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1 **APC-targeted proinsulin expression inactivates insulin-specific memory CD8⁺**
2 **T cells in NOD mice.**

3

4

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23

24 **ABSTRACT**

25 Type 1 diabetes (T1D) results from T-cell mediated autoimmune destruction of pancreatic β
26 cells. Effector T-cell responses emerge early in disease development and expand as disease
27 progresses. Following β cell destruction, a long-lived T-cell memory is generated that represents
28 a barrier to islet transplantation and other cellular insulin-replacement therapies. Development of
29 effective immunotherapies that control or ablate β cell destructive effector and memory T cell
30 responses has the potential to prevent disease progression and recurrence. Targeting antigen
31 expression to antigen-presenting cells inactivates cognate $CD8^+$ effector and memory T-cell
32 responses and has therapeutic potential. Here we investigated this in the context of insulin-
33 specific responses in the non-obese diabetic mouse where genetic immune tolerance defects
34 could impact on therapeutic tolerance induction. Insulin-specific $CD8^+$ memory T cells
35 transferred to mice expressing proinsulin in antigen-presenting cells proliferated in response to
36 transgenically-expressed proinsulin and the majority were rapidly deleted. A small proportion of
37 transferred insulin-specific Tmem remained undeleted and these were antigen-unresponsive,
38 exhibited reduced TCR expression and H-2K^d/insB₁₅₋₂₃ tetramer binding and expressed co-
39 inhibitory molecules. Expression of proinsulin in antigen-presenting cells also abolished the
40 diabetogenic capacity of $CD8^+$ effector T cells. Therefore, destructive insulin-specific $CD8^+$ T
41 cells are effectively inactivated by enforced proinsulin expression despite tolerance defects that
42 exist in diabetes-prone NOD mice. These findings have important implications in developing
43 immunotherapeutic approaches to T1D and other T cell-mediated autoimmune diseases.

44 INTRODUCTION

45 Type 1 diabetes (T1D) results from the progressive inflammatory destruction of insulin-secreting
46 pancreatic β cells. Many immune cell types are implicated in promoting β -cell destructive
47 inflammation but T cells are critical. In humans and mice, T cells specific for pancreatic β cell
48 antigens are recruited into effector populations early during the prodromal, pre-clinical phase of
49 autoimmune diabetes where disease is progressing¹. Responses directed at (pro)insulin epitopes
50 are prominent early in disease and determinant spreading ultimately leads to the targeting of a
51 wide array of β -cell antigens^{2,3}. Along with this, effector T cells expand as disease progresses⁴⁻
52 ⁶ and ultimately form memory populations⁶ that persist long after β -cell destruction is complete.
53 These contribute to recurrent autoimmune rejection of replacement insulin-secreting tissues such
54 as islet transplants⁷⁻⁹. One of the key challenges for control of memory T cell responses and
55 particularly those in T1D is the resistance of memory T cells to regulatory T cells (Treg),
56 conventional immunosuppression, chemotherapeutic agents^{7, 10-14} and some methods of
57 tolerance induction^{15, 16}. Understanding how to effectively control effector and memory T cell
58 responses will provide opportunities for therapeutic interventions to interrupt disease progression
59 in T1D by limiting epitope and determinant spreading or purging established effector and
60 memory populations and to alleviate autoimmune resistance to cellular insulin-replacement
61 therapies.

62 Targeting antigen expression to antigen presenting cell (APC) populations effectively ablates
63 antigen-specific memory and effector $CD4^+$ and $CD8^+$ T cell responses through deletion and
64 induction of unresponsiveness¹⁷⁻¹⁹. Depending on the APC type targeted, inactivation of $CD8^+$
65 effector/memory T cells occurs slowly when antigen is expressed in DC or much more rapidly
66 when antigen is targeted to MHC class II⁺ APC¹⁸, but studies have focussed on non-autoimmune

67 prone strains of mice. It remains unclear whether defects that exist in immune regulation in the
68 NOD mouse, that replicate those in T1D-prone individuals with or at-risk of T1D²⁰⁻²³, impact on
69 the effectiveness of immunotherapy focussed on memory T cells. Additionally, experimental
70 systems employed to explore tolerance induction in T cells typically employ model antigens
71 matched to high-affinity TCR transgenic T cells rather than physiological antigens and T cells
72 with relevant disease-associated TCR and TCR affinities.

73
74 Here we sought to gain insight into the applicability of targeted antigen expression for
75 inactivation of memory T cell responses where a natural islet antigen was targeted and
76 TCR/pMHC affinity reflected that occurring naturally during T1D development. We also asked
77 whether inactivation of memory CD8⁺ T cell responses was effective in the presence of genetic
78 defects in immune regulation that underlie disease susceptibility in a relevant autoimmune-prone
79 model of spontaneous diabetes. To achieve this, we used CD8⁺ T cells expressing a
80 physiologically-relevant intermediate affinity TCR recognizing insulin B₁₅₋₂₃²⁴ in conjunction
81 with mice where proinsulin is expressed in diverse APC types by an MHC class II promoter²⁵.
82 We found in the autoimmune-prone NOD strain, the majority of memory insulin-specific CD8⁺
83 T cells transferred to mice expressing proinsulin were deleted and the remainder rapidly
84 inactivated. Additionally, expression of proinsulin in APC ablated the diabetogenic capacity of
85 activated insulin-specific cytotoxic T lymphocytes (CTL). Overall, these findings were
86 remarkably similar to those reported in analogous studies where the model antigen ovalbumin
87 (OVA) was used in conjunction with high-affinity TCR Tg T cells¹⁸. This indicates that, at least
88 in the NOD mouse model, perturbations of immune regulation that underlie spontaneous diabetes
89 susceptibility do not limit the capacity of APC-expressed antigen to inactivate memory CD8⁺ T

90 cell responses. Knowledge gained here in the NOD mouse model may extend our understanding
91 of tolerance induction and guide development of therapies to control deleterious anti- β cell
92 effector and memory T cell responses.

93 RESULTS

94 In vitro generation of Tmem

95 We employed an in-vitro differentiation procedure¹⁷ where G9 T cells were activated in the
96 presence of cognate antigen (insB₁₅₋₂₃) and IL-2 for 3 days and then washed and recultured in IL-
97 15 to induce central memory differentiation. We have previously validated memory T cells
98 (herein termed Tmem) generated using this and similar procedures in tolerance and other studies
99^{17, 19, 26} and their behaviour is similar to in-vivo derived memory cells¹⁷. During in-vitro
100 differentiation, G9 T cells underwent blastogenesis and acquired high levels of CD44 expression
101 during insB₁₅₋₂₃ and IL-2 stimulation (**Suppl. 1A,B**). After removal of insB₁₅₋₂₃ and IL-2, and
102 culture in IL-15, the majority of cells reacquired high levels of CD62L expression such that
103 approximately two-thirds expressed a CD44^{hi}CD62L^{hi} Tcm phenotype (**Suppl. 1B-D**).
104 Following in vitro differentiation G9 Tmem showed high levels of TCR Vβ6 expression and H-
105 2K^d/insB₁₅₋₂₃ tetramer binding similar to naïve G9 T cells (**Suppl. 2A,B**).

106

107 G9 Tmem activation by transgenically-expressed proinsulin leads to rapid deletion

108 To determine if G9 Tmem were activated by endogenous or transgenically-expressed
109 (pro)insulin, CFSE-labelled G9 Tmem were transferred to non-Tg and proinsulin-expressing PI-
110 Tg NOD along with B16 mice that lack the G9-recognised determinant. Three days later, G9
111 Tmem showed little evidence of division in B16 recipients (**Fig. 1A,B**). In NOD recipients, a
112 small, but statistically-significant proportion of G9 Tmem showed evidence of division in
113 pancreatic LN (pLN) but not skin-draining LN (sdLN) or spleen (**Fig. 1A,B**). Based on the
114 proliferation index, the extent of proliferation was low in NOD pLN and at 3 days after transfer
115 did not differ significantly to B16 mice (**Fig. 1B**). In contrast, G9 Tmem showed evidence of

116 several rounds of division in spleen, sdLN and pLN of PI-Tg recipients (**Fig. 1A,B**). Both the
117 proportion of cells entering division within 3 days and the extent of division in PI-Tg recipients
118 was reduced for G9 Tmem relative to that observed for their naïve counterparts in a similar
119 setting (submitted). Interestingly, reduced proliferation of Tmem has been observed in a similar
120 settings where Tn and Tm have been compared¹⁸ and is in line with other observations of
121 reduced proliferation or expansion capacity of memory relative to naïve CD8⁺ T cells^{27,28}.

122

123 Analysis of population kinetics showed G9 Tmem became distributed throughout all lymphoid
124 tissues examined (spl, sdLN and pLN) in non-Tg and PI-Tg recipients within 1 to 2 days of
125 transfer (**Fig. 1C**). In spleen, there was a noticeable transient accumulation of G9 Tmem 1 day
126 after transfer but this diminished, possibly through redistribution, by 2 days after transfer.
127 Between days 2 and 7 after transfer the population of G9 Tmem remained relatively stable in size
128 in spleen of non-Tg NOD recipients. However, in spleens of PI-Tg recipients, the G9 Tmem
129 population diminished slowly such that by 7 days after transfer, the population was significantly
130 reduced in size compared to 2 days after transfer (**Fig. 1C**, d2 > d7, p<0.05) and G9 Tmem were
131 significantly less frequent in PI-Tg than in non-Tg spleen 3, 5 and 7 days after transfer (p<0.01).
132 In sdLN and pLN of non-Tg NOD recipients, stable populations of G9 Tmem became
133 established and these remained relatively stable in number across the analysis period. In PI-Tg
134 recipients, significantly fewer G9 Tmem accumulated in sdLN or pLN than in non-Tg recipients
135 (**Fig. 1C**, p<0.05 or greater at all time points except d2 in pLN) and the number of G9 Tmem
136 decreased in PI-Tg pLN over time (d1 > d3, d5, d7; p<0.01 or greater, d2 > d5, d7; p< 0.05).
137 When accumulation of G9 Tmem was analysed as a proportion of the total CD8⁺ population
138 (**Suppl. 3**), a similar pattern was observed. Accumulation of G9 T cells was not increased in PI-

139 Tg relative to non-Tg recipients despite the substantial proliferation indicated by CFSE dilution.
140 Overall, far fewer G9 Tmem were recovered from PI-Tg than non-Tg recipients indicating G9
141 Tmem were deleted when proinsulin was expressed in MHC class II⁺ APC. This, together with
142 the relatively low numbers of G9 recovered suggests that onset of deletion may have been very
143 rapid.

144

145 **G9 TCR expression is reduced in PI-transgenic recipients**

146 Enumeration suggested that G9 Tmem were rapidly deleted in PI-Tg recipients, however, a small
147 residual population of undeleted G9 T cells remained. Expression of TCR Vβ6 by G9 Tmem
148 remained relatively unchanged after transfer to non-Tg NOD recipients (**Fig. 2A,B**, all n.s.
149 except spleen d1>d7, p<0.05). However, at each time point tested, expression of TCR Vβ6 by
150 G9 Tmem recovered from PI-Tg recipient spleen, sdLN and pLN was reduced compared to non-
151 Tg recipients (**Fig. 2B**; all p<0.01 or greater). Interestingly, the reduction in TCR Vβ6
152 expression in PI-Tg recipients occurred principally within the first 3 days after transfer and
153 remained relatively stable thereafter (**Fig. 2B**). TCR down-regulation is prominent in tolerance
154 models where antigen is expressed in a high proportion of APC^{17,29}. To define whether
155 proinsulin expression in a high proportion of APC was a prerequisite for TCR down-regulation
156 we generated mixed PI-Tg/non-Tg bone marrow (BM) chimeras where proinsulin-encoding BM
157 was titrated to control the proportion of PI-Tg APC arising in recipients. When G9 Tmem were
158 transferred to chimeric recipients, TCR expression on G9 was down-regulated in recipients with
159 greater than 20% PI-Tg engraftment and in 2 of 4 recipients where there was approximately 10%
160 PI-Tg engraftment (**Fig. 2C**), indicating that proinsulin expression was required in only a minor
161 proportion of APC to induce TCR down-regulation.

162

163 **G9 tetramer binding is reduced in PI-transgenic recipients**

164 We previously showed for naïve insulin-specific CD8⁺ T cells that down-regulation of TCR after
165 transfer to PI-Tg mice was associated with loss of H-2K^d/insB₁₅₋₂₃ tetramer binding (manuscript
166 submitted). Here, for G9 Tmem, those transferred to non-Tg recipients retained high levels of H-
167 2K^d/insB₁₅₋₂₃ tetramer binding (**Fig. 3A-C**) with more than three quarters binding H-2K^d/insB<sub>15-
168 23</sub> tetramer at the high levels (**Fig. 3A,B**). In contrast, in PI-Tg recipients the majority of G9
169 Tmem bound less H-2K^d/insB₁₅₋₂₃ tetramer (**Fig 3A-C**) resulting in a lower proportion binding
170 H-2K^d/insB₁₅₋₂₃ tetramer at high levels compared to non-Tg recipients (**Fig. 3B**). In PI-Tg
171 recipients reduced H-2K^d/insB₁₅₋₂₃ tetramer binding correlated with reduced TCR expression
172 (**Fig. 3C**). In conjunction with the overall reduction in G9 Tmem number (**Fig. 1**), G9 T cells
173 binding high levels of H-2K^d/insB₁₅₋₂₃ tetramer were present only as a very low, significantly
174 reduced, proportion of total CD8⁺ T cells in PI-Tg compared to non-Tg recipients (**Fig. 3D,E**).

175

176 **G9 Tmem are rendered unresponsive in proinsulin-expressing recipients**

177 To determine whether G9 Tmem were rendered unresponsive in PI-Tg recipients, G9 Tmem
178 were transferred to NOD and PI-Tg recipients. **Five** days later recipients were immunised or not
179 with insB₁₅₋₂₃/IFA/poly IC and the response of G9 Tmem analysed. **In NOD recipients, G9**
180 **Tmem were readily detectable in spleens of non-Tg recipients of G9 Tmem (Fig. 4A) and robust**
181 **expansion of G9 in response to immunisation was observed between immunised and**
182 **unimmunised non-Tg recipients (Fig. 4A).** In contrast to non-Tg recipients, G9 Tmem were
183 infrequent in spleens of PI-Tg recipients (**Fig. 4A**) and present at close to the limit of flow
184 cytometric detection determined by analysis of untransferred controls (**Fig. 4A**). No

185 immunisation-associated expansion of G9 T cells was evident in PI-Tg recipients (**Fig. 4A**).
186 InsB₁₅₋₂₃-restimulated ELISpot assays revealed that insB₁₅₋₂₃-responsive, IFN- γ -producing G9
187 were readily detectable in non-Tg recipients, relative to no-transfer controls **and** their frequency
188 was increased by immunisation (**Fig. 4C**). Notably, insB₁₅₋₂₃ stimulation revealed virtually no
189 insB₁₅₋₂₃-responsive IFN- γ -producing cells in spleen cells of PI-Tg recipients **whether immunised**
190 **or not**, indicating ablation of G9 responses. Overall the data indicates that G9 Tmem are mostly
191 deleted in PI-Tg recipients and any residual undeleted G9 T cells are rendered unresponsive.

192

193 **G9 Tmem show increased expression of ‘exhaustion’ markers**

194 Increased expression of ‘co-inhibitory’ or ‘exhaustion’ markers is associated with many settings
195 in which T-cells are inactivated. When analysed, PD-1 (CD279) , CD160 and LAG-3 (CD223)
196 were upregulated on a significantly greater proportion of G9 Tmem in PI-Tg compared to non-
197 Tg recipients (Fig. 5A-H) such that the proportion of PD-1⁺CD160⁺ and PD-1⁺LAG-3⁺ G9
198 Tmem was significantly increased (**Fig. 5B,C**). CD244 was largely unchanged although a small
199 proportion (approximately 25%) of PD-1⁺ G9 Tmem in PI-Tg pLN, but not elsewhere, co-
200 expressed CD244 (not shown). These differences were observed even though G9 Tmem acquired
201 higher levels of PD-1, CD160 and LAG-3 during in vitro differentiation. CD5 expression by G9
202 Tmem was increased in non-Tg pLN relative to spleen, but not in PI-Tg relative to non-Tg.

203 **APC-targeted expression of proinsulin limits G9 effector function**

204 As the data indicated transgenic expression of proinsulin terminated insulin-specific memory
205 CD8⁺ T cell responses, we examine whether effector function could also be directly modulated.
206 G9 T cells were cultured for 2 days to generate CTL that were transferred to PI-Tg or non-Tg

207 control recipients. In non-Tg recipients, transfer of 10^7 G9 CTL induced diabetes in
208 approximately one-quarter of recipients (**Fig. 6A**) in line with the diabetogenicity of these cells²⁴
209 and the reduced incidence that would be predicted in immune-competent recipients compared to
210 NOD.scid mice that have been used previously for similar experiments²⁴. In contrast, after
211 parallel transfers to PI-Tg recipients, no mice developed diabetes over the 8-week monitoring
212 period (**Fig. 6A**). In conclusion expression of proinsulin by APC not only terminated insulin-
213 specific CD8⁺ memory T cells responses but also inhibited the diabetogenicity of insulin-specific
214 CTL. To further understand the mechanisms of protection we determined whether G9 CTL were
215 deleted and TCR down-regulated in PI-Tg recipients. Three days after G9 CTL transfer, the
216 number of G9 T cells was significantly reduced in spleens, sdLN and pLN of PI-Tg recipients
217 compared to non-Tg controls (**Fig. 6B**) indicating that deletion had indeed occurred. Analysis
218 of TCR V β 6 expression revealed that although TCR expression by G9 was reduced in PI-Tg
219 relative to non-Tg control recipients, the degree of downregulation (**Fig. 6C**) was not as
220 prominent as that observed for G9 Tmem (**Fig. 2**).

221

222 **DISCUSSION**

223 In T1D, effector and memory T cell responses directed at pancreatic β cells sustain disease
224 progression and give rise to autoimmune memory that persists as a long-term barrier to cellular
225 insulin replacement therapies such as islet transplantation. No immunotherapies have yet been
226 developed for clinical application that effectively prevent T1D progression or reverse established
227 anti- β -cell memory. This reflects the unmet need to effectively terminate difficult-to-control
228 effector and memory T-cell responses. Here we show that enforced expression of proinsulin
229 targeted to APC rapidly and efficiently ablates insulin-specific memory CD8⁺ T-cell responses
230 and disables the diabetogenic capacity of insulin-specific CTL.

231

232 Enforced expression of antigen is a potent means to induce T-cell tolerance which is as effective
233 for effector and memory T cells as it is for naive T cells in non-autoimmune-prone mice¹⁷⁻¹⁹. In
234 humans and NOD mice, genetically-determined defects in tolerance that contribute to the
235 development of pathogenic anti- β -cell responses are present. These defects, that include
236 perturbations in apoptotic pathways and alterations in the makeup and function of Treg and APC
237 that impair normal tolerance induction^{21, 30} could negatively impact on induction of therapeutic
238 tolerance. Indeed, establishment of transplantation tolerance meets with particular challenges³¹⁻
239³³. However, some forms of therapeutic manipulation for tolerance induction may overcome
240 these effects. We have found enforced antigen expression is extremely robust and not reliant on
241 the presence of intact immunoregulatory circuits provided by, for example, NKT cells
242 (unpublished) or Treg³⁴ unlike some other forms of therapy^{35, 36}. Here we found that enforced
243 expression of proinsulin by APC effectively curtailed insulin-specific memory CD8⁺ T cell
244 responses and restrained diabetes induction by insulin-specific CTL. This therapeutic approach

245 is therefore effective in the face of genetic perturbations of tolerance and immune regulatory
246 networks that contribute to diabetes development. While detailed mechanistic studies have not
247 have been performed in many instances, enforced expression of β -cell antigens other than
248 proinsulin, such as IGRP and GAD, appears to effectively induce tolerance in naïve $CD4^+$ and
249 $CD8^+$ T cells even though this may not influence the course of autoimmune diabetes in NOD
250 mice^{3, 37}. Together these studies and our findings here establish the universality of enforced
251 antigen expression for tolerance induction across a range of β -cell antigens meaning that the
252 findings presented here for memory T cells and CTL would also extend to other β -cell antigens.
253 **Whether low levels of proinsulin expression and presentation by, for instance fibroreticular or**
254 **other cells, that appear to modulate the responsiveness of naïve insulin-specific $CD8^+$ T cells³⁸**
255 **can similarly influence the function of insulin-specific Tmem or CTL remains to be determined.**

256

257 Most insulin-specific memory $CD8^+$ T cells were rapidly deleted after encountering proinsulin-
258 expressing APC and those remaining undeleted were rendered unresponsive. The substantial
259 TCR down-regulation we observed indicates that loss of antigen signalling likely plays a key role
260 in maintaining unresponsiveness but is potentially reinforced by the actions of co-inhibitory
261 molecules such as PD-1, CD160, LAG-3 and CD244 that are also upregulated. These
262 observations indicate ‘adaptive tuning’ of residual undeleted insulin-specific $CD8^+$ Tmem to the
263 proinsulin expressed in PI-Tg recipients. An unexpected finding was that CD5, which is an
264 indicator of antigen-sensing³⁹ and upregulated by tolerant T cells in many settings^{26, 40} was not
265 upregulated by inactivated G9 Tmem in PI-Tg recipients. This could perhaps reflect altered
266 regulation of CD5 in Tmem, particularly as CD5 expression was reduced during in vitro Tm

267 differentiation (**Suppl. 2**). Alternatively downstream signalling may be blocked proximally to
268 the TCR⁴¹ thereby preventing CD5 upregulation.

269
270 Here, enforced proinsulin expression was controlled by an MHC class II promoter, targeting
271 proinsulin expression to DC, B cells and possibly other APC. In other studies, targeting
272 expression of a model antigen using an MHC class II promoter also led to rapid deletion and
273 TCR down-regulation, and loss of effector function but in high-affinity ovalbumin (OVA)-
274 specific memory CD8⁺ T cells¹⁸. Rapid deletion and TCR down-regulation appears to be the
275 consequence of extensive antigen expression in such settings^{18, 29} and perhaps this is mediated
276 by inclusion of B cells as antigen-expressing APC^{42, 43}. Whether ‘high dose’ antigen is critical
277 for effective inactivation of insulin-specific CD8⁺ memory T cells is yet to be fully explored, but
278 inactivation of insulin-specific CD8⁺ memory T cells proceeded even when low proportions of
279 APC expressed proinsulin as this was similar to studies using OVA¹⁸ suggesting the altered
280 immunoregulatory environment of NOD mice has little influence on this. One key component of
281 tolerance induction by enforced antigen expression is persistent exposure to antigen⁴⁴, making
282 gene therapy approaches that achieve long-term antigen expression highly applicable. Certainly,
283 transfer of genetically-modified hematopoietic stem cells shows gene therapy has the potential to
284 be an effective treatment^{26, 44, 45} and this is capable of preventing recurrent autoimmune attack of
285 transplanted islets²⁶. **Here we found no inflammatory infiltrates in pancreatic islets of PI-Tg**
286 **recipients of G9 Tmem, even after immunization (e.g. Fig. 4) or CTL (e.g. Fig. 6) suggesting**
287 **that a component of protection may be mediated through limiting T-cell traffic to target tissues**
288 **as described previously¹⁸.** Importantly, the mechanisms that we find underlie tolerance
289 induction by enforced antigen expression and HSC-mediated gene therapy appear to replicate

290 those found in some clinical studies ⁴⁶ emphasizing the potential clinical applicability of this
291 approach. Interestingly, plasmid vaccines show promise as a gene-therapy approach and this has
292 been exploited by a proinsulin encoding DNA vaccine that displays a degree of efficacy ⁴⁷ even
293 though expression of encoded antigen is likely transient. Whether transient expression of antigen
294 carries a risk of boosting rather than inhibiting memory responses remains unclear, as does the
295 minimum period of ‘antigen exposure’ required to induce Tmem inactivation. However,
296 approaches exploiting enforced antigen expression for tolerance induction, under some
297 circumstances, may not need to induce long-term antigen expression if a physiological source of
298 antigen is present to ‘maintain’ tolerance ⁴⁸. Such approaches may be ideally suited for early
299 stages of T1D where endogenous islet antigens present in residual islets can ‘maintain’ tolerance,
300 once established.

301

302 It is worthy of consideration that PI-Tg mice are normally devoid of islet inflammation ^{25, 45} and
303 this could influence the process of tolerance induction in transferred insulin-specific CD8⁺ T
304 cells. In the absence of islet inflammation, trafficking of G9 Tmem or CTL to pLN or pancreatic
305 islets may be reduced, perhaps contributing to reduced pathogenicity. Other than an initial
306 accumulation in spleen, G9 Tm are equally infrequent in the tissues examined in PI-Tg
307 recipients although there may be a propensity for G9 Tmem to accumulate in pLN (**Fig. 1,**
308 **Suppl. 3**), and possibly pancreatic islets, in non-Tg NOD recipients. Activated/effector islet-
309 antigen specific CD4⁺ T cells might act to limit CD8⁺ T-cell tolerance ⁴⁹ in non-Tg NOD mice
310 and the absence of these cells in PI-Tg mice facilitate the ready induction of tolerance in G9
311 Tmem by proinsulin-expressing APC. It would be interesting to determine the effect of co-
312 transferred activated islet-antigen specific CD4⁺ T cells, or even other specificities of islet-

313 antigen specific CD8⁺ T cells, on tolerance induction to understand the influence of ‘help’ on
314 tolerance induction and to understand the potential for tolerance induction in an ‘inflamed’
315 setting. Interestingly, we have recently that memory/effector Th2-skewed CD4⁺ T cells are
316 readily inactivated by enforced antigen expression in APC⁵⁰ and this could potentially extend to
317 diabetogenic CD4⁺ T cells.

318

319 A key challenge for therapy of T1D is that epitope and determinant spreading occurs as diseases
320 develops, necessitating the development of immunotherapeutic approaches that are able to
321 control the diverse anti-islet effector, and ultimately, memory T cell specificities that emerge
322 during disease development. As T cell responses to (pro)insulin play such an important role
323 early in disease development, our findings indicate that approaches where proinsulin is
324 overexpressed early during disease development may hold great therapeutic promise.

325 **METHODS**

326 *Mice*

327 Transgenic mice (PI-Tg) expressing mouse proinsulin II under an MHC class II (I-E α^k) promoter
328 have been described²⁵. G9C8 (G9) mice carry insulin B₁₅₋₂₃-specific TCR transgenic (V α 18,
329 V β 6) T cells²⁴ and NOD.CD45.2 mice carry a congenic CD45 allele⁵¹. B16 mice lack both
330 insulin 1 and insulin 2 (PI1^{-/-} PI2^{-/-}) and express a mutated preproinsulin (B16A) under a rat
331 insulin promoter⁵². Non-transgenic NOD/ShiLtJArc (CD45.1⁺) mice were purchased from the
332 Animal Resources Facility (Perth, WA). Non-Tg and PI-Tg mice were crossed to NOD.CD45.2
333 mice to obtain (CD45.1⁺/CD45.2⁺) F1 offspring. All mice were bred and/or housed under
334 specific pathogen-free conditions in the Princess Alexandra Hospital Biological Resources
335 Facility (Brisbane, Australia), the Translational Research Institute Biological Resources Facility
336 (Brisbane, Australia). Male mice 8-12 weeks of age randomly assigned to boxes were used for
337 all experiments except analysis of diabetes onset after transfer of G9 CTL where 11-12 week-old
338 female mice were used. All animal experiments were approved by The University of Queensland
339 Animal Ethics Committee (Projects 164/12, 144/15).

340

341 *Adoptive transfers and in vivo assays*

342 For in vitro-generated G9 Tmem, LN (axillary, brachial, inguinal, mesenteric) cells were
343 cultured in the presence of insB₁₅₋₂₃ (10 μ g/ml; Auspep, Australia) and rhIL-2 (10ng/ml;
344 PeproTech, Australia) in complete RPMI/5% HI-FCS for 3 days, washed and recultured with IL-
345 15 (10ng/ml; PeproTech, Australia) for an additional 2 days as described¹⁷. To generate CTL,
346 G9 LN cells were cultured in the presence of insB₁₅₋₂₃ (10 μ g/ml; Auspep, Australia) and rhIL-2
347 (10ng/ml; PeproTech, Australia) for 2 days. Cultured cells (>90% G9 T cells) were washed prior

348 to i.v. injection (lateral tail vein, 2×10^6 unless specified otherwise). Labelling with
349 carboxyfluorescein diacetate succinimidyl ester (CFSE) was as described previously⁵³. To test
350 antigen-responsiveness some mice were immunised i.p. with insB₁₅₋₂₃/IFA/poly IC
351 (50 μ g/100 μ g). Mice were screened for glycosuria weekly using Diastix Reagent Strips (Bayer,
352 Leverkusen, Germany). Blood glucose was then determined for glycosuric mice using an Accu-
353 Chek Go glucometer (Roche, Basel, Switzerland) and mice were deemed diabetic following two
354 consecutive blood glucose readings >12mM. To generate mixed bone marrow (BM) chimeras,
355 graded mixtures of PI-Tg (CD45.1⁺CD45.2⁺) and non-Tg (CD45.2⁺) BM were transferred to
356 lethally irradiated NOD.CD45.2 recipients using procedures described elsewhere¹⁸.

357

358 *Flow cytometry and in vitro assays*

359 At indicated timepoints, recipient spleens, skin-draining LN (sdLN; pooled inguinal, axillary,
360 brachial) and pancreatic LN (pLN) were harvested, disrupted by pressing through cell strainers
361 (BD Falcon) and erythrocytes lysed with hypotonic NH₄Cl/Tris buffer (spleens only). Cells were
362 washed, and prepared for further analysis. Fluorochrome-conjugated antibodies were purchased
363 from BioLegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or produced and
364 conjugated in-house (listed in **Suppl. 4**). PE-conjugated tetramers loaded with the insB₁₅₋₂₃
365 (LYLVCGERV) APL that exhibits high affinity for H2-K^d⁵⁴ (K^d-insB₁₅₋₂₃) or listeriolysin
366 (LLO)₉₁₋₉₉ (GYKDGNEYI) were sourced from the NIH Tetramer Facility. G9 T cells were
367 typically defined as CD45.1⁺CD45.2⁻CD8⁺V β 6⁺ cells although when B16 recipients were used,
368 G9 were defined as CFSE⁺CD8⁺V β 6⁺ in these mice. Data were collected using FACSCanto,
369 LSRII (BD Biosciences) or Gallios (Beckman Coulter) cytometers and analyzed with FACSDiva
370 (BD Biosciences), Kaluza (Beckman Coulter) or FlowJo (TreeStar Inc) software. To enumerate

371 cell number, cytometric bead-based counting assays were performed as described ⁵¹.
372 Proliferation index for CFSE dilution assays was calculated as described ⁵⁵. ELISpots were
373 performed as described previously ⁵³ using insB₁₅₋₂₃ at 10µg/ml. Data are displayed as Δ spot
374 forming cells (Δ SFC = # spots with stimulation - # spots in no stimulation control).

375

376 *Statistical Analysis*

377 Pairwise comparison of means was performed with appropriate use of a two-tailed Student's t-
378 test and multiple groups with a one-way ANOVA followed by Tukey post-test (GraphPad
379 Prism). Analyses of diabetes development following G9 CTL transfer were performed by Log-
380 rank (Mantel Cox) test (GraphPad Prism). Exact sample numbers/group and sampling procedure
381 is listed in each Figure legend. No blinding of groups was performed. Sample sizes were based
382 on prior experience with these models and the minimum number of animals were used to obtain
383 statistically significant differences where these existed.

384

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390 and MRC [G0901155, FSW].

391

392 **CONFLICTS OF INTEREST**

393 The authors declare no conflicts of interest.

394

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606 **Figure Legends**

607 **Figure 1. G9 Tmem are deleted in PI-Tg recipients**

608 **A,B)** CFSE-labelled G9 Tmem were transferred to B16A, non-Tg or PI-Tg recipients and three
609 days later spleen, skin-draining LN (sdLN) and pancreatic LN (pLN) harvested and dye dilution
610 determined using flow cytometry. Representative histograms show the extent of division,
611 proportion divided (mean \pm SD) (**A**) and proliferation index (**B**). **C)** G9 T cells were transferred
612 to congenically-distinct (CD45.1⁺/CD45.2⁺) NOD or PI-Tg recipients and G9 T cells
613 (CD45.1⁺/CD45.2⁻CD8⁺/V β 6⁺) enumerated as indicated. Data (**A,B**) are pooled from 2
614 independent experiments and cytometry plots (**B**) are representative of 4-6 individual mice or for
615 (**C**) data are pooled from at least two independent experiments per timepoint (**day 1 n=5, day 2**
616 **n=6, day 3 n=5, day 5 n=6, day 7 n=11**). Plots show individual mice and mean \pm SD (**B**) or mean
617 \pm SD (**C**). ANOVA with Tukey's post-test.

618

619 **Figure 2. G9 Tmem TCR expression is reduced in PI-Tg recipients.**

620 **A,B)** G9 Tmem were transferred to NOD or PI-Tg recipients and seven days later (**A**) or at the
621 indicated times (**B**) TCR V β 6 expression was determined by flow cytometry and plotted relative
622 to that of host CD8⁺ V β 6 T cells. **C)** Mixed chimeras were generated and 6 weeks later G9
623 Tmem transferred. Three days later mice were analysed for engraftment proportions and V β 6
624 TCR expression. Data are representative FACS plots (**A**), mean \pm SD (**day 1 n=5, day 2 n=6,**
625 **day 3 n=5, day 5 n=6, day 7 n=11**) pooled from 2-3 experiments per time point (**B**) or individual
626 values pooled from 2 experiments showing 4 parameter non-linear curve fit (**C**). ANOVA with
627 Tukey's post-test was used to compare means (**B**).

628

629 **Figure 3. Tetramer binding by G9 Tmem is reduced in PI-Tg recipients.**

630 **A-E)** G9 Tmem cells were transferred to non-Tg or PI-Tg recipients and three days later G9 T
631 cell (CD45.1⁺/CD45.2⁻CD8⁺/V β 6⁺) K^d-insB₁₅₋₂₃ tetramer binding and V β 6 expression were
632 determined by flow cytometry. Depicted is K^d-insB₁₅₋₂₃-binding by G9 T cells (solid lines) or
633 host PI-Tg CD8⁺ V β 6⁺ T cells where the vertical dotted line denotes the cut-off used to define
634 K^d-insB₁₅₋₂₃^{hi} T cells (**A**). The proportion of G9 T cells binding high levels of K^d-insB₁₅₋₂₃ G9
635 cells was calculated (**B**). V β 6 expression on G9 relative to host CD8⁺ and K^d-insB₁₅₋₂₃-binding
636 normalised to the mean value for non-Tg recipients in each experiment was plotted (**C**). Total

637 number of K^d-insB₁₅₋₂₃^{hi} G9 T cells (**D**) or their proportion of total CD8⁺ T cells was calculated.
638 Data are representative FACS plots (**A**), data points for individual organs from individual mice
639 (**D**) or individual mice with mean ± SD pooled from 2 or more experiments (**B,D,E**). ANOVA
640 with Tukey's post-test or Student's t-test (**D,E**).

641

642 **Figure 4 G9 Tmem are rendered unresponsive in PI-Tg recipients**

643 (**A,B**) G9 Tmem were transferred to congenically-distinct (CD45.1⁺/CD45.2⁺) non-Tg or PI-Tg
644 recipients that were **five** days later immunised or not with insB₁₅₋₂₃/IFA/poly I:C. A further 5
645 days later G9 T cells (CD45.1⁺/CD45.2⁻/CD8⁺/Vβ6⁺) were enumerated in spleen using flow
646 cytometry (**A**) and insB₁₅₋₂₃-responsive IFN-γ-producing cells determined by ELISpot (**B**). Data
647 are pooled from 2 experiments and show individual mice and mean ± SD. ANOVA with Tukey's
648 post-test.

649

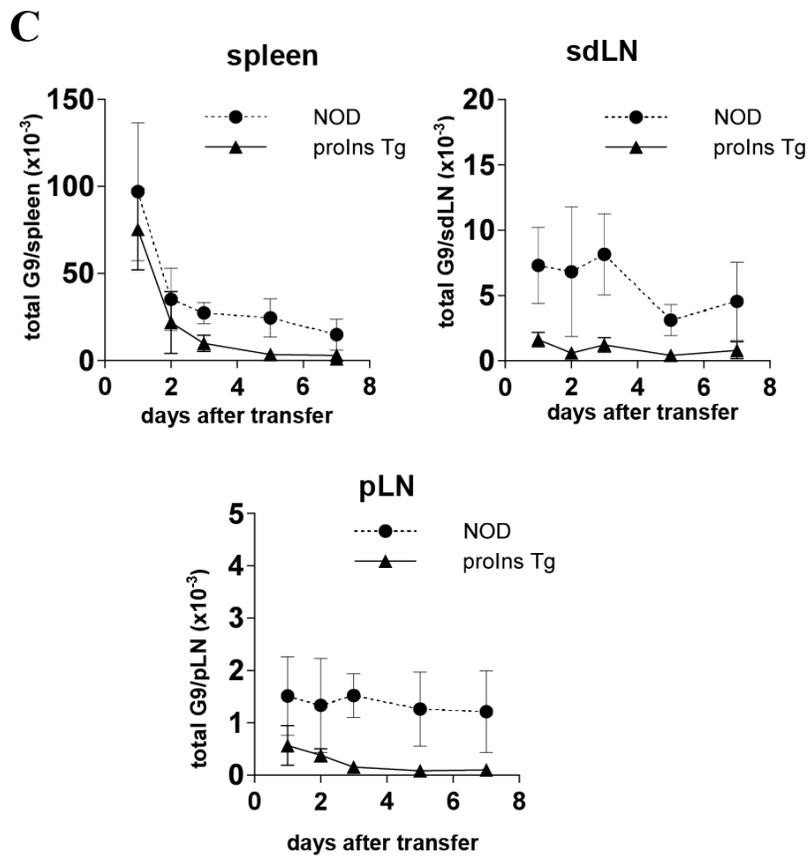
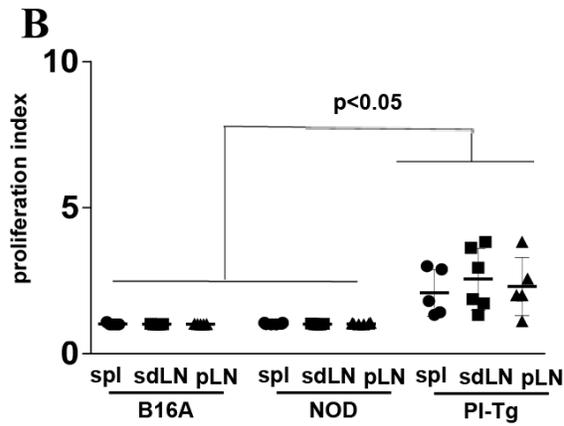
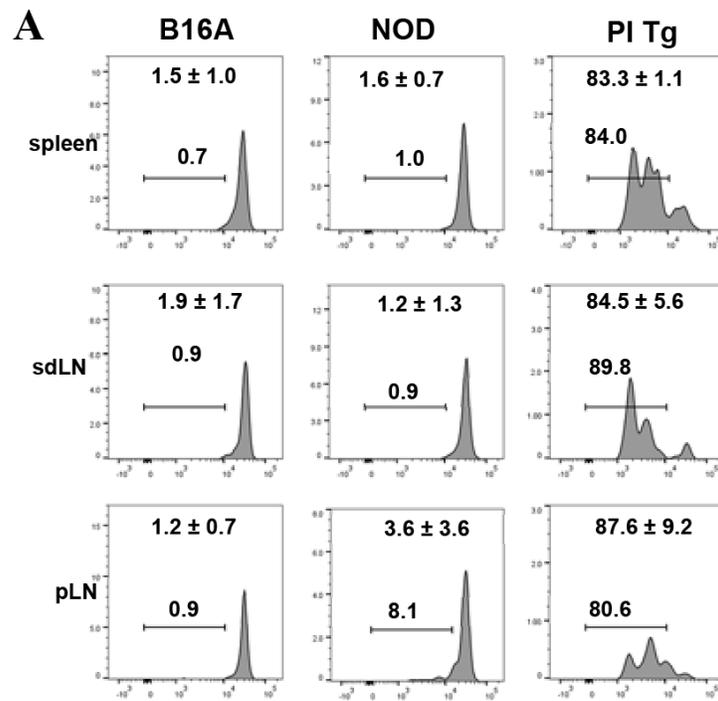
650 **Figure 5. G9 Tmem express elevated levels of co-inhibitory molecules in PI-Tg recipients**

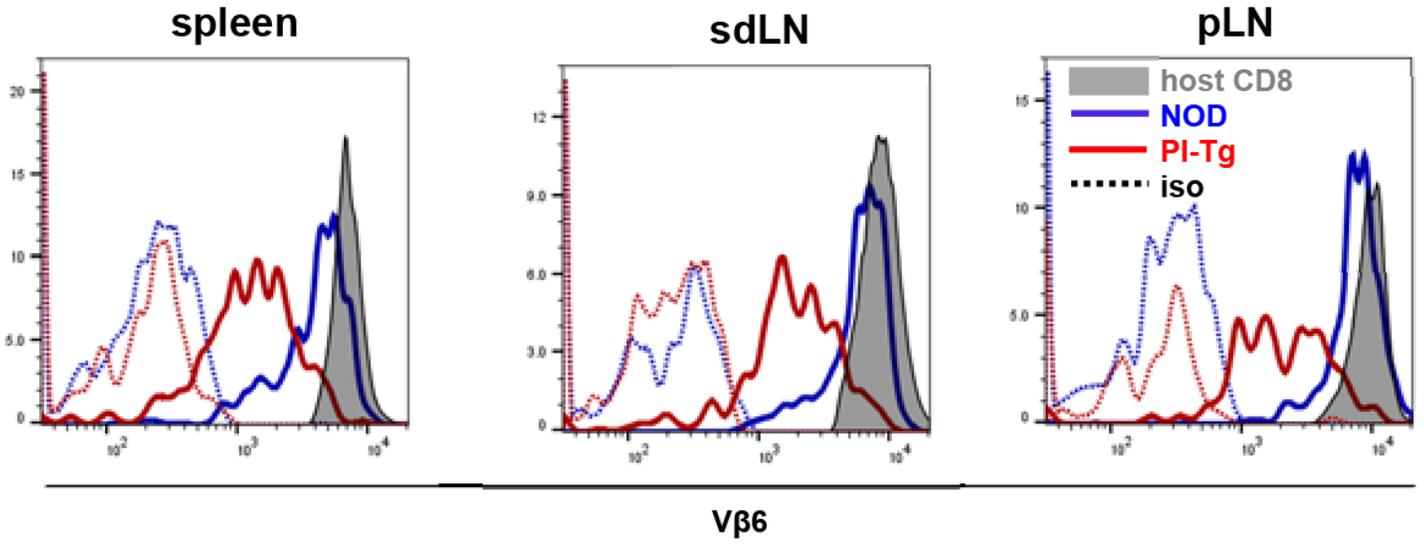
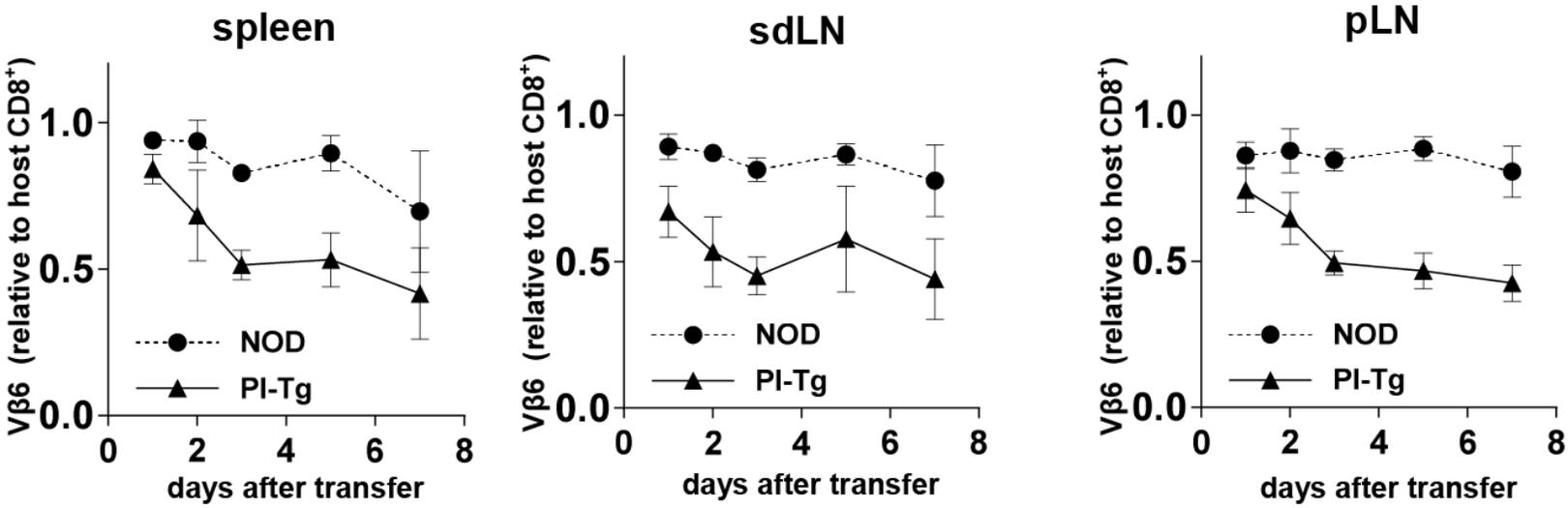
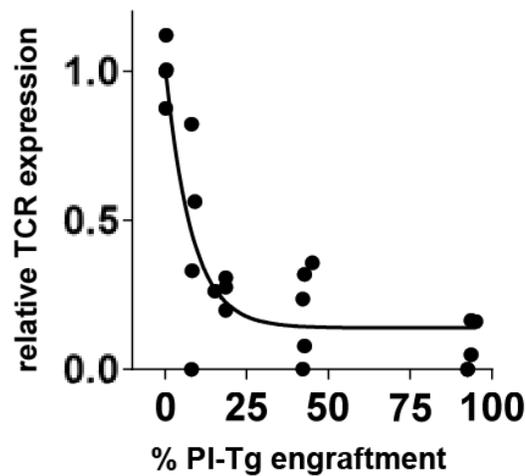
651 **A-K**) G9 Tmem were transferred to congenically-distinct (CD45.1⁺/CD45.2⁺) non-Tg or PI-Tg
652 recipients and 5 days later G9 T cells (CD45.1⁺/CD45.2⁻/CD8⁺/Vβ6⁺) were analysed by flow
653 cytometry. Data points show individual mice pooled from 2 experiments with mean ± SD.
654 ANOVA with Tukey's post-test.

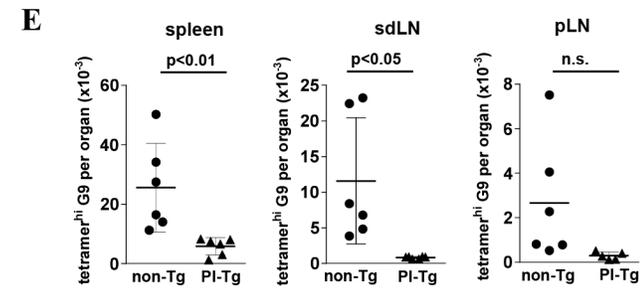
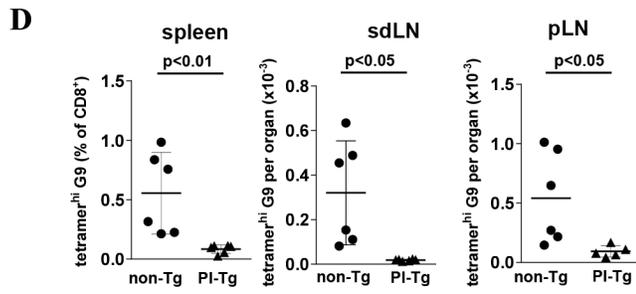
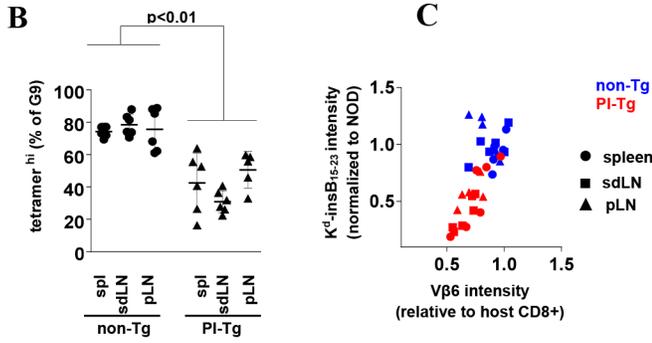
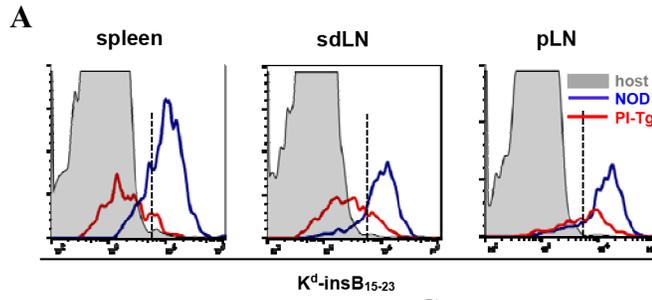
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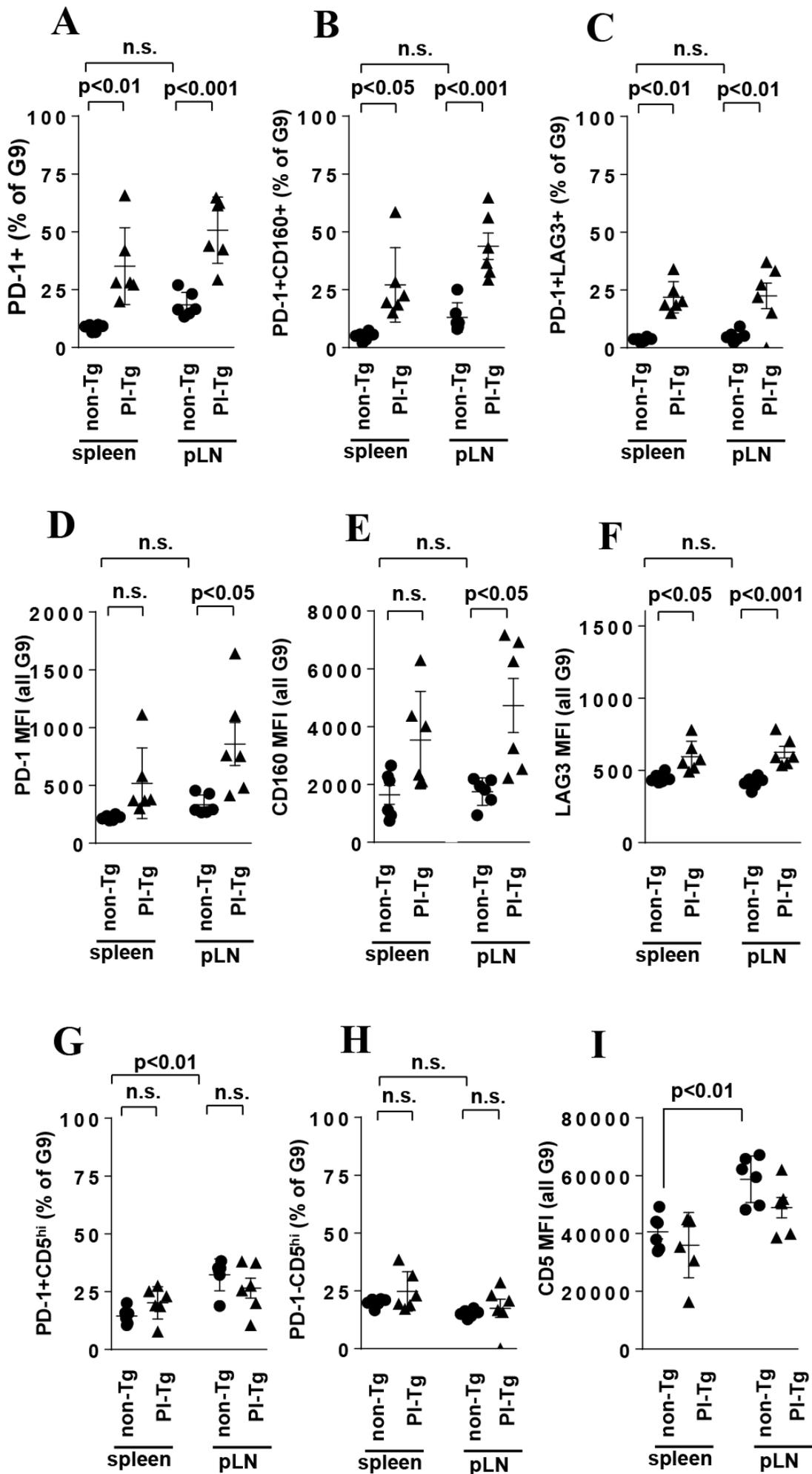
656 **Figure 6. Transgenic proinsulin expression inhibits insulin-specific CTL effector function**

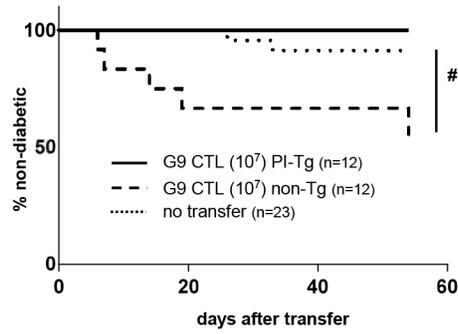
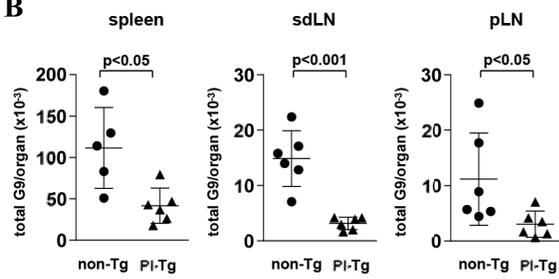
657 **A**) G9 LN and spleen cells were cultured with B₁₅₋₂₃ and IL-2 for 2 days and transferred i.v. to
658 11-12 week-old female non-Tg or PI-Tg recipients and diabetes onset compared to
659 contemporaneous untransferred controls. Urinary glucose was monitored twice-weekly and
660 diabetes onset confirmed when 2 consecutive blood glucose were > 12mM. Mantel Cox log-
661 rank test #: p=0.0078, †: p=0.0371. **B,C**) G9 LN and spleen cells were cultured with B₁₅₋₂₃ and
662 IL-2 for 2 days and transferred i.v. to 9 week-old female non-Tg (NOD.CD45.2) or PI-Tg (PI-Tg
663 x NOD.CD45.2) mice. Three days later tissues were harvested and G9 (CD45.1⁺/CD45.2⁻
664 /CD8⁺/Vβ6⁺) T cells analysed by flow cytometry. Data points show individual mice pooled from
665 2 experiments with mean ± SD. t-test was used to compare means.



A**B****C**





A**B****C**