Target Journal: *Islets - Technical Note*

Title: Beta-cell Cre expression and reduced *Ins1* gene dosage protect mice from type 1 diabetes

Authors: Søs Skovsø[1]*, Peter Overby[1]*, Jasmine Memar-Zadeh[1], Jason Lee[1], Iryna Shanina[1], Vaibhav Sidarala[2], Elena Levi-D'Ancona[2], Jie Zhu[2], Scott A. Soleimanpour[2], Marc Horwitz[1], James D. Johnson*[1]

* These authors contributed equally

Affiliation: Life Sciences Institute, University of British Columbia, Vancouver, BC, Canada

Division of Metabolism, Endocrinology, and Diabetes of the Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA

Correspondence: J.D.J.

Keywords: type 1 diabetes animal models, Cre recombinase, knock-in mice, pancreatic β-cell

Funding: This project was primarily supported by an operating grant to J.D.J. from the Canadian Institutes for Health Research (PJT-152999) and the JDRF Centre of Excellence at UBC (3-COE-2022-1103-M-B). S.A.S. was supported by the JDRF (CDA-2016-189, COE-2019-861), the NIH (R01 DK108921, U01 DK127747), and the Department of Veterans Affairs (I01 BX004444).

Acknowledgements: We thank colleagues in the β-cell biology field for helpful discussions, including on Twitter.

Author Contributions:
S. S. co-conceived experiments, designed and executed in vivo experiments, analyzed data, edited the manuscript
P. O. executed in vivo experiments, analyzed data, co-wrote the manuscript
J. M-Z. analyzed data and co-wrote the manuscript
J. L. designed and analyzed flow cytometry experiments
I. S. designed and executed flow cytometry experiments
V. S. conducted *in vivo* validation experiments
E. L-D. conducted *in vivo* validation experiments
J. Z. conducted *in vivo* validation experiments
S. A. S. oversaw and interpreted *in vivo* validation experiments
M. H. oversaw and interpreted *in vivo* experiments
J. D. J. designed the model, co-conceived experiments, analyzed data, co-wrote the manuscript, and is the ultimate guarantor of this work.

Abstract word count: 300
Body word count: 2704
Reference count: 31
Figure count: 6

Abstract

Cre-mediated recombineering is the main tool of choice for cell type-specific analysis of gene function in pre-clinical models. In the type 1 diabetes research field, multiple lines of NOD mice have been generated that express Cre in pancreatic β-cells using insulin promoter fragments, but tissue promiscuity remains a concern. The constitutive *Ins1*$^{tm1.1(cre)Thor}$ (*Ins1*^{Cre}) mouse on a C57/bl6-J background has been shown to have high β-cell specificity and with no off-target effects reported. Therefore, we explored if *Ins1*^{Cre} mice on an NOD background could be used as a novel tool to investigate β-cell specific gene deletion in a type 1 diabetes setting. Here, we examine a new NOD mouse model in which Cre is inserted into the endogenous *Ins1* locus for ideal β-cell specificity. Fully wildtype (*Ins1*$^{WT/WT}$), *Ins1* heterozygous (*Ins1*$^{Cre/WT}$ or *Ins1*$^{Neo/WT}$), and *Ins1* null mice (*Ins1*$^{Cre/Neo}$) littermate mice had either none, one or two *Ins1* alleles replaced with Cre or a neomycin cassette. Female *Ins1* $^{Neo/WT}$ mice exhibited significant protection from type 1 diabetes. *Ins1*$^{Cre/WT}$ knock-in mice were further protected. The effects of combined neo and Cre knock-in in *Ins1*$^{Neo/Cre}$ mice were additive and resulted in near-complete type 1 diabetes prevention up to one year of age. In *Ins1*$^{Neo/Cre}$ mice, protection from diabetes was associated with reduced in insulitis at 12 weeks of age. We were unable to find significant differences in insulin auto-antibodies or in immune populations between genotypes in the pancreatic lymph node or spleen in 50 weeks old mice, suggesting a β-cell specific mechanism. Collectively, these data confirm previous reports that the loss of *Ins1* alleles protects NOD mice from diabetes and demonstrates, for the first time, that Cre itself may have additional protective effects. This has significant implication for the experimental design and interpretation of pre-clinical type 1 diabetes studies using β-cell-specific Cre in NOD mice.

Introduction

Type 1 diabetes (T1D) is a chronic disorder precipitated by immune-mediated pancreatic β-cell destruction and associated with the presence of anti-islet autoantibodies[1,2]. Due to the progressive loss of β-cells and consequent insulin deficiency, individuals living with type 1 diabetes have a lifelong dependency on exogenous insulin[3]. Higher levels of endogenous insulin secretion are associated with better short- and long-term outcomes in type 1 diabetes. Preservation of residual β-cells and islet function at the time of diagnosis is therefore imperative[4].

Exogenous insulin administration is not a cure of type 1 diabetes. Research efforts using pre-clinical animal models therefore continue to produce new therapeutic possibilities. Initially developed as a model for spontaneous onset of cataracts, the female non-obese diabetic mouse (NOD) is the most well-established and extensively used model of type 1 diabetes[5]. This mouse model recapitulates multiple pathophysiological features of human type 1 diabetes, including the development of autoantibodies in prediabetic NOD mice[6], circulating autoreactive T cells[7], and the subsequent hyperglycemia and progressive β-cell loss[8]. While the onset of hyperglycemia most often occurs between 12-15 weeks of age, islet immune infiltration is established earlier at 8-12 weeks with insulitis present across the pancreas[8]. The cleanliness of housing facilities and the animal breeding approach are just two factors which affect the age at which these features present.

Mice express two nonallelic insulin genes. Located on chromosome 7, the insulin 2 gene (*Ins2*) is the murine homologue of the human insulin gene[9]. The insulin 1 gene (*Ins1*) is found on the murine chromosome 19, is the result of an RNA-mediated gene duplication event, and has a simpler gene structure lacking the second intron present in *Ins2*[9]. *Ins1* is expressed specifically in pancreatic β-cells, whereas *Ins2* is expressed in the pancreas, thymus, and the brain[10]. Previous studies have examined the effects of insulin gene knockout in NOD mice. Complete knockout of *Ins2* on the NOD background accelerated type 1 diabetes[2], a phenomenon attributed to a failure in central tolerization to insulin. Thymus-specific deletion of *Ins2* was reported to be sufficient to cause spontaneous diabetes, even outside the NOD background[11], although we have not observed autoimmune diabetes in globally deficient *Ins2*$^{-/-}$ mice[12]. Male, but not female, NOD:*Ins1*$^{Neo/WT}$;*Ins2*$^{-/-}$ mice, with a single remaining *Ins1* allele have been shown to succumb to insulin insufficiency[13], a finding we confirmed on other backgrounds[14], depending on housing conditions[10]. In contrast, NOD:*Ins1* knockout mice have previously been shown to be protected from type 1 diabetes[7], which was proposed to be due to the loss of autoantigenic *Ins1*-derived peptides. Insulin is a primary auto-antigen in both murine and human type

1 diabetes pathogenesis[1]. Replacing *Ins1* with non-antigenic human insulin also protects NOD mice from the onset of type 1 diabetes[8]. Together, these previous observations indicate that insulin gene dosage is a key player the diabetes incidence in NOD mice.

For the purpose of this study, we backcrossed the original *Ins1*$^{tm1.1(cre)Thor}$ (*Ins1*^{Cre}) mouse from the C57/bl6-J/N background 12 times onto an NOD background. Subsequently, we characterized the new NOD *Ins1*^{Cre} knock-in mouse line that was generated for truly β-cell specific expression of Cre-recombinase. We compared the type 1 diabetes incidence, insulitis, and immune activation in NOD *Ins1*^{Cre} mice with a different knock-in cassette (neo) to determine whether Cre itself affect the diabetes incidence. Our data demonstrate that Cre expression has further protective effects beyond the loss of a single *Ins1* allele. Carefully chosen proper controls and caution are therefore required when interpreting experiments including β-cell specific Cre expression in mice on an NOD background.

Methods

Mice

All animal procedures and ethical standards were in accordance with the Canadian Council for Animal Care guidelines. All animal studies and protocols were approved by the University of British Columbia Animal Care Committee and Institutional Care and Use Committee (IACUC) at the University of Michigan. In Vancouver, mice were house in the Centre for Disease Modelling Specific Pathogen-Free (SFP) facility on a standard 12-h light/12-h dark cycle with ad libitum access to chow diet (PicoLab, Mouse Diet 20–5058).

To generate NOD:*Ins1*^{Cre} and NOD:*Ins1*^{Neo} mice, we contracted Jax labs to backcross (>12 times) *Ins1*^{Cre} and *Ins1*^{Neo} mice onto a NOD/ShiLtJ (commonly known as NOD). The *Ins1*^{Cre} and *Ins1*^{Neo} mice were originally on a mixed, largely C57Bl/6J, background. Subsequently we designed a strict breeding strategy for our study. An *Ins1*^{Cre} maternal parent colony as well as a *Ins1*^{Neo} paternal parent colony was established. Each parent colony was backcrossed every five generations. Female *Ins1*$^{Cre/WT}$ and male *Ins1*$^{Neo/WT}$ mice were set up as breeders to generate experimental mice at 7-8 weeks of age. Mice from the parental colonies were only included once as breeders to eliminate risk of onset of hyperglycemia during pregnancy and weaning at later ages. This breeding strategy generated littermates of four genotypes: wildtype NOD:*Ins1*$^{WT/WT}$ mice with both insulin 1 alleles, heterozygous NOD:*Ins1*$^{Cre/WT}$ mice with an *Ins1* replaced with Cre-recombinase, heterozygous NOD:*Ins1*$^{Neo/WT}$ mice with one *Ins1* allele replaced with a neomycin cassette, and full *Ins1* null mice with both *Ins1* alleles replaced NOD:*Ins1*$^{Neo/Cre}$. Specific cohorts were monitored twice per week for the sole purpose of determining hyperglycemia onset incidence and body mass changes. Any mice that developed diabetes, defined as two consecutive blood glucose measurements of 16 mmol/L or one measurement higher than 22 mmol/L, were euthanized. Specific cohorts were generated for tissue analysis terminated at 12 weeks of age in a pre-diabetic phase (for the Vancouver housing facility) or at 1 year of age, all animals were monitored for diabetes prior to euthanasia.

An additional colony of NOD:*Ins1*^{Cre} mice were generated independently through in-house backcrossing at a second site (University of Michigan) via the speed congenic approach in consultation with Charles River Laboratories (Wilmington, MA). Following each backcross, *Ins1*$^{Cre/WT}$ offspring with allelic profiles most closely matching the NOD strain (determined by MAX-BAX mouse 384 SNP panel screening), were selected as breeders for the subsequent backcross. Following 8 generations of backcrossing, animals with an allelic profile percent match >99.9% were utilized to generate *Ins1*$^{WT/WT}$ and *Ins1*$^{Cre/WT}$ experimental mice. Animals were housed on a standard 12-h light/12-h dark cycle with access to ad libitum chow diet (LabDiets, Rodent Diet 5L0D) and acidified water (pH 2.5 - 3.0) in an SPF facility. Blood glucose measurements were taken once-twice per week. Incidence of diabetes, defined as blood glucose levels > 16 mM for 5 consecutive measurements, was recorded. Diabetic mice were euthanized and excluded from future analysis.

Tissue processing and histology

Immediately following euthanasia, pancreata were collected according to a pre-established protocol with the exception that extracted pancreases were not further treated to obtain isolated islets and were

instead processed as a whole[15]. The dissected pancreata were fixed in 10% formalin and embedded in 4% PFA for 24 hours prior to storage in 70% ethanol at 4 °C. Paraffin-embedded sections were prepared, stained with hematoxylin and eosin (H&E), and imaged by WaxIT Histology Services Inc. (Vancouver, BC).

Islet infiltration scoring

Images from H&E stained pancreatic sections were analyzed with QuPath software[16]. Islets in pancreatic sections were scored blindly for pancreatic islet infiltration of mononuclear immune cells according to the previously established 4-point scale[17]. In brief, 0- no insulitis, 1- peri-insulitis marked by less than 25% peripheral immune-islet infiltration, 2- insulitis marked by 25-75% immune cell infiltration, 3- severe insulitis marked by greater than 75% immune-islet infiltration. Random samples were scored by a second person to ensure consistency. 25-30 islets per mouse.

Insulin auto-antibodies

Blood was collected at study endpoint by cardiac puncture from anesthetized Animals (2.5% isoflurane) and put directly on ice. Samples were shipped to the Insulin Antibody Core Lab at the University of Colorado, Barbara Davis Diabetes Centre for auto-antibody analysis.

Flow cytometry

Immediately following euthanasia lymph nodes and spleen were isolated and splenic single-cell suspensions were counted and stained with fluorescently conjugated monoclonal antibodies (mAbs) for cell-surface markers (see Table 1 for list of antibodies used). Following staining, cells were analyzed by flow cytometry and Flow Jo software (Tree Star Inc., Ashland, Oregon)

Statistics

Statistical significance was assessed using 2-way ANOVA analysis with Tukey's multiple comparisons test, at a threshold of $p < 0.05$. Prism 9 (GraphPad Software Inc., USA) was used for statistical analyses and generation of most figure panels. Data are expressed as mean ± SEM unless otherwise specified.

Results

Type 1 diabetes incidence

It is well established that the type 1 diabetes incidence exhibits a female bias in NOD mice. As expected, we observed a 75% diabetes incidence (19 of 39 mice) in female NOD:*Ins1*$^{WT/WT}$ mice tracked for 1 year (Fig. 1A). We observed significant protection in female littermate NOD:*Ins1*$^{Neo/WT}$ mice, lacking an *Ins1* allele, with a delay in average onset and a 60% diabetes incidence by 1 year (Fig. 1A). In female NOD:*Ins1*$^{Cre/WT}$ littermates, with Ins1 replaced by Cre-recombinase, there was a further significant delay in diabetes onset and only a 40% diabetes incidence NOD:*Ins1*$^{Cre/WT}$ by 1 year (Fig. 1A). Double mutant female NOD:*Ins1*$^{Neo/Cre}$ mice had an even further delay in type 1 diabetes incidence, with the first case of hyperglycemia being observed at 30 weeks of age and a final diabetes incidence of only 20% (Fig. 1A), demonstrating an additive protective effect. In female mice, random blood glucose (Fig. 1B,C) and body mass (Fig. 1D,E) prior to diabetes onset were not different between any groups. Together, these data confirm the previous findings that reduced *Ins1* gene dosage protects NOD mice from diabetes, but also reveal a further, additive protective effect of β-cell Cre expression.

In male littermates, as expected, diabetes was rare (20% diabetes incidence) in the NOD:*Ins1*$^{WT/WT}$ group. We observed no cases of hyperglycemia in any of the male mice with reduced *Ins1* gene dosage (Fig. 2A). We observed no differences in random blood glucose (Fig. 2 B,C) or body mass (Fig. 2D,E) between any of the groups in males.

Insulitis and Insulin auto-antibodies

Next, we examined the effects of reduced *Ins1* gene dosage and Cre expression on insulitis in female mice, the pathological evidence of islet directed autoimmunity. H&E-stained pancreas sections (Fig. 3A)

were blindly scored for immune islet infiltration in a pre-diabetic cohort of littermates euthanized at 12 weeks of age, and also in the mice that survived to 1 year. Insulitis scores from 12-week-old littermates were not significantly different, although we observed the least amount of immune islet infiltration in double mutant NOD:*Ins1*$^{Neo/Cre}$ mice (Fig. 3B), consistent with their more complete protection from diabetes. For the 1-year-old cohort, we observed no statistically significant differences in insulitis scores (Fig. 3C). However, it should be noted that the sample size for the surviving NOD:*Ins1*$^{WT/WT}$ mice was only 3. We were unable to identify differences in insulin auto-antibodies in the 1 year-old mice (Fig. 3D), and unfortunately we did not collect blood for this analysis at the 12 week timepoint for all cohorts.

Immune cell characterization

We next used a panel of validated antibodies and FACS to assess immune cell populations in the pancreatic lymph nodes and spleen at 50 weeks of age. While we were able to confidently identify many key immune cell populations (Fig. 4), there were no significant differences between groups (Fig. 5). These observations demonstrate that β-cell specific insulin gene manipulations alter type 1 diabetes incidence without robust effects on the lymphocytes found in the pancreatic lymph nodes and spleen.

Independent Validation Cohort

To ensure that protective effects of β-cell Cre expression were not solely limited to a single animal facility, we additionally studied female NOD:*Ins1*$^{Cre/WT}$ mice that were independently generated at a separate site (Fig. 6) in parallel to the cohorts studied in Figure 1. Overall incidence of diabetes development in female NOD mice in this second animal colony was as expected (approximately 65-80% by 25 weeks of age, Fig. 6A). Similar to studies in Figure 1, we observed that female NOD:*Ins1*$^{Cre/WT}$ animals were protected from diabetes incidence (25% by 1 year) and had significantly improved mean blood glucose (Fig. 6C) when compared to NOD:*Ins1*$^{WT/WT}$ littermates (75% incidence by 1 year). Again, these studies confirm the protective effects of reduced Ins1 gene dosage and β-cell Cre expression in NOD mice and suggest that these findings are not due to a consequence of environment or housing.

Discussion

In this study, we examined the effects of replacing either one or two *Ins1* alleles with either Cre-recombinase and/or a neomycin-resistance cassette. We found a reduction in the diabetes incidence in female *Ins1*$^{Cre/WT}$ mice and *Ins1*$^{Neo/Cre}$ mice when compared to littermate control *Ins1*$^{WT/WT}$ mice. This work has implications for our understanding of the pathogenesis of type 1 diabetes, as well as for the use of Cre-recombinase as a tool for *in vivo* genome engineering in mouse models of the disease.

Our findings support previous work that demonstrated a similar reduction in diabetes incidence in female NOD mice lacking 1 or 2 alleles of *Ins1*[9,13]. Similarly, replacing the murine *Ins1* gene with the human *INS* gene was found to protect female NOD mice from diabetes in both heterozygous and homozygous states[18]. We observed no diabetes incidence, and therefore no differences in male *Ins1*^{WTWT}, *Ins1*$^{Cre/WT}$, *Ins1*$^{Neo/WT}$, and *Ins1*$^{Neo/Cre}$ NOD mice. A previous study found that removal of a single *Ins1* allele is sufficient to abolish spontaneous diabetes in 50-week-old male NOD mice[9]. Together with the work of others, our experiments support the contention that proinsulin 1 is a key player in the generation of autoimmunity. While there are several autoantigens targeted by autoreactive T cells in type 1 diabetes[2], insulin and proinsulin are particularly common autoantibody targets in prediabetic humans[19,20]. Our experiments were underpowered to detect subtle differences in the levels of insulin autoantibodies, as we were limited by only examining a single, late time point. However, we did examine insulitis at two time points, and insulin antibodies are often correlated with insulitis[21]. In our hands, there was a qualitative difference in the number of islets that did not exhibit insulitis at 50 weeks in mice with at least 1 *Ins1* allele replaced. At 12 weeks, there was a slight trend towards more insulitis-free islets in the *Ins1*$^{Neo/Cre}$ mice, consistent with the greater protection from type 1 diabetes incidence. These observations are consistent with previous studies showing that *Ins1* knockout in NOD mice is protective against the development and severity of insulitis[9]. Thus, our results show that reducing the *Ins1* gene dosage lowers the threshold required for diabetes onset, likely by removing the source of primary autoantigens and suppressing insulitis.

The Cre-loxP systems are vital tools for research, however, there are multiple caveats that should be considered related to side effects and the determination of correct controls. To examine this, we utilized a different knock-in cassette (neo) and found a qualitative difference in diabetes incidence between Ins*1*$^{Cre/WT}$ and *Ins1*$^{Neo/WT}$ mice, with a 62.5 and 41.7 survival proportion respectively. These findings suggest that Cre itself affects diabetes rates in female NOD mice. Mechanistically, we are able to attribute this effect to clear differences in insulitis, suggesting the possibility of a β-cell autonomous effect of Cre expression. Previous studies highlighted the potential of Cre recombinase to result in toxicity due to DNA damage[22,23]. Mammalian genomes contain pseudo loxP sites, and even though these sequences can deviate considerably from the consensus loxP site, they can still serve as functional recognition sites for Cre[24]. It is predicted that the frequency of pseudo-loxP sites could be as many as 250 and 300 in mouse and human genomes, respectively[25]. The sustained presence of high levels of Cre in fibroblasts can cause growth arrest and chromosomal abnormalities[26,27]. Cre-dependent DNA damage and accumulation of cytoplasmic DNA have been shown to initiate a STING-dependent immune response[28]. STING is an intracellular adaptor molecule, associated with the endoplasmic reticulum membrane[29], that can play a critical role in detecting pathogen-derived DNA in the cytoplasm[30]. Theoretically, Cre expressed in β-cells could delay the onset of diabetes modulating the cell cycle. There is precedence for diabetes protection in NOD mice with early exposure to pathogen in the coxsackievirus mode[31]. Future studies, beyond the scope of this work, will be required to delineate the molecular mechanisms by which Cre expression induces further protection that *Ins1* loss in the NOD mouse model.

In summary, our observations suggest caution when interpreting experiments that involve Cre recombinase in NOD mice. At the bare minimum, Cre-only controls are essential. Additional tools for in vivo genome engineering are required to advance the field. Many studies will need to be re-interpreted.

References:

1 Daniel, D., Gill, R. G., Schloot, N. & Wegmann, D. Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur J Immunol* **25**, 1056-1062, doi:10.1002/eji.1830250430 (1995).

2 Thebault-Baumont, K. *et al.* Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice. *J Clin Invest* **111**, 851-857, doi:10.1172/JCI16584 (2003).

3 Atkinson, M. A., Eisenbarth, G. S. & Michels, A. W. Type 1 diabetes. *Lancet* **383**, 69-82, doi:10.1016/S0140-6736(13)60591-7 (2014).

4 Ehlers, M. R. Immune interventions to preserve beta cell function in type 1 diabetes. *J Investig Med* **64**, 7-13, doi:10.1097/JIM.0000000000000227 (2016).

5 Makino, S. *et al.* Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu* **29**, 1-13, doi:10.1538/expanim1978.29.1_1 (1980).

6 Melanitou, E., Devendra, D., Liu, E., Miao, D. & Eisenbarth, G. S. Early and quantal (by litter) expression of insulin autoantibodies in the nonobese diabetic mice predict early diabetes onset. *J Immunol* **173**, 6603-6610, doi:10.4049/jimmunol.173.11.6603 (2004).

7 Gregori, S., Giarratana, N., Smiroldo, S. & Adorini, L. Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. *J Immunol* **171**, 4040-4047, doi:10.4049/jimmunol.171.8.4040 (2003).

8 Chen, Y. G., Mathews, C. E. & Driver, J. P. The Role of NOD Mice in Type 1 Diabetes Research: Lessons from the Past and Recommendations for the Future. *Front Endocrinol (Lausanne)* **9**, 51, doi:10.3389/fendo.2018.00051 (2018).

9 Moriyama, H. *et al.* Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse. *Proc Natl Acad Sci U S A* **100**, 10376-10381, doi:10.1073/pnas.1834450100 (2003).

10 Mehran, A. E. *et al.* Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. *Cell Metab* **16**, 723-737, doi:10.1016/j.cmet.2012.10.019 (2012).

11 Fan, Y. *et al.* Thymus-specific deletion of insulin induces autoimmune diabetes. *EMBO J* **28**, 2812-2824, doi:10.1038/emboj.2009.212 (2009).

12 Martin-Pagola, A. *et al.* Insulin2 gene (Ins2) transcription by NOD bone marrow-derived cells does not influence autoimmune diabetes development in NOD-Ins2 knockout mice. *Scand J Immunol* **70**, 439-446, doi:10.1111/j.1365-3083.2009.02316.x (2009).
13 Babaya, N. *et al.* A new model of insulin-deficient diabetes: male NOD mice with a single copy of Ins1 and no Ins2. *Diabetologia* **49**, 1222-1228, doi:10.1007/s00125-006-0241-4 (2006).
14 Zhang, A. M. Y. *et al.* Endogenous Hyperinsulinemia Contributes to Pancreatic Cancer Development. *Cell Metab* **30**, 403-404, doi:10.1016/j.cmet.2019.07.003 (2019).
15 Villarreal, D. *et al.* A Simple High Efficiency Protocol for Pancreatic Islet Isolation from Mice. *J Vis Exp*, doi:10.3791/57048 (2019).
16 Bankhead, P. *et al.* QuPath: Open source software for digital pathology image analysis. *Sci Rep* **7**, 16878, doi:10.1038/s41598-017-17204-5 (2017).
17 Lee, J. T. C., Shanina, I., Chu, Y. N., Horwitz, M. S. & Johnson, J. D. Carbamazepine, a beta-cell protecting drug, reduces type 1 diabetes incidence in NOD mice. *Sci Rep* **8**, 4588, doi:10.1038/s41598-018-23026-w (2018).
18 Elso, C. M. *et al.* Replacing murine insulin 1 with human insulin protects NOD mice from diabetes. *PLoS One* **14**, e0225021, doi:10.1371/journal.pone.0225021 (2019).
19 Kuglin, B., Gries, F. A. & Kolb, H. Evidence of IgG autoantibodies against human proinsulin in patients with IDDM before insulin treatment. *Diabetes* **37**, 130-132, doi:10.2337/diab.37.1.130 (1988).
20 Yu, L. *et al.* Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci U S A* **97**, 1701-1706, doi:10.1073/pnas.040556697 (2000).
21 Robles, D. T., Eisenbarth, G. S., Dailey, N. J., Peterson, L. B. & Wicker, L. S. Insulin autoantibodies are associated with islet inflammation but not always related to diabetes progression in NOD congenic mice. *Diabetes* **52**, 882-886, doi:10.2337/diabetes.52.3.882 (2003).
22 Schmidt-Supprian, M. & Rajewsky, K. Vagaries of conditional gene targeting. *Nat Immunol* **8**, 665-668, doi:10.1038/ni0707-665 (2007).
23 Janbandhu, V. C., Moik, D. & Fassler, R. Cre recombinase induces DNA damage and tetraploidy in the absence of loxP sites. *Cell Cycle* **13**, 462-470, doi:10.4161/cc.27271 (2014).
24 Thyagarajan, B., Guimaraes, M. J., Groth, A. C. & Calos, M. P. Mammalian genomes contain active recombinase recognition sites. *Gene* **244**, 47-54, doi:10.1016/s0378-1119(00)00008-1 (2000).
25 Karimova, M. *et al.* Vika/vox, a novel efficient and specific Cre/loxP-like site-specific recombination system. *Nucleic Acids Res* **41**, e37, doi:10.1093/nar/gks1037 (2013).
26 Silver, D. P. & Livingston, D. M. Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. *Mol Cell* **8**, 233-243, doi:10.1016/s1097-2765(01)00295-7 (2001).
27 Loonstra, A. *et al.* Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc Natl Acad Sci U S A* **98**, 9209-9214, doi:10.1073/pnas.161269798 (2001).
28 Pepin, G. *et al.* Cre-dependent DNA recombination activates a STING-dependent innate immune response. *Nucleic Acids Res* **44**, 5356-5364, doi:10.1093/nar/gkw405 (2016).
29 Barber, G. N. STING: infection, inflammation and cancer. *Nat Rev Immunol* **15**, 760-770, doi:10.1038/nri3921 (2015).
30 Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674-678, doi:10.1038/nature07317 (2008).
31 Serreze, D. V. *et al.* Diabetes acceleration or prevention by a coxsackievirus B4 infection: critical requirements for both interleukin-4 and gamma interferon. *J Virol* **79**, 1045-1052, doi:10.1128/JVI.79.2.1045-1052.2005 (2005).

Figure Legends

Figure 1. *Ins1* replacement with Cre and Neo protects female NOD mice from type 1 diabetes.
(A) Kaplan-Meier plot denoting diabetes incidence in NOD mice by *Ins1* genotype.
(B<C) Individual and mean random blood glucose of female mice. The mean blood glucose of the *Ins1*$^{Neo/Cre}$ was significantly lower than that of the *Ins1*$^{WT/WT}$ colonies, with an adjusted p-value of 0.0023. Moreover, the mean blood glucose of the *Ins1*$^{Neo/WT}$ colony was higher than those of the *Ins1*$^{WT/WT}$ and *Ins1*$^{Cre/WT}$ colonies, with adjusted p-values of 0.0002 and 0.0058 respectively.
(D,E) Individual and mean body mass traces female mice. The mean body mass of the *Ins1*$^{Neo/Cre}$ mice was significantly lower compared to the *Ins1*$^{Cre/WT}$ colony (adjusted p-value<0.0001). The mean blood glucose of the *Ins1*$^{Cre/WT}$ was also higher than the *Ins1*$^{WT/WT}$ and the *Ins1*$^{Cre/WT}$ colonies, both with an adjusted p-value<0.0001. Error bars represent SEM

Figure 2. Effects of *Ins1* replacement with Cre and Neo in male NOD mice.
(A) Kaplan-Meier plot denoting diabetes incidence in NOD mice by *Ins1* genotype.
(B,C) Individual and mean random blood glucose in male mice.
(D,E) Individual and mean body mass traces in male mice . Error bars represent SEM.

Figure 3. Insulitis scoring in female NOD mice with *Ins1* replacement.
(A) Representative images of H&E stained pancreata used for insulitis scoring. Scale bars are 100 µm.
(B) Mean percent insulitis scores at 12-week-old and 1-year-old time points. Error bars represent SEM.

Figure 4. Gating strategy for flow cytometry of pancreatic lymph node and spleen cells.
(A) Singlets were obtained with use of FSC-A x FSC-H parameters and viable cells were identified by selecting viability dye negative cells for subsequent analysis. The populations were subsequently split into three groups of interest with dendritic cells identified by CD11c, B cells identified by CD19, and T cells identified by CD3. T cells were further categorized into cytotoxic and helper phenotypes with use of CD4 and CD8 markers and their respective single marker populations were assessed for activation and priming status (naïve, effector, memory) with the use of CD69, CD44, and CD62L. Regulatory T cell populations were further selected for with the use of a Foxp3 marker.

Figure 5. Immune profiling in female NOD mice with *Ins1* replacement.
Flow cytometric analysis of cell populations within the pancreatic lymph node and spleen at 50 weeks of age.

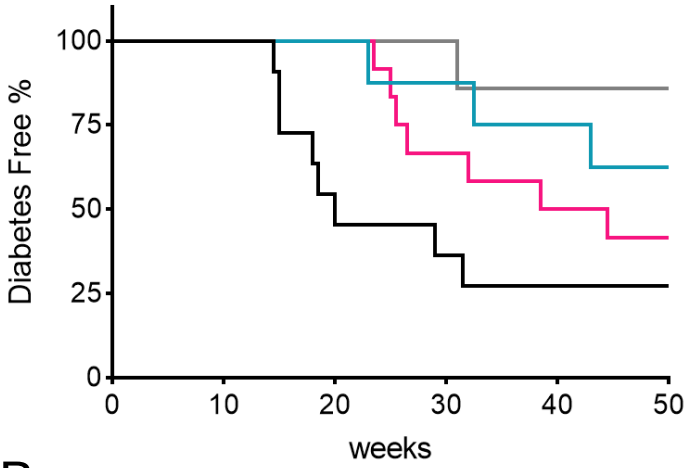
Figure 6. *Ins1* replacement with Cre protects female NOD mice from type 1 diabetes in an independent facility.
(A) Kaplan-Meier plot denoting diabetes incidence. **(B,C)** Individual and mean random blood glucose of female NOD colonies from a second, independent site. The mean blood glucose of the *Ins1*$^{Neo/Cre}$ (green) was significantly lower than that of the *Ins1*$^{WT/WT}$ (blue) littermates, with an adjusted p-value < 0.05. Female NOD non-littermate controls used to track overall diabetes incidence in the colony are listed in red. Error bars represent SEM.

Table 1. Summary of Antibodies used for Flow cytometry

A

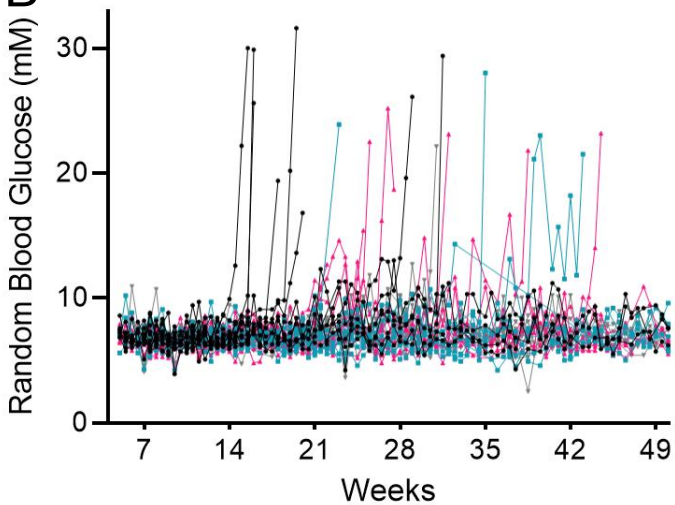
♂ *Ins1*$^{neo/wt}$ x *Ins1*$^{cre/wt}$ ♀

Ins1$^{wt/wt}$ (n=11)
Ins1$^{cre/wt}$ (n=8)
Ins1$^{neo/wt}$ (n=12)
Ins1$^{neo/cre}$ (n=7)

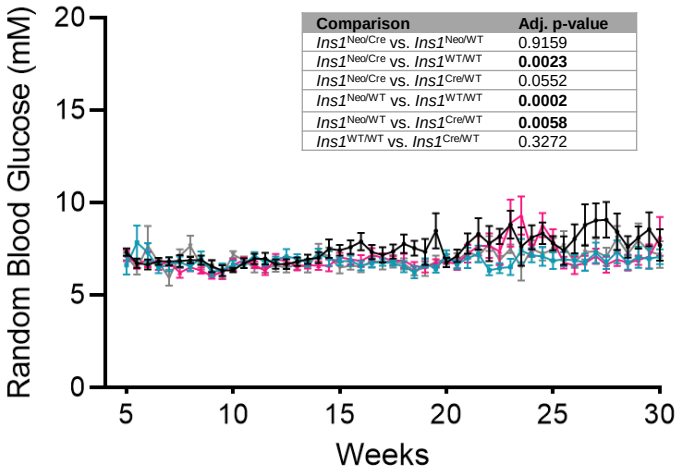


Comparison	p-value
Ins1$^{wt/wt}$ vs. *Ins1*$^{Cre/wt}$	0.0531
Ins1$^{wt/wt}$ vs. *Ins1*$^{Neo/wt}$	0.0566
Ins1$^{wt/wt}$ vs. *Ins1*$^{Cre/Neo}$	**0.0159**
Ins1$^{Cre/wt}$ vs. *Ins1*$^{Neo/wt}$	0.3924

B

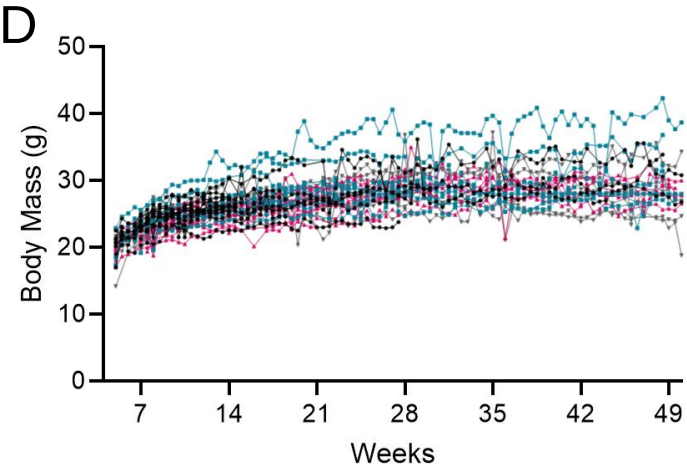


C

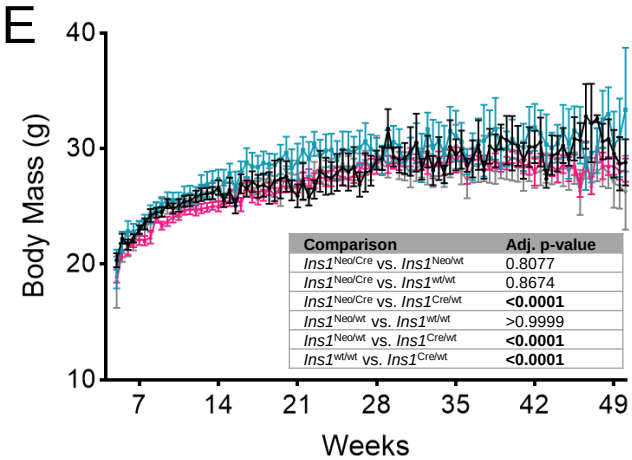


Comparison	Adj. p-value
Ins1$^{Neo/Cre}$ vs. *Ins1*$^{Neo/WT}$	0.9159
Ins1$^{Neo/Cre}$ vs. *Ins1*$^{WT/WT}$	**0.0023**
Ins1$^{Neo/Cre}$ vs. *Ins1*$^{Cre/WT}$	0.0552
Ins1$^{Neo/WT}$ vs. *Ins1*$^{WT/WT}$	**0.0002**
Ins1$^{Neo/WT}$ vs. *Ins1*$^{Cre/WT}$	**0.0058**
Ins1$^{WT/WT}$ vs. *Ins1*$^{Cre/WT}$	0.3272

D



E



Comparison	Adj. p-value
Ins1$^{Neo/Cre}$ vs. *Ins1*$^{Neo/wt}$	0.8077
Ins1$^{Neo/Cre}$ vs. *Ins1*$^{wt/wt}$	0.8674
Ins1$^{Neo/Cre}$ vs. *Ins1*$^{Cre/wt}$	**<0.0001**
Ins1$^{Neo/wt}$ vs. *Ins1*$^{wt/wt}$	>0.9999
Ins1$^{Neo/wt}$ vs. *Ins1*$^{Cre/wt}$	**<0.0001**
Ins1$^{wt/wt}$ vs. *Ins1*$^{Cre/wt}$	**<0.0001**

Figure 1

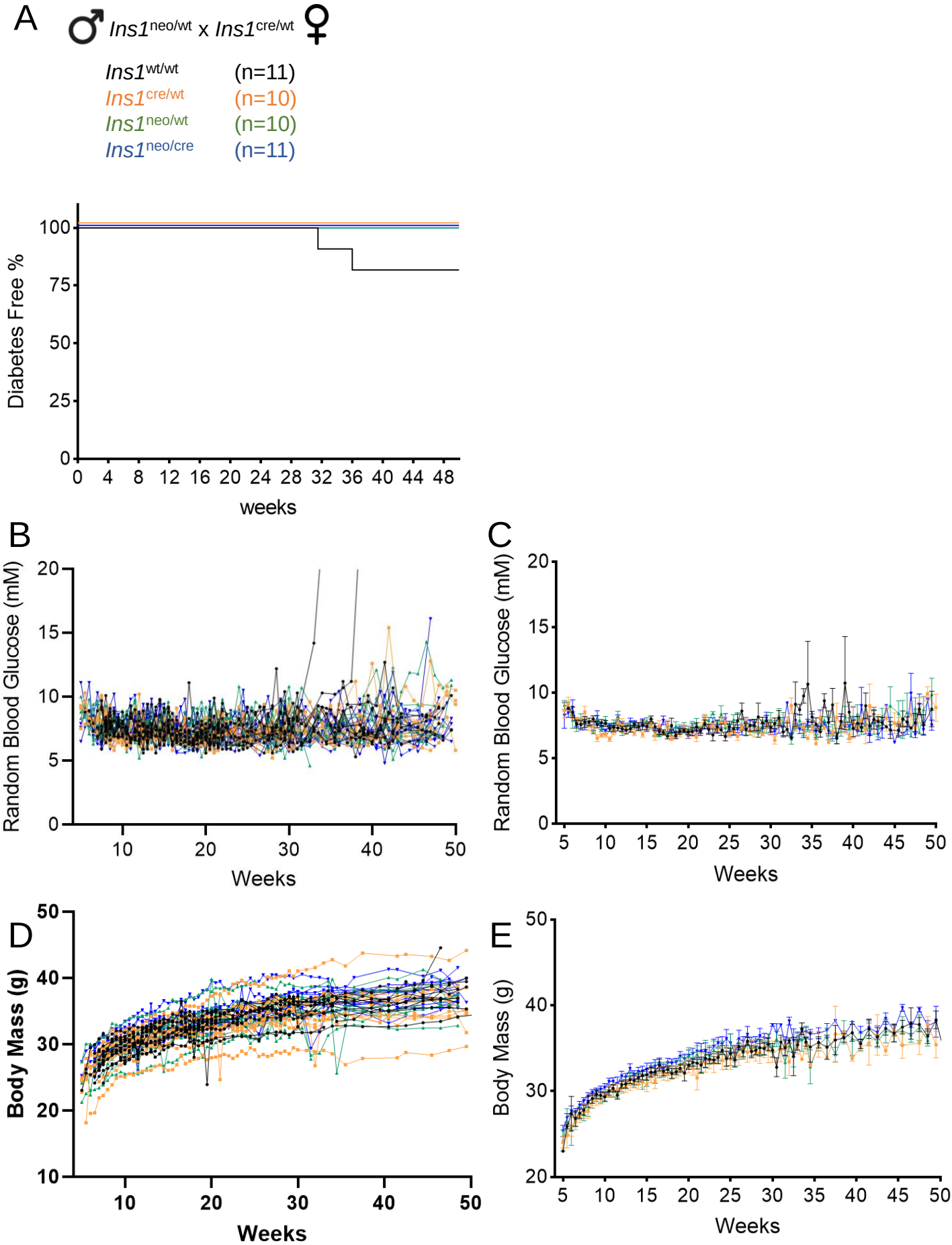
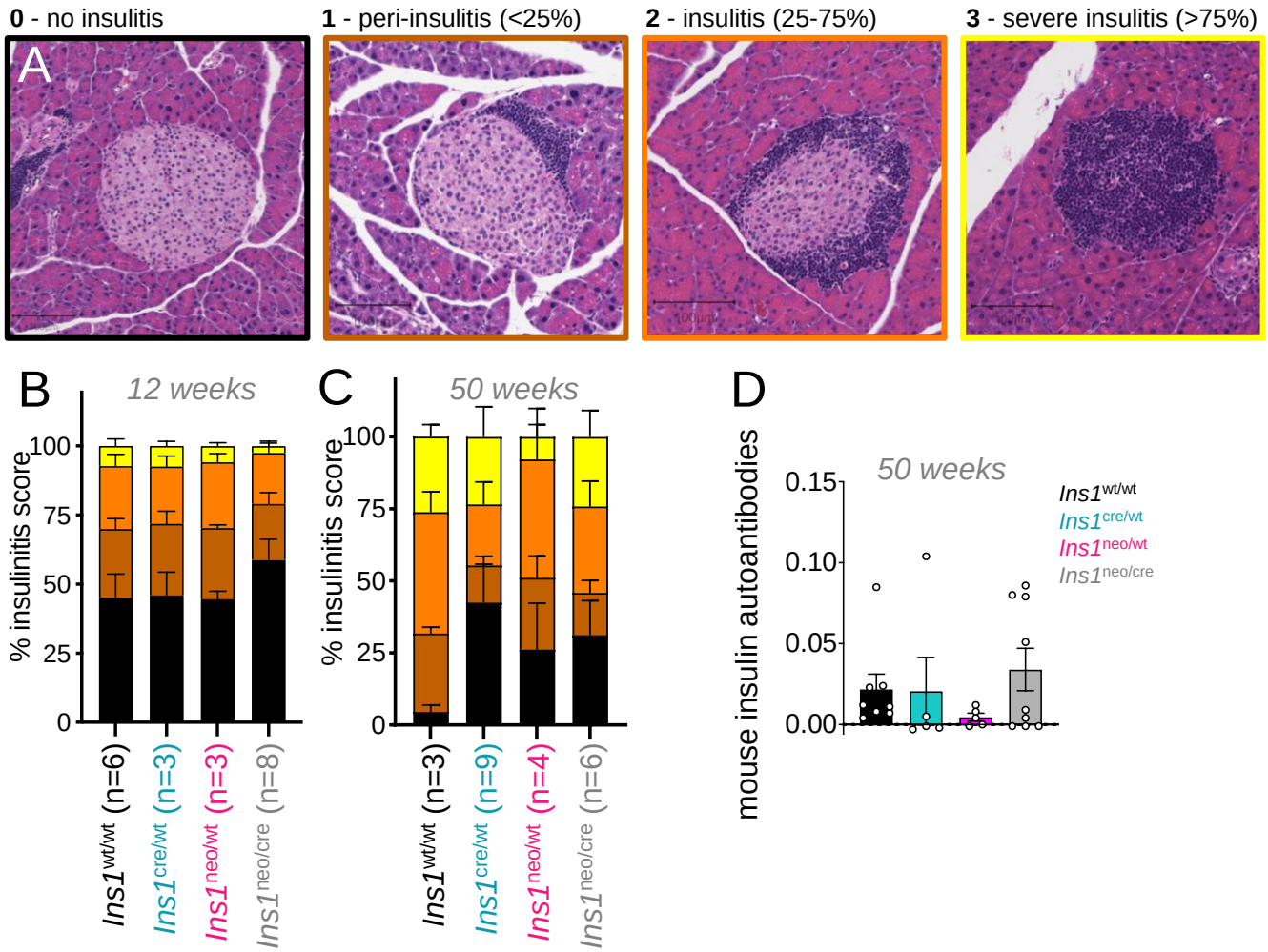
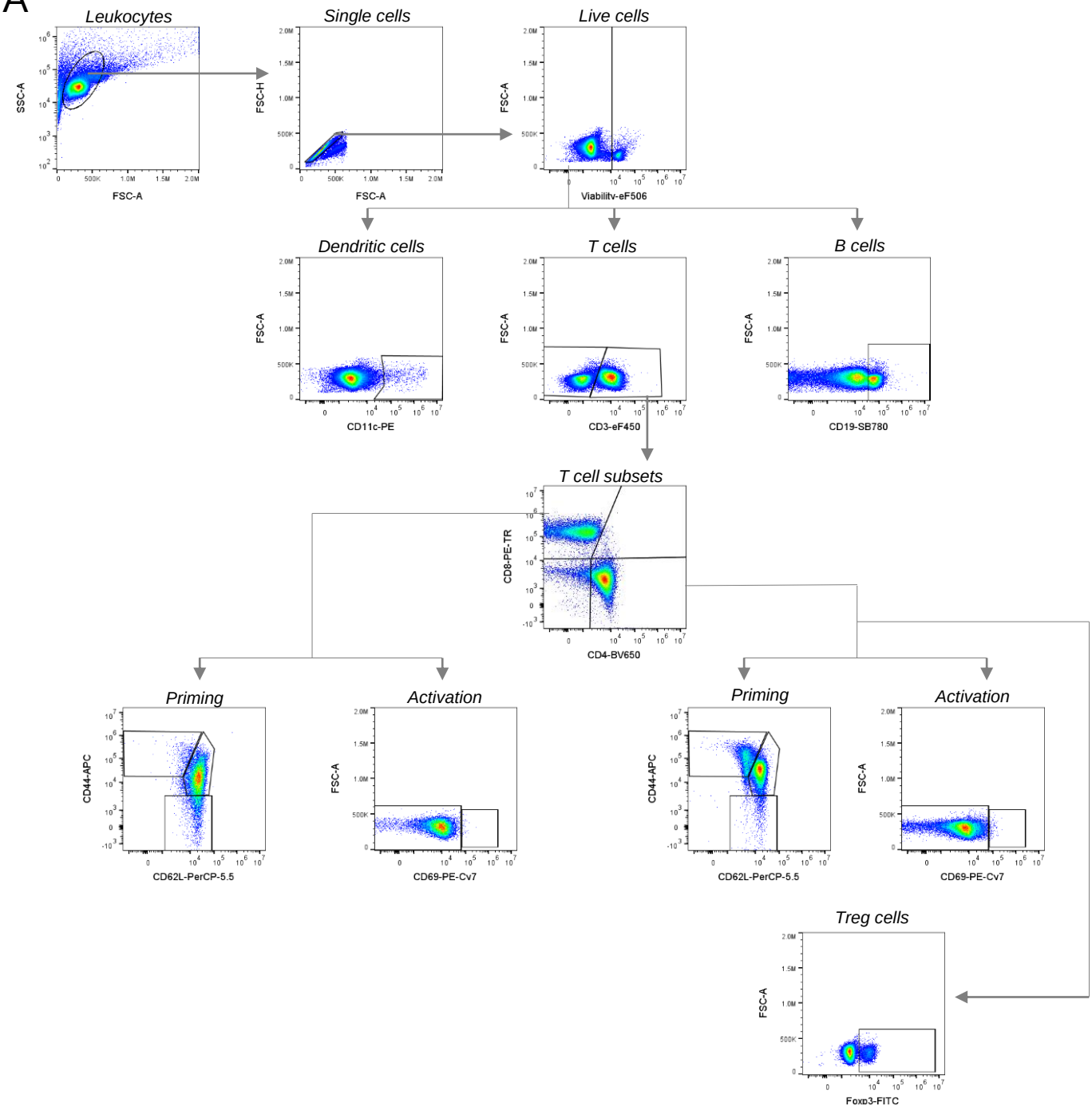
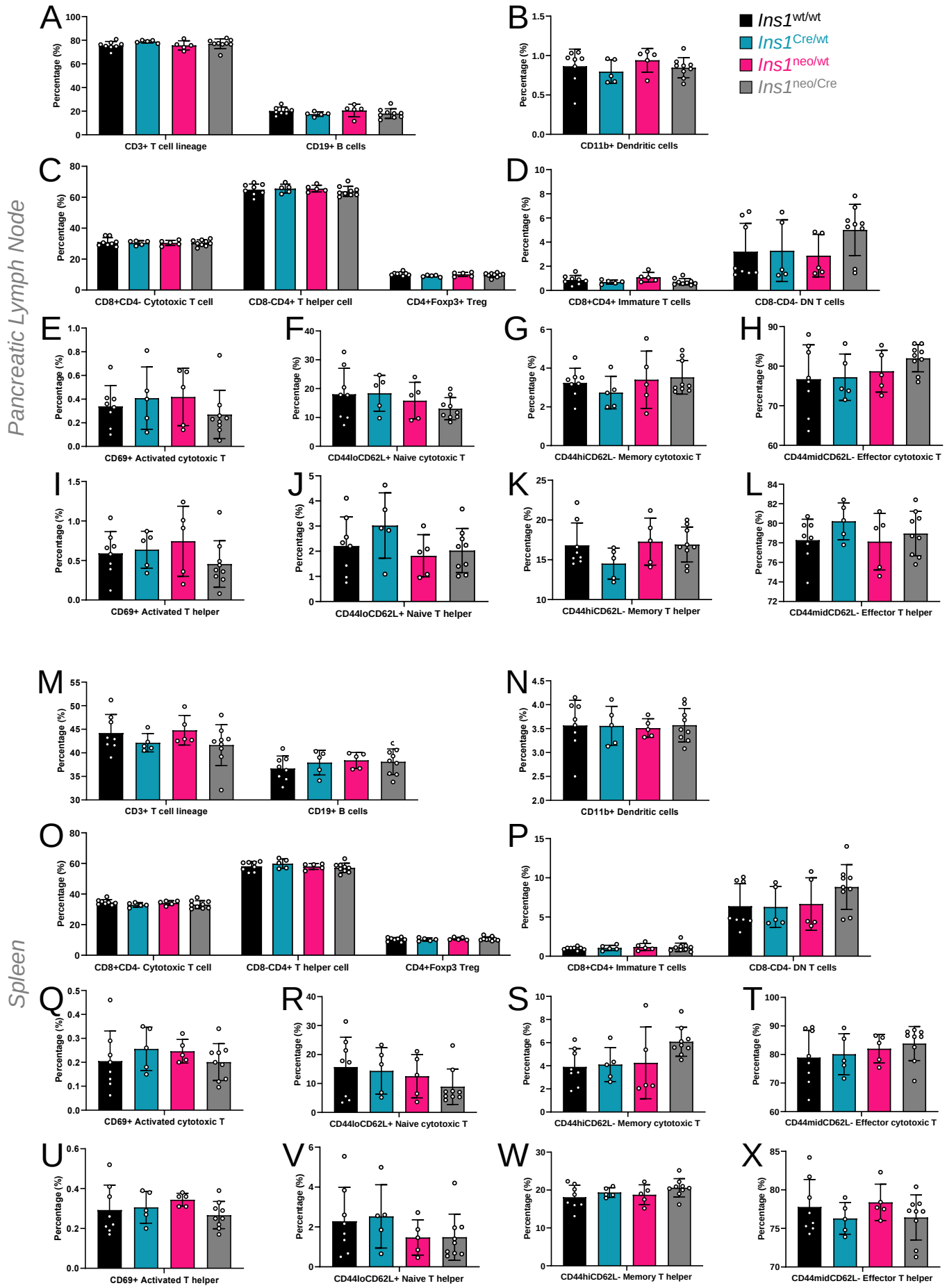

A
♂ Ins1neo/wt x Ins1cre/wt ♀
Ins1wt/wt (n=11)
Ins1cre/wt (n=10)
Ins1neo/wt (n=10)
Ins1neo/cre (n=11)
Diabetes Free %
weeks
B
Random Blood Glucose (mM)
Weeks
C
Random Blood Glucose (mM)
Weeks
D
Body Mass (g)
Weeks
E
Body Mass (g)
Weeks

Figure 2


0 - no insulitis
1 - peri-insulitis (<25%)
2 - insulitis (25-75%)
3 - severe insulitis (>75%)
A
B
12 weeks
% insulinitis score
100
75
50
25
0
Ins1wt/wt (n=6)
Ins1cre/wt (n=3)
Ins1neo/wt (n=3)
Ins1neo/cre (n=8)
C
50 weeks
% insulinitis score
100
75
50
25
0
Ins1wt/wt (n=3)
Ins1cre/wt (n=9)
Ins1neo/wt (n=4)
Ins1neo/cre (n=6)
D
50 weeks
mouse insulin autoantibodies
0.15
0.10
0.05
0.00
Ins1wt/wt
Ins1cre/wt
Ins1neo/wt
Ins1neo/cre

A


Leukocytes
SSC-A
FSC-A
Single cells
FSC-H
FSC-A
Live cells
FSC-A
Viability-eF506
Dendritic cells
FSC-A
CD11c-PE
T cells
FSC-A
CD3-eF450
B cells
FSC-A
CD19-SB780
T cell subsets
CD8-PE-TR
CD4-BV650
Priming
CD44-APC
CD62L-PerCP-5.5
Activation
FSC-A
CD69-PE-Cv7
Priming
CD44-APC
CD62L-PerCP-5.5
Activation
FSC-A
CD69-PE-Cv7
Treg cells
FSC-A
Foxp3-FITC

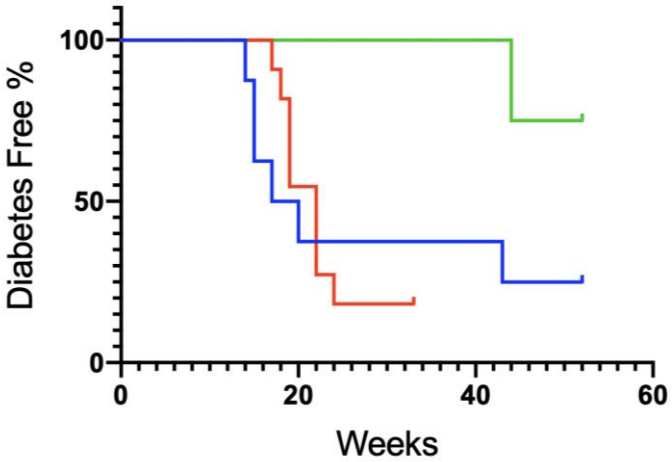

Pancreatic Lymph Node
Spleen
Ins1wt/wt
Ins1Cre/wt
Ins1neo/wt
Ins1neo/Cre
Percentage (%)
CD3+ T cell lineage
CD19+ B cells
CD11b+ Dendritic cells
CD8+CD4- Cytotoxic T cell
CD8-CD4+ T helper cell
CD4+Foxp3+ Treg
CD8+CD4+ Immature T cells
CD8-CD4- DN T cells
CD69+ Activated cytotoxic T
CD44loCD62L+ Naive cytotoxic T
CD44hiCD62L- Memory cytotoxic T
CD44midCD62L- Effector cytotoxic T
CD69+ Activated T helper
CD44loCD62L+ Naive T helper
CD44hiCD62L- Memory T helper
CD44midCD62L- Effector T helper
CD4+Foxp3 Treg

A

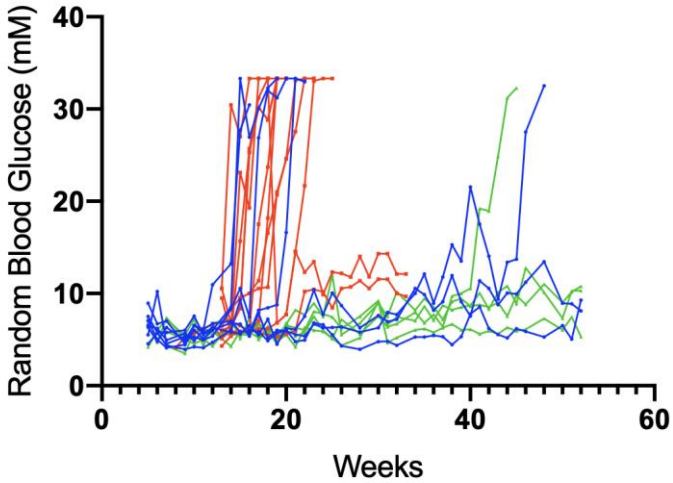
Michigan cohort

♂ *Ins1*$^{wt/wt}$ x *Ins1*$^{cre/wt}$ ♀

Ins1$^{cre/wt}$ (n=4)
Ins1$^{wt/wt}$ Littermates (n=7)
Ins1$^{wt/wt}$ Non-Littermates (n=11)



B



C

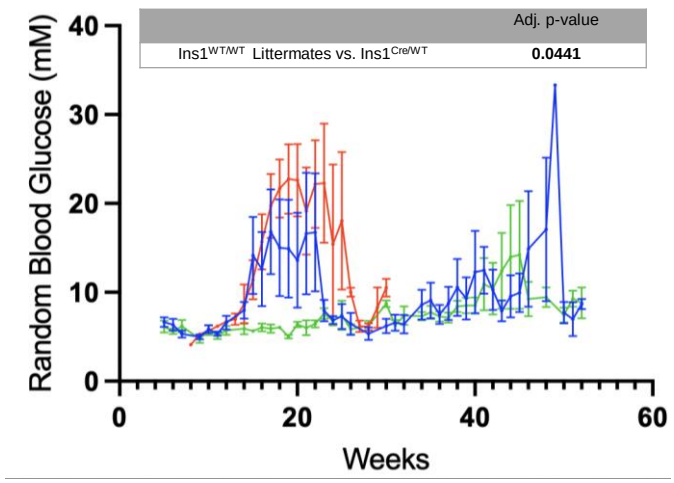


Figure 6

Table 1

Antibody	Manufacturer	Colour	Laser (nm)	Filter	Catalogue Number
CD3	ThermoFisher	eFluor450	405	450/45	48-0033-82
CD4	ThermoFisher	BV650	405	660/10	64-0042-82
CD8	BioLegend	PE-TR	561	610/20	100762
CD11c	ThermoFisher	PE	561	585/42	12-0114-81
CD19	ThermoFisher	SB780	405	763/43	78-0193-82
CD44	ThermoFisher	APC	633	660/10	17-0441-82
CD62L	ThermoFisher	PerCP-Cy5.5	488	690/50	45-0621-82
CD69	ThermoFisher	FITC	488	525/40	11-0692-82
Foxp3	ThermoFisher	AlexaFluor 700	633	712/25	565773-82
Viability	ThermoFisher	eFluor 506	405	525/40	65-0866-14