Peripheral proinsulin expression controls low avidity proinsulin-reactive CD8 T-cells in type 1 diabetes.

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Abstract

Low-avidity autoreactive CD8 T-cells (CTL) escape from thymic negative selection and peripheral tolerance mechanisms are essential for their regulation. We report the role of proinsulin (PI) expression on the development and activation of insulin-specific CTL, in the NOD mouse model of type 1 diabetes (T1D). We studied insulin B chain-specific CD8 T-cells from different T-cell receptor transgenic mice (G9C$\alpha^{-/-}$) expressing normal PI1 and PI2 or altered proinsulin expression levels. In the absence of PI2 (*Ins2*$^{-/-}$), CTL in pancreatic lymph nodes (PLN) were more activated and male G9C$\alpha^{-/-}$ mice developed T1D. Furthermore, when the insulin-specific CTL developed in transgenic mice lacking their specific PI epitope, the CTL demonstrated increased cytotoxicity and proliferation *in vitro* and *in vivo* in the PLN after adoptive transfer into NOD recipients. DC-stimulated proliferation of insulin-specific T-cells was reduced in the presence of lymph node stromal cells (LNSC) from NOD mice but not from mice lacking the PI epitope. Our study shows that LNSC regulate CTL activation and suggests that exposure to proinsulin in the periphery is very important in maintenance of tolerance of autoreactive T-cells. This is relevant for human T1D and has implications for the use of antigen-specific therapy in tolerance induction.

Type 1 diabetes is a multifactorial immune-mediated disease (1). In both humans and mice, CD8 T-cells play an important role in both early events and the final effector phases of diabetes development (reviewed in (2)).

Proinsulin (PI), the larger prohormone of the active hormone insulin, is targeted in the autoimmune attack in type 1 diabetes in both mouse and man (reviewed in (3)). Self-antigens, including proinsulin, are expressed in the thymus in mice and humans (4-6), controlled by Aire, and in *aire*$^{-/-}$ mice, PI is not detectable in the thymus (7). In humans, the second most important genetic susceptibility gene after the MHC (*Insulin Dependent Diabetes Mellitus2, IDDM*2), is the insulin 5'VNTR region, controlling expression of thymic and pancreatic PI (8). Mice express proinsulin 1 (PI1) and proinsulin 2 (PI2, homologous to human PI) differing by 2 amino acids (aa) in the B chain and 3aa in the C-peptide. While PI1 is expressed predominantly in the pancreas (4; 9), expression of PI1 in the thymus is much lower than PI2 (10). As PI2 expression is greater in the thymus, it is likely that PI2 influences central T cell tolerance to proinsulin.

However, PI-specific T-cells escape from thymic negative selection. In the NOD mouse, when PI2 was overexpressed in the thymus on the MHC class II promoter (PI2tg mice), diabetes incidence was decreased (11; 12). Interestingly, NOD*Ins1*$^{-/-}$ mice have reduced diabetes (13), whereas diabetes is accelerated in NOD*Ins2*$^{-/-}$ mice, with 100% developing disease (13; 14). PI2 is expressed in pancreatic lymph nodes (15), and is weakly expressed in lymph node stromal cells (LNSC) (16). Peripheral Aire-expressing cells in the secondary lymphoid organs also interact with autoreactive T-cells (7). These heterogeneous LNSC, which function in a similar manner to medullary thymic epithelial cells, may be very important in mediating peripheral tolerance (16). Peripheral tissue antigen expression, like PI2, is regulated by deformed epidermal autoregulatory factor (Deaf1) and its splice variants, and the expression of these factors also correlates with severity of disease in NOD mice (17; 18).

We have previously cloned highly diabetogenic CD8 T-cells (G9C8) from the islets of young pre-

diabetic NOD mice (19) which rapidly transferred diabetes to NOD and NOD.scid mice (2; 19). These G9C8 cells recognise insulin B chain, aa15-23, restricted by MHC class I K^d. In NOD mice, such T-cells infiltrate islets at 4 weeks of age (20; 21), representing a substantial proportion of the few cells present in the very early infiltrate. We believe that they are very important in the early events leading to diabetes. Interestingly, unlike CD8 T-cells recognising other islet autoantigens, the percentage of the insulin-reactive cells does not increase with time, and T-cells responsive to other auto-antigens, predominate later (22). The insulin B15-23 peptide, common to both mouse PI1 and PI2, binds poorly to K^d and mutation at position 16 from Y to A abolished the antigen recognition (23).

Both CD8 and CD4 T-cells recognising insulin have been implicated in pathogenesis of diabetes in mice and in humans (20; 24-26). The dominant CD8 epitope (B15-23) overlaps the CD4 epitope of insulin (B9-23) (20). Nakayama and colleagues showed that when both PI1 and 2 were knocked out and insulin expressing alanine at position B16 instead of tyrosine – designated Y16A - was substituted for the native insulin, the mutated mice (designated *Ins1*$^{-/-}$*Ins2*$^{-/-}$.Ins2*Y16A mice) were protected from diabetes. This mutation removes both the CD4 and CD8 epitopes in the B chain of insulin (25) but is metabolically active, thus preventing death from insulin deficiency.

Our study examines a central question - how is tolerance maintained to autoreactive T-cells that respond to low-affinity peptides? We tested the hypothesis that tolerance of insulin-reactive CD8 cells is influenced by the expression of PI, both centrally and peripherally. We developed model systems to directly study the effect of differing PI expression on the selection and reactivity, *in vivo*, of the insulin B15-23-reactive CD8 T cell. Using mice that express different levels of proinsulin, we found that decreased PI expression was associated with heightened CTL effector function and diabetes development, related to regulation by LNSC activity.

Material and Methods

Mice

Mice were housed in microisolators or scantainers in the specific pathogen-free facility at Cardiff University. All procedures were performed in accordance with protocols approved by the UK Home Office. NOD/Caj mice were originally obtained from Yale University. NOD*Ins2*$^{-/-}$, NOD*Ins1*$^{-/-}$, NODY16Atg mice were obtained from the Jackson Laboratory. NOD-PI2tg mice, with transgenic overexpression of proinsulin 2 (PI2) on the MHC class II promoter (11) were kindly provided by Prof L. Harrison. G9Cα$^{-/-}$ mice were generated as previously described (2). The mice with mutations in proinsulin genes were bred with TCRα and TCRβ lines generating G9Cα$^{-/-}$*Ins2*$^{-/-}$ and G9Cα$^{-/-}$*Ins1*$^{-/-}$*Ins2*$^{-/-}$ Y16A (G9Cα$^{-/-}$Y16A) mice (Table 1). The PI2tg was introduced in the TCRα line to generate G9Cα$^{-/-}$ PI2tg mice. C57BL/6 mice were purchased from Harlan (UK).

Diabetes incidence

Mice were monitored weekly for glycosuria (Bayer Diastix) from 6 weeks of age. Diabetes was diagnosed after two consecutive blood glucose levels above 13.9mM.

Flow cytometry

Lymphoid tissues including thymus, spleen, pancreatic (PLN), mesenteric (MLN), and para-aortic lymph nodes (ALN) were collected from age- and gender-matched G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, and G9Cα$^{-/-}$Y16A. Lymphocytes were labelled with antibodies against CD4 (GK1.5,) CD8 (53-6.7), CD62L (MEL14), CD69 (H1.2F3) all from Biolegend, Vβ6 (RR47, BD), and live/dead exclusion (eBioscience). Regulatory T-cells (Tregs) were identified as CD4, CD25 (ebio3C7) and Foxp3 (FJK-16s, eBioscience) positive. Stromal cells were labelled with CD45 (30-F11), CD31, and gp38/podoplanin (Biolegend) CD11c (BD), UEA-1 (Vector). Flow cytometric samples were collected using the BD Canto II and analysed with FlowJo software v7.6.5 (Treestar Inc.).

***In vitro* T cell proliferation assays**

For proliferation against islets, splenic CD8 T-cells from G9Cα$^{-/-}$ mice were selected using MACS CD8 microbeads (Miltenyi). Islets from TCRCα$^{-/-}$ or Y16A mice were isolated (27; 28), trypsinized and irradiated (4000 Rad); 20 islets were cultured with 10^5 CD8 T-cells for 4 days, before overnight pulse with ^{3}H-thymidine. Proliferation (CPM) was determined using the Microbeta2 Plate counter (PerkinElmer).

For proliferation assays against insulin B15-23 peptide (LYLVCGERG), PLN were collected from age- and gender-matched mice. Lymphocytes were labelled with 0.5μM CFDA SE (Molecular Probes) following the manufacturer's protocol. Cells were stimulated with a range (0-4μg/mL) of peptide for 72 hours at 37°C and 5% CO_2. After stimulation, cells were labelled with anti-CD8, anti-CD69, and live/dead exclusion. Proliferation and activation of CD8 lymphocytes were analysed by flow cytometry.

***In vivo* T cell proliferation assay**

Splenic CD8 T-cells from age- and gender-matched G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, and G9Cα$^{-/-}$Y16A mice were selected using MACS CD8 microbeads (Miltenyi) and labelled with 2μM CFDA-SE (Molecular Probes) following the manufacturers' protocols. Labelled cells, (10^7), were transferred intravenously to 6-week-old female NOD recipients. After 4 days, recipient spleen and LNs were collected, cells were labelled with CD8, CD69, and live/dead exclusion, and analysed for proliferation and activation by flow cytometry as above.

CD8 T cell cytotoxicity assay

Splenic CD8 T-cells were positively selected from age- and gender-matched G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, and G9Cα$^{-/-}$Y16A mice. Thymocytes were incubated with plate-bound anti-CD4 (GK1.5, Biolegend) for 1 hour at 4°C. Non-adherenT-cells were collected and single positive CD8 T-cells were magnetically sorted. P815 cells were labelled with PKH-26 (Sigma) following the manufacturer's instructions. CTLs

were incubated with peptide-loaded P815 cells for 16-18 hours at 37°C and labelled with TOPRO-3 (Molecular Probes) immediately before flow cytometric analysis to identify dead cells and determine specific lysis of target P815 cells (2).

T cell Receptor Surface Expression

TCR expression levels were compared in age- and gender-matched G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, and G9Cα$^{-/-}$ Y16A mice. CD8 T-cells were positively selected from PLN and spleens. Lipopolysaccharide (LPS)-activated DCs from NOD mice were used to stimulate CD8 T-cells with a range (0-4 μg/mL) of insulin peptide B15-23 (LYLVEGERG) over 72 hours. Cells were labelled with CD4, CD8, Vβ6, and live/dead exclusion. Mean fluorescence intensity (MFI) of TCR was analysed by flow cytometry.

LNSC preparation and culture

LNSC were prepared from NODCα$^{-/-}$ (WT), NOD*Ins1-/-*, NOD*Ins2-/-*, Y16A, and C57BL/6 mice. PLN were pooled from 18-20 mice and teased open using a 30g needle. Tissue was digested with 0.5mg/ml Dispase II (Sigma), 0.5mg/ml collagenase P (Roche), and 100U/ml DNase I (Roche) in RPMI-1640. Samples were digested at 37°C in a shaking water bath for 5-minute intervals and tissue debris was allowed to settle and the supernatant containing single cells was collected (29). Hematopoietic cells were removed using complement depletion following the manufacturer's protocol. Briefly, cells were labelled with purified anti-CD45 for 45min at 4°C. After washing, cells were incubated for 60min at 37°C in Low-Tox®-M Rabbit Complement (Cedar Lane), then plated at 2 million cells/well in 6-well plates in RPMI-1640 with 10% FBS. Non-adherenT-cells were removed after 48 hours and stromal cells were grown at 37°C, 5% CO_2 for 7-10 days.

T cell proliferation with DC and LNSC

Bone marrow DCs, from NOD-PI2tg mice, were grown in medium containing GM-CSF (1.5ng/ml) and stimulated overnight with LPS (1μg/ml). Splenocyte CD8 T-cells were negatively selected using MACS CD8 T cell-isolation kit II (Miltenyi) and labelled with 0.5μM CFDA. Pancreatic LNSC cells were collected from 6-well plates with 0.25% trypsin and seeded at 10^4cells/well in 96-well round-bottomed plates for 24-48 hours to allow adhesion. LPS-activated DCs ($2X10^5$/well) and CFDA-SE-labelled CD8 T-cells ($1X10^5$/well) were added to wells containing LNSC. T-cells were collected after 72 and 96 hours, labelled with anti-CD8, anti-CD69, and live/dead exclusion. Proliferation and activation of T-cells were analysed by flow cytometry as above.

Statistical analysis

Means were compared using One-way Anova with $p<0.05$ considered significant. Kaplan-Meier survival analysis was used to evaluate onset of diabetes (GraphPad Prism).

Results

Mouse model development

TCR transgenic mice expressing the clonotypic insulin-reactive G9C8 TCR on the NOD.TCRC$\alpha^{-/-}$ genetic background (2) were designated G9C$\alpha^{-/-}$ mice. G9C$\alpha^{-/-}$ mice were crossed to NOD*Ins2*$^{-/-}$ mice (14), to generate G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ mice. Thus, we studied cells that developed in the absence of PI2. In addition, we also crossed G9C$\alpha^{-/-}$ mice to the *Ins1*$^{-/-}$*Ins2*$^{-/-}$.Ins2*Y16A mice (25) to generate G9*Ins1*$^{-/-}$ *Ins2*$^{-/-}$.Ins2*Y16A mice (designated G9C$\alpha^{-/-}$Y16A mice) expressing only mutated insulin that cannot be recognized by the G9C8 T-cells. The loss of recognition of the insulin expressed in the *Ins1*$^{-/-}$*Ins2*$^{-/-}$ .Ins2*Y16A mice is shown in Supplementary Fig.1 with much reduced G9 CTL proliferation to Y16A islets compared to NOD islets. Finally, the G9C$\alpha^{-/-}$ mice were crossed to mice that expressed PI2 as a transgene on the MHC class II promoter (11). These mice were all bred in-house and are shown in

table1.

G9Cα$^{-/-}$ mice develop diabetes in the absence of PI2

Although G9Cα$^{-/-}$ mice do not develop spontaneous diabetes (2), here we found that in the absence of PI2, 25% of the male, but not female G9Cα$^{-/-}$*Ins2*$^{-/-}$ mice developed spontaneous diabetes, by 10 weeks of age (Fig.1). The diabetes incidence contrasts with wild-type male NOD mice where diabetes only occurs after 18 weeks in our colony. In previous studies, diabetes was accelerated in NOD*Ins2*$^{-/-}$ female mice compared to NOD mice (13; 14).

Insulin-reactive CD8 T-cells are selected in the thymus

To further investigate the selection of insulin-reactive CTL, we examined the development of CD8 T-cells in the thymus of G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, G9Cα$^{-/-}$Y16A mice by flow cytometry (Fig.2A-C). Overall, no significant differences were seen in the CD8 T cell frequency (Fig.2D), or in the total numbers of thymocytes (Fig.2E) and no differences between male and female mice were seen (data not shown). Specifically, neither lack of PI2, nor lack of the specific epitope recognized by the insulin-reactive T-cells altered thymic selection. Furthermore, no deletion of the CD8 thymocytes was seen in the G9Cα$^{-/-}$PI2tg mice that over-expressed PI2.

Insulin-reactive CD8 T-cells are deleted in the peripheral lymphoid tissues

We next studied G9 cells in the peripheral lymphoid tissues, focusing on the spleen, PLN, MLN and distant ALN. There were clear differences in the frequency of the G9 CD8 T-cells between the G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, and G9Cα$^{-/-}$Y16A mice (Fig.3) in the spleen and the different lymph nodes. Unlike the thymus, the percentage of the G9Cα$^{-/-}$PI2tg CD8 T-cells was considerably reduced in the periphery, suggesting that these cells had been deleted (Fig.3). As there were few G9 cells in the periphery of G9Cα$^{-/-}$PI2tg mice, these mice were not a focus for further analysis. Conversely, G9 cells were increased in the lymph nodes of the G9Cα$^{-/-}$*Ins2*$^{-/-}$ mice, which may reflect the fact that when *Ins2* is deficient, the

G9 cells are released from regulation, become activated and expand. In contrast, the spleen of the G9Cα$^{-/-}$Y16A mice was smaller as described for the original Y16 mice (25), possibly due to the metabolic requirement for insulin to maintain immune cells, in addition to the need for T-cells to recognise antigen for survival. Although the mutated insulin is metabolically active, it may be less efficient or not as highly expressed as native PI2.

Activation of insulin-reactive CD8 T-cells

To investigate maturation of the insulin-reactive CTL, G9 CD8 T-cells were tested for proliferation to insulin B15-23 peptide in culture. As indicated in Fig.4A, CFDA SE-labelled PLN CD8 T-cells from G9Cα$^{-/-}$Y16A mice proliferated more than cells from G9Cα$^{-/-}$*Ins2*$^{-/-}$ mice, which in turn had a greater response than the cells from G9Cα$^{-/-}$ mice. This result was recapitulated *in vivo* (Fig.4B) when the CFDA SE-labelled CD8 T-cells were transferred into 6-week old NOD female mice to test for proliferation to endogenously-presented antigen in the PLN as described previously (2). CD8 T-cells isolated from an environment lacking normal insulin expression exhibited the most reactivity to endogenous insulin.

Furthermore, we examined cytotoxicity of the G9 CD8 T-cells towards insulin-peptide-loaded targets. The CD8 T-cells from G9Cα$^{-/-}$Y16A mice exhibited greater cytotoxicity in response to the target antigenic peptide compared with the CD8 T-cells from G9Cα$^{-/-}$ or G9Cα$^{-/-}$*Ins2*$^{-/-}$ mice (Fig.4C), similar to the proliferative response. This was only seen with peripheral lymphoid cells - there was no difference in the cytotoxic capacity of single-positive CD8 thymocytes (Fig.4D), indicating that the cells acquired this difference in their activation after release from the thymus. Thus, peripheral, but not central, insulin-reactive CD8 T-cells from mice lacking their target antigenic epitope were more cytotoxic on encountering their cognate antigen.

Phenotype of peripheral G9 cells in different insulin-expressing environments

To further investigate the phenotype of G9 CD8 T-cells in the different lymphoid tissues, we assessed the cells in the PLN, MLN and ALN, testing for intrinsic differences in activation status. There was a significant reduction in CD62L expression in CD8 T-cells of the PLNs in the G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ mice (Fig.5A), indicating a greater shift in the balance of activated memory T-cells. In the G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ mice, although PI2 is not expressed, PI1 is still present in the mice and therefore, the cells will be exposed to endogenous antigen (the B15-23 epitope is identical in PI1 and PI2). This alteration in the CD62L expression was not seen in cells from the G9C$\alpha^{-/-}$Y16A mice, which lacked the antigenic epitope recognized by G9CD8 T-cells and therefore had no cognate antigenic stimulation in the periphery (Fig.5).

Altered activation of peripheral insulin-reactive T-cells is not related to TCR levels or presence of regulatory T-cells (Tregs)

To assess whether the different levels of activation of the G9 T-cells shown in Figs.4 and 5 were associated with TCR expression, we tested the baseline level TCR in the different strains. We found no overall difference in the levels of the clonotypic TCR, measured by Vβ6 monoclonal antibody staining, shown at day 0, which is the baseline before stimulation, in both the spleen and PLNs. Similarly, there were no significant differences overall, after stimulation of the cells with peptide (Fig.6A and B). No differences in the frequency of CD25+FoxP3+ Tregs indicated that altered frequencies of Tregs was not respsonsible for these differences (Fig.6C, D)

LNSC suppress proliferation of peripheral insulin-reactive T-cells.

As ectopic antigen expression in LNSC has been implicated in peripheral tolerance (7; 16; 30; 31), we tested the G9 T cell interactions with LNSC from mice with different levels of proinsulin expression. We extracted LNSC which, are a heterogeneous group of cells (7; 29; 32) that are CD45- and CD11c-, with

the majority identified as fibroblastic reticular cells (FRC), defined as $gp38^+$ (podoplanin) and $CD31^-$ (Fig.7A). We then investigated the effects of LNSC on G9 CD8 T cell proliferation. Our earlier experiments suggested that chronic exposure to PI in development influenced the baseline reactivity of the G9C8 CD8 T-cells (Fig.4). We hypothesized that expression of PI in LNSC influenced this reactivity. Thus, we tested proliferation of the G9 CD8 T-cells from each of the different proinsulin-expressing strains of mice in response to specific antigen, in the presence of pancreatic LNSC derived from each of the different proinsulin-expressing mouse strains.

To ensure that we were testing the effect of endogenous proinsulin in the LNSC, we stimulated G9C8 cells in the absence of exogenous peptide. For this, we used PI2 transgenic DCs that express proinsulin transgene in APCs and present the insulin peptide, removing any possibility of additional antigen processing of exogenous antigen by the LNSC. This allowed us to study the cumulative effects of DC stimulation and potential LNSC impact on T cell activity (Fig 7B). We showed that the proliferation of each of the insulin-reactive CD8 T-cells was inhibited in the presence of wild-type stromal cells that express both PI1 and PI2, or in the absence of either PI1 (from *Ins1*$^{-/-}$) or PI2 (from *Ins2*$^{-/-}$) alone (Fig.7C). There was no obvious inhibition by the stromal cells from the *Ins1*$^{-/-}$*Ins2*$^{-/-}$.Ins2*Y16A mice, where no proinsulin expressed was recognizable by the G9 CD8 T-cells. In addition, there was no obvious inhibition by LNSC extracted from C57BL/6 mice, which express K^b, an MHC class I molecule not recognized by G9 cells.

Discussion

We have studied the development and activation of low-avidity insulin-reactive CTLs (23) that become highly diabetogenic when fully activated. We show that, irrespective of the endogenous PI expression in the mice, the thymic CD8 T cell frequency and function was not different. However, in the periphery, although similar in number, the insulin-reactive CTLs demonstrated increased proliferative

and cytotoxic capacity when they developed in the absence of PI2 (G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ mice), and even more when cognate peptide was absent, in the Y16A mice. Furthermore, male G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ mice developed spontaneous autoimmune diabetes. Why this has occurred only in male mice is currently not known, but it is possible that the male mice may be more sensitive due to hormonal effects. Conversely, where PI2 was overexpressed, the G9 cells were deleted in the periphery. Pancreatic LNSC from mice expressing normal PI levels had inhibitory effects on insulin-reactive T cell proliferation and there was no inhibition by LNSC from mice expressing mutated proinsulin that the T-cells could not recognize. Our results indicate LNSC regulate the activation and function of insulin-reactive G9 T-cells.

We focused on these low-avidity insulin-reactive T-cells, a prevalent population in early insulitis (20; 33), that do not expand later in the disease process, as do IGRP-responsive T-cells (22; 34). In addition, diabetes requires proinsulin autoreactivity, even if responses to IGRP become dominant later on (35). We observed that in the G9C$\alpha^{-/-}$ mice, where the vast majority of the cells are G9 insulin-reactive CD8 T-cells, immunizing the mice with peptide and adjuvant induced diabetes (2). Furthermore, purified splenic G9 cells, once activated, can transfer diabetes very rapidly. This suggests that, under normal circumstances, in the absence of other cell specificities, tolerance is maintained and additional stimuli are required to raise the cells over the threshold of activation. Our current study demonstrates that when expression of PI2 was reduced, G9 cells proliferate more and are more potently cytotoxic both *in vitro* and *in vivo*.

How is this tolerance effected? Previous investigators have suggested that thymically-expressed insulin modulates insulin autoreactivity (10) and other evidence suggests that this is important for CD4 T-cells (11). PI2 is expressed in the thymus, whereas PI1 expressed at a lower/undetectable level (4-6). However, for these low-avidity CD8 T-cells, central tolerance does not play a role, as no differences in their frequency were seen in the thymus, nor in their cytotoxic potential, irrespective of whether PI

expression was increased or decreased. For these low-avidity CD8 T-cells, peripheral tolerance mechanisms are dominant and they do not cause diabetes.

Peripheral tolerance mechanisms include induction of ignorance, anergy, and deletion (reviewed in (36)) or regulation induced by Tregs. Here, tolerance was not related to frequency of Tregs. Although the expression level of TCR (37) or CD8 co-receptor (38) may contribute to CD8 T cell responsiveness including down-regulation on activation (37), this was not a mechanism that contributed to differences in responsiveness here either, as the levels of TCR were not higher in those cells that responded more to antigen.

Whilst DCs play a major role in determining activation or tolerance of peripheral T-cells, in recent years, ectopic expression of antigen in peripheral LNSC modulates immune function (39). A comprehensive study by Yip and colleagues (40) showed that PI2 is expressed in PLN. In this study, we demonstrated that in the absence of PI2, but in the presence of PI1, more CD8 T-cells in the PLN became effector memory cells, with a reduction in CD62L expression. This phenomenon was not seen in the G9C$\alpha^{-/-}$Y16A mice, in which, G9 cells are completely unable to target the endogenous insulin expressed. Therefore, the activation of the insulin-reactive CTLs requires not only release from tolerance mechanisms but also peripheral stimulation. The cells unable to recognise endogenous insulin may be released from inhibition, but they cannot recognize endogenous antigen, and thus do not differentiate into effector memory cells.

LNSC have been shown to induce tolerance to CD4 T-cells (15; 17) and are able to present antigen (31). We have demonstrated that the level of antigen expressed in these LNSC may be very important in determining the outcome of tolerance for autoreactive CTLs. In this case, the more PI antigen expressed, the more effective the tolerance exerted by the LNSC on the peripheral T-cells. When

the insulin-reactive CD8 T-cells were given the same antigenic stimulus, the reduced proliferation was greater in the presence of the LNSC derived from mice expressing more endogenous PI. Although the methods we used did not show direct antigen presentation by the LNSC to the T-cells, we only observed the inhibitory effect on G9 T-cells when MHC-matched stromal cells were used. This suggests that the tolerance is exerted by the LNSC directly, and relates to their ability to interact with the T-cells and present antigen as control LNSC expressing non-matched MHC had no suppressive effect.

Low affinity CD8 T-cells have also been found in humans (26; 41). The expression of proinsulin, controlled by an upstream variable nucleotide tandem repeat (VNTR) is an important genetic susceptibility factor, second only to the MHC (6; 8; 42). Humans have only a single proinsulin, unlike mice. Nevertheless, our mice that express varying levels of proinsulin may recapitulate some of the expression differences seen in humans. We suggest that, in individuals expressing different levels of proinsulin, there may be ectopically expressed antigen outside the islet beta cells, encoded by the VNTR, and a similar mechanism of tolerance may apply. In susceptible people who express IDDM2 class I VNTR that leads to increased susceptibility to type 1 diabetes, this may lead to increased activity of insulin-reactive T-cells as the peripheral tolerance mechanisms may be decreased. This remains to be tested in humans.

In conclusion, our studies have indicated that for low-avidity autoreactive CD8 T-cells, mechanisms of central tolerance causing negative selection are insufficient to remove such cells, even when antigen expression is increased. It is clear that peripheral tolerance is the main method by which such cells are controlled. Expression of self-antigen recognised by the T-cells in the peripheral lymph nodes, specifically in the stromal cells, plays an important role in reducing autoreactivity. Further understanding of critical tolerogenic pathways will be important in identifying how these mechanisms may be boosted for the development and application of immune-modulating therapeutics for T1D.

Author Contributions

TCT, JAP, EDL, SJH, JD, JB, AT, SA, PE, and LKS carried out experiments; TCT, JAP, EDL, SJH, LW, and FSW analyzed data; TCT and FSW wrote the manuscript; TCT, JAP, SJH, LW, and FSW edited the manuscript. The project was conceived by FSW, who assumes responsibility for the work.

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Author Disclosures

There are no competing financial conflicts of interest.

Prior Presentation

Parts of this study were presented in abstract form at the Diabetes UK Annual Professional Conference, 2011 and the Immunology of Diabetes Conference 2013.

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Genotype	T cell repertoire	Insulin expression	Diabetes
G9Cα$^{-/-}$	G9 TCR transgenic insulin-specific	Normal	No diabetes
G9Cα$^{-/-}$*Ins2*$^{-/-}$	G9 TCR transgenic insulin-specific	Proinsulin 2 deficient	Accelerated diabetes
G9Cα$^{-/-}$*Ins1*$^{-/-}$*Ins2*$^{-/-}$ Ins2*Y16A^{tg} (G9Cα$^{-/-}$ Y16A)	G9 TCR transgenic insulin-specific	Lack both native insulin genes, mutated insulin transgene positive	No diabetes
G9Cα$^{-/-}$PI2^{tg}	G9 TCR transgenic insulin-specific	Overexpression of insulin by APC	No diabetes
NODCα$^{-/-}$	αβT cell deficient	Normal	No diabetes
NOD*Ins1*$^{-/-}$	Polyclonal	Proinsulin 1 deficient	No diabetes
NOD*Ins2*$^{-/-}$	Polyclonal	Proinsulin 2 deficient	Accelerated diabetes
NOD*Ins1*$^{-/-}$*Ins2*$^{-/-}$ Ins2*Y16A^{tg} (Y16A)	Polyclonal	Lack both native insulin genes, mutated insulin transgene positive	No diabetes
NOD-PI2^{tg}	Polyclonal	Overexpression of insulin by APC	No diabetes
C57BL/6	Polyclonal	Normal	No diabetes

Table 1: List of transgenic and knock-out mice used

Figure legends

Figure 1: **Spontaneous development of autoimmune diabetes in G9Cα$^{-/-}$*Ins2*$^{-/-}$ male mice.** Mice were monitored for the onset of diabetes by weekly urinalysis and diabetes confirmed by blood glucose measurement. Diabetes was diagnosed after two positive urinalysis readings and blood glucose was confirmed above 13.9 mM. Only G9Cα$^{-/-}$Ins2$^{-/-}$ male mice (~25%) developed hyperglycemia (p<0.0071).

Figure 2: **Modified insulin expression does not decrease the development of G9C8 CD8^{+} T-cells in the thymus.** Representative thymic dot plots for (A) G9Cα$^{-/-}$, (B) G9Cα$^{-/-}$*Ins2*$^{-/-}$, (C) G9Cα$^{-/-}$Y16A are shown. (D) Thymocytes from 5-10 week old male mice were assessed for the frequency of SP CD8 cells comparing mice with varying levels of insulin expression. Total (D) thymocyte counts comparing G9Cα$^{-/-}$, G9Cα$^{-/-}$*Ins2*$^{-/-}$, G9Cα$^{-/-}$Y16A, and G9Cα$^{-/-}$PI2tg.

Figure 3: **Peripheral effects of insulin expression on the frequency of G9C8 CD8^{+} T-cells.** 5-10 week old male mice were evaluated for the frequency of CD8^{+} T-cells in (A, B) Spleen, (C) Pancreatic LN, (D) Mesenteric LN, and (E) Para-aortic LN. There was no significant elevation in CD8 T-cells in PLN that could contribute to increase autoimmune diabetes in the G9Cα$^{-/-}$*Ins2*$^{-/-}$ male mice. Overexpression of PI2 (G9Cα$^{-/-}$PI2^{tg}) resulted in significant deletion of insulin-reactive CD8^{+} T-cells. ***p<0.01

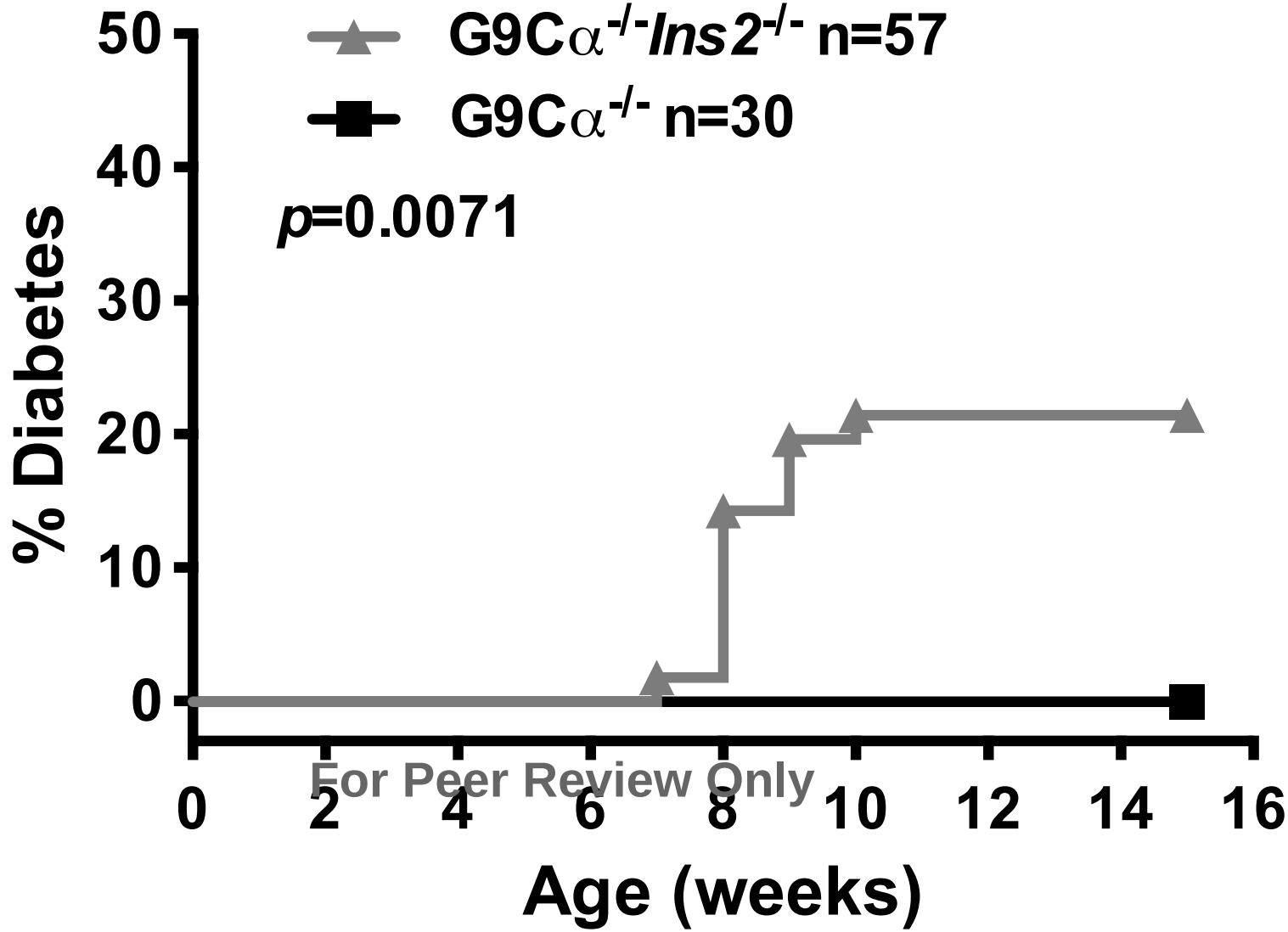
Figure 4: **Elevated responses of G9C8 CD8^{+} T-cells to peptide when insulin expression is reduced during development**. (A) Pancreatic lymphocytes were labelled with CFDA-SE (0.5 μM) and stimulated *in vitro* without (left panel) or with 2 μg/ml peptide (middle and right panels). Proliferation of CD8^{+} T-cells from G9Cα$^{-/-}$ (black), G9Cα$^{-/-}$*Ins2*$^{-/-}$ (red), and G9Cα$^{-/-}$Y16A (blue) was assessed by CFDA-SE dilution after 72 hours. Representative histograms of four independent experiments are shown. (B)

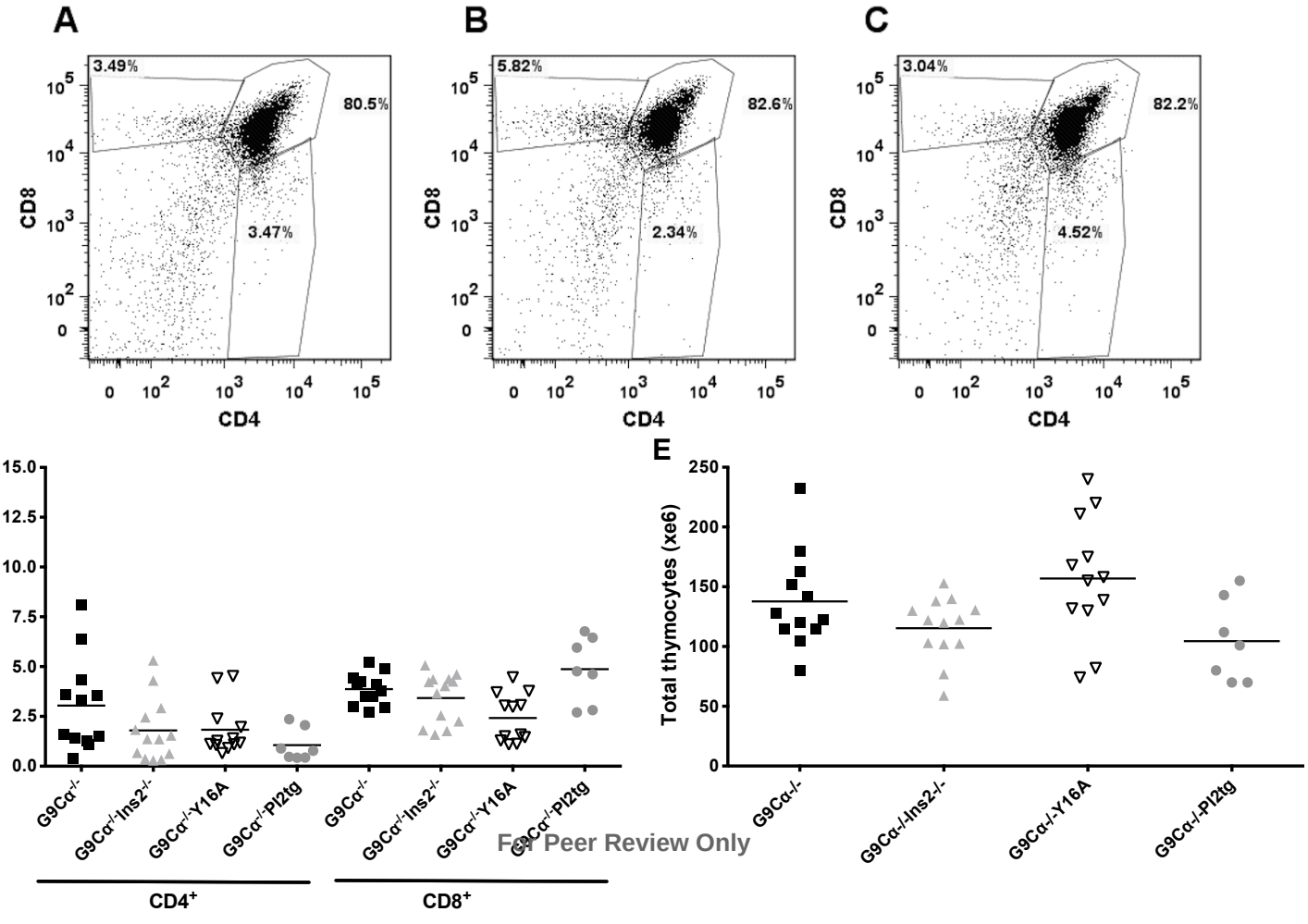
In vivo responses to insulin were assessed by adoptive transfer of CFDA-SE-labelled (2 μM) $1x10^7$ $CD8^+$ T-cells to 6-week old female NOD recipients. After 4 days, proliferation of transferred cells in the mesenteric LN (left panel) and pancreatic LN (middle and right panel) of recipient mice was compared. $CD8^+$ T lymphocytes from G9C$\alpha^{-/-}$Y16A (blue) exhibited the most robust proliferative response (A) *in vitro* and (B) *in vivo* (mean ± SEM n=4). (C&D) Cytotoxic capabilities were assessed by a flow-based method. PKH-labelled targets were co-cultured with (C) splenic $CD8^+$ T-cells and (D) SP $CD8^+$ thymocytes as effectors for 16 hours and target cell death was determined by TOPRO-3 staining. G9C$\alpha^{-/-}$ Y16A $CD8^+$ splenocytes exhibited increased cytotoxicity, specifically killing peptide-loaded P815 targets, whereas this elevation was not witnessed when SP $CD8^+$ thymocytes were used as effectors. Splenocyte cytotoxicity was repeated 6-8 times in duplicate, thymic 3-5 replicates performed in duplicate (mean ± SEM). *p<0.05, **p<0.01, *** p<0.001

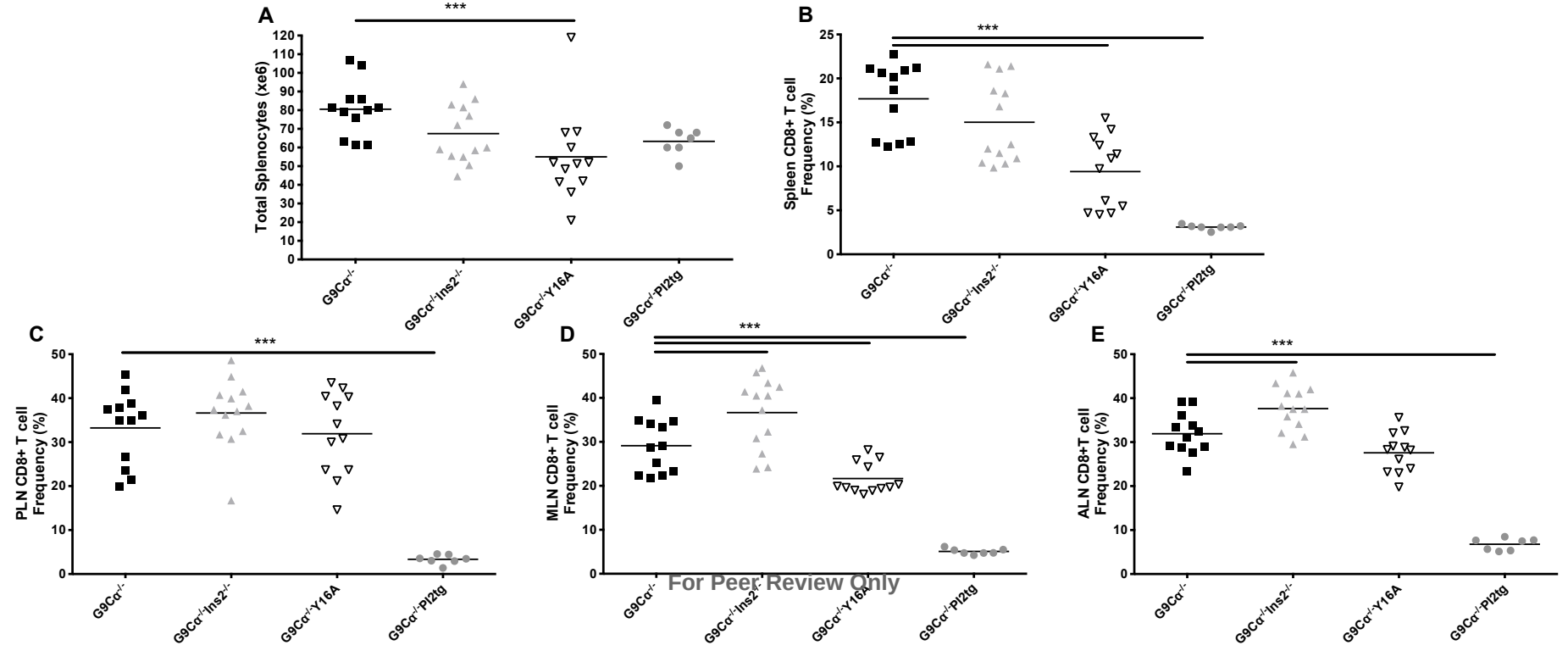
Figure 5**: Increased activation status of $CD8^+$ T lymphocytes.** $CD8^+$ T-cells were stained for CD62L expression in (A) pancreatic lymph nodes, (B) mesenteric lymph nodes and (C) para-aortic lymph nodes. CD62L was significantly downregulated on lymphocytes from the pancreatic LN of 5-10 week old G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ male mice (A). There was no downregulation observed on lymphocytes isolated from the mesenteric LN (B) or para-aortic LN (C) (mean ± SEM). (D) Histograms are representative of 13 mice.

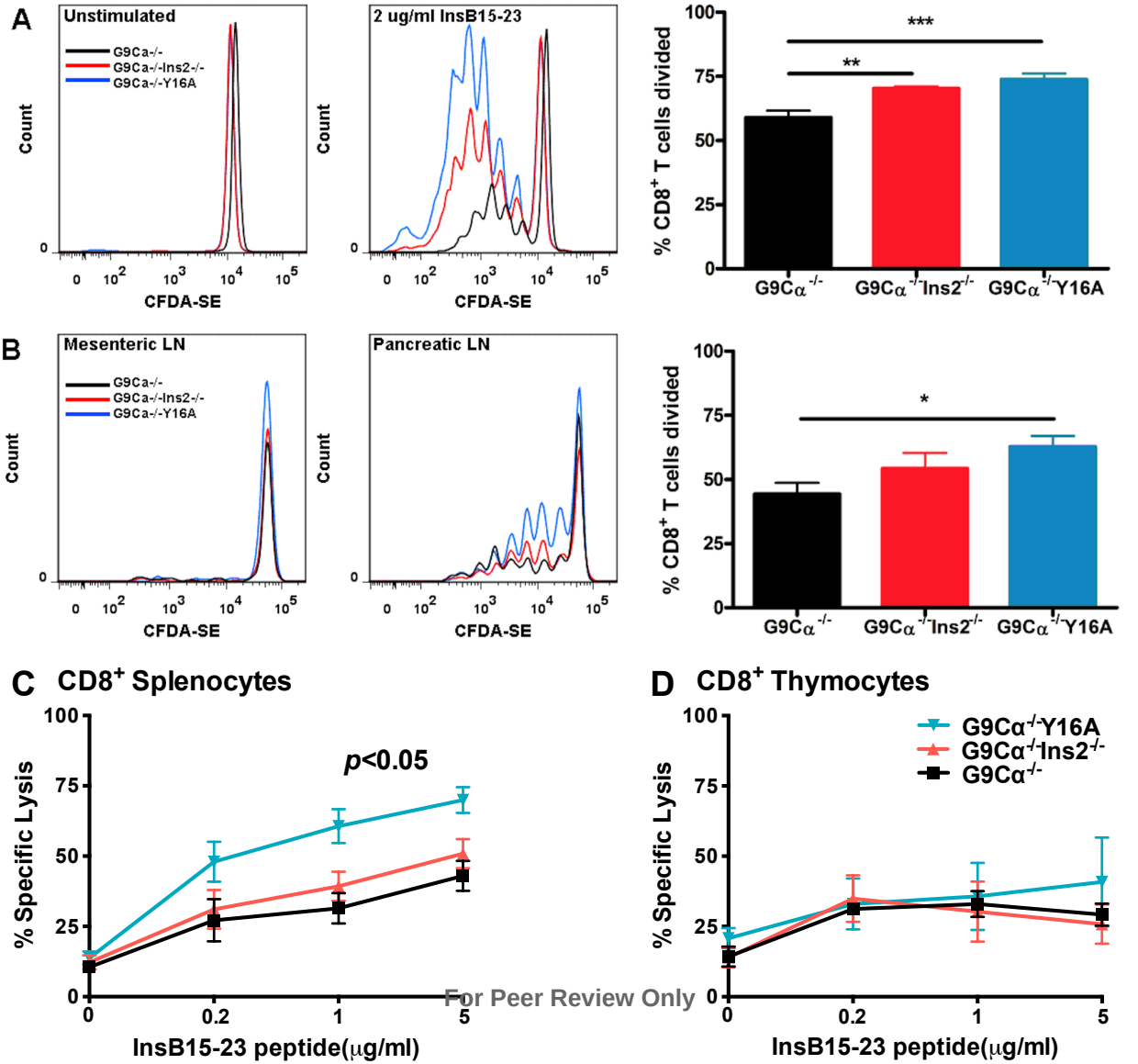
Figure 6: **TCR expression on insulin-reactive CD8 T-cells**. (A) Pancreatic LN or (B) Splenic $CD8^+$ T-cells from G9C$\alpha^{-/-}$, G9C$\alpha^{-/-}$*Ins2*$^{-/-}$, and G9C$\alpha^{-/-}$Y16A mice were activated with 4 μg/ml InsulinB15-23 peptide over 3 days, mean ± SEM of n=4. No elevations in TCR expression were seen to explain increased activation and cytolytic capacity. Frequency of Tregs (CD4+CD25+FoxP3+) cells was assessed from (C) PLN (n=4) and (D) spleen ((mean ± SEM of n=8) from G9C$\alpha^{-/-}$, G9C$\alpha^{-/-}$*Ins2*$^{-/-}$, and G9C$\alpha^{-/-}$Y16A mice.

Figure 7: **Association of insulin expression with the suppressive capacity of pancreatic lymph node stromal cells**. (A) LNSC were CD45- after complement depletion. Cells were further characterized as UEA-1 expressing. Evaluating CD31 and gp38 identified a heterogeneous population. The majority of cells were gp38+CD31-, typical of FRC (right panel). (B) G9C$\alpha^{-/-}$ T-cells were co-cultured with PI2tg-expressing DCs and LNSC from mice with various levels of proinsulin expression. The cumulative effects on T cell activation could then be measured to determine if LNSC suppression could reduce DC-mediated activation of G9 T-cells. (C) Pancreatic LNSC (10^4 cells) were co-cultured with NOD-PI2^{tg} bone marrow-derived DC (2X10^5cells) and CFDA-SE-labelled CD8$^+$ T-cells (10^5 cells) from G9C$\alpha^{-/-}$ (black bars), G9C$\alpha^{-/-}$*Ins2*$^{-/-}$ (grey bars), and G9C$\alpha^{-/-}$Y16A (open bars) mice. The origin of the pancreatic LNSC used is shown on the x axis, with the expression of proinsulin within the LNSC marked beneath the graphs. No exogenous peptide was used in order to prevent antigen processing by the LNSC. Proliferation was assessed after 72 hours of stimulation as a percentage of control set as 100% (T-cells stimulated by DCs without LNSC present, which is represented by the dashed horizontal line). Baseline proliferation in the absence of DCs was <4% divided cells. Data represents four independent experiments performed in duplicate (mean ± SEM). One-way ANOVA analysis compared each T cell group with the internal control of T-cells stimulated with DCs. *p<0.05, **p<0.01, ***p<0.001








A
Unstimulated
G9Ca-/-
G9Ca-/-Ins2-/-
G9Ca-/-Y16A
Count
CFDA-SE
2 ug/ml InsB15-23
% CD8+ T cells divided
G9Cα-/-
G9Cα-/-Ins2-/-
G9Cα-/-Y16A
B
Mesenteric LN
Pancreatic LN
C CD8+ Splenocytes
p<0.05
% Specific Lysis
InsB15-23 peptide(μg/ml)
D CD8+ Thymocytes
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