APC-targeted proinsulin expression inactivates insulin-specific memory CD8$^+$ T cells in NOD mice.

Peta L. S. Reeves[1], Rajeev Rudraraju[1], Xiao Liu[1], F. Susan Wong[2], Emma E. Hamilton-Williams[1], and Raymond J. Steptoe[1]

[1]*The University of Queensland Diamantina Institute, Brisbane QLD 4102, Australia*

[2] *Institute of Molecular & Experimental Medicine, Cardiff University School of Medicine, Cardiff, Wales*

Please address correspondence to:

Raymond J. Steptoe

The University of Queensland Diamantina Institute,

TRI, Level 6

37 Kent Street

Woolloongabba, QLD 4102,

Australia

email: r.steptoe@uq.edu.au

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ABSTRACT

Type 1 diabetes (T1D) results from T-cell mediated autoimmune destruction of pancreatic β cells. Effector T-cell responses emerge early in disease development and expand as disease progresses. Following β cell destruction, a long-lived T-cell memory is generated that represents a barrier to islet transplantation and other cellular insulin-replacement therapies. Development of effective immunotherapies that control or ablate β cell destructive effector and memory T cell responses has the potential to prevent disease progression and recurrence. Targeting antigen expression to antigen-presenting cells inactivates cognate $CD8^+$ effector and memory T-cell responses and has therapeutic potential. Here we investigated this in the context of insulin-specific responses in the non-obese diabetic mouse where genetic immune tolerance defects could impact on therapeutic tolerance induction. Insulin-specific $CD8^+$ memory T cells transferred to mice expressing proinsulin in antigen-presenting cells proliferated in response to transgenically-expressed proinsulin and the majority were rapidly deleted. A small proportion of transferred insulin-specific Tmem remained undeleted and these were antigen-unresponsive, exhibited reduced TCR expression and H-$2K^d$/ins$B_{15\text{-}23}$ tetramer binding and expressed co-inhibitory molecules. Expression of proinsulin in antigen-presenting cells also abolished the diabetogenic capacity of $CD8^+$ effector T cells. Therefore, destructive insulin-specific $CD8^+$ T cells are effectively inactivated by enforced proinsulin expression despite tolerance defects that exist in diabetes-prone NOD mice. These findings have important implications in developing immunotherapeutic approaches to T1D and other T cell-mediated autoimmune diseases.

INTRODUCTION

Type 1 diabetes (T1D) results from the progressive inflammatory destruction of insulin-secreting pancreatic β cells. Many immune cell types are implicated in promoting β-cell destructive inflammation but T cells are critical. In humans and mice, T cells specific for pancreatic β cell antigens are recruited into effector populations early during the prodromal, pre-clinical phase of autoimmune diabetes where disease is progressing [1]. Responses directed at (pro)insulin epitopes are prominent early in disease and determinant spreading ultimately leads to the targeting of a wide array of β-cell antigens [2, 3]. Along with this, effector T cells expand as disease progresses [4-6] and ultimately form memory populations [6] that persist long after β-cell destruction is complete. These contribute to recurrent autoimmune rejection of replacement insulin-secreting tissues such as islet transplants [7-9]. One of the key challenges for control of memory T cell responses and particularly those in T1D is the resistance of memory T cells to regulatory T cells (Treg), conventional immunosuppression, chemotherapeutic agents [7, 10-14] and some methods of tolerance induction [15, 16]. Understanding how to effectively control effector and memory T cell responses will provide opportunities for therapeutic interventions to interrupt disease progression in T1D by limiting epitope and determinant spreading or purging established effector and memory populations and to alleviate autoimmune resistance to cellular insulin-replacement therapies.

Targeting antigen expression to antigen presenting cell (APC) populations effectively ablates antigen-specific memory and effector $CD4^+$ and $CD8^+$ T cell responses through deletion and induction of unresponsiveness [17-19]. Depending on the APC type targeted, inactivation of $CD8^+$ effector/memory T cells occurs slowly when antigen is expressed in DC or much more rapidly when antigen is targeted to MHC class II^+ APC [18], but studies have focussed on non-autoimmune

prone strains of mice. It remains unclear whether defects that exist in immune regulation in the NOD mouse, that replicate those in T1D-prone individuals with or at-risk of T1D [20-23], impact on the effectiveness of immunotherapy focussed on memory T cells. Additionally, experimental systems employed to explore tolerance induction in T cells typically employ model antigens matched to high-affinity TCR transgenic T cells rather than physiological antigens and T cells with relevant disease-associated TCR and TCR affinities.

Here we sought to gain insight into the applicability of targeted antigen expression for inactivation of memory T cell responses where a natural islet antigen was targeted and TCR/pMHC affinity reflected that occurring naturally during T1D development. We also asked whether inactivation of memory $CD8^+$ T cell responses was effective in the presence of genetic defects in immune regulation that underlie disease susceptibility in a relevant autoimmune-prone model of spontaneous diabetes. To achieve this, we used $CD8^+$ T cells expressing a physiologically-relevant intermediate affinity TCR recognizing insulin B_{15-23} [24] in conjunction with mice where proinsulin is expressed in diverse APC types by an MHC class II promoter [25]. We found in the autoimmune-prone NOD strain, the majority of memory insulin-specific $CD8^+$ T cells transferred to mice expressing proinsulin were deleted and the remainder rapidly inactivated. Additionally, expression of proinsulin in APC ablated the diabetogenic capacity of activated insulin-specific cytotoxic T lymphocytes (CTL). Overall, these findings were remarkably similar to those reported in analogous studies where the model antigen ovalbumin (OVA) was used in conjunction with high-affinity TCR Tg T cells [18]. This indicates that, at least in the NOD mouse model, perturbations of immune regulation that underlie spontaneous diabetes susceptibility do not limit the capacity of APC-expressed antigen to inactivate memory $CD8^+$ T

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

RESULTS

In vitro generation of Tmem

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

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

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

several rounds of division in spleen, sdLN and pLN of PI-Tg recipients (**Fig. 1A,B**). Both the proportion of cells entering division within 3 days and the extent of division in PI-Tg recipients was reduced for G9 Tmem relative to that observed for their naïve counterparts in a similar setting (submitted). Interestingly, reduced proliferation of Tmem has been observed in a similar settings where Tn and Tm have been compared [18] and is in line with other observations of reduced proliferation or expansion capacity of memory relative to naïve $CD8^+$ T cells [27, 28].

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

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

G9 TCR expression is reduced in PI-transgenic recipients

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

G9 tetramer binding is reduced in PI-transgenic recipients

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

G9 Tmem are rendered unresponsive in proinsulin-expressing recipients

To determine whether G9 Tmem were rendered unresponsive in PI-Tg recipients, G9 Tmem were transferred to NOD and PI-Tg recipients. Five days later recipients were immunised or not with $insB_{15-23}$/IFA/poly IC and the response of G9 Tmem analysed. In NOD recipients, G9 Tmem were readily detectable in spleens of non-Tg recipients of G9 Tmem (**Fig. 4A**) and robust expansion of G9 in response to immunisation was observed between immunised and unimmunised non-Tg recipients (**Fig. 4A**). In contrast to non-Tg recipients, G9 Tmem were infrequent in spleens of PI-Tg recipients (**Fig. 4A**) and present at close to the limit of flow 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 InsB$_{15\text{-}23}$-restimulated ELISpot assays revealed that insB$_{15\text{-}23}$-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, insB$_{15\text{-}23}$ stimulation revealed virtually no
189 insB$_{15\text{-}23}$-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

control recipients. In non-Tg recipients, transfer of 10^7 G9 CTL induced diabetes in approximately one-quarter of recipients (**Fig. 6A**) in line with the diabetogenicity of these cells [24] and the reduced incidence that would be predicted in immune-competent recipients compared to NOD.scid mice that have been used previously for similar experiments [24]. In contrast, after parallel transfers to PI-Tg recipients, no mice developed diabetes over the 8-week monitoring period (**Fig. 6A**). In conclusion expression of proinsulin by APC not only terminated insulin-specific $CD8^+$ memory T cells responses but also inhibited the diabetogenicity of insulin-specific CTL. To further understand the mechanisms of protection we determined whether G9 CTL were deleted and TCR down-regulated in PI-Tg recipients. Three days after G9 CTL transfer, the number of G9 T cells was significantly reduced in spleens, sdLN and pLN of PI-Tg recipients compared to non-Tg controls (**Fig. 6B**) indicating that deletion had indeed occurred. Analysis of TCR Vβ6 expression revealed that although TCR expression by G9 was reduced in PI-Tg relative to non-Tg control recipients, the degree of downregulation (**Fig. 6C**) was not as prominent as that observed for G9 Tmem (**Fig. 2**).

DISCUSSION

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

Enforced expression of antigen is a potent means to induce T-cell tolerance which is as effective for effector and memory T cells as it is for naive T cells in non-autoimmune-prone mice [17-19]. In humans and NOD mice, genetically-determined defects in tolerance that contribute to the development of pathogenic anti-β-cell responses are present. These defects, that include perturbations in apoptotic pathways and alterations in the makeup and function of Treg and APC that impair normal tolerance induction [21, 30] could negatively impact on induction of therapeutic tolerance. Indeed, establishment of transplantation tolerance meets with particular challenges [31-33]. However, some forms of therapeutic manipulation for tolerance induction may overcome these effects. We have found enforced antigen expression is extremely robust and not reliant on the presence of intact immunoregulatory circuits provided by, for example, NKT cells (unpublished) or Treg [34] unlike some other forms of therapy [35, 36]. Here we found that enforced expression of proinsulin by APC effectively curtailed insulin-specific memory $CD8^+$ T cell responses and restrained diabetes induction by insulin-specific CTL. This therapeutic approach

is therefore effective in the face of genetic perturbations of tolerance and immune regulatory networks that contribute to diabetes development. While detailed mechanistic studies have not have been performed in many instances, enforced expression of β-cell antigens other than proinsulin, such as IGRP and GAD, appears to effectively induce tolerance in naïve $CD4^+$ and $CD8^+$ T cells even though this may not influence the course of autoimmune diabetes in NOD mice [3, 37]. Together these studies and our findings here establish the universality of enforced antigen expression for tolerance induction across a range of β-cell antigens meaning that the findings presented here for memory T cells and CTL would also extend to other β-cell antigens. Whether low levels of proinsulin expression and presentation by, for instance fibroreticular or other cells, that appear to modulate the responsiveness of naïve insulin-specific CD8+ T cells [38] can similarly influence the function of insulin-specific Tmem or CTL remains to be determined.

Most insulin-specific memory $CD8^+$ T cells were rapidly deleted after encountering proinsulin-expressing APC and those remaining undeleted were rendered unresponsive. The substantial TCR down-regulation we observed indicates that loss of antigen signalling likely plays a key role in maintaining unresponsiveness but is potentially reinforced by the actions of co-inhibitory molecules such as PD-1, CD160, LAG-3 and CD244 that are also upregulated. These observations indicate 'adaptive tuning' of residual undeleted insulin-specific $CD8^+$Tmem to the proinsulin expressed in PI-Tg recipients. An unexpected finding was that CD5, which is an indicator of antigen-sensing [39] and upregulated by tolerant T cells in many settings [26, 40] was not upregulated by inactivated G9 Tmem in PI-Tg recipients. This could perhaps reflect altered regulation of CD5 in Tmem, particularly as CD5 expression was reduced during in vitro Tm

differentiation (**Suppl. 2**). Alternatively downstream signalling may be blocked proximally to the TCR [41] thereby preventing CD5 upregulation.

Here, enforced proinsulin expression was controlled by an MHC class II promoter, targeting proinsulin expression to DC, B cells and possibly other APC. In other studies, targeting expression of a model antigen using an MHC class II promoter also led to rapid deletion and TCR down-regulation, and loss of effector function but in high-affinity ovalbumin (OVA)-specific memory $CD8^+$ T cells [18]. Rapid deletion and TCR down-regulation appears to be the consequence of extensive antigen expression in such settings [18, 29] and perhaps this is mediated by inclusion of B cells as antigen-expressing APC [42, 43]. Whether 'high dose' antigen is critical for effective inactivation of insulin-specific $CD8^+$ memory T cells is yet to be fully explored, but inactivation of insulin-specific $CD8^+$ memory T cells proceeded even when low proportions of APC expressed proinsulin as this was similar to studies using OVA [18] suggesting the altered immunoregulatory environment of NOD mice has little influence on this. One key component of tolerance induction by enforced antigen expression is persistent exposure to antigen [44], making gene therapy approaches that achieve long-term antigen expression highly applicable. Certainly, transfer of genetically-modified hematopoietic stem cells shows gene therapy has the potential to be an effective treatment [26, 44, 45] and this is capable of preventing recurrent autoimmune attack of transplanted islets [26]. Here we found no inflammatory infiltrates in pancreatic islets of PI-Tg recipients of G9 Tmem, even after immunization (e.g. **Fig. 4**) or CTL (e.g. **Fig. 6**) suggesting that a component of protection may be mediated through limiting T-cell traffic to target tissues as described previously [18]. Importantly, the mechanisms that we find underlie tolerance 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 APC [50] 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.

METHODS

Mice

Transgenic mice (PI-Tg) expressing mouse proinsulin II under an MHC class II ($I\text{-}E_\alpha^k$) promoter have been described [25]. G9C8 (G9) mice carry insulin $B_{15\text{-}23}$-specific TCR transgenic (Vα18, Vβ6) T cells [24] and NOD.CD45.2 mice carry a congenic CD45 allele [51]. B16 mice lack both insulin 1 and insulin 2 ($PI1^{-/-}$ $PI2^{-/-}$) and express a mutated preproinsulin (B16A) under a rat insulin promoter [52]. Non-transgenic NOD/ShiLtJArc ($CD45.1^+$) mice were purchased from the Animal Resources Facility (Perth, WA). Non-Tg and PI-Tg mice were crossed to NOD.CD45.2 mice to obtain ($CD45.1^+/CD45.2^+$) F1 offspring. All mice were bred and/or housed under specific pathogen-free conditions in the Princess Alexandra Hospital Biological Resources Facility (Brisbane, Australia), the Translational Research Institute Biological Resources Facility (Brisbane, Australia). Male mice 8-12 weeks of age randomly assigned to boxes were used for all experiments except analysis of diabetes onset after transfer of G9 CTL where 11-12 week-old female mice were used. All animal experiments were approved by The University of Queensland Animal Ethics Committee (Projects 164/12, 144/15).

Adoptive transfers and in vivo assays

For in vitro-generated G9 Tmem, LN (axillary, brachial, inguinal, mesenteric) cells were cultured in the presence of $insB_{15\text{-}23}$ (10μg/ml; Auspep, Australia) and rhIL-2 (10ng/ml; PeproTech, Australia) in complete RPMI/5% HI-FCS for 3 days, washed and recultured with IL-15 (10ng/ml; PeproTech, Australia) for an additional 2 days as described [17]. To generate CTL, G9 LN cells were cultured in the presence of $insB_{15\text{-}23}$ (10μg/ml; Auspep, Australia) and rhIL-2 (10ng/ml; PeproTech, Australia) for 2 days. Cultured cells (>90% G9 T cells) were washed prior

to i.v. injection (lateral tail vein, 2 x 10^6 unless specified otherwise). Labelling with carboxyfluorescein diacetate succinimidyl ester (CFSE) was as described previously [53]. To test antigen-responsiveness some mice were immunised i.p. with $insB_{15-23}$/IFA/poly IC (50µg/100µg). Mice were screened for glycosuria weekly using Diastix Reagent Strips (Bayer, Leverkusen, Germany). Blood glucose was then determined for glycosuric mice using an Accu-Chek Go glucometer (Roche, Basel, Switzerland) and mice were deemed diabetic following two consecutive blood glucose readings >12mM. To generate mixed bone marrow (BM) chimeras, graded mixtures of PI-Tg ($CD45.1^+CD45.2^+$) and non-Tg ($CD45.2^+$) BM were transferred to lethally irradiated NOD.CD45.2 recipients using procedures described elsewhere [18].

Flow cytometry and in vitro assays

At indicated timepoints, recipient spleens, skin-draining LN (sdLN; pooled inguinal, axilliary, brachial) and pancreatic LN (pLN) were harvested, disrupted by pressing through cell strainers (BD Falcon) and erythrocytes lysed with hypotonic NH_4Cl/Tris buffer (spleens only). Cells were washed, and prepared for further analysis. Fluorochrome-conjugated antibodies were purchased from BioLegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or produced and conjugated in-house (listed in **Suppl. 4**). PE-conjugated tetramers loaded with the $insB_{15-23}$ (LYLVCGERV) APL that exhibits high affinity for H2-K^d [54] (K^d-$insB_{15-23}$) or listeriolysin $(LLO)_{91-99}$ (GYKDGNEYI) were sourced from the NIH Tetramer Facility. G9 T cells were typically defined as $CD45.1^+CD45.2^-CD8^+V\beta6^+$ cells although when B16 recipients were used, G9 were defined as $CFSE^+CD8^+V\beta6^+$ in these mice. Data were collected using FACSCanto, LSRII (BD Biosciences) or Gallios (Beckman Coulter) cytometers and analyzed with FACSDiva (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 $insB_{15-23}$ 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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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Figure Legends

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

A,B) CFSE-labelled G9 Tmem were transferred to B16A, non-Tg or PI-Tg recipients and three days later spleen, skin-draining LN (sdLN) and pancreatic LN (pLN) harvested and dye dilution determined using flow cytometry. Representative histograms show the extent of division, proportion divided (mean ± SD) (**A**) and proliferation index (**B**). **C**) G9 T cells were transferred to congenically-distinct ($CD45.1^+/CD45.2^+$) NOD or PI-Tg recipients and G9 T cells ($CD45.1^+/CD45.2^-CD8^+/V\beta6^+$) enumerated as indicated. Data (**A,B**) are pooled from 2 independent experiments and cytometry plots (**B**) are representative of 4-6 individual mice or for (**C**) data are pooled from at least two independent experiments per timepoint (day 1 n=5, day 2 n=6, day 3 n=5, day 5 n=6, day 7 n=11). Plots show individual mice and mean ± SD (**B**) or mean ± SD (**C**). ANOVA with Tukey's post-test.

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

A,B) G9 Tmem were transferred to NOD or PI-Tg recipients and seven days later (**A**) or at the indicated times (**B**) TCR Vβ6 expression was determined by flow cytometry and plotted relative to that of host $CD8^+$ Vβ6 T cells. **C**) Mixed chimeras were generated and 6 weeks later G9 Tmem transferred. Three days later mice were analysed for engraftment proportions and Vβ6 TCR expression. Data are representative FACS plots (**A**), mean ± SD ((day 1 n=5, day 2 n=6, day 3 n=5, day 5 n=6, day 7 n=11) pooled from 2-3 experiments per time point (**B**) or individual values pooled from 2 experiments showing 4 parameter non-linear curve fit (**C**). ANOVA with Tukey's post-test was used to compare means (**B**).

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

A-E) G9 Tmem cells were transferred to non-Tg or PI-Tg recipients and three days later G9 T cell ($CD45.1^+/CD45.2^-CD8^+/V\beta6^+$) K^d-$insB_{15-23}$ tetramer binding and Vβ6 expression were determined by flow cytometry. Depicted is K^d-$insB_{15-23}$-binding by G9 T cells (solid lines) or host PI-Tg $CD8^+$ $V\beta6^+$ T cells where the vertical dotted line denotes the cut-off used to define K^d-$insB_{15-23}^{hi}$ T cells (**A**). The proportion of G9 T cells binding high levels of K^d-$insB_{15-23}$ G9 cells was calculated (**B**). Vβ6 expression on G9 relative to host CD8+ and K^d-$insB_{15-23}$-binding normalised to the mean value for non-Tg recipients in each experiment was plotted (**C**). Total

number of K^d-ins$B_{15\text{-}23}^{hi}$ G9 T cells (**D**) or their proportion of total $CD8^+$ T cells was calculated. Data are representative FACS plots (**A**), data points for individual organs from individual mice (**D**) or individual mice with mean ± SD pooled from 2 or more experiments (**B,D,E**). ANOVA with Tukey's post-test or Student's t-test (**D,E**).

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

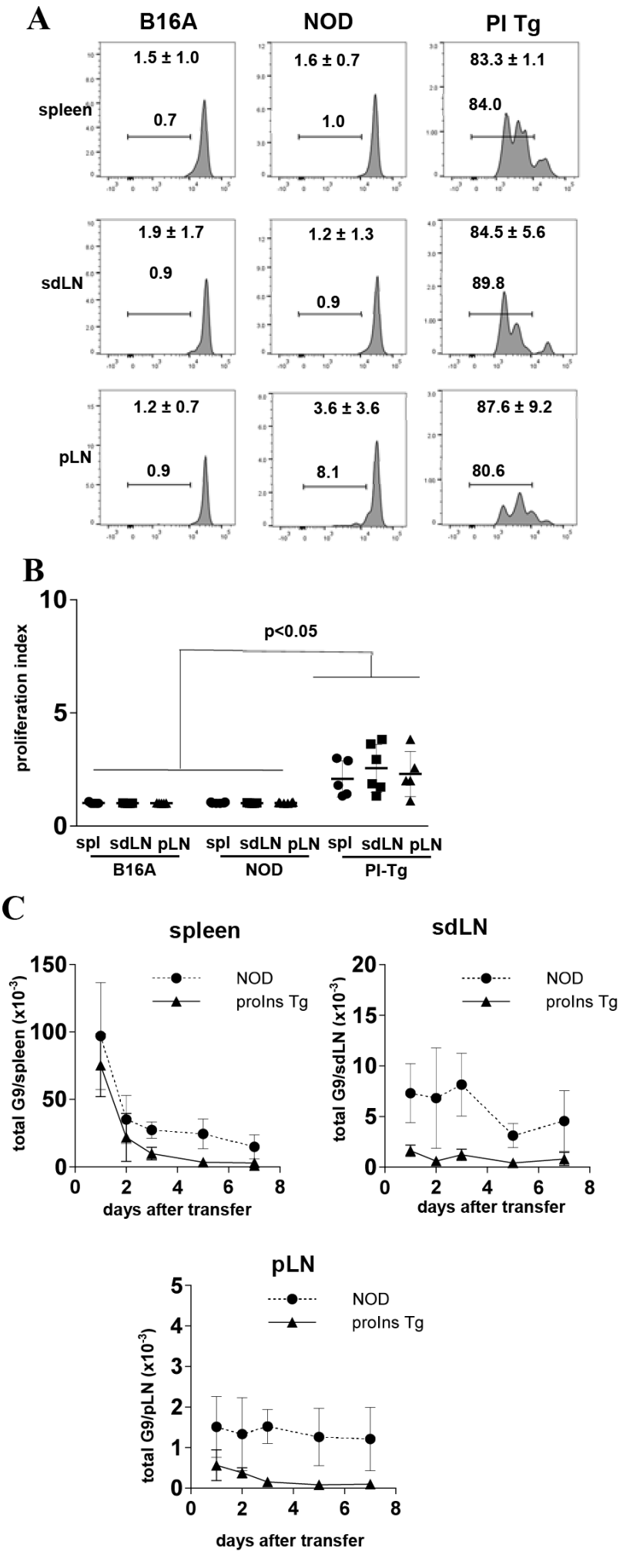
(**A,B**) G9 Tmem were transferred to congenically-distinct ($CD45.1^+/CD45.2^+$) non-Tg or PI-Tg recipients that were five days later immunised or not with ins$B_{15\text{-}23}$/IFA/poly I:C. A further 5 days later G9 T cells ($CD45.1^+/CD45.2^-/CD8^+/V\beta6^+$) were enumerated in spleen using flow cytometry (**A**) and ins$B_{15\text{-}23}$-responsive IFN-γ-producing cells determined by ELISpot (**B**). Data are pooled from 2 experiments and show individual mice and mean ± SD. ANOVA with Tukey's post-test.

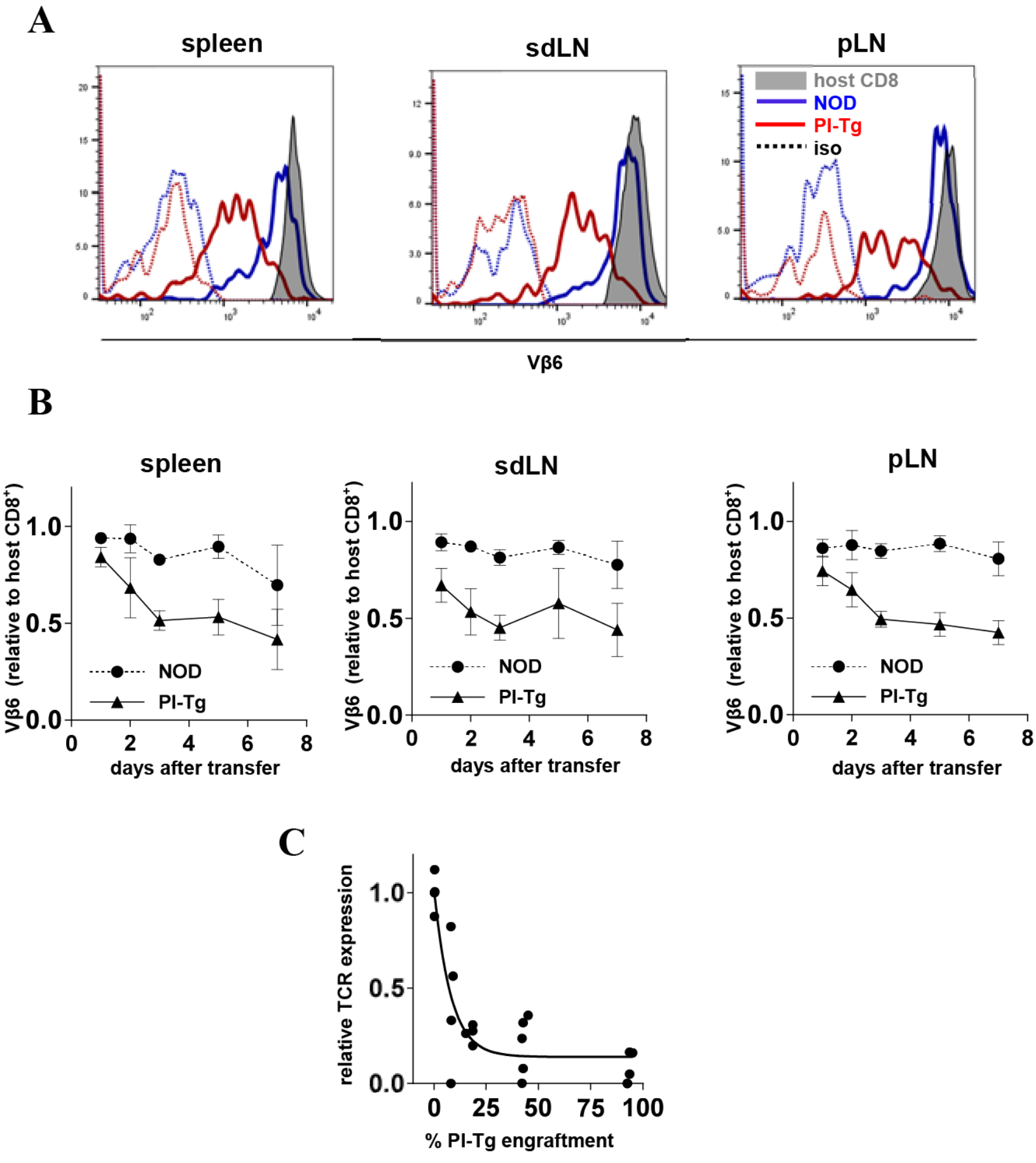
Figure 5. G9 Tmem express elevated levels of co-inhibitory molecules in PI-Tg recipients

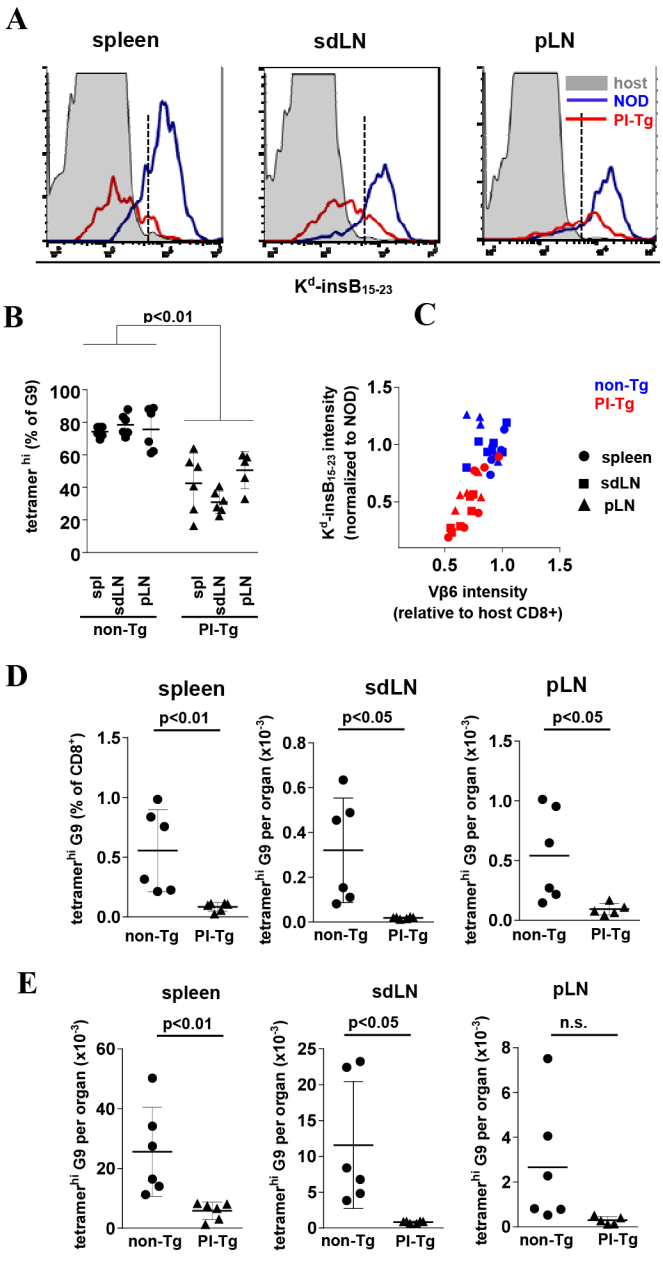
A-K) G9 Tmem were transferred to congenically-distinct ($CD45.1^+/CD45.2^+$) non-Tg or PI-Tg recipients and 5 days later G9 T cells ($CD45.1^+/CD45.2^-/CD8^+/V\beta6^+$) were analysed by flow cytometry. Data points show individual mice pooled from 2 experiments with mean ± SD. ANOVA with Tukey's post-test.

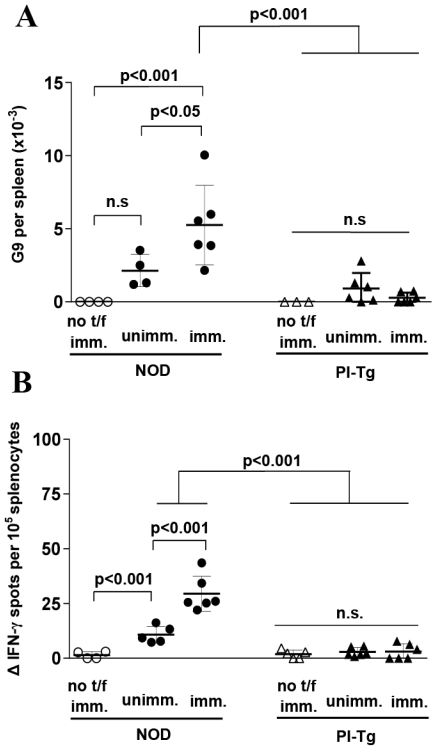
Figure 6. Transgenic proinsulin expression inhibits insulin-specific CTL effector function

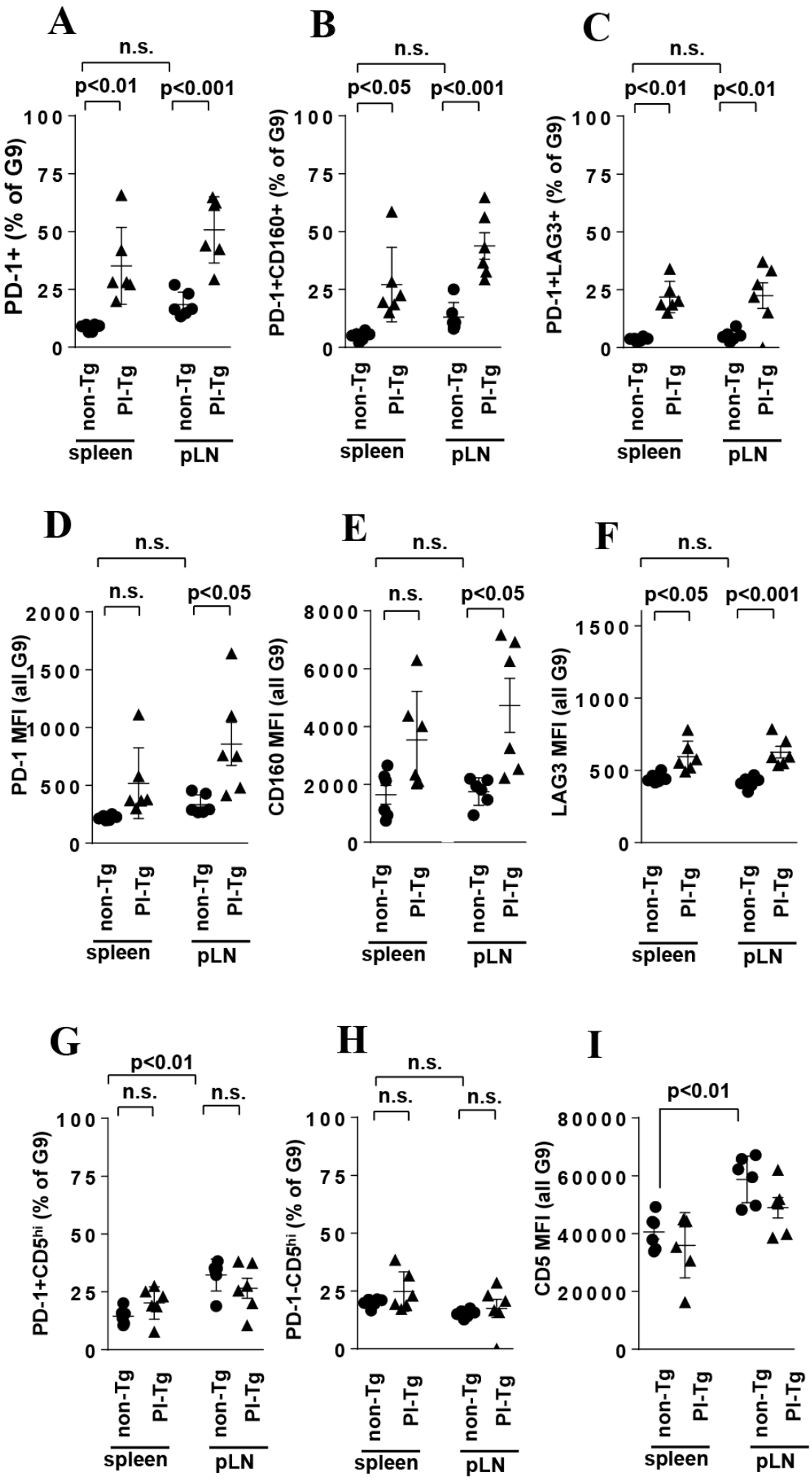
A) G9 LN and spleen cells were cultured with $B_{15\text{-}23}$ and IL-2 for 2 days and transferred i.v. to 11-12 week-old female non-Tg or PI-Tg recipients and diabetes onset compared to contemporaneous untransferred controls. Urinary glucose was monitored twice-weekly and diabetes onset confirmed when 2 consecutive blood glucose were > 12mM. Mantel Cox log-rank test #: p=0.0078, †: p=0.0371. **B,C**) G9 LN and spleen cells were cultured with $B_{15\text{-}23}$ and IL-2 for 2 days and transferred i.v. to 9 week-old female non-Tg (NOD.CD45.2) or PI-Tg (PI-Tg x NOD.CD45.2) mice. Three days later tissues were harvested and G9 ($CD45.1^+/CD45.2^-/CD8^+/V\beta6^+$) T cells analysed by flow cytometry. Data points show individual mice pooled from 2 experiments with mean ± SD. t-test was used to compare means.

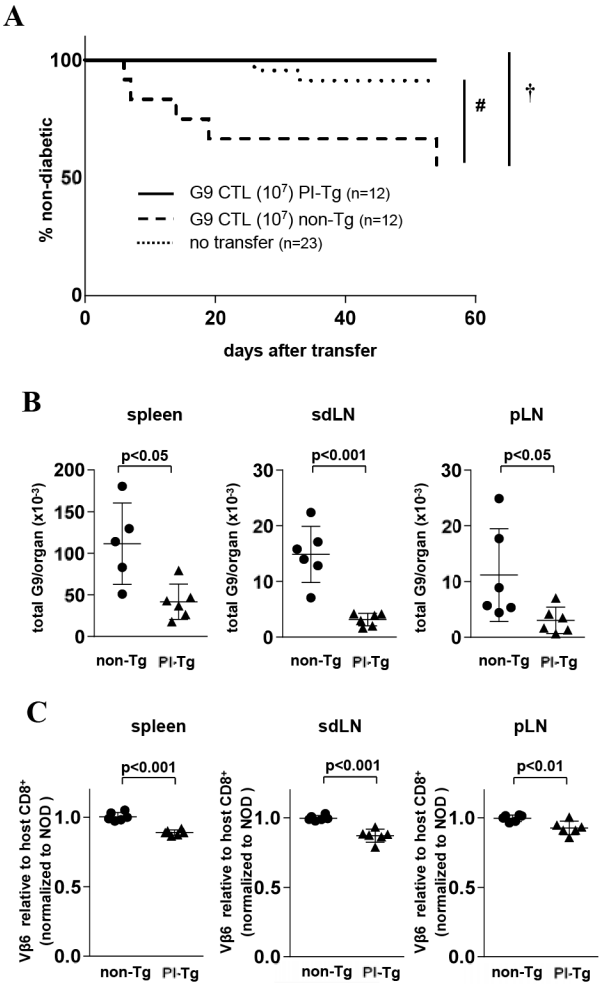

A
B16A
NOD
PI Tg
spleen
sdLN
pLN
1.5 ± 1.0
0.7
1.6 ± 0.7
1.0
83.3 ± 1.1
84.0
1.9 ± 1.7
0.9
1.2 ± 1.3
0.9
84.5 ± 5.6
89.8
1.2 ± 0.7
0.9
3.6 ± 3.6
8.1
87.6 ± 9.2
80.6
B
proliferation index
p<0.05
spl sdLN pLN spl sdLN pLN spl sdLN pLN
B16A
NOD
PI-Tg
C
spleen
sdLN
pLN
NOD
proIns Tg
total G9/spleen (x10-3)
total G9/sdLN (x10-3)
total G9/pLN (x10-3)
days after transfer


A
spleen
sdLN
pLN
host CD8
NOD
PI-Tg
iso
Vβ6
B
spleen
sdLN
pLN
Vβ6 (relative to host CD8+)
1.0
0.5
0.0
NOD
PI-Tg
0
2
4
6
8
days after transfer
C
relative TCR expression
1.0
0.5
0.0
0
25
50
75
100
% PI-Tg engraftment


A
spleen
sdLN
pLN
host
NOD
PI-Tg
Kd-insB15-23
B
p<0.01
tetramer hi (% of G9)
spl
sdLN
pLN
non-Tg
PI-Tg
C
non-Tg
PI-Tg
spleen
sdLN
pLN
Kd-insB15-23 intensity (normalized to NOD)
Vβ6 intensity (relative to host CD8+)
D
spleen
sdLN
pLN
p<0.01
p<0.05
p<0.05
tetramerhi G9 (% of CD8+)
tetramerhi G9 per organ (x10-3)
non-Tg
PI-Tg
E
spleen
sdLN
pLN
p<0.01
p<0.05
n.s.
tetramerhi G9 per organ (x10-3)
non-Tg
PI-Tg


A
p<0.001
p<0.001
p<0.05
n.s
n.s
G9 per spleen (x10-3)
15
10
5
0
no t/f imm.
unimm.
imm.
no t/f imm.
unimm.
imm.
NOD
PI-Tg
B
Δ IFN-γ spots per 10^5 splenocytes
100
75
50
25
0
p<0.001
p<0.001
p<0.001
n.s.
no t/f imm.
unimm.
imm.
no t/f imm.
unimm.
imm.
NOD
PI-Tg


A
n.s.
p<0.01
p<0.001
PD-1+ (% of G9)
100
75
50
25
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
B
n.s.
p<0.05
p<0.001
PD-1+CD160+ (% of G9)
100
75
50
25
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
C
n.s.
p<0.01
p<0.01
PD-1+LAG3+ (% of G9)
100
75
50
25
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
D
n.s.
n.s.
p<0.05
PD-1 MFI (all G9)
2000
1500
1000
500
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
E
n.s.
n.s.
p<0.05
CD160 MFI (all G9)
8000
6000
4000
2000
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
F
n.s.
p<0.05
p<0.001
LAG3 MFI (all G9)
1500
1000
500
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
G
p<0.01
n.s.
n.s.
PD-1+CD5hi (% of G9)
100
75
50
25
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
H
n.s.
n.s.
n.s.
PD-1-CD5hi (% of G9)
100
75
50
25
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN
I
p<0.01
CD5 MFI (all G9)
80000
60000
40000
20000
0
non-Tg
PI-Tg
non-Tg
PI-Tg
spleen
pLN


A
100
50
0
% non-diabetic
0
20
40
60
days after transfer
G9 CTL (10^7) PI-Tg (n=12)
G9 CTL (10^7) non-Tg (n=12)
no transfer (n=23)
#
†
B
spleen
sdLN
pLN
p<0.05
p<0.001
p<0.05
total G9/organ (x10^-3)
non-Tg
PI-Tg
C
spleen
sdLN
pLN
p<0.001
p<0.001
p<0.01
Vβ6 relative to host CD8+ (normalized to NOD)
non-Tg
PI-Tg