Antigen presenting cell-targeted proinsulin expression converts insulin-specific CD8$^+$ T-cell priming to tolerance in autoimmune-prone NOD mice

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Abbreviations:

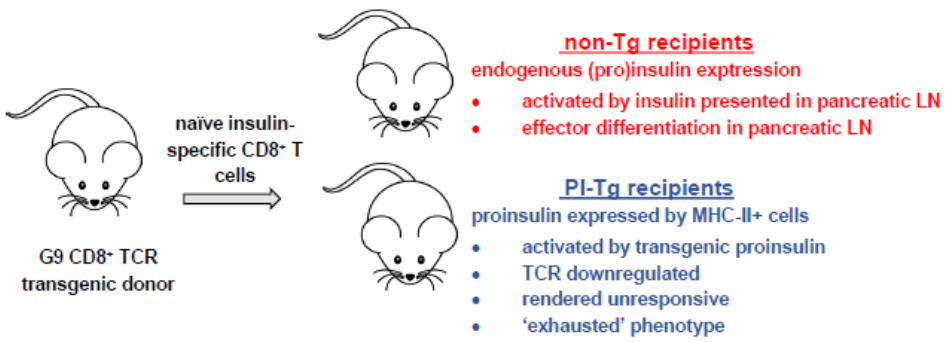
G9	G9C8 TCR transgenic mice
APC	antigen-presenting cell
CFSE	carboxyfluorescein succinimidyl ester
DC	dendritic cell
IFN-ɣ	interferon gamma
NOD	non-obese diabetic mouse
non-Tg	non-transgenic NOD mouse
PI-Tg	proinsulin transgenic
pLN	pancreatic lymph node
sdLN	skin-draining lymph node
MHC	major histocompatibility complex
T1D	type 1 diabetes
TCR	T cell receptor
Treg	regulatory T cell

ABSTRACT

Type 1 diabetes (T1D) results from autoimmune destruction of insulin-producing pancreatic β cells. Therapies need to incorporate strategies to overcome the genetic defects that impair induction or maintenance of peripheral T-cell tolerance and contribute to disease

development. We tested whether the enforced expression of an islet autoantigen in antigen-presenting cells (APC) counteracted peripheral T-cell tolerance defects in autoimmune-prone NOD mice. We observed that insulin-specific $CD8^+$ T cells transferred to mice in which proinsulin was transgenically expressed in APCs underwent several rounds of division and the majority were deleted. Residual insulin-specific $CD8^+$ T cells were rendered unresponsive and this was associated with TCR downregulation, loss of tetramer binding and expression of a range of co-inhibitory molecules. Notably, accumulation and effector differentiation of insulin-specific $CD8^+$ T cells in pancreatic lymph nodes was prominent in non-transgenic recipients but blocked by transgenic proinsulin expression. This shift from T-cell priming to T-cell tolerance exemplifies the tolerogenic capacity of autoantigen expression by APC and the capacity to overcome genetic tolerance defects.

Therapies are required to overcome the T-cell responses that underlie type 1 diabetes. Transgenic expression of proinsulin in antigen-presenting cells induces tolerance in naïve insulin-specific $CD8^+$ T cells through mechanisms that TCR downregulation and, potentially, expression of co-inhibitory molecules. In contrast in non-Tg mice insulin-specific $CD8^+$ T cells appeared to undergo effector differentiation in pancreatic lymph nodes.



INTRODUCTION

Type 1 diabetes (T1D) is a progressive inflammatory disease where pancreatic β cell-specific T cells participate in immune-mediated β cell destruction. Immune responses to insulin are critical in evolution of the autoimmune T-cell response in mice and humans [1] and in the NOD mouse (pro)insulin is likely the primary target responsible for disease initiation [2]. $CD8^+$ T cells directly contribute to β-cell killing [3] and appear to play a crucial role in disease initiation, possibly by promoting antigen release from damaged β cells.

β cell-specific T cells are recruited into effector and memory populations because, at one or more levels, immune tolerance fails. Invariably, some self-specific T cells escape central tolerance induction making peripheral tolerance mechanisms crucial for prevention of autoimmunity. Alterations in immune cell function in NOD mice have been associated with insulin-dependent diabetes (*Idd*) risk allele effects [4-8] and similar effects are reported in humans with or at risk of T1D [5, 9]. Several defects that adversely impact thymic negative selection are present in NOD mice [10, 11] and these could potentially affect peripheral tolerance induction. Development of antigen-presenting cell (APC), such as DC and B cells is also altered in NOD mice and humans [12-15] potentially contributing to impaired T-cell deletion [7]. Additionally, regulatory T (Treg) cell function is compromised [16, 17], through quantitative and qualitative changes [18-21] and decreased activation by APC [22, 23]. The effect of genetic alterations in pathways controlling peripheral tolerance induction on therapeutic approaches aimed at reinstating peripheral T-cell tolerance, however, remains undefined.

Targeting antigen expression to dendritic cells (DC) or other APC in adoptive transfer or inducible experimental models is robustly tolerogenic and induces peripheral tolerance in naïve and memory antigen-specific $CD8^+$ T cells [24-26] and has therapeutic potential. Most

studies, however, have focused on foreign or model antigens and high-affinity T cells [24, 26, 27]. Here we used adoptive transfer of $CD8^+$ T cells expressing a physiologically-relevant intermediate affinity TCR recognizing insulin B_{15-23} [28] to compare the outcome of activation in response to (pro)insulin expressed physiologically or transgenically. In response to physiologically-expressed (pro)insulin, insulin-specific $CD8^+$ T cells preferentially accumulated in pLN and developed characteristics of effector $CD8^+$ T cells consistent with a propensity for tolerance defects and development of autoimmunity. In contrast, when proinsulin expression was enforced in APC, insulin-specific $CD8^+$ T cells were abortively activated and proliferation was followed by deletion of most cells. Residual undeleted insulin-specific $CD8^+$ were rendered unresponsive and this was associated with TCR-downregulation and expression of a range of co-inhibitory molecules typical of 'exhausted' T cells. Accumulation of insulin-specific T cells in pLN was substantially inhibited.

RESULTS

G9 T cells respond to PI transgene-derived determinants

Insulin-specific G9 $CD8^+$ T cells develop, undergo thymic selection and are maintained in the periphery of mice naturally expressing cognate antigen [28] which could influence their activation state. Therefore, G9 T cells were examined for signs of antigen-induced activation. G9 T cells ($CD8^+V\beta6^+$) and the analogous $CD8^+V\beta6^+$ T-cell population from wild-type NOD mice showed similar TCR Vβ6 staining (**Supporting Information Fig. 1A,B**) and specific staining with K^d-insB_{15-23} tetramer **(Supporting Information Fig 1C,D)**. Based on CD44, CD62L (**Supporting Information Fig. 1E**) and CD69 (not shown) there was no indication of increased accumulation of activated or post-activated $CD8^+$ T cells in G9 mice. G9 T cells exhibited a typical naïve phenotype and were therefore deemed suitable for studies requiring naïve T cells.

To determine the location, extent and specificity of G9 T-cell activation by endogenous or transgenically-expressed (pro)insulin, CFSE-labelled G9 T cells were transferred to B16 mice which lack the G9 T-cell determinant, non-Tg NOD/ShiLtJ (non-Tg) and proinsulin-Tg (PI-Tg) recipients. Three days after transfer and as expected, G9 T cells showed little evidence of division in B16 recipients (**Fig. 1A, gating shown in Supporting Information Fig. 2A**). Similar results were found for spleen and skin-draining lymph nodes (sdLN) of non-Tg NOD recipients, but in pLN approximately 20% of G9 T cells (**Fig. 1A**) showed evidence of division ($p<0.001$ compared to NOD spl, NOD sdLN). Proliferation was limited, with G9 T cells having undergone only one or two divisions (**Fig. 1A**). In contrast, in spleen, sdLN and pLN of PI-Tg recipients almost all G9 T cells had divided several times (**Fig. 1A**). Quantitative comparison confirmed division was much more extensive in PI-Tg than non-Tg recipients (**Fig. 1B**). The data demonstrate functional presentation of transgene-derived T-cell determinants in PI-Tg mice.

APC-expressed proinsulin deletes G9 T cells

We next analysed the population kinetics of G9 T cells after transfer. In non-Tg recipients, G9 T cells were distributed throughout all lymphoid tissues examined (spleen, sdLN and pLN) within 1 to 2 days of transfer and formed stable populations that did not significantly change in number over the time-points analysed (**Fig. 1C, gating shown in Supporting Information Fig. 2B**). In non-Tg recipients, the total number of G9 in each lymphoid site examined reflected total lymphoid organ cellularity (**Fig. 1C**). In PI-Tg recipients, the population of G9 T cells in spleen expanded rapidly between 1 and 2 days after transfer ($8.5\pm4.2\text{x}10^3$ vs $28.6\pm6.7\text{x}10^3$ [mean±SD], $p<0.05$, **Fig. 1C**), exceeding the number in non-Tg recipients at day 2 ($12.6\pm7.1\text{x}10^3$). The number of G9 T cells then declined significantly to very low levels (e.g. $2.3\pm1.5\text{x}10^3$ at day 7, d2>d3 $p<0.01$; d2&d3>d5&d7 $p<0.01$). Similarly, in sdLN, G9 T cells accumulated transiently, peaking in number ($2.5\pm0.5\text{x}10^3$) 3

days after transfer (d3>d1, $p<0.01$) and then declining to low levels (e.g $1.4\pm1.0x10^3$ at day 7, d3> d5&d7 $p<0.01$). In sdLN, G9 T cells were most numerous in non-Tg recipients (**Fig. 1C**) despite the substantial proliferation in this site in PI-Tg recipients (**Fig. 1A**). Few G9 T cells accumulated in pLN of PI-Tg recipients (**Fig. 1C**) and, overall, G9 T cells were substantially more numerous in pLN of non-Tg recipients ($p<0.01$ at d1,3,5,7). To compare accumulation of G9 independently of total cellularity, their frequency as a proportion of the total $CD8^+$ T cell pool in each lymphoid site was also calculated. This revealed that G9 T cells selectively accumulated in pLN (e.g. 0.4±0.1% of total $CD8^+$ at day 3) compared to the spleen and sdLN of non-Tg recipients (e.g. 0.2±0.1% or 0.2±0.1% of total CD8+ at day 3 respectively, $p<0.01$; d3,5,7) (**Supporting Information Fig. 3**). In PI-Tg recipients, G9 accumulation relative to the total $CD8^+$ pool reflected their absolute number and there was no enrichment of G9 T cells in pLN of PI-Tg recipients (**Supporting Information Fig. 3**). Therefore, transgenic expression of proinsulin limited accumulation of G9 T cells in pLN that was otherwise prominent in non-Tg wild-type NOD recipients.

Transgenic proinsulin induces G9 T-cell unresponsiveness

Population kinetics indicated that G9 T cells were largely deleted in PI-Tg recipients but a small residual population remained. The residual G9 T cells were likely rendered unresponsive, but to formally test this non-Tg and PI-Tg recipients of G9 T cells were immunised or not with $insB_{15\text{-}23}$/polyI:C 7 days after transfer and the response of G9 T cells analysed. Immunisation led to a significant expansion of the G9 T-cell population in the spleen of non-Tg recipients, whereas in PI-Tg recipients the G9 T-cell population was small and no expansion was detected **(Fig. 2A).** This indicated G9 T cells were 'inactivated' and had lost the capacity to proliferate in response to immunogenic challenge in PI-Tg mice. To determine whether inactivation extended to effector cytokine production, the ability to produce IFN-γ was tested. ELISpot assays revealed that cells producing IFN-γ in response to

insB$_{15-23}$ restimulation were present in spleens of non-Tg recipients of G9 cells, but not no-transfer control mice (**Fig. 2B**). Immunisation in this setting did not lead to an overall increase in the frequency of IFN-γ-producing insB$_{15-23}$-responsive cells in non-Tg recipients (11.0±8.7 v 9.8±2.7 unimm. vs imm.) suggesting these cells may have already been antigen-activated by encounter with physiologically-expressed (pro)insulin. Significantly, few insB$_{15-23}$-responsive, IFN-γ-producing cells were detected in PI-Tg recipients regardless of immunisation (0.7±1.4 vs 1.0±2.2, unimm. vs imm.). Overall, the frequency did not differ from no-transfer controls indicating that those few residual G9 T cells in PI-Tg recipients had been rendered incapable of producing this important effector cytokine.

Transgenic proinsulin down modulates G9 TCR expression

In settings where antigen is widely expressed, cognate TCR down-regulation occurs as a component of T-cell inactivation [29, 30]. Contraction of the G9 population in PI-Tg recipients along with their lack of responsiveness indicated deletion and inactivation was occurring, so we determined whether TCR downregulation contributed. For these studies, TCR Vβ6 expression by G9 T cells was normalised to that of recipient CD8$^+$Vβ6$^+$ T cells. Overall, there was a slight trend to reduced expression of TCR Vβ6 expression by G9 T cells in spleen and sdLN of non-Tg recipients (**Fig 3A,B**), but this was significant only in spleen at day 7 (1.04±0.05 vs 0.74±0.25, d3 vs d7, $p<0.05$). In pLN of non-Tg recipients, no such trend was observed and, notably, G9 TCR Vβ6 expression was higher than in spleen and sdLN (0.95±0.12 vs 0.74±0.25, 0.84±0.12 respectively, $p<0.001$) 7 days after transfer. In PI-Tg recipients, TCR Vβ6 expression was significantly reduced in spleen, sdLN and pLN at all time points compared to non-Tg recipients ($p<0.001$ except spleen day 2 and pLN day 1 where $p<0.05$). Therefore the outcome of antigen presentation to G9 T cells in non-Tg pLN differs not only to that in spleen and sdLN of non-Tg mice but also pLN and other lymphoid tissues of PI-Tg mice.

As TCR downregulation could inhibit the capacity of G9 T cells to bind cognate antigen we probed antigen recognition using K^d-insB_{15-23} tetramer. Prior to transfer, almost all $CD8^+V\beta6^+$ G9 T cells bound K^d-insB_{15-23} tetramer mostly at uniformly high levels (**Supporting Information Fig. 1)**. Three days after transfer, the majority of G9 T cells in non-Tg recipients bound high levels of K^d-insB_{15-23} tetramer (**Fig. 4A,B**) whereas far fewer G9 T cells in PI-Tg recipients bound K^d-insB_{15-23} tetramer at similarly high levels (**Fig. 4A,B**). Overall, this was reflected in a reduced MFI which correlated with reduced TCR Vβ6 expression (**Fig. 4C**). Accordingly, lymphoid organs of PI-Tg recipients contained considerably fewer K^d-insB_{15-23} $tetramer^{hi}$ cells (**Fig. 4D**). In non-Tg recipients where G9 T cells were enriched within $CD8^+$ T cells of pLN compared to other lymphoid tissues (**Fig. 1, Supporting Information Fig 3**) and TCR expression was higher (**Fig. 3B**), G9 T cells stained more intensely with K^d-insB_{15-23} tetramer in pLN (MFI: 3456±266) than sdLN (MFI: 2543±247; $p<0.01$) and spleen (MFI: 2607±255; $p<0.05$) (**Fig. 4C**). No such stratification was observed in PI-Tg recipients (**Fig. 4C**). TCR downregulation and loss of tetramer binding in PI-Tg recipients is not merely a consequence of antigen sensing *per se* because antigen recognition also occurs in pLN of non-Tg recipients, so must therefore reflect the unique nature of (pro)insulin presentation in PI-Tg mice. Indeed, this suggests that activation of G9 T cells by physiologically-expressed (pro)insulin in non-Tg, wild-type NOD and transgenically-expressed (pro)insulin in PI-Tg mice leads to distinct functional consequences for G9 T cells .

Effector differentiation of G9 T cells in non-Tg NOD mice

We investigated the nature of G9 T cells that accumulated in pLN of non-Tg recipients. The early activation marker CD69 showed that G9 T cells were activated in the pLN but not spleen or sdLN of non-Tg recipients (**Fig. 5A**) consistent with CFSE dilution (**Fig. 1**) and CD69 expression was sustained for at least 7 days (e.g 31.4±13.7% vs 4.9±1.3%, 5.7±2.3%

pLN vs spl, sdLN at day 7, $p<0.01$ or greater relative to spleen, sdLN). In PI-Tg recipients, a high proportion of G9 T cells initially expressed CD69 in all sites examined but this diminished such that by 7 days after transfer the proportion was significantly reduced ($p<0.05$ compared to d1,2, 3 for spleen, sdLN) (**Fig. 5A**). Analysis of CD44 expression showed that post-activated $CD44^{hi}$ G9 accumulated to the greatest extent in PI-Tg mice but also in pLN of non-Tg recipients (**Supporting Information Fig. 4**). $CD62L^{lo}$ 'effector' phenotype cells were prevalent within the $CD44^{hi}$ population in the pLN of non-Tg recipients (**Fig. 5B**) but not in PI-Tg recipients where they waned with time (**Fig. 5B**). Numerically, G9 $CD44^{hi}CD62L^{lo}$ effector T cells were highly abundant in pLN of non-Tg recipients compared to PI-Tg recipients and were significantly overrepresented compared to spleen (**Fig. 5C,** 0.60 ± 0.05 $x10^3$ vs 0.10 ± 0.04 $x10^3$, $p<0.001$). Similarly, analysis of CD127 which typifies memory T cells and their precursors and KLRG1 that typifies terminally-differentiated effector T cells revealed that terminal effector differentiation of G9 T cells was apparent in pLN of non-Tg recipients (**Fig. 5D**). This was further reflected by the reduced proportion of $CD127^{+}KLRG1^{-}$ memory-phenotype G9 T cells in pLN of non-Tg recipients (**Fig 5D**). No differences in these cell subsets in spleen between non-Tg and PI-Tg recipients were apparent indicating a localised effect. These detailed analyses reveal effector differentiation of G9 T cells in pLN of non-Tg recipients, but not in this or other sites of PI-Tg counterparts where tolerance appears to predominate.

Expression of co-inhibitory receptors by G9 T cells in PI-Tg NOD mice

Expression of co-inhibitory receptors is a useful means to define the functional properties of T cells, and here, might provide useful insights into the mechanisms of unresponsiveness. The co-inhibitory molecule PD-1, a key indicator of antigen sensing, and in some instances, T-cell exhaustion, was highly expressed on a substantial proportion of G9 T cells in spleen, pLN (**Fig. 6A, Supporting Information Fig. 5**) and sdLN (**Supporting Information Fig 5**)

of PI-Tg recipients. Other key indicators of 'exhaustion' CD160, LAG3 (**Fig 6B,C, Supporting Information Fig. 5**) and to a lesser extent CD244 (**Supporting Information Fig. 5**) were all co-expressed by PD-1$^+$ G9 T cells in PI-Tg recipients, but Tim-3 was not (not shown). These markers were largely absent from G9 T cells in spleen of non-Tg recipients. Notably, in pLN of non-Tg recipients a moderate portion (26.9±6.8%) of G9 T cells expressed elevated levels of PD-1 (**Fig. 6A,D, Supporting Information Fig. 5**), consistent with antigen-sensing and activation of G9 T cells in this site. Interestingly, in pLN, CD160, LAG3 and CD244 were not significantly increased relative to spleen for G9 in non-Tg recipients indicating that expression of CD160, LAG3 and CD244 was apparently not linked with that of PD-1 and suggesting that although sensing antigen, these cells were not 'exhausted'. CD5, which is a reliable reporter of antigen-sensing [31], was elevated on most PD-1$^+$ G9 T cells in PI-Tg recipients (**Fig. 6G, Supporting Information Fig. 5**). Some G9 T cells in pLN of non-Tg recipients co-expressed PD-1 and high levels of CD5 (**Fig. 6H, Supporting Information Fig. 5**), although remarkably, most showed high levels of CD5 expression equivalent to G9 T cells in PI-Tg recipients (**Fig. 6I, Supporting Information Fig. 5**) but without the high levels of PD-1 observed in PI-Tg recipients (**Fig. 6 D,H, Supporting Information Fig. 5**). Overall, G9 T cells in pLN of non-Tg recipients appear to have sensed antigen, divided weakly and undergone an activation process that leads to effector differentiation with little co-inhibitory molecule expression. In contrast in PI-Tg mice, this process is blocked.

DISCUSSION

Insulin is an important β cell autoantigen and is a target of immune responses early in development of T1D in humans. Insulin or its prohormonal precursor proinsulin has been implicated as the initial target of anti-β cell T-cell responses in NOD mice. Understanding how T-cell responses to this critical antigen unfold in autoimmune diabetes-prone mice and

how insulin-specific T-cell tolerance might be promoted, in order to prevent disease initiation is crucial to development of effective preventative therapies. Here we show that enforced expression of proinsulin, targeted to MHC class II^+ APC leads to effective tolerance induction in insulin-specific $CD8^+$ T cells which is unimpeded by diabetes-promoting tolerance defects in NOD mice.

Much of the understanding of peripheral T-cell tolerance is formulated from models employing highly-immunogenic neoantigens in conjunction with high-affinity TCR Tg T cells. TCR/ligand affinity influences the outcome of T-cell activation through, for example, T-cell/DC interaction times and effector versus memory differentiation programs, T-cell polarisation and differential regulation of cell death pathways. However, less is known of whether low or intermediate affinity T cells are as susceptible to peripheral tolerance induction as those with high affinity TCR and whether affinity influences the mechanisms of tolerance induction. Some evidence suggests that high affinity TCR signals might lead to 'anergy' rather than deletion [32]. The G9 T cells used here express an intermediate affinity TCR recognizing $K^d/insB_{15\text{-}23}$ taken from a $CD8^+$ T cell cloned from the islet infiltrate of young prediabetic mice [28]. Our data demonstrate low/intermediate affinity $CD8^+$ T cells specific for an endogenous islet antigen are highly susceptible to peripheral tolerance induction when cognate antigen is expressed by APC. Furthermore, the kinetics of tolerance induction and the apparent mechanisms (deletion/TCR downregulation) appear remarkably similar to that observed where TCR affinity is $\sim 10^4$-fold higher [29]. In both these cases, cognate antigen was expressed under an MHC class II promoter. Under these conditions, antigen is expressed in a diverse range of APC and at high abundance which appears to underlie the potent tolerogenicity [29] and suggests that 'antigen dose' rather than affinity could be crucial for determining the outcome of tolerance. Indeed other studies using G9 T cells indicate that responses of insulin-specific CTL in wild-type NOD mice are modulated

by the level of endogenous proinsulin expressed in these mice [33]. Our data indicate that enforcing expression of higher levels of proinsulin, here targeted to APC, which presumably allows maximal tolerogenic effectiveness and ablates the functional proinsulin-reactive T-cell repertoire. Whether targeting expression to APC is crucial or whether other approaches such as plasmid vaccines might be equally effective is yet to be determined. These studies show that naïve insulin-specific $CD8^+$ T cells are highly susceptible to tolerance induction. While this models an intervention that might be applied before the onset of pre-diabetes, the clinical challenge is understanding if responses can be controlled once T-cell priming has occurred, i.e. in memory T cells. This is a question we are actively pursuing.

After adoptive transfer, extensive proliferation of G9 T cells was elicited by APC-expressed proinsulin that was followed by deletion. G9 T cells acquired a range of phenotypic characteristics associated with peripheral tolerance including almost uniform expression of high levels of CD5, down-regulation of TCR and up-regulation of a range of co-inhibitory molecules including PD-1, CD160, LAG-3 and CD244. Notably also, while transiently expressing CD69, G9 T cells reverted mostly to a $CD69^{lo}$ phenotype and converted from a naïve to central memory phenotype. We interpret that this reflects the widespread expression and presentation of transgenic proinsulin. In the absence of signals such as co-stimulation, IL-12 or type I IFN that are required for upregulation of anti-apoptotic molecules or to prevent TCR downregulation in $CD8^+$ effector differentiation during pathogen-associated responses, this leads to tolerance induction. In wild-type, non-Tg NOD mice, CD69 expression, upregulation of CD5, which is a reliable reporter of cognate TCR ligation [31], and CD44 upregulation all indicated antigen-sensing by a substantial proportion of G9 T cells in pLN but not other sites. Despite this, only a small proportion of G9 had proliferated, mostly only 1-2 divisions within 3 days of transfer. Overtly, this is consistent with a much lower level of $insB_{15\text{-}23}$ presentation than in PI-Tg recipients. Interestingly, despite minimal

proliferation, antigen sensing led to acquisition of phenotypic characteristics by G9 T cells that were consistent with activation and effector differentiation, but without substantial upregulation of co-inhibitory molecules or an apparent increase in avidity based on increased tetramer binding. Whether this is a trait of NOD $CD8^+$ T cells or a consequence of low-affinity antigen recognition of low levels of antigen is as yet undetermined. It is interesting, however, to speculate that under these conditions, where low levels of antigen determinants are presented, local innate inflammation or endogenous danger-associated molecular patterns (DAMPs) are effective in promoting the $CD8^+$ effector differentiation we observed. Whether the rapid and extensive proliferation promoted by transgenic proinsulin is an essential prerequisite for peripheral tolerance is unclear, but is a widely-reported precursor to peripheral tolerance induction in vivo. The outcomes do, however, suggest that presentation of transgenically-expressed proinsulin might overcome local DAMPs or other signals that would otherwise promote effector differentiation when low levels of cognate antigen determinants are presented in pLN.

Expression of proinsulin by hematopoietic or other cells is an important modulator of insulin-specific T-cell responses. Transgenic expression of proinsulin prevents diabetes development in NOD mice [34, 35] through preventing the initial T-cell priming events that lead to determinant spreading and the recruitment of additional pathogenic antigen specificities [36]. For this preventative effect, timing is crucial and expression of proinsulin throughout gestation and until weaning is effective [37]. In a more physiological setting, normal expression of proinsulin regulates the responsiveness of insulin-specific $CD8^+$ T cells. Deficiency in proinsulin1 or proinsulin2 leads to an increased frequency of insulin B15-23-reactive $CD8^+$ T cells and deficiency in both promotes CTL activity of insulin-specific $CD8^+$ T cells [33, 38]. These effects may be mediated by central and or peripheral effects, but a substantial component appears to be mediated peripherally [33]. In this setting where a low

level of proinsulin is expressed, most likely in lymph node stromal cells or peripheral AIRE-expressing cells, the inhibitory effect appears greatest for higher avidity cells [33]. Whether a difference exists in the effects of enforced proinsulin expression on $CD8^+$ and $CD4^+$ T cells remains unclear. However, in other settings some induction of Treg cells occurs when antigen-specific $CD4^+$ T cells are present [39], although if deletion is highly effective[40] development/expansion of Treg cells is limited. Here, where proinsulin expression was enforced within MHC class II+ APC, T-cell exhaustion appeared to play a key role in maintaining the unresponsive state of those G9 T cells that persisted after initial antigen encounter when the majority of cells are deleted. From this it might be predicted that administration of anti-PD-1 antibodies would reverse unresponsiveness as reported in a similar setting by others [41]. However, in a similar model of tolerance induction by enforced antigen expression, PD-1 blockade more effectively promoted development of effector function during tolerance induction than reversal of unresponsiveness [42]. Extending this, it remains unclear whether precipitation of T1D by PD-1/PD-L1 blockade in humans [43] results either from reversal of tolerance or from promotion of effector function in spontaneously-activated islet-specific T cells, but this warrants clarification.

Genetic alterations reported to impair induction and maintenance of tolerance in NOD mice might impact on induction of peripheral tolerance through several pathways. Whether these pathways contribute to the propensity for G9 effector differentiation in pLN of non-Tg recipients would require additional analyses employing a range of congenic strains to identify the contributions of individual *Idd* loci. That G9 T cells were effectively tolerised when proinsulin was expressed in APC indicates that genetically-determined impairments that might limit peripheral tolerance induction are overcome when this approach is used. This is consistent with previous observations that peripheral tolerance induction by APC- or DC-targeted antigen in non-autoimmune strains of mice is not perturbed by the absence of

important immunoregulatory cells populations such as regulatory T cells [44] or NKT cells (unpublished). As the effectiveness of peripheral tolerance induction by APC-directed antigen expression is determined by the frequency of antigen-expressing APC [29] whether genetic alterations in NOD alter the sensitivity to peripheral tolerance might be revealed by studying the frequency of antigen-expression APC required for tolerance induction. Our unpublished preliminary data, however, suggest that the sensitivity is similar for NOD and non-autoimmune-prone mice.

Overall, an important observation here is that insulin-specific $CD8^+$ T cells are responsive to peripheral tolerance induction when antigen is over-expressed despite the demonstrated tolerance defects that are present in NOD mice. This demonstrates that not all forms of T-cell tolerance induction are negatively impacted by the genetic defect present in NOD mice and has important implications for the successful development of immunotherapeutic approaches to T1D and other T cell-mediated autoimmune diseases.

MATERIALS AND METHODS

Mice

G9C8 (G9) insulin $B_{15\text{-}23}$-specific TCR (Vα18, Vβ6) transgenic mice, mice expressing mouse proinsulin II under the control of an MHC class II ($I\text{-}E_\alpha^k$) promoter and NOD.CD45.2 congenic mice have all been described [28, 34, 45]. B16 mice lack both insulin 1 and insulin 2 but express mutated (B16A) preproinsulin [2]. NOD/ShiLtJArc ($CD45.1^+$) mice were purchased from the Animal Resources Facility (Perth, WA). Proinsulin transgenic (PI-Tg) and NOD/ShiLtJArc (non-Tg) mice were crossed to NOD.CD45.2 mice to obtain $CD45.1^+/CD45.2^+$ offspring. Young (6-8 week-old) male mice were studied to minimise the effects of islet inflammation. Mice were bred and/or housed under specific pathogen-free conditions at the PAH Biological Resources Facility (Brisbane, Australia), TRI Biological Resources Facility (Brisbane, Australia) or PACE Facility (Brisbane, Australia). Experiments

were approved by The University of Queensland Animal Ethics Committee (Projects 164/12,144/15).

Adoptive transfers

An overview of the adoptive transfers is shown in (**Supporting InformationFig 6**). For adoptive transfer of naïve G9 T cells, single cell suspensions were prepared from pooled axillary, brachial, inguinal and mesenteric lymph nodes (LN). In some experiments $CD8\beta^+$ G9 T cells were purified from pooled LN cells by high-speed cell sorting. G9 T cells ($5x10^6$ LN, approximating $2x10^6$ $CD8^+V\beta6^+$ T cells or 2 x 10^6 $CD8\beta^+$T cells) were injected i.v. (lateral tail vein). In some experiments LN cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as described [24] prior to transfer.

Flow cytometry, in vivo and in vitro assays

Antibodies were purchased from BioLegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or produced and conjugated in-house (antibodies used are listed in **Supporting Information Table 1**). PE-conjugated tetramers loaded with the $insB_{15-23}$(23V) with high affinity for H2-K^d [46] or listeriolysin $(LLO)_{91-99}$ were sourced from the NIH Tetramer Facility. Spleens, skin-draining LN (sdLN; pooled axillary, brachial, inguinal) and pancreatic LN (pLN) were harvested, disrupted by pressing through cell strainers (Falcon) and erythrocytes lysed with hypotonic NH_4Cl/Tris buffer (spleens only). Tetramer staining was as described [47]. 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 $CFSE^+CD8^+V\beta6^+$ cells. Cytomety was performed 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 cell number, cytometric bead-based counting assays were performed as described [45]. To test antigen-responsiveness some mice were immunised i.p. with $insB_{15-23}$/poly IC (50µg/100µg). ELISpot assays were

performed as described previously [24] using $insB_{15-23}$ (10µg/ml) stimulation. Data are presented as Δ spot forming cells (Δ SFC: # spots with stimulation - # spots in no stimulation control).

Statistical Analysis

Comparison of means was performed using Student t-test and multiple groups were compared using one-way ANOVA followed by Tukey post-test (GraphPad Prism).

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflicts of interest.

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FIGURE LEGENDS

Figure 1. Naive G9C8 T cells are deleted in proinsulin-transgenic recipients.

A,B) CFSE-labelled G9 LN cells were transferred to B16A, NOD or PI-Tg recipients and three days later CFSE dilution in CFSE-labelled $CD8^+$ T cells recovered from skin-draining LN (sdLN), pancreatic LN (pLN) or spleen was determined using flow cytometry. Representative histograms (**A**) show the percent divided for that sample along with mean ± SD proportion divided. Proliferation index is shown in (**B**). **C**) G9 T cells were transferred to congenically-distinct ($CD45.1^+/CD45.2^+$) NOD or PI-Tg recipients and as indicated G9 T cells ($CD45.1^+/CD45.2^-CD8^+/V\beta6^+$) enumerated. Data are representative of at least six individual mice from 2 experiments with 3 recipient mice per group per experiment (A), individual mice pooled from four independent experiments with 1-2 recipient mice per group per experiment (B) or pooled (C) from at least two independent experiments per timepoint with 2 recipient mice per group per experiment and show mean ±SD (n=4-8/group at each time point). ANOVA with Tukey's post-test, $p<0.05$ considered significant.

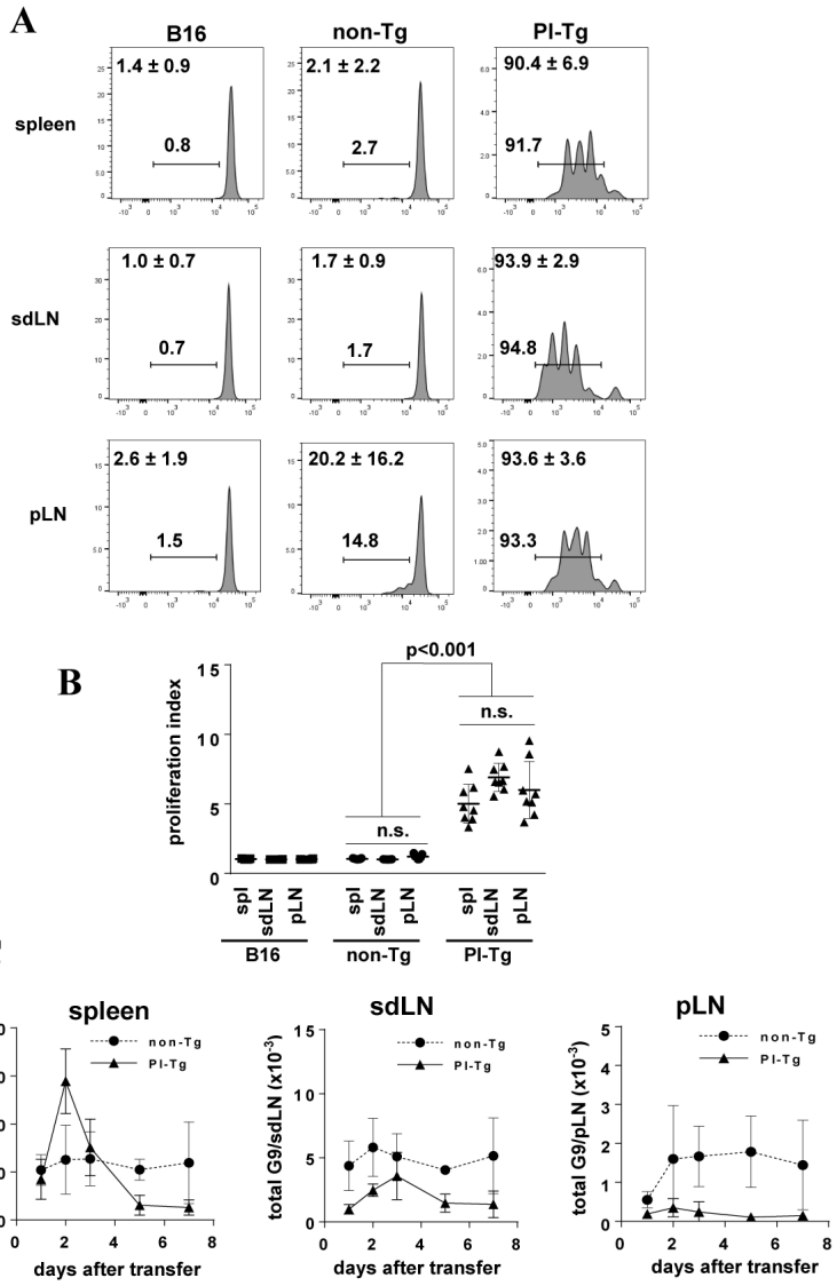


Figure 2. G9 T cells are rendered unresponsive in proinsulin-transgenic recipients.

(**A,B**) G9 LN cells (CD45.1$^+$) were transferred to congenically-distinct (CD45.1$^+$/CD45.2$^+$) NOD or PI-Tg recipients and seven days later recipients were immunised or not with insB$_{15\text{-}23}$/poly I:C. Five days later G9 T cells (CD45.1$^+$/CD45.2$^-$/CD8$^+$/Vβ6$^+$) were enumerated in spleen (**A**) and insB$_{15\text{-}23}$-responsive IFN-γ-producing cells assayed by ELISpot (**B**). Data show individual mice and mean ±SD (n=2-3 mice for untransferred controls, n=6 for unimmunised non-Tg, n=5 for unimmunised PI-Tg, n=7 for immunised non-Tg & PI-Tg, pooled from 2-3 independent experiments with 1-3 recipient mice per group per experiment). ANOVA with Tukey's post-test, $p<0.05$ considered significant.

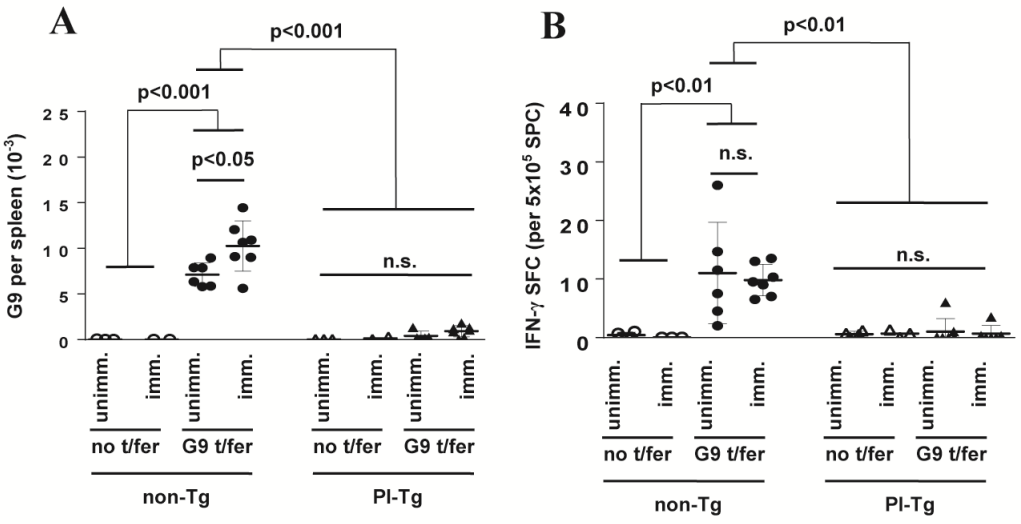


Figure 3. TCR expression on G9 T cells is reduced in proinsulin-transgenic recipients.

A,B) G9 T cells were transferred to non-Tg or PI-Tg recipients and seven days later (**A**) or at the indicated times (**B**) TCR Vβ6 expression was determined on G9 T cells (CD45.1+CD8+ Vβ6+) recovered from spleen, sdLN or PLN as indicated by flow cytometry and normalised relative to that of host $CD8^+$ Vβ6 T cells. Data are representative of 2-3 experiments with 2-3 mice per group per experiment FACS plots (A) or mean ± SD pooled from 2 or 3 independent experiments per timepoint with 2-3 recipient mice per group per experiment (n=4-9/group at each time point) (B). ANOVA with Tukey's post-test, $p<0.05$ was considered significant.

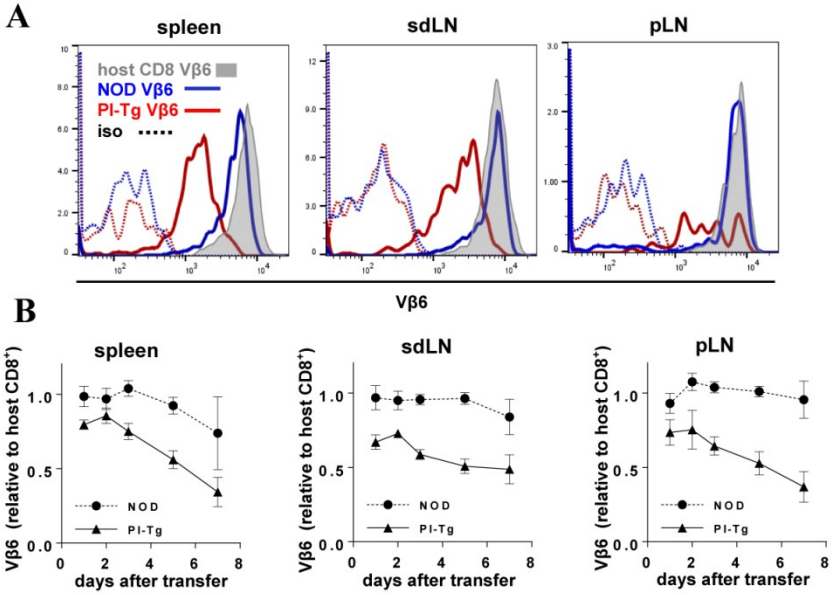


Figure 4. Tetramer binding by G9 T cells is reduced in proinsulin-Tg recipients.

A-D) Three days after transfer, G9 T cell ($CD45.1^+/CD45.2^-CD8^+/V\beta6^+$) Vβ6 expression and K^d-$insB_{15\text{-}23}$ binding was determined by flow cytometry. (**A**) K^d-$insB_{15\text{-}23}$-binding by G9 T cells (solid lines) or host PI-Tg $CD8^+$ $V\beta6^+$ T cells. Dotted line denotes the cut-off used to define K^d-$insB_{15\text{-}23}^{hi}$ T cells (**A**). The proportion and total number of K^d-$insB_{15\text{-}23}^{hi}$ G9 cells was calculated (**B,D**) and Vβ6 expression and K^d-$insB_{15\text{-}23}$-binding plotted (**C**). Data are representative (**A**) or show individual mice pooled from 2 experiments, with 3 mice per group per experiment (n=6 for all groups) (**B-D**). Bars show mean ± SD. ANOVA with Tukey's post-test or students t-test, $p<0.05$ was considered significant.

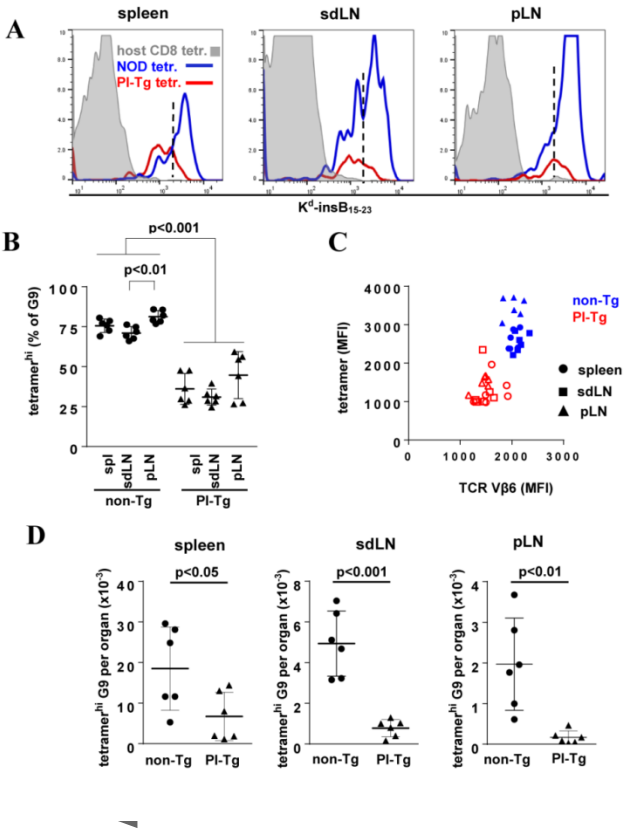


Figure 5. G9 T cells undergo effector differentiation in pLN of non-transgenic recipients.

(**A,B**) G9 LN cells ($CD45.1^+$) were transferred to congenically-distinct ($CD45.1^+/CD45.2^+$) non-Tg or PI-Tg recipients. As shown, the proportion of $CD69^+$ (**A**) and $CD44^{hi}/CD62L^{hi/lo}$ G9 T cells was determined. $CD62L^{lo}$ cells as a proportion of $CD44^{hi}$ G9 T cells (**B**) or total number of $CD44^{hi}/CD62L^{hi/lo}$ cells at day 7 were plotted. (**C**). Five days after transfer expression of markers defining memory and effector populations was determined (**D**). Data are mean ± SD (n=4-6) pooled from 2 or 3 more experiments (**A-C**) or 2 experiments (n=5-6) with 2-3 mice per group per experiment (**D**). ANOVA with Tukey's post-test or students t-test, $p<0.05$ was considered significant. *:$p<0.05$

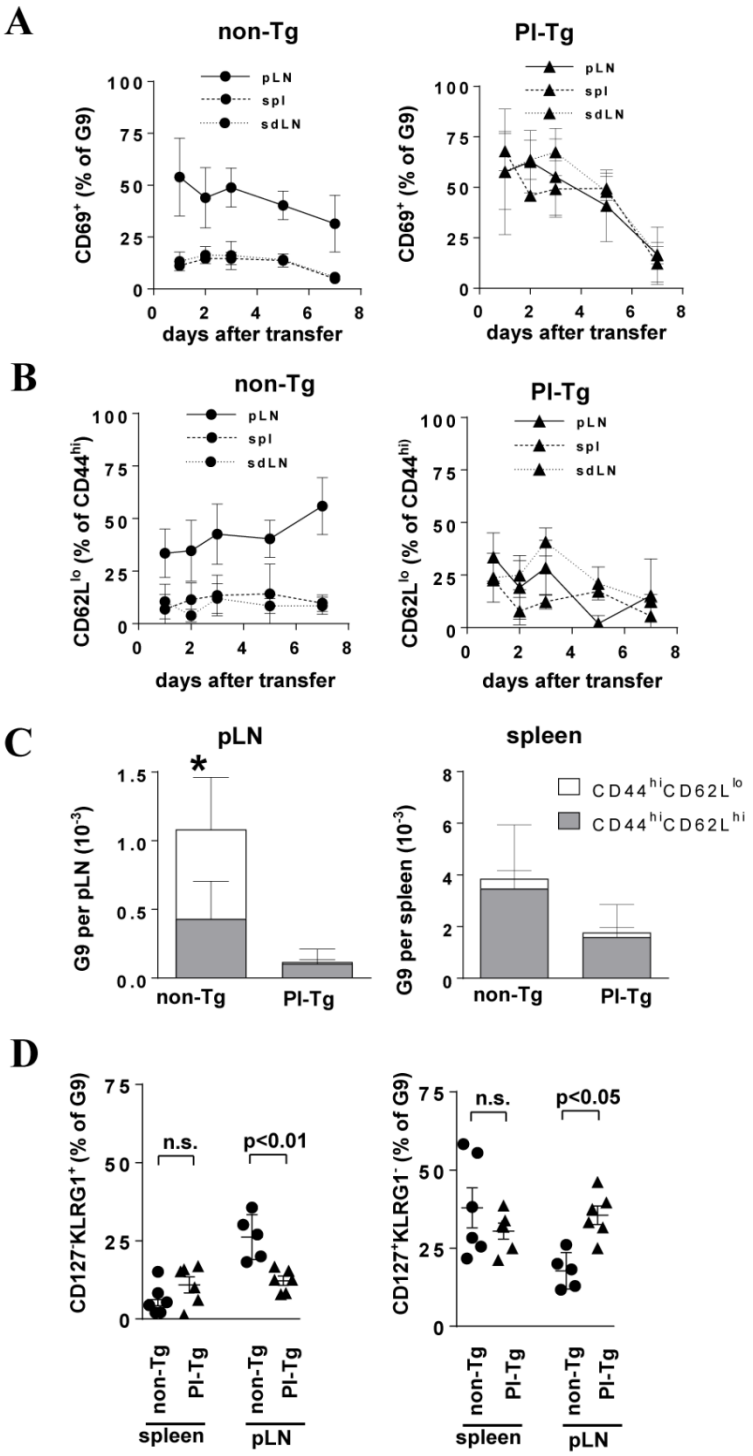
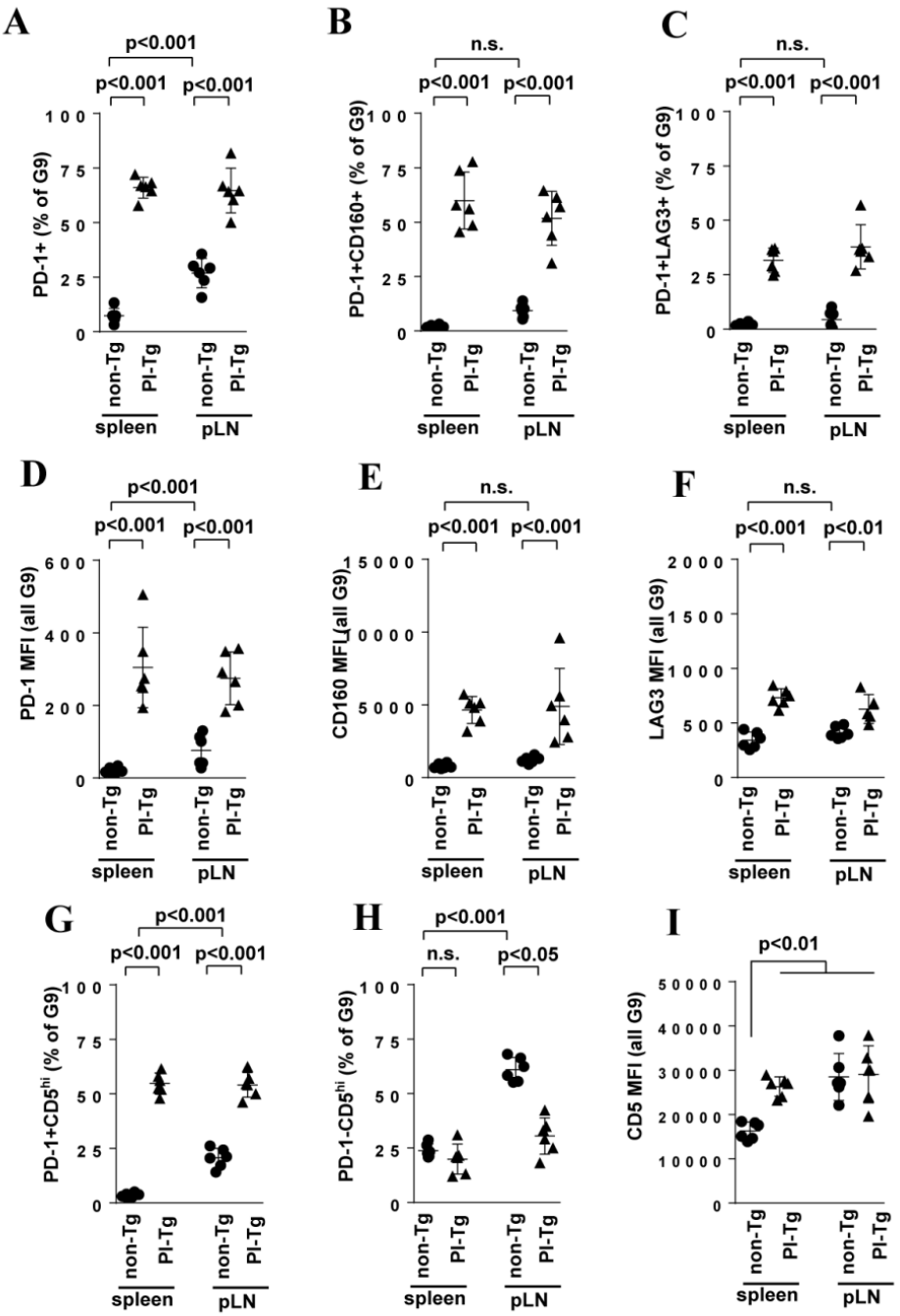


Figure 6. G9 T cells in PI-Tg recipients express elevated levels of co-inhibitory molecules.

(A-I) G9 LN cells 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 are show individual mice pooled from 2 experiments with 3 mice per group per experiment (n=6/group). Bars show mean ± SD. ANOVA with Tukey's post-test, $p<0.05$ was considered significant.



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