Internal regulation between constitutively expressed T cell coinhibitory receptors BTLA and CD5 and tolerance in recent thymic emigrants

Adeolu O. Adegoke[1], Govindarajan Thangavelu[1,2], Ting-Fang Chou[3], Marcos Petersen[1,5], Kiyokazu Kakugawa[4], Julia F. May[5], Kevin Joannou[5], Qingyang Wang[3], Kristofor K. Ellestad[2,5], Louis Boon[6], Peter A. Bretscher[7], Hilde Cheroutre[3,4], Mitchell Kronenberg[3,8], Troy A. Baldwin[5], and Colin C. Anderson[1,2,5*].

[1]Department of Surgery, University of Alberta, Edmonton, AB, Canada
[2]Alberta Diabetes and Transplant Institutes, University of Alberta, Edmonton, AB, Canada;
[3]La Jolla Institute for Immunology, La Jolla, CA 92037, USA
[4]Laboratory for Immune Crosstalk, RIKEN Center for Integrative Medical Sciences, 1-7-22 Suehiro, Tsurumi-ku, Yokohama 230-0045, Japan
[5]Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada
[6]JJP Biologics, Warsaw, Poland
[7]Department of Microbiology and Immunology, College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada.
[8]Department of Molecular Biology, University of California San Diego, La Jolla CA 92093 USA

*Corresponding author

E-mail: colinand@ualberta.ca (CCA)

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Abbreviations: DN, double negative; DP, double positive; SP, single positive; WT, wild type; RTE, recent thymic emigrants; FLC, fetal liver cells

Abstract

Several coinhibitory receptors are upregulated upon activation, whereas a small number of coinhibitory receptors are expressed constitutively by naive T cells. The relationship between constitutively expressed coinhibitors is unknown. We found an inverse relationship between two constitutively expressed coinhibitors, CD5 and BTLA; BTLA expression was low in the thymus and high in the periphery, corresponding respectively with high and low CD5 expression. Germline or induced deletion of *Btla* in somatic cells demonstrated a causal relationship between BTLA expression and CD5 levels in T cells of central and peripheral lymphoid tissues. The effect of BTLA on CD5 expression on thymic and peripheral CD4 T cells was due to BTLA signaling, rather than signaling by its ligand, the herpes virus mediator (HVEM). Regulation was maintained in mice with a non-signaling HVEM mutant but was lost in *Tnfrsf14*$^{-/-}$ (*Hvem*$^{-/-}$) mice. Increased CD5 levels have been positively associated with increased recognition of self-peptide MHC complexes. Thus, control of CD5 expression by BTLA signals early in T cell ontogeny suggested that BTLA might be important for establishing self-tolerance in newly generated T cells. Consistent with this concept, we found that BTLA, as well as the inducible coinhibitor PD-1, were needed post thymic selection in recent thymic emigrants (RTE) to establish self-tolerance. RTE lacking BTLA caused a multiorgan autoimmune disease whose development required CD4 T cells and MHC class II. Together, our findings identify a negative regulatory pathway allowing constitutively expressed coinhibitory receptors to calibrate their expression in thymic T cell differentiation. Expression of constitutive and induced coinhibitory receptors is needed to establish tolerance in the periphery for RTE.

Introduction

To generate repertoire diversity, developing T cells in the thymus express receptors generated from random recombination of TCR gene segments [1]. The outcome of this stochastic TCR gene segment rearrangement is the generation of T cells bearing receptors that recognize foreign antigens or self-antigens as agonists, with the latter potentially promoting autoimmune diseases, although varying degrees of beneficial autoreactivity are present in regulatory T cells (Treg) and innate-like T cells, such as natural killer T cells (NKT cells). Positive selection of T cells creates an additional potential danger, as conventional $CD4^+$ and $CD8\alpha\beta^+$ T cells are positively selected based on an ability to recognize a self-peptide–MHC complex. Hence, a critical function of the immune system is to discriminate self from non-self, failure of which will result in detrimental autoimmunity. Thus, most T cells that bind self-peptide–MHC with sufficient affinity need to be made tolerant, and all T cells need to be maintained in a state where they perceive low-affinity interactions with self-peptide–MHC ligand (e.g., the positively selecting peptide) as a 'tonic' signal rather than an agonist signal. Although T cell activation and tonic signaling are determined by the interaction of TCR with specific antigenic peptide–MHC complexes, the functional outcome of the T cell response is strongly influenced by costimulatory and coinhibitory signals [2,3]. Such coinhibitory receptors, like programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), are absent from naïve T cells and are upregulated upon activation [4,5]. In contrast, there are a small number of coinhibitory receptors expressed constitutively by naïve T cells, including CD5, B and T lymphocyte attenuator (BTLA), and V-domain immunoglobulin suppressor of T cell activation (VISTA) [6–9] .

75 CD5 is a coinhibitory receptor constitutively expressed on post-selected thymocytes,
76 mature T cells, and a subset of B cells (B1a cells) [6,9–12]. CD5 is expressed as a 67-kDa type I
77 transmembrane glycoprotein (gp), which belongs to the highly conserved superfamily of protein
78 receptors known as the scavenger receptor cysteine rich (SRCR) superfamily [13]. The proposed
79 ligands for CD5 include: CD72 [14], IL-6 [15,16], gp40–80 [17–19], gp150 [20], gp200 [21],
80 IgV_H framework region [22], and CD5 itself [23]; however, their physiological relevance and
81 relative importance in interacting with CD5 remains an active area of investigation. CD5 also
82 appears to function without its extracellular domain, questioning whether a ligand for CD5 is
83 relevant. During T cell activation, CD5 is rapidly recruited and co-localizes with the TCR/CD3
84 complex at the immune synapse, dampening downstream TCR signals [24,25].
85 BTLA (CD272) is a negative regulator of antigen receptors on B and T cells, dendritic
86 cells, macrophages, and NK cells [7,26–28]. The BTLA cytoplasmic region contains both ITIM
87 and ITSM motifs and a Grb2 recognition motif [7]. Following T cell activation and the
88 interaction of BTLA with its ligand, the herpesvirus entry mediator (HVEM or CD270), the
89 tyrosine residues in ITIM and ITSM of BTLA are phosphorylated and then recruit SH2-
90 containing tyrosine phosphatase 1 (SHP-1) and SHP-2 phosphatases to dampen TCR signaling
91 [27,29]. A T cell can express both BTLA and HVEM, and HVEM interactions with BTLA
92 between cells, in *trans*, or *cis* interactions in which one cell expresses both binding partners, are
93 inhibitory [30]. Consistent with its coinhibitory function, mice lacking BTLA develop systemic
94 autoimmune disease and multiorgan lymphocytic infiltration [31]; they also have increased
95 frequencies of T follicular helper cells in Peyer's patches and increased IgG and IgA, the latter
96 affecting the homeostasis of gut bacteria [32]. In contrast, $Cd5^{-/-}$ mice remain relatively healthy,
97 even in late-life [33]. Although constitutively expressed BTLA and CD5 on T cells have unique

98 non-redundant roles, the relationship between these coinhibitors is not well defined. It was
99 recently reported that CD5 expression levels increased in SHP-1 knockout T cells relative to WT
100 T cells [34]. Since BTLA and CD5 exert their inhibitory function through lymphocyte activation-
101 induced recruitment of SHP-1 to their cytoplasmic tail tyrosine residues, these two coinhibitors
102 might have some overlapping or counter-regulatory functions. In addition, CD5 expression is set
103 during T cell development in the thymus and finely adjusted throughout the life of the T cell
104 [35,36], with CD5 surface expression correlating directly with the avidity or signaling intensity
105 of TCR–self-peptide MHC interaction [35,37]. CD5 is expressed most highly on T cells in the
106 thymus [35,38] and on recent thymic emigrants in the periphery [39]. CD5 levels are modified
107 by the autoimmune disease associated H-2^{g7} haplotype [40]. Given the above, CD5 levels might
108 identify stages where the self-reactive potential of the T cell repertoire is greatest. Herein, we
109 explored the relationship between these coinhibitors and found that BTLA broadly controls CD5
110 expression levels from early in T cell ontogeny and that both BTLA and PD-1 are needed to
111 establish peripheral tolerance at the recent thymic emigrant stage.
112

113 # Results

114

115 ## Negative regulation of CD5 expression by BTLA

116

117 To examine the relationship between CD5 and BTLA, we assessed CD5 and BTLA expression
118 in the steady state in both TCRβ^{hi} single positive (SP) T cells in the thymus and splenic T cells
119 from 7-10-week-old C57BL/6 mice (S1A Fig). Consistent with earlier studies [35,38], thymic SP
120 T cells expressed higher levels of CD5 relative to splenic SP T cells (Fig 1A). In contrast,

121 expression of BTLA was lower in the thymic SP T cells relative to splenic SP T cells (Fig 1A).
122 Analysis of the proportion of BTLA$^+$ cells among thymic and splenic SP T cells also revealed a
123 reduced frequency of BTLA$^+$ SP T cells in the thymus relative to the spleen (Fig 1B). This
124 inverse relationship between BTLA and CD5 was apparent when comparing expression in
125 central vs peripheral lymphoid organs but not within each tissue individually; T cells with the
126 highest BTLA expression in each tissue did not have reduced CD5 expression (S1B Fig). These
127 data suggested that BTLA and CD5 have opposite trajectories of expression as T cells mature.

128 A small fraction of splenic T cells are recent thymic emigrants (RTE). We hypothesized
129 that these newly generated T cells would also express lower levels of BTLA relative to their
130 established or more mature T cell counterparts. To examine this, we used the *Rag2p*^{GFP} mice,
131 where GFP expression is restricted to thymocytes, RTE and newly generated B cells [41,42].
132 Analysis of BTLA expression comparing the GFP$^+$ (RTE) and GFP$^-$ (mature) T cells in the
133 spleen revealed that BTLA expression was significantly lower in RTE relative to their mature T
134 cell counterparts (Fig 1C, D); BTLA expression on T cells also increased as GFP expression
135 decreased in both the spleen and thymus (S2 Fig). In contrast, CD5 expression was significantly
136 higher on RTE (Fig 1C, D). Collectively, these data indicated an inverse relationship between
137 CD5 and BTLA expression as T cells mature.

138 To determine if there was a causal relationship between BTLA expression and CD5
139 expression, we compared CD5 expression between polyclonal WT and coinhibitor BTLA
140 deficient T cells in the periphery. We analyzed PD-1 in parallel, because we showed earlier that
141 it plays a role in the establishment of tolerance in newly generated T cells [43,44]. The
142 expression of CD5 was significantly higher in *Btla*$^{-/-}$ splenic CD4 and CD8 T cells relative to
143 their WT and PD-1 deficient (*Pdcd1*$^{-/-}$) counterparts; the overall increase in CD5 mean

144 fluorescence intensity (MFI) of *Btla*$^{-/-}$ T cells was due to a reduction in CD5 low cells (Fig 1E).
145 Congenital absence of the inducible coinhibitor PD-1 resulted in significantly increased CD5
146 expression in splenic CD8 T cells (Fig 1E). PD-1 deficiency did not alter BTLA expression
147 (S3A Fig). To assess whether the elevated CD5 expression in *Btla*$^{-/-}$ splenic T cells originated
148 from the thymus or preferentially increased in the periphery, we compared CD5 expression
149 between the WT, *Pdcd1*$^{-/-}$, and *Btla*$^{-/-}$ thymic TCRβ^{hi} SP cells. These cells in the *Btla*$^{-/-}$ but not
150 *Pdcd1*$^{-/-}$ mice expressed higher CD5 than their WT counterparts (Fig 1E). A possible
151 explanation is that BTLA is expressed on a much higher percentage of thymocytes than PD-1.
152 WT, *Btla*$^{-/-}$, and *Pdcd1*$^{-/-}$ T cells expressed a similar level of CD5 in DN and DP populations
153 (S3B, C Fig), indicating that CD5 expression in the *Btla*$^{-/-}$ T cells increased only post thymic
154 selection. Although only a small fraction of thymic or splenic T cells expressed the inducible
155 coinhibitor PD-1, BTLA deficiency also increased its expression (S3D Fig).
156 Treg cells make up a relatively small proportion of the bulk CD4^{+} T cell population, but
157 the preferential expression of higher levels of CD5 in Treg cells [45] may skew the CD5
158 expression levels in the *Btla*$^{-/-}$ CD4^{+} T cells. Therefore, we analyzed Treg numbers [46] and
159 CD5 expression in splenic and thymic TCRβ^{+} CD4^{+} Foxp3^{+} (Treg) or TCRβ^{+} CD4^{+} Foxp3^{-} (non-
160 Treg) cells of WT and *Btla*$^{-/-}$ mice (S4 Fig). CD5 expression was significantly higher in both
161 Treg and non-Treg cells of the *Btla*$^{-/-}$ mice in the spleen and thymus (S4C, D Fig).
162 Since CD5 expression is correlated with the signaling strength of TCR:self-antigen (self-
163 pMHC) interactions [35,37] and Nur77 serves as a specific reporter of antigen receptor signaling
164 in murine and human T and B cells [47–53], we hypothesized that Nur77^{GFP} expression would be
165 enhanced in *Btla*$^{-/-}$ T cells. We generated *Btla*$^{-/-}$ *Nur77*^{GFP} mice and compared their GFP
166 expression levels in the splenic and thymic T cells to that in BTLA sufficient *Nur77*^{GFP} mice.

167 Thymic and splenic *Btla*$^{-/-}$ CD8 T cells expressed higher levels of GFP relative to the WT CD8
168 T cells (Fig 1F). In contrast, GFP expression in the thymic and splenic *Btla*$^{-/-}$ CD4 T cells was
169 not significantly different from their WT CD4 T cell counterparts (Fig 1F). Since Nur77^{GFP}
170 expression in B cells also correlates to the B cell receptor affinity for antigen [51], we compared
171 GFP expression levels in B cells from *Btla*$^{-/-}$ *Nur77*^{GFP} mice and WT *Nur77*^{GFP} mice and found
172 no significant difference between the two groups (S4E Fig). Together, these data suggest that
173 BTLA expression directly or indirectly determined the level of CD5 expression across the major
174 conventional T cell subsets, while having a more limited effect on Nur77 expression.
175

176 ## BTLA regulates CD5 expression in adult mice

177

178 We determined whether the lack of BTLA from early in T cell ontogeny, as in the case of the
179 germline *Btla* knockout mice, is important for producing heightened T cell CD5 expression. For
180 example, the lack of BTLA during the neonatal period might alter the TCR repertoire when it is
181 first generated, indirectly affecting CD5 levels. We therefore assessed the effect of deleting *Btla*
182 in young adult mice by crossing *B6.Btla*$^{fl/fl}$ mice to a tamoxifen-inducible Cre recombinase
183 expressing strain, *B6*$^{Cre/ERT2}$, to generate *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ mice (S5 Fig). We injected adult 7-
184 week-old *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ and WT control *B6*$^{Cre/ERT2+/-}$ mice intraperitoneally with tamoxifen
185 (Fig 2A). At one-week post-tamoxifen injection only about a third of T cells circulating in the
186 blood had lost BTLA expression. However, the BTLA deficient fraction of CD4 T cells had
187 significantly heightened CD5 expression (Fig 2B). By two weeks post-tamoxifen, *B6*$^{Cre/ERT2+/-}$
188 splenic CD4 and CD8 SP T cells, were 97 ± 2% and 94 ± 4% positive for BTLA, respectively,
189 while *B6*$^{Cre/ERT2+}$*Btla*$^{fl/fl}$ T cells were reduced to 2 ± 1% and 3 ± 2% positive for BTLA (Fig 2C).

190 CD5 expression on thymic and splenic T cells had not yet significantly changed at this time point
191 relative to WT controls (data not shown). By four weeks post-tamoxifen CD5 and PD-1
192 expression in the thymic and splenic CD4 T cells and thymic CD8 T cells of *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$
193 mice had increased relative to their *B6*$^{Cre/ERT2+/-}$ counterparts (Fig 2D). These data indicated that
194 the impact of BTLA deficiency on CD5 and PD-1 expression is not specific to T cell ontogeny
195 early in life; rather, it can be instigated acutely in adulthood. The rapid increase in CD5 expression
196 on peripheral circulating CD4 T cells, induced by BTLA deletion, suggested that BTLA may be
197 capable of regulating CD5 expression after the early generation of the TCR repertoire and in
198 mature, peripheral T cells. Consistent with the possibility that BTLA regulates CD5 independent
199 of TCR repertoire changes, we did not detect any major differences in the repertoire of sorted
200 thymic CD4 SP T cells of WT and *Btla*$^{-/-}$ mice assessed by single cell RNA sequencing (S6 Fig);
201 however, these findings do not rule out potential subtle differences in the repertoire of *Btla*$^{-/-}$
202 mice. Therefore, we tested the effect of BTLA on CD5 expression when the TCR repertoire is
203 fixed. We crossed the *Btla* gene knockout strain to OT-II [54] and OT-I [55] TCR transgenic
204 mice. Complete BTLA deficiency (OT-II.*Btla*$^{-/-}$) led to heightened CD5 expression in OT-II T
205 cells in the periphery and the thymus (Fig 3). OT-II T cells that expressed high levels of the
206 transgenic TCR lacked endogenous TCR expression [56]. The heightened CD5 in BTLA
207 deficient OT-II T cells was evident also in those CD4 SP thymocytes and splenocytes expressing
208 high levels of the transgenic TCR (Vβ5Vα2), indicating that endogenous TCR expression and
209 TCR repertoire changes were not responsible for the altered CD5 expression (Fig 3). In a
210 preliminary analysis of OT-I CD8 T cells, that naturally expressed very high levels of CD5 [57],
211 BTLA deficiency further increased CD5 expression (S7 Fig).
212

213 # HVEM signals do not net set the level of CD5 expression

214

215 The only known ligand for BTLA is HVEM, a member of the tumor necrosis factor superfamily
216 (TNFRSF) and therefore is designated as TNFRSF14. HVEM also functions as a signaling
217 receptor, recruiting TRAF2 and activating NK-κβ Rel A, to co-stimulate T cells [58]. HVEM
218 signaling is also involved in survival of memory CD4 [59] and memory CD8 T cells [60,61].
219 Given the potential for bi-directional signaling, regulation of CD5 and PD-1 expression by
220 BTLA could be due to BTLA signals, HVEM-mediated signals, or both. We therefore examined
221 CD5 and PD-1 expression in germline HVEM knockout mice and mice engineered to express a
222 'tail mutant' HVEM with the intact ectodomain but lacking the normal sequence of the
223 intracytoplasmic domain and therefore incapable of activating NF-κB ($Hvem^{tm/tm}$).
224 Using CRISPR-Cas9 technology, we generated an *Hvem* mutant lacking exon 7 with a
225 cytoplasmic tail sequence created by splicing exon 6 to exon 8 (S8A Fig). This sequence lacks
226 the proline-x- glutamate amino acid motif likely required for tumor necrosis factor receptor-
227 associated factor (TRAF) binding and HVEM signaling [62]. We validated the lack of HVEM
228 signaling by the mutant HVEM in vitro in transfected cells using cells expressing HVEM ligands
229 and interacting with cells expressing the tail mutant HVEM with an NF-κB reporter as a readout
230 (S8B Fig). In vivo, the tail mutant HVEM had lower surface expression than either WT mice or
231 $Hvem^{WT/tm}$ hemizygous mice, (S8C Fig). Despite this, we validated that the reduced amount of
232 surface HVEM was sufficient in vivo in a liver injury model induced by activating invariant
233 natural killer T (iNKT) cells with the potent agonist α-galactosylceramide (αGalCer). Previous
234 studies show that either BTLA or CD160 expressed serve as attenuators of αGalCer-mediated
235 acute hepatic injury [63–65], and this occurs via engagement of HVEM [66]. To test the

236 importance of HVEM signaling function in this hepatic injury model, we co-housed littermate
237 WT control, *Hvem*$^{tm/+}$ hemizygous, and *Hvem*$^{tm/tm}$ mice that were injected with αGalCer and
238 analyzed 24h later. *Hvem*$^{tm/tm}$ mice displayed a similar serum alanine aminotransferase (ALT)
239 level compared to WT or hemizygous mice (S8D Fig), indicating HVEM acts as a ligand for
240 signaling inhibitory receptors in this model.

241 We found that CD4 SP T cells from *Hvem*$^{-/-}$ mice expressed higher CD5 in the thymus
242 and spleen as well as higher PD-1 in the thymus (Fig 4A). This elevated CD5 and PD-1
243 expression occurred despite thymic and splenic CD4 and CD8 SP T cells also having heightened
244 BTLA expression in *Hvem*$^{-/-}$ mice, in agreement with a previous report [67]. The heightened
245 BTLA expression, however, could not compensate for the lack of the only known BTLA binding
246 partner. In contrast, despite having lower levels of HVEM than either WT mice or *Hvem*$^{WT/tm}$
247 hemizygous mice, HVEM expression by *Hvem*$^{tm/tm}$ mice was sufficient to keep CD5 and PD-1
248 expression at a level similar to that in WT mice (Fig 4B and S8E Fig). This suggests it is
249 engagement with BTLA that underlies HVEM's influence on CD5 and PD-1 expression, not
250 signaling through the HVEM cytoplasmic domain.

251

252 BTLA signaling in newly generated T cells blocks autoimmune 253 disease

254

255 Expression of BTLA and its effects on CD5 levels early in T cell ontogeny (Fig 1B, D) may
256 reflect a need for this coinhibitor to 'tune' developing T cells to establish tolerance to self-
257 peptide/MHC complexes. Previously, we showed that newly generated T cells depended on PD-1
258 to broadly establish self-tolerance; transfer of PD-1 deficient fetal liver cells (FLC) to syngeneic

259 *Rag*$^{-/-}$ recipients led rapidly to a lethal multiorgan autoimmune disease as newly generated T
260 cells emerged from the thymus [43,44]. To test if BTLA expression is similarly functionally
261 important in newly generated T lymphocytes, we used three approaches that included either
262 stimulating BTLA signaling or removing it. First, we treated recipients of *Pdcd1*$^{-/-}$ FLC with an
263 agonistic antibody to BTLA. In this hematopoietic stem cell (HSC) transfer model, T cells
264 typically begin to seed the periphery approximately three weeks post-FLC injection [43]. Shortly
265 after T cell generation recipients of *Pdcd1*$^{-/-}$ HSC developed symptoms of disease. The agonistic
266 anti-BTLA antibody significantly delayed disease development (S9A Fig). In the two additional
267 approaches, we adoptively transferred FLC that were either congenitally deficient in BTLA or
268 BTLA was inducibly deleted after transfer (Fig 5A). We transferred FLC from *B6*$^{Cre/ERT2+/-}$
269 *Btla*$^{fl/fl}$ or *B6*$^{Cre/ERT2+/-}$ control mice to adult *Rag*$^{-/-}$ mice, followed by injection of tamoxifen to
270 induce *Btla* gene deletion in the transferred cells. FLC was used as a source of HSC in this
271 experiment, allowing for the deletion of BTLA in T cell progenitors pre-thymic selection.
272 Similar to the germline *Btla*$^{-/-}$ T cells, peripheral T cells in the recipients of FLC from
273 *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ mice had increased CD5 expression level through the 8-week experimental
274 period (Fig 5B). T cells were detected in the peripheral blood of FLC recipient mice around four
275 weeks post FLC transfer, which coincided with the onset of disease in recipients of *B6*$^{Cre/ERT2+/-}$
276 *Btla*$^{fl/fl}$ FLC (Fig 5C). All the recipients of *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ FLC were diseased before the
277 experimental end-point while their *B6*$^{Cre/ERT2+/-}$ counterparts remained free of disease (Fig 5C).
278 Lymphocytes populating the periphery of recipients of *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ FLC were BTLA
279 negative while lymphocytes populating *B6*$^{Cre/ERT2+/-}$ FLC recipients expressed BTLA (Fig 5D).
280 Although BTLA expression has been seen on non-hematopoietic cells in some settings [68,69],

281 these data indicated that loss of BTLA in fetal liver derived cells alone was sufficient for the
282 generation of multi-organ autoimmune disease in the recipients.
283 To examine in more detail the autoimmune disease generating effects of BTLA
284 deficiency in newly generated vs. established *Btla*$^{-/-}$ T cells, we compared transfers of FLC,
285 thymocytes, whole splenocytes or sorted splenic T cells to syngeneic *Rag*$^{-/-}$ recipients. Most of
286 the recipients of *Btla*$^{-/-}$ thymocytes or FLC demonstrated severe morbidity, while recipients of
287 the WT control FLC, FLC deficient in Fas (*lpr* FLC), and recipients of *Btla*$^{-/-}$ whole splenocytes
288 or purified splenic T cells remained free of disease (Fig 6A). Histological analysis of tissue
289 sections obtained from sick mice 60-65 days post cell transfer revealed lymphocytic infiltrations
290 in major organs, including the liver, kidney, and pancreas of *Btla*$^{-/-}$ FLC recipients (S9B Fig).
291 Lymphocytic infiltration was present in the liver of *Btla*$^{-/-}$ FLC but not WT FLC recipients and
292 included CD4 and CD8 T cells (Fig 6B). The liver was the most frequently affected internal
293 organ examined in *Rag*$^{-/-}$ recipients of *Btla*$^{-/-}$ FLC, consistent with the late life spontaneous
294 hepatitis that has been observed in unmanipulated *Btla*$^{-/-}$ mice [31]. Newly generated *Btla*$^{-/-}$ T
295 cells demonstrated substantially increased proliferation compared to WT T cells (Fig 6C).
296 Collectively, these data demonstrated a requirement for BTLA in newly generated T cells to
297 establish tolerance and prevent lymphopenia-potentiated autoimmune disease, while BTLA was
298 not required for maintaining tolerance of established T cells under these conditions.
299

300 CD4^{+} T cells and MHC II are required for autoimmune disease

301

302 Having shown that loss of BTLA in newly generated T cells leads to autoaggressive T cells, we
303 asked what T cell subset(s) is required for disease generation. We adoptively transferred sorted

304 CD4 or CD8 SP thymocytes from *Btla*$^{-/-}$ or WT mice into *Rag*$^{-/-}$ mice (Fig 7A and S10A Fig).
305 We monitored the recipient mice for several weeks or until losing ≥ 20% of baseline body
306 weight, whichever came first. Recipients of *Btla*$^{-/-}$ CD4 SP T cells started losing weight as early
307 as 21 days post cell transfer, which continued for up to 53 days when they had lost ≥ 20% of
308 their baseline body weight (Fig 7A). All of the *Btla*$^{-/-}$ CD4 SP T cell recipient mice had a
309 hunched appearance, piloerection, diarrhea and some had dermatitis. Disease occurred in male
310 and female *Rag*$^{-/-}$ recipients of *Btla*$^{-/-}$ CD4 SP T cells. In contrast, none of the recipients of
311 *Btla*$^{-/-}$ CD8 SP T cells showed signs of morbidity, and their body weight increased throughout
312 the experiment. Although recipients of WT SP CD4 or CD8 T cells had an initial decline in body
313 weight, they quickly recovered and showed an improvement in body weight and no signs of
314 disease (Fig 7A). To examine what MHC molecule is required for this autoimmune disease, we
315 adoptively transferred whole *Btla*$^{-/-}$ SP thymocytes to *K*^{b}*D*$^{b-/-}$ *Rag*$^{-/-}$ or *CiiTA*$^{-/-}$ *Rag*$^{-/-}$ mice
316 that lacked both Rag and MHC class I genes or lacked both Rag and MHC class II protein
317 expression [70], respectively. All *CiiTA*$^{-/-}$ *Rag*$^{-/-}$ mice were free of disease, while all *K*^{b}*D*$^{b-/-}$
318 *Rag*$^{-/-}$ recipients had signs of morbidity within 3 weeks post T cell transfer, regardless of sex
319 (Fig 7B). Similarly, sorted CD4 *Btla*$^{-/-}$ SP thymocytes transferred to *K*^{b}*D*$^{b-/-}$ *Rag*$^{-/-}$ caused
320 disease, while sorted CD8 *Btla*$^{-/-}$ SP thymocytes transferred to *CiiTA*$^{-/-}$ *Rag*$^{-/-}$ mice did not
321 (S10B, C Fig)**.** Thus, MHC class II and MHC class II-restricted CD4 T cells were required to
322 induce disease.
323

324 BTLA and PD-1 signaling are needed in newly generated T cells to
325 block autoimmune disease

326

327 The current studies identified that, like PD-1, BTLA was important for the establishment of
328 tolerance in newly generated T cells; however, they did not address whether these coinhibitory
329 receptors were needed during thymic selection or post thymic selection, when the newly
330 generated T cells seed the periphery. To examine if autoimmune disease occurs when BTLA or
331 PD-1 is deleted post thymic selection, thymocytes from adult (7 – 9-week-old) *B6*$^{Cre/ERT2+/-}$
332 *Btla*$^{fl/fl}$ or *B6*$^{Cre/ERT2+/-}$ *Pdcd1*$^{fl/fl}$ mice were adoptively transferred via the tail vein to adult *Rag*$^{-/-}$
333 mice, followed by tamoxifen injection to induce gene deletion in the transferred thymocytes. The
334 control group received *B6*$^{CreERT2+/-}$ thymocytes and tamoxifen injection (Fig 8A). There was a
335 near complete deletion of BTLA and PD-1 by day 7 post the last dose of tamoxifen in the T cells
336 of *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ and *B6*$^{Cre/ERT2+/-}$ *Pdcd1*$^{fl/fl}$ thymocyte recipients, respectively, while
337 expression of these coinhibitors was maintained in the *B6*$^{CreERT2+/-}$ thymocyte recipients (Fig
338 8B). All of the *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ and *B6*$^{Cre/ERT2+/-}$ *Pdcd1*$^{fl/f}$ thymocyte recipients, but not their
339 *B6*$^{CreERT2+/-}$ counterparts, developed inflammatory disease. They began to lose body weight at
340 days 7 - 14 post thymocyte transfer and showed additional signs of disease at days 12 - 17 (Fig
341 8C). Histological examination showed CD4 SP T cell infiltration in the kidney and liver of
342 *B6*$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ thymocyte recipients and the kidney, liver, and lung of *B6*$^{Cre/ERT2+/-}$ *PD-1*$^{fl/fl}$
343 thymocyte recipients (S11 Fig). Since BTLA and PD-1 were present during thymic selection and
344 deleted only post cell transfer, these coinhibitors were needed at the RTE stage to establish
345 tolerance.

346 # Discussion

347

348 In this study, we aimed to understand the relationship between the constitutively expressed T cell
349 coinhibitory molecules BTLA [71,72] and CD5, and an induced coinhibitor, PD-1, in the steady

350 state and under conditions that promote multi-organ autoimmune disease. Our data on steady
351 state CD5 expression in the thymus and spleen were consistent with findings in the literature
352 [35,38] and when compared with BTLA expression, revealed an inverse relationship. Our data
353 showed that peripheral T cells expressed higher levels of BTLA than thymocytes. This is a result
354 of a maturation process that began in the thymus and continued in the periphery, with RTE in the
355 spleen expressing lower levels of BTLA relative to the mature splenic T cells. In contrast,
356 splenic RTE expressed higher levels of CD5 relative to their mature T cell counterparts, as seen
357 previously [39]. High CD5 on RTE may reflect increased sensitivity to self-peptide-MHC
358 complexes, consistent with the greater self-reactivity and autoimmune potential of RTE that we
359 have observed in the context of PD-1 deficiency [44].

360 Analysis of CD5 expression in the *Btla*$^{-/-}$ mice or upon induced BTLA deletion showed
361 that the overall mean CD5 expression was enhanced in BTLA deficient CD4 and CD8 T cells,
362 indicating that BTLA negatively regulates CD5 expression. Heightened CD5 expression was a
363 result of a reduction in CD5 low cells. This suggests that BTLA was either needed for survival of
364 CD5 low cells or it enhanced CD5 expression in the CD5 low cells. While a role for BTLA in T
365 cell survival during chronic stimulation has been shown [73], we found increased expression of
366 CD5 on BTLA deficient OT-II CD4 T cells, a T cell that normally expresses relatively low CD5
367 levels [37]. This indicated BTLA may be able to negatively regulate CD5 expression
368 independently from any effects it might have on the generation of the TCR repertoire early in
369 life. CD5 expression on differentiating thymocytes reflects the TCR affinity for self-peptide
370 MHC complexes. Regulation of CD5 expression in thymocytes appeared to be specific to BTLA,
371 as a deficiency in another coinhibitor (PD-1) had no effect on CD5 expression. Complementing
372 the CD5 expression data, the *Btla*$^{-/-}$ thymic and splenic CD8 T cells displayed enhanced

373 Nur77^{GFP} expression. Although our data showed a disparity between CD5 and Nur77^{GFP}
374 expression levels in the *Btla*$^{-/-}$ thymic and splenic CD4 T cells, Zinzow-Kramer *et al*., had
375 previously reported that a broad range exists in the expression levels of CD5, Ly6C, and
376 Nur77^{GFP} in vivo for naïve T cells even within the same TCR niche [74]. This suggests that CD5
377 and Nur77 can be regulated differently from each other in response to TCR signals. Heightened
378 PD-1 expression in CD4 and CD8 SP thymocytes in mice deficient for either BTLA or HVEM
379 further supported the concept that loss of BTLA signaling leads to enhanced self-recognition
380 early in T cell ontogeny. Strikingly, even the decreased levels of HVEM expression, as occurred
381 in *Hvem*$^{tm/tm}$ mice, were sufficient to negatively regulate both BTLA and PD-1. Given that
382 HVEM interacts with several different ligands, it could not be anticipated whether HVEM
383 deficiency would mimic BTLA deficiency in the regulation of CD5 and PD-1 expression.
384 HVEM deficiency mimicked BTLA deficiency closely, however, for control of CD5 expression
385 in CD4 T cells but not in CD8 T cells. This suggested the other ligands for HVEM, including
386 LIGHT and CD160, may have differential effects on CD5. Together these findings suggest that
387 expression of some co-inhibitors are calibrated by BTLA signals triggered through a highly
388 sensitive engagement by HVEM, acting not as a signaling receptor but purely as a ligand or
389 binding partner for BTLA.

390 Mice deficient in BTLA develop autoimmune disease only later in life [31], suggesting
391 that either other coinhibitors compensate for the loss of BTLA in early life, or early events take
392 time for their consequences to become apparent. Increased CD5 expression in the *Btla*$^{-/-}$ mice
393 suggested that CD5 may serve as a compensatory coinhibitory receptor. Despite this, increased
394 CD5 on newly generated T cells was not sufficient for preventing autoimmune disease following
395 transfer to immune deficient mice. The conditions in *Rag*$^{-/-}$ recipients, however, in terms of the

396 extent or rapidity of lymphopenia induced proliferation, changes in the microbiome, and others,
397 might not fully represent the conditions in perinatal immune competent mice. Compensatory
398 coinhibitor expression may limit T cell autoreactivity in other contexts. In cancer therapy it can
399 limit the efficacy of treatment [75]. BTLA blockade has been shown to limit tumour growth and
400 improve survival in a murine model [76] and it is a target in clinical trials [77]. The effect of
401 blocking both CD5 and BTLA have yet to be determined. Supporting our findings in the
402 *Pdcd1*$^{-/-}$ mice, where CD5 expression was elevated in the splenic CD8^{+} T cells, a recent report
403 showed that blocking CD5 together with PD-1 substantially enhanced survival and tumour cell
404 killing [78].

405 We examined the role of BTLA in newly generated T cells and found that BTLA, like
406 PD-1 [43,44], was important for establishing tolerance in newly generated, polyclonal T cells. In
407 contrast, BTLA signaling appears to be dispensable for establishing tolerance in newly generated
408 TCR transgenic CD8 T cells that recognize a tissue-specific antigen expressed in the thymus and
409 pancreas [79]. This might reflect a more critical role for BTLA in CD4 T cells. Consistent with
410 this idea, we found that sorted *Btla*$^{-/-}$ CD4 but not CD8 T cells could generate multi-organ
411 autoimmune disease, and disease development did not occur in MHC class II deficient recipients.
412 BTLA was also previously reported to regulate CD4 T cell alloreactivity and proliferative
413 responses to MHC class II antigens [80]. CD4 T cells and/or MHC class II are also required for
414 disease development caused by newly generated *Pdcd1*$^{-/-}$ T cells [43,44].

415 To understand at what point during T cell development BTLA and PD-1 may be needed
416 to establish tolerance, we deleted each of these coinhibitors individually in RTE that had the
417 capacity to express them during thymic development. We found that loss of BTLA or PD-1 post
418 thymic selection in RTE resulted in autoimmune disease in lymphopenic host mice, associated

419 with loss of weight, dermatitis, diarrhea, and T cell infiltration of organs. Thus, BTLA and PD-1
420 were needed post thymic selection to establish peripheral tolerance in newly generated T cells, at
421 least in *Rag*$^{-/-}$ mice. Our current study does not exclude a role for these coinhibitors during
422 central tolerance. A role for BTLA in central tolerance has not yet been examined. We
423 previously found that PD-1 was not needed for central tolerance in CD4 or CD8 T cells specific
424 to a ubiquitous self-antigen [43], however, PD-1 was needed for non-deletional central tolerance
425 in CD8 T cells that recognize a tissue-restricted antigen [79]. The current data suggest that any
426 role the coinhibitors might have in inducing central tolerance is not by itself sufficient to broadly
427 establish a durable self-tolerance. The need for these coinhibitors in RTE suggests that BTLA
428 and PD-1 will be critical to establishing tolerance during the neonatal period when all peripheral
429 T cells are RTE [81,82]. Consistent with this idea, a substantial proportion of neonatal CD4 T
430 cells, those undergoing natural lymphopenia-induced-proliferation, were found to express PD-1
431 [44]. Our current studies are further addressing this important question.

432 Interestingly, adult mouse splenic T cells from mice with germline BTLA or PD-1 [43]
433 deficiency did not cause overt autoimmune diseases in the recipients. From *Pdcd1*$^{-/-}$
434 splenocytes, only the sorted RTE subpopulation could generate overt disease; sorted established
435 T cells did not cause disease [44]. A key question for future studies is whether the seeming lack
436 of need for these coinhibitors in established T cells indicates that the coinhibitors are not
437 required to maintain a tolerant state to tonic self-peptide/MHC ligands. An alternative possibility
438 is that these coinhibitors are needed to maintain tolerance when T cells have developed in their
439 presence. Although our study establishes the importance of BTLA and PD-1 signaling in RTE to
440 block autoimmune disease development in lymphopenic recipients, the germline BTLA or PD-1
441 knockout mice seem to be protected from overt autoimmune disease during the neonatal period

442 when there is physiological lymphopenia. This is likely to result from two factors: (1) their T cell
443 repertoire is first generated early in life, a period naturally deficient in lymph node stroma [83],
444 and (2) early disease-causing events normally take time for their consequences to become
445 apparent. Consistent with the first idea, we previously showed that neonatal *Rag*$^{-/-}$ recipients of
446 *Pdcd1*$^{-/-}$ FLC were relatively resistant to disease, as were adult recipients that lacked or had
447 reduced lymph nodes [43]. Supporting the second idea, T cells generated early in life in the NOD
448 mice play an important role in the initiation of insulitis long before the onset of overt diabetes
449 that manifests much later in life [84,85].

450 Taken together, our data identify a regulatory axis between BTLA and CD5 and a critical
451 need for both constitutive and inducible coinhibitors (BTLA and PD-1) when T cells initially
452 seed the periphery.

453

454 # Materials and methods

455

456 ## Animals

457

458 Mice used in this study included male and female B6.129S7-*Rag1*^{tm1Mom}/J (Rag$^{-/-}$), B6.Cg-
459 *Foxp3*$^{tm2(EGFP)Tch}$/J (*Foxp3*^{EGFP}) (34,35), B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J (B6$^{Cre/ERT2+}$),
460 C57BL/6J (H-2^{b}; B6), B6.129S2-Ciita^{tm1Ccum}/J (*CiiTA*$^{-/-}$), and B6.MRL-*Tnfrsf6*^{lpr}/J (*lpr*) mice
461 were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The C57BL/6
462 H-2K^{btm1}-H-2D^{btm1}N12 (*K*^{b}*D*$^{b-/-}$) were originally obtained from the National Institute of Allergy
463 and Infectious Diseases (NIAID) Exchange Program (NIH: 004215) [86]. We generated the
464 *CiiTA*$^{-/-}$ *Rag*$^{-/-}$ mice and *K*^{b}*D*$^{b-/-}$ *Rag*$^{-/-}$ mice by crossing the *Rag*$^{-/-}$ mice with *CiiTA*$^{-/-}$ mice

465 and $K^bD^{b-/-}$ mice, respectively. B6.*Rag2p*^{GFP} mice [41,42] were kindly provided by Dr. Pamela
466 Fink (University of Washington, Seattle, WA), and we previously generated B6.*Rag2p*^{GFP}
467 *Pdcd1*$^{-/-}$ mice [44]. C57BL/6-*Btla*$^{-/-}$ (abbreviated as *Btla*$^{-/-}$) were originally provided by Dr.
468 Kenneth Murphy (Washington University, St. Louis, MO), and C57BL/6-*Pdcd1*$^{-/-}$ (abbreviated
469 as *Pdcd1*$^{-/-}$; backcrossed 11 generations to C57BL/6; originally generated by Prof. T. Honjo and
470 colleagues [87]) mice were bred at the University of Alberta. We crossed OT-II mice (kindly
471 provided by Dr Sue Tsai, University of Alberta) and OT-I mice (kindly provided by Dr. M.
472 McGargill, St. Jude Children's Research Hospital, Memphis, TN) to *Btla*$^{-/-}$ mice, and crossed
473 *Foxp3*^{EGFP} mice to *Btla*$^{-/-}$ and *Pdcd1*$^{-/-}$ mice [46]. The B6.*Pdcd1*$^{fl/fl}$ mice were purchased from
474 Taconic Biosciences (Rensselaer, NY, USA). B6.*Btla*$^{fl/fl}$ mice [32,88] generated by us (M.K.)
475 and were kindly provided by John R Šedý and Carl Ware (Sanford Burnham Prebys Medical
476 Discovery Institute, La Jolla, United States). B6.*Nur77*^{GFP} mice [53] were provided by Dr Kristin
477 Hogquist (University of Minnesota, Minneapolis, MN) and we generated *Btla*$^{-/-}$ *Nur77*^{GFP} mice.
478 *Hvem*$^{-/-}$, *Hvem*$^{fl/fl}$ and mice with the cytoplasmic tail of *Hvem* deleted mice were bred and
479 analyzed at La Jolla Institute for Immunology, La Jolla, CA, United States. All mice were
480 between 7 – 24 weeks old. Animal care was in accordance with the Canadian Council on Animal
481 Care guidelines. The studies were performed under Animal Use Protocol 215, approved by the
482 Animal Care and Use Committee Health Sciences of the University of Alberta. Mice were
483 housed under clean conventional housing conditions at the University of Alberta Health Sciences
484 Laboratory Animal Services (HSLAS) facilities. Mouse studies carried out at La Jolla Institute
485 for Immunology used animals bred and housed under specific pathogen–free conditions and were
486 approved by the La Jolla institute for Immunology Animal Care and Use Committee under
487 protocol AP00001007.

488

489 ## Generation of HVEM mutant mice

490 Mouse HVEM cytoplasmic tail mutant (*Hvem*^{tm}) mice were generated by the CRISPR/Cas9
491 system by injection of a sgRNA-Cas9 complex plus a donor specific single-stranded DNA
492 (ssDNA) into C57BL/6 pronuclear embryos. All materials for the CRISPR/Cas9 system were
493 purchased from Integrated DNA Technologies (IDT, Newark, NJ). The specific sgRNA targeted
494 the front of the *Tnfrsf14* exon 7: 5'-AGAACAUCAAGUCAUGGGAG-3'. The ssDNA
495 homology directed repair (HDR) template has a stop codon in the exon 7 of the *Tnfrsf14* locus:
496 5'-CCATAAGCATATGCCAGTTGGAACTTCCTCCCCGACCCAGTTATACCTGGAAAGG
497 CTCCAGCTCCTTAGTCACTTAGCCTGTAACACAAGAACATCAAGTCATGGGAGAGCT
498 GAAGCAAGAGGGGAGGGAGACGGGCACACAGCAATGAAAAACCCACATTCTGGGA
499 TTCCAGCTGTGTGATCTACCTCCCAAGTCTGAC-3'. The HDR repair did not occur, but we
500 obtained an F0 founder that has an allele with a deletion of the whole exon 7 and a read-through
501 out-of-frame amino acid sequence by splicing exon 6 to exon 8. The F0 founder was backcrossed
502 to the WT C57BL/6 strain for at least 2 generations. We obtained homozygous offspring
503 (*Hvem*^{WT} and *Hvem*^{tm}) by intercrossing the N2 generation of *Hvem*$^{+/tm}$ mice. The mice were
504 generated at the RIKEN Center for Integrative Medical Sciences (IMS), Yokohama, Japan. All
505 procedures related to strain construction were approved by the RIKEN IMS Animal Care and
506 Use Committee. We confirmed that the tail-mutated HVEM protein was incapable of signaling in
507 vitro and in vivo, as described previously [66]. For in vitro testing, the pGL4.32[luc2P/NF-κB-
508 RE/Hygro] (NF-κB–driven firefly luciferase; Promega) and pRL-TK (*Renilla* luciferase as an
509 internal control; Promega) plasmids were co-transfected with mouse *Hvem*^{WT}, or the *Hvem*^{tm}
510 sequence with deletion of exon 7, or control vector (EGFP) plasmids into 293T cells by TransIT-

511 LT1 (Mirus). One day later, transfected cells were co-cultured with transfected 293T cells
512 expressing mouse LIGHT, CD160, or BTLA. On the next day, firefly and *Renilla* luciferase
513 activity were measured through the Dual-Glo Luciferase Assay System (Promega) and detected
514 by the EnVision 2104 Multimode Plate Reader (PerkinElmer). For in vivo testing, we used a
515 hepatic injury model. Co-housed female littermates were injected with 2 µg αGalCer (KRN7000;
516 Kyowa Kirin Research) in a total volume of 200 µl PBS by retro-orbital injection. Serum ALT
517 activity was measured using a colorimetric/fluorometric assay kit (ab105134; Abcam) at 24 h
518 after injection as described previously [66].
519

520 Cell transfer, tamoxifen-induced conditional deletion, and disease
521 criteria

522

523 Thymocytes or splenocytes containing the indicated number of SP T cells were injected into
524 adult *Rag*$^{-/-}$ mice. Briefly, thymuses and spleens were removed from the donors and mashed
525 with glass slides on ice to make a single-cell suspension, followed by filtration with a 70 µm cell
526 strainer (Fisherbrand™). CD4 and CD8 SP T cells were isolated by negative selection from a
527 single cell thymocyte suspension using EasySep™ Mouse Naïve CD4^{+} T Cell Isolation Kit (#
528 19765) and EasySep™ Mouse Naïve CD8^{+} T Cell Isolation Kit (# 19858), respectively, from
529 STEMCELL Technologies (Vancouver, Canada) according to manufacturer's instructions. The
530 purity of the sorted cell population was > 96%. Viability was assessed by trypan blue exclusion
531 and > 90% viable cells were used for experiments. Where indicated, TCR^{+} CD24^{low} cells were
532 sorted from splenocytes of six-week-old *Btla*$^{-/-}$ mice on a FACS BD influx^{TM} cell sorter (BD
533 Biosciences). The purity of the sorted cell populations was 92%. Fetal liver cells (FLC) were used as a

534 source of hematopoietic stem cells (HSC) and were harvested from embryonic day 14 – 16
535 fetuses. A single-cell suspension was made on ice by gently pipetting the fetal livers and
536 filtration through a 70 µm cell strainer (Fisherbrand™). Viability was assessed by trypan blue
537 exclusion and > 90% viable cells were used for experiments. Six-eight-week-old male and
538 female *Rag*$^{-/-}$ mice were used as recipients, and each recipient received 10 - 20 x 10^6 FLC,
539 followed by tamoxifen injection as described below. Some HSC recipients also received agonist
540 anti-BTLA antibody (6A6; 10µg/g body weight) or hamster IgG isotype control antibody once
541 per week beginning 18 days post fetal liver injection. To induce BTLA or PD-1 deletion,
542 B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ mice or adult *Rag*$^{-/-}$ recipients of B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ or B6$^{Cre/ERT2+/-}$
543 *Pdcd1*$^{fl/fl}$ FLC or thymocytes were intraperitoneally injected with 1.4 mg tamoxifen (Sigma-
544 Aldrich) in corn oil [+5% (vol/vol) ethanol] on days 0, 1, 3, 5, and 6. Recipients of control
545 B6$^{CreERT2+/-}$ FLC or thymocytes also received tamoxifen injection. Signs of disease included loss
546 of weight, hunched appearance, piloerection, diarrhea, and dermatitis. Recipient mice were no
547 longer considered disease-free when two or more of the above signs were evident, or mice had
548 lost ≥ 20% of baseline body weight. In addition, for mice to be classified as diseased, disease
549 signs must persist for at least two weeks. Immunohistochemistry staining was performed on
550 tissues from multiple organs collected from recipient mice.
551

552 **Immunohistochemistry**

553

554 Mice were euthanized and transcardially perfused with phosphate buffer saline (PBS), followed
555 by 4% paraformaldehyde (PFA) in PBS. Harvested non-lymphoid organs (heart, kidneys, liver,
556 and lungs) were immersed in 4% PFA in PBS overnight at 4°C and then transferred into fresh

557 30% sucrose in PBS every day for two consecutive days at 4°C. Tissues were embedded in
558 optimum cutting temperature compound (TissueTek OCT, Sakura Finetek, 4583), frozen on
559 liquid nitrogen, and cryosectioned (Leica CM1950) at –20°C with a thickness of 5 or 10 µM on
560 glass slides. Following three washes in 1x PBS, tissue sections were blocked in 10% normal goat
561 serum for 1 h at room temperature and incubated in rat anti-mouse CD4 (1:200; MCA2691; Bio-
562 Rad) or CD8a (Biolegend, San Diego, CA) antibody overnight at 4°C. Slides were washed in
563 PBS-Tween (0.5% Tween 20 in 1x PBS) and incubated in goat anti-rat IgG Alexa Fluor® 488
564 (1:200; A11006; Life Technologies) antibody for 45 min at room temperature. To visualize
565 cellular nuclei, tissue sections were counterstained with VECTASHIELD mounting medium
566 with DAPI (Vector Laboratories, H-1200) (42,43). Spleens from *Rag*$^{-/-}$ and WT mice were used
567 as the CD4 negative and positive control, respectively. The negative control for primary antibody
568 specificity was omitting the primary antibody in the staining. Immunofluorescence images were
569 taken using either a Leica DM IRB Microscope and Open Lab software or an Axioplan,
570 Axiovision 4.1 software (Carl Zeiss, Toronto, ON). An average of three images per section were
571 examined.
572

573 **Flow cytometry and BrdU incorporation**

574

575 Fluorochrome conjugated antibodies for flow cytometry staining used in this study were
576 purchased from ThermoFisher Scientific: murine anti-TCRβ (H57-597), CD4 (RM4-5), CD5
577 (53-7.3), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14), FoxP3 (FJK-16s), BTLA (6F7), PD-1
578 (J43); or BioLegend: CD19 (6D5), HVEM (HMHV-1B18). GFP expression was also analyzed in
579 mice expressing the GFP transgene. Peripheral blood samples, thymocytes, and splenocytes were

stained after incubation with FcR block, which was a cocktail of anti-CD16/32 antibody (2.4G2; Bio Express, West Lebanon, NH) and mouse, rat, and hamster sera. Staining was done at 4°C for 20 minutes, followed by washing and resuspension in Hanks' Balanced Salt Solution (HBSS) supplemented with 2% fetal bovine serum (FBS). A BD LSR II (BD Biosciences) with FlowJo software was used for data acquisition and analyses. To assess proliferation in vivo, experimental mice were treated with 2 mg BrdU in PBS by i.p. injection. BrdU incorporation was assessed in splenic T cells 24 hours after injection using a BrdU flow cytometry kit (BD PharMingen™).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data of biological replicates are depicted as mean ± standard error. Data were statistically analyzed using Student's t-test with Welch's correction or Wilcoxon matched-pairs signed rank test or Mann-Whitney test. Statistical analysis in experiments with one variable and three groups was performed using one-way ANOVA with Dunn's multiple comparisons test or Kruskal-Wallis test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). Disease onset/incidence was compared by the Kaplan–Meier method. Probability values reported for survival curve comparisons were calculated using the Mantel-Cox method.

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Author Contributions

Conceptualization: Colin C. Anderson, Adeolu O. Adegoke, Troy A. Baldwin, Mitchell Kronenberg

Formal Analysis: Adeolu O. Adegoke, Govindarajan Thangavelu, Ting-Fang Chou, Marcos Petersen, Kevin Joannou, Qingyang Wang

Funding Acquisition: Colin C. Anderson, Troy A. Baldwin, Peter A. Bretscher, Hilde Cheroutre, Mitchell Kronenberg

Investigation: Adeolu O. Adegoke, Govindarajan Thangavelu, Ting-Fang Chou, Marcos Petersen, Julia F. May, Kevin Joannou, Qingyang Wang, Kristofor K. Ellestad

Methodology: Kiyokazu Kakugawa, Hilde Cheroutre

Resources: Kiyokazu Kakugawa, Hilde Cheroutre, Louis Boon

Project Administration: Colin C. Anderson

Supervision: Colin C. Anderson, Troy A. Baldwin, Mitchell Kronenberg, Peter A. Bretscher

Writing – original draft: Colin C. Anderson, Adeolu O. Adegoke, Govindarajan Thangavelu

Writing – review & editing: All authors

Conflict of interest statement

Louis Boon is CSO and board member at JJP Biologics, a company developing HVEM targeting approaches in oncology.

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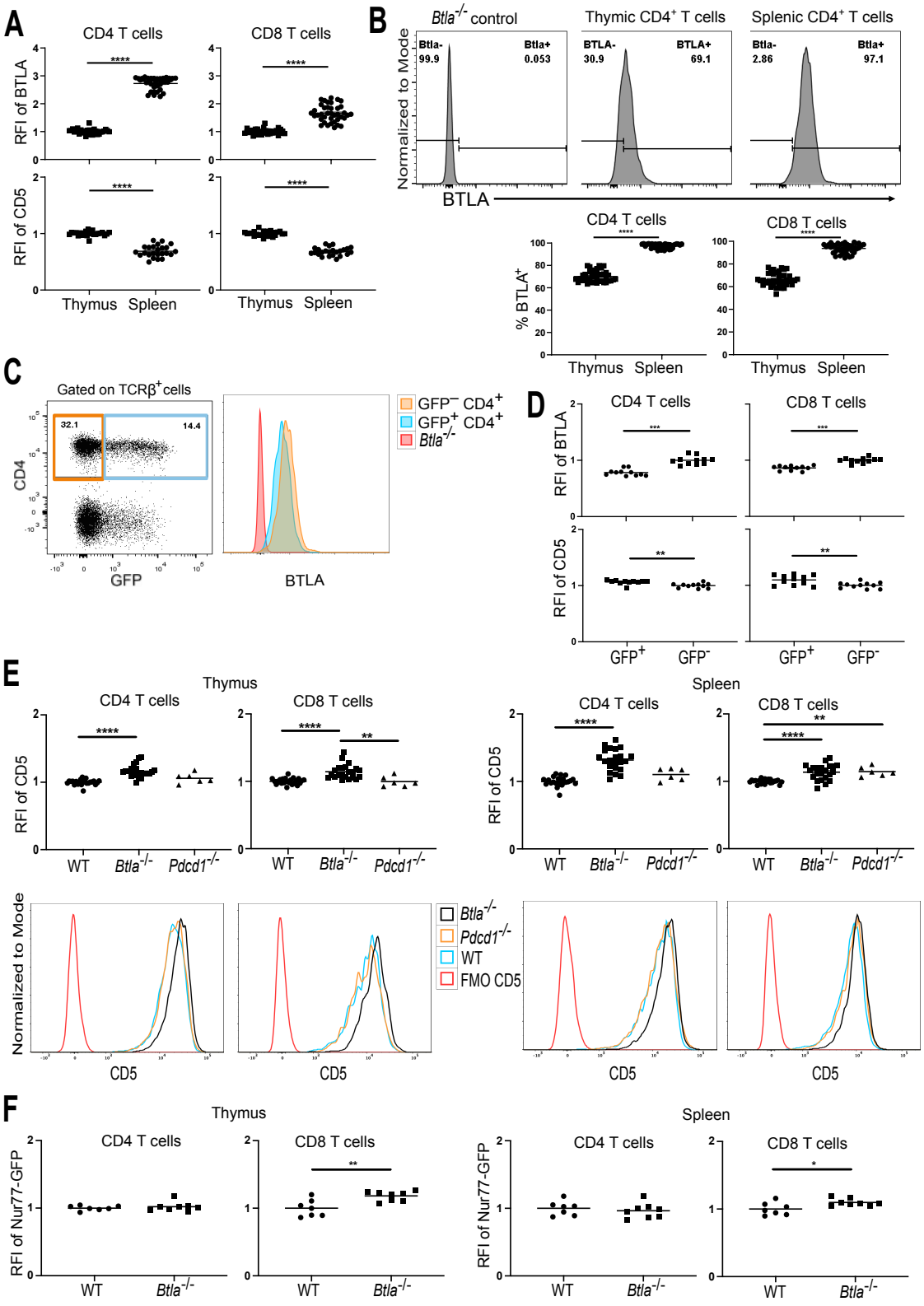

895

896 **Fig 1. BTLA expression in the thymus and spleen is inversely related to CD5 expression.** [A]

897 Relative fluorescent intensities (RFIs) of BTLA (upper row) or CD5 (lower row) in CD4 SP T cells (left

898 column) and CD8 SP T cells (right column) in the thymus and spleen (BTLA, n = 39; CD5, n = 24). To
899 calculate the RFI of BTLA or CD5, mean fluorescence intensity (MFI) data were normalized to the
900 average MFI of BTLA or CD5 of the thymic SP T cells in each individual experiment. [**B**] Representative
901 histograms showing the proportion of $BTLA^+$ CD4 SP T cells (upper row) in the thymus and spleen.
902 Lower row shows the proportion of $BTLA^+$ CD4 SP T cells (lower left) and $BTLA^+$ CD8 SP T cells
903 (lower right) in the thymus and spleen (n = 39). [**C**] Representative flow cytometry dot plot (left) and
904 histogram (right) of the indicated markers in splenic $TCR\beta^+$ cells from 7 -10-wk old B6.*Rag2p*^{GFP} mice.
905 [**D**] RFIs of BTLA (top panels) and CD5 (lower panels) on mature (GFP^-) or newly generated (GFP^+) SP
906 T cells in the spleen (n = 11). Data are normalized to the average BTLA MFI or CD5 MFI of the GFP
907 negative splenic SP T cells in each individual experiment. [**E**] CD5 expression on CD4 SP T cells and
908 CD8 SP T cells from thymus (left) or spleen (right) and their corresponding representative histograms
909 (lower rows) from WT (n = 24; B6.*Foxp3*^{GFP}, and B6.*Nur77*^{GFP}), *Btla*$^{-/-}$ (n = 22; B6.*Foxp3*^{GFP} *Btla*$^{-/-}$ and
910 B6.*Nur77*^{GFP} *Btla*$^{-/-}$), and *Pdcd1*$^{-/-}$ (n = 6; B6.*Foxp3*^{EGFP} *Pdcd1*$^{-/-}$) mice. [**F**] Nur77-GFP expression.
911 Dots indicate individual mice from six – nine independent experiments (A – B, E) or two independent
912 experiments (C – D, F). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

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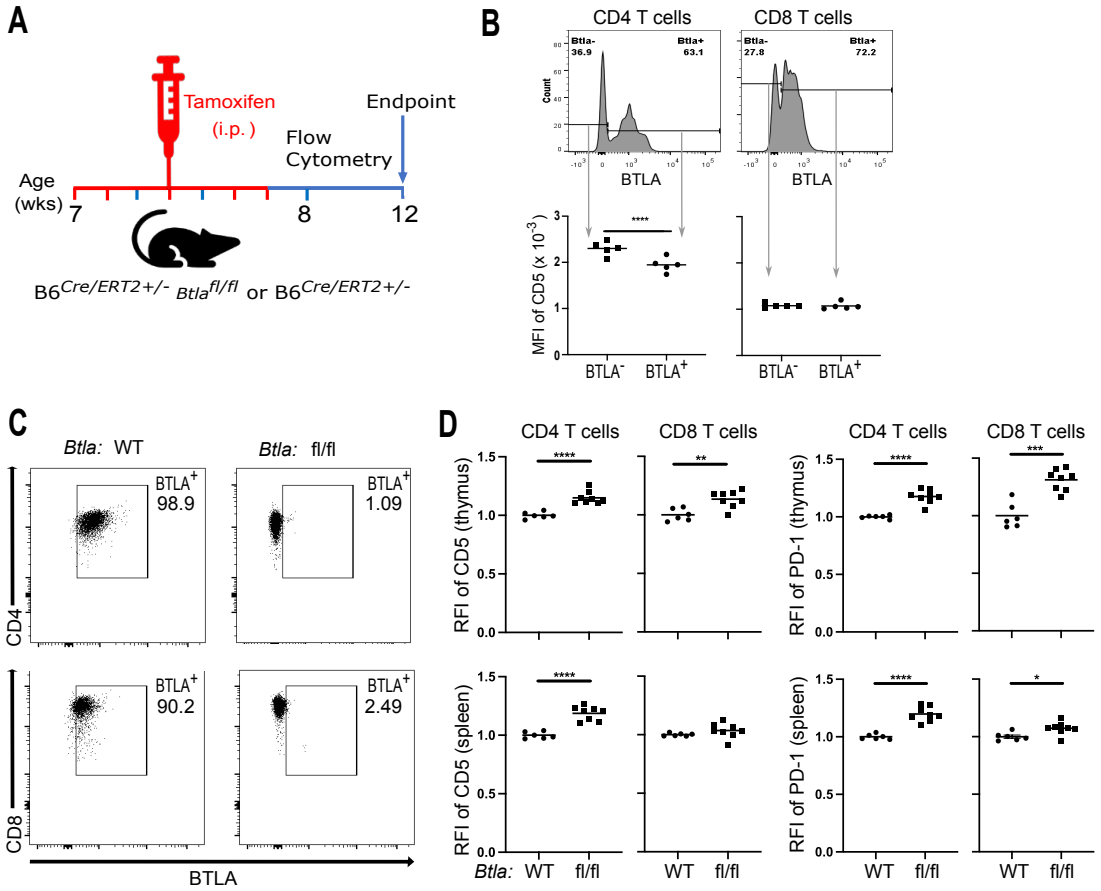

Fig 2. Loss of BTLA in adult mice leads to increased T cell CD5 and PD-1 expression. [**A**] Adult B6$^{Cre/ERT2+/-}$ or B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ mice received five doses of tamoxifen on days 0, 1, 3, 5, and 6 (highlighted in red). [**B**] Representative histograms (top) of BTLA expression and the MFI of CD5 (bottom) of BTLA^{+} and BTLA^{-} cells in the CD4 gated (left) and CD8 gated (right) T cells in the peripheral blood at one week post tamoxifen. [**C**] Representative dot plots of BTLA expression in the splenic CD4 SP T cells (top) and CD8 T cells (bottom) in the B6$^{Cre/ERT2+/-}$ mice (left) and B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ mice (right) at two weeks post tamoxifen. [**D**] RFI of CD5 and PD-1 in the thymic (top row) CD4 and CD8 or splenic (bottom row) CD4 and CD8 T cells of B6$^{Cre/ERT2+/-}$ (WT) and B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ (fl/fl) mice at four weeks post tamoxifen. Dots indicate individual mice from two independent experiments. $^{**}p < 0.01$, $^{****}p < 0.0001$.

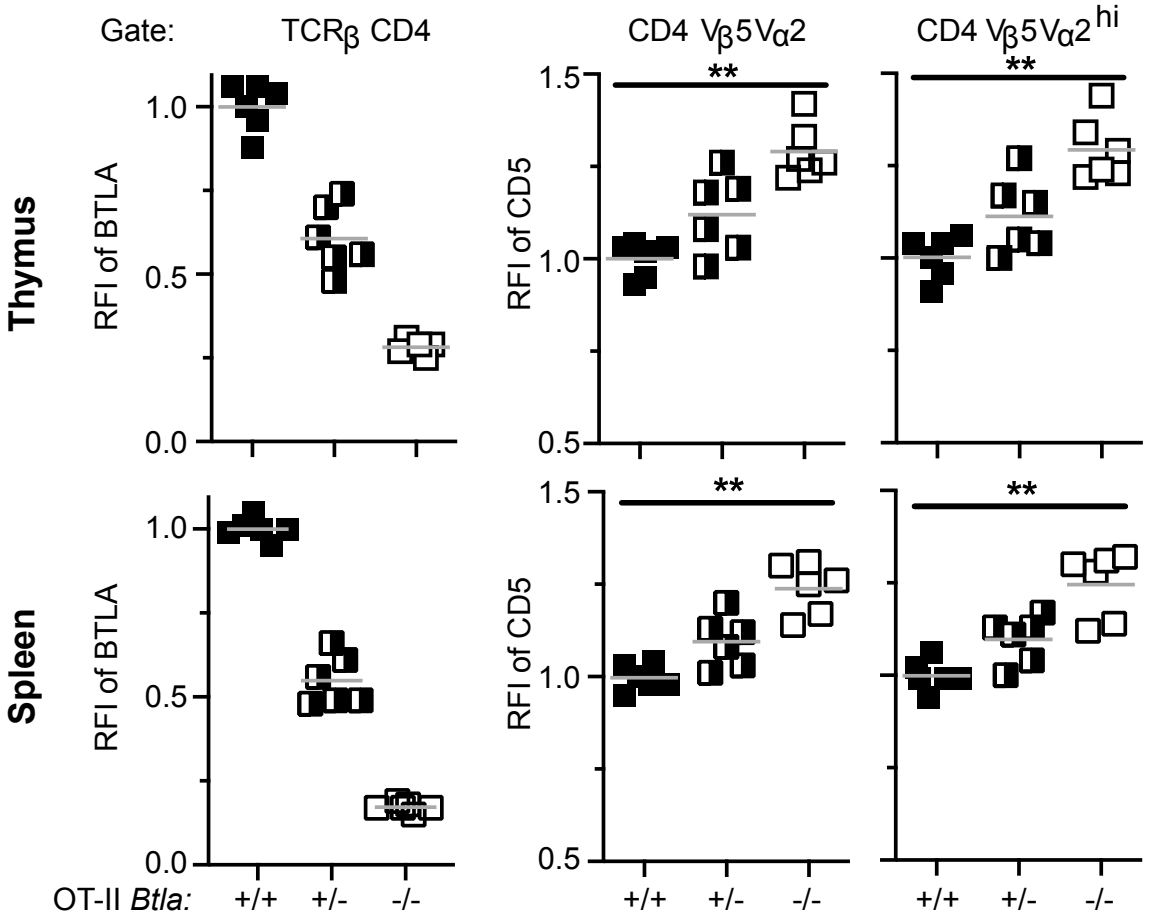

Fig 3. BTLA deficiency increases CD5 levels in TCR transgenic OT-II CD4 T cells in thymus and spleen. RFI of BTLA gated on TCRβ$^+$ CD4$^+$ SP cells (left) and RFI of CD5 in all CD4$^+$ TCR-Vβ5Vα2$^+$ cells (middle) and RFI of CD5 in CD4$^+$ SP cells expressing high levels TCR-Vβ5Vα2 (right; gate included only the top approximately 50% of TCR-Vβ5Vα2 expressing cells). Analysis of T cells from the thymus (top) and spleen (bottom) of OT-II.*Btla*$^{+/+}$ (WT OT-II; n = 6), OT-II.*Btla*$^{+/-}$ (n = 6) and OT-II.*Btla*$^{-/-}$ mice (n = 6). The grey line is the mean of the RFI. CD5 expression on OT-II.*Btla*$^{+/+}$ T cells was significantly lower than on BTLA deficient (*Btla*$^{-/-}$) OT-II T cells, $^{**}p < 0.01$.

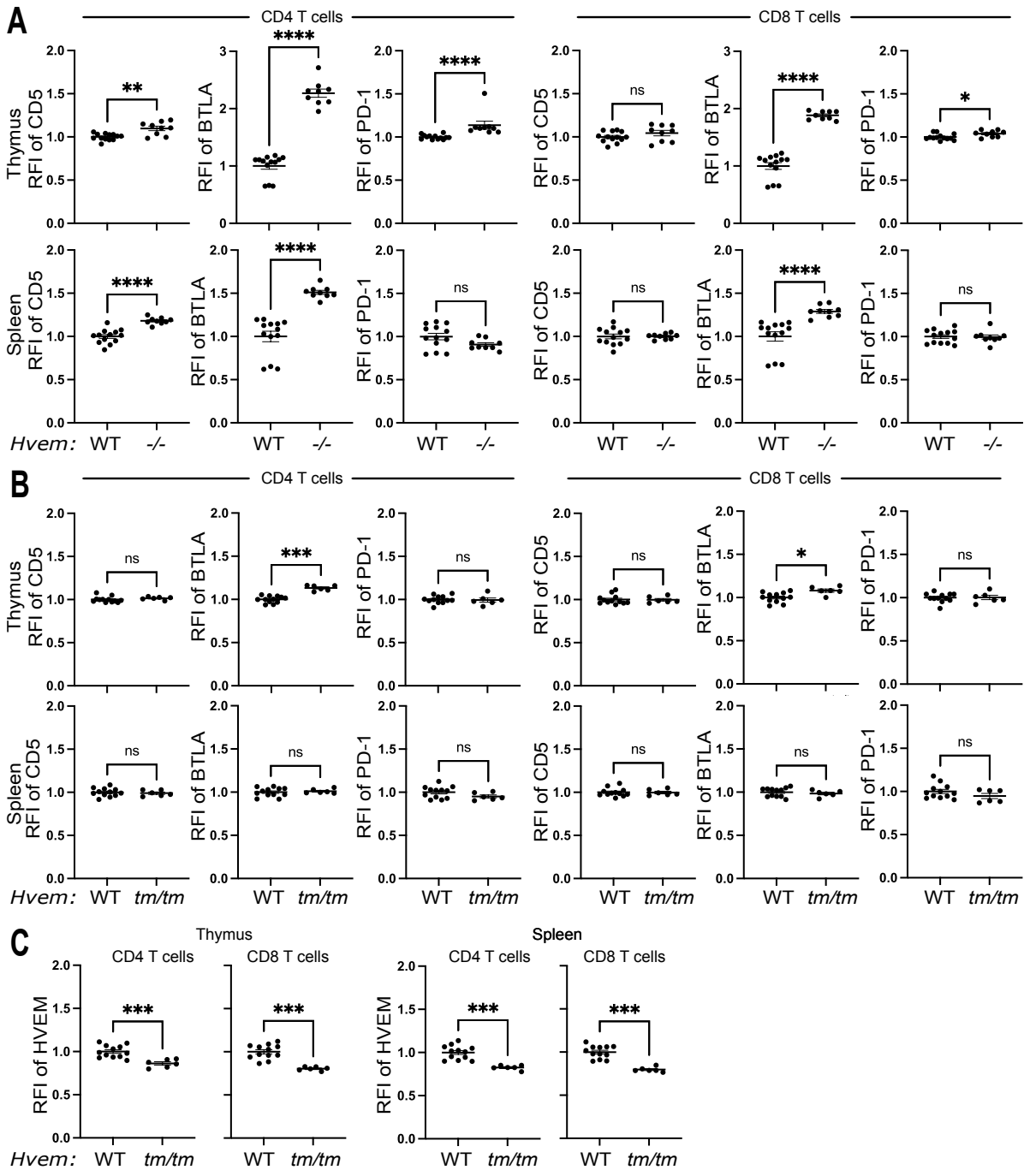

Fig 4. HVEM dependent control of CD5 and PD-1 expression from early in CD4 T cell ontogeny does not require HVEM signaling. Thymocytes and splenocytes from WT and *Hvem*$^{-/-}$ [**A**] or *Hvem*$^{tm/tm}$ mice [**B, C**], gated on SP CD4 and SP CD8 T cells, were examined by flow cytometry for expression of CD5, BTLA, PD-1 and HVEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

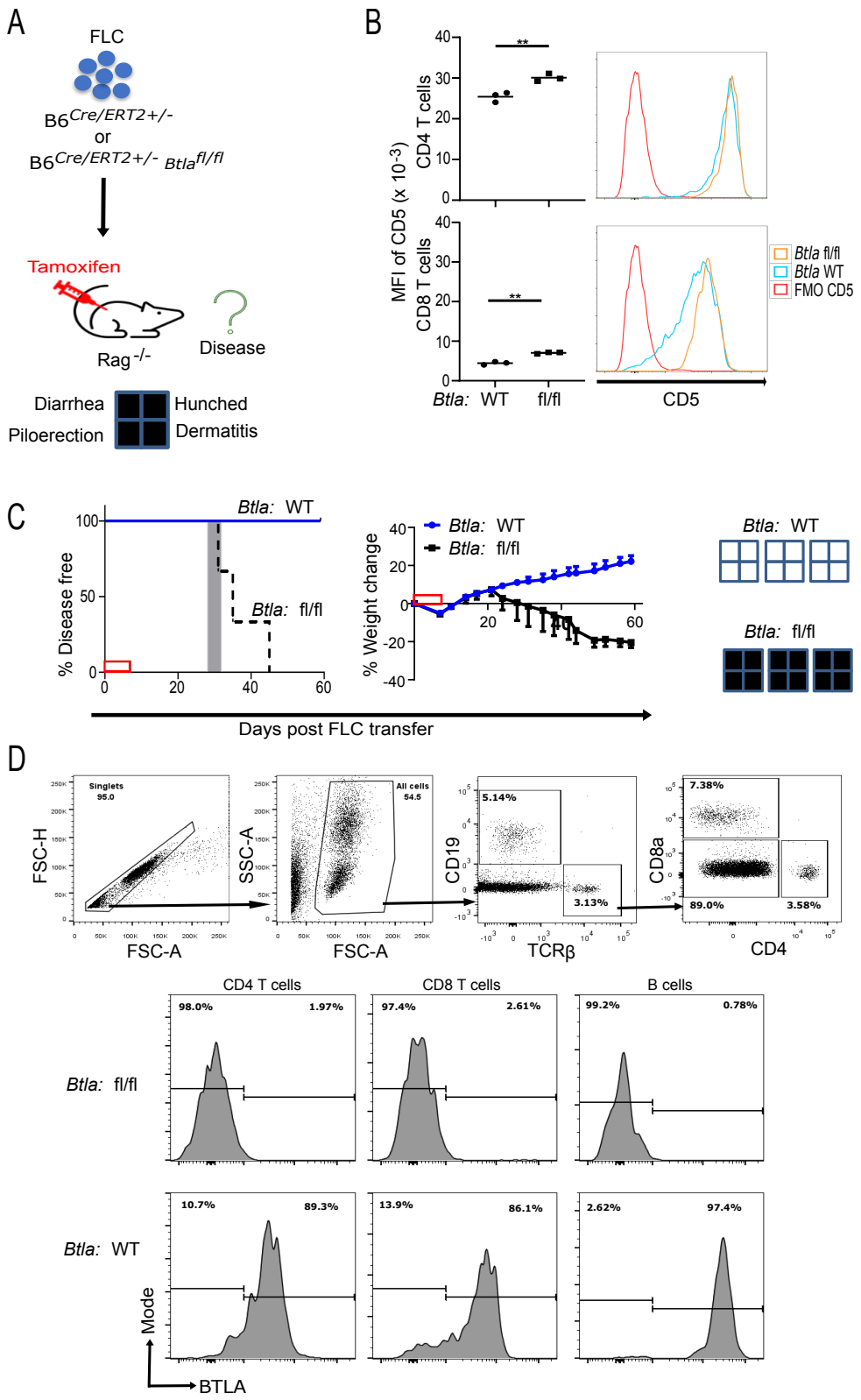

Fig 5. Loss of BTLA early in T cell ontogeny generates autoimmune disease. [**A**] We adoptively transferred 20 x 10^6 FLC pooled from 8-10 embryonic day 14 - 16 B6$^{Cre/ERT2+/-}$ (WT) or B6$^{Cre/ERT2+/-}$

945 *Btla*$^{fl/fl}$ (fl/fl) fetuses to 7-wk old *Rag*$^{-/-}$ mice on day 0 (n = 3 recipients per group), followed by tamoxifen
946 injection on days 0, 1, 3, 5, and 6. Recipient mice were monitored for signs of disease for eight weeks
947 post FLC transfer. [**B**] MFI of CD5 in CD4 T cells (top) and CD8 T cells (bottom) with respective
948 representative histograms of peripheral T cells in the recipients of FLC from B6$^{Cre/ERT2+/-}$ and B6$^{Cre/ERT2+/-}$
949 *Btla*$^{fl/fl}$ at eight weeks post tamoxifen. Dots indicate data from individual mice; $^{**}p < 0.01$. [**C**] Left panel:
950 disease incidence in recipients of B6$^{Cre/ERT2+/-}$ (blue line) B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ (black dashed line) FLC.
951 Survival curves were significantly different, $p = 0.02$. The grey rectangle indicates the range, in days, at
952 which the first T cells were detected in the peripheral blood after FLC transfer. Right panel: weight
953 changes in recipients of B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ FLC or B6$^{Cre/ERT2+/-}$ FLC. The red box on the X-axes indicates
954 the tamoxifen treatment period. The presence (filled) or absence (empty) of disease signs is depicted on
955 the far-right panel. [**D**] Flow cytometry gating (top). A representative histogram of BTLA expression in
956 the T and B cells populating the periphery of B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ FLC recipient mice (middle) or
957 B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ FLC recipient mice (bottom) at four weeks post FLC transfer is shown.

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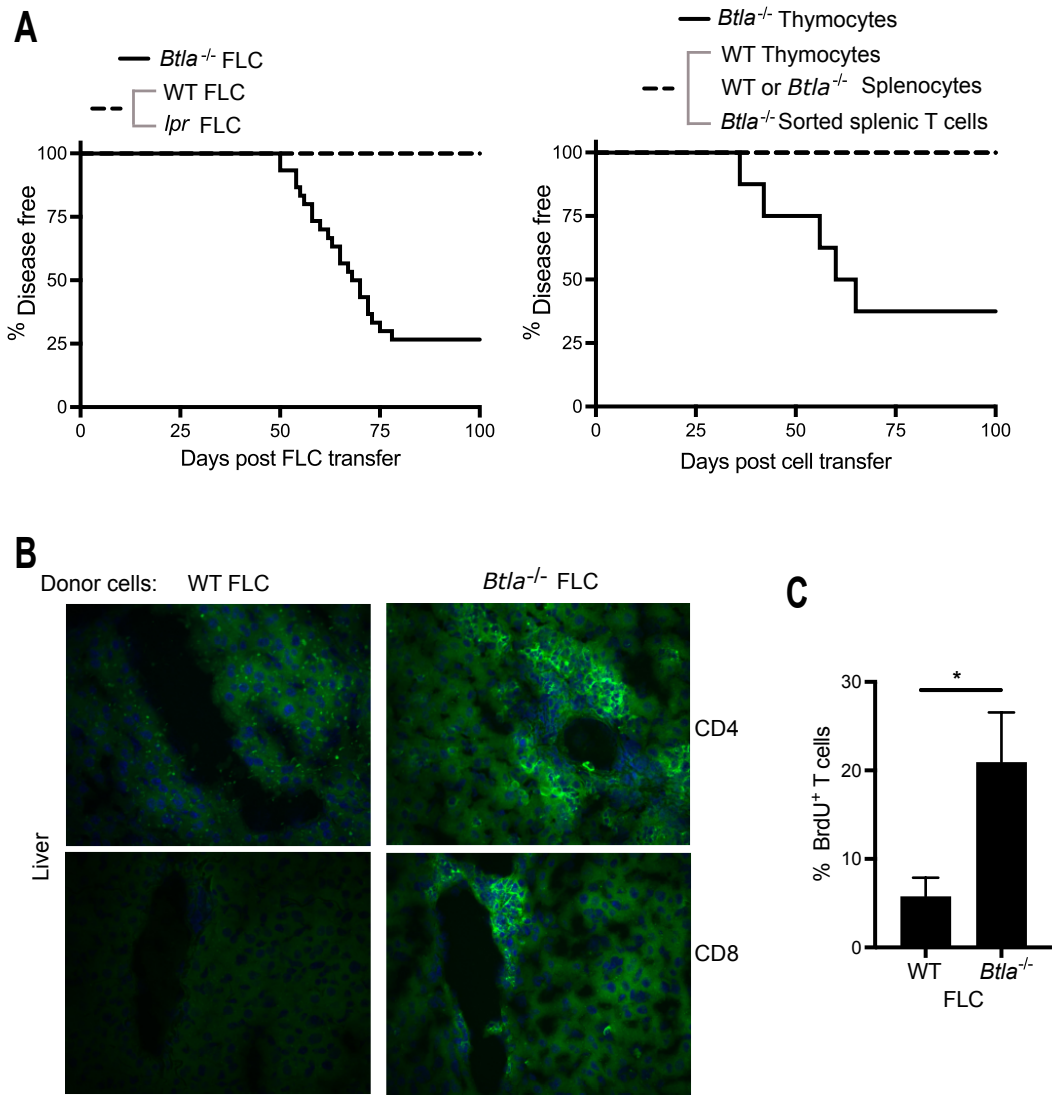

Fig 6. BTLA is needed for establishment of tolerance in newly generated T cells. [**A**] *Left:* Adult *Rag*$^{-/-}$ mice were given fetal liver cells from *Btla*$^{-/-}$ (n = 30) or WT (n = 10) or *lpr* fetuses (n = 3) and disease was monitored. *Right:* Adult *Rag*$^{-/-}$ mice were given one of the following cell populations (all populations contained 2-2.5 x 10^6 single positive T cells), *Btla*$^{-/-}$ (n = 8) or WT thymocytes (n=4), or WT or *Btla*$^{-/-}$ splenocytes (n = 4), or FACS sorted *Btla*$^{-/-}$ splenic TCR$^+$ CD24^{low} T cells (n = 3) and were monitored for disease incidence (diseased mice exhibited two or more of the following symptoms: loss of weight, hunched posture, piloerection, dermatitis and diarrhea.). Disease was only observed in *Btla*$^{-/-}$ FLC or thymocyte groups, with all other groups surviving to day 100 disease-free. [**B**] Representative

970 immunofluorescence (n = 5-6/group; original magnification × 400) of liver from individual recipients of

971 WT or *Btla*$^{-/-}$ FLC. Blue: staining with the nuclear marker 4',6'-diamidino-2-phenylindole (DAPI);

972 green: CD4 or CD8 staining. [**C**] The mean percentage of BrdU^{+} T cells in the spleen of *Rag*$^{-/-}$ recipients

973 (n = 4/group) of *Btla*$^{-/-}$ FLC was significantly higher (*$p < 0.05$) than that of WT FLC recipients

974 examined 65 days post-FLC transfer.

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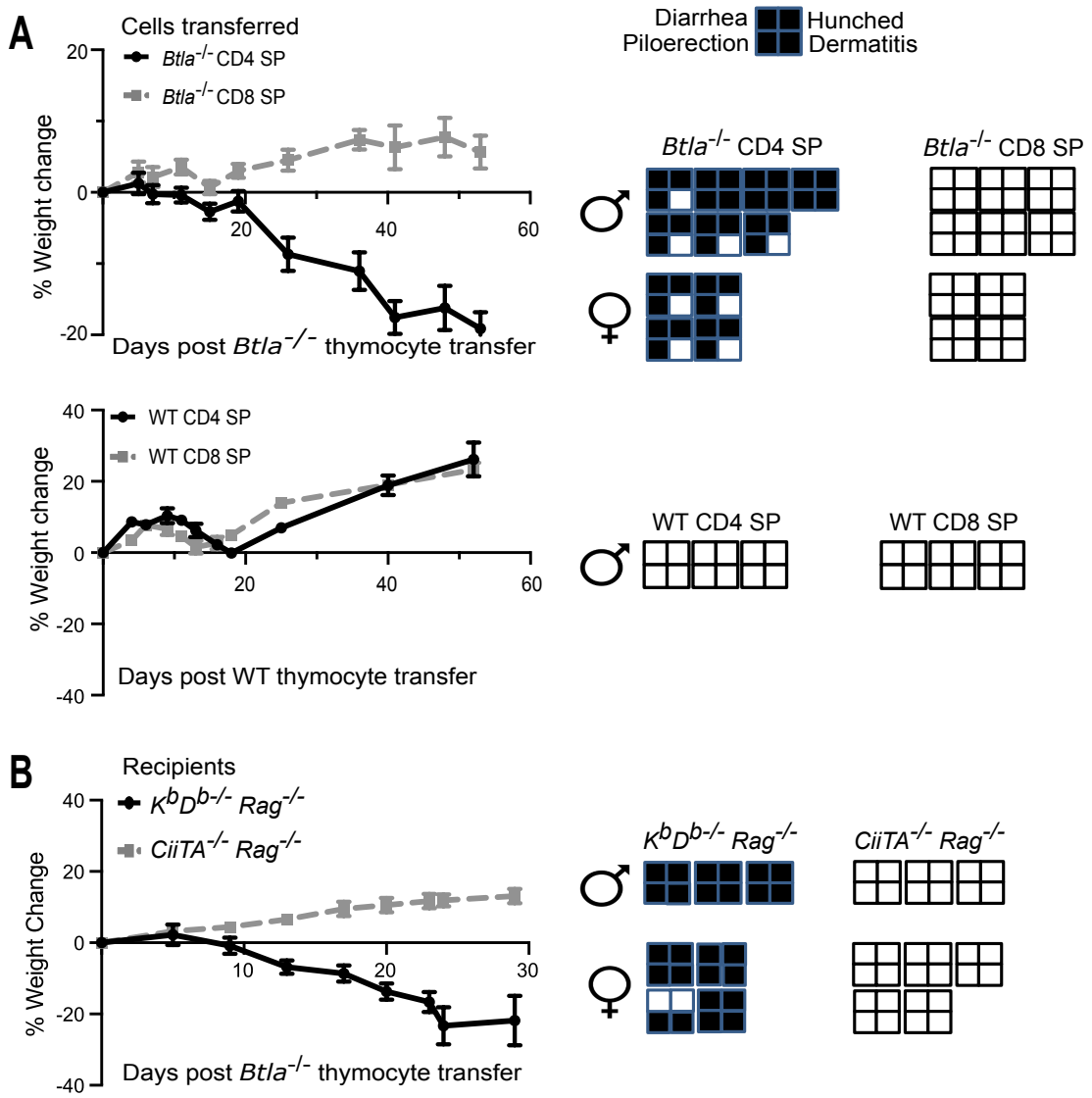

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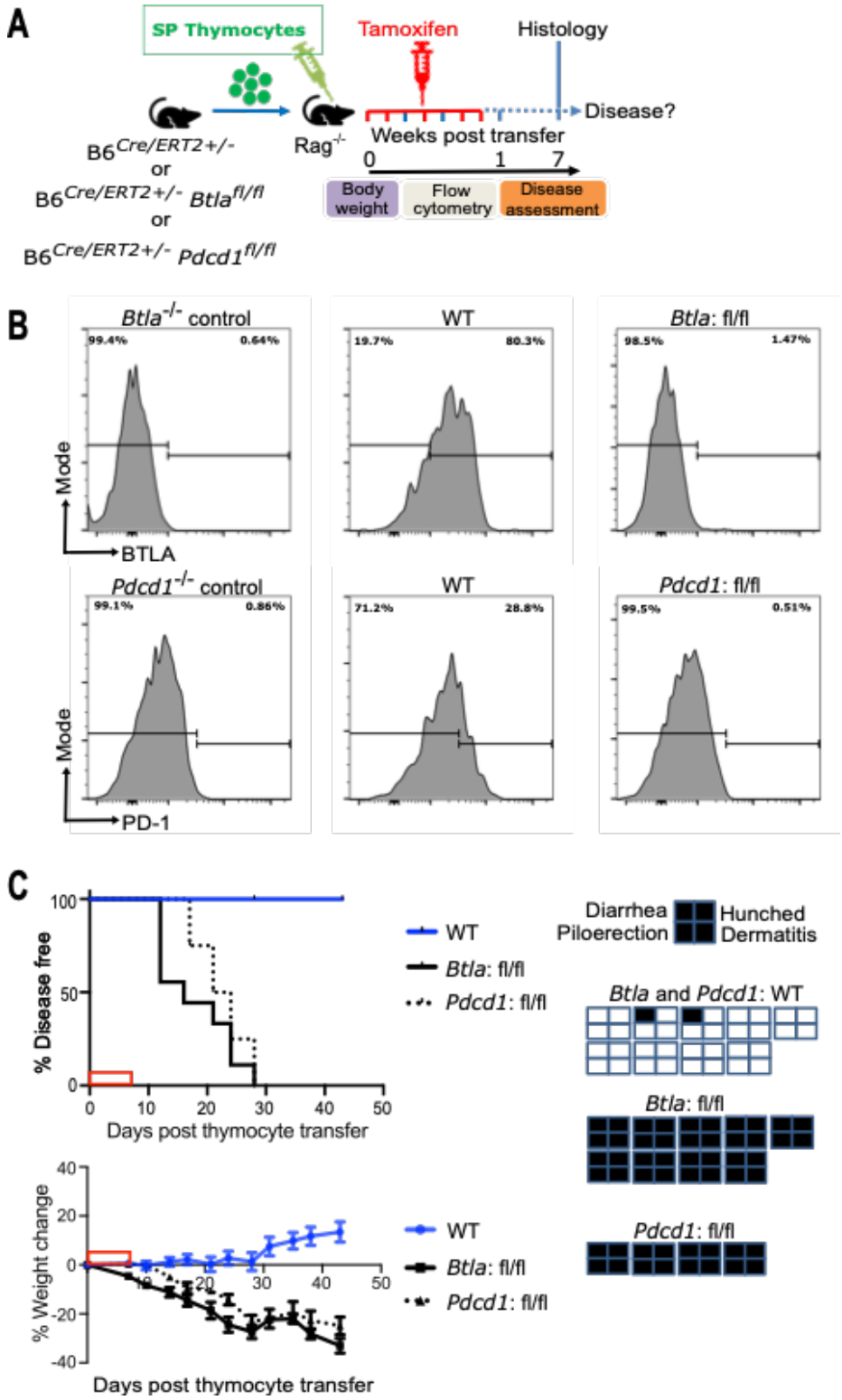
977 **Fig 7. Autoimmune disease in Btla$^{-/-}$ thymocyte recipients requires CD4^{+} T cells and MHC II.** [**A**]

978 We adoptively transferred 3 x 10^6 MACS-sorted CD4 or CD8 SP thymocytes pooled from seven 8 – 10-

979 wk old B6.*Foxp3*^{EGFP} x *Btla*$^{-/-}$ (left column) or B6.Foxp3^{EGFP} (right column) mice i.v. to 8 – 10-wk old
980 *Rag*$^{-/-}$ mice (*Btla*$^{-/-}$ thymocyte recipients, n = 10-11 mice/group; WT thymocyte recipients, n=3
981 mice/group) and monitored for several weeks or after losing ≥ 20% of baseline body weight, whichever
982 came first. Body weight change of the *Btla*$^{-/-}$ SP thymocyte recipients (data was from three independent
983 experiments) or WT SP thymocyte recipients is shown, and the presence (shaded) or absence (unshaded)
984 of disease signs is depicted to the right of the graphs. [**B**] Thymocytes containing 3 x 10^6 SP cells (i.e.,
985 non-sorted) pooled from seven 8 – 10-wk old B6.*Foxp3*^{EGFP} x *Btla*$^{-/-}$ mice were injected via tail vein to 8
986 – 10-wk old *K*^{b}*D*$^{b-/-}$ *Rag*$^{-/-}$ mice (n = 7) or *CiiTA*$^{-/-}$ *Rag*$^{-/-}$ (n = 8) mice, which were then monitored for
987 several weeks or until after losing ≥ 20% of baseline body weight, whichever came first. Body weight
988 change of recipient mice is shown. Data are from two independent experiments. The presence (shaded) or
989 absence (unshaded) of disease signs is depicted to the right of the graph. Cell donors and recipients were
990 of the sex indicated.



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992 **Fig 8. BTLA and PD-1 are needed post thymic selection in newly generated T cells to block**

993 **autoimmune disease.** [**A**] We adoptively transferred thymocytes containing 5 x 10^6 SP (non-pooled)

994 from 7 – 12-week-old B6$^{Cre/ERT2+/-}$ (WT) or B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ (fl/fl) mice or B6$^{Cre/ERT2+/-}$ *Pdcd1*$^{fl/fl}$ (fl/fl)
995 mice to 7 - 12-week-old *Rag*$^{-/-}$ mice on day 0, followed by tamoxifen injection on days 0, 1, 3, 5, and 6.
996 Mice were then monitored for signs of disease for seven weeks. [**B**] Flow cytometry gated on TCRβ^{+}
997 cells. Representative histograms of BTLA (top row) or PD-1 (lower row) expression in the T cells of
998 germline gene knockout control mice and B6$^{Cre/ERT2+/-}$ or B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ or B6$^{Cre/ERT2+/-}$ *Pdcd1*$^{fl/fl}$
999 thymocyte recipients at two weeks post thymocyte transfer (i.e., seven days post tamoxifen) is shown. [**C**]
1000 Top Left panel: disease incidence in recipients of B6$^{Cre/ERT2+/-}$ (n = 9) or B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ (n = 9) or
1001 B6$^{Cre/ERT2+/-}$ *Pdcd1*$^{fl/fl}$ (n = 4) thymocytes. Disease free survival curve comparison demonstrated a
1002 significant difference between both fl/fl groups and the WT, with $p < 0.0001$. Data are combined from
1003 three independent experiments (two for *Btla*: fl/fl and one for PD-1: fl/fl; WT were included in all three
1004 experiments). Bottom left panel: weight changes in the indicated of B6$^{Cre/ERT2+/-}$ *Btla*$^{fl/fl}$ or B6$^{Cre/ERT2+/-}$
1005 *Pdcd1*$^{fl/fl}$ or B6$^{Cre/ERT2+/-}$ thymocyte recipients. The presence (shaded) or absence (unshaded) of disease
1006 signs is depicted on the far-right panels. The red box on the X-axis indicates the tamoxifen treatment
1007 period.

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