 (B12). Untreated mice (NT) served as control group. Mice were sacrificed at day 21 and  
310 spleens were harvested and fixed for immunohistological analysis. **A)** Serial sections were

311 stained with hemotoxylin and eosin (x20), anti-human CD20 / CD5 (x20) B) and anti-human  
312 CD38 / CD138 C) anti-human PD1/ ICOS / Bcl6/ CXCL13 (x100 or x200). Histological  
313 quantification of human CD5+ T cells, ICOS+ or PD1+ Tfh cells, BCL6+ and CXCL13+ GC  
314 cells, CD19+ B cells, CD27+/CD38+ B differentiated cells and CD138+ cells per mm<sup>2</sup>. The  
315 cumulative data of two independent experiments are shown. Each point represents a single  
316 xenograft; data are presented as mean percentage  $\pm$  SEM. Statistical significance from the  
317 controls (NT) was determined using one-way anova and Tukey's multiple comparisons test. p  
318  $\leq$  \*0.05, \*\*0.01 and \*\*\*0,005.

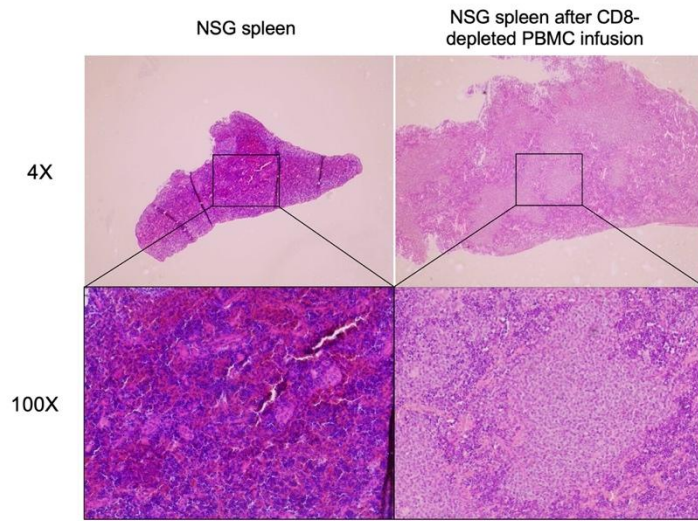
319

320 **Figure 4: Belatacept treatment affect the plasma cells' ability to secrete**  
321 **immunoglobulins.**

322 Intraspenic injection of NSG mice were realize with  $10 \times 10^6$  of CD8 depleted PBMCs (CD8-  
323 ). Belatacept (5mg/kg) were injected either at the same day of cells injection (B0) or day 12  
324 (B12) Untreated mice (NT) served as control group. **A)** Serum of mice were collected at day  
325 21 and human immunoglobulin, IgG (1,2,3 and 4), IgA, IgE, and IgM were analysis by  
326 Luminex assay. The cumulative data of five independent experiments are shown. Each point  
327 represents a single xenograft; data are presented as mean percentage  $\pm$  SEM. Statistical  
328 significance from the controls (NT) was determined using one-way anova test and Tukey's  
329 multiple comparisons test. p  $\leq$  \*0.05, \*\*0.01, \*\*\*0.001 and \*\*\*\*0,0001. **B)** Pie chart of IgGs  
330 repartition between complement binding IgGs (IgG1,IgG3) and non complement binding  
331 IgGs (IgG2,IgG4) for the three groups.

332

333 **Supplemental figure 1: Comparision between NSG spleen before and after human cells**  
334 **infusion.** Serial sections of normal NSG spleen and CD8-depleted PBMC infused mice at d21  
335 were stained with hemotoxylin and eosin (x4 and x100).



**Supplemental Figure 1**

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