nature portfolio

Peer Review File

TGF-β-mediated crosstalk between TIGIT+ Tregs and CD226+CD8+ T cells in the progression and remission of type 1 diabetes

Corresponding Author: Professor Xia Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Zhong et al. reports on a costimulatory and co-inhibitory axis reported to by involved in modulating CD8+ T cell and Treg function during the pathogenesis of T1D. The authors use a combination of clinical patient samples and two animal models to conduct a series of mechanistic studies using flow cytometric analyses and single cell sequencing technologies. Overall, the study presents a large number of proposed mechanisms of regulation relating to the balance between CD226 and TIGIT in T cell subsets but falls somewhat short of offering compelling data that these mechanisms are essential in disease progression. The manuscript would be bolstered by narrowing the focus bolstering the validation experiments. The authors jump between human samples, NOD mice, and C57BL/6 mouse models without a clear rationale for the change. Indeed, there is no clear rationale provided for why the STZ model was used in a non-autoimmune prone stain of mice would be an appropriate model system to explain the mechanisms of regulation by this costimulatory axis. Those experiments could have been conducted in the NOD mouse during the natural progression of the disease. A number of additional concerns are outlined below that will require clarification:

It is not clearly presented how TGF-β1 clusters of interest CD226+CCR7- CD8 and TIGIT+CCR7- Tregs were selected, besides an association shown in their MLM and serum levels for their groups. It would have been more informative to show differences in TGF-β in TIGIT+CCR7- vs TIGIT-CCR7+ Tregs.

The human subject cohorts are not sufficiently characterized. What is the HLA genotypes of the patient cohorts? The subjects appear negative for ZnT8 autoantibodies when the proportion of subjects is typically ~70% in new onset T1D subjects and declines to ~50% in a general T1D populations post onset. How was this assay conducted and has it been validated in clinical consortia programs (e.g., IASP)? The manuscript is not clear on how new onsets are clearly delineated from patients undergoing partial clinical remission.

One of the major conclusions proposed by the authors states that TIGIT+CCR7- Tregs and CD226+CCR7- CD8s are “implicated in T1D remission”, since these subsets fluctuate during the honeymoon period. There is no clear mechanism proposed for why this might occur. Is this a metabolic response feature to exogenous insulin, or an immune resolution during a period of waxing and waning autoimmunity? It is concerning that the longitudinal studies in the NOD do not show the same trends with disease progression. These concepts need to be clarified.

Minor corrections:

-Non-standard abbreviations need to be written out in the text at first occurrence
-Single cell subsets appear to be manually annotated. The authors should clarify if this correlates with other single cell datasets for cell annotation, such as azimuth.
-Data shown in figures should indicate comparisons that reached statistical significance and clearly state the tests and thresholds employed for the comparison in the appropriate figure legend.
-The authors should clearly indicate what cellular phenotypes are age dependent, and correct if needed in the comparisons. This is particularly important when considering the expression of both CD226 and TIGIT are highly enriched on memory T cell subsets relative to naïve T cells, and these ratios change with chronological age following exposures.

-A Table outlining key subject demographic information would improve the overall cohort classifications.
-Given CD226 is a candidate risk gene in T1D, the authors should analyze the subjects studies for the potential impact of susceptibility versus protective alleles on the phenotypes observed.

Reviewer #2

(Remarks to the Author)
Zhong and colleagues identified TIGIT+ CCR7-Tregs through cohort validation and functional analysis, and showed that these cells may inhibit CD226+ CCR7- CD8+ T cells via TGF-β. The involvement of TIGIT+ Tregs in the suppression of autoimmune disease pathogenesis is already known, but it is worthwhile to demonstrate the importance of TIGIT+ CCR7- Tregs from the analysis of patient cohorts. However, there are several issues to be addressed. The specific comments are as follows:

Major points,
1, It is unclear how the anti-CD226 antibodies used in the in vivo experiments in Fig. 8 suppress disease. CD226 expressed on CD8+ T cells is known as an activating receptor.
However, in Fig. S8, anti-CD226 antibodies had no effect on CD8+ T cell activation. This issue should be discussed. In addition, the authors should clarify whether the anti-CD226 antibodies used deplete CD226+ cells.

2, Ex vivo experiments of Figure 8 should include the analysis of Tregs in addition to CD8+ T cells. In particular, it is important to analyze whether TIGIT+ CCR7-Tregs are increased by the administration of anti-CD226 antibodies.

3, The authors claim that TIGIT+ CCR- Tregs suppress CD226+CD8+ T cells through TGF-β, but there is no direct evidence. Co-culture experimental systems may be useful.
In addition, the authors should demonstrate the functional differences in the effects on CD226+CD8+ T cells between TIGIT-CCR+ Tregs and TIGIT+ CCR- Tregs.

4, Do the expression levels of CD155, the common ligand for TIGIT and CD226, differ between TD1 patients and HD? It is important to analyze the dynamics of CD155 expression levels when considering the activation kinetics of TIGIT+ and CD226+ cells.

Minor points
1, This manuscript should be further revised in English. For example, in line 43 (... peripheral blood would ideal due...) needs a 'be' after 'would'. Other parts of the text should also be carefully checked.

2, Abbreviations in the figures should be clearly stated in the respective figure legends. For example, FCP, SCP, and GADA in Figure 1. The legends of other diagrams should also be carefully modified.

3, Figures do not match the textual description; it is assumed that Fig2I and Figu2J are reversed.

4, The explanations in the Figure legend are not adequate. For example, which dot plot in Fig7AB shows which patient? What do the numbers 0-3 indicate in Fig7C? The legend to Figure 8 should state the numbers of mice used and the experiments performed, etc.

5, Lines 237-238: "Additionally, there were no significant differences in the functions of the two cell subsets between the T1D and HD groups (Fig. 5E-J)", but Fig 5 shows the results comparing CD226+CD8+ T cells with TIGIT+CD8+ T cells and does not contain data comparing T1D with HD.

Reviewer #3

(Remarks to the Author)
In this MS, Zhong and colleagues perform a detailed single-cell RNAseq analysis of circulating immune cells in new-onset T1D children with and without partial remission and in healthy controls. Starting from a limited set of samples, they describe a population of TIGIT+ Tregs and CD226+ CD8+ T cells and suggest that there may be a TGF-β-mediated crosstalk between the two. They then build on a much larger sample set to confirm these results by flow cytometry, showing that these TIGIT+ Treg and CD226+ CD8+ T-cell population are respectively positively and negatively correlated with stimulated C peptide levels and respectively enriched and depleted in patients under partial remission. They conclude with studies in a low-dose STZ model of autoimmune diabetes by showing that a CD226 antibody reduced disease penetrance and insulitis. The study is rigorously conducted and, despite its complexity, well written and easy to follow throughout. Clearly, these results are of importance. There are some points that however deserve attention to make the conclusions more convicing.

1. The definition of remission as a MMTT-stimulated C-peptide level ≥300 pM seems very loose. On these grounds, most of these patients would have significant insulin needs, while modest needs are the essence of the clinical definition of the honeymoon period. This definition, as the other one used of an IDAA1C ≤9, seems rather to indicate a partial remission, and this should be clearly stated from start (and indeed it is the definition used later in the paper). Indeed, stimulated C-peptide is ultimately the most distinctive feature between the 2 groups, while other parameters show some degree of overlap.

Stimulated C- peptide is also the parameter better correlated with TIGIT+ Treg increase and TIGIT- Treg decrease (Fig. 3C-D), and with CD226+CD8+ decrease and TIGIT+CD8+ increase (Fig. 5C-D).
2. Fig. 2H: the fact that TIGIT+CCR7- Tregs decline post-remission is mentioned in the text but now shown. Moreover, these differences across groups are quite modest and should be statistically supported.
3. Fig. 3B: the rationale of excluding CCR7+KLRG1- CD8+ T cells as 'non-effectors' is not clear – couldn't these cells also include CM subsets?
4. Fig. 4E-I: the legend should indicate that red lines are T1D patients, blue lines are HDs. What is the statistical test to which the asterisks refer to? It is said that there is a difference between T1D and HDs in the marker/cytokine profile of TIGIT+ but not TIGIT- Tregs, but the test seems then to compare the 2 groups of Tregs but not the 2 groups of donors – the statistical comparison of the 2 donor groups should also be shown in these figures. The same applies to Fig. 5E-J.
5. Fig. S5C: the gating strategy would be clearer with a dot plot depicting TIGIT and C226 simultaneously, so to appreciate the distinction and overlap of the 2 subsets (as done in Fig. S6 for Tregs). It would also make sense to invert these 2 supplementary Figs as Tregs are presented first in the text.
6. Was the machine learning algorithm also applied to TIGIT+ Tregs, and what was the result? This should also be showed even if negative.
7. Fig. 7C: the correlations of TIGIT+ Tregs and CD226+ CD8+ T cells with the severity of insulitis should be shown similarly to what done with C-peptide in T1D patients.
8. Finally and more importantly, the immunosuppressive activity of human TIGIT+ Tregs should be validated with functional suppression assays and compared with that of TIGIT- Tregs. The same applies for CD226+ vs. TIGIT+ CD8+ T cells for their cytotoxic activity. These experiments could also allow to validate the TGF-beta-mediated cross-talk between the Treg and CD8+ T-cell subsets.

Minor points:
- Abstract is missing from the MS.
- The Introduction is quite comprehensive but may need some shortening.
- Gene names should be in italics.
- The "lymphocyte activation cocktail" used to stimulate PBMCs should be detailed.
- Line 280: "cytokine screening ability" should read "cytokine secretion ability".

Reviewer #4

(Remarks to the Author)
Zhong et al. submitted a paper titled "TGF-β-mediated crosstalk between TIGIT1 + Tregs and CD226+ CD8+ T cells in the progression and remission of type 1 diabetes" for publication in Nature Communications. The authors used scRNA-seq to identify Treg and CD8+ T cell subtypes that were demonstrated to have different abundance levels in the different disease stages of patients diagnosed with type 1 diabetes. The authors used an extensive number of approaches to demonstrate the clinical significance of these T cell subtypes. Overall, I find this study to be well executed and highly interesting. However, I have some major and minor concerns related to the data analysis and the manuscript.

Major comments

1. Figure 1C shows the UMAP plot of the whole scRNA-seq dataset with the main cell types colored. While the authors have done good job at annotating most of the main cell types, they reported a few unusual cell types, namely granulocytes, double-negative T (DNT) cells and NKT cells. Since the cells should be PBMCs (Fig. 1B), why were there granulocytes?
2. Please provide the complete list of the cell type markers results obtained using Seurat's FindAllMarkers function. The dotplot (Fig. 1D) that shows a few selected genes per cluster is not enough. This could be a supplementary file (Excel file), and it would give readers a better sense of the different cell types and their gene markers.
3. The authors should provide violin plots of key quality control metrics for the main cell types (Fig. 1C) in the supplement. Please include the percentage of mitochondrial reads per cell, the percentage of ribosomal reads per cell, the total number of UMIs per cell, and the number of genes per cell. The violin plots must be grouped by the cell type so that it's possible to compare the metrics between the cell types. If some of the cell types, such as DNTs or c5 Tregs, exhibit highly aberrant quality metrics, they should preferably be removed or the very least be marked as poor-quality cells.
4. Related to the 3rd comment, the authors should explain in more detail how they performed quality control to remove bad cells.
5. How did the authors deal with doublets in scRNA-seq analysis? Please use doublet detection methods such as scDblFinder to detect doublets and remove any cells that are doublets with high probability (based on the tool's prediction and gene markers).
6. The authors should provide some independent cell type assignment for the main cell types (Fig. 1C). This can be easily done using tools such as CellTypist (https://www.celltypist.org/). This may also help annotate the three unusual cell types (granulocytes, DNT, NKT).
7. One major limitation in the machine learning model is that the authors did not validate their model using an independent cohort. The model was just built based on a single cohort, and the authors basically performed cross validation to evaluate the model's performance, which should generally be only used to optimize the model and not as the final performance assessment. A completely independent test set that the model has never seen before is crucial for assessing its generalization to new, unseen data. This set is used only once after the model is trained and validated. Please discuss this limitation.
8. When the authors performed GSEA analysis, they performed prior gene filtering based on adjusted p-value of 0.05 (rows 485, 486). To my understanding, this gene filtering is not something that is required or usually done. What impact does this have on the result?

9. The codes for reproducing the analyses should be provided.

Minor comments

1. Although some software versions are marked, some are not. Please add the missing software versions.
2. Cellranger provides a set of quality control metrics in the HTML web summary files. Please provide a summary of these metrics in the supplement.
3. “Perform dimensional reduction and clustering” part of the method chapter requires clarification. Prior to PCA, Seurat selects a fixed number of highly variable genes (HVGs). What is the final number of HVGs? What was the HVG selection method used in FindVariableFeatures? How many principal components were selected after HVG selection? I find “non-linear t-SNE” as a term a bit weird. I think simply “t-SNE” would be better.
4. In “Batch effect correction”, please mention the batch variable name that was used for correction, such as sample.
5. In “Detection of differentially expressed genes and functional enrichment analysis”, how was the pseudo-bulk analysis exactly done? What statistical test, how was the pseudo-bulk aggregation done (mean or sum aggregation)? In the same section, the authors mention both Seurat’s FindMarkers and pseudo-bulk, but no clear explanation is given as to what comparisons were done using each method. Please explain this further.
6. In “RNA-seq” part, no details of the data analysis are given. Please explain how the analysis was done. I think it would also make sense to rename the title to “Bulk RNA-seq”.
7. In “Construction and evaluation of machine learning model”, what are the different outcomes in “features, with different outcome events serving as the target variable”?
8. In “Statistical analysis”, how exactly was “the adjustment for potentially confounding variables” done prior to ANOVA?
9. I found the main text to be well written. However, the data analysis part of the Methods chapter had clear grammatical errors.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors should be commended for the efforts to address prior review concerns. The resulting manuscript is much improved, however, a number of questions remain.

-The authors show representative plots of the frequency of cytokine-producing CD8s for each cytokine assayed, but instead plot the gMFI data for each cytokine. Plotting the differences between frequencies of cytokine-producing cells may be more physiologically relevant than switching from frequency to gMFI between the representative plots and data.

-Suppression assays using dye dilution approaches need to account for both the number of cells that enter a proliferative cycle, as well as the number of cell divisions. That data is not accessible from Ki-67 analysis alone. This draws in concerns for the suppression assay employed that measured Ki-67 gMFI in the effectors at 48 hrs. Data may also need to be normalized to the 1:0 control condition.

-The new figure 8G-H should have representative gating for CCR7/TIGIT, rather than just SSC/TIGIT

-It is not clear what “CCS” is on the y-axis for 8F.

-The new Figure 8N-O should have representative gating for CD8/TIGIT, rather than just TIGIT.

-The authors show that there is a decrease in the frequency of CD226+CD8+ and an increase in TIGIT+CD8+ in the spleen following in vivoTx, but suggest that there isn’t any functional changes in the CD226+CD8+ population. The authors should clarify what they hypothesize the antibody is doing to that subset. To this point, the authors should clarify whether the anti-CD226 mAb is depleting or blocking in action. This notion of epitope specificity should also be considered for flow staining antibodies relative to the therapeutic mAb.

-The authors indicate there isn’t any impact in the pLN, so seeing if there are differences in the pancreas-infiltrating CTL populations would be of interest.

Reviewer #2

(Remarks to the Author)
No further comments from the reviewer.

Reviewer #3

(Remarks to the Author)
The Authors have satisfactorily addressed most of the points raised.

A major issue remains in the suppression assays presented in Fig. 7G-I using sorted TIGIT+CCR7- and TIGIT-CCR7+ Tregs. The sorting of these populations only included a preliminary magnetic isolation of CD4+ T cells, which were subsequently stained for TIGIT and CCR7 for sorting. Thus, these cells do not qualify as Tregs, as they have not been sorted on additional Treg markers, e.g. CD25+CD127- as done in Fig. S8. It is thus not surprising to see that TIGIT+CD4+ T cells are more suppressive than CCR7+CD4+ T cells, as the latter are likely to be composed mainly of naïve and central memory conventional T cells. These experiments should be repeated by gating on CD25+CD127- CD4+ Tregs before sorting TIGIT+CCR7- and TIGIT-CCR7+ subsets.

The same may apply to the suppression assays presented in Fig. 4J using the same Treg subsets. There is no detail on how these subsets were sorted and it is assumed that the same strategy was used.

Reviewer #4

(Remarks to the Author)
Congratulations on this excellent revision. The authors have addressed my concerns about the manuscript adequately. I have no further comments.

Version 4:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed a number of key comments from the original review. However, there remain a number of items that either need to be directly addressed, or the language and conclusions need to be modified to more accurately reflect the data presented. Specifically, the following items should be addressed:
1. For insulitis scoring, the authors demonstrate that CD8+ T cells are associated with increased insulitis and Tregs are associated with decreased insulitis; however, the authors do not directly compare how frequencies of TIGIT+CCR7- Tregs versus TIGIT-CCR7+ Tregs may impact insulitis. These cells need to be directly assessed in the pancreas and pLN or tested formally through adoptive co-transfer experiments to definitely support the claims of direct regulation.
2. The authors initially state in the abstract and introduction that anti-CD226 reduces diabetes in the NOD, without clarifying it is Cyclophosphamide-induced. This is an important point because the treatment with Cyclophosphamide alters the normal distribution of cells and does not reflect the same processes underway in spontaneous disease.
3. The authors still did not actually perform dye dilution suppression assays to evaluate the amount of Treg or effector T cell proliferation. Instead, the authors showed alterations in pro-inflammatory cytokines and changes in Ki-67 before concluding differences in suppressive capacity. While in vitro assays of suppression are not without their own limitations, they do provide important information on numbers of cell divisions, cell death, and the potential for observing consumption over direct suppression.
4. In the last figure, the authors suggest that anti-CD226 improves beta cell function and alters glucose tolerance. The authors do not have any metabolic data (e.g., glucose tolerance tests), so they should instead state that it reduces insulitis. The killing assay was conducted using a mast cell line (P815).

Reviewer #3

(Remarks to the Author)
The Authors have clarified the pending issue highlighted by this Reviewer. Thank you for this additional revision and congratulations on this nice piece of work.

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RE: Manuscript ID: NCOMMS-23-50175

Dear Reviewers:

Thank you very much for taking the time to review our manuscript titled "TGF-β-mediated crosstalk between TIGIT$^+$ Tregs and CD226$^+$ CD8$^+$ T cells in the progression and remission of type 1 diabetes". We greatly appreciate the valuable comments from you. We have meticulously revised the manuscript to address each query and suggestion. Our detailed point-by-point responses are provided below.

Reviewer #1: The manuscript by Zhong et al. reports on a costimulatory and co-inhibitory axis reported to by involved in modulating CD8$^+$ T cell and Treg function during the pathogenesis of T1D. The authors use a combination of clinical patient samples and two animal models to conduct a series of mechanistic studies using flow cytometric analyses and single cell sequencing technologies.
Overall, the study presents a large number of proposed mechanisms of regulation relating to the balance between CD226 and TIGIT in T cell subsets but falls somewhat short of offering compelling data that these mechanisms are essential in disease progression. The manuscript would be bolstered by narrowing the focus bolstering the validation experiments.
The authors jump between human samples, NOD mice, and C57BL/6 mouse models without a clear rationale for the change. Indeed, there is no clear rationale provided for why the STZ model was used in a non-autoimmune prone stain of mice would be an appropriate model system to explain the mechanisms of regulation by this costimulatory axis. Those experiments could have been conducted in the NOD mouse during the natural progression of the disease. A number of additional concerns are outlined below that will require clarification:

Response 1: Thank you for your critical evaluation of our manuscript and for your constructive comments. We have made substantial changes to strengthen the proposed mechanisms of regulation concerning the CD226 and TIGIT in T cell subsets.

To further support our findings, we have included additional validation experiments:
-We assessed the supernatants from cultured TIGIT$^+$ CCR7$^-$ Treg cell subpopulations and established that they indeed secrete higher levels of TGF-β1 compared to their TIGIT$^-$ CCR7$^+$ Treg counterparts, as depicted in the new Figure 7D (Figure R1). This is consistent with the results of our intracellular staining: TIGIT$^+$ CCR7$^-$ Tregs have higher levels of TGF-β1 than TIGIT$^-$ CCR7$^+$ Tregs (main figure 4I).

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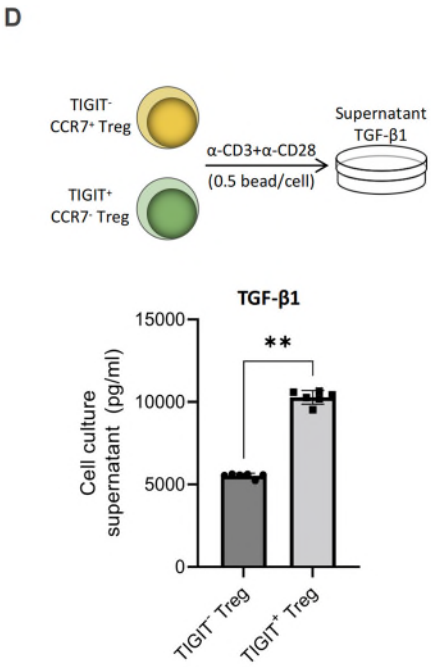

Figure R1 (Figure 7D in the main text) TGF-β1 secretion by TIGIT and CCR7 defined Treg subsets from T1D patients. Sorted TIGIT$^+$ CCR7$^-$ Tregs and TIGIT$^-$ CCR7$^+$ Tregs from T1D patients (n = 6) were incubated with α-CD3/CD28 antibodies (0.5 bead/cell) for 48 h at 37°C. The TGF-β1 levels in the supernatants were measured by ELISA. Two-tailed unpaired t-test; data are shown as the mean ± SD. $^{**}P < 0.01$.

-We demonstrated that treating CD226$^+$ CCR7$^-$ CD8$^+$ T cells with TGF-β1 in vitro markedly inhibited their functional capacity, which is detailed in the new Figures 7E-F (Figure R2).

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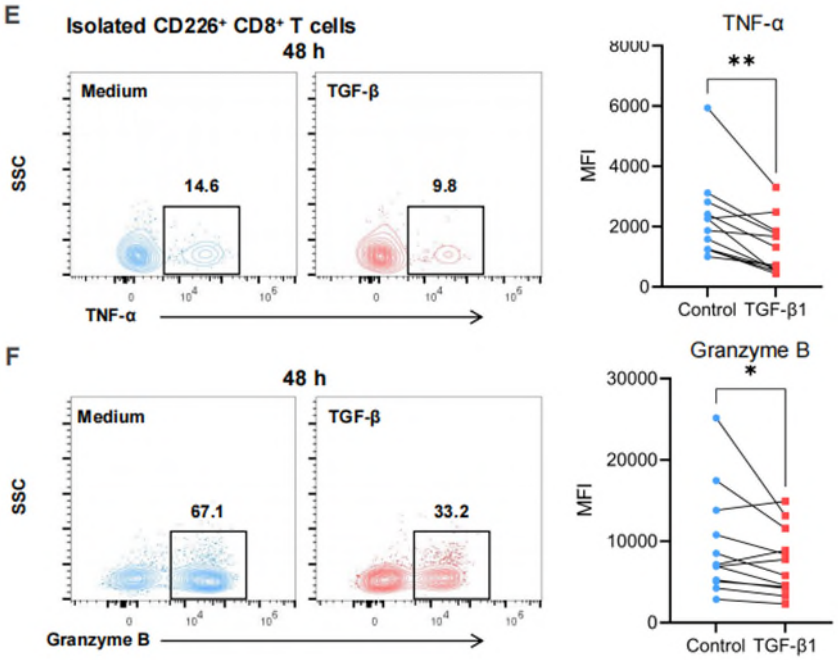

Figure R2 (Figure 7E&F in the main text) TGF-β1 suppressed the activity of CD226$^+$ CCR7$^-$ CD8$^+$ T cells in vitro. Representative flow plots and expression levels of (E) TNF-α , and (F) granzyme B in CD226$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients following 48 hours of stimulation with or without 10 ng/ml TGF-β1 (n = 12). Two-tailed paired t-test. *P < 0.05, **P < 0.01.

For the assessment of cytotoxic activity, CD8$^+$ T cells were initially enriched through positive selection using magnetic CD8$^+$ beads provided by Miltenyi Biotec. Following enrichment, CD226$^+$ and TIGIT$^+$ CD8$^+$ T cell subsets were isolated via flow cytometry sorting. These isolated subsets were subsequently cocultured with P815 mastocytoma cells for a duration of 48 hours to evaluate apoptotic induction in the target cells. The resulting data, illustrated in the corresponding Figure R3, demonstrate that CD226$^+$ CD8$^+$ T cells exert a significantly enhanced cytotoxic effect on P815 cells when compared to TIGIT$^+$ CD8$^+$ T cells, as indicated by an increased rate of apoptosis in the P815 cell population.

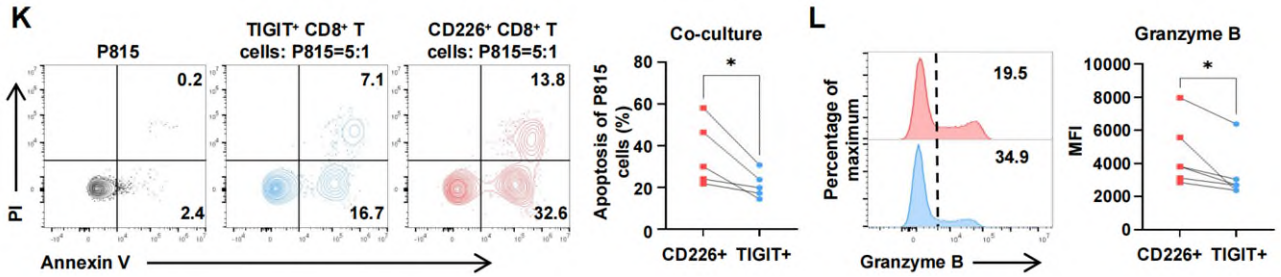

Figure R3 (now Figure 5K-L in the main text) Assessment of cytotoxic activity of CD8$^+$ T-cell subsets. CD226$^+$ and TIGIT$^+$ CD8$^+$ T cells, isolated from T1D patients (n = 6), were cocultured with

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fluorescently-labeled P815 cells at a ratio of 5:1 for 5 h at 37°C. (**K**) Representative flow cytometry plots and quantification of apoptosis in P815 cells. (**L**) Representative peak plots and quantification of granzyme B expression within $CD8^+$ T-cell subsets. Two-tailed paired t-test. *$P < 0.05$.

-Lastly, through co-culture experiments of $TIGIT^+$ $CCR7^-$ Tregs with $CD226^+$ $CCR7^-$ $CD8^+$ T cells, we directly observed the suppressive effort exerted by the $TIGIT^+$ $CCR7^-$ Tregs on the $CD226^+$ $CCR7^-$ $CD8^+$ T cells, these results are presented in new Figures 7G-I (Figure R4).

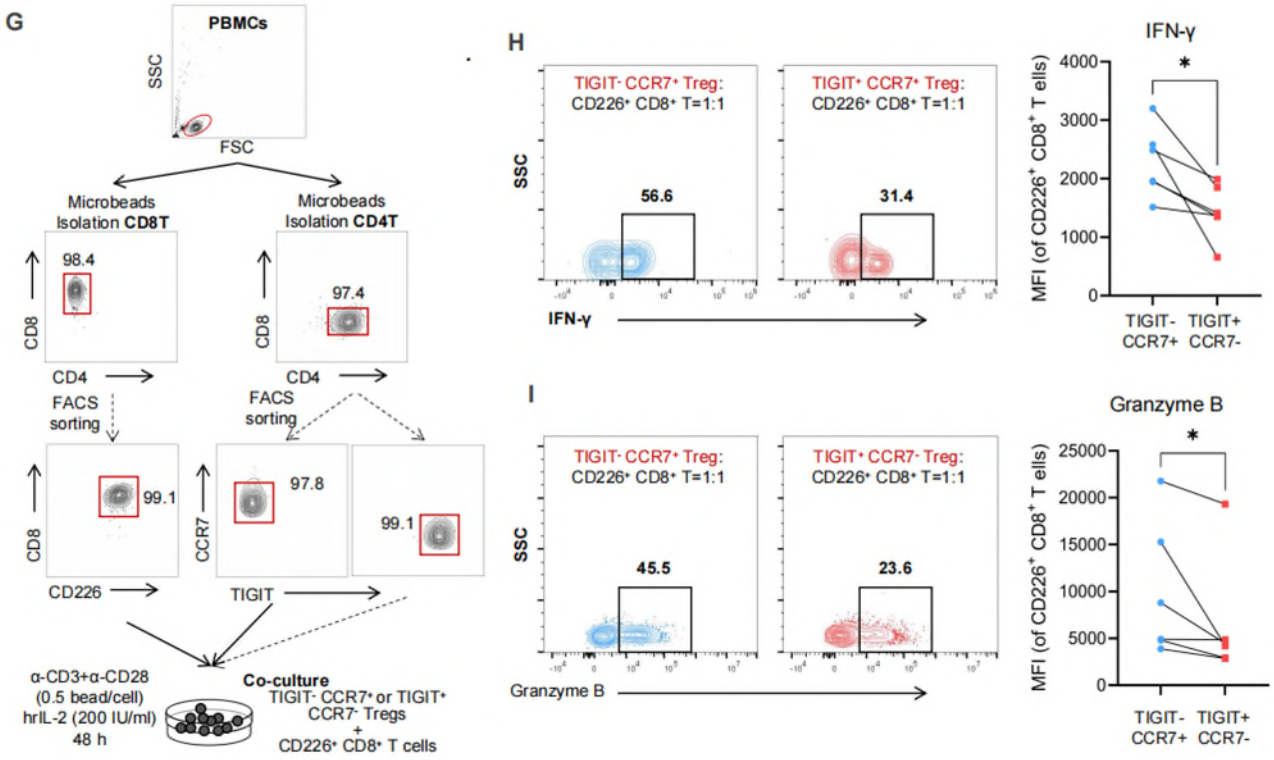

Figure R4 (Figure 7G-I in the main text) Co-culture of $TIGIT^+$ $CCR7^-$ Tregs with $CD226^+$ $CCR7^-$ $CD8^+$ T cells. (**G**) Diagram illustrating the experimental setup. Representative flow plots and expression levels of (**H**) IFN-γ , and (**I**) granzyme B in $CD226^+$ $CCR7^-$ $CD8^+$ T cells from T1D patients following 48 hours of co-culture with $TIGIT^+$ $CCR7^-$ or $TIGIT^-$ $CCR7^+$ Tregs at a 1:1 ratio (n = 6). Two-tailed paired t-test. *$P < 0.05$.

We agree that additional experiments should also be conducted in the NOD mouse models alongside the model of STZ. We analyzed the features of $TIGIT^+$ $CCR7^-$ Tregs and $CD226^+$ $CCR7^-$ $CD8^+$ T cells within the spleens and PLNs of NOD mice across different age groups to establish a correlation between these cellular subpopulations and the extent of insulitis. This section of the results has been moved to the supplementary files. Furthermore, we employed the NOD cyclophosphamide-accelerated model and verified that diminishing $CD226^+$ $CD8^+$ T cell expression can postpone the development of T1D (Figure R5; main Figure 8J-O) according to suggestions. We opted for the

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cyclophosphamide-accelerated NOD model over the spontaneous NOD model to ensure a more consistent and rapid onset of T1D, enabling us to efficiently evaluate the therapeutic efficacy of anti-CD226 monoclonal antibodies. This model has been widely used in various studies (*Li S, et al. Antioxidants. 2021; Louvet C, et al. PNAS. 2008*) and this approach not only increases the incidence and synchronicity of disease onset but also intensifies the disease's progression, providing a stringent test for our therapeutic intervention within a practical timeframe.

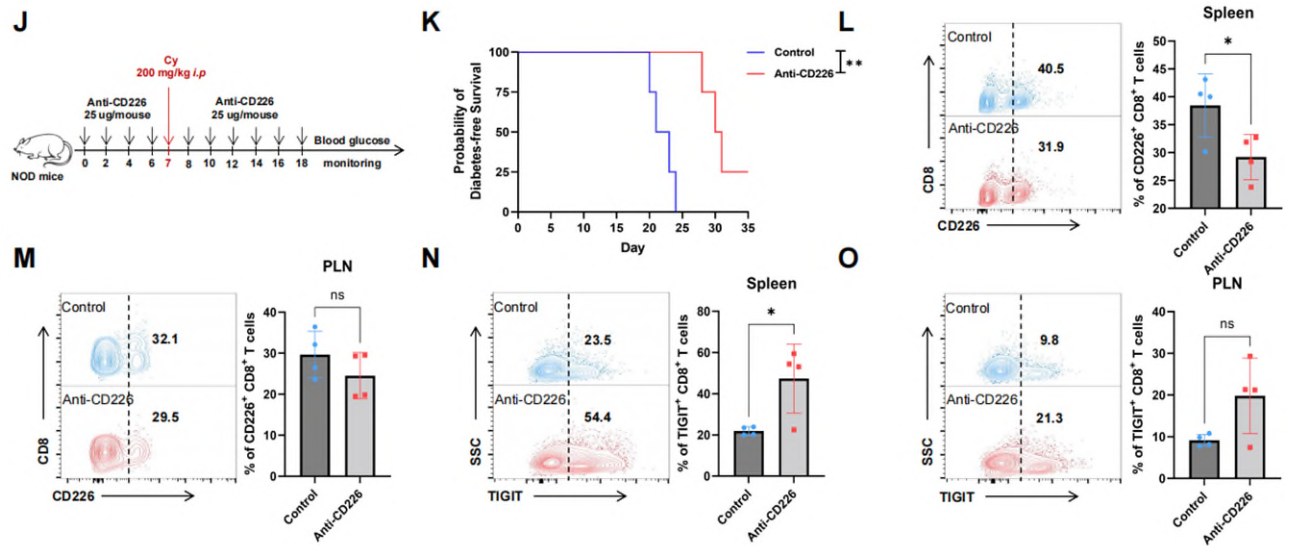

Figure R5 (Figure 8J-O in the main text) Anti-CD226 therapy alleviates hyperglycemia and promotes immune equilibrium in Cy-induced T1D mice. Female NOD mice aged 12 weeks (n=8 per group) were administered a single Cy dose of 400 mg/kg intraperitoneally to precipitate diabetes. The Anti-CD226 group was treated with 25 µg of anti-CD226 mAb every other day for 10 doses, while the control group received isotype injections. Four mice from each group were sacrificed for immune profiling post-treatment, with the remainder observed subsequently to assess the diabetic incidence. (**J**) Schematic diagram for the therapeutic experiment. (**K**) Survival curves comparing the incidence of diabetes between the two groups (n = 4). Significance determined by Log-rank (Mantel-Cox) test. (**L-O**) The proportion of $CD226^+$/$TIGIT^+$ $CD8^+$ T cells in the spleen and PLN are depicted for the two groups. Two-tailed unpaired t-test; data are shown as the mean ± SD. *P < 0.05. Cy, cyclophosphamide.

We believe that the integration of these two animal models provides a more comprehensive assessment of the therapeutic impact of anti-CD226 monoclonal antibodies, as well as insight into the regulatory mechanisms exerted by this costimulatory axis in T1D. These revisions and additional data significantly enhance the manuscript's strength and scientific rigor. We thank you again for your valuable feedback.

Major revisions:

-It is not clearly presented how TGF-β1 clusters of interest $CD226^+$ $CCR7^-$ CD8 and $TIGIT^+$ $CCR7^-$

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Tregs were selected, besides an association shown in their MLM and serum levels for their groups. It would have been more informative to show differences in TGF-β in TIGIT$^+$ CCR7$^-$ vs TIGIT$^-$ CCR7$^+$ Tregs.

Response 2: We appreciate your thoughtful and constructive comments on our study. Following your advice, we sorted TIGIT$^+$ CCR7$^-$ Treg and TIGIT$^-$ CCR7$^+$ Treg cells and cultured them in vitro for 48 hours, after which we measured the levels of TGF-β1 in the culture supernatants. As delineated in Figure R1 (refer to main figure 7D), the TGF-β1 concentration detected in the supernatant from TIGIT$^+$ CCR7$^-$ Treg cells was significantly elevated (P<0.05) in comparison to that of the TIGIT$^-$ CCR7$^+$ Treg cells. These findings align with the data obtained from their intracellular protein level assessments (see Figure R6, main figure 4I).

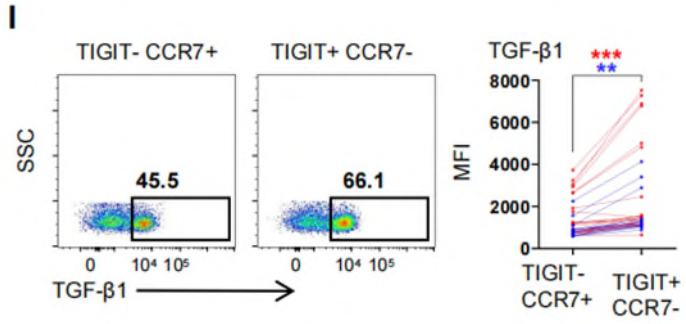

Figure R6 (Figure 4I in the main text) Representative flow cytometry plots and quantification of (**H**) TGF-β1 in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs from T1D patients (red line, n = 40) and HD (blue line, n = 14). Two-tailed paired t-test. ****P < 0.0001.

-The human subject cohorts are not sufficiently characterized. What is the HLA genotypes of the patient cohorts?

Response 3: Thanks. We have added a detailed characterization about the T1D cohorts. In terms of HLA genotype distribution, we observed the following within the cross-sectional cohort: DR3/DR4 at 16.7%, DR3/DR9 at 8.3%, DR4/DR4 at 8.3%, DR4/DRX at 16.7%, DR9/DR9 at 41.7%, and DR9/DRX at 8.3%. Conversely, the longitudinal cohort exhibited a varied distribution, with DR3/DR3 (4.3%), DR3/DR4 (23.9%), DR3/DR9 (15.2%), DR3/DRX (4.3%), DR4/DR9 (8.7%), DR4/DRX (2.2%), DR9/DR9 (17.4%), DR9/DRX (21.7%), DRX/DRX (2.2%). However, the autoimmune nature of our T1D cases were defined by the presence of islet related autoantibodies and not by HLA genotypes.

-The subjects appear negative for ZnT8 autoantibodies when the proportion of subjects is typically ~70% in new onset T1D subjects and declines to ~50% in a general T1D populations post onset. How was this assay conducted and has it been validated in clinical consortia programs (e.g., IASP)?

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Response 4: Thank you. Regarding the ZnT8A values for Cohort I, we have been negligent in the graphical transformation, the data is as follows, and Figure 1A has been corrected. Positive threshold: GADA > 0.05; IA-2A > 0.02; ZnT8A > 0.011. Marked with a red background.

Table R1 Autoantibodies of Cohort I.

	GADA	IA2A	ZnT8A
NEW1	0.0057	1.3942	0.0032
NEW2	-0.0014	0.1797	0.6879
NEW3	-0.0010	0.3824	0.1029
NEW4	0.3354	1.9253	0.5993
NEW5	0.3598	0.5923	0.0925
PR1	0.4180	0.6116	0.0967
PR2	0.1412	0.7075	-0.0001
PR3	0.3249	-0.0038	-0.0036
PR4	1.7272	1.6028	0.1085

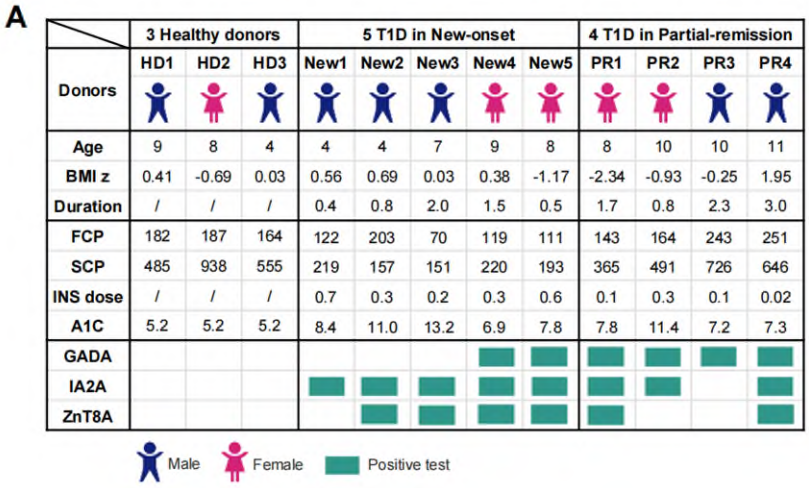

Figure R7. Schematic illustration highlighting the clinical information for the Cohort I.

The determination of GADA, IA-2A, and ZnT8A was conducted through radioimmunoassay, as previously reported (*Shi X, et al. Diabetologia. 2019*). Samples that initially tested positive were re-evaluated with a second test to confirm their status. Only patients with confirmed positive results were included in the study. The sensitivities of the assays for GADA, IA-2A, and ZnT8A were found to be 82%, 76%, and 76%, respectively. Specificities reached 96.7%, 100%, and 100%, respectively, as determined by the Islet Autoantibody Standardization Program (IASP 2020). This information has been incorporated into the Methods section.

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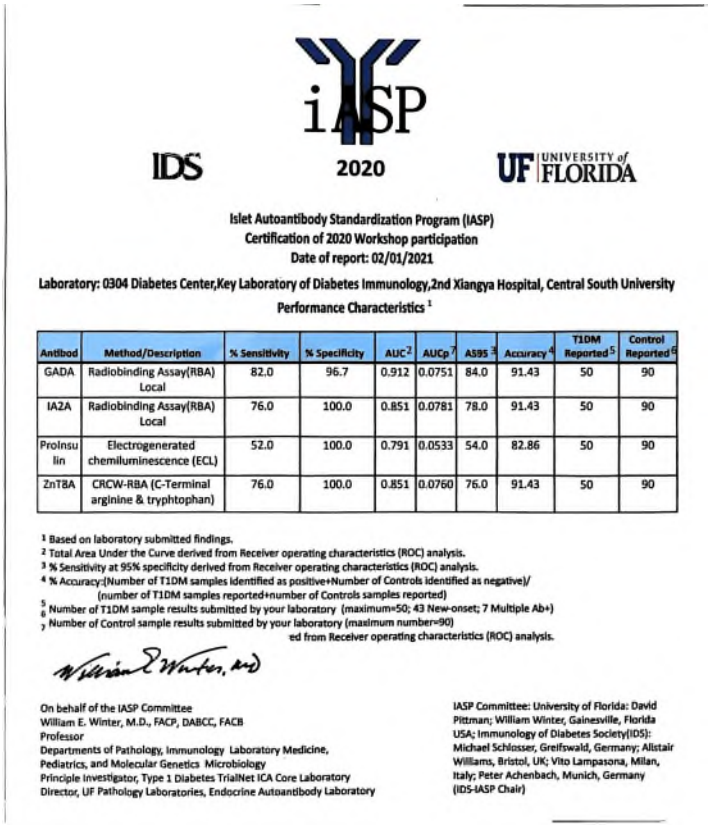

IDS 2020 UF UNIVERSITY of FLORIDA

Islet Autoantibody Standardization Program (IASP)
Certification of 2020 Workshop participation
Date of report: 02/01/2021

Laboratory: 0304 Diabetes Center,Key Laboratory of Diabetes Immunology,2nd Xiangya Hospital, Central South University

Performance Characteristics [1]

Antibod	Method/Description	% Sensitivity	% Specificity	AUC[2]	AUCp[7]	AS95[3]	Accuracy[4]	T1DM Reported[5]	Control Reported[6]
GADA	Radiobinding Assay(RBA) Local	82.0	96.7	0.912	0.0751	84.0	91.43	50	90
IA2A	Radiobinding Assay(RBA) Local	76.0	100.0	0.851	0.0781	78.0	91.43	50	90
Proinsulin	Electrogenerated chemiluminescence (ECL)	52.0	100.0	0.791	0.0533	54.0	82.86	50	90
ZnT8A	CRCW-RBA (C-Terminal arginine & tryphtophan)	76.0	100.0	0.851	0.0760	76.0	91.43	50	90

[1] Based on laboratory submitted findings.
[2] Total Area Under the Curve derived from Receiver operating characteristics (ROC) analysis.
[3] % Sensitivity at 95% specificity derived from Receiver operating characteristics (ROC) analysis.
[4] % Accuracy:(Number of T1DM samples identified as positive+Number of Controls identified as negative)/ (number of T1DM samples reported+number of Controls samples reported)
[5] Number of T1DM sample results submitted by your laboratory (maximum=50; 43 New-onset; 7 Multiple Ab+)
[6] Number of Control sample results submitted by your laboratory (maximum number=90)
[7] ed from Receiver operating characteristics (ROC) analysis.

William E. Winter, MD

On behalf of the IASP Committee
William E. Winter, M.D., FACP, DABCC, FACB
Professor
Departments of Pathology, Immunology Laboratory Medicine, Pediatrics, and Molecular Genetics Microbiology
Principle Investigator, Type 1 Diabetes TrialNet ICA Core Laboratory
Director, UF Pathology Laboratories, Endocrine Autoantibody Laboratory

IASP Committee: University of Florida: David Pittman; William Winter, Gainesville, Florida USA; Immunology of Diabetes Society(IDS): Michael Schlosser, Greifswald, Germany; Allstair Williams, Bristol, UK; Vito Lampasona, Milan, Italy; Peter Achenbach, Munich, Germany (IDS-IASP Chair)

Figure R8. IASP certification

-The manuscript is not clear on how new onsets are clearly delineated from patients undergoing partial clinical remission.

Response 5: Thank you. In this study, "new-onset" pertains to patients who were newly diagnosed within three months and haven't gone through the honeymoon phase yet (did not meet the criteria of honeymoon phase); and those who met the criteria of honeymoon were assigned to the "honeymoon phase" category.

-One of the major conclusions proposed by the authors states that TIGIT$^+$ CCR7$^-$ Tregs and CD226$^+$ CCR7$^-$ CD8s are “implicated in T1D remission”, since these subsets fluctuate during the honeymoon period. There is no clear mechanism proposed for why this might occur. Is this a metabolic response feature to exogenous insulin, or an immune resolution during a period of waxing and waning autoimmunity?

Response 6: We appreciate your excellent comments. The fluctuations of TIGIT$^+$ CCR7$^-$ Tregs and CD226$^+$ CCR7$^-$ CD8T cells during the honeymoon period indeed suggest their involvement in T1D remission. However, the mechanism for these changes remains elusive. And the mechanisms of the occurrence of honeymoon phase are poorly understood.

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Our current hypothesis leans towards an immunological resolution, supported by studies from our team and others that show a transit restoration of immune tolerance and a reduced autoimmune attack during the honeymoon period (*Zhong T, et al. J Autoimmun. 2024*). However, we cannot rule out the possibility of a metabolic response to exogenous insulin therapy, which may also play a role in modulating immune cell dynamics.
Our team is committed to demystifying the honeymoon phase in T1D—its triggers, its cessation, and the feasibility of its induction or extension. Leveraging our initial discoveries, we have performed fecal 16S rRNA sequencing and serum metabolomic/proteomic profiling in a subset of T1D patients. We are now exploring further the impact of the gut microbiota, serum metabolic and proteomic profiles, and islet regeneration on initiating the honeymoon phase and fostering immune tolerance.

-It is concerning that the longitudinal studies in the NOD do not show the same trends with disease progression. These concepts need to be clarified.

Response 7: Thank you for pointing this out. To establish a correlation between the proportion of $TIGIT^+$ $CCR7^-$ Tregs and $CD226^+$ $CCR7^-$ $CD8^+$ T cells and the severity of insulitis, we evaluated these cellular subpopulations in the spleens and PLNs of NOD mice across various age groups. We found that **these two cell types exhibited inverse and positive correlations, respectively, with the severity of insulitis (Fig. S11A–D). This is consistent with the results in the patient cohort.** However, the lack of an animal model that accurately recapitulates the "honeymoon period" presents a major challenge to mimic the immunological resolution observed in human studies. Despite our attempts to initiate remission in NOD mice through insulin therapy, the immune activation and destruction in these mice proved to be persistently irreversible, contrasting with the clinical course in T1D patients. Future work is warranted to establish a T1D mouse model that resembles the human “honeymoon period”.

Minor revisions:
-Non-standard abbreviations need to be written out in the text at first occurrence.

Response 8: Thank you for pointing out the use of non-standard abbreviations in our manuscript. We have ensured that all non-standard abbreviations are fully written out at their first occurrence in the text.

-Single cell subsets appear to be manually annotated. The authors should clarify if this correlates with other single cell datasets for cell annotation, such as azimuth.

Response 9: Thank you. We confirm that the initial annotation was indeed performed manually, based on known markers and the expression profiles of the cells. To ensure the validity and reliability of our annotations, we have cross-referenced our manually annotated cell subsets with established single cell datasets, including but not limited to those available in Azimuth.

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-Data shown in figures should indicate comparisons that reached statistical significance and clearly state the tests and thresholds employed for the comparison in the appropriate figure legend.

Response 10: Thank you. We have updated our figures to include markers denoting statistical significance and have clarified the statistical tests and thresholds used in the figure legends for precise and transparent comparison.

-The authors should clearly indicate what cellular phenotypes are age dependent, and correct if needed in the comparisons. This is particularly important when considering the expression of both CD226 and TIGIT are highly enriched on memory T cell subsets relative to naïve T cells, and these ratios change with chronological age following exposures.

Response 11: Thank you for your valuable comment. We acknowledge the importance of accurately defining cellular phenotypes in the context of age, especially given that the expression of markers such as CD226 and TIGIT varies considerably within memory T cell subsets when compared to naïve T cells, and these subsets themselves are subject to change with aging.
As indicated in Table S4, we applied a multivariate logistic regression analysis to adjust for variables such as age, gender, and BMI. Following this adjustment, the frequencies of $TIGIT^+$ $CCR7^-$ Tregs and $CD226^+$ $CCR7^-$ CD8T cells remained significantly different between patients in the honeymoon phase and those outside of it, suggesting a statistically significant association.

Table S4

Multivariate logistic regression analysis to identify the variables independently associated with remission in T1D.

	Multivariate analysis			Multivariate analysis	
	Odds ratio (CI 95%)	*P*		Odds ratio (CI 95%)	*P*
Age	1.01 (0.92–1.09)	0.91	Age	1.05 90.93–1.18）	0.43
Gender	2.26 (0.71–7.18)	0.17	Gender	1.71 (0.44–6.74)	0.44
BMI	1.17 (0.93–1.46)	0.19	BMI	1.27 (0.95–1.69)	0.11
			$CD226^+$ $CCR7^-$		
$CCR7^+$ $TIGIT^-$ Treg	0.86 (0.78–0.04)	0.00	$CD8^+$ T cells	0.74 (0.65–0.86)	0.00
Age	1.04 (0.95–1.14)	0.43	Age	1.07 (0.98–1.16)	0.11
Gender	1.52 (0.47–4.92)	0.46	Gender	1.74 (0.62–4.87)	0.29
BMI	1.06 (0.85–1.33)	0.61	BMI	1.10 (0.89–1.37)	0.39
			$TIGIT^+$ $CCR7^-$		
$CCR7^-$ $TIGIT^+$ Treg	1.31 (1.15–1.48)	0.00	$CD8^+$ T cells	1.06 (1.01–1.10)	0.01

The model includes 90 patients; 34/90 (37.8%) patients are in the remission phase. T1D, type 1 diabetes; remission, partial remission; BMI, body mass index; Gender is a dichotomous variable; all other variables are continuous.

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-A Table outlining key subject demographic information would improve the overall cohort classifications.

Response 12: Thank you. This study encompasses three principal cohorts: Cohort I, intended for single-cell sequencing, with demographic details depicted in Figure 1A; Cohort II, designated for the cross-sectional validation of the frequency changes in TIGIT^{+} CCR7^{-} Tregs and CD226^{+} CCR7^{-} CD8T cells corresponding to the "honeymoon phase", with demographics outlined in Table S3; and Cohort III, which is applied for predictive modeling using machine learning, with its population demographics detailed in Table S5–7.

-Given CD226 is a candidate risk gene in T1D, the authors should analyze the subjects studies for the potential impact of susceptibility versus protective alleles on the phenotypes observed.

Response 13: We appreciate your recommendation to explore the impact of CD226 allelic variations on T1D phenotypes. However, our current study is primarily focused on the immunological aspects, specifically the subpopulations of immune cells. We believe that the immunophenotypic data will provide valuable insights independent of the genetic variations in CD226.
We understand the importance of genetic contributions to T1D and the specific influence of CD226 allelic variants. To that end, we are planning subsequent studies dedicated to examining the interplay between CD226 gene mutations and disease manifestations. This planned research will enable a thorough exploration of how CD226 susceptibility and protective alleles contribute to the development and progression of T1D, as well as their impact on therapeutic interventions. We are committed to investigating these genetic factors in due course and sharing our comprehensive findings. And we added the potential impact of CD226 susceptibility versus protective alleles on the phenotypes observed in the discussion part according to your suggestions.

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Reviewer #2 Zhong and colleagues identified TIGIT$^+$ CCR7$^-$ Tregs through cohort validation and functional analysis, and showed that these cells may inhibit CD226$^+$ CCR7$^-$ CD8$^+$ T cells via TGF-β. The involvement of TIGIT$^+$ Tregs in the suppression of autoimmune disease pathogenesis is already known, but it is worthwhile to demonstrate the importance of TIGIT$^+$ CCR7$^-$ Tregs from the analysis of T1D patient cohorts in different stages of disease progression. However, there are several issues to be addressed. The specific comments are as follows:

Response 1: Thank you very much for reviewing our study and providing us with valuable feedback. **We utilized three cohorts to address the importance of TIGIT$^+$ CCR7$^-$ Tregs and CD226$^+$ CCR7$^-$ CD8$^+$ T cell in different stages of T1D.** Cohort I, intended for single-cell sequencing, with demographic details depicted in Figure 1A; Cohort II, designated for the cross-sectional validation of the frequency changes in TIGIT$^+$ CCR7$^-$ Tregs and CD226$^+$ CCR7$^-$ CD8T cells corresponding to the "honeymoon phase", with demographics outlined in Table S3; and Cohort III, which is applied for predictive modeling using machine learning, with its population demographics detailed in Table S5–7.We have addressed each of the comments below and have revised the manuscript accordingly.

Major points,

1. It is unclear how the anti-CD226 antibodies used in the in vivo experiments in Fig. 8 suppress disease. CD226 expressed on CD8$^+$ T cells is known as an activating receptor. However, in Fig. S8, anti-CD226 antibodies had no effect on CD8$^+$ T cell activation. This issue should be discussed. In addition, the authors should clarify whether the anti-CD226 antibodies used deplete CD226$^+$ cells.

Response 2: Thank you. We utilized the anti-mouse CD226 functional grade (Clone: 10E5, eBioscience, Cat #: 16-2261) for therapeutic intervention. The 10E5 clone was initially reported in its application to experimental autoimmune encephalomyelitis (EAE), where its administration delayed disease onset and lessened the severity of Th1-mediated autoimmune pathology (*Dardalhon et al, J Immunol, 2005*). Subsequently, it has also been documented to suppress antigen-specific T cell proliferation and IFN-γ production in vivo, as well as to extend the survival of skin allografts (*Liu et al, Arthritis Research & Therapy, 2018*).

Our recent studies have reaffirmed that CD226 functions as an activating receptor on CD8$^+$ T cells. The addition of new Figure S13 (referred to as Figure R9) demonstrates that treatment with **anti-CD226 antibodies led to a reduction in both proliferation and cytokine production of CD8$^+$ T cells**, consistent with existing literature (*Johnston RJ, et al. Cancer Cell. 2014*). This finding aligns with our initial reports and provides further evidence of the antibody's impact on T cell responses.

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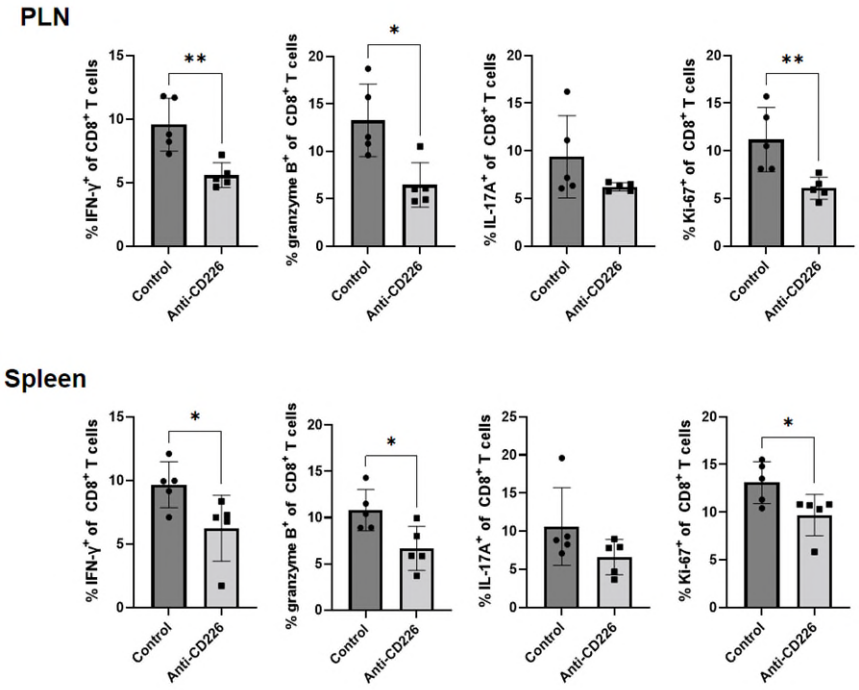

Figure R9 (new Figure S13) . Administration of Anti-CD226 mAb in vivo inhibits the expansion of CD8⁺ T cells and cytokines-production. The expression levels of IFN-γ, granzyme B, IL-17A and Ki-67 within $CD8^+$ T cells in the spleen and PLN are depicted for the two groups (n = 5). Two-tailed unpaired t-test; data are shown as the mean ± SD. $*P < 0.05$, $**P < 0.01$.

Interestingly, despite these observed effects on proliferation and cytokine production, our functional analysis as shown in old Figure S8 (now Figure S14 or Figure R10 in the response letter) indicates that **the overall functional capacity of $CD226^+$ $CD8^+$ T cells is not compromised by the anti-CD226 treatment**. This uncoupling of proliferation and cytokine production from other aspects of $CD8^+$ T cell function suggests a more complex role of CD226 in T cell regulation.

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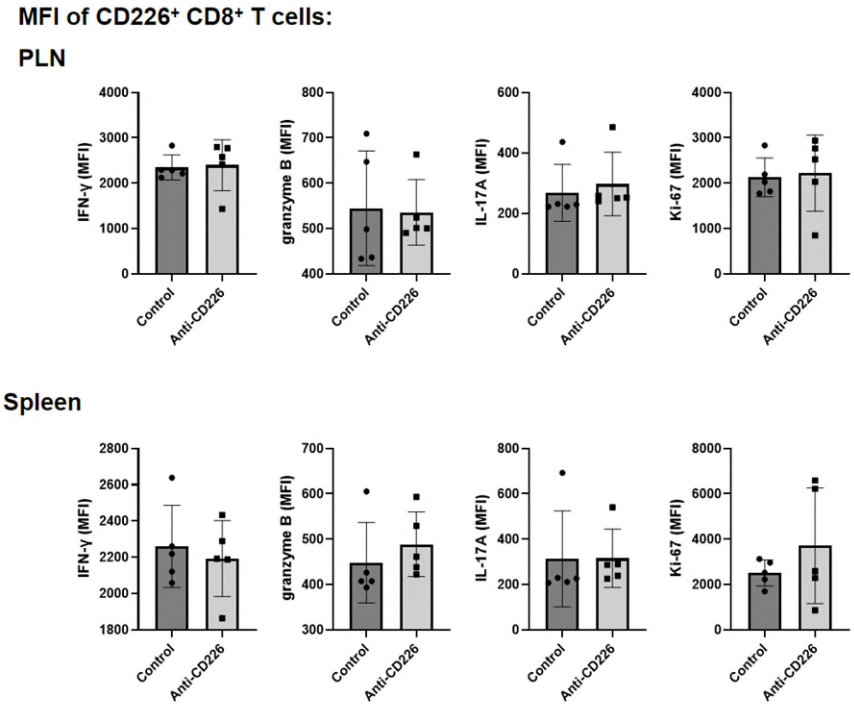

Figure R10 (new Figure S14) Anti-CD226 treatment does not change the function of CD226$^+$ CD8$^+$ T cells. The expression levels of IFN-γ, granzyme B, IL17A, and KI67 within CD226$^+$ CD8$^+$ T cells in the spleen and PLN are depicted for the two groups (n = 5). Two-tailed unpaired t-test; data are shown as the mean ± SD.

In humans, CD226 is found on a variety of immune cells, including CD4+ and CD8+ T cells, NK cells, monocytes, as well as certain subsets of B cells and platelets. The expression profile of CD226 in mice differs notably; it is primarily present on T cells (CD8+ and CD4+), with only modest levels observed on select NK cell subsets and macrophages, and it is notably absent on B cells. The administration of anti-CD226 mAb could interfere with the interaction between CD226 and its natural ligands, potentially inhibiting the activation of macrophages that is crucial for effective antigen presentation and subsequent T cell activation. **This interference could lead to a reduction in CD226 expression and the consequent depletion of a subset of CD226-positive cells, likely impacting CD226-positive CD8$^+$ T cells predominantly.**
In this study, we sought to dissect the intricate balance between CD226$^+$ CD8$^+$ T cells and TIGIT$^+$ CCR7$^-$ Treg cells that orchestrates immune tolerance during the 'honeymoon phase'. To achieve this, we meticulously assessed the dynamic equilibrium of these two pivotal cell subpopulations in response to precise modulation with anti-CD226 therapeutic intervention. Our revised manuscript will include a detailed discussion of these findings, emphasizing the nuanced role of CD226 in T cell biology and the potential implications for antibody-based therapies targeting CD226.

-Ex vivo experiments of Figure 8 should include the analysis of Tregs in addition to CD8$^+$ T cells. In

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particular, it is important to analyze whether TIGIT$^+$ CCR7$^-$ Tregs are increased by the administration of anti-CD226 antibodies.

Response 3: Thank you for emphasizing this crucial aspect. In response to the suggestion, we revisited our ex vivo data in Figure 8 and included an analysis of Tregs along with CD8$^+$ T cells. We examined the effects of anti-CD226 antibody treatment on TIGIT$^+$ CCR7$^-$ Tregs (Figure R11; new Figure 8G-H). The results demonstrated that the application of anti-CD226 intervention not only led to a reduction in the population of CD226$^+$ CD8$^+$ T cells but also results in an upregulation of TIGIT$^+$ CCR7$^-$ Treg cells. This dual effect highlights the potential of anti-CD226 antibodies to modulate the immune response by influencing key regulatory and effector cell populations.

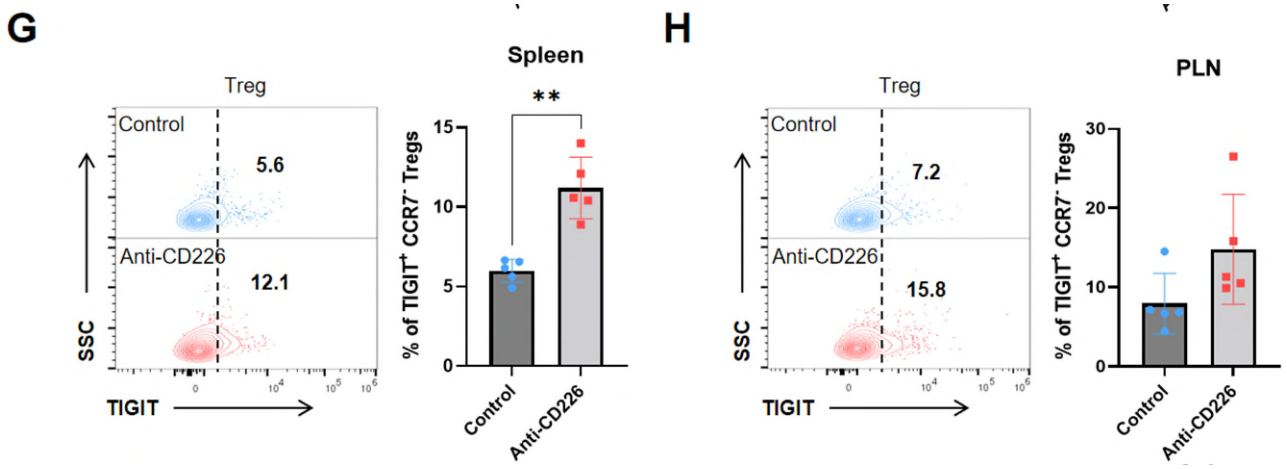

Figure R11 (new Figure 8G-H) Anti-CD226 treatment leads to an upregulation of TIGIT$^+$ Treg cells. The expression levels of TIGIT within Tregs in the spleen and PLN are depicted for the two groups (n = 5). Two-tailed unpaired t-test; data are shown as the mean ± SD. $^{**}P<0.01$.

-3. The authors claim that TIGIT$^+$ CCR7$^-$ Tregs suppress CD226$^+$ CD8$^+$ T cells through TGF-β, but there is no direct evidence. Co-culture experimental systems may be useful. In addition, the authors should demonstrate the functional differences in the effects on CD226$^+$ CD8$^+$ T cells between TIGIT$^-$ CCR7$^+$ Tregs and TIGIT$^+$ CCR7$^-$ Tregs.

Response 4: We appreciate your suggestion and have performed the following experiments to address this.

Firstly, we assessed the supernatants from cultured TIGIT$^+$ CCR7$^-$ Treg cell subpopulations and established that they indeed secrete higher levels of TGF-β1 compared to their TIGIT$^-$ CCR7$^+$ Treg counterparts, as depicted in the new Figure 7D (Figure R1). This is consistent with the results of our intracellular staining: TIGIT$^+$ CCR7$^-$ Tregs have higher levels of TGF-β1 than TIGIT$^-$ CCR7$^+$ Tregs (main figure 4I).

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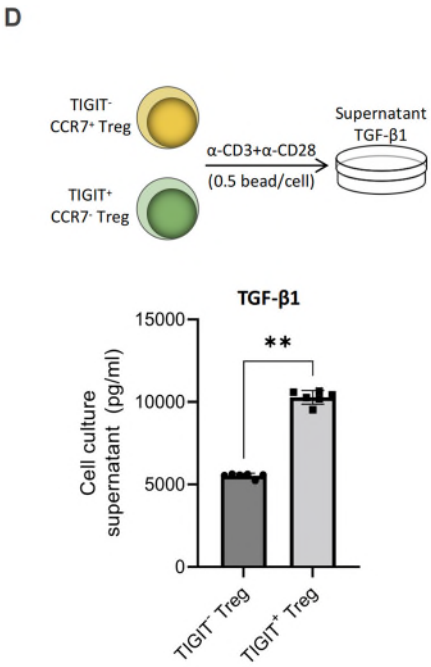

Figure R1 (Figure 7D in the main text) TGF-β1 secretion by TIGIT and CCR7 defined Treg subsets from T1D patients. Sorted TIGIT$^+$ CCR7$^-$ Tregs and TIGIT$^-$ CCR7$^+$ Tregs from T1D patients (n = 6) were incubated with α-CD3/CD28 antibodies (0.5 bead/cell) for 48 h at 37°C. The TGF-β1 levels in the supernatants were measured by ELISA. Two-tailed unpaired t-test; data are shown as the mean ± SD. $^{**}P < 0.01$.

Then, we demonstrated that treating CD226$^+$ CCR7$^-$ CD8$^+$ T cells with TGF-β1 in vitro markedly inhibited their functional capacity, which is detailed in the new Figures 7E-F (Figure R2).

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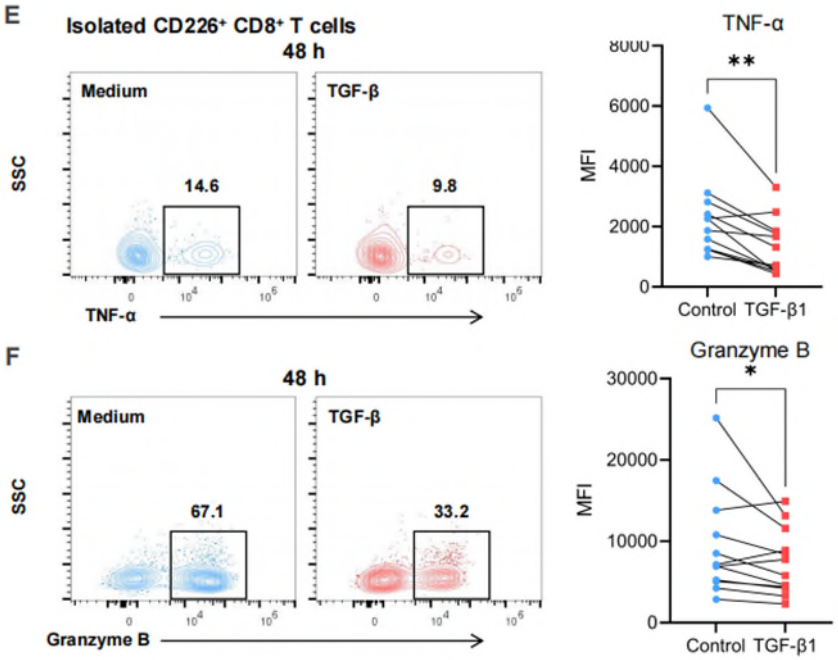

Figure R2 (Figure 7E&F in the main text) TGF-β1 suppressed the activity of CD226$^+$ CCR7$^-$ CD8$^+$ T cells in vitro. Representative flow plots and expression levels of (E) TNF-α , and (F) granzyme B in CD226$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients following 48 hours of stimulation with or without 10 ng/ml TGF-β1 (n = 12). Two-tailed paired t-test. *P < 0.05, **P < 0.01.

Lastly, we sorted TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ Treg subsets, and co-culturing them with CD226$^+$ CCR7$^-$ CD8$^+$ T cells. Our results indicate that TIGIT$^+$ CCR7$^-$ Tregs exert a more potent suppressive effect on CD226$^+$ CCR7$^-$ CD8$^+$ T cells compared to their TIGIT$^-$ CCR7$^+$ counterparts. These findings have been detailed and are now presented in the updated Figures 7G-I (Figure R4) in the revised manuscript.

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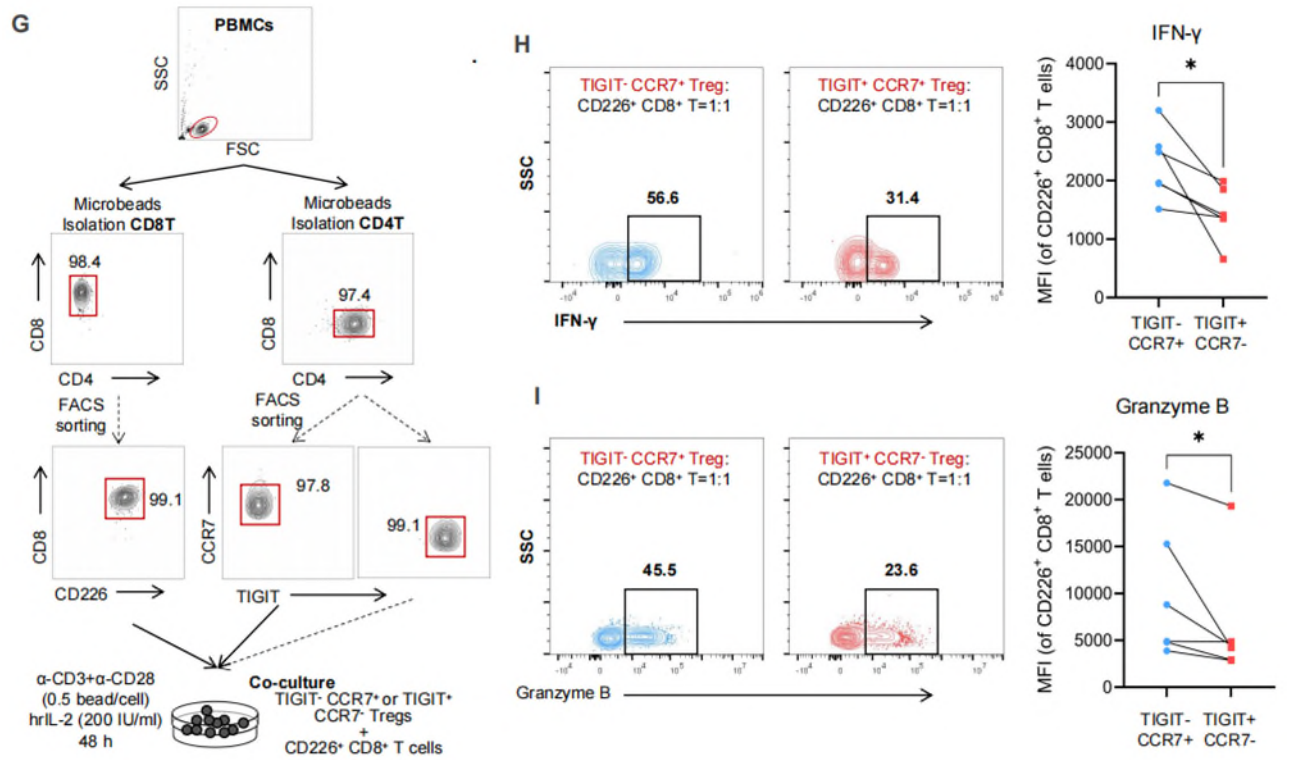

Figure R4 (Figure 7G-I in the main text) Co-culture of TIGIT$^+$ CCR7$^-$ Tregs with CD226$^+$ CCR7$^-$ CD8$^+$ T cells. (**G**) Diagram illustrating the experimental setup. Representative flow plots and expression levels of (**H**) IFN-γ , and (**I**) granzyme B in CD226$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients following 48 hours of co-culture with TIGIT$^+$ CCR7$^-$ or TIGIT$^-$ CCR7$^+$ Tregs at a 1:1 ratio (n = 6). Two-tailed paired t-test. *P < 0.05.

-4. Do the expression levels of CD155, the common ligand for TIGIT and CD226, differ between T1D patients and HD? It is important to analyze the dynamics of CD155 expression levels when considering the activation kinetics of TIGIT$^+$ and CD226$^+$ cells.

Response 5: Thanks. CD155 is widely expressed on APCs, such as dendritic cells, macrophages, and B cells. The binding of CD155 on APCs to CD226 on CD8 T cells leads to T cell activation and can enhance the cytotoxic function of these cells. Conversely, engagement with TIGIT allows CD155 to convey inhibitory signals that attenuate T cell activation and proliferation, fostering immune tolerance. In the context of T1D, the balance between costimulatory and coinhibitory signals mediated by CD155 and its receptors can significantly impact the progression and severity of the disease. Dysregulation in this signaling pathway could contribute to the autoimmune attack on pancreatic beta cells.

Nevertheless, this study focuses in on the intrinsic mechanisms of T cells rather than on APCs. Therefore, we only briefly mentioned the CD155-related results in the manuscript (line 186-189, Figure R12). To address this question about CD155, **we performed additional analysis which**

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suggested that CD155 is primarily expressed on monocytes, and the expression of CD155 on monocytes of patients in the honeymoon period is significantly lower than that of newly diagnosed patients. Confirmation and functional characterization of CD155+ Monocytes as well as their role in T1D will be examined in detail in a follow up investigation.

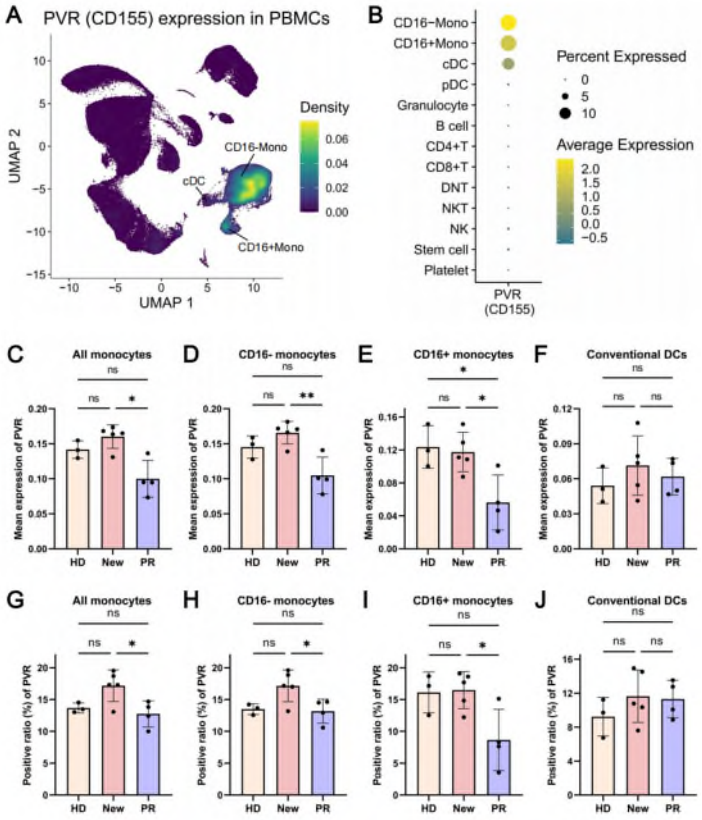

Figure R12 (Fig S7) The expression of CD155. (**A**) The UMAP plot highlights the expression levels of PVR in PBMCs. (**B**) Dot plot showing the expression levels of the PVR in each cell type. (**C-F**) Comparisons of the mean PVR expression among the three groups in (**C**) all monocytes, (**D**) CD16- monocytes, (**E**) CD16+ monocytes, and (**F**) conventional DCs. (**G-J**) Comparisons of the positive ratio of PVR among the three groups in (**G**) all monocytes, (**H**) CD16- monocytes, (I) CD16+ monocytes, and (**J**) conventional DCs .

Minor points

-5. This manuscript should be further revised in English. For example, in line 43 (... peripheral blood would ideal due...) needs a 'be' after 'would'. Other parts of the text should also be carefully checked.

Response 6: Thank you for your valuable feedback. We have revised the sentence in line 43 as follows:

"... peripheral blood would be ideal due ..."

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We have also checked the entire manuscript carefully, making necessary corrections to grammar and spelling. We hope that these changes will enhance the quality and clarity of our manuscript.

-6. Abbreviations in the figures should be clearly stated in the respective figure legends. For example, FCP, SCP, and GADA in Figure 1. The legends of other diagrams should also be carefully modified.

Response 7: Thank you for pointing this out. We have defined those abbreviations, such as FCP, SCP, and GADA, in the legends of Figure 1 and all other figures in the manuscript.

-7. Figures do not match the textual description; it is assumed that Fig2I and Figu2J are reversed.

Response 8: Thank you for pointing this out. The error has been rectified.

8.The explanations in the Figure legend are not adequate. For example, which dot plot in Fig7AB shows which patient? What do the numbers 0-3 indicate in Fig7C? The legend to Figure 8 should state the numbers of mice used and the experiments performed, etc.

Response 9: Thank you for your valuable feedback regarding the figure legends. We have revised them to make the figures clearer and more understandable.
The revised version is as follows:
"Representative flow cytometry plots and quantification for (**A**) TIGIT$^+$ CCR7$^-$ Tregs, and (**B**) CD226$^+$ CCR7$^-$ CD8$^+$ T cells in PLN and spleen of 4-, 8-, and 12-week-old NOD mice, compared with C57 controls (n=4–7). Symbols represent each group: C57 mice (circles), 4-week NOD mice (squares), 8-week (upright triangles), and 12-week NOD mice (inverted triangles). (**C**) HE staining in four groups. Insulitis scoring across the four groups (n=4–7) is depicted as follows: Grade 0, no lymphocytic infiltration (grey bars); grade 1, <30% infiltration (dark grey bars); grade 2, <50% islet infiltration (blue bars); grade 3, >50% islet infiltration (red bars)."
As for Figure 8, each group—control and Anti-CD226—started with 10 C57 mice. Diabetes was induced with 40 mg/kg STZ injections daily for 5 days. The Anti-CD226 group then received 25 μg/mouse of anti-CD226 mAb every three days for a total of four doses, while controls received Isotype injections. We euthanized half the mice from each group post-treatment to assess immune changes and the rest at the observation period's end for immune phenotype analysis. Initially, data from all 10 mice were analyzed. However, due to the lack of long-term immune tolerance from anti-CD226 treatment and potential variation in the immune landscape at different post-treatment intervals, the revised analysis includes only data from the first 5 mice. We have clarified this point in the revised version.
Given that the C57BL/6 strain lacks a natural predisposition for autoimmunity, we adopted the NOD Cy-accelerated model and confirmed that reducing CD226$^+$ CD8$^+$ T cell expression delays the onset of T1D (Figure R5; see also Figure 8J-O in the main text). However, due to the significant impact of Cy on Treg cells in NOD mice, the study did not investigate changes in the Treg subpopulations.

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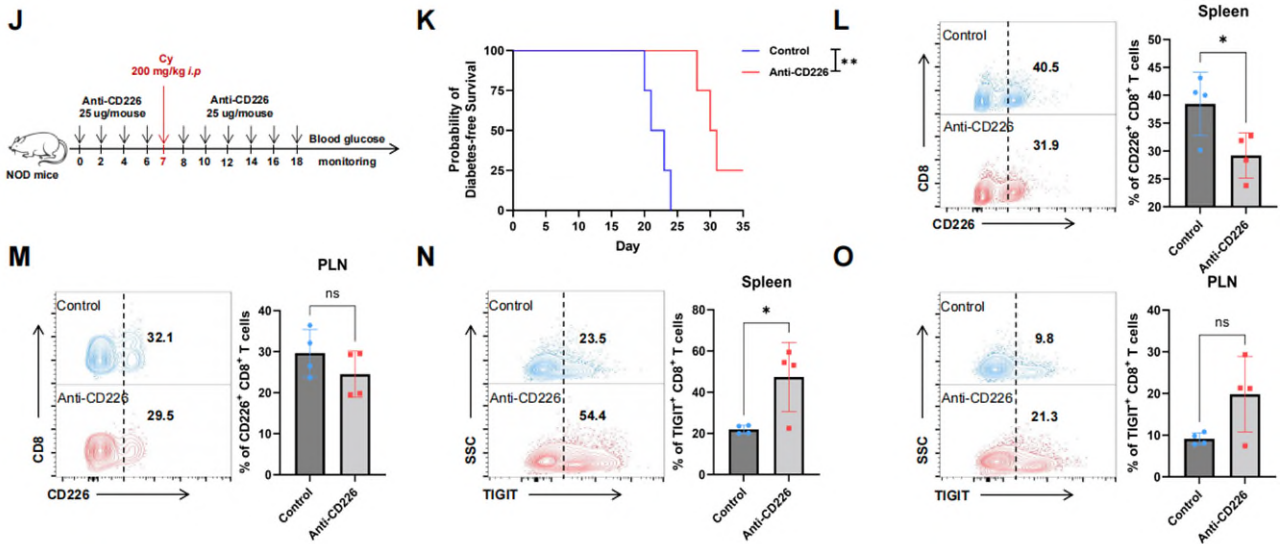

Figure R5 (Figure 8J-O in the main text) Anti-CD226 therapy alleviates hyperglycemia and promotes immune equilibrium in Cy-induced T1D mice. Female NOD mice aged 12 weeks (n=8 per group) were administered a single Cy dose of 400 mg/kg intraperitoneally to precipitate diabetes. The Anti-CD226 group was treated with 25 μg of anti-CD226 mAb every other day for 10 doses, while the control group received isotype injections. Four mice from each group were sacrificed for immune profiling post-treatment, with the remainder observed subsequently to assess the diabetic incidence. (**J**) Schematic diagram for the therapeutic experiment. (**K**) Survival curves comparing the incidence of diabetes between the two groups (n = 4). Significance determined by Log-rank (Mantel-Cox) test. (**L-O**) The proportion of $CD226^+$/$TIGIT^+$ $CD8^+$ T cells in the spleen and PLN are depicted for the two groups. Two-tailed unpaired t-test; data are shown as the mean ± SD. *P < 0.05. Cy, cyclophosphamide.

-9. Lines 237-238: "Additionally, there were no significant differences in the functions of the two cell subsets between the T1D and HD groups (Fig. 5E-J)", but Fig 5 shows the results comparing $CD226^+$ $CD8^+$ T cells with $TIGIT^+$ $CD8^+$ T cells and does not contain data comparing T1D with HD.

Response 10: Thank you. We acknowledge that the initial versions of these figures focused on comparing $CD226^+$ $CD8^+$ T cells with $TIGIT^+$ $CD8^+$ T cells within the T1D patient group and inadvertently omitted the direct comparative analysis between T1D patients and healthy donors. We have clarified the group analyses in the revised figures and provided detailed explanations in the accompanying legends.
As indicated in the legend of the figure below: Red and blue "*" denote statistical significance within T1D and HDs, respectively, between $CD226^+$ and $TIGIT^+$ $CCR7^-$ $CD8^+$ T cell subpopulations; "#" and "†" signify statistical significance of marker expression in $CD226^+$ and $TIGIT^+$ $CCR7^-$ $CD8^+$ T cells, respectively, between T1D and HDs.

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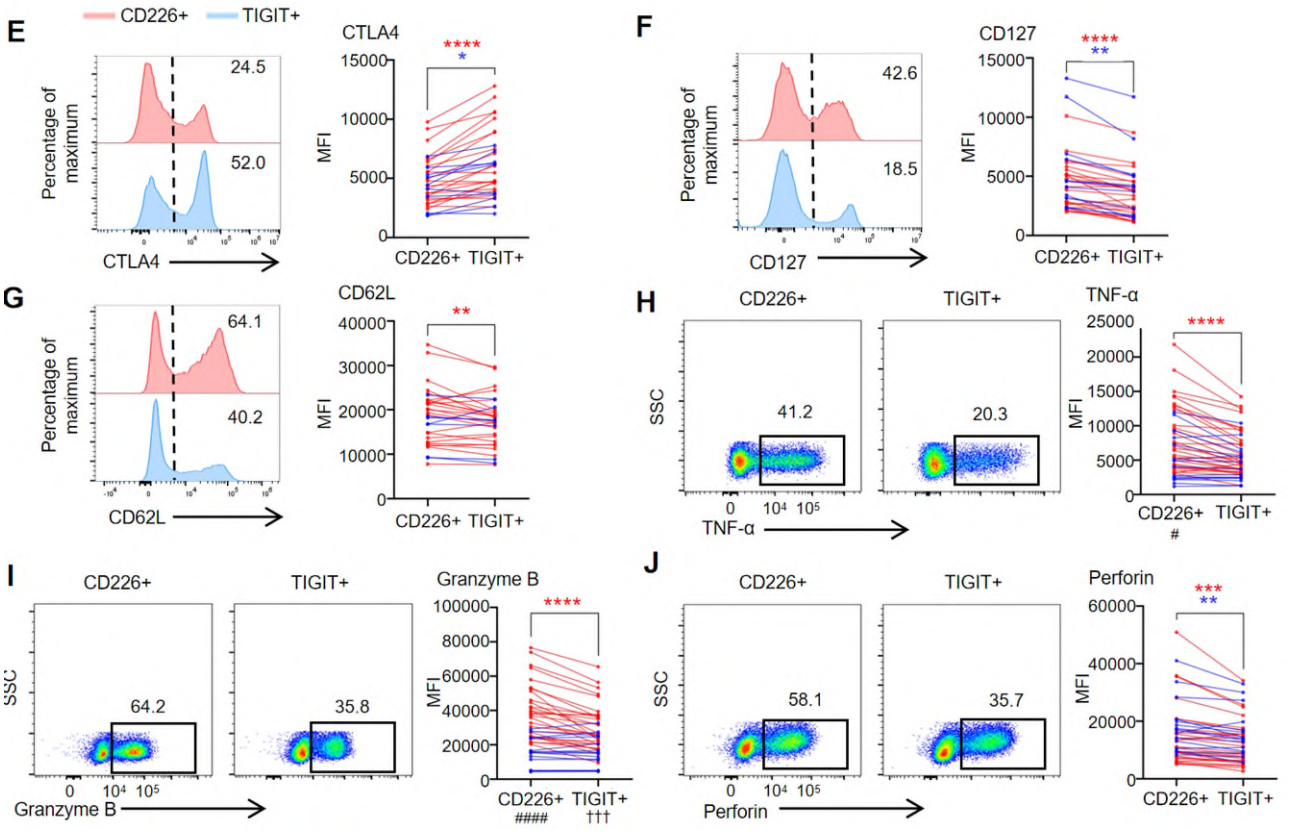

Figure R13 (now Figure 5E-J in the main text) Changes in the functional makers between two distinctive CD8$^+$ T subsets. (**E-J**) Representative diagrams and quantification of (**E**) CTLA4, (**F**) CD127, (**G**) CD62L, (**H**) TNF-α, (**I**) Granzyme B, and (**J**) perforin in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cell subpopulations; "#" and "†" signify statistical significance of marker expression in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells, respectively, between T1D and HDs. */#$P < 0.05$; **$P < 0.01$; ***/†††$P < 0.001$; ****/####$P < 0.0001$.

Reviewer #3: In this MS, Zhong and colleagues perform a detailed single-cell RNAseq analysis of circulating immune cells in new-onset T1D children with and without partial remission and in healthy controls. Starting from a limited set of samples, they describe a population of TIGIT$^+$ Tregs and CD226$^+$ CD8$^+$ T cells and suggest that there may be a TGF-β-mediated crosstalk between the two. They then build on a much larger sample set to confirm these results by flow cytometry, showing that these TIGIT$^+$ Treg and CD226$^+$ CD8$^+$ T-cell population are respectively positively and negatively correlated with stimulated C peptide levels and respectively enriched and depleted in patients under partial remission. They conclude with studies in a low-dose STZ model of autoimmune diabetes by

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showing that a CD226 antibody reduced disease penetrance and insulitis.The study is rigorously conducted and, despite its complexity, well written and easy to follow throughout. Clearly, these results are of importance. There are some points that however deserve attention to make the conclusions more convincing.

Response 1: Thank you for your thorough evaluation of our manuscript. We are pleased to hear that you appreciate the significance of our findings for the field. Please see our detailed, point-by-point response that follows.

-1. The definition of remission as a MMTT-stimulated C-peptide level ≥300 pM seems very loose. On these grounds, most of these patients would have significant insulin needs, while modest needs are the essence of the clinical definition of the honeymoon period. This definition, as the other one used of an IDAA1C ≤9, seems rather to indicate a partial remission, and this should be clearly stated from start (and indeed it is the definition used later in the paper). Indeed, stimulated C-peptide is ultimately the most distinctive feature between the 2 groups, while other parameters show some degree of overlap. Stimulated C- peptide is also the parameter better correlated with $TIGIT^+$ Treg increase and $TIGIT^-$ Treg decrease (Fig. 3C-D), and with $CD226^+$ $CD8^+$ decrease and $TIGIT^+$ $CD8^+$ increase (Fig. 5C-D).

Response 2: We greatly appreciate your insightful comments regarding our definition of remission based on MMTT-stimulated C-peptide levels. We understand your concern that our criteria for remission, defined as a stimulated C-peptide level ⩾300 pmol/L, may appear to be less stringent than the clinical definition typically associated with the honeymoon phase, which is characterized by modest insulin requirements.
Upon reflection, we concur that the term "partial remission" more accurately characterizes the status of the patients and aligns with the clinical observations of their insulin needs. We have revised our manuscript to consistently refer to this state as "partial remission" throughout the text. This change will clarify the clinical status of the patients under study and ensure that our terminology reflects the most appropriate clinical context.
Furthermore, we agree that the distinction between full and partial remission is crucial, particularly since stimulated C-peptide levels serve as a key differentiator between the groups we examined. As you have pointed out, this parameter not only distinguishes the groups more clearly but also shows a strong correlation with the changes in $TIGIT^+$ Treg and $CD226^+$ $CD8^+$ T cell populations (as demonstrated in Figures 4C-D and 5C-D).

-2. Fig. 2H: the fact that $TIGIT^+$ $CCR7^-$ Tregs decline post-remission is mentioned in the text but now shown. Moreover, these differences across groups are quite modest and should be statistically supported.

Response 3: In consideration of the limited sample size in our single-cell sequencing analysis, we

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identified trends in the $CCR7^+$ $TIGIT^-$ Tregs without achieving statistically significant differences. To ensure the reliability of our findings, we subsequently conducted extensive flow cytometry validation in a cohort with a larger sample size, confirming the observed trends. In the manuscript on Line 149-151, we have modified the relevant statement to: "In contrast, we noted an increasing trend in the proportion of $CCR7^+$ $TIGIT^-$ Tregs in the new-onset group, but a decreasing trend during the PR period (Fig. 2H)".

-3. Fig. 3B: the rationale of excluding $CCR7^+$ $KLRG1^-$ $CD8^+$ T cells as 'non-effectors' is not clear – couldn't these cells also include CM subsets?

Response 4: Thanks. The exclusion of $CCR7^+$ $KLRG1^-$ $CD8^+$ T cells as 'non-effectors' was based on our aim to focus specifically on effector memory (EM) and terminally differentiated effector memory (TEMRA) subsets, which are usually characterized by the downregulation of CCR7 and high KLRG1 expression. In consideration of your suggestion, we have revised the nomenclature for the $CCR7^+$ $KLRG1^-$ $CD8^+$ T cells in Figure 3B to '$CCR7^+$ $CD8^+$ Tn/Tcm' to more precisely reflect the potential inclusion of naive (Tn) and central memory (Tcm) subsets within this population. This adjustment aims to ensure the accuracy and rigor of our results. We appreciate your insightful feedback, and this modification will be incorporated into the updated manuscript (Line177-178).

-4. Fig. 4E-I: the legend should indicate that red lines are T1D patients, blue lines are HDs. What is the statistical test to which the asterisks refer to? It is said that there is a difference between T1D and HDs in the marker/cytokine profile of $TIGIT^+$ but not $TIGIT^-$ Tregs, but the test seems then to compare the 2 groups of Tregs but not the 2 groups of donors – the statistical comparison of the 2 donor groups should also be shown in these figures. The same applies to Fig. 5E-J.

Response 5: Thank you for your attention to detail in our figure legends and your constructive comments regarding the statistical analysis presented in Figures 4E-I and 5E-J. We have amended the legends to clearly denote that the red lines represent T1D patients and the blue lines represent HDs. We have also clarified the group analyses directly in the updated figures and provided detailed explanations in the accompanying legends. The specifics are shown in the following figures:

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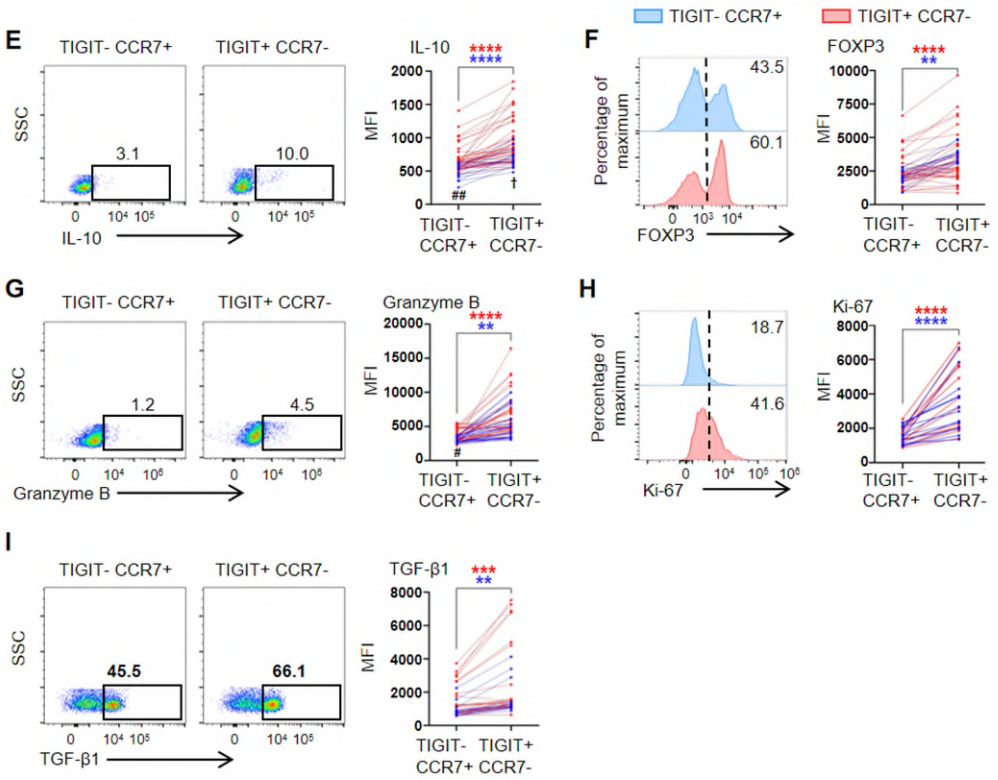

Figure R14 (now Figure 4E-I in the main text) Changes in the functional makers between two distinctive Treg subsets. (**E-I**) Representative diagrams and quantification of (**E**) IL-10, (**F**) FOXP3, (**G**) Granzyme B, (**H**) Ki-67 and (**I**) TGF-β1 in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Treg subpopulations; "#" and "†" signify statistical significance of marker expression in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs, respectively, between T1D and HDs. */†$P < 0.05$; **/##$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.

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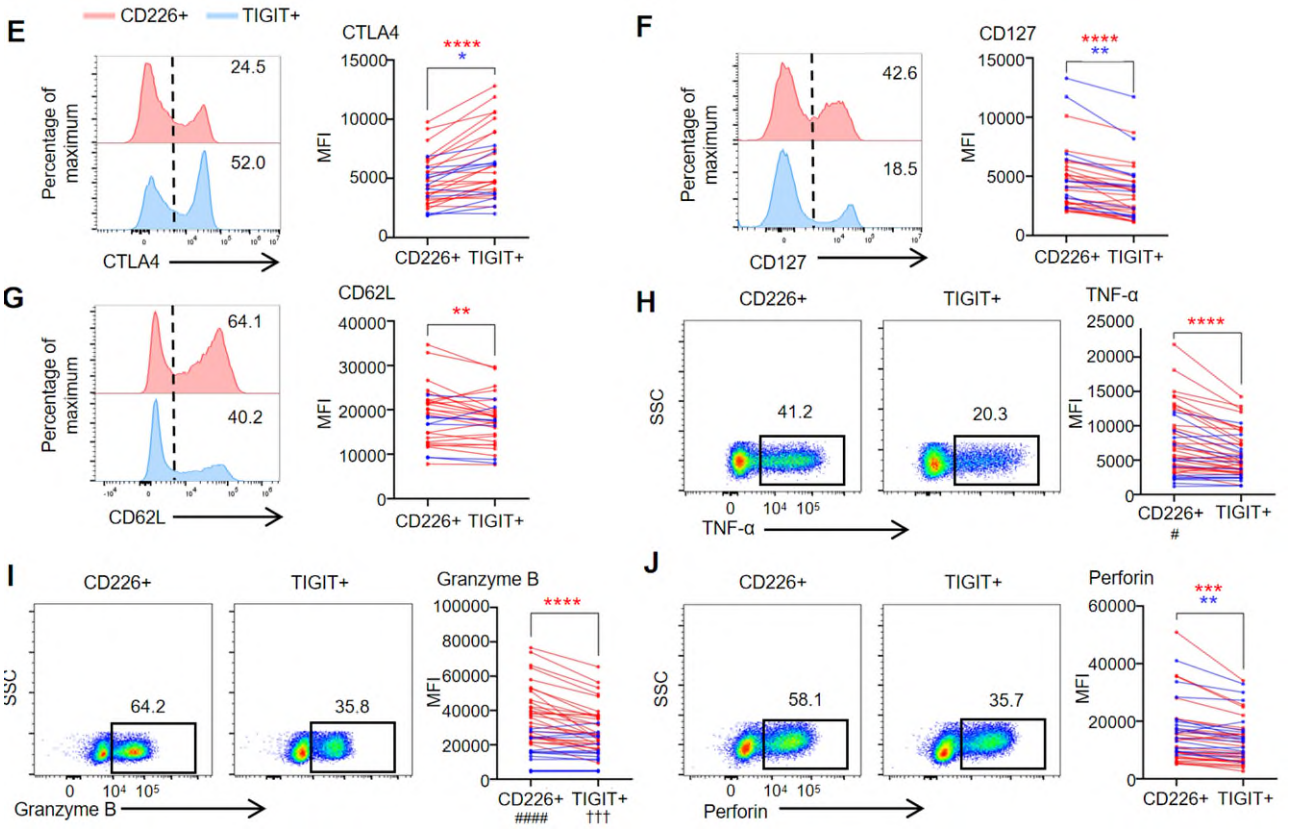

Figure R13 (now Figure 5E-J in the main text) Changes in the functional makers between two distinctive CD8$^+$ T subsets. (**E-J**) Representative diagrams and quantification of (**E**) CTLA4, (**F**) CD127, (**G**) CD62L, (**H**) TNF-α, (**I**) Granzyme B, and (**J**) perforin in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cell subpopulations; "#" and "†" signify statistical significance of marker expression in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells, respectively, between T1D and HDs. */#$P < 0.05$; **$P < 0.01$; ***/†$P < 0.001$; ****/####$P < 0.0001$.

-5. Fig. S5C: the gating strategy would be clearer with a dot plot depicting TIGIT and C226 simultaneously, so to appreciate the distinction and overlap of the 2 subsets (as done in Fig. S6 for Tregs). It would also make sense to invert these 2 supplementary Figs as Tregs are presented first in the text.

Response 6: Thank you. We agree that including a dot plot showing TIGIT and CD226 simultaneously would better illustrate the distinction and potential overlap between the two CD8$^+$ T cell subsets. Regarding the order of the supplementary figures, we inverted their positions in the supplementary material so that the presentation of Tregs precedes that of the CD8$^+$ T cell subsets,

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aligning with their discussion in the manuscript. The revised gating strategy for the $CD8^+$ T cell subpopulations is depicted in the diagram below:

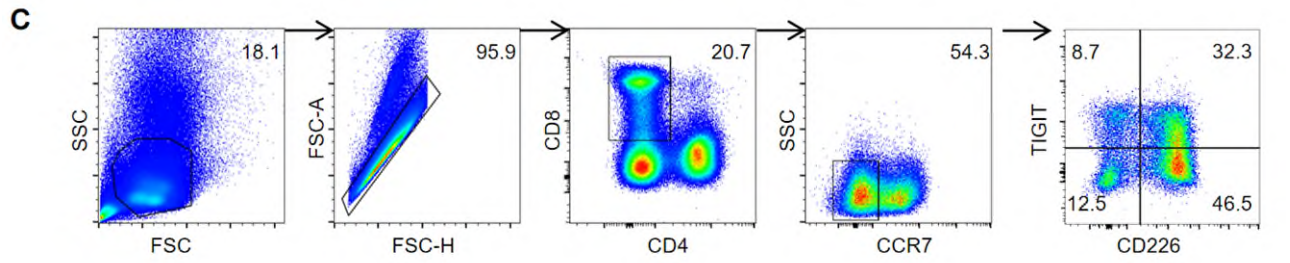

Figure R15 (now Figure S9C) Gating strategy of $CD226^+$ and $TIGIT^+$ $CCR7^-$ $CD8^+$ T-cell populations.

-6. Was the machine learning algorithm also applied to $TIGIT^+$ Tregs, and what was the result? This should also be showed even if negative.

Response 7: We explored the predictive value of TIGIT-associated Treg subsets in additional cohorts (Fig. S10A-B) through similar methods to the CD226 clusters mentioned in the text (Method "Construction and evaluation of machine learning model" part and Fig. 6). The results showed that the classifier constructed by the CatBoost algorithm can distinguish T1D patients with rapid islet function failure with a good performance of an average AUC of 0.73 (Fig. S10C-E). However, due to sample limitations, we were unable to validate this model with an external cohort.

-Fig. 7C: the correlations of $TIGIT^+$ Tregs and $CD226^+$ $CD8^+$ T cells with the severity of insulitis should be shown similarly to what done with C-peptide in T1D patients.

Response 8: Thanks. In response to your comment regarding Figure 7C, we adjusted our presentation to show the correlations of $TIGIT^+$ Tregs and $CD226^+$ $CD8^+$ T cells with the severity of insulitis, as we did for C-peptide levels in T1D patients. As depicted in the accompanying Figure R16, Spearman's correlation analysis was employed to assess the association between T cell subsets and the severity of insulitis. Our results indicate an inverse correlation between $TIGIT^+$ Tregs, obtained from both pancreatic lymph nodes (PLNs) and spleen, and insulitis severity. In contrast, $CD226^+$ $CD8^+$ T cells exhibited a positive correlation with the progression of insulitis. These modifications have been incorporated into the updated manuscript.

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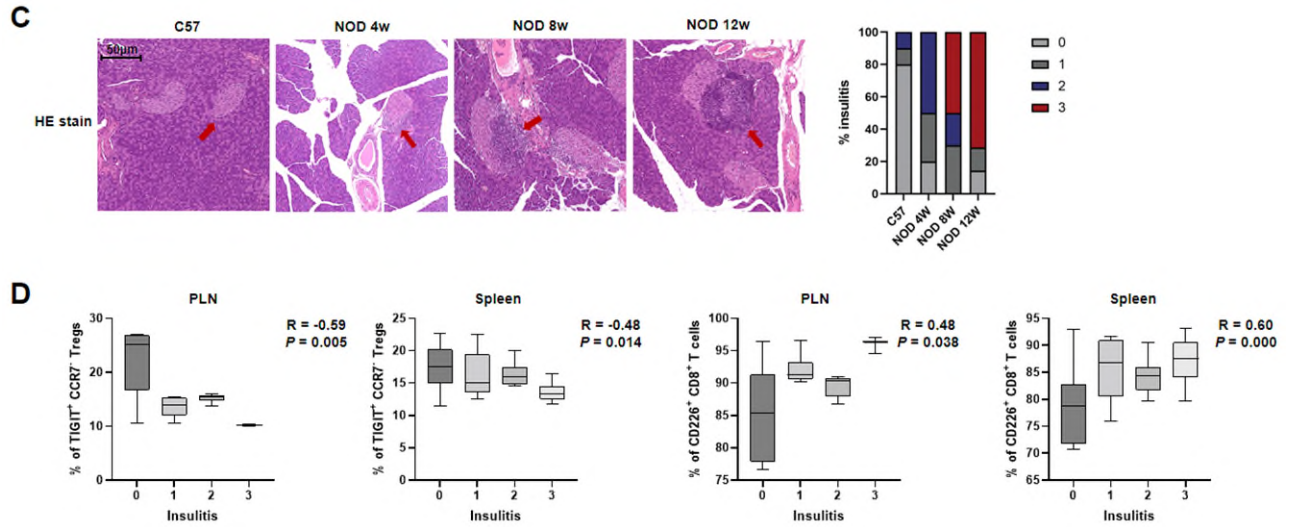

Figure R16 (now Figure S11) Correlations of TIGIT$^+$ CCR7$^-$ Tregs and CD226$^+$ CD8$^+$ T cells with insulitis in NOD mice. (**C**) HE staining in four groups. Insulitis scoring across the four groups (n=4–7) is depicted as follows: Grade 0, no lymphocytic infiltration (grey bars); grade 1, <30% infiltration (dark grey bars); grade 2, <50% islet infiltration (blue bars); grade 3, >50% islet infiltration (red bars). (**D**) Box plots showing the association between T cell subsets and the severity of insulitis. Data are presented as the mean ± SD. *P* values were calculated using Spearman's correlation analysis.

-8. Finally and more importantly, the immunosuppressive activity of human TIGIT$^+$ Tregs should be validated with functional suppression assays and compared with that of TIGIT$^-$ Tregs. The same applies for CD226$^+$ vs. TIGIT$^+$ CD8$^+$ T cells for their cytotoxic activity. These experiments could also allow to validate the TGF-beta-mediated cross-talk between the Treg and CD8$^+$ T-cell subsets.

Response 9: Thank you for your suggestion. We sorted TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ Tregs and co-cultured them with CD8$^+$ T cells at ratios of 1:1, 1:2, 1:4, and 1:8 for 48 hours to assess the proliferation of the CD8$^+$ T cells. As illustrated in the Figure R16, TIGIT$^+$ CCR7$^-$ Tregs exhibited a stronger immunosuppressive effect on CD8$^+$ T cells compared to TIGIT$^-$ CCR7$^+$ Tregs, as indicated by the levels of Ki-67 within the CD8$^+$ T cells.

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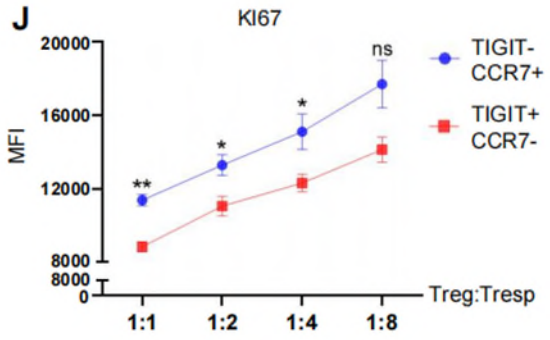

Figure R17 (now Figure 4J in the main text) Suppression assay of Treg subsets. TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Treg cells, isolated from T1D patients (n = 6), were cocultured with autologous CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 48 h at 37°C. Ki-67 in CD8$^+$ T cells was measured. Two-tailed unpaired t-test; data are shown as the mean ± SEM. *P < 0.05; **P < 0.01

For the assessment of cytotoxic activity, CD8$^+$ T cells were initially enriched through positive selection using magnetic CD8$^+$ beads provided by Miltenyi Biotec. Following enrichment, CD226$^+$ and TIGIT$^+$ CD8$^+$ T cell subsets were precisely isolated via flow cytometry sorting. These isolated subsets were subsequently cocultured with P815 mastocytoma cells for a duration of 48 hours to evaluate apoptotic induction in the target cells. The resulting data, illustrated in the corresponding Figure R3, demonstrate that CD226$^+$ CD8$^+$ T cells exert a significantly enhanced cytotoxic effect on P815 cells when compared to TIGIT$^+$ CD8$^+$ T cells, as indicated by an increased rate of apoptosis in the P815 cell population.

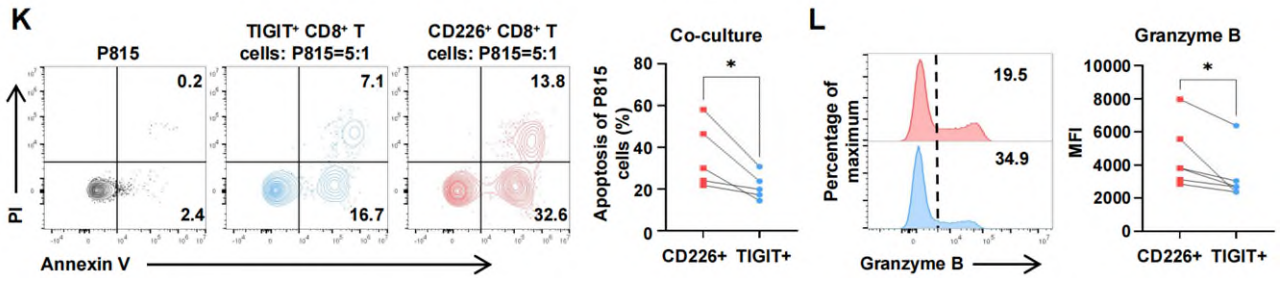

Figure R3 (now Figure 5K-L in the main text) Assessment of cytotoxic activity of CD8$^+$ T-cell subsets. CD226$^+$ and TIGIT$^+$ CD8$^+$ T cells, isolated from T1D patients (n = 6), were cocultured with fluorescently-labeled P815 cells at a ratio of 5:1 for 5 h at 37°C. (**K**) Representative flow cytometry plots and quantification of apoptosis in P815 cells. (**L**) Representative peak plots and quantification of granzyme B expression within CD8$^+$ T-cell subsets. Two-tailed paired t-test. *P < 0.05.

We also conducted precise sorting of TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ Treg subsets, followed by their respective co-culturing with CD226$^+$ CCR7$^-$ CD8$^+$ T cells. Our observations revealed that TIGIT$^+$ CCR7$^-$ Tregs exert a more potent suppressive effect on CD226$^+$ CCR7$^-$ CD8$^+$ T cells compared to their TIGIT$^-$ CCR7$^+$ counterparts. These findings have been detailed and are now presented in the updated Figures 7G-I (Figure R4) in the revised manuscript.

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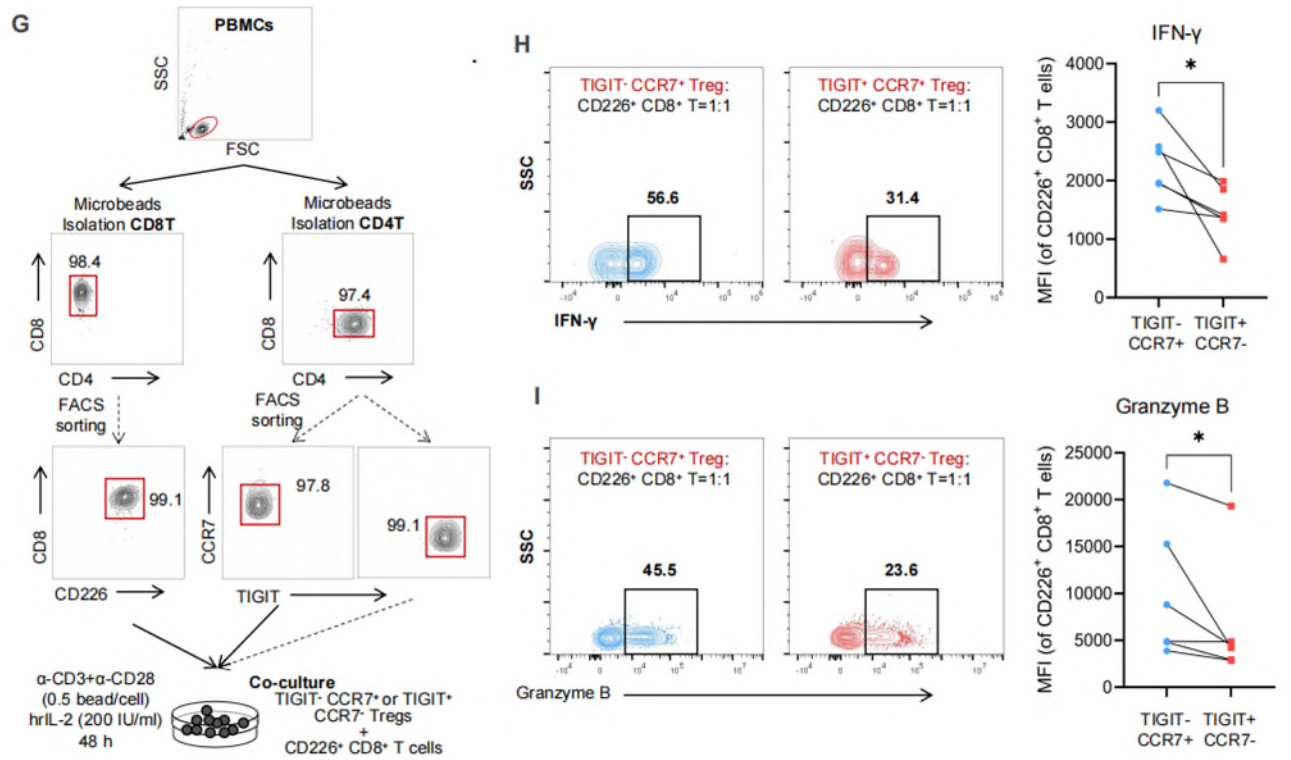

Figure R4 (Figure 7G-I in the main text) Co-culture of TIGIT$^+$ CCR7$^-$ Tregs with CD226$^+$ CCR7$^-$ CD8$^+$ T cells. (**G**) Diagram illustrating the experimental setup. Representative flow plots and expression levels of (**H**) IFN-γ , and (**I**) granzyme B in CD226$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients following 48 hours of co-culture with TIGIT$^+$ CCR7$^-$ or TIGIT$^-$ CCR7$^+$ Tregs at a 1:1 ratio (n = 6). Two-tailed paired t-test. *P < 0.05.

We have incorporated the results of these functional assays into the revised manuscript and expect that they will provide valuable insights into the immunoregulatory dynamics between these T cell subsets.

Minor points:
9. Abstract is missing from the MS.

Response 10: Apologies for uploaded an incomplete version of the manuscript. We have fixed this.

10. The Introduction is quite comprehensive but may need some shortening.

Response 11: Thank you for the feedback. We revised the Introduction and condense it, ensuring that it remains comprehensive while focusing on the most essential points and background necessary for understanding the context and significance of the study.

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11. Gene names should be in italics.

Response 12: Thank you for bringing this to our attention. We have reviewed the manuscript and corrected the formatting of all gene names by italicizing them, in accordance with the standard scientific nomenclature.

12. The “lymphocyte activation cocktail” used to stimulate PBMCs should be detailed.

Response 13: Thanks. We have revised the Methods section accordingly. The updated protocol is as follows: "PBMCs were stimulated for 4–6 hours using a lymphocyte activation cocktail (BD Bioscience, San Jose, CA, USA), which included PMA (Phorbol 12-Myristate 13-Acetate), a calcium ionophore (Ionomycin), and the protein transport inhibitor BD GolgiPlug™ (Brefeldin A), in accordance with the manufacturer's recommendations."

Line 280: “cytokine screening ability” should read “cytokine secretion ability”.

Response 14: Thank you for pointing out the typo. The text on Line 328 was corrected to "cytokine secretion ability" to accurately reflect the intended meaning.

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Reviewer #4 Zhong et al. submitted a paper titled "TGF-β-mediated crosstalk between TIGIT$^+$ Tregs and CD226$^+$ CD8$^+$ T cells in the progression and remission of type 1 diabetes" for publication in Nature Communications. The authors used scRNA-seq to identify Treg and CD8$^+$ T cell subtypes that were demonstrated to have different abundance levels in the different disease stages of patients diagnosed with type 1 diabetes. The authors used an extensive number of approaches to demonstrate the clinical significance of these T cell subtypes. Overall, I find this study to be well executed and highly interesting. However, I have some major and minor concerns related to the data analysis and the manuscript.

Response 1: We greatly appreciate your constructive feedback on our manuscript. We have meticulously addressed each of the major and minor concerns in our revised manuscript, as detailed below.

Major comments

-1. Figure 1C shows the UMAP plot of the whole scRNA-seq dataset with the main cell types colored. While the authors have done good job at annotating most of the main cell types, they reported a few unusual cell types, namely granulocytes, double-negative T (DNT) cells and NKT cells. Since the cells should be PBMCs (Fig. 1B), why were there granulocytes?

Response 2: Thanks. Following your suggestion, we annotated the cells using CellTypist, and the "Immune_All_Low" model categorized these granulocytes as Mast cells, a subtype of granulocytes. Regarding the presence of granulocytes in the PBMC dataset, we think that this may be related to technical factors introduced during the collection and processing of samples. In our study, we employed the standard Ficoll-Paque Plus density-gradient centrifugation method, as detailed in the Methods section. Theoretically, granulocytes should sediment to the bottom during centrifugation; however, practical considerations, such as variations in individual immune states and operating conditions, may result in a small collection of lighter granulocytes within the lymphocyte layer. Nevertheless, their presence is extremely limited. Additionally, quality control analysis reveals that cells labeled as granulocytes exhibit lower total UMI and gene features (Fig. S1). We explained this phenomenon in the text at Line 120-123 and marking these cells as of lower quality.
DNTs exhibit the CD3+CD4-CD8- phenotype, and quality control procedures did not reveal any anomalies in their quality. CellTypist annotated DNT as gdT (an innate immune lymphocyte which containing a high proportion of CD4- CD8- T cells), however, DNT cells also contain cells with abTCR. Regarding NKT cells in our dataset, CellTypist annotated this group of cells as NK (using the "Healthy_COVID19_PBMC" model) or as a mixture of NK and cytotoxic T cells (using the "Immune_All_Low" model). In our dataset, NKT cells exhibit features of NK cells but also express high levels of CD3 and low levels of CD8. Hence, we consider these cells to represent NKT characteristics. Additionally, existing literature supports the presence of a certain number of NKT

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cells in peripheral blood (PMID: 32528956).
To ensure caution and rigor in our analysis, we mentioned in the manuscript the need for careful consideration when analyzing the three unusual cell types (granulocytes, DNTs, NKTs). For a comprehensive overview, we have included the complete list of cell type markers for various immune cells in the supplementary file (sup-file-1).

-2. Please provide the complete list of the cell type markers results obtained using Seurat's FindAllMarkers function. The dotplot (Fig. 1D) that shows a few selected genes per cluster is not enough. This could be a supplementary file (Excel file), and it would give readers a better sense of the different cell types and their gene markers.

Response 3: Thanks. We appreciate your suggestion to provide a more comprehensive list of cell type markers obtained using Seurat's FindAllMarkers function. We have provided a detailed list of cell type markers and present it as a supplementary file (sup-dataset-1. This additional resource will offer readers a more thorough understanding of the different cell types and their associated gene markers.

-3. The authors should provide violin plots of key quality control metrics for the main cell types (Fig. 1C) in the supplement. Please include the percentage of mitochondrial reads per cell, the percentage of ribosomal reads per cell, the total number of UMIs per cell, and the number of genes per cell. The violin plots must be grouped by the cell type so that it's possible to compare the metrics between the cell types. If some of the cell types, such as DNTs or c5 Tregs, exhibit highly aberrant quality metrics, they should preferably be removed or the very least be marked as poor-quality cells.

Response 4: Thanks. We appreciate your guidance in improving the presentation of our data and ensuring transparency in the quality assessment of the cell types. We have incorporated violin plots of key quality control metrics for the main cell types in the supplementary material (Fig. S1). The violin plots are grouped by cell type, allowing for a direct comparison of metrics across different cell types. Additionally, we have provided a more detailed description of the quality control procedures in the Methods section to enhance clarity on the applied measures.

-4. Related to the 3rd comment, the authors should explain in more detail how they performed quality control to remove bad cells.

Response 5: Thanks. To ensure data reliability, we conducted at least two rounds of filtering for low-quality cells, doublet detection, and cell type annotation. Detailed information has been supplemented in the Methods section, with a corresponding flowchart provided in Fig. S1A. Specifically, after the initial quality control on cells processed by Cell Ranger, we performed the first round of filtering based on the conditions of removing genes detected in fewer than 3 cells and excluding cells with fewer than 200 detected genes (min_genes=200, min_cells=3). Subsequently, we applied scDblFinder for doublet detection. The criteria for inclusion in the second round of filtering include the number of

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genes between 200 and 6000, the total number of UMIs ranging from 500 to 34000, and mitochondrial gene percentages below 13% (200<nFeature<6000, 500<nCount<34000, and Percent.mt<13). Following these steps, we initiated the clustering and manual annotations process on the overall cell population, guided by CellTypist's two models for major cell types (Fig. S2). Notably, the second-round filtering already addressed the exclusion of a portion of doublets (Fig. S1), often characterized by exceptionally high UMI and gene counts. Subsequently, during annotation, we carefully detected and removed remaining doublets, informed by scDblFinder results, gene markers, and clustering outcomes. For major cell subgroups, as mentioned in the manuscript (e.g., $CD8^+$ T and $CD4^+$ T), we performed secondary clustering to refine immune phenotyping.

5. How did the authors deal with doublets in scRNA-seq analysis? Please use doublet detection methods such as scDblFinder to detect doublets and remove any cells that are doublets with high probability (based on the tool's prediction and gene markers).

Response 6: Thank you for your inquiry regarding the handling of doublets in our scRNA-seq analysis. Following the initial filtration step (min_genes=200, min_cells=3) outlined in the methods, we employed scDblFinder for doublet detection. Subsequently, we conducted a second round of filtering based on the conditions mentioned earlier (200<nFeature<6000, 500<nCount<34000, and Percent.mt<13). This second round of filtering effectively addressed the exclusion of a subset of doublets, as illustrated in Fig. S1. We believe that the remaining doublets marked by scDblFinder should be handled with caution. During the annotation phase, we meticulously detected and removed any lingering doublets, guided by scDblFinder predictions, gene markers, and clustering results.

6. The authors should provide some independent cell type assignment for the main cell types (Fig. 1C). This can be easily done using tools such as CellTypist (https://www.celltypist.org/). This may also help annotate the three unusual cell types (granulocytes, DNT, NKT).

Response 7: Thanks. Following your suggestion, we annotated the cells based on two models ("Immune_All_Low" and "Healthy_COVID19_PBMC") of CellTypist. Guided by the CellTypist results, we conducted two rounds of annotation on major cell types (the second round of cell types was more refined). Comparison of manually annotated results with CellTypist is shown in Sup-Fig. The "Immune_All_Low" model categorized granulocytes as Mast cells, a subtype of granulocytes. Regarding the presence of granulocytes in the PBMC dataset, we think that this may be related to technical factors introduced during the collection and processing of samples. DNTs exhibit the $CD3^+$ $CD4^-$ $CD8^-$ phenotype, and quality control procedures did not reveal any anomalies in their quality. CellTypist annotated DNT as gdT (an innate immune lymphocyte which containing a high proportion of CD4-CD8- T cells), however, DNT cells also contain cells with abTCR. Regarding NKT cells in our dataset, CellTypist annotated this group of cells as NK (using the "Healthy_COVID19_PBMC" model) or as a mixture of NK and cytotoxic T cells (using the "Immune_All_Low" model). In our dataset, NKT cells exhibit features of NK cells but also express high levels of CD3 and low levels of

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CD8. Hence, we consider these cells to represent NKT characteristics. Additionally, existing literature supports the presence of a certain number of NKT cells in peripheral blood (PMID: 32528956).

7. One major limitation in the machine learning model is that the authors did not validate their model using an independent cohort. The model was just built based on a single cohort, and the authors basically performed cross validation to evaluate the model's performance, which should generally be only used to optimize the model and not as the final performance assessment. A completely independent test set that the model has never seen before is crucial for assessing its generalization to new, unseen data. This set is used only once after the model is trained and validated. Please discuss this limitation.

Response 8: We acknowledge the limitation in our machine learning model due to the absence of an independent validation cohort. Recognizing the importance of a rigorous approach, we have addressed this limitation by acquiring a new, independent prospective cohort (n=40) for external validation. This addition allows for a more comprehensive demonstration of the generalization capabilities of our study data and model. We ensured consistency in covariates between the discovery and external validation cohorts and supplemented the understanding of feature importance by calculating SHAP values. The details are shown in the results section "Predicting the pace of T1D β-cell function decline by using CD226 and TIGIT-associated T cell subsets" and Fig.6. Your valuable advice has prompted us to enhance the robustness of our analysis.

8. When the authors performed GSEA analysis, they performed prior gene filtering based on adjusted p-value of 0.05 (rows 485, 486). To my understanding, this gene filtering is not something that is required or usually done. What impact does this have on the result?

Response 9: We appreciate your meticulous review. The gene filtering based on an adjusted p-value of 0.05 was implemented as a cautious measure to focus the Gene Set Enrichment Analysis (GSEA) on a subset of genes with heightened statistical significance. While we recognize that this may not be a standard practice in all GSEA analyses, it was done to ensure a conservative approach. To address your concern, we have carefully reassessed the impact of this gene filtering on our results. We found that GSEA results without prior gene filtering are consistent with our initial findings, and there is no substantial alteration in the conclusions. But in order to more standardize our analysis process, we performed GSEA analysis using all genes without p-value filtering (Fig.4H) according to your suggestion. Your insights have been invaluable in refining our analysis approach.

-9. The codes for reproducing the analyses should be provided.

Response 10: Thank you. The code for the analysis is available at: "https://github.com/Lxy-

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xyeyy/scRNAseq-T1D".

Minor comments

-1. Although some software versions are marked, some are not. Please add the missing software versions.

Response 11: Thank you for bringing this to our attention. We have now updated the manuscript to include all the missing software version details, ensuring complete transparency and reproducibility of our computational analyses. We appreciate your thorough review and guidance.

-2. Cellranger provides a set of quality control metrics in the HTML web summary files. Please provide a summary of these metrics in the supplement.

Response 12: Thank you for highlighting the importance of quality control metrics. We have now included a summary of the relevant metrics from the Cellranger analysis in Table.S1 as supplementary materials.

-3. “Perform dimensional reduction and clustering” part of the method chapter requires clarification. Prior to PCA, Seurat selects a fixed number of highly variable genes (HVGs). What is the final number of HVGs? What was the HVG selection method used in FindVariableFeatures? How many principal components were selected after HVG selection? I find “non-linear t-SNE” as a term a bit weird. I think simply “t-SNE” would be better.

Response 13: Prior to PCA for the overall PBMC cell subpopulations, the final number of Highly Variable Genes (HVGs) selected was set at 4000. In the subsequent round of clustering for specific cell types like CD8+ T cells, 3000 HVGs were employed. The method for HVG selection used in FindVariableFeatures was the Variance Stabilizing Transformation (VST) method. Following PCA, the top 30 principal components were utilized for UMAP, and graph-based clustering was performed with a resolution parameter set at 1.2. Based on your suggestion, we have updated "non-linear t-SNE" to simply "t-SNE" for clarity. These additions and modifications have been incorporated into the "Perform dimensional reduction and clustering" section of the Methods.

-4. In “Batch effect correction”, please mention the batch variable name that was used for correction, such as sample.
Response 14: The batch variable used for correction is labeled as "Batch," representing the batch associated with sample collection and sequencing. We have supplemented the batch information for each sample in Table-S1 as supplementary materials.

-5. In “Detection of differentially expressed genes and functional enrichment analysis”, how was the

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pseudo-bulk analysis exactly done? What statistical test, how was the pseudo-bulk aggregation done (mean or sum aggregation)? In the same section, the authors mention both Seurat's FindMarkers and pseudo-bulk, but no clear explanation is given as to what comparisons were done using each method. Please explain this further.

Response 15: In the "Detection of Differentially Expressed Genes and Functional Enrichment Analysis" section, we conducted pseudo-bulk analysis using the muscat R package to explore differences in specific cell types between different disease groups, such as T1D-New vs T1D-PR. Pseudo-bulk analysis involved aggregating individual cell expressions at the sample level, and we utilized the sum aggregation method with default parameters. This approach aimed to capture group-level differences in gene expression. Simultaneously, Seurat's FindMarkers function was employed to detect differentially expressed genes (DEGs) at the single-cell level, focusing on identifying DEGs between specific cell types or conditions. For Seurat's FindMarkers, we employed the Wilcoxon rank-sum test as the statistical test. We appreciate your feedback, and provided a clearer and more concise explanation of these analyses in the revised manuscript (method section "Detection of differentially expressed genes and functional enrichment analysis").

-6. In "RNA-seq" part, no details of the data analysis are given. Please explain how the analysis was done. I think it would also make sense to rename the title to "Bulk RNA-seq".

Response 16: For the RNA-seq analysis, we followed a comprehensive workflow which includes the following steps:

Quality Control: Raw sequencing reads were first subjected to quality control using FastQC, to ensure data integrity.

Read Mapping: Clean reads were then aligned to the reference genome using the Spliced Transcripts Alignment to a Reference (STAR) aligner.

Quantification: Transcript abundance was quantified using featureCounts, which provides read counts for each gene.

Normalization and Differential Expression Analysis: The read counts were normalized to account for library size and other technical variations using the DESeq2 package. Differential expression analysis was then performed to identify genes with statistically significant changes in expression between conditions.

Functional Enrichment Analysis: Genes found to be differentially expressed were subjected to enrichment analysis using tools such as GOseq, to identify overrepresented biological processes and pathways.

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Data Visualization: We visualized the expression data through various plots, including heatmaps and volcano plots, to represent the expression patterns and significance of differentially expressed genes. We have amended the manuscript to include these details. Additionally, we agree with your suggestion to rename the section title to "Bulk RNA-seq" to accurately reflect the nature of the sequencing approach used. The title has been updated to avoid any confusion with single-cell RNA-seq methodologies.

-7. In “Construction and evaluation of machine learning model”, what are the different outcomes in “features, with different outcome events serving as the target variable”?

Response 17: We acknowledge the previous lack of clarity in our explanation. The target predictive variables were categorized based on two outcome groups: patients with a decline in C-peptide area under the curve (CP-AUC) exceeding 50% from baseline at the 18-month follow-up were classified as the "Fast" group, while those with a decline below 50% were categorized as the "Slow" group. In each repetition, we trained multiple classifiers on the training set, utilizing the frequencies of target cell types (derived from flow cytometry) as features, with different outcome events ("Fast group" or "Slow group") serving as the target variable. Based on your suggestion, we've revised this section (Lines 635-643) to provide more detailed information.

-8. In “Statistical analysis”, how exactly was “the adjustment for potentially confounding variables” done prior to ANOVA?

Response 18: We appreciate your interest in the statistical methodologies underpinning our study. To ensure a rigorous control of potential confounders, we employed a "matching" strategy that carefully balanced critical factors, including gender, age, and BMI, across comparative groups. Subsequently, we applied "multivariate logistic regression" to account for any remaining confounding influences, thereby sharpening the accuracy of our results.

-9. I found the main text to be well written. However, the data analysis part of the Methods chapter had clear grammatical errors.

Response 19: We are grateful for your positive comments about the main text of our manuscript. We acknowledge the grammatical issues you've identified in the Data Analysis section of the Methods chapter and have since conducted a thorough review and correction of this section.

We hope our response is helpful in addressing your concerns.
We are looking forward to hearing from you soon.

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Sincerely yours,

Zhiguang Zhou, Bin Zhao, Xia Li

_____

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RE: Manuscript ID: NCOMMS-23-50175A

Dear Reviewers:

Thank you very much for taking the time to review our revised manuscript titled "TGF-β-mediated crosstalk between TIGIT$^+$ Tregs and CD226$^+$ CD8$^+$ T cells in the progression and remission of type 1 diabetes". We sincerely appreciate the constructive feedback provided. Following a thorough review of each comment, we have undertaken substantial additional work to specifically address the concerns raised.

A comprehensive point-by-point response has been compiled, detailing our approach and the amendments made in response to your invaluable comments.

Reviewer #1: The authors should be commended for the efforts to address prior review concerns. The resulting manuscript is much improved, however, a number of questions remain.
-The authors show representative plots of the frequency of cytokine-producing CD8s for each cytokine assayed, but instead plot the gMFI data for each cytokine. Plotting the differences between frequencies of cytokine-producing cells may be more physiologically relevant than switching from frequency to gMFI between the representative plots and data.

Response 1: Thank you for your valuable suggestion. In accordance with your advice, we have updated Figures 4 and 5 to present the frequencies of cytokine-producing CD4/8$^+$ T cells corresponding to each assessed cytokine, instead of utilizing the gMFI data.

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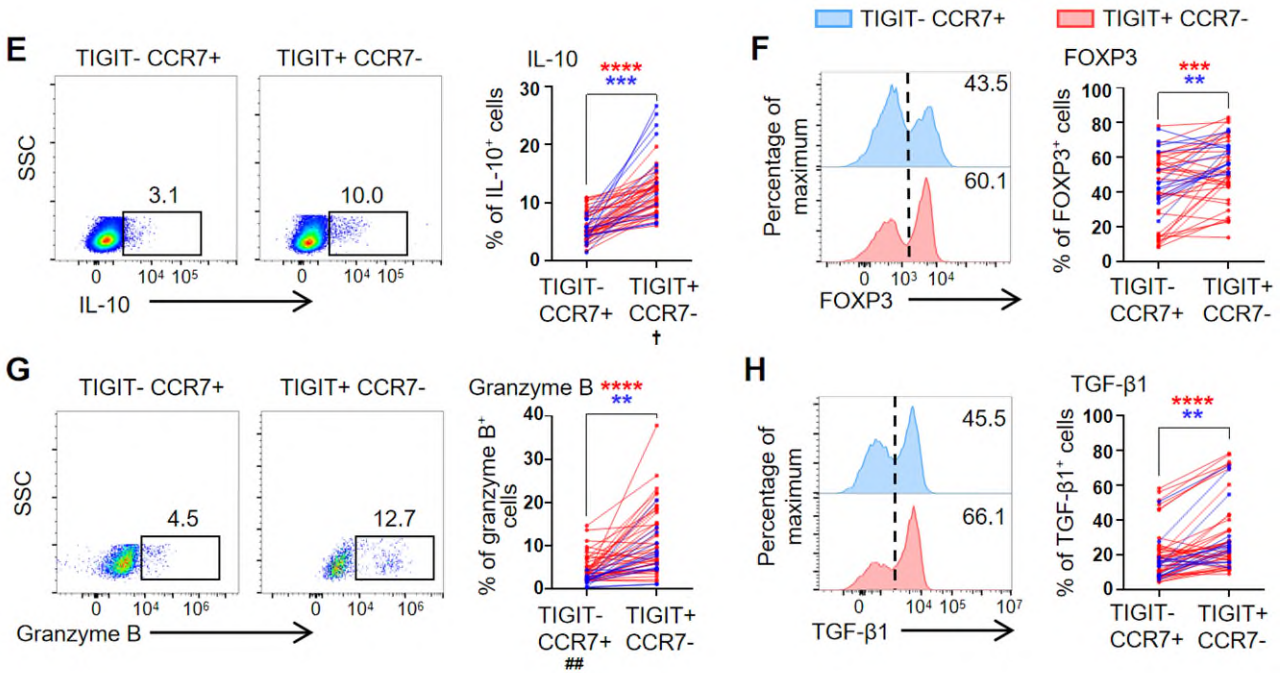

Revised Figure 4 E–H The function of two distinctive Treg subsets. (**E-H**) Representative diagrams and quantification of (**E**) IL-10, (**F**) FOXP3, (**G**) Granzyme B, and (**H**) TGF-β1 in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Treg subpopulations; "#" and "†" signify statistical significance of marker expression in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs, respectively, between T1D and HDs. (**I**) TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Tregs, isolated from T1D patients (n=4), were cocultured with CFSE labeled CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 72 h at 37°C. The proliferation of CD8$^+$ T cells was measured. Two-tailed unpaired t-test; data are shown as the mean ± SEM. */†P < 0.05; **/##P < 0.01; ***P < 0.001; ****P < 0.0001. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.

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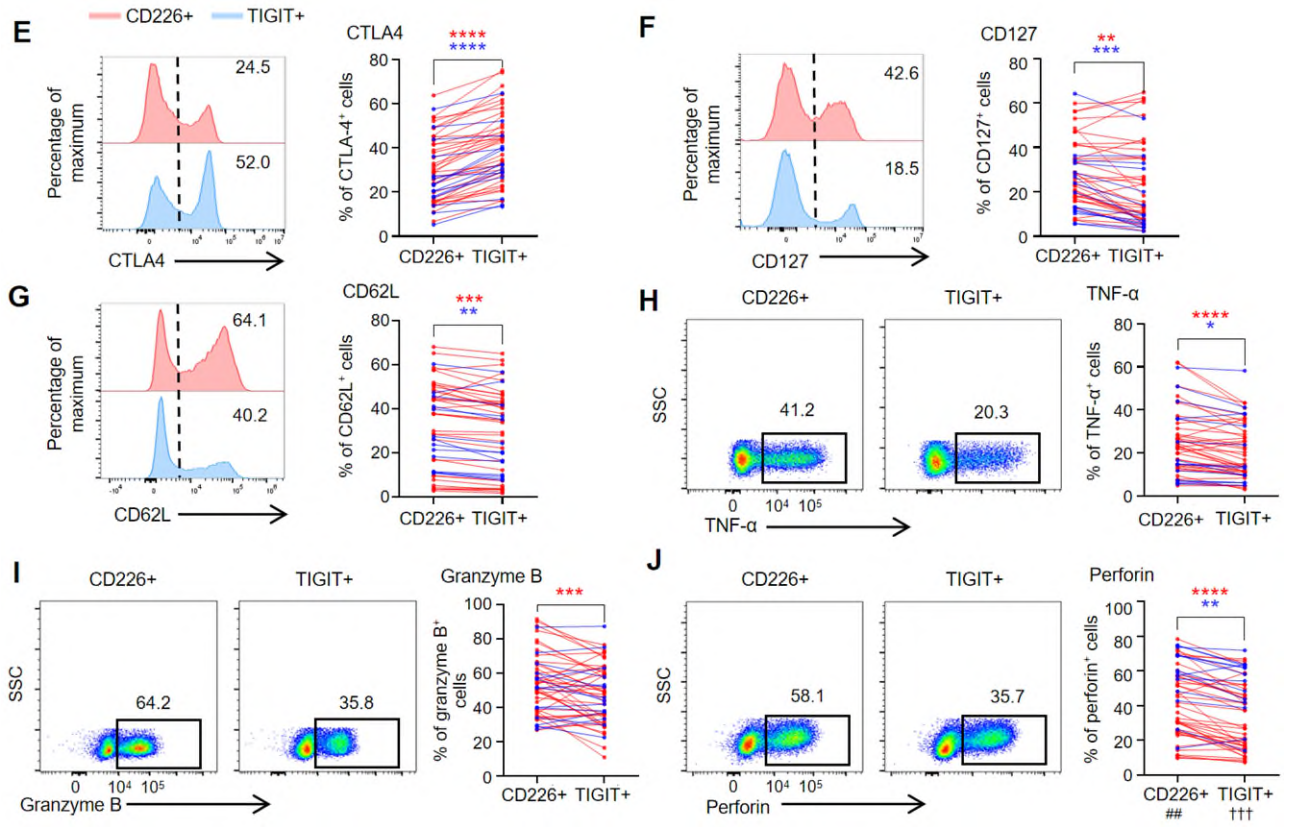

Revised Figure 5 E–J The function between two T1D-stage-associated CD8$^+$ T subsets. (**E-J**) Representative diagrams and quantification of (**E**) CTLA4, (**F**) CD127, (**G**) CD62L**,** (**H**) TNF-α, (**I**) Granzyme B, and (**J**) perforin in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cell subpopulations; "#" and "†" signify statistical significance of marker expression in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells, respectively, between T1D and HDs. (**K-L**) CD226$^+$ and TIGIT$^+$ CD8$^+$ T cells, isolated from T1D patients (n = 6), were cocultured with fluorescently-labeled P815 cells at a ratio of 5:1 for 5 h at 37°C. (**K**) Representative flow cytometry plots and quantification of apoptosis in P815 cells. (**L**) Representative peak plots and quantification of granzyme B expression within CD8$^+$ T-cell subsets. */#P < 0.05; **P < 0.01; ***/†P < 0.001; ****/####P < 0.0001. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.

-Suppression assays using dye dilution approaches need to account for both the number of cells that enter a proliferative cycle, as well as the number of cell divisions. That data is not accessible from Ki-67 analysis alone. This draws in concerns for the suppression assay employed that measured Ki-67 gMFI in the effectors at 48 hrs. Data may also need to be normalized to the 1:0 control condition.

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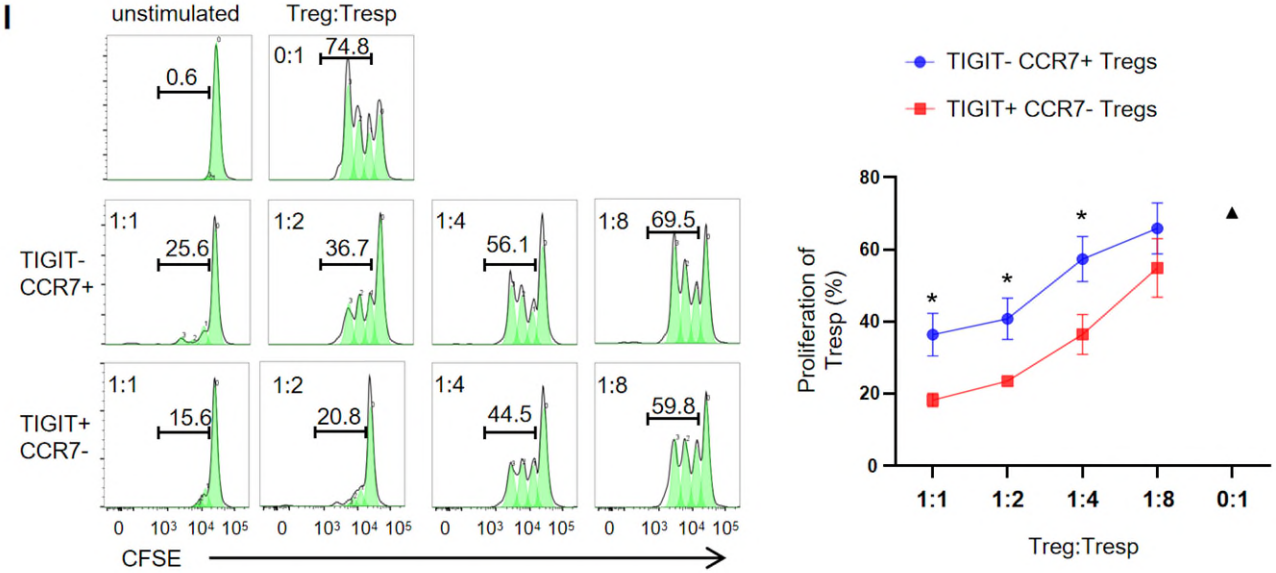
Response 2: Thank you for pointing this out. In our efforts to elucidate the differential suppressive functions of Treg subsets on $CD8^+$ T cell expansion, we have supplemented the results of CFSE staining. Briefly, two subsets of sorted $TIGIT^+$ $CCR7^-$ and $TIGIT^-$ $CCR7^+$ Tregs were co-cultivated with CFSE-stained $CD8^+$ T cells across a gradient of ratios (0:1, 1:1, 1:2, 1:4, and 1:8), sustaining the culture over a continuous period of 72 hours. Notably, the $TIGIT^+$ $CCR7^-$ Treg fraction exhibited a pronounced immunosuppressive influence, significantly curtailing the proliferation of the $CD8^+$ T cells. This effect was documented by the observed attenuation in cell division among the $CD8^+$ T cells, as depicted in Figure 4I. Regarding the Ki-67 analysis, we had indeed conducted control experiments with a Tregs-to-Responder T ratio of 0:1 (Figure R 1) , but the results were not previously displayed in the manuscript.



Revised Figure 4 I Treg suppression assay. $TIGIT^+$ $CCR7^-$ Treg cells and $TIGIT^-$ $CCR7^+$ Tregs, isolated from T1D patients (n=4), were cocultured with CFSE labeled $CD8^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 72 h at 37°C. The proliferation of $CD8^+$ T cells was measured. Two-tailed unpaired t-test; data are shown as the mean ± SEM. $*P < 0.05$.

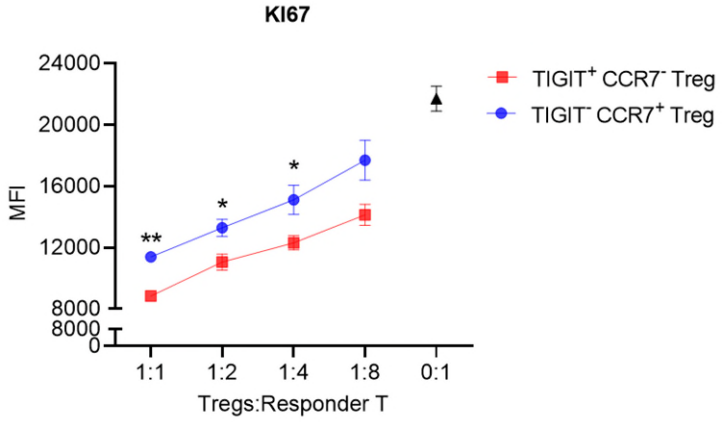


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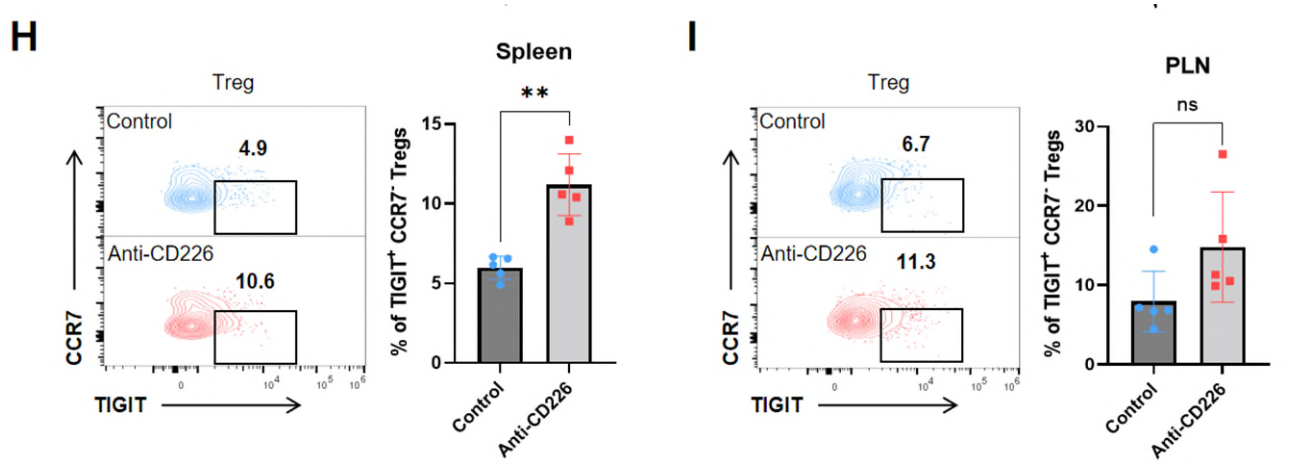
Figure R1 Suppression assay of Treg subsets. TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Treg cells, isolated from T1D patients (n = 6), were cocultured with autologous CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 48 h at 37°C. Ki-67 in CD8$^+$ T cells was measured. Two-tailed unpaired t-test; data are shown as the mean ± SEM. *P < 0.05; **P < 0.01

-The new figure 8G-H should have representative gating for CCR7/TIGIT, rather than just SSC/TIGIT

Response 3: We appreciate your suggestion regarding the representative flow cytometry plots on the left should match the statistical gating shown on the right. Accordingly, we have made revisions to the new Figure 8G-H to display the representative gating for CCR7/TIGIT instead of SSC/TIGIT. Thank you for bringing this to our attention.



Revised Figure 8H-I Frequencies of TIGIT$^+$ CCR7$^-$ Tregs in spleen and PLN are shown (n = 5).

-It is not clear what "CCS" is on the y-axis for 8F.

Response 4: We appreciate your attention to detail in identifying the ambiguity. "CCS" on the y-axis of Figure 8F was a typographical error. It has been corrected to "CD8" in the revised figure. We apologize for any confusion this may have caused and assure that the updated figure now clearly conveys the intended information.

-The new Figure 8N-O should have representative gating for CD8/TIGIT, rather than just TIGIT.

Response 5: Thank you for clarifying the situation with the representative gating in Figures 8N-O. We have corrected this labeling error in the revised figures to accurately depict the gating strategy for CD8/TIGIT and CD8/CD226. The corrected figures should now properly reflect our analysis of the CD8$^+$ T cell subsets.

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-The authors show that there is a decrease in the frequency of $CD226^+$ $CD8^+$ and an increase in $TIGIT^+$ $CD8^+$ in the spleen following in vivoTx, but suggest that there isn't any functional changes in the $CD226^+$ $CD8^+$ population. The authors should clarify what they hypothesize the antibody is doing to that subset. To this point, the authors should clarify whether the anti-CD226 mAb is depleting or blocking in action. This notion of epitope specificity should also be considered for flow staining antibodies relative to the therapeutic mAb.

Response 6: Thank you. We utilized the anti-mouse CD226 functional grade (Clone: 10E5, eBioscience, Cat #: 16-2261) for therapeutic intervention. The 10E5 clone was initially reported in its application to experimental autoimmune encephalomyelitis (EAE), where its administration delayed disease onset and lessened the severity of Th1-mediated autoimmune pathology (*Dardalhon et al, J Immunol, 2005*). Subsequently, it has also been documented to suppress antigen-specific T cell proliferation and IFN-γ production in vivo, as well as to extend the survival of skin allografts (*Liu et al, Arthritis Research & Therapy, 2018*). However, none of the above studies directly tested the functional changes in $CD226^+$ $CD8^+$ T cells.

Our data demonstrated that treatment with **anti-CD226 antibodies led to a reduction in both proliferation and cytokine production of the total $CD8^+$ T cells (Fig. S13)**, consistent with existing literature (*Johnston RJ, et al. Cancer Cell. 2014*). This finding aligns with our initial reports and provides further evidence of the antibody's impact on T cell responses.

Interestingly, despite these observed effects on proliferation and cytokine production, our functional analysis as shown in Figure S14 indicated that **the overall functional capacity of $CD226^+$ $CD8^+$ T cells is not compromised by the anti-CD226 treatment (Fig. S14)**. This uncoupling of proliferation and cytokine production from other aspects of $CD8^+$ T cell function suggests a more complex role of CD226 in T cell regulation.

The administration of anti-CD226 mAb could interfere with the interaction between CD226 and its natural ligands, potentially inhibiting its activation by APC such as macrophages, which is crucial for effective antigen presentation and subsequent $CD8^+$ T cell activation. **This interference could lead to a reduction in CD226 expression and the consequent depletion of a subset of $CD226^+$ $CD8^+$ T cells.** Hence, while therapeutic application of anti-CD226 mAb is expected to result in the depletion of $CD226^+$ $CD8^+$ T cells, it may not suppress the activation of $CD226^+$ $CD8^+$ T cells. This is likely due to, as a co-stimulatory molecule, the functional regulation of CD226 may involve negative feedback loops, or its effects could be compensated by other co-stimulatory or co-inhibitory molecules.

In response to the concern regarding epitope specificity, we verified the CD226 antibodies used for flow cytometry staining in this study have not been reported to possess any cell inhibitory or depletive functions. These staining antibodies are typically designed and validated to bind specifically to their target epitopes without inducing functional changes in the cells they label, ensuring that the observed effects in our experiments can be attributed to the therapeutic mAb rather than the flow cytometry staining process.

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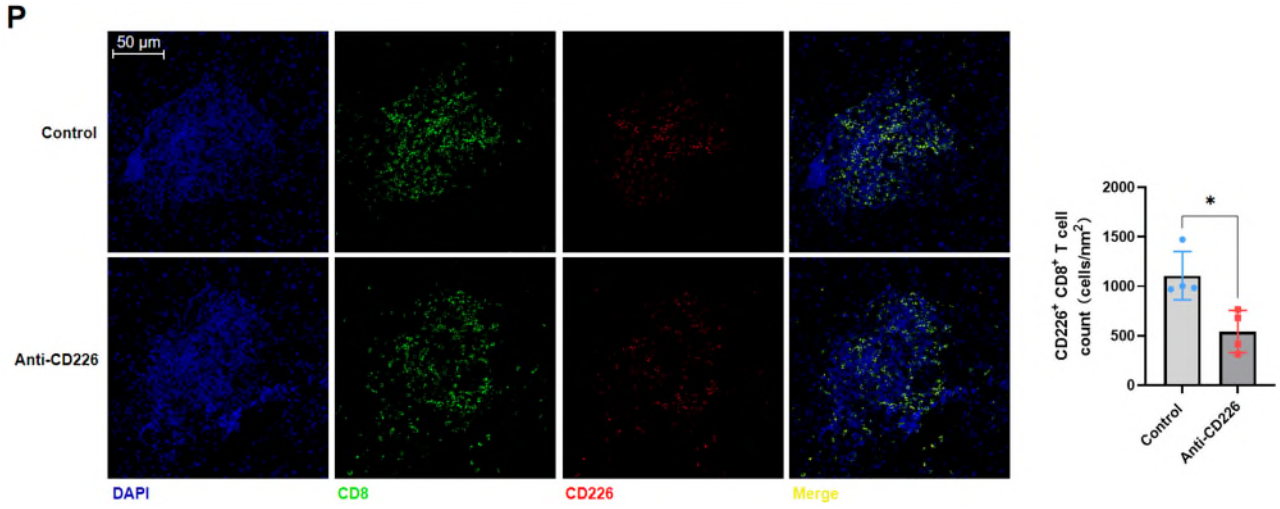
-The authors indicate there isn't any impact in the pLN, so seeing if there are differences in the pancreas-infiltrating CTL populations would be of interest.

Response 7: In response to the question regarding potential differences in CTL populations, despite the lack of observed effects in the PLN, we have conducted a focused immunofluorescent staining analysis on pancreatic tissues. Our results have revealed that treatment with Anti-CD226 does indeed ameliorate the infiltration of CTLs ($CD226^+$ CD8T) in NOD mice.



Revised Figure 8P Anti-CD226 therapy alleviated the accumulation of local cytotoxic $CD226^+$ $CD8^+$ T cells in islets. (**P**) Multiplex fluorescence immunohistochemistry staining was used to evaluate the presence of $CD226^+$ $CD8^+$ T cells within the islets of NOD mice. Representative multiplex fluorescence images of $CD226^+$ $CD8^+$ T cells in the pancreas specimen. Nuclei, CD8 and CD226 within the cells, are visualized in blue, green, and red, respectively. Assessment of the density of $CD226^+$ $CD8^+$ T cells within the islets. *P* values were calculated using the unpaired t-test, $*P<0.05$.

Reviewer #3: The Authors have satisfactorily addressed most of the points raised.

A major issue remains in the suppression assays presented in Fig. 7G-I using sorted $TIGIT^+$ $CCR7^-$ and $TIGIT^-$ $CCR7^+$ Tregs. The sorting of these populations only included a preliminary magnetic isolation of $CD4^+$ T cells, which were subsequently stained for TIGIT and CCR7 for sorting. Thus, these cells do not qualify as Tregs, as they have not been sorted on additional Treg markers, e.g. $CD25^+$ $CD127^-$ as done in Fig. S8. It is thus not surprising to see that $TIGIT^+$ $CD4^+$ T cells are more suppressive than $CCR7^+$ $CD4^+$ T cells, as the latter are likely to be composed mainly of naïve and central memory conventional T cells. These experiments should be repeated by gating on $CD25^+$ $CD127^-$ $CD4^+$ Tregs before sorting $TIGIT^+$ $CCR7^-$ and $TIGIT^-$ $CCR7^+$ subsets.

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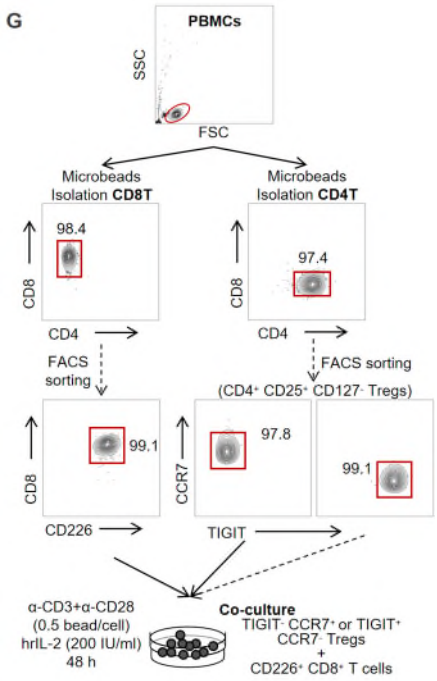
Response 1: Thank you. In our flow cytometric sorting process, we thoroughly examined a comprehensive panel of Treg-specific markers, including CD4, CD25, CD127, CCR7, and TIGIT. It appears there was a miscommunication in Figure 7G, resulting in unclear labeling and subsequent misunderstanding. To rectify this, we have made the corrections to the figure to accurately reflect the inclusion of all relevant Treg markers in the sorting process. This ensures that the sorted TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ cells are appropriately identified as Tregs, addressing the concerns about the specificity of the cell populations used in our assays.



Revised Figure 7G Diagram illustrating the experimental setup.

-The same may apply to the suppression assays presented in Fig. 4J using the same Treg subsets. There is no detail on how these subsets were sorted and it is assumed that the same strategy was used.

Response 2: Thank you. Consistent with the previous experiments, the sorting of Tregs throughout the study was performed as described in the methods section: "We initially performed positive magnetic bead enrichment using anti-CD4 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to purify CD4$^+$ T cells from T1D patients, followed by cell sorting of TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ Treg (CD4$^+$ CD25$^+$ CD127$^-$) subsets using the FACS Aria II cell sorter (BD Biosciences)."

We hope our response is helpful in addressing your concerns.
We are looking forward to hearing from you soon.

With warm regards,

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RE: Manuscript ID: NCOMMS-23-50175B

Dear Reviewers:

Thank you very much for taking the time to review our revised manuscript titled "TGF-β-mediated crosstalk between TIGIT$^+$ Tregs and CD226$^+$ CD8$^+$ T cells in the progression and remission of type 1 diabetes". We sincerely appreciate the constructive feedback provided. Following a thorough review of each comment, we have undertaken substantial additional work to specifically address the concerns raised. **For clarity, in the manuscript, initial revisions are marked in red, and the second round of revisions are marked in blue.**

A comprehensive point-by-point response has been compiled, detailing our approach and the amendments made in response to your invaluable comments.

Reviewer #1: The authors should be commended for the efforts to address prior review concerns. The resulting manuscript is much improved, however, a number of questions remain.
-The authors show representative plots of the frequency of cytokine-producing CD8s for each cytokine assayed, but instead plot the gMFI data for each cytokine. Plotting the differences between frequencies of cytokine-producing cells may be more physiologically relevant than switching from frequency to gMFI between the representative plots and data.

Response 1: Thank you for your valuable suggestion. In accordance with your advice, we have updated Figures 4 and 5 to present the frequencies of cytokine-producing CD4/8$^+$ T cells corresponding to each assessed cytokine, instead of utilizing the gMFI data.

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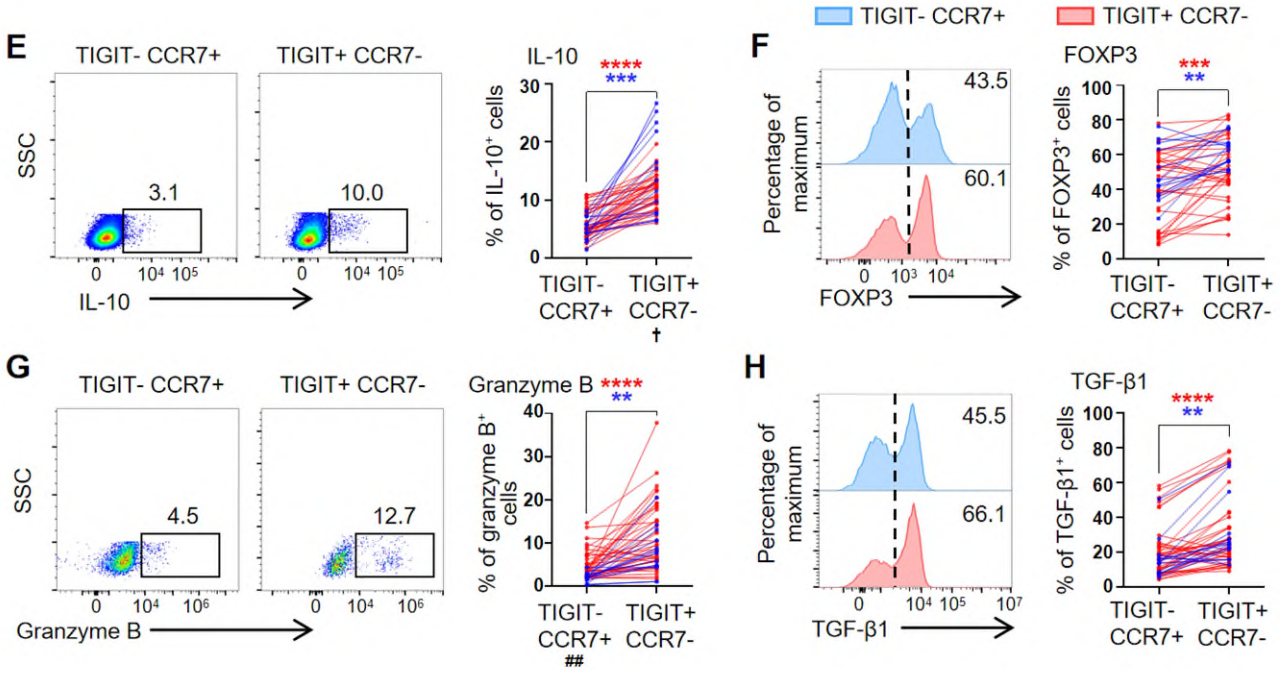

Revised Figure 4 E–H The function of two distinctive Treg subsets. (**E-H**) Representative diagrams and quantification of (**E**) IL-10, (**F**) FOXP3, (**G**) Granzyme B, and (**H**) TGF-β1 in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Treg subpopulations; "#" and "†" signify statistical significance of marker expression in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs, respectively, between T1D and HDs. Two-tailed unpaired t-test; data are shown as the mean ± SEM. */†$P < 0.05$; **/##$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.

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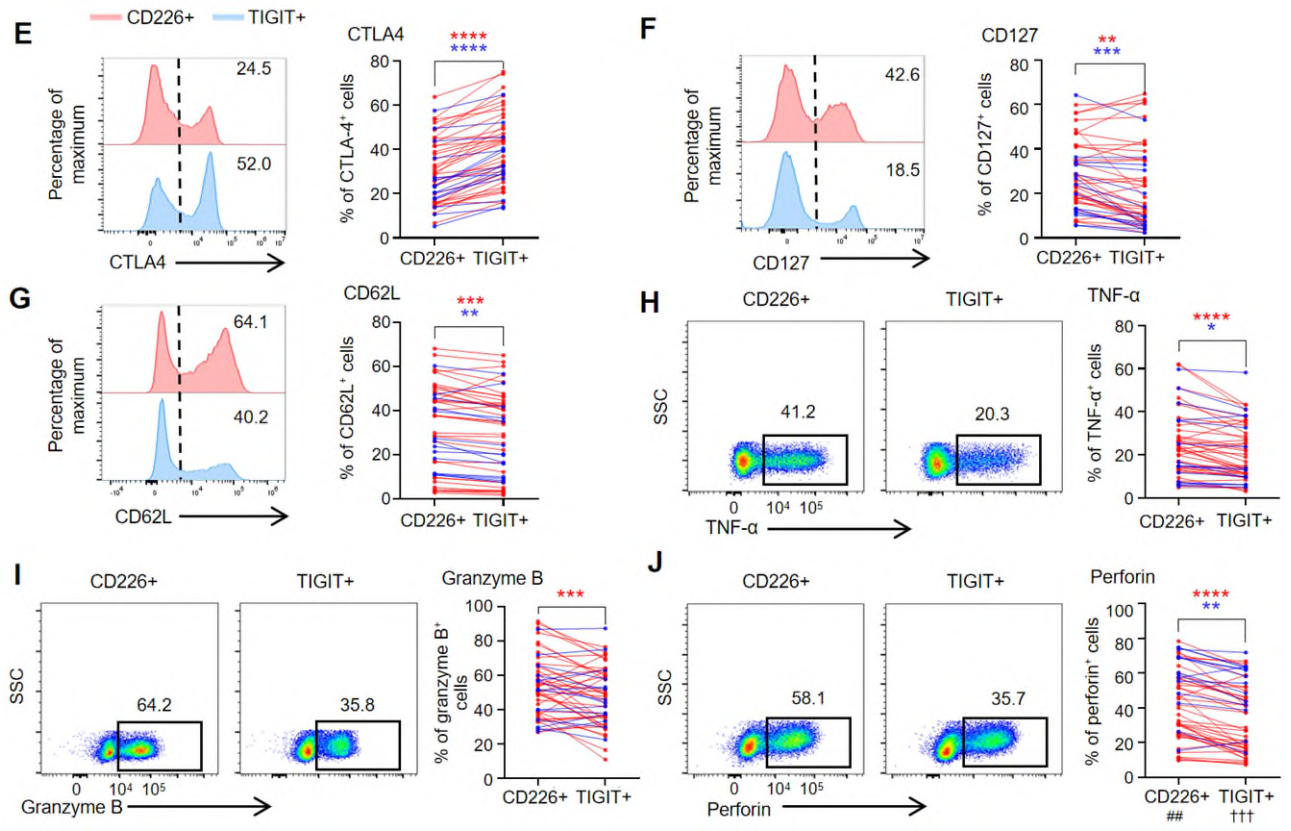

Revised Figure 5 E–J The function between two T1D-stage-associated CD8$^+$ T subsets. (**E–J**) Representative diagrams and quantification of (**E**) CTLA4, (**F**) CD127, (**G**) CD62L**,** (**H**) TNF-α, (**I**) Granzyme B, and (**J**) perforin in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cell subpopulations; "#" and "†" signify statistical significance of marker expression in CD226$^+$ and TIGIT$^+$ CCR7$^-$ CD8$^+$ T cells, respectively, between T1D and HDs. */#$P < 0.05$; **$P < 0.01$; ***/†$P < 0.001$; ****/####$P < 0.0001$. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.

-Suppression assays using dye dilution approaches need to account for both the number of cells that enter a proliferative cycle, as well as the number of cell divisions. That data is not accessible from

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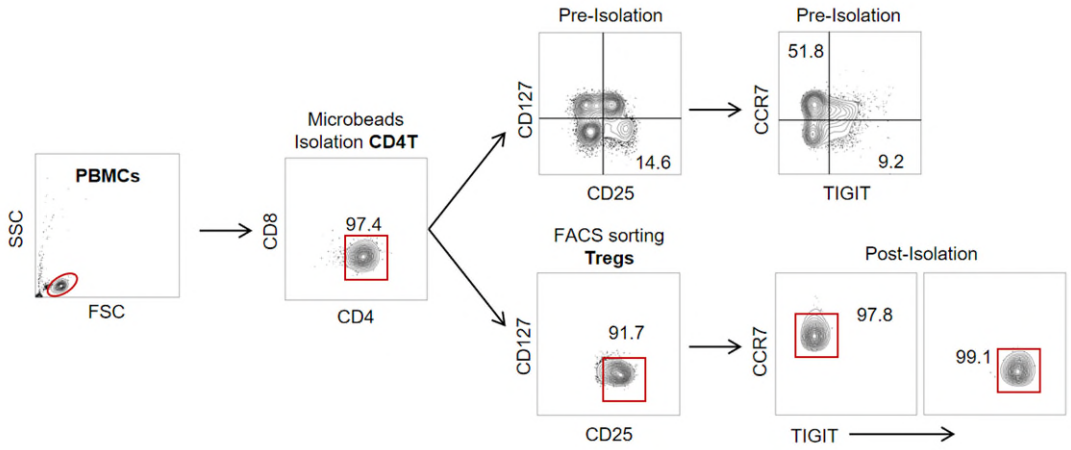
Ki-67 analysis alone. This draws in concerns for the suppression assay employed that measured Ki-67 gMFI in the effectors at 48 hrs. Data may also need to be normalized to the 1:0 control condition.

Response 2: Thank you for pointing this out. In our efforts to elucidate the differential suppressive functions of Treg subsets on $CD8^+$ T cell expansion, we have supplemented our study with additional results from CFSE staining. Briefly, two subsets of sorted $TIGIT^+$ $CCR7^-$ and $TIGIT^-$ $CCR7^+$ Tregs were co-cultivated with CFSE-stained $CD8^+$ T cells across a gradient of ratios (0:1, 1:1, 1:2, 1:4, and 1:8), sustaining the culture over a continuous period of 72 hours. Notably, the $TIGIT^+$ $CCR7^-$ Treg fraction exhibited a pronounced immunosuppressive effect, significantly inhibiting the proliferation of the $CD8^+$ T cells. This effect was documented by the inhibition in cell division among the $CD8^+$ T cells, as depicted in Figure 4I. Regarding the Ki-67 analysis, although we had conducted control experiments with a Tregs-to-Responder T ratio of 0:1 (Figure R1), these results were not previously displayed in the manuscript.



Revised Figure S8C The sorting process for $TIGIT^+$ $CCR7^-$ Tregs and $TIGIT^-$ $CCR7^+$ Tregs.

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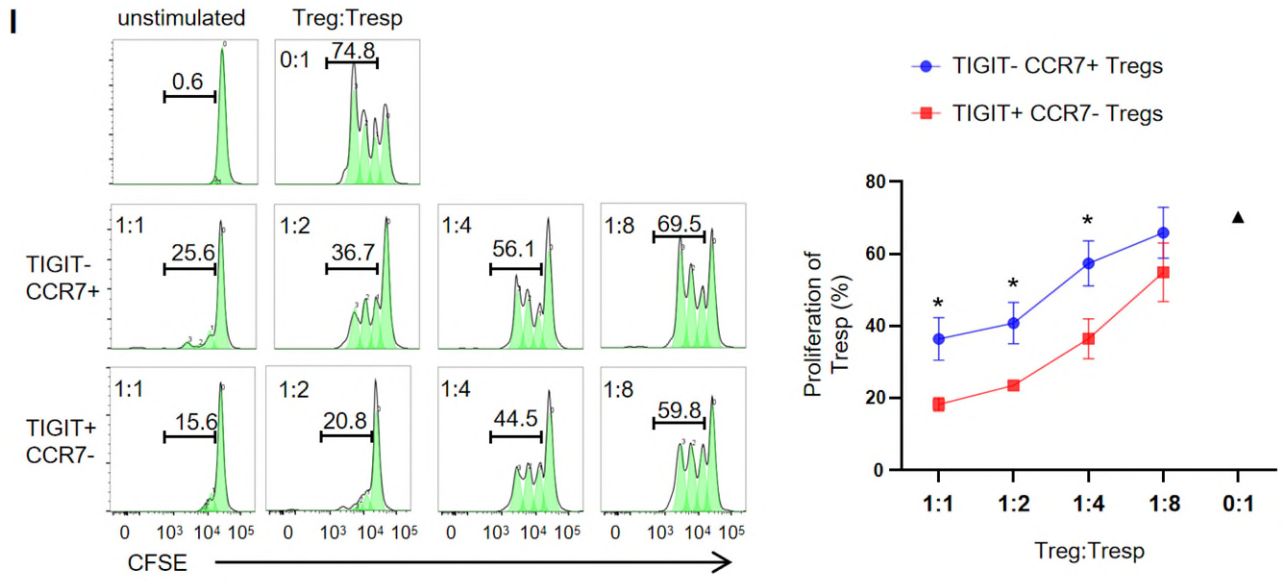

Revised Figure 4 I Treg suppression assay. TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Tregs, isolated from T1D patients (n=4), were cocultured with CFSE labeled CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 72 h at 37°C. The proliferation of CD8$^+$ T cells was measured. The triangle symbolizes the control group with a Tregs-to-Responder T ratio of 0:1. Two-tailed unpaired t-test; data are shown as the mean ± SEM. *P < 0.05.

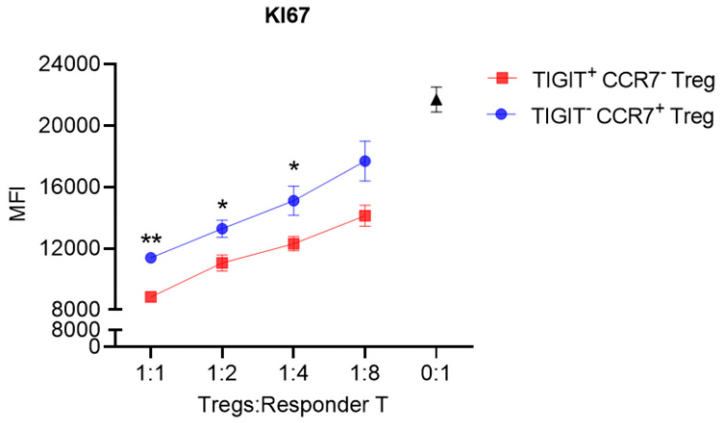

Figure R1 Suppression assay of Treg subsets. TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Treg cells, isolated from T1D patients (n = 6), were cocultured with autologous CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 48 h at 37°C. Ki-67 in CD8$^+$ T cells was measured. The triangle symbolizes the control group with a Tregs-to-Responder T ratio of 0:1. Two-tailed unpaired

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t-test; data are shown as the mean ± SEM. *$P < 0.05$; **$P < 0.01$

-The new figure 8H-I should have representative gating for CCR7/TIGIT, rather than just SSC/TIGIT

Response 3: We appreciate your suggestion regarding the representative flow cytometry plots on the left should match the statistical gating shown on the right. Accordingly, we have made revisions to the original Figure 8H&I to display the representative gating for CCR7/TIGIT instead of SSC/TIGIT. The original Figure 8H&I has been changed to Figure S13E&F.

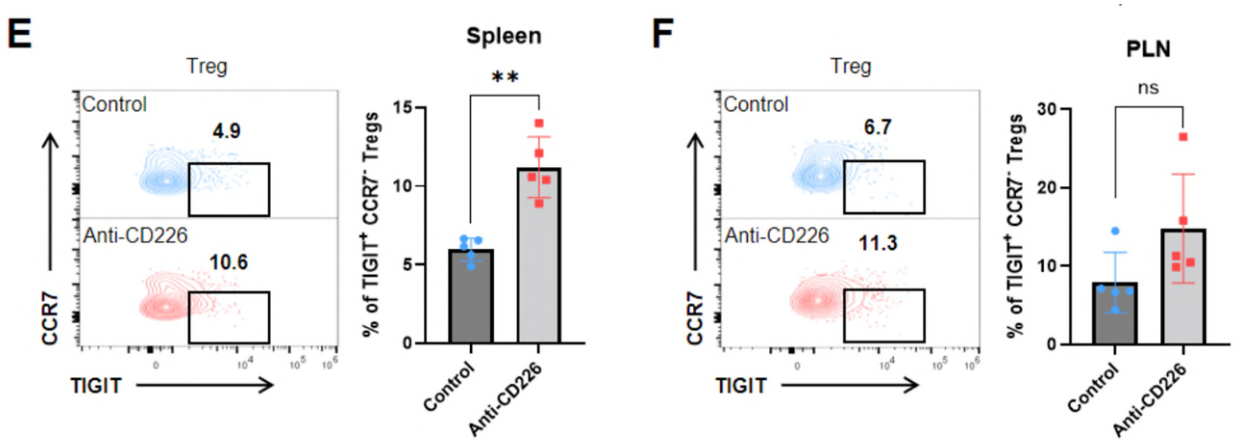

Figure S13E&F Frequencies of TIGIT$^+$ CCR7$^-$ Tregs in spleen and PLN from STZ-induced mouse model are shown (n = 5).

-It is not clear what "CCS" is on the y-axis for 8F.

Response 4: We appreciate your attention to detail in identifying the ambiguity. "CCS" on the y-axis of original Figure 8F was a typographical error. It has been corrected to "CD8" in the revised figure. We apologize for any confusion this may have caused and assure that the updated figure now clearly conveys the intended information.

-The new Figure 8N-O should have representative gating for CD8/TIGIT, rather than just TIGIT.

Response 5: Thank you for clarifying the situation with the representative gating in original Figures

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8N-O. We have corrected this labeling error in the revised figures to accurately depict the gating strategy for CD8/TIGIT and CD8/CD226.

-The authors show that there is a decrease in the frequency of $CD226^+$ $CD8^+$ and an increase in $TIGIT^+$ $CD8^+$ in the spleen following in vivoTx, but suggest that there isn't any functional changes in the $CD226^+$ $CD8^+$ population. The authors should clarify what they hypothesize the antibody is doing to that subset. To this point, the authors should clarify whether the anti-CD226 mAb is depleting or blocking in action. This notion of epitope specificity should also be considered for flow staining antibodies relative to the therapeutic mAb.

Response 6: Thank you. We utilized functional grade anti-mouse CD226 (Clone: 10E5, eBioscience, Cat #: 16-2261) for therapeutic intervention. The 10E5 clone was initially reported in its application in experimental autoimmune encephalomyelitis (EAE), where its administration delayed disease onset and decreased the severity of Th1-mediated autoimmune pathology (*Dardalhon et al, J Immunol, 2005*). Subsequently, it has also been documented to suppress antigen-specific T cell proliferation and IFN-γ production in vivo, as well as to extend the survival of skin allografts (*Liu et al, Arthritis Research & Therapy, 2018*). Recently, this antibody has also been applied to additional disease models as well (*Sakano Y, et al. J Allergy Clin Immunol. 2024*). However, none of the above studies directly tested the functional changes in $CD226^+$ $CD8^+$ T cells.

Our data demonstrated that treatment with anti-CD226 antibodies led to a reduction in both proliferation and cytokine production of the total $CD8^+$ T cells in STZ-induced diabetic mice (now Fig. 8C-F), consistent with existing literature (*Johnston RJ, et al. Cancer Cell. 2014;*). This finding aligns with our initial reports and provides further evidence of the antibody's impact on T cell responses.

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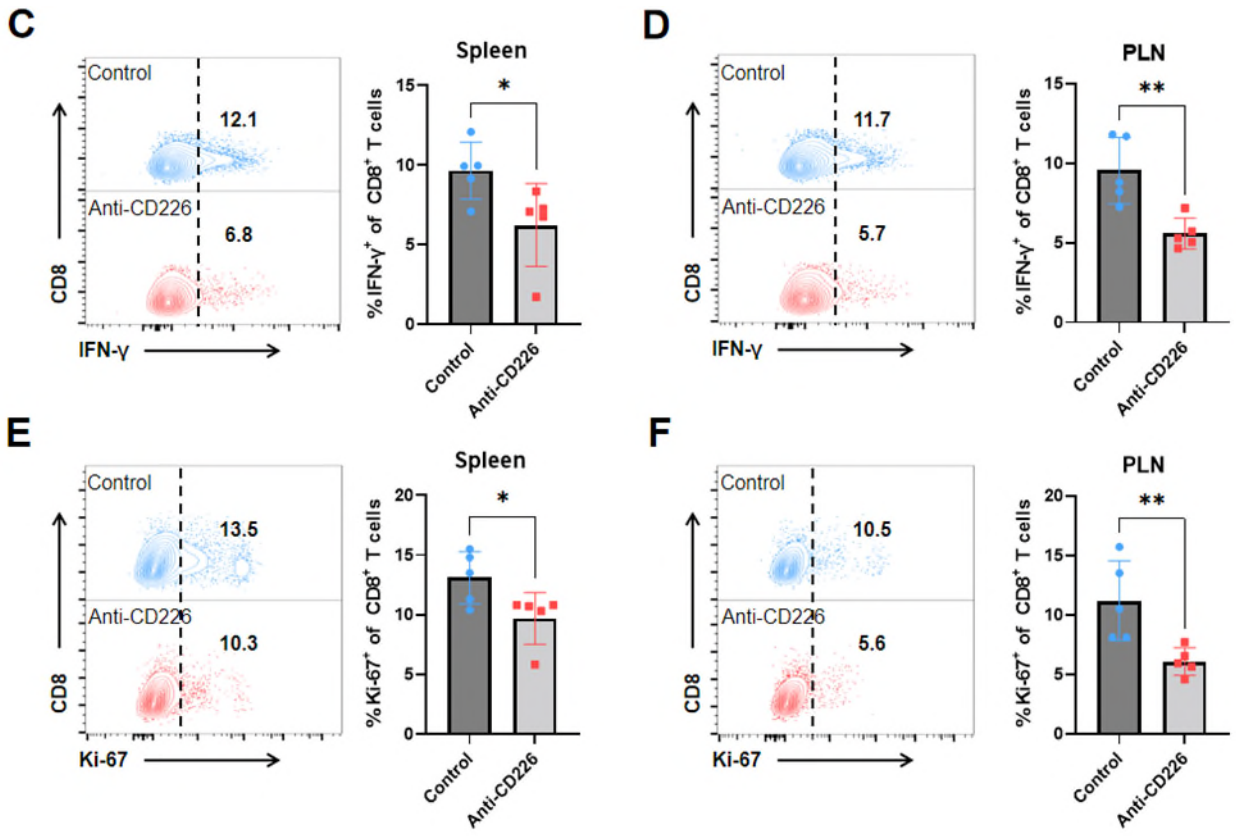

Now Fig. 8C-F Administration of Anti-CD226 mAb in vivo inhibits the expansion of CD8$^+$ T cells and cytokine-production in STZ-induced diabetic mice. The expression levels of IFN-γ, and KI67 within CD8$^+$ T cells in the spleen and PLN were depicted for the two groups (n=5). Data are shown as the mean ± SD; two-tailed unpaired t-test. *P<0.05; **P<0.01.

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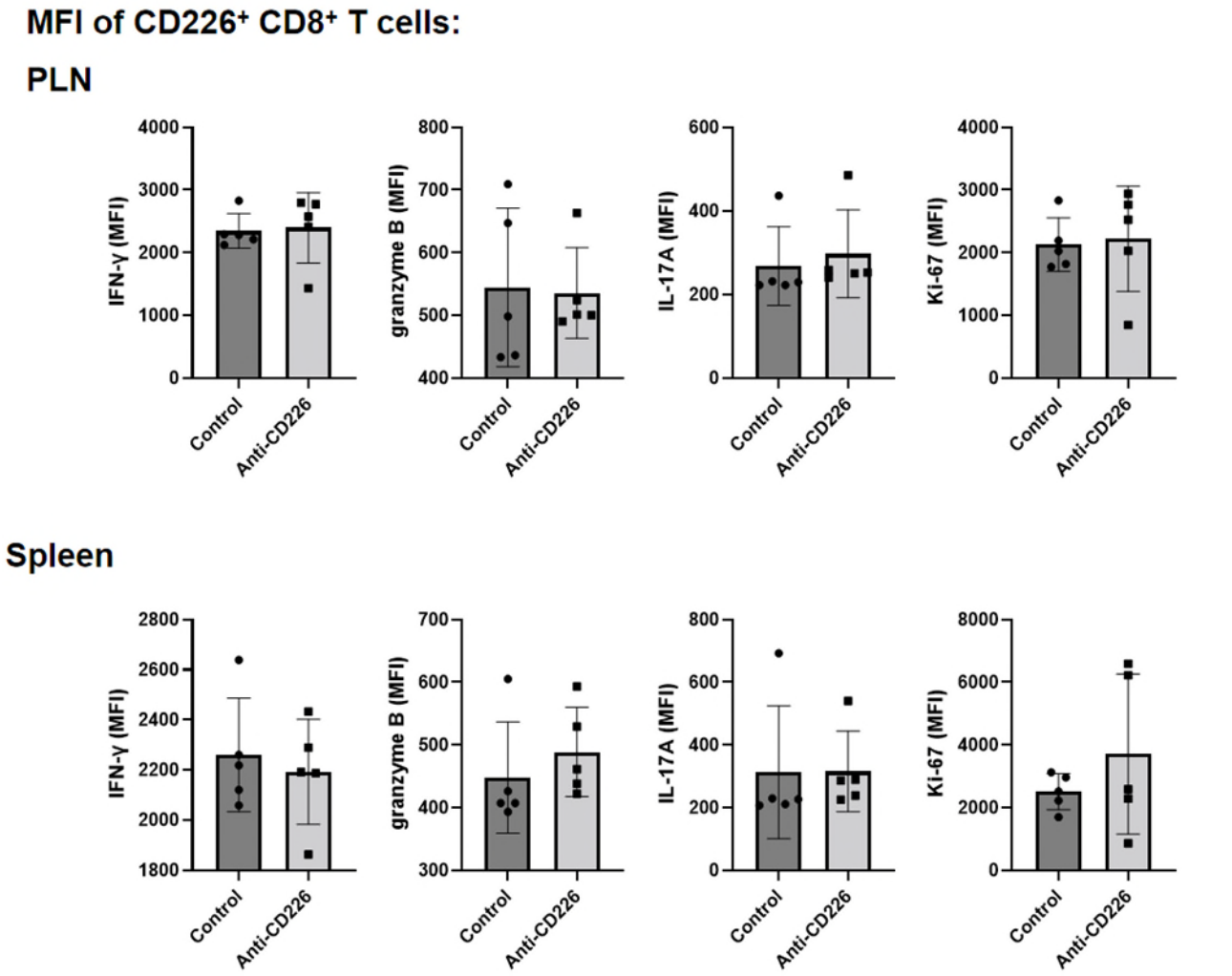

Fig. R2 Anti-CD226 treatment does not change the function of CD226$^+$ CD8$^+$ T cells in STZ-induced diabetic mice. The expression levels of IFN-γ, granzyme B, IL17A, and KI67 within CD226$^+$ CD8$^+$ T cells in the spleen and PLN are depicted for the two groups. Two-tailed unpaired t-test; data are shown as the mean ± SD.

However, functional analysis showed that the overall functional capacity of CD226$^+$ CD8$^+$ T cells is not compromised by the anti-CD226 treatment in STZ-induced diabetic mice (Fig. R2).

To further substantiate the mechanism of anti-CD226 mAb, we assessed the alterations in CD8$^+$ T cell functionality following CD226 blockade using the cyclophosphamide-accelerated NOD mouse

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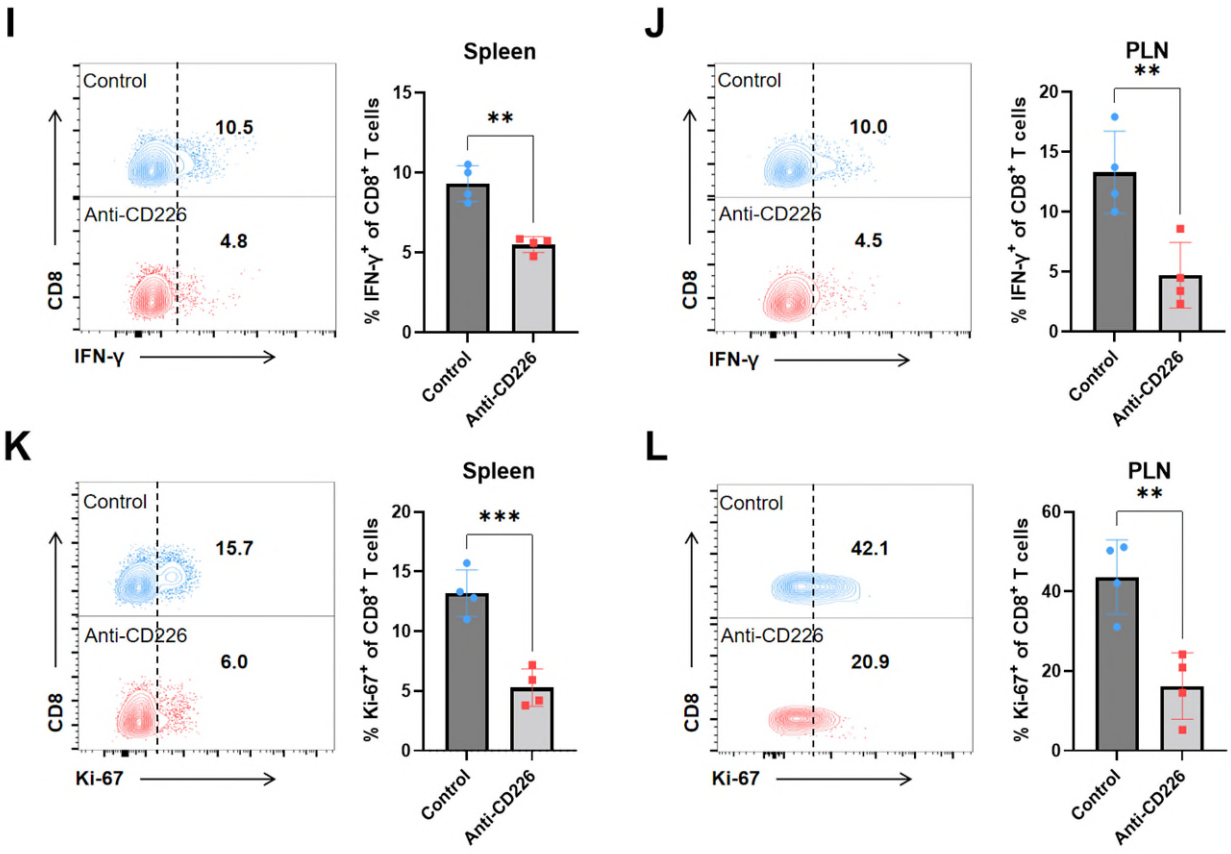
model, which provides a more accurate representation of the autoimmune milieu associated with T1D. **The findings demonstrated that intervention with anti-CD226 mAb significantly attenuated the proliferation and cytokine secretion of both total CD8$^+$ T cells (now Fig. 8I-L) and CD226-expressing CD8$^+$ T subsets (Fig. R3).**



Now Fig. 8I-L Anti-CD226 treatment inhibited the proliferation and cytokine-production of CD8$^+$ T cells in Cy-induced NOD mice. The expression levels of (**I, J**) IFN-γ, and (**K, L**) KI67 within CD8$^+$ T cells in the spleen and PLN were depicted for the two groups (n = 4). Two-tailed unpaired t-test; data were shown as the mean ± SD. ***P*<0.01; ****P*<0.001.

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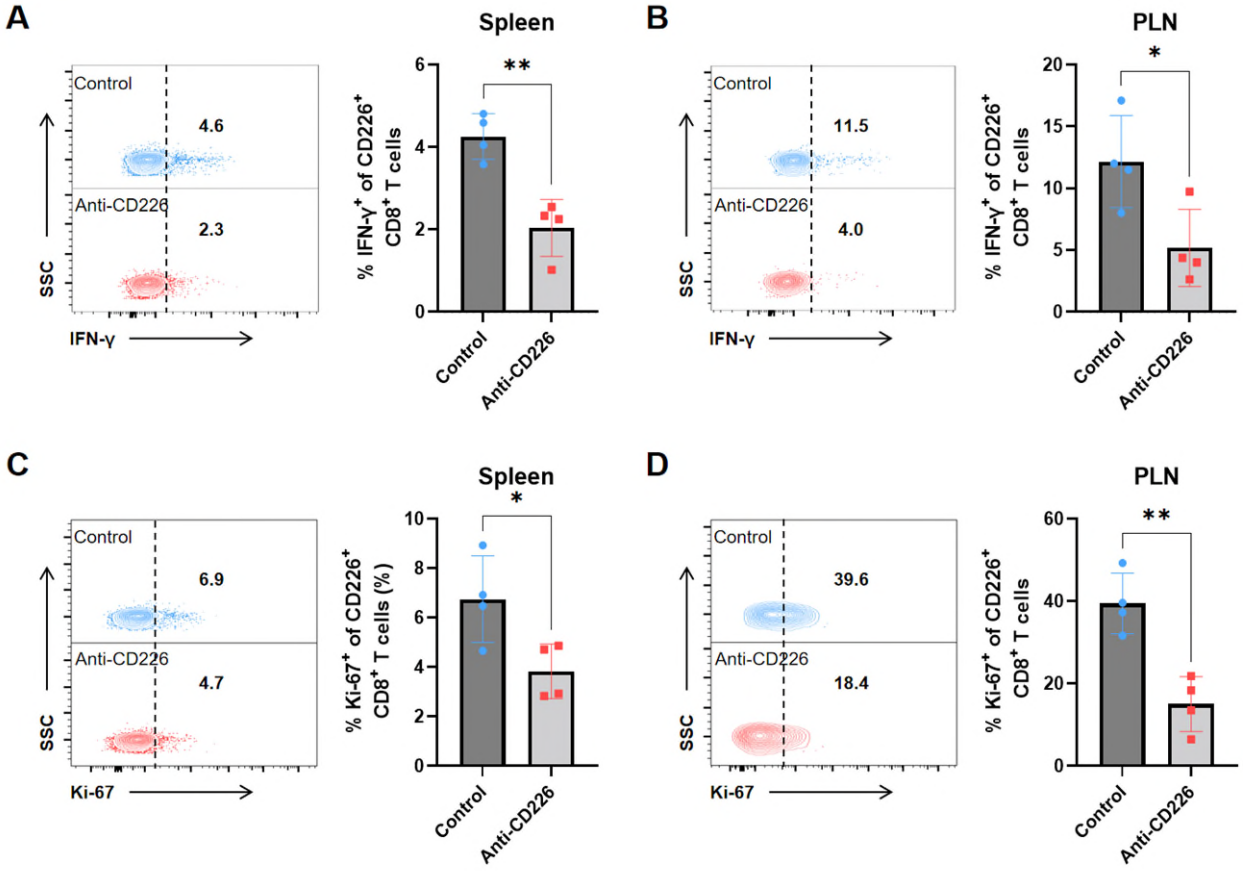

Fig. R3 Anti-CD226 treatment inhibited the proliferation and cytokine-production of CD226$^+$ CD8$^+$ T cells in Cy-induced NOD mice. The expression levels of (**A, B**) IFN-γ, and (**C, D**) KI67 within CD226$^+$ CD8$^+$ T cells in the spleen and PLN were depicted for the two groups (n=4). Two-tailed unpaired t-test; data were shown as the mean ± SD. *P<0.05; **P<0.01.

We also conducted a focused immunofluorescent staining analysis on pancreatic tissues, and revealed that treatment with anti-CD226 mAb does indeed ameliorated the immune infiltration in NOD mice (Fig.S14E).

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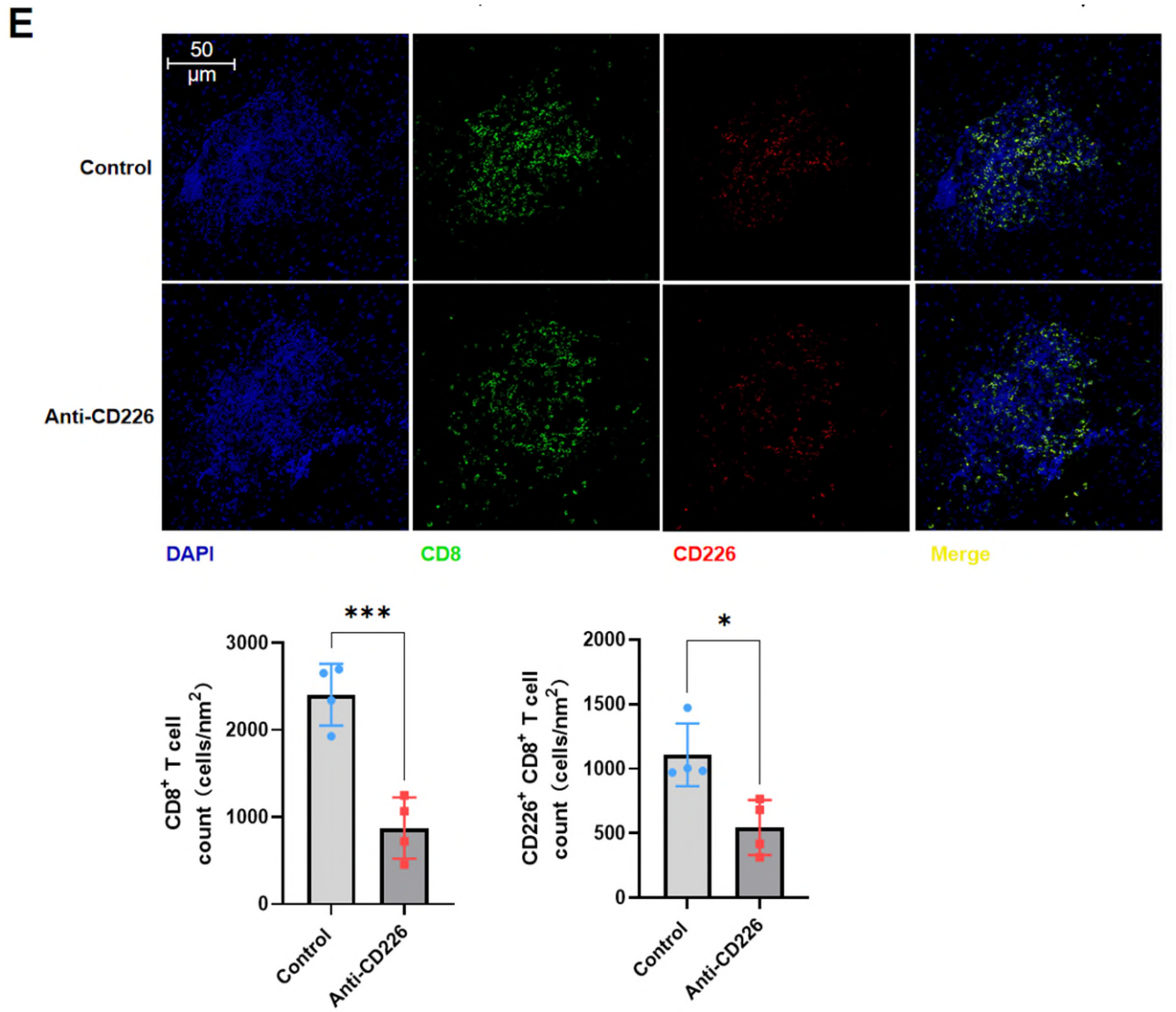

Fig.S14E Anti-CD226 therapy alleviated local immune infiltration in the pancreatic islets. (**E**) Multiplex fluorescence immunohistochemistry staining was used to evaluate the presence of total $CD8^+$ T cells and $CD226^+$ $CD8^+$ T cells within the islets of NOD mice. Representative multiplex fluorescence images in the pancreas specimen were shown. Nuclei, CD8 and CD226 within the cells, are visualized in blue, green, and red, respectively. Assessment of the density of total $CD8^+$ T cells and $CD226^+$ $CD8^+$ T cells within the islets. *P* values were calculated using the unpaired t-test, $*P<0.05$.

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To elucidate the potential mechanistic action of anti-CD226 mAb, we evaluated the phosphorylation levels of signaling proteins within $CD8^+$ T cells post-CD226 blockade. Our findings demonstrated a marked decrease in the phosphorylation of AKT and m-TOR proteins (Fig. S15A-C).

To further validate the blocking effect of CD226, we conducted additional in vitro experiments. $CD8^+$ T cells, isolated from the spleens of NOD mice, were cultured in vitro and exposed to anti-CD3 (5 μg/mL) and mPVR-Fc (5 μg/mL), either alone or in combination with anti-CD226 mAb (5 μg/mL), for a period of 24 h (Fig. S15D-F). These experiments confirmed that CD226 blockade significantly suppresses both cell proliferation and cytokine production, mirroring the inhibitory effects observed in vivo. Based on these observations, we propose that **anti-CD226 mAb impairs $CD8^+$ T cell functionality predominantly through the inhibition of AKT-mTOR signaling pathways.**

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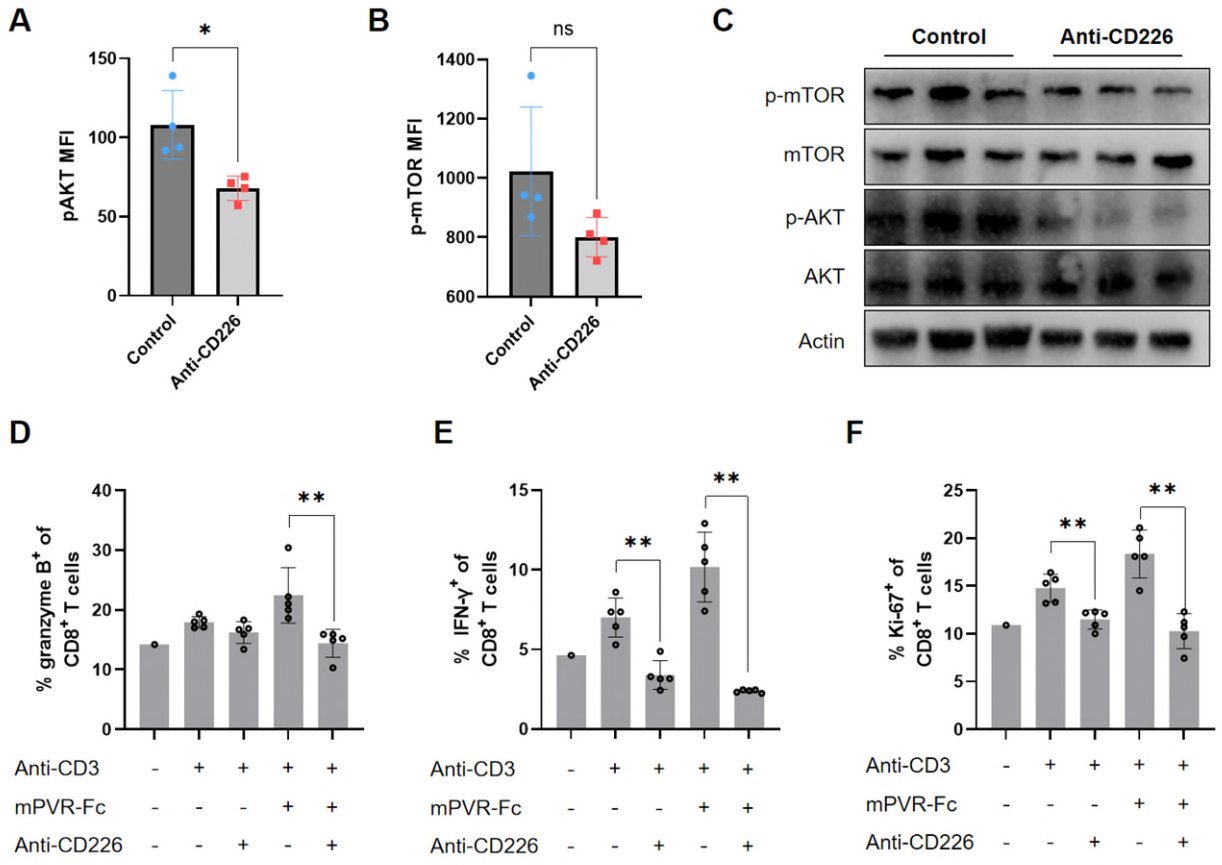

Fig. S15 (related to Fig.8) CD226 blockade inhibited the proliferation and cytokine-production of CD8$^+$ T cells via AKT-mTOR signaling. The expression levels of (**A**) pAKT, and (**B**) p-mTOR within splenic CD8$^+$ T cells from Cy-induced mouse model were depicted (n=4). (**C**) CD8$^+$ T cells from the spleens of the two groups were sorted and the levels of pAKT and p-mTOR were determined by western blots. (**D–F**) Splenic CD8$^+$ T cells from NOD mice were enriched and stimulated for 24 h in the presence of anti-CD3 (5 μg/mL), mPVR-Fc (5 μg/mL), and/or anti-CD226 (5 μg/mL) antibodies as indicated. The expression levels of (**D**) granzyme B, (**E**) IFN-γ, and (**F**) Ki-67 within CD8$^+$ T cells were shown (n=5). Two-tailed unpaired t-test; data were shown as the mean ± SD. **P*<0.05; ***P*<0.01.

In response to the concern regarding epitope specificity, we verified that the CD226 antibodies used for flow cytometry staining in this study have not been reported to possess any cell inhibitory or depletive functions. These staining antibodies are typically designed and validated to bind specifically to their target epitopes without inducing functional changes in the cells they label, ensuring that the observed effects in our experiments can be attributed to the therapeutic mAb rather than the flow

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cytometry staining process.

-The authors indicate there isn't any impact in the pLN, so seeing if there are differences in the pancreas-infiltrating CTL populations would be of interest.

Response 7: In response to the question regarding potential differences in CTL populations, despite the lack of observed effects in the PLN, we have conducted a focused immunofluorescent staining analysis on pancreatic tissues. Our results have revealed that treatment with anti-CD226 does indeed ameliorated the infiltration of CD8 CTLs in NOD mice.

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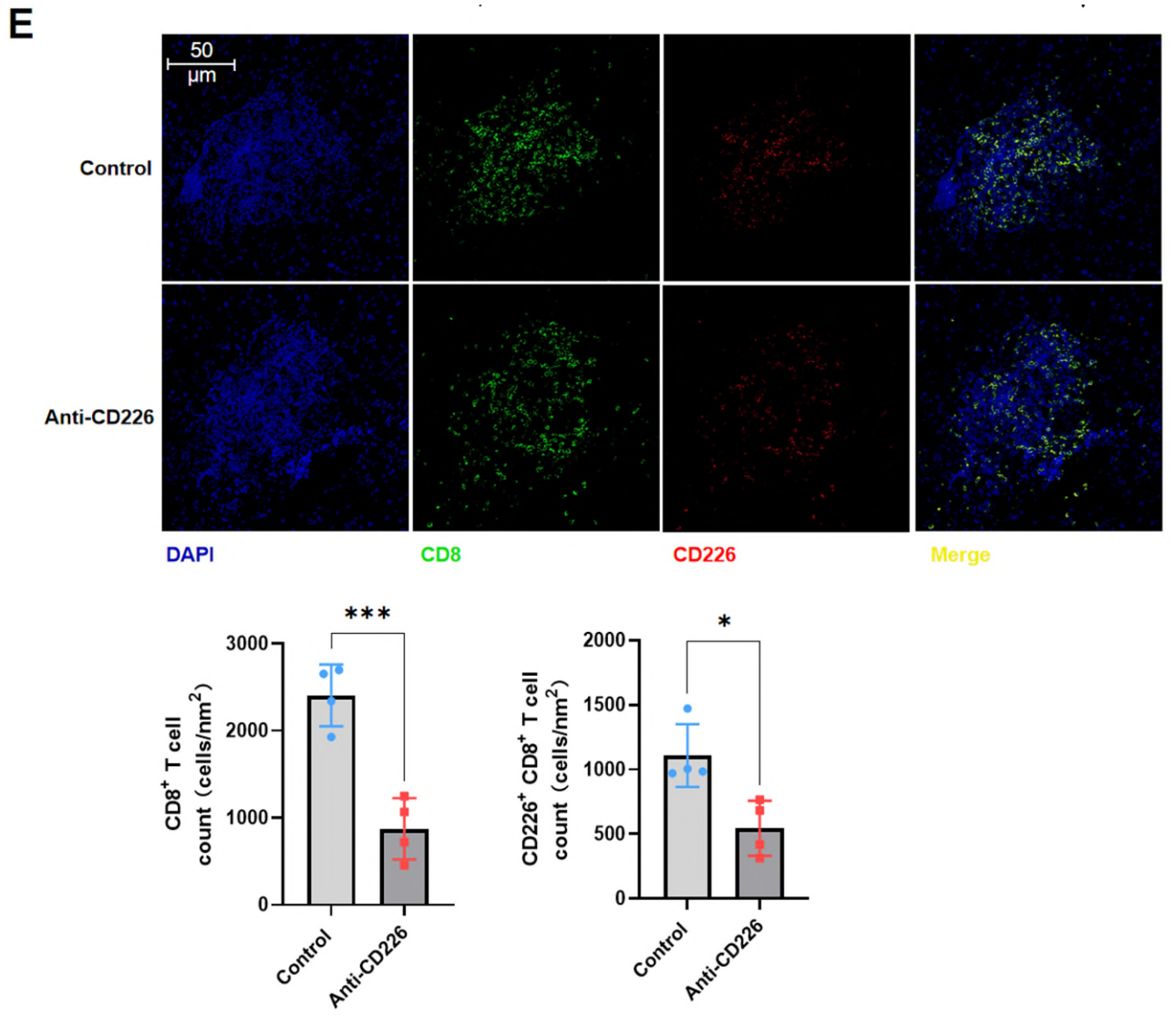

Fig.S14E Anti-CD226 therapy alleviated local immune infiltration in the pancreatic islets. (**E**) Multiplex fluorescence immunohistochemistry staining was used to evaluate the presence of total $CD8^+$ T cells and $CD226^+$ $CD8^+$ T cells within the islets of NOD mice. Representative multiplex fluorescence images in the pancreas specimen were shown. Nuclei, CD8 and CD226 within the cells, are visualized in blue, green, and red, respectively. Assessment of the density of total $CD8^+$ T cells and $CD226^+$ $CD8^+$ T cells within the islets. *P* values were calculated using the unpaired t-test, $*P<0.05$.

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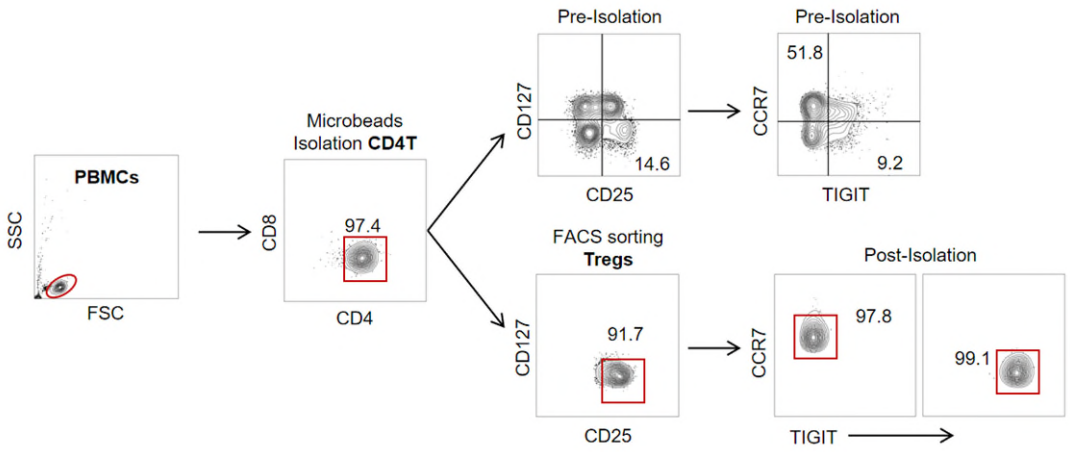
Reviewer #3: The Authors have satisfactorily addressed most of the points raised.

A major issue remains in the suppression assays presented in Fig. 7G-I using sorted TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ Tregs. The sorting of these populations only included a preliminary magnetic isolation of CD4$^+$ T cells, which were subsequently stained for TIGIT and CCR7 for sorting. Thus, these cells do not qualify as Tregs, as they have not been sorted on additional Treg markers, e.g. CD25$^+$ CD127$^-$ as done in Fig. S8. It is thus not surprising to see that TIGIT$^+$ CD4$^+$ T cells are more suppressive than CCR7$^+$ CD4$^+$ T cells, as the latter are likely to be composed mainly of naïve and central memory conventional T cells. These experiments should be repeated by gating on CD25$^+$ CD127$^-$ CD4$^+$ Tregs before sorting TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ subsets.

Response 1: Thank you. In our flow cytometric sorting process, we thoroughly examined a comprehensive panel of Treg-specific markers, including CD4, CD25, CD127, CCR7, and TIGIT. It appears there was a miscommunication in Figure 7G, resulting in unclear labeling and subsequent misunderstanding. To rectify this, we have made the corrections to the figure to accurately reflect the inclusion of all relevant Treg markers in the sorting process. This ensures that the sorted TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ cells are appropriately identified as Tregs, addressing the concerns about the specificity of the cell populations used in our assays.



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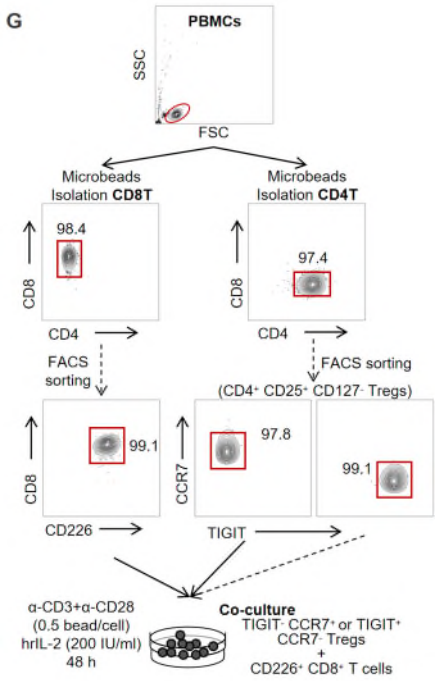
Revised Figure S8C The sorting process for TIGIT$^+$ CCR7$^-$ Tregs and TIGIT$^-$ CCR7$^+$ Tregs



Revised Figure 7G Diagram illustrating the experimental setup.

-The same may apply to the suppression assays presented in Fig. 4J using the same Treg subsets. There is no detail on how these subsets were sorted and it is assumed that the same strategy was used.

Response 2: Thank you. Consistent with the previous experiments, the sorting of Tregs throughout the study was performed as described in the methods section: "We initially performed positive magnetic bead enrichment using anti-CD4 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to purify CD4$^+$ T cells from T1D patients, followed by cell sorting of TIGIT$^+$ CCR7$^-$ and TIGIT$^-$ CCR7$^+$ Treg (CD4$^+$ CD25$^+$ CD127$^-$) subsets using the FACS Aria II cell sorter (BD Biosciences)."

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Reviewer #4: Congratulations on this excellent revision. The authors have addressed my concerns about the manuscript adequately. I have no further comments.

Thank you for your positive feedback and for confirming that all concerns have been adequately addressed. We appreciate your support.

We hope our response is helpful in addressing your concerns.

We are looking forward to hearing from you soon.

With warm regards,

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RESPONSE TO REVIEWERS' COMMENTS

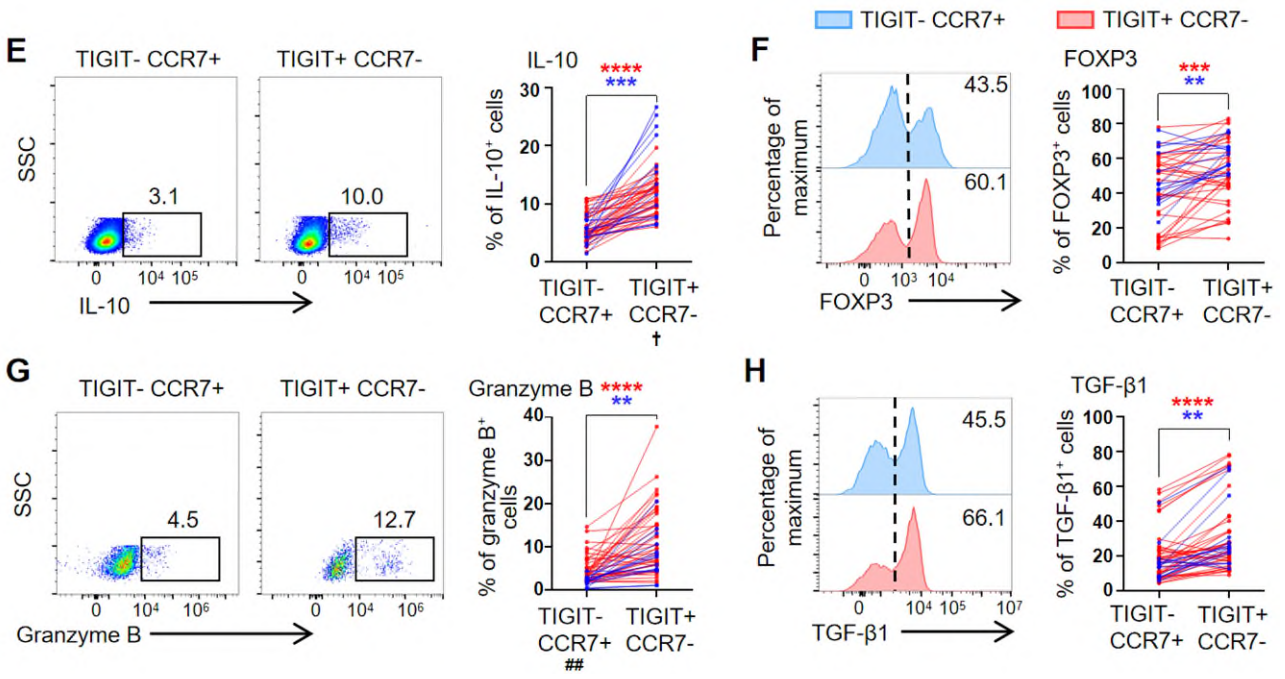
Dear Reviewers:

Thank you very much for taking the time to review our revised manuscript titled "TGF-β-mediated crosstalk between TIGIT$^+$ Tregs and CD226$^+$ CD8$^+$ T cells in the progression and remission of type 1 diabetes". We sincerely appreciate the constructive feedback provided. Following a thorough review of each comment, we have undertaken substantial additional work to specifically address the concerns raised. **For clarity, in the manuscript, initial revisions are marked in red, and the second round of revisions are marked in blue.**

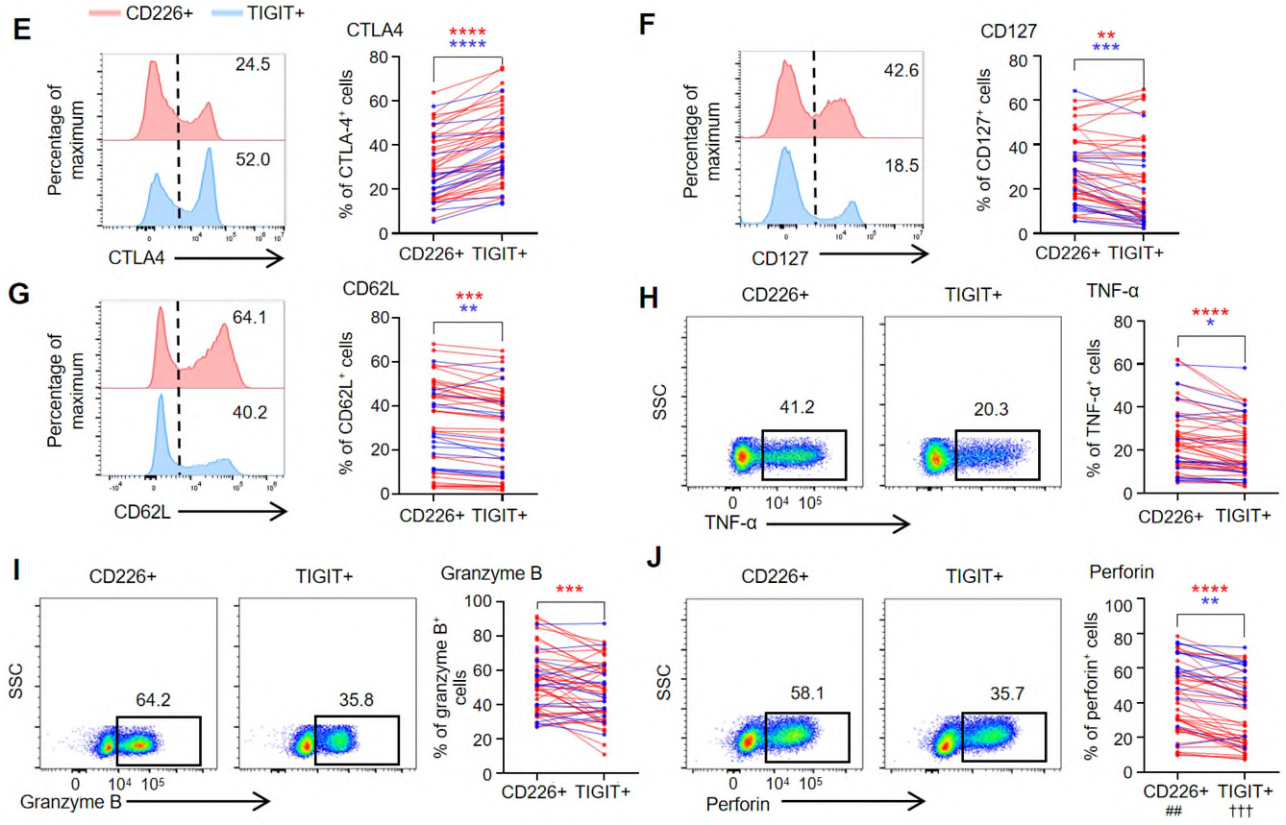
A comprehensive point-by-point response has been compiled, detailing our approach and the amendments made in response to your invaluable comments.

Reviewer #1: The authors should be commended for the efforts to address prior review concerns. The resulting manuscript is much improved, however, a number of questions remain.
-The authors show representative plots of the frequency of cytokine-producing CD8s for each cytokine assayed, but instead plot the gMFI data for each cytokine. Plotting the differences between frequencies of cytokine-producing cells may be more physiologically relevant than switching from frequency to gMFI between the representative plots and data.

Response 1: Thank you for your valuable suggestion. In accordance with your advice, we have updated Figures 4 and 5 to present the frequencies of cytokine-producing CD4/8$^+$ T cells corresponding to each assessed cytokine, instead of utilizing the gMFI data.



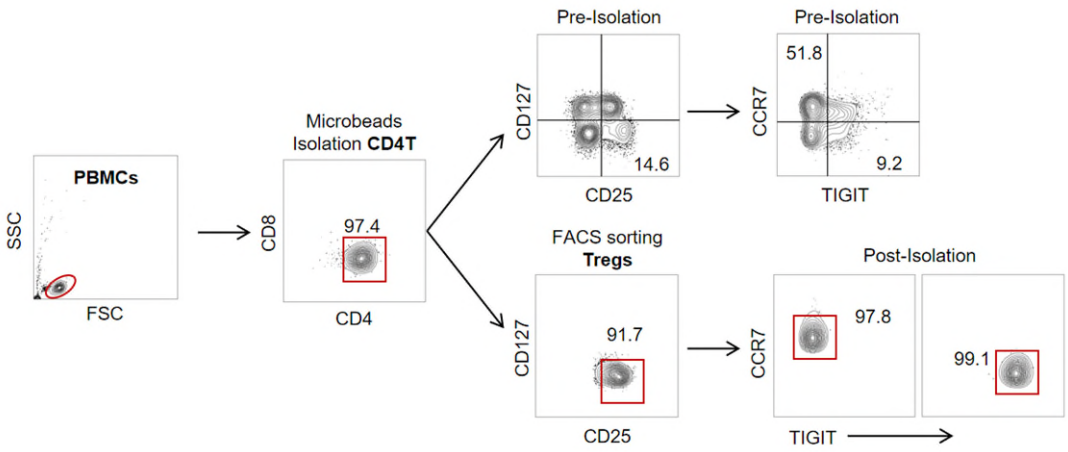
Revised Figure 4 E–H The function of two distinctive Treg subsets. (**E-H**) Representative diagrams and quantification of (**E**) IL-10, (**F**) FOXP3, (**G**) Granzyme B, and (**H**) TGF-β1 in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Treg subpopulations; "#" and "†" signify statistical significance of marker expression in TIGIT$^-$ CCR7$^+$ and TIGIT$^+$ CCR7$^-$ Tregs, respectively, between T1D and HDs. Two-tailed unpaired t-test; data are shown as the mean ± SEM. */†P < 0.05; **/##P < 0.01; ***P < 0.001; ****P < 0.0001. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.



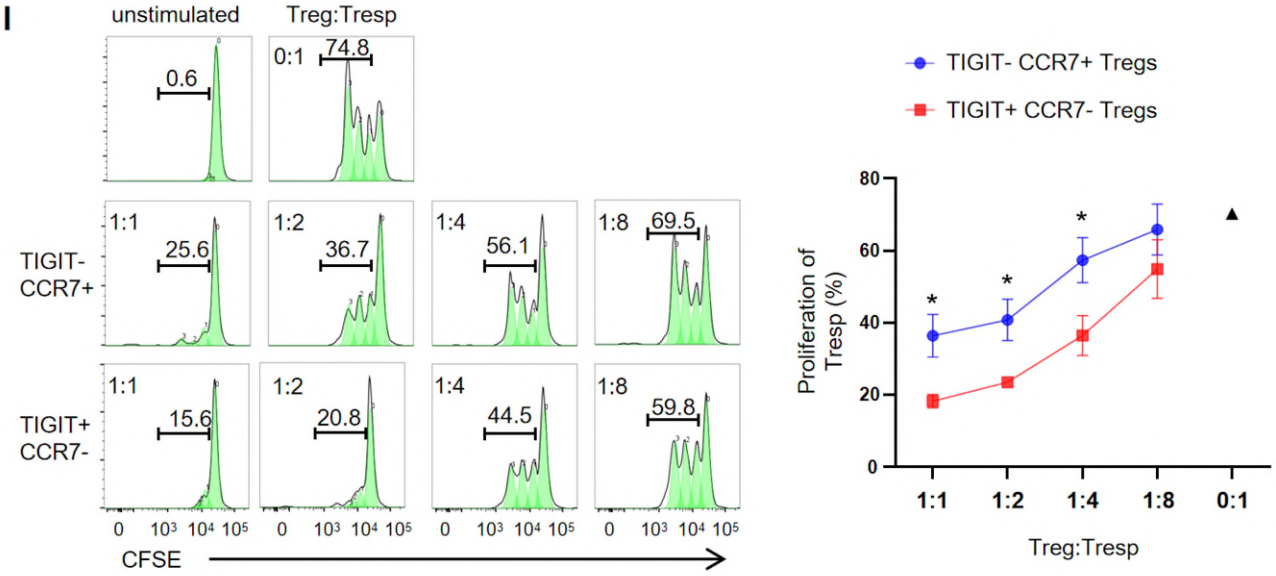
Revised Figure 5 E–J The function between two T1D-stage-associated CD8⁺ T subsets. (**E–J**) Representative diagrams and quantification of (**E**) CTLA4, (**F**) CD127, (**G**) CD62L**,** (**H**) TNF-α, (**I**) Granzyme B, and (**J**) perforin in CD226⁺ and TIGIT⁺ CCR7⁻ CD8⁺ T cells from T1D patients (red line, n=40) and HD (blue line, n=14). The *P* value for each protein was calculated using a paired/unpaired t-test. Red and blue "*" denote statistical significance within T1D and HDs, respectively, between CD226⁺ and TIGIT⁺ CCR7⁻ CD8⁺ T cell subpopulations; "#" and "†" signify statistical significance of marker expression in CD226⁺ and TIGIT⁺ CCR7⁻ CD8⁺ T cells, respectively, between T1D and HDs. */#$P < 0.05$; **$P < 0.01$; ***/†$P < 0.001$; ****/####$P < 0.0001$. T1D, type 1 diabetes; PR, partial remission; HD, healthy donor; PCP, postprandial C-peptide.

-Suppression assays using dye dilution approaches need to account for both the number of cells that enter a proliferative cycle, as well as the number of cell divisions. That data is not accessible from Ki-67 analysis alone. This draws in concerns for the suppression assay employed that measured Ki-67 gMFI in the effectors at 48 hrs. Data may also need to be normalized to the 1:0 control condition.

Response 2: Thank you for helping improve our data. Yes, the number and state of cells matters a lot in interpreting the results of suppression assays. Also, Ki-67 measuring is not the best way for suppression assays. In our efforts to elucidate the differential suppressive functions of Treg subsets on $CD8^+$ T cell expansion, we provided a more solid results with CFSE staining and deleted the Ki-67 part with attention to the matching of cell number and cell divisions. Briefly, two subsets of sorted $TIGIT^+$ $CCR7^-$ and $TIGIT^-$ $CCR7^+$ Tregs (5×10^4) were co-cultivated with CFSE-stained $CD8^+$ T cells across a gradient of ratios (0:1, 1:1, 1:2, 1:4, and 1:8), sustaining the culture over a continuous period of 72 hours. Notably, the $TIGIT^+$ $CCR7^-$ Treg fraction exhibited a pronounced immunosuppressive effect, significantly inhibiting the proliferation of the $CD8^+$ T cells. This effect was documented by the inhibition in cell division among the $CD8^+$ T cells, as depicted in Figure 4I. Regarding the Ki-67 analysis, we had conducted control experiments with a Tregs-to-Responder T ratio of 0:1 (Figure R1). However, Ki-67 results were not displayed in the revised manuscript .



Revised Figure S8C The sorting process for $TIGIT^+$ $CCR7^-$ Tregs and $TIGIT^-$ $CCR7^+$ Tregs.



Revised Figure 4 I Treg suppression assay. TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Tregs, isolated from T1D patients (n=4), were cocultured with CFSE labeled CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 72 h at 37°C. The proliferation of CD8$^+$ T cells was measured. The triangle symbolizes the control group with a Tregs-to-Responder T ratio of 0:1. Two-tailed unpaired t-test; data are shown as the mean ± SEM. *P < 0.05.

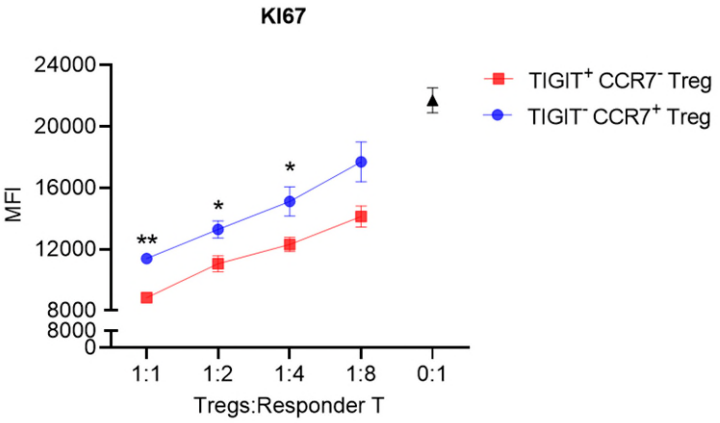


Figure R1 Suppression assay of Treg subsets. TIGIT$^+$ CCR7$^-$ Treg cells and TIGIT$^-$ CCR7$^+$ Treg cells (5×10^4), isolated from T1D patients (n = 6), were cocultured with autologous CD8$^+$ T cells in the presence of α-CD3/CD28 antibodies (0.5 bead/cell) for 48 h at 37°C. Ki-67 in CD8$^+$ T cells was measured. The triangle symbolizes the control group with a Tregs-to-Responder T ratio of 0:1. Two-tailed unpaired t-test; data are shown as the mean ± SEM. *P < 0.05; **P < 0.01

-The new figure 8H-I should have representative gating for CCR7/TIGIT, rather than just SSC/TIGIT

Response 3: We appreciate your suggestion regarding the representative flow cytometry plots on the left should match the statistical gating shown on the right. Accordingly, we have made revisions to the original Figure 8H&I to display the representative gating for CCR7/TIGIT instead of SSC/TIGIT. The original Figure 8H&I has been changed to Figure S13E&F.

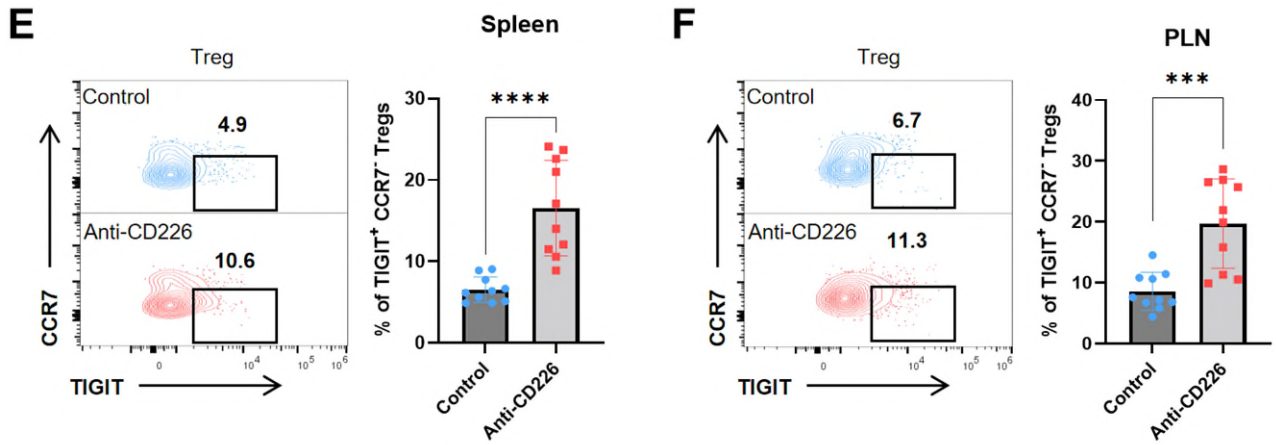


Figure S13 E&F Frequencies of TIGIT^{+} CCR7^{-} Tregs in spleen and PLN from STZ-induced mouse model are shown (n = 10).

-It is not clear what "CCS" is on the y-axis for 8F.

Response 4: We appreciate your attention to detail in identifying the ambiguity. "CCS" on the y-axis of original Figure 8F was a typographical error. It has been corrected to "CD8" in the revised figure. We apologize for any confusion this may have caused and assure that the updated figure now clearly conveys the intended information.

-The new Figure 8N-O should have representative gating for CD8/TIGIT, rather than just TIGIT.

Response 5: Thank you for clarifying the situation with the representative gating in original Figures 8N-O. We have corrected this labeling error in the revised figures to accurately depict the gating strategy for CD8/TIGIT and CD8/CD226.

-The authors show that there is a decrease in the frequency of $CD226^+$ $CD8^+$ and an increase in $TIGIT^+$ $CD8^+$ in the spleen following in vivoTx, but suggest that there isn't any functional changes in the $CD226^+$ $CD8^+$ population. The authors should clarify what they hypothesize the antibody is doing to that subset. To this point, the authors should clarify whether the anti-CD226 mAb is depleting or blocking in action. This notion of epitope specificity should also be considered for flow staining antibodies relative to the therapeutic mAb.

Response 6: Thank you for making us think deeper. In response to your suggestions, we firstly searched published literature for reference. We utilized functional grade anti-mouse CD226 (Clone: 10E5, eBioscience, Cat #: 16-2261) for therapeutic intervention. The 10E5 clone was initially reported in its application in experimental autoimmune encephalomyelitis (EAE), where its administration delayed disease onset and decreased the severity of Th1-mediated autoimmune pathology (*Dardalhon et al, J Immunol, 2005*). Subsequently, it has also been documented to suppress antigen-specific T cell proliferation and IFN-γ production in vivo, as well as to extend the survival of skin allografts (*Liu et al, Arthritis Research & Therapy, 2018*). Recently, this antibody has also been applied to additional disease models as well (*Sakano Y, et al. J Allergy Clin Immunol. 2024*). Through literature reviewing, we found that none of the above studies directly tested whether the function of the anti-CD226 mAb is blocking or depleting, so we designed some testing to verify the function of anti-CD226 mAb in vitro and in vivo after discussion with some immunologists.

To elucidate the mechanism of action of the anti-CD226 mAb, we conducted a binding assay to evaluate the ability of the rat anti-mouse CD226 mAb (10E5) to block the binding of CD226 to its ligand PVR, an experiment frequently used for testing neutralization & blocking antibodies. In short, 2×10^5 splenic $CD8^+$ T cells were incubated with different concentrations of antibodies (10E5) and 5 μg/mL mPVR-hFc fusion protein. DyLight 488-conjugated goat anti-human IgG Fc antibody (ab98619, abcam, Cambridge, MA, USA) was used to detect the binding frequency of mPVR-Fc fusion protein. As shown in ***Figure S13A***, the anti-CD226 mAb can efficiently block the binding or CD226 to PVR. To further validate the blocking effect of anti-CD226 mAb, $CD8^+$ T cells, isolated from the spleens of NOD mice, were cultured in vitro and exposed to anti-CD3 (5 μg/mL) and mPVR-Fc (5 μg/mL), either alone or in combination with anti-CD226 mAb (5

μg/mL), for a period of 24 h (***Fig. S13B–D***). We confirmed that while PVR binding to CD226 promotes T cell proliferation and cytokine production, **CD226 blockade significantly suppresses both cell proliferation and cytokine production of CD8$^+$ T cells in vitro.**

Next, we validated the function and therapeutic potential of anti-CD226 mAb in vivo. Firstly, our data demonstrated that treatment with anti-CD226 antibodies led to a reduction in both proliferation and cytokine production of the total CD8$^+$ T cells in STZ-induced diabetic mice, consistent with existing literature (*Johnston RJ, et al. Cancer Cell. 2014*). As regarding to the previous findings of no significant changes in the function of CD226$^+$ CD8$^+$ population, we constructed a larger sample size and discovered that the functional capacity of CD226$^+$ CD8$^+$ T cells is also compromised by anti-CD226 treatment (see revised Fig. S14). Secondly, we assessed the alterations in CD8$^+$ T cell functionality following CD226 blockade using the cyclophosphamide-accelerated NOD mouse model, which provides a more accurate representation of the autoimmune milieu associated with T1D. The findings demonstrated that intervention with anti-CD226 mAb significantly attenuated the proliferation and cytokine secretion of both total CD8$^+$ T cells (now *Fig. 8I-L*) and CD226-expressing CD8$^+$ T subsets (*Fig. S15*). Finally, to elucidate the effect of anti-CD226 mAb on signaling pathways of CD8+ T cells, we evaluated the phosphorylation levels of signaling proteins AKT and mTOR. Our findings demonstrated a marked decrease in the phosphorylation of AKT and m-TOR proteins after CD226 blockage compared to control (***Fig. S13E–G***).

In summary, these experiments confirmed that CD226 mAb blockade significantly suppresses both T cell proliferation and cytokine production by disrupting the interaction between CD226 and PVR.

In response to the concern regarding epitope specificity, we verified that the CD226 antibody (**TX42.1**) used for flow cytometry staining and the therapeutic CD226 antibody (**10E5**) in this study bind to different epitopes of CD226. And the TX42.1 have not been reported to possess any cell inhibitory or depletive functions. The observed effects in our experiments can be attributed to the therapeutic mAb rather than the flow cytometry staining process.

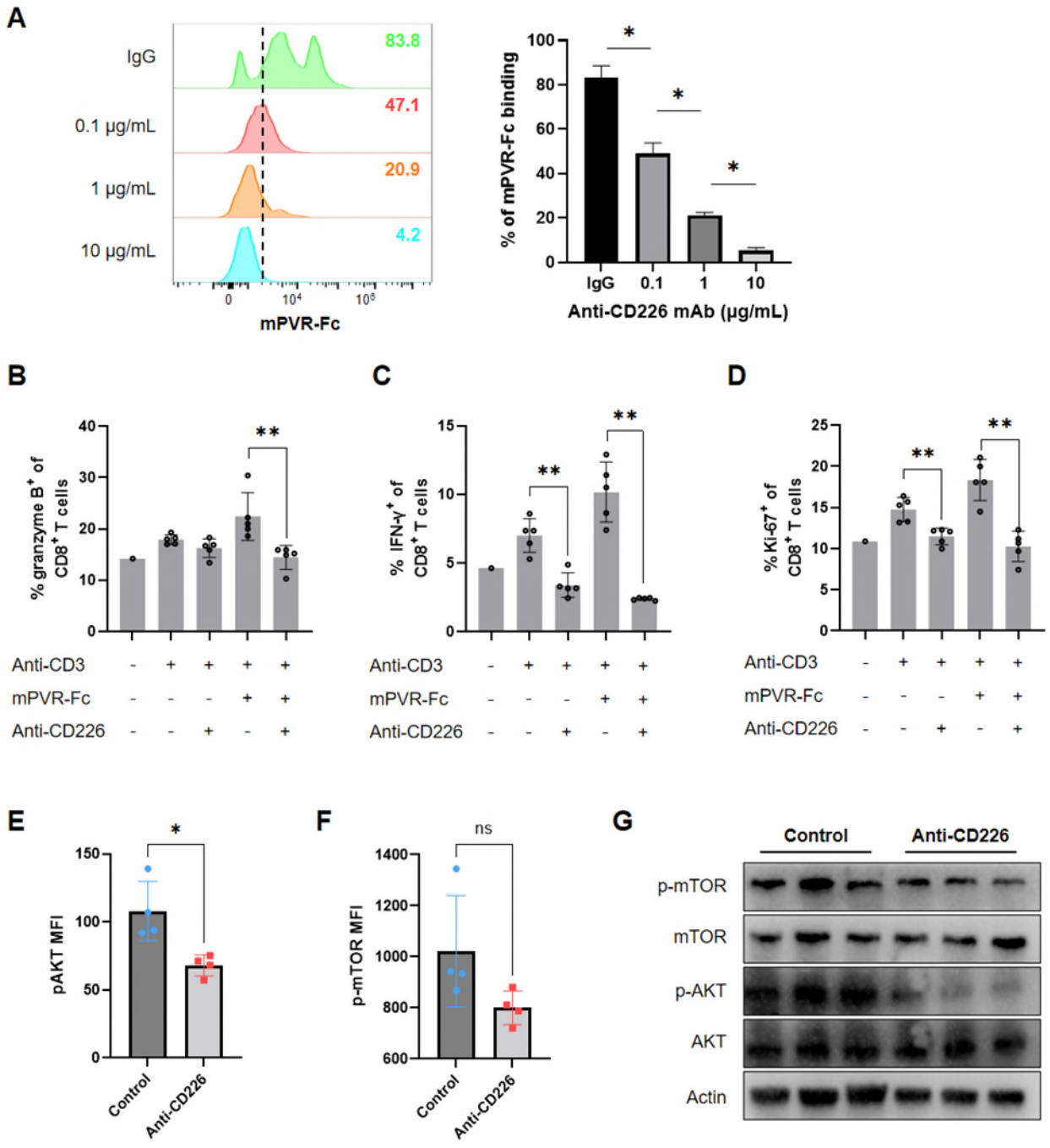
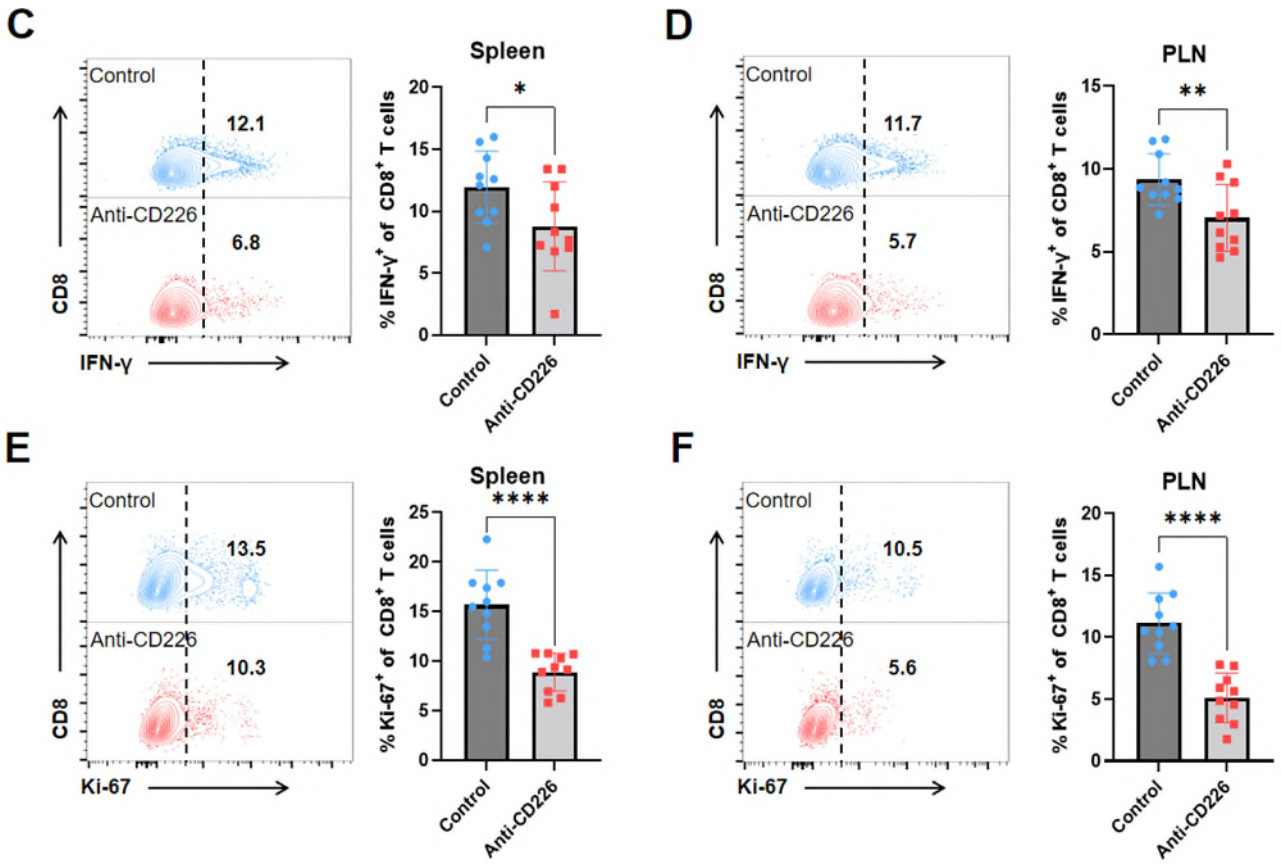


Fig. S13 (related to Fig.8) CD226 blockade inhibited the proliferation and cytokine-production of CD8⁺ T cells. (**A**) Identification of rat anti-mouse CD226 monoclonal antibody. Representative histogram (left) and quantification (right) of mouse PVR-hFc fusion protein binding to mouse CD8⁺ T cells at indicated anti-mCD226 concentrations. (**B–D**) Splenic CD8⁺ T cells from NOD mice were enriched and stimulated for 24 h in the presence of anti-CD3 (5 μg/mL), mPVR-Fc (5 μg/mL), and/or anti-CD226 (5 μg/mL) antibodies as indicated. The expression levels of (**B**) granzyme B, (**C**) IFN-γ, and (**D**) Ki-67 within CD8⁺ T cells were

shown (n=5). The expression levels of (**E**) pAKT, and (**F**) p-mTOR within splenic $CD8^+$ T cells from Cy-induced mouse model were depicted (n=4). (**G**) $CD8^+$ T cells from the spleens of the two groups were sorted and the levels of pAKT and p-mTOR were determined by western blots.Two-tailed unpaired t-test; data were shown as the mean ± SD. *P<0.05; **P<0.01.



Now Fig. 8 C-F Administration of Anti-CD226 mAb in vivo inhibits the expansion of $CD8^+$ T cells and cytokine-production in STZ-induced diabetic mice. The expression levels of IFN-γ, and KI67 within $CD8^+$ T cells in the spleen and PLN were depicted for the two groups (n=10). Data are shown as the mean ± SD; two-tailed unpaired t-test. *P<0.05; **P<0.01.

STZ-induced mouse model:

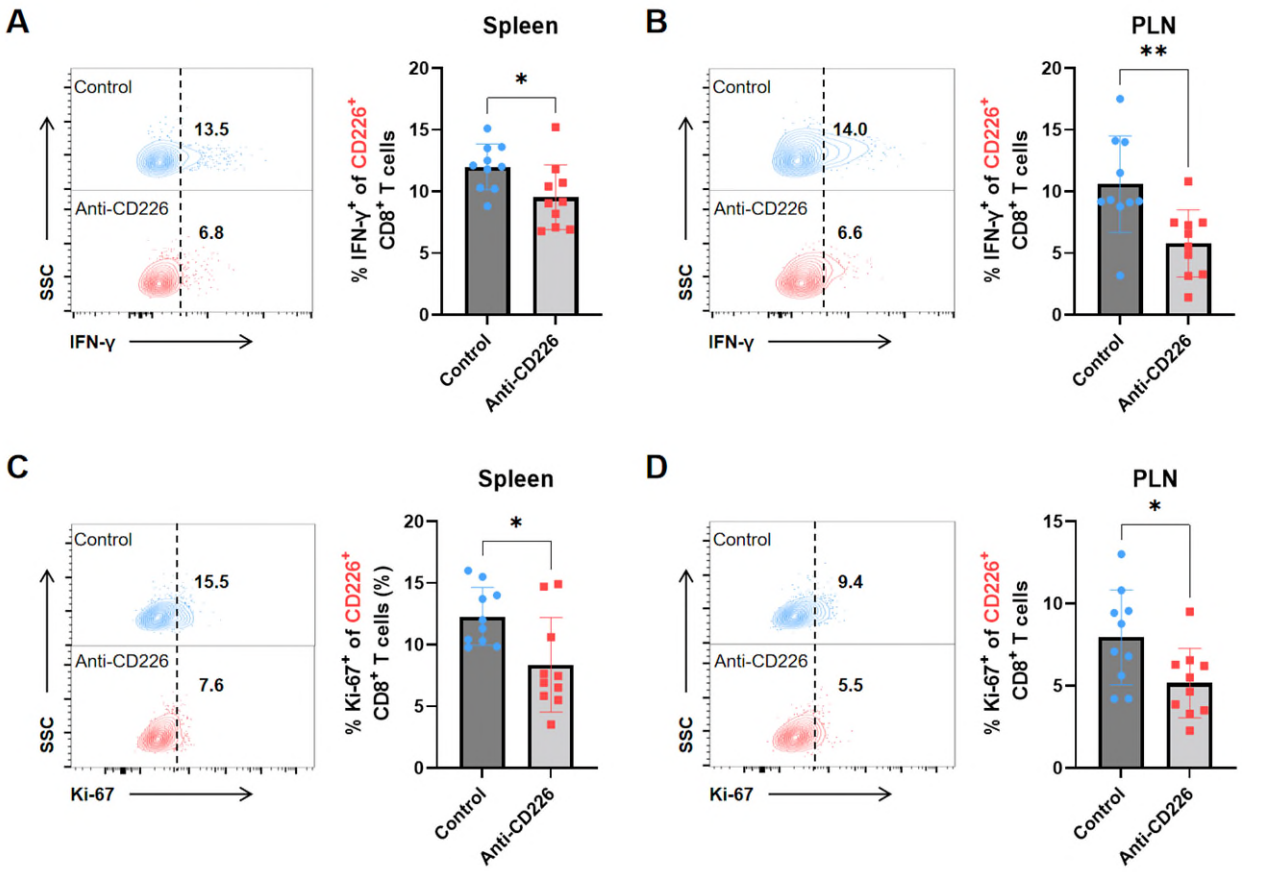
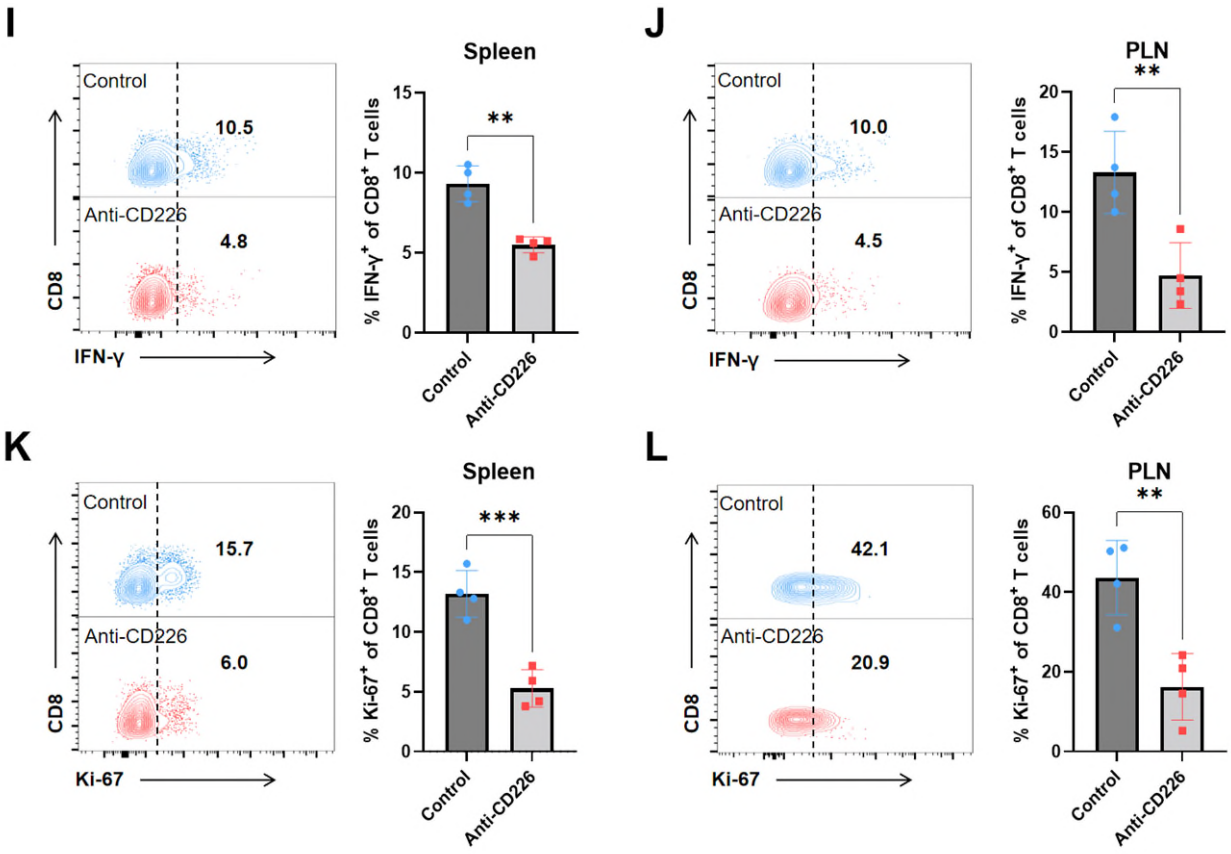


Fig. S14 Anti-CD226 treatment does change the function of CD226$^+$ CD8$^+$ T cells in STZ-induced diabetic mice. The expression levels of (**A, B**) IFN-γ, and (**C, D**) KI67 within CD226$^+$ CD8$^+$ T cells in the spleen and PLN are depicted for the two groups (n=10). Two-tailed unpaired t-test; data are shown as the mean ± SD.



Now Fig. 8I-L Anti-CD226 treatment inhibited the proliferation and cytokine-production of $CD8^+$ T cells in Cy-induced NOD mice. The expression levels of (I, J) IFN-γ, and (K, L) KI67 within $CD8^+$ T cells in the spleen and PLN were depicted for the two groups (n = 4). Two-tailed unpaired t-test; data were shown as the mean ± SD. $^{**}P<0.01$; $^{***}P<0.001$.

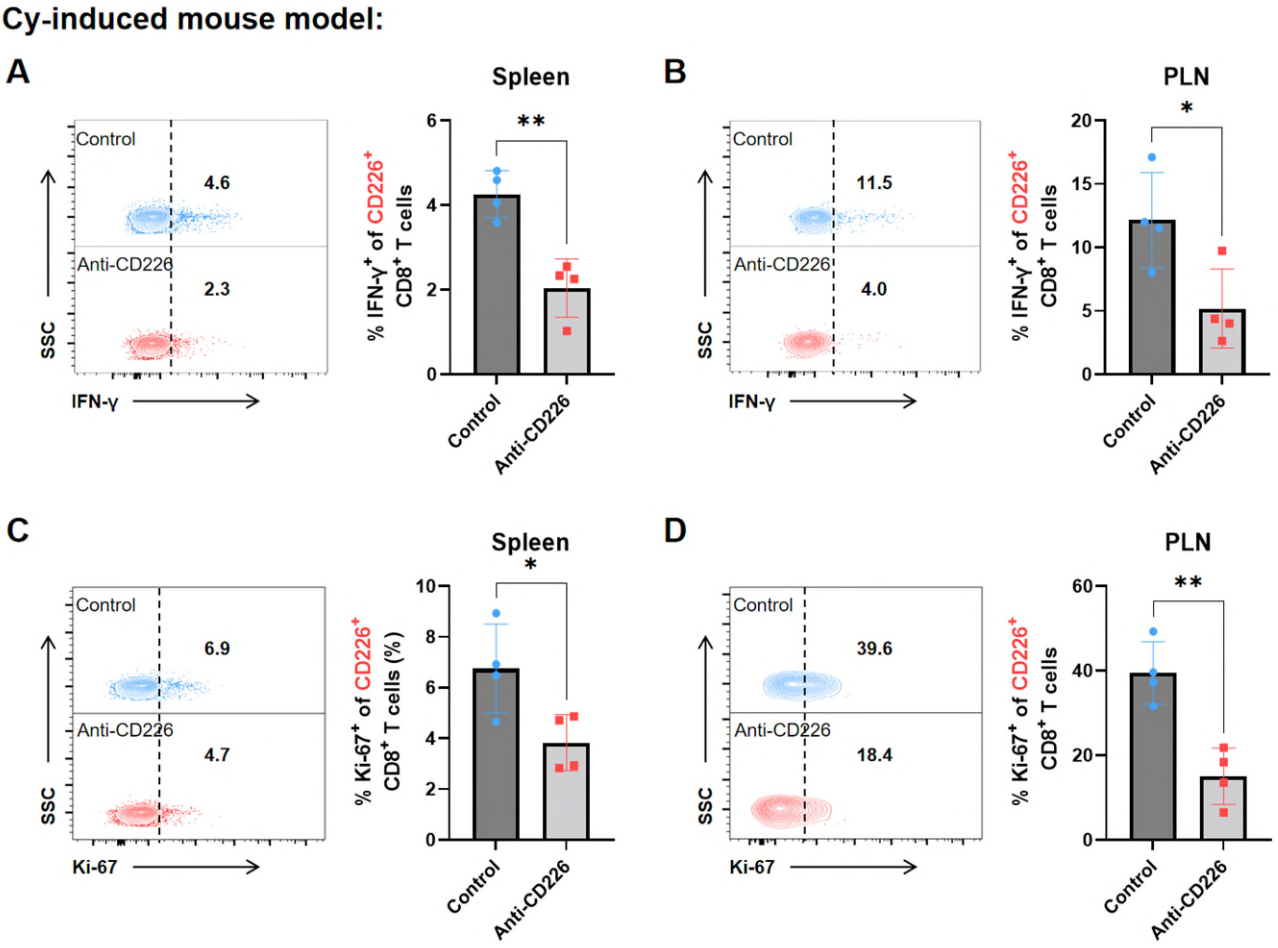


Fig. S15 Anti-CD226 treatment inhibited the proliferation and cytokine-production of $CD226^+$ $CD8^+$ T cells in Cy-induced NOD mice. The expression levels of (A, B) IFN-γ, and (C, D) KI67 within $CD226^+$ $CD8^+$ T cells in the spleen and PLN were depicted for the two groups (n=4). Two-tailed unpaired t-test; data were shown as the mean ± SD. *P<0.05; **P<0.01.

-The authors indicate there isn't any impact in the pLN, so seeing if there are differences in the pancreas-infiltrating CTL populations would be of interest.

Response 7: In response to the question regarding potential differences in CTL populations, despite the lack of observed effects in the PLN, we have conducted a focused immunofluorescent staining analysis on pancreatic tissues. Our results have revealed that treatment with anti-CD226 does indeed ameliorate the infiltration of CD8 CTLs in NOD mice.

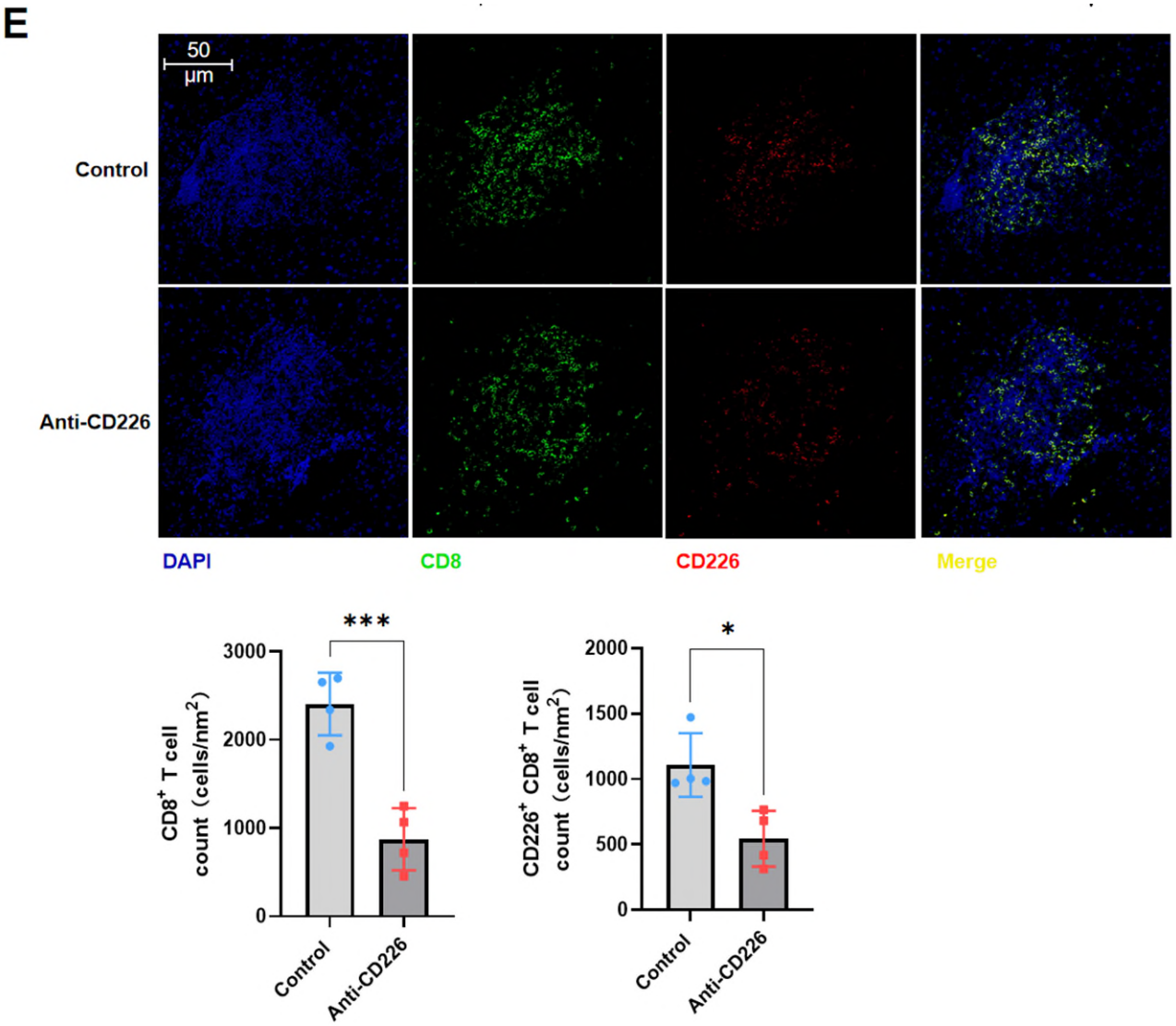
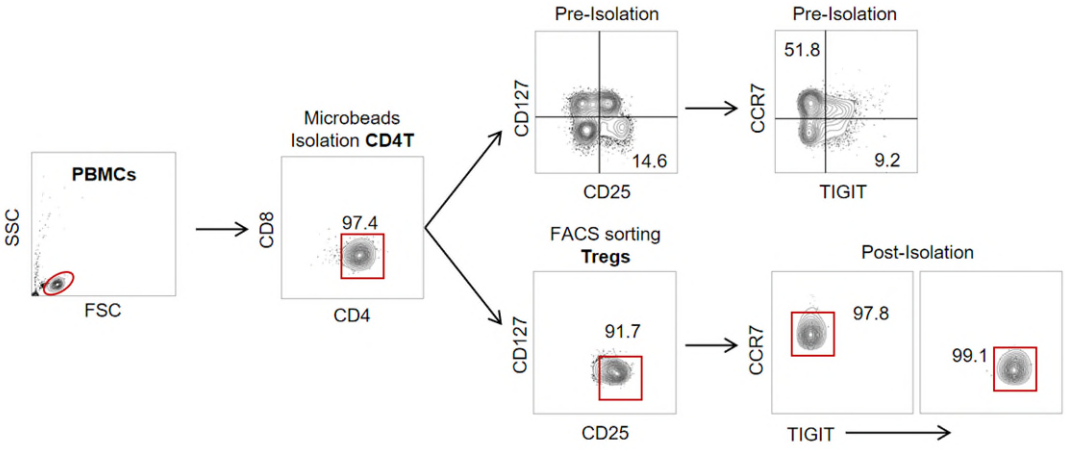


Fig.S17E Anti-CD226 therapy alleviated local immune infiltration in the pancreatic islets. (**E**) Multiplex fluorescence immunohistochemistry staining was used to evaluate the presence of total $CD8^+$ T cells and $CD226^+$ $CD8^+$ T cells within the islets of NOD mice. Representative multiplex fluorescence images in the pancreas specimen were shown. Nuclei, CD8 and CD226 within the cells, are visualized in blue, green, and red, respectively. Assessment of the density of total $CD8^+$ T cells and $CD226^+$ $CD8^+$ T cells within the islets. *P* values were calculated using the unpaired t-test, $*P<0.05$.

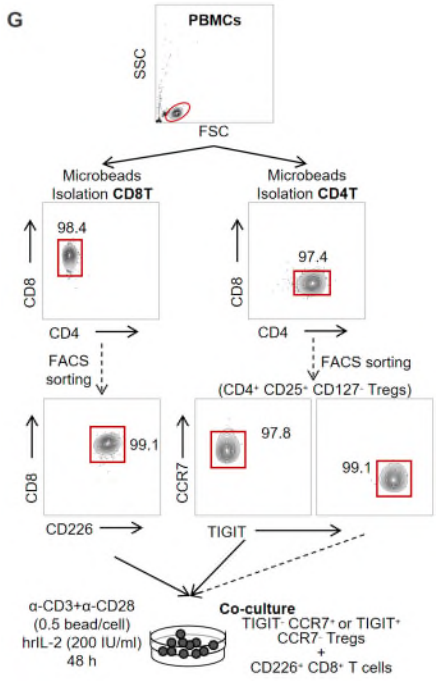
Reviewer #3: The Authors have satisfactorily addressed most of the points raised.

A major issue remains in the suppression assays presented in Fig. 7G-I using sorted TIGIT^{+} CCR7^{-} and TIGIT^{-} CCR7^{+} Tregs. The sorting of these populations only included a preliminary magnetic isolation of CD4^{+} T cells, which were subsequently stained for TIGIT and CCR7 for sorting. Thus, these cells do not qualify as Tregs, as they have not been sorted on additional Treg markers, e.g. CD25^{+} CD127^{-} as done in Fig. S8. It is thus not surprising to see that TIGIT^{+} CD4^{+} T cells are more suppressive than CCR7^{+} CD4^{+} T cells, as the latter are likely to be composed mainly of naïve and central memory conventional T cells. These experiments should be repeated by gating on CD25^{+} CD127^{-} CD4^{+} Tregs before sorting TIGIT^{+} CCR7^{-} and TIGIT^{-} CCR7^{+} subsets.

Response 1: Thank you. In our flow cytometric sorting process, we thoroughly examined a comprehensive panel of Treg-specific markers, including CD4, CD25, CD127, CCR7, and TIGIT. It appears there was a miscommunication in Figure 7G, resulting in unclear labeling and subsequent misunderstanding. To rectify this, we have made the corrections to the figure to accurately reflect the inclusion of all relevant Treg markers in the sorting process. This ensures that the sorted TIGIT^{+} CCR7^{-} and TIGIT^{-} CCR7^{+} cells are appropriately identified as Tregs, addressing the concerns about the specificity of the cell populations used in our assays.



Revised Figure S8C The sorting process for TIGIT^{+} CCR7^{-} Tregs and TIGIT^{-} CCR7^{+} Tregs



Revised Figure 7G Diagram illustrating the experimental setup.

-The same may apply to the suppression assays presented in Fig. 4J using the same Treg subsets. There is no detail on how these subsets were sorted and it is assumed that the same strategy was used.

Response 2: Thank you. Consistent with the previous experiments, the sorting of Tregs throughout the study was performed as described in the methods section: "We initially performed positive magnetic bead enrichment using anti-CD4 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to purify $CD4^+$ T cells from T1D patients, followed by cell sorting of $TIGIT^+$ $CCR7^-$ and $TIGIT^-$ $CCR7^+$ Treg ($CD4^+$ $CD25^+$ $CD127^-$) subsets using the FACS Aria II cell sorter (BD Biosciences).

Reviewer #4: Congratulations on this excellent revision. The authors have addressed my concerns about the manuscript adequately. I have no further comments.

Thank you for your positive feedback and for confirming that all concerns have been adequately addressed. We appreciate your support.

We hope our response is helpful in addressing your concerns.

We are looking forward to hearing from you soon.

With warm regards,

Zhiguang Zhou, Bin Zhao, Xia Li

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RE: Manuscript ID: NCOMMS-23-50175D-Z

Dear Reviewer #1:

We are grateful for the opportunity to submit our revised manuscript, "TGF-β-mediated Crosstalk between TIGIT$^+$ Tregs and CD226$^+$CD8$^+$ T Cells in the Progression and Remission of Type 1 Diabetes", and we appreciate your constructive feedback and suggestions which help in enhancing the quality and clarity of our work.

We have carefully addressed each point raised, and have made the necessary revisions to address your concerns. Here are our detailed responses to each question raised:

1. For insulitis scoring, the authors demonstrate that CD8$^+$ T cells are associated with increased insulitis and Tregs are associated with decreased insulitis; however, the authors do not directly compare how frequencies of TIGIT$^+$CCR7$^-$ Tregs versus TIGIT$^-$CCR7$^+$ Tregs may impact insulitis. These cells need to be directly assessed in the pancreas and pLN or tested formally through adoptive co-transfer experiments to definitely support the claims of direct regulation.

Response: Thanks. As shown in **Supplementary Fig. 12**, we conducted additional statistical analyses using existing data to directly compare the frequencies of TIGIT$^+$CCR7$^-$ Tregs versus TIGIT$^-$CCR7$^+$ Tregs in the spleen and PLN of NOD mice of various ages, evaluating their correlation with the severity of insulitis. Our findings revealed a significant negative correlation between TIGIT$^+$CCR7$^-$ Tregs and insulitis severity ($r = -0.48$, $p = 0.014$ in the spleen; $r = -0.59$, $p = 0.005$ in PLN), whereas TIGIT$^-$CCR7$^+$ Tregs showed a significant positive correlation ($r = 0.47$, $p = 0.015$ in the spleen; $r = 0.62$, $p = 0.003$ in PLN).

Building on our prior functional experiments (**Fig. 4&7**), it can be concluded that TIGIT$^+$CCR7$^-$ Tregs possess enhanced immunosuppressive capabilities compared to TIGIT$^-$CCR7$^+$ Tregs.

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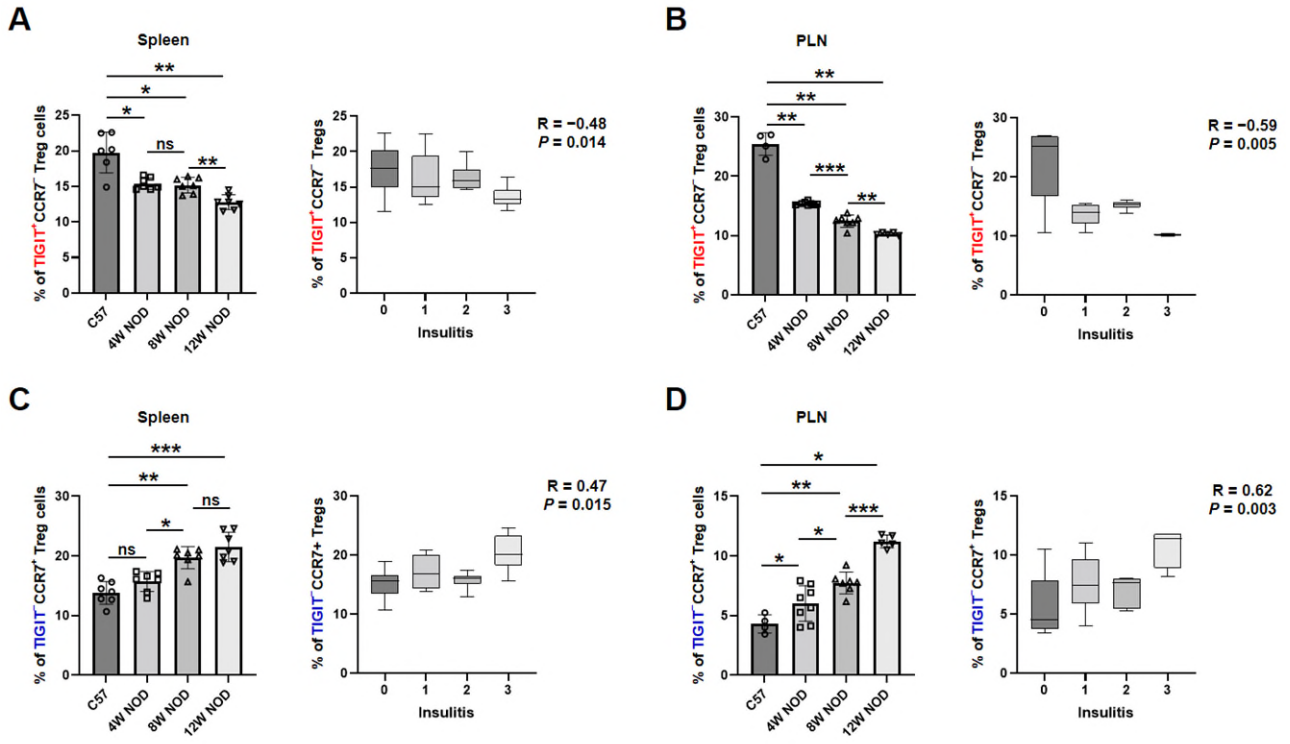

Supplementary Fig. 12 Dynamics of TIGIT$^+$CCR7$^-$ versus TIGIT$^-$CCR7$^+$ Tregs in NOD mice. Association between TIGIT$^+$CCR7$^-$ Tregs (**A–B**) and TIGIT$^-$CCR7$^+$ Tregs (**C–D**) with the severity of insulitis in the spleen and PLN of NOD mice aged 4 (n=7), 8 (n=7), and 12 (n=5) weeks, compared to age-matched C57BL/6 controls (n=4). Symbols represent each group: C57BL/6 mice (circles), 4-week NOD mice (squares), 8-week (upright triangles), and 12-week NOD mice (inverted triangles). *P* values were calculated using a t-test or Spearman's correlation analysis. Data are presented as the mean ± SD. n indicates the number of biologically independent samples examined. Source data are provided as a Source Data File.

2. The authors initially state in the abstract and introduction that anti-CD226 reduces diabetes in the NOD, without clarifying it is Cyclophosphamide-induced. This is an important point because the treatment with Cyclophosphamide alters the normal distribution of cells and does not reflect the same processes underway in spontaneous disease.

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Response: Thank you for pointing out. We acknowledge that the treatment effects described were observed in Cyclophosphamide (CY)-induced diabetes in NOD mice, which differs from spontaneous diabetes due to the drug's impact on immune cell distribution.

CY accelerates diabetes in NOD mice, providing an acute model to assess the potential protective effects of various molecules (*Proc Natl Acad Sci U S A. 2008; Biomed Pharmacother. 2024*). As documented in previous studies (*Journal of Autoimmunity. 2005*), CY injection leads to a dramatic reduction in total cell numbers in the spleen and PLN around days 6–7 post-injection. However, this effect diminishes significantly by day 10. We conducted our assessment of immune cell changes approximately two weeks after CY administration, at which point the effects of CY are considerably reduced.

In response, we have revised our abstract and introduction to explicitly state that our findings are specific to CY-induced conditions.

3. The authors still did not actually perform dye dilution suppression assays to evaluate the amount of Treg or effector T cell proliferation. Instead, the authors showed alterations in pro-inflammatory cytokines and changes in Ki-67 before concluding differences in suppressive capacity. While in vitro assays of suppression are not without their own limitations, they do provide important information on numbers of cell divisions, cell death, and the potential for observing consumption over direct suppression.

Response: Thanks. Regarding this question, we have conducted those experiments in our July 2024 revision (NCOMMS-23-50175C).

In addition to classical proliferation suppression experiment using Ki-67 (**Supplementary Figure 8D**), we performed the Treg suppression assay with CFSE to measure cell divisions. Briefly, two subsets of sorted $TIGIT^{+}CCR7^{-}$ and $TIGIT^{-}CCR7^{+}$ Tregs (5×10^{4}) were co-cultivated with CFSE-

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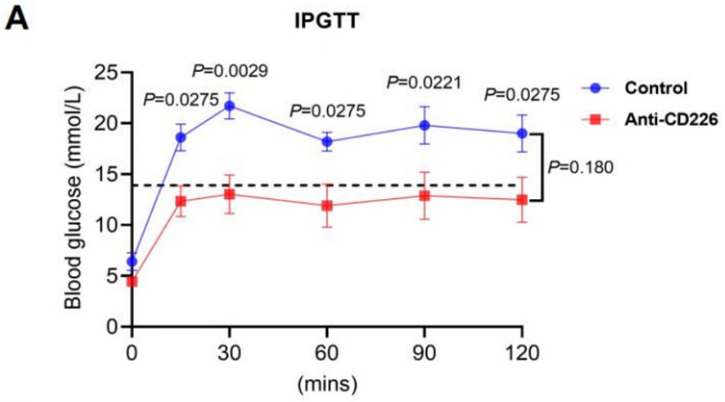
stained $CD8^+$ T cells across a gradient of ratios (0:1, 1:1, 1:2, 1:4, and 1:8), sustaining the culture over a continuous period of 72 hours. Notably, the $TIGIT^+CCR7^-$ Treg fraction exhibited a pronounced immunosuppressive effect, significantly inhibiting the proliferation of the $CD8^+$ T cells. This effect was documented by the inhibition in cell division among the $CD8^+$ T cells, as depicted in **Figure 4I**. In summary, the data of in vitro assays of suppression are available and our results demonstrated that $TIGIT^+CCR7^-$ Tregs have stronger inhibitory functions.

4. In the last figure, the authors suggest that anti-CD226 improves beta cell function and alters glucose tolerance. The authors do not have any metabolic data (e.g., glucose tolerance tests), so they should instead state that it reduces insulitis. The killing assay was conducted using a mast cell line (P815).

Response: Thanks. This result was initially displayed in the **main Figure 8C** of NCOMMS-23-50175A but was transferred to **Supplementary Figure 14A** during the revision process. It demonstrated that the application of anti-CD226 mAb improved glucose tolerance.
Additionally, we acknowledge the oversight in our description of the cell line used in the killing assay. We have corrected this in the manuscript to specify that the assay was conducted using the P815 mast cell line, and we have ensured that this detail is clear in the methods section of our paper.



Supplementary Figure 14A Anti-CD226 therapy alleviates glucose tolerance in STZ-induced diabetic mice. (A) IPGTT comparing the control group and the anti-CD226 mAb group (n=5),

analyzed via mixed-effects model. Data are shown as the mean ± SEM.

We believe these modifications have strengthened the manuscript and better aligned our conclusions with the data presented. We look forward to your feedback and are happy to make further changes if necessary.

Thank you once again for reviewing our revised manuscript and appreciate your constructive feedback.

Sincerely,

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