Soluble factors secreted by T-cells promote β-cell proliferation

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Running Title: T-cells mediate β-cell proliferation

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Word Count

Abstract: 162

Main Text: 4885

Diabetes Publish Ahead of Print, published online October 2, 2013

ABSTRACT

Type 1 diabetes is characterized by infiltration of pancreatic islets with immune cells leading to insulin deficiency. While infiltrating immune cells are traditionally considered to negatively impact β-cells by promoting their death, their contribution to proliferation is not fully understood. Here we report that islets exhibiting insulitis also manifested proliferation of β-cells which positively correlated with the extent of lymphocyte infiltration. Adoptive transfer of diabetogenic $CD4^+$ and $CD8^+$ T-cells, but not B-cells, selectively promoted β-cell proliferation *in vivo* independent from the effects of blood glucose, circulating insulin or by modulating apoptosis. Complementary to our *in vivo* approach co-culture of diabetogenic $CD4^+$ and $CD8^+$ T-cells with NOD.$RAG1^{-/-}$ islets in an *in vitro* transwell system led to a dose-dependent secretion of candidate cytokine/chemokines (IL-2, IL-6, IL-10, MIP-1α and RANTES) that together enhanced β-cell proliferation. These data suggest that soluble factors secreted from T cells are potential therapeutic candidates to enhance β-cell proliferation in efforts to prevent and/or delay the onset of type 1 diabetes.

INTRODUCTION

Type 1 Diabetes (T1D) is a chronic T-cell mediated autoimmune disease characterized by selective destruction of β-cells resulting in hyperglycemia (1). A major limitation to successful therapy has been a lack of complete understanding of the precise pathways and mechanisms that trigger T1D compounded by the polygenic nature of the disease and the influence of environmental and/or stochastic factors (2).

Studies using the non-obese diabetic (NOD) mice have identified roles for CD4+ and CD8+ T-cells, and macrophages in β-cell destruction. Other cell types including B-cells, natural killer (NK) cells, NKT-cells and the DC subsets have also been detected in the pancreatic infiltrate and draining lymph nodes and could contribute to β-cell death (3).

While immune-cells are generally considered to promote β-cell death, some studies argue that they also enhance their replication. For example, Sreenan et al. have reported increased β-cell proliferation in NOD mice which exhibit infiltration of pancreatic islets prior to the onset of diabetes (4). In addition, von Herrath et al. reported that non-diabetic RIP-LCMV x SV129 mice, where the numbers and effector functions of autoaggressive CD4 and CD8 lymphocytes were not decreased, have increased β-cell regeneration compared to non-diabetic C57BL/6 controls (5). In other studies, Sherry et al. suggested the increased β-cell proliferation that occurs after arresting the autoimmune process is secondary to effects of the inflammatory infiltrate. The latter study also showed that reversal of infiltration by anti-CD3 mAb or T-reg therapy was associated with reduced β-cell proliferation (6). A notable study that partially addressed the mechanism is that by Dor and colleagues who reported that the use of standard immunosuppression drugs abolished β-cell proliferation and recovery from diabetes (7). Recent studies have also reported that humans with T1D exhibit persistent mature β-cells in the pancreas that may be secondary to protective factors that prevent their destruction (8; 9). A n understanding of how these β-cells survive and/or regenerate is an exciting and timely area of interest.

Notwithstanding the scant information on the ability of human β-cells to replicate (10; 11), studies in rodent models indicate that β-cell proliferation is increased in physiologic conditions, pathophysiologic states, and in injury models(7; 12-15). In these models, glucose, insulin, IGFs (insulin like growth factors), GH (growth hormone), GLP-1 (glucagon-like-peptide-1), adipokines such as leptin, HGF (hepatocyte growth factor) and lactogens such as prolactin (PRL) have all been implicated in regulating β-cell proliferation (16).

In addition to the factors noted above, cytokines derived from the inflammatory response itself have been reported to stimulate islet-cell replication (17; 18), and treatment with IL-4 or

IL-10 has been reported to inhibit the development and prevent the recurrence of T1D in NOD mice (19; 20).

In this study, we tested the hypothesis that one or more lymphocytes, or their secretions, promote β-cell regeneration *in vivo*. We report, for the first time to our knowledge, that $CD4^+$ and $CD8^+$ T-cell subsets, but not B-lymphocytes, secrete soluble factors and are potential novel targets that can be harnessed to promote β-cell proliferation to counter the progression of T1D.

RESEARCH DESIGN AND METHODS

Mice: Female NOD/shiLTJ mice, 20 weeks of age were used as splenocyte donors and NOD.RAG1$^{-/-}$ mice, 5-6 week of age were used as recipients for adoptive transfer studies and islet donors for splenocyte-islet co-culture experiments. Male C57BL/6J (B6) mice islets, 5-6 weeks of age, were used for recombinant protein treatments. Blood glucose was measured under *ad libidum* conditions and mice were considered diabetic when two consecutive measurements of blood glucose exceeded 200 mg/dl.

Adoptive transfer of diabetes and depletion of splenocytes: A total of 10^7 splenocytes (SPLs) were purified from NOD mice with diabetes and injected intravenously into a single NOD.RAG1$^{-/-}$ mouse. To obtain SPL preparations devoid of B-cells, $CD4^+$, $CD8^+$ T cells, they were incubated with anti-B220-PE, anti-CD4-PE and anti-CD8a-PE (Biolegend), respectively. The cells were washed in PBS and resuspended in MACS (magnetic activated cell sorter) buffer and anti-PE Microbeads and run on the AutoMACS system (Miltenyi Biotec). Samples from the B-cell, $CD4^+$-, $CD8^+$ -depleted splenocyte aliquots were stained with anti-mouse CD19-PE, anti-$CD4^-$Pasific Blue and anti-CD8a-FITC (Biolegend), respectively, analyzed with a FACSAria (BD Biosciences) and determined to be >98%

depleted (data not shown). For $CD4^+$ and $CD8^+$ double-depletion, fractionated depleted cells were injected into NOD.$RAG1^{-/-}$ mice. We also used *in vivo* depletion by injecting 0.5 mg of anti-CD4, anti-CD8 or both mAb into NOD.$RAG1^{-/-}$ mice every three days after depleted-splenocyte transfer. Three weeks post-injection of total or depleted splenocytes, pancreas was harvested and prepared for β-cell morphometry. To track lymphocytes homing to host pancreatic islets in adoptively transferred mice we used NOD.Raspberry splenocytes from mice generated by microinjection of a β-actin/mRaspberry construct in the pronucleus of fertilized NOD mouse eggs.

Streptozotocin injection and insulitis scoring: Eighteen female NOD mice 6 weeks of age were injected intraperitoneally with streptozotocin (STZ, Sigma) at a concentration of 75 or 100 mg/kg/BW. Day 0 was defined as the first day of injection and pancreas was harvested from 3 mice every other day starting at day 1 until day 7. Insulitis was evaluated as reported (21).

Immunohistochemistry: Pancreata were harvested, fixed and embedded in paraffin 6 h post-injection with BrdU (100mg/kg/BW). Sections were stained using antibodies to BrdU, Ki67, phosphohistone H3), or insulin, appropriate secondary antibodies and counterstained with DAPI. At least, 1,000-2,000 β-cell nuclei were counted per animal and data were expressed as % of BrdU+, Ki67+ or pHH3+ β-cells. Cell death was detected by TUNEL assay (Chemicon, ApopTag S7100). Frozen sections were co-immunostained for insulin and a DSRed polyclonal antibody (Clontech) to detect mRaspberey protein followed by appropriate secondary antibodies.

Islet isolation and mixed lymphocyte-islet cell culture: Islets were isolated from 5-6 weeks old NOD.$RAG1^{-/-}$ or B6 mice and cultured as described previously (22). In parallel we prepared depleted or total lymphocyte cell suspensions from 20 week old diabetic or 7-8 week

old pre-diabetic female NOD mice (23). After starvation, 150 size-matched islets were co-cultured with splenocytes or treated with recombinant proteins in 5mM glucose. Contact between islets and splenocytes placed above the transwell membrane was prevented by using a 0.4 μm Transwell insert (Corning Life Sciences). Forty-eight hours after co-culture, medium was collected for luminex assay and islets were embedded in agar for β-cell morphometry. At least 1000-2000 β-cell nuclei were counted for quantifying proliferation and apoptosis.

Islet dispersion and cell sorting: Overnight cultured islets were dispersed and β-cells were sorted as described previously (24). Sorted β-cells were washed and stained with anti-CD45 (eBioscience) followed by fixation, permeabilization and staining with anti-BrdU (BrdU staining kit-APC, eBioscience). Cells were analyzed with BD LSR II analyzer.

Recombinant protein treatment: One hundred and fifty handpicked islets isolated from B6 mice were cultured in the absence or presence of IL-2 (5 pg/ml or: 500pg/ml), IL-6 (200 pg/ml or 200 ng/ml), IL-10 (4 pg/ml or 400 pg/ml), MIP-1α (10 pg/ml or10 ng/ml), RANTES (5 pg/ml or 500 pg/ml), a low dose combination of all the cytokines/chemokines or 15% FBS (positive control). Low doses were selected from our luminex assay results and high doses were based on manufacturer's recommendations (R&D Systems). To determine whether the proliferative effects are direct, islets treated with either recombinant proteins or total DM splenocytes were cultured in the presence of specific inhibitors/neutralizing molecules: Ro 26-4550 (IC_{50}=3μM), anti-IL6 (ND_{50}=0.005μg/ml), anti-IL10 (ND_{50}=0.045μg/ml), Maraviroc (IC_{50}=3.3nM) and Maraviroc (IC_{50}=5.2nM) to block IL-2, IL-6, IL-10, MIP-1α and RANTES, respectively

Statistics: Data are expressed as means ±SEM following a 2-tailed Student's *t* test, and considered significant at P value ≤ 0.05.

Study Approval: All animal experiments were conducted following approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center in accordance with NIH guidelines.

RESULTS

Adoptive transfer of diabetogenic splenocytes promote β-cell replication in NOD.RAG1$^{-/-}$ mice

To directly examine whether splenocytes induce proliferation of β-cells we performed adoptive transfer experiments (6; 25). We used 20 week old hyperglycemic (Diabetic mice, DM) or 7-8 week old normoglycemic NOD mice (Pre-diabetic mice, Pre-DM) as splenocyte donors. Since the kinetics of disease transfer are dependent on the age of the mice when the splenocytes are transferred (26) and most islets in pre-diabetic animals have only peri-insulitis (Supplemental Fig. 1*A*) we considered Pre-DM as the control cohort. To confirm that splenocytes after adoptive transfer target host pancreatic islets we injected splenocytes derived from hyperglycemic NOD.Raspberry mice congenically marked with mRaspberry fluorescent protein, a far red protein which is generally preferred for *in vivo* imaging, into female NOD mice. We visualized that marked cells accumulate and infiltrate into host islets (Supplemental Fig. 1*B*).Intravenous injection of freshly isolated total DM splenocytes or control Pre-DM splenocytes into 5-6 week old immune-deficient NOD.RAG1$^{-/-}$ mice (Fig. 1*A*) showed a significant increase in β-cell mitosis in the group injected with DM splenocytes compared with controls. To ascertain that we were counting only proliferating β-cells and not overlapping immune cells we used confocal microscopy z-stack, 3D and orthographic imaging and double staining for insulin and CD3, a T-cell receptor marker, or F4/80, a common macrophage marker (Fig. 2, *A-N*). A 5-fold increase in BrdU incorporation indicated β-cells in the S-phase of the cell cycle (Fig. 1, *B* and *C*), while an augmentation in pHH3

immunostaining suggested progression into the G2 or M phases (Fig. 1, *B* and *D*). The enhanced mitosis was confirmed using Ki67 (Fig. 1, *B* and *E*). To confirm our immunohistological findings we performed FACS analysis of dispersed islets for β-cell sorting according to autoflourescence and size (Fig. 2, *O* and *P*). We used CD45 staining to gate out immune cells and quantified BrdU positive β-cells (Fig. 2*Q*). We examined BrdU immunostaining in the CD45 positive cell population as an internal control (Supplemental Fig. 1 *C-F*). The increase in proliferating β-cells was consistent with the immunohistological data (Fig. 2, *R-T*). In addition, co-immunostaining for BrdU and insulin or GLUT2 (β-cell membrane marker) confirmed the identity of the cells (Supplemental Fig. 2, *A* and *B*). TUNEL immunostaining did not reveal significant differences in β-cell apoptosis between groups (Fig. 1, *F* and *G*). Together these data indicate that injection of splenocytes isolated from DM promotes β-cell replication *in vivo*.

T-cells, but not B-cells, are the dominant players in β-cell proliferation

Previous studies have reported that T1D is primarily a T-cell mediated autoimmune disease (27-29). To evaluate the effect of B-cells on β-cell proliferation we injected total (10^7) or B-cell depleted (6.4×10^6) cell populations intravenously into female NOD.RAG1$^{-/-}$ mice (Fig. 1*A*) followed, three weeks later, by harvesting of pancreas, liver and epididymal fat. Co-immunostaining of BrdU and insulin in pancreas sections from animals receiving total splenocytes from diabetic animals and animals administered B-cell depleted splenocytes showed no significant difference in β-cell replication (Fig. 3, *A* and *B*). Furthermore, co-immunostaining of pancreas sections for PDX1 (Pancreatic and duodenal homebox-1), a β-cell transcription factor, and BrdU did not reveal differences between groups (Supplemental Fig. 2*C*). The β-cell specificity of the effects on proliferation was confirmed by a virtual lack

of proliferation in hepatocytes or adipocytes (Supplemental Fig. 2, *D* and *E*). These data demonstrate that B-cells are unlikely to contribute to β-cell proliferation in this model.

We next evaluated the relative importance of T-cells for β-cell proliferation using a similar approach. We considered $CD4^+$ and $CD8^+$ T cells to be likely candidate(s) because they are the major T-cell subsets infiltrating in or around the islets and are the final executors of β-cell destruction (30). In addition to *in vitro* depletion, we injected NOD.RAG1$^{-/-}$ mice receiving *in vitro* depleted splenocytes with anti-CD4, anti-CD8 or both mAbs to promote *in vivo* depletion. The groups receiving the individual $CD4^+$ and $CD8^+$ depleted splenocytes as well as the CD4/CD8 double depleted splenocytes exhibited dramatically decreased β-cell proliferation compared to the groups injected either with whole splenocytes from diabetic animals or B-cell depleted splenocytes. Moreover, mice which received $CD8^+$ depleted splenocytes showed a slightly greater β-cell proliferation compared to animals administered $CD4^+$ or double-depleted splenocytes ($p<0.05$) (Fig. 3B). Apoptosis trended to be higher in the total DM splenocyte and B-cell depleted splenocyte-administered animals but did not reach statistical significance (Supplemental Fig 5*A*). These results suggest that $CD4^+$ and $CD8^+$ T-cells act together to stimulate β-cell replication in animals injected with diabetogenic splenocytes.

β-cell proliferation is positively correlated with islet infiltration

Infiltration in the pancreatic islets with mononuclear inflammatory cells is a key feature in T1D in NOD mice. An interesting observation during the analyses of sections for β-cell proliferation in the different groups discussed above was a striking difference in the percentage of infiltrated cells (Fig. 4*A*). Scoring for insulitis revealed that while the animals receiving total DM splenocytes and B-cell depleted DM splenocytes contained islets with moderate to severe insulitis, the number of affected islets was significantly reduced in animals

receiving Pre-DM splenocytes. In contrast, all the groups that received T-cell subtype(s)-depleted splenocytes were virtually free of insulitis with a few scattered islets exhibiting minimal infiltration (Fig. 4, *A* and *B*). We observed a linear and significant correlation between the islets manifesting insulitis and β-cell proliferation (r=0.71; p=0.004) (Fig. 4*C*). To confirm this finding we used an alternative model that promotes infiltration in islets, namely the streptozotocin (STZ)-induced diabetic NOD mouse. Examination of pancreas sections in mice that receive intra-peritoneal injection of a single dose (75 or 100 mg/kg/b.wt) of STZ again revealed a positive correlation between β-cell proliferation and mononuclear cell infiltration beginning on day one and peaking on day five or day three after injection (Fig. 4, *D* and *E*). The lack of significant alterations in blood glucose and insulin levels at the peak of the proliferation effect suggested the proliferation was independent of the effects of glycemia or insulin (Supplemental Fig. 3, *A* and *B*). The mice that were subjected to the adoptive transfer experiments over the three week period following splenocyte injection were also normoglycemic (Supplemental Fig. 3*C*). The virtual absence of mononuclear immune-cell infiltration in liver and adipose confirmed the β-cell specificity (Supplemental Fig. 3*D*). Together these results suggest that β-cell proliferation occurs soon after immune-cell infiltration, prior to the onset of diabetes, and is independent of the effects of glucose and insulin.

Soluble factors secreted by lymphocytes promote β-cell proliferation

Our *in vivo* data indicated that increasing numbers of infiltrating lymphocytes positively correlated with β-cell proliferation. To determine whether this direct effect is observed *in vitro* we designed mixed lymphocyte-islet culture (MLIC) experiments to examine whether splenocytes (total or T-cell depleted) from diabetic NOD mice promoted β-cell proliferation.

Briefly, islets were isolated from NOD.RAG1$^{-/-}$ mice and cultured overnight; in parallel, we isolated aliquots of diabetogenic total or T-cell depleted splenocytes (Fig. 5*A*).

Prior to co-culture of lymphocytes with islets, we hypothesized that a specific ratio of islet cells to lymphocytes is critical to promote proliferation of β-cells and that soluble factors mediate the proliferation. Analyses of β-cell proliferation in single islets showed a positive correlation with lymphocyte infiltration, (Fig. 5*B*), that was similar to the *in vivo* studies (Fig. 4*C*). To determine the ratio between infiltrating immune cells vs insulin(+) β-cells pancreas sections from total splenocyte- or STZ-injected mice were examined for both cell types; the infiltrating cells were between 4 to 10 times greater than β-cells in the islets that exhibited proliferation (Fig. 5*C*). Therefore, we co-cultured freshly isolated splenocytes with 150 islets in varying ratios (see below) for 48 hours followed by embedding the islets in agar for immunohistochemical analyses (Fig. 5*A*). To address whether the effects are mediated by soluble factors or by direct contact we co-cultured the lymphocytes with the islets either in the presence or absence of microporous transwell inserts which prevent direct splenocyte-islet contact while allowing soluble factors to diffuse across (Fig. 5*D*). We first assessed the capacity of total DM and Pre-DM splenocytes to stimulate β-cell proliferation at an increasing islet-cell to splenocyte ratio (1:1, 1:2, 1:5, 1:10). Forty-eight hours after co-culture, we observed that total splenocytes from diabetic mice, at a ratio of 1:5 and 1:10, significantly induced β-cell proliferation in a dose-dependent manner compared to islets co-cultured with Pre-DM splenocytes in transwell conditions, suggesting a role for soluble factors secreted from lymphocytes isolated from DM in β-cell proliferation (Fig. 5*D*). On the other hand, non-transwell conditions which permitted cell-cell contact also revealed an effect on β-cell proliferation that was slightly higher compared to the transwell studies likely due to the increased cell-cell contact (Fig. 5*E*).

Next, we performed a second set of co-culture experiments to examine the effects of selected T-cell subtype(s) on β-cell proliferation *in vitro* (Fig. 5*F*). A 1:1 and 1:10 islet-cell to splenocyte ratio in the transwell system revealed that islets co-cultured with total DM splenocytes promoted a 3 to 10 fold higher β-cell proliferation at the 1:10 ratio compared to Pre-DM splenocytes or splenocytes that are depleted for T-cell subtype(s). The proliferation was either very low or undetectable (ND) at the 1:1 ratio. Among the groups treated with splenocytes that are depleted of the T-cell subtype(s) the $CD8^+$ depleted group revealed statistically significant higher β-cell proliferation compared to $CD4^+$ only or $CD4^+/CD8^+$ double depleted ($p<0.05$) groups. Moreover, in positive selection experiments, islets cultured with only $CD4^+$ cells (1 to 10 ratio of islet cells to CD4+ cells) showed higher ($p=0.07$) β-cell proliferation compared to $CD8^+$ only co-cultured groups (Fig. 5*F*) and was consistent with our negative selection experiments exhibiting low proliferation in the $CD4^+$ depleted group. Evaluation of β-cell death by TUNEL assay did not reveal significant differences between groups (Supplemental Fig. 5*B*). These results support our *in vivo* findings that $CD4^+$ and $CD8^+$ T-cells act together and that $CD4^+$ T-cells are likely more effective in stimulating β-cell proliferation by secreting soluble factors and independent of cell-cell contact.

Effects of cytokines on β-cell proliferation

To identify the soluble factor(s) that drive β-cell proliferation in pancreatic islets we analyzed media in the co-culture experiments to detect potential cytokines/chemokines/growth factors released by the splenocytes. We ranked the cytokines/chemokines from 1 to 4 according to their significant differences between DM and Pre-DM splenocyte treatments in the two transwell conditions (Supplemental Table 1). Among them group 1 cytokines/chemokines included candidate molecules (IL-2, IL-6, IL-10, MIP-1α and RANTES)which showed a dose-dependent higher concentration in the group treated with splenocytes from the DM

compared to mice treated with splenocytes from Pre-DM in both co-culture conditions (Fig. 6, *A-E*). We ruled out IP-10 as a candidate since it is not expressed on lymphocytes, is a known chemoattractant for immune cells and has been shown to be produced by the β-cells. The candidate molecules in the other groups (groups 2,3 and-4) were not significantly increased between DM and Pre-DM splenocytes treated mice at least in one or both co-culture conditions and were therefore not studied in detail. While some of the candidates (IL-2 and IL-6) were increased in both 'with' and 'without' transwell conditions, others (IL-10, MIP-1α and RANTES) were higher in the 'without' transwell condition probably due to direct cell-cell contact that potentially allows immune cells to respond rapidly via multiple pathways. In support of a potential proliferative role for each of the five cytokines/chemokines on β-cells we first confirmed expression of their receptors on sorted β-cells. (Fig. 7*A*). Second, we investigated the effects of the individual cytokines/chemokines on pancreatic islets isolated from B6 mice in the presence or absence of specific inhibitors and/or neutralizing antibodies over a range of concentrations (18; 31; 32). Low doses of IL-6 (200 pg/ml) strongly induced β-cell proliferation and increased up to 10-fold at the higher dose (200 ng/ml) ($p<0.05$)(Supplemental Fig. 4*B*). Moreover, IL-2, IL-10 and MIP-1α demonstrated significantly higher proliferation even at low levels with 8-, 4- and 3-fold increases respectively ($p<0.05$) (Supplemental Fig. 4, *A, C* and *D*). On the other hand, treatment with RANTES resulted in β-cell proliferation at lower doses (5 and 500 pg/ml) but the effect was surprisingly reduced at 50 ng/ml (Supplemental Fig. 4*E*). To determine specificity we examined the effects of specific inhibitors or neutralizing antibodies in islets treated with low dose cytokines/chemokines. In all cases we observed neutralization of the proliferative effects of the respective cytokine/chemokine (Fig. 7 *B-F*). In some cases (IL-2 inhibitor and Maraviroc 3.3nM) the neutralization was not complete and is likely due to variable IC50s of the compounds (Fig 7G). Cytokines and/or chemokines are known to be secreted by

macrophages or dendritic cells to impact T-cell function and conversely secretions from T-cells can also impact macrophages (27; 33). To examine whether the cytokine/chemokines act synergistically to enhance β-cell proliferation we compared the individual effects versus a combination (Supplemental Fig. 4*F*). In addition to their significant individual effects on proliferation a combination of the cytokines/chemokines ,at doses used in the individual treatments, showed a significant increase ($p<0.001$) but was not dramatically different from the individual effects likely because some of the cytokines share common downstream pathways to stimulate proliferation. Finally, to confirm the recombinant protein treatment findings we undertook an independent experiment wherein total DM splenocytes served as the source of cytokines/chemokines. Islet-cells and splenocytes were cultured in a 1:10 ratio in a transwell system in the presence or absence of inhibitory/neutralizing antibodies against the candidate factors. While splenocytes alone significantly increased β-cell proliferation compared to untreated islets (Fig. 8 *A* and *B*), adding the inhibitory/neutralizing molecule reversed this effect. Consistent with our previous observations theses data suggested that each candidate has a potential to induce β-cell regeneration (Fig. 8 *C-H*). In summary, cytokines/chemokines that are secreted from lymphocytes in close proximity to islet cells promote detectable β-cell proliferation.

DISCUSSION

Although immune-cells have been implicated in the proliferation of β-cells during the progression of T1D (4; 6), the cell type(s) and mechanisms that control β-cell regeneration remain unknown. Here we report that soluble factors secreted from $CD4^+$ and $CD8^+$ T-cells directly stimulate β-cell proliferation.

NOD.$RAG1^{-/-}$ mice injected with DM splenocytes exhibited a significant elevation in all markers of β-cell proliferation compared to controls. Adoptive transfer of diabetes to

immunocompromised syngenic recipients can be achieved only when a combination of splenic $CD4^+$ and $CD8^+$ T-cells from donor NOD mice is used and not by either T-cell subsets alone (34). Injecting $CD4^+$-, $CD8^+$- or $CD4^+/CD8^+$ double-depleted splenocytes into NOD.RAG1$^{-/-}$ animals resulted in a dramatic decrease in β-cell proliferation compared to the animals that received total DM splenocytes. In addition, we also observed that animals in whom T-cell subtype(s) were depleted exhibited minimal pancreatic islet infiltration compared to animals receiving total splenocytes or B-cell depleted splenocytes. Considering that the development of T1D requires the presence of both $CD4^+$ and $CD8^+$ T-cells, depleting one or both of these cells would impact the inflammatory response and alter β-cell replication. This possibility was supported by a linear and significant correlation between β-cell proliferation and immune cell infiltration in our studies. Among the T-cell subtypes, both $CD8^+$ T-cell depleted splenocyte injection (*in vivo*) and islet co-culture (*in vitro*) studies demonstrated higher proliferation compared to $CD4^+$ depleted or $CD4^+/CD8^+$ double-depleted cohorts. Similar results were observed when islets were cultured with $CD4^+$ T-cell subset alone signifying their role in β-cell proliferation. We did not detect a significant difference in β-cell apoptosis between the groups either because the duration following injection of splenocytes is not sufficient to promote significant apoptosis and/or because it is often difficult to detect dead β-cells due to their rapid engulfment and disposal (35).

While our studies point to $CD4^+$ and $CD8^+$ T-cells as critical for β-cell proliferation during T1D progression, we cannot exclude the potential contribution of macrophages or dendritic cells and their secreted products (36). Our data suggests that B-cells are unlikely to play a role in β-cell replication. Depletion of macrophages prevents T1D development (37) and macrophages have been reported to impact β-cells by producing pro-inflammatory cytokines (38). One possible role for macrophages in β-cell proliferation is that T-cell subtypes,

especially $CD4^+$, recruit additional $CD4^+$, $CD8^+$ or granulocytes into the infiltrate and contribute to local secretion of cytokines and chemokines. Indeed, T-cells secrete soluble factors such as GM-CSF (granulocyte macrophage-colony stimulating factor) (39) to influence leukocytes and recruit them to inflammatory sites during inflammation (40; 41). Thus, in addition to their direct effect on β-cell proliferation by cytokine secretion, it is possible that T-cells act indirectly by triggering mononuclear cells to secrete soluble factors, via the classical or non-classical pathways, that can in turn enhance β-cell proliferation.

While earlier studies implicate a T-cell dependent proliferative effect on aortic smooth muscle (42) and orbital fibroblasts (43), the mechanism(s) remains unclear. Careful analyses of our data from the transwell experiments indicate that CD4+ and CD8+ lymphocytes secrete IL-2, IL-6, IL-10, MIP-1α and RANTES, each of which showed a dose-dependent effect on β-cell proliferation. Some of these factors have been associated with proliferation of other cell types. For example, IL-2, regulates the growth and function of T-cells (44), while IL-6 stimulates α- and β-cell proliferation *in vitro* (18) and is known to reinforce the effects of IL-2 in promoting the differentiation of $CD4^+$ cells into Type-2 T helper cells (T_H2) (45). RANTES acts with IL-2 to induce the proliferation and activation of natural-killer (NK) cells to form chemokine-activated killer cells (46). Since β-cells themselves have been reported to produce inflammatory cytokines such as IL-1β (47) we do not exclude the β-cell as a source of some of these molecules. Cytokines, such as IL-1β and INF-γ, when used in combination, can induce de-differentiation of newly generated β-cells mediated by re-expression of the Notch-Delta pathway (48). Whether the soluble factors detected in our experiments are also involved in similar pathways to modulate β-cell mass warrants further investigation.

Although, the capacity to proliferate are strikingly different between rodents and human the observations that β-cells can regenerate in humans have prompted studies to investigate safe

approaches to enhance their functional mass. Whether the candidate molecules identified in our study can be used either individually or in combination with an appropriate immunosuppressive regimen to preserve β-cell mass requires further research. In the context of cytokines, IL-6 has been reported to stimulate human islet proliferation (18). Although attempts at expansion *ex-vivo* resulted in a change in the β-cell phenotype, lineage-tracing studies suggest that de-differentiated human β-cells are able to survive and replicate *in vitro*(49). Thus, testing the candidates we have identified in *ex-vivo* conditions can be a first step to evaluate their ability to expand human β-cells. However, a role for these molecules as 'therapeutic' agents has to be viewed with caution due to their well-established roles in the immune network. For example, rapamycin, an immunosuppressant drug used to protect rejection in organ transplantations, inhibits lymphocyte proliferation by inhibiting their response to IL-2(50). Despite IL-2 being important in lymphocyte activation, it also contributes to the development and expansion of $CD4^+$ $CD25^+$ regulatory T-cells, which promote self-tolerance by suppressing T-cell responses *in vivo*. Thus, extensive and careful dosing studies are necessary to examine the potential of the candidate cytokines/chemokines for β-cell expansion.

We propose that some of the pro- and anti-inflammatory cytokines/chemokines secreted in the islet microenvironment during insulitis have the potential to promote proliferation by activating diverse signaling cascades (e.g. JAK/STAT, MAPK or PI3K/AKT). This potential beneficial effect triggers the islets to secrete chemoattractant molecules [e.g. eotaxin (CCL11), IP-10 (CXCL-10) and MCP-1(CCL2)] which in turn amplify the recruitment of mononuclear cells and the release of multiple cytokines/chemokines. The detection of increased levels of chemotactic molecules in our experiments, especially IP-10 and eotaxin

likely amplifies the number of immune cells that secrete soluble factors in the inflamed area to further promote β-cell proliferation and prevent progression of diabetes.

In summary, we report that $CD4^+$ and $CD8^+$ T-cells secrete soluble factors that promote β-cell replication in the NOD model of T1D. Therapeutic targeting of one or a combination of these soluble factors may prove useful to delay and/or counter the progression of T1D by enhancing functional β-cell mass.

ACKNOWLEDGMENTS

This work was supported by a Juvenile Diabetes Research Foundation (3-2010-474) Fellowship and Turkish Diabetes, Obesity and Nutrition Association (Novartis Science Award) to E.D.; some of the reagents were supported by National Institutes of Health Grant RO1 DK 67536 to R.N.K. and the Joslin Diabetes Center Flow Cytometry Cores supported by Diabetes Research Center Grant (NIH P30DK036836) and NIH PO1 AI054904 to D.M.

No potential conflicts of interest relevant to this article were reported.

E.D. and R.N.K. conceived the idea and designed the experiments. E.D. performed all experiments, analyzed the data and wrote the manuscript; W.J. assisted in the adoptive transfer experiments; S.K. and D.J.D.F. contributed to animal maintenance; S.K. and A.E.O. assisted in islet isolation; A.K.K.T. assisted in the real time-PCR experiments; J.H. and D.K. assisted in the immunohistochemical experiments; J.L.G. contributed to the flow cytometry analysis and NOD.Raspberry studies; D.M. contributed to designing the experiments, troubleshooting and the NOD.Raspberry studies and R.N.K supervised the project and wrote the manuscript. R.N.K and E.D. are the guarantors of this work and, as such, had full access to all the data in the study and takes the responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Gordon C. Weir, MD (Joslin Diabetes Center and Harvard Medical School, Boston, MA), and Fumihiko Urano, MD, PhD (Washington University School of Medicine, St.Louis, MO) for discussions and advice, and Olivia Lovegreen (Joslin Diabetes Center, Boston, MA) for editorial assistance and the Specialized Assay Core (Joslin Diabetes Center, MA) for hormone and metabolite analyses.

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FIGURE LEGENDS

Figure 1. **Adoptive transfer of diabetes stimulates β-cell proliferation in NOD.RAG1$^{-/-}$ recipients.** (**A**) Experimental strategy showing total splenocyte (DM or Pre-DM) or depleted splenocyte (diabetic mice) transfer (1 x 10^7 cells) into NOD.RAG1$^{-/-}$ mice. Bromodeoxyuridine (BrdU; 100mg/kg/b.wt) was injected three weeks post-transfer and 6 hours later the pancreases were harvested for immunohistochemical analyses. (**B**) Paraffin-embedded sections of pancreas from mice receiving DM or Pre-DM splenocytes, co-stained with proliferation markers BrdU, pHH3 or Ki67 with insulin and DAPI. (Scale bar 50 μm) Arrows indicate proliferating β-cells (BrdU+/insulin+). Inset shows magnified view of representative proliferating β-cells. (**C-E**) Quantification of data shown in **B** for BrdU (**C**), pHH3 (**D**) and Ki67 (**E**) (n=4-16 mice each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (student's *t*-test). (**F**) TUNEL staining of paraffin-embedded sections of pancreatic tissues obtained from recipient mice receiving DM or Pre-DM splenocytes for apoptosis detection. (Scale bar 100 μm) Arrows indicate TUNEL+/β-cell+ cells undergoing apoptosis. Inset shows a magnified representative image of TUNEL(+) β-cell. Lower image represents positive control of TUNEL staining in rat tumor tissue. (**G**) Quantification of data in **F**. (n=4-6 mice each group). Data are expressed as means ±SEM.

Figure 2. Confirmation of proliferating β-cells. (**A-E**) 2D (**A**) and 3D (**B**) confocal microscopy view of pancreatic section derived from total diabetic NOD mouse splenocyte injected animal. Magnified area highlighted from **B** shows 3D imaging of insulin (red) (**C**), Ki67 (green) (**D**) and DAPI (blue) (**E**). (Scale bar 100 μm) Arrows indicate proliferating β-cells. (**F**) Orthographic image of the same pancreatic section in **A** shows the horizontal and vertical view of a proliferating β-cell in a circle (ZEN-2009). (**G**) Mouse spleen section stained as positive control for F4/80 (green), common macrophage marker, and Ki67 (red). (**H**) Total diabetic splenocyte injected NOD.RAG1$^{-/-}$ pancreatic section stained for F4/80 (green), insulin (blue) and Ki67 (red) (Scale bar 100 μm). Arrows indicate proliferating β-cells. (**I,J**) Magnified view of proliferating (**I**) and non-proliferating (**J**) macrophages from **H**. (**K**) Mouse spleen section stained as positive control for CD3 (red), T-cell marker, and Ki67 (green). (**L**) Total diabetic splenocyte injected NOD.RAG1$^{-/-}$ pancreatic section stained for CD3 (red), insulin (blue) and Ki67 (green) (Scale bar 100 μm). (**M,N**) Magnified view of proliferating T-cell (**M**) and β-cell (**N**) from **L**. (**O**) Sorting of β- and non-β-cells from dispersed islets from mice following adoptive transfer of Pre-DM or DM splenocytes by flow cytometry based on size (FS Lin) and autoflourescence (FL1). (**P**) Sorted β-cells stained for CD45 and BrdU. (**Q-S**) Dot-plot showing gated out CD45+ cells (**Q**), and BrdU+ β-cells from Pre-DM (**R**) or DM (**S**) splenocyte transferred mice. (**T**) Quantification of data in **R** and **S**. (n=3 each group). ** $P < 0.01$. Experiment was performed in triplicate. Data are expressed as means ±SEM.

Figure 3. **T-cell subsets play a major role in β-cell proliferation.** (**A**) Paraffin-embedded sections of pancreatic tissues derived from recipient mice receiving total diabetic or B-cell depleted diabetic NOD mouse splenocytes, co-stained for the proliferation marker BrdU (green) with insulin (red) and DAPI (blue). (Scale bar 50 μm) Arrows indicate proliferating β-

cells (BrdU+/insulin+). Inset shows a magnified representative image of a proliferating β-cell. (**B**) Quantification of proliferating β-cells in pancreatic sections obtained from mice receiving total (DM or Pre-DM) or B-cell-, $CD4^+$-, $CD8^+$- and $CD4^+/CD8^+$ double-depleted diabetic NOD splenocytes. (n=4-6 mice each group). * $P < 0.05$ and *** $P < 0.001$ (student's *t*-test). Data are expressed as means ±SEM.

Figure 4. Pancreatic islet infiltration positively correlates with β-cell proliferation. (**A**) Immunofluorescence and hematoxylin and eosin staining of consecutive pancreatic sections harvested from NOD.RAG1$^{-/-}$ mice three weeks after receiving total (DM or Pre-DM) or B-cell-, $CD4^+$-, $CD8^+$- and $CD4^+/CD8^+$ double-depleted diabetic NOD splenocytes. (Scale bar 400 μm) Pancreatic islets are outlined with dotted lines for ease of comprehension. (**B**) Pancreatic islets showing insulitis expressed as a percentage in the treated groups in **A**. (**C**) Linear regression of islet infiltration and BrdU(+) β-cells in pancreas sections harvested from NOD.RAG1$^{-/-}$ mice transferred with total diabetic splenocytes. Each square represents a mouse (n=9) scored for insulitis in at least 20 islets (n=9). *r=0.713, p=0.004* (**D**) Pancreatic sections harvested from STZ-induced diabetic NOD mice at day 3 and 7, co-stained for the proliferation markers BrdU (green), with insulin (red) and DAPI (blue). (Scale bar 50 μm). Arrows indicate proliferating β-cells (BrdU+/insulin+). Inset shows a magnified view of a representative proliferating β-cell. (**E**) Quantification of β-cell proliferation (bars) and insulitis scores (red dots) in the pancreatic islets in streptozotocin (STZ)-injected NOD mice at 1-7 days post STZ administration (n=3 mice for each time point). Data are expressed as means ±SEM.

Figure 5. **β-cell proliferation is stimulated by infiltrating lymphocytes via soluble factors.** (**A**) Experimental strategy showing total splenocytes (DM or Pre-DM) or depleted splenocytes (diabetic mice), co-cultured with 5-6 weeks old NOD.RAG1$^{-/-}$ mice islets (150 islets/condition) for 48h in 5 mM glucose in the presence or absence of transwell inserts. (**B**) Linear regression of insulitis score and BrdU(+) β-cells in single pancreatic islets harvested from NOD.RAG1$^{-/-}$ mice transferred with total diabetic splenocytes. Each square represents a single islet out of 120 analyzed islets. *r=0.80*, *p<0.001*. (**C**) Representing pancreatic sections derived from total splenocyte- or STZ-injected mice used for determining the ratio between infiltrating immune cells vs insulin(+) β-cells. Islets are indicated by dotted lines in the right panel. The area of infiltration is shown around the islet in the left panel. (**D,E**) β-cell proliferation in agar-embedded NOD.RAG1$^{-/-}$ islets co-cultured with total splenocytes from DM or Pre-DM at a ratio of 1:1, 1:2, 1:5, 1:10 or without splenocytes in the presence (**D**) or absence (**E**) of transwell inserts (n=3-4). * $P < 0.05$ and ** $P < 0.01$ (student's *t*-test). (**F**) Quantification of proliferating β-cells in pancreatic islets co-cultured with total (DM or Pre-DM), negatively or positively selected DM splenocytes at 1:1 or 1:10 ratio with transwell conditions. (n=3-6 each condition) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (student's *t*-test). ND, not detected. Data are expressed as means ±SEM.

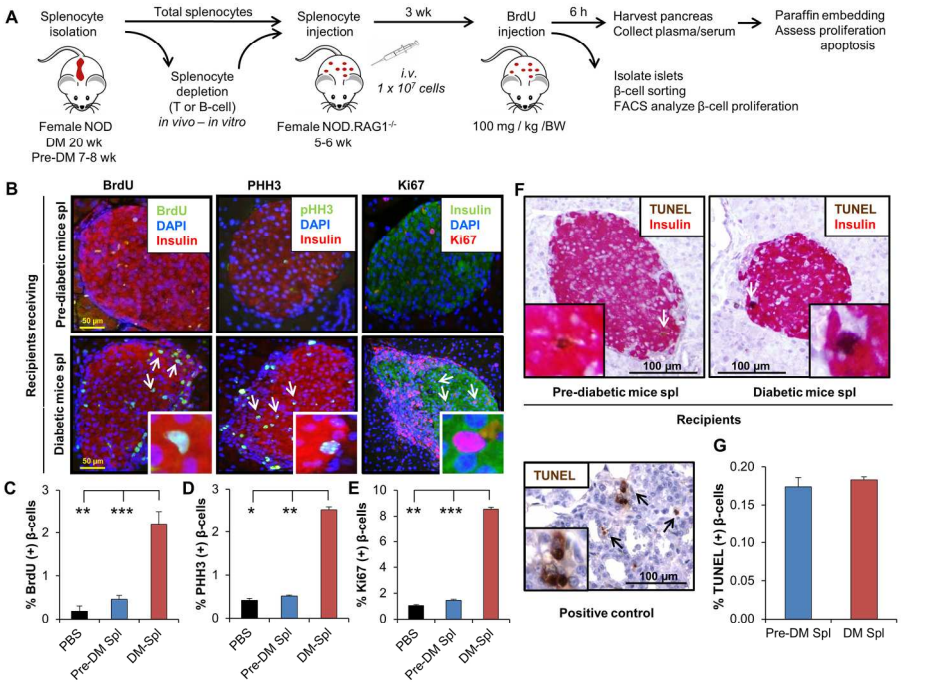
Figure 6. **Lymphocyte secreted soluble factors that drive β-cell proliferation**. (**A-E**) Luminex assay results from culture medium obtained 48h after co-culturing NOD.RAG1$^{-/-}$ islet with NOD splenocytes (DM or Pre-DM) at a ratio of 1:1, 1:2, 1:5, 1:10 or only

splenocytes at 10x in the presence or absence of transwell for IL-2 (**A**), IL-6 (**B**), IL-10 (**c**), MIP-1α (**D**) and RANTES (**E**) (n=3-4 each condition). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (student's *t*-test). Comparison: *, diabetic vs diabetic and §, diabetic vs pre-diabetic. Data are expressed as means ±SEM.

Figure 7. Effect of soluble factors on β-cell proliferation is reversed by inhibitory/neutralizing antibody treatment *in vitro*. (**A**) Detection of the cytokine/chemokine receptor subunit mRNAs by RT-PCR from sorted β-cells and splenocytes harvested from C57BL/6 mice (**B-F**) Agar-embedded pancreatic islets from C57BL/BJ mouse treated in the absence (control) or presence of low dose recombinant proteins with or without inhibitory/neutralizing molecules (as described in *Methods*) for 48h (150 islets/condition, 3-4 replicates). Representative sections are shown. Islets were co-stained for the proliferation marker Ki67 (green) with insulin (red) and DAPI (blue). Arrows indicate proliferating β-cells (Ki67+/insulin+) (Scale bar 200 μm). Inset shows a magnified image of a representative proliferating β-cell. (**G**) Quantification of data in **B-F** (n=3-4 each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (student's *t*-test). Comparison: *, untreated vs cytokine/chemokine or inhibitory/neutralizing antibody treated and #, cytokine/chemokine treated vs inhibitory/neutralizing antibody treated. Data are expressed as means ±SEM.

Figure 8. Lymphocyte secreted soluble factors enhance β-cell proliferation *in vitro*. (**A-G**) One hundred and fifty agar embedded islets harvested from C57BL/6 mice co-cultured without (untreated control) (**A**) or with total DM splenocytes in the absence (treated control) (**B**) or presence of inhibitory/neutralizing molecule Ro 26-4550 (**C**), anti-IL6 (**D**), anti-IL10 (**E**), Maraviroc 3.3nM (**F**), or Maraviroc 5.2nM (G). Islets were co-stained for the proliferation marker Ki67 (green), insulin (red), and DAPI (blue). Arrows indicate proliferating β-cells (Ki67+/insulin+) (Scale bar 200 μm). Inset shows magnified image of a representative proliferating β-cell. (**H**) Quantification of proliferating β-cells in **A-G** (n=3-4 each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (student's *t*-test). Comparison: *, untreated vs DM splenocyte treated and #, DM splenocyte treated vs inhibitory/neutralizing Ab treated. Data are expressed as means ±SEM.

Figure 1 Dirice E et al.



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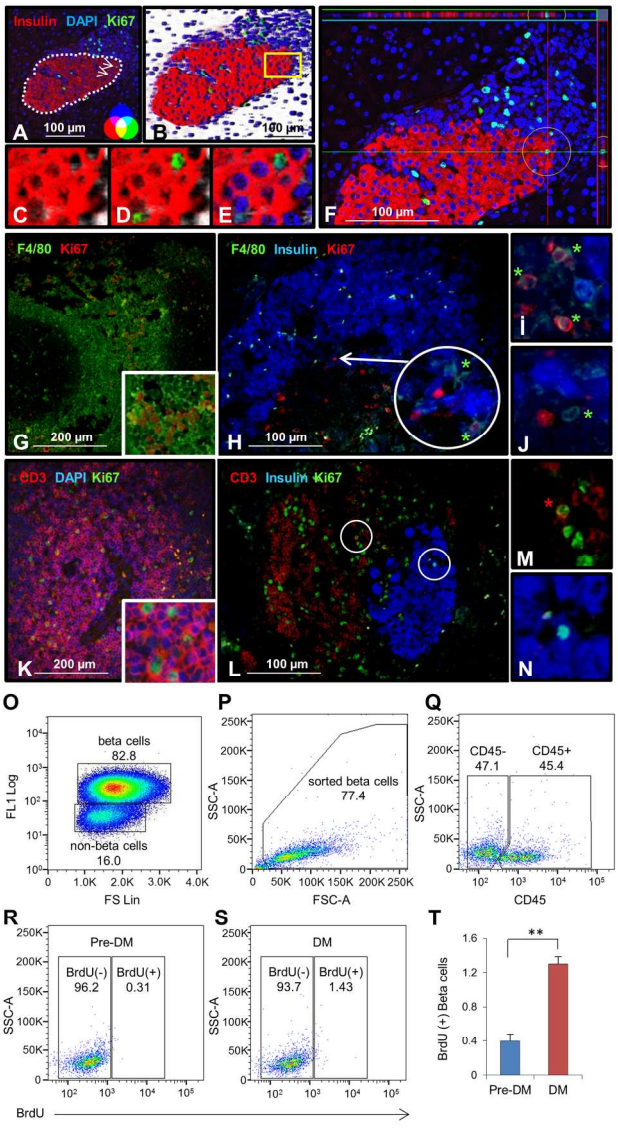

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Figure 3 Dirice E et al.

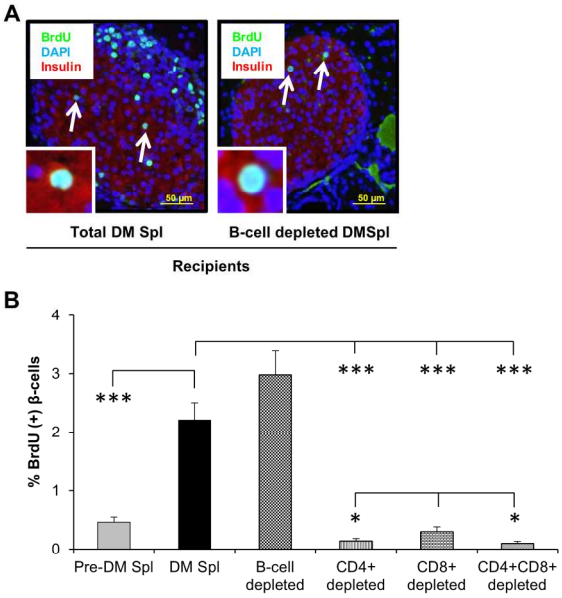

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Figure 4 Dirice E et al.

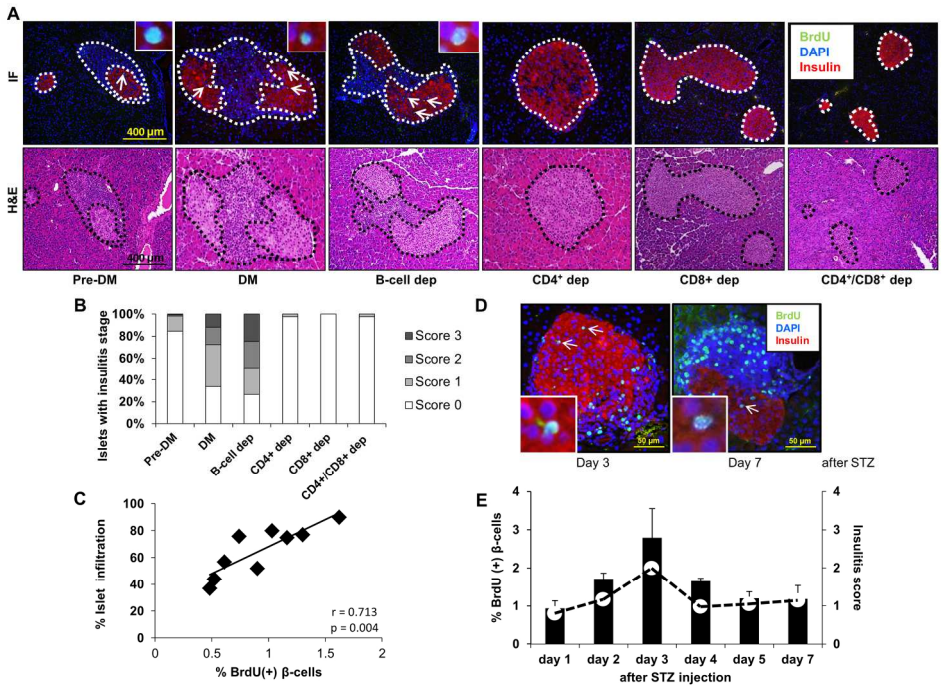

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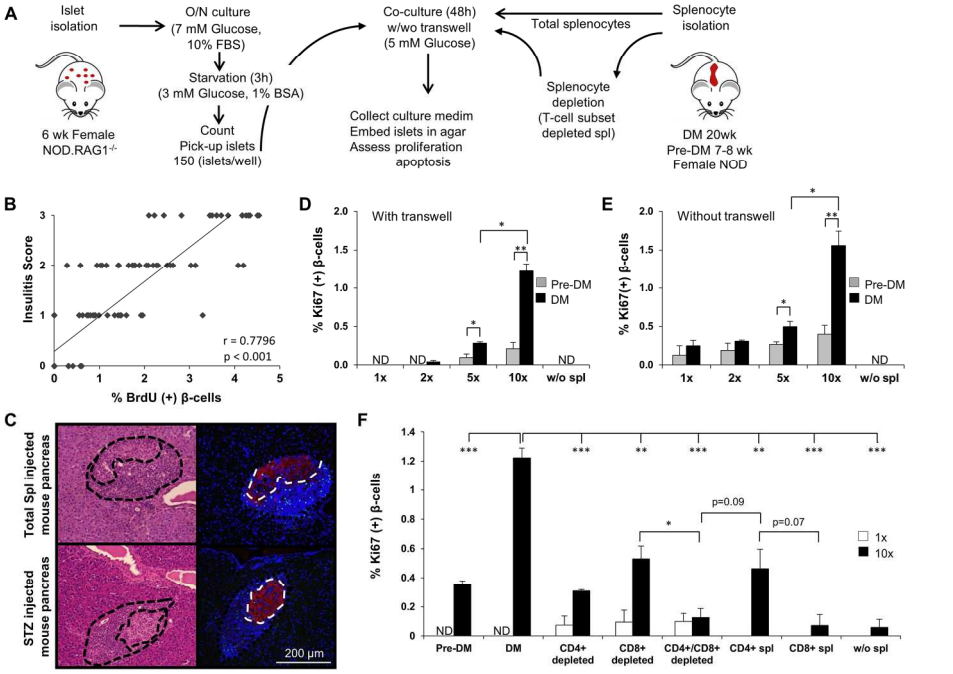
Figure 5 Dirice E et al.



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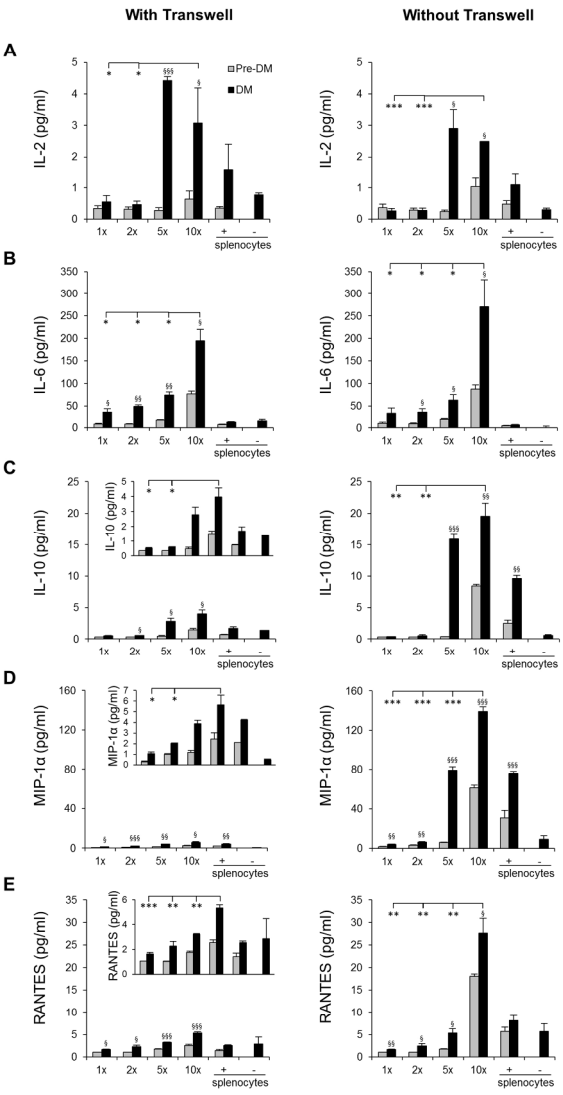

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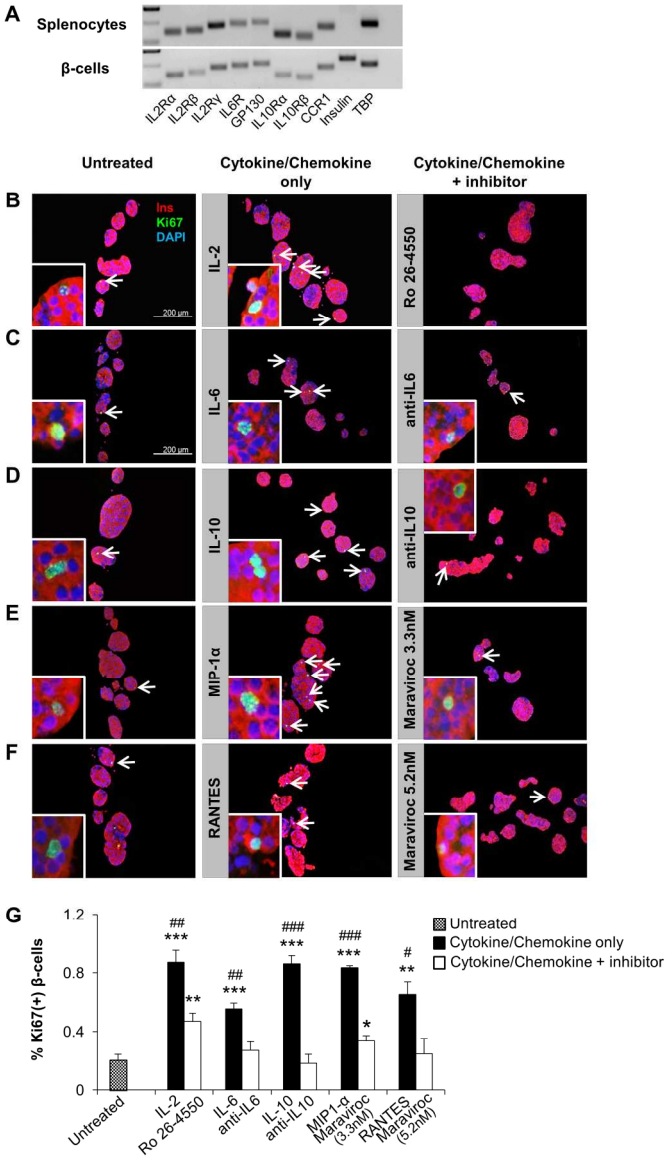

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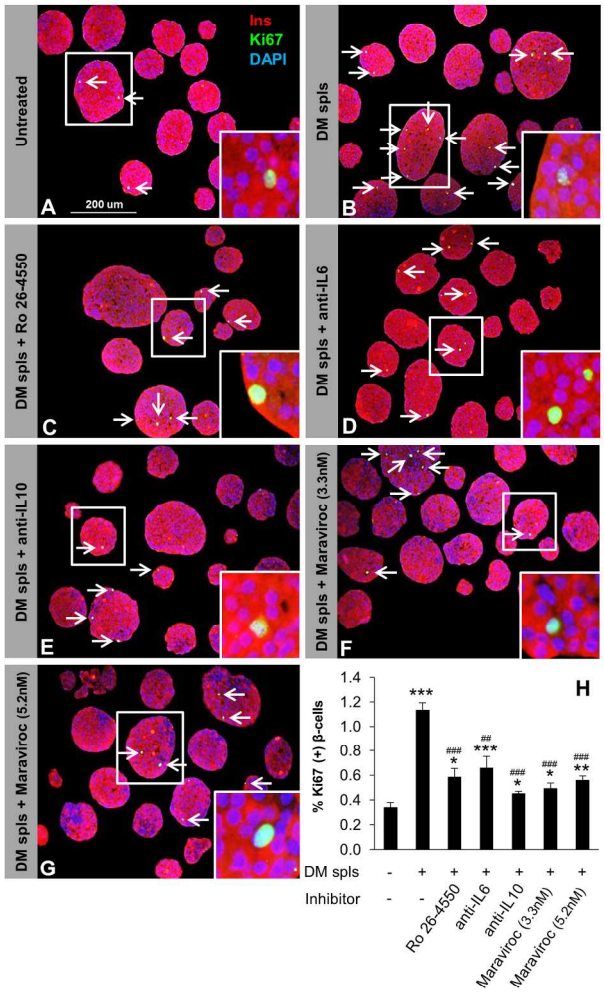

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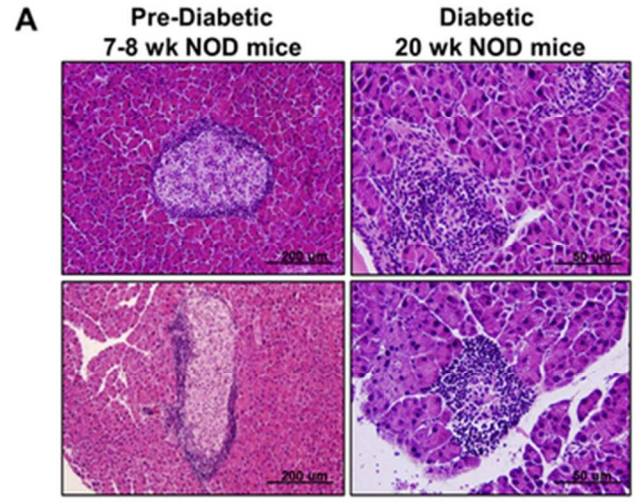
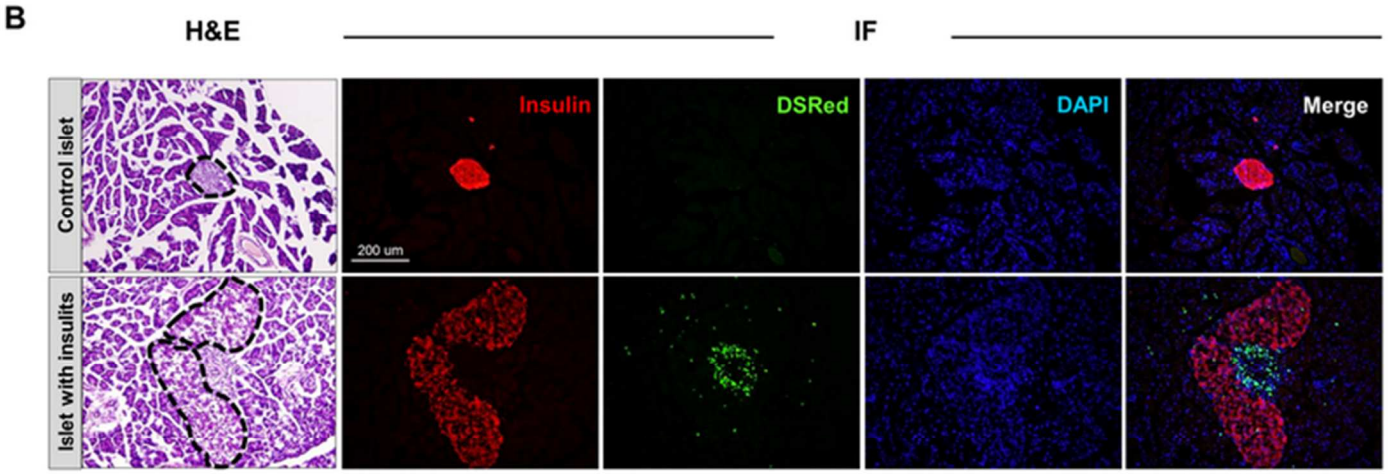
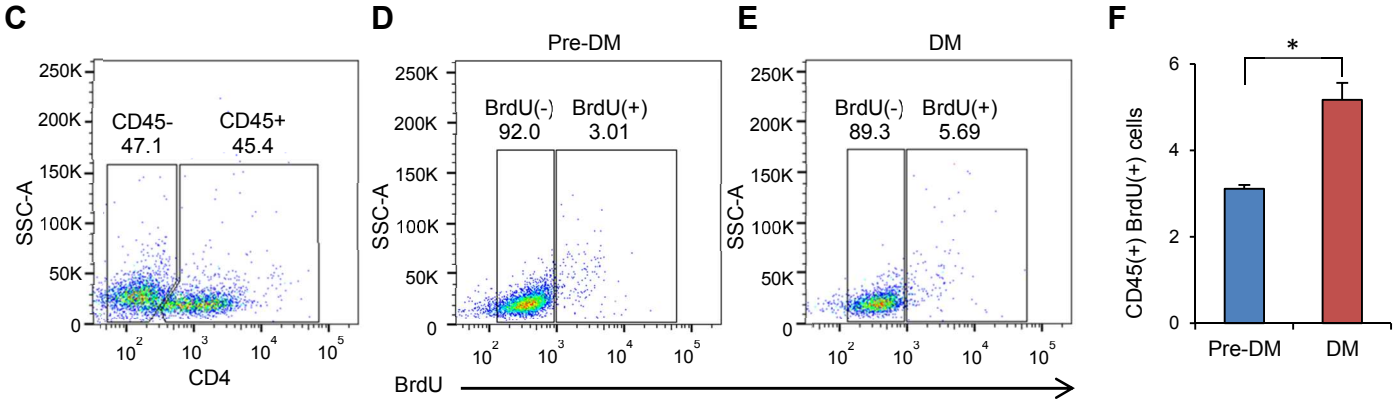
Supplemental Table 1. Statistical comparisons of cytokine/chemokine concentrations obtained from Luminex assay. Luminex assay was used to determine cytokine/chemokine levels from medium collected 48h after culturing NOD.RAG1$^{-/-}$ islet and NOD splenocyte (pre-DM or DM) at a ratio of 1-1, 1-2, 1-5, 1-10 in the presence or absence of transwell inserts. All cytokines/chemokines were grouped according to the 'p' value in both (group 1), with transfer (group 2), without transwell (group 3) or not significant in either condition (group 4). Significance between pre-DM and DM splenocyte transferred groups was determined by using the 1-10 islet cell to splenocytes ratio and analyzed by student's *t*-test. ND, not-detected.

		Pre-DM vs DM, p value	
		With transwell	Without transwell
Group1: Significant in both conditions	IL-2 (pg/ml)	0.041	0.035
	IL-6 (pg/ml)	0.036	0.049
	IL-10 (pg/ml)	0.040	0.013
	MIP-1α (pg/ml)	0.042	4.71E-05
	RANTES (pg/ml)	0.001	0.050
	IP-10 (pg/ml)	0.047	0.034
Group2: Significant in ''with transwell'' conditions	MCP-1 (pg/ml)	0.017	0.058
Group3: Significant 'in "without transwell'' conditions	IL-5 (pg/ml)	0.134	0.044
	IL-12(p70) (pg/ml)	0.275	0.026
	IL-17 (pg/ml)	ND	0.014
	TNF-α (pg/ml)	0.759	0.007
	KC (pg/ml)	0.080	0.048
	MIP-2 (pg/ml)	0.462	0.045
	MIG (pg/ml)	ND	0.020
Group4: Not significant in either condition	IL-1α (pg/ml)	ND	ND
	IL-1β (pg/ml)	ND	ND
	IL-3 (pg/ml)	ND	ND
	IL-4 (pg/ml)	0.090	0.424
	IL-7 (pg/ml)	ND	ND
	IL-9 (pg/ml)	ND	ND
	IL-12(p40) (pg/ml)	ND	ND
	IL-13 (pg/ml)	ND	0.172
	IL-15 (pg/ml)	0.232	0.174
	IFN-γ (pg/ml)	0.870	0.283
	G-CSF (pg/ml)	0.214	0.289
	GM-CSF (pg/ml)	0.096	0.683
	M-CSF (pg/ml)	0.112	0.115
	Eotaxin (pg/ml)	0.247	0.192
	MIP-1β (pg/ml)	ND	ND
	LIF (pg/ml)	ND	0.527
	LIX (pg/ml)	ND	ND
	VEGF (pg/ml)	ND	ND

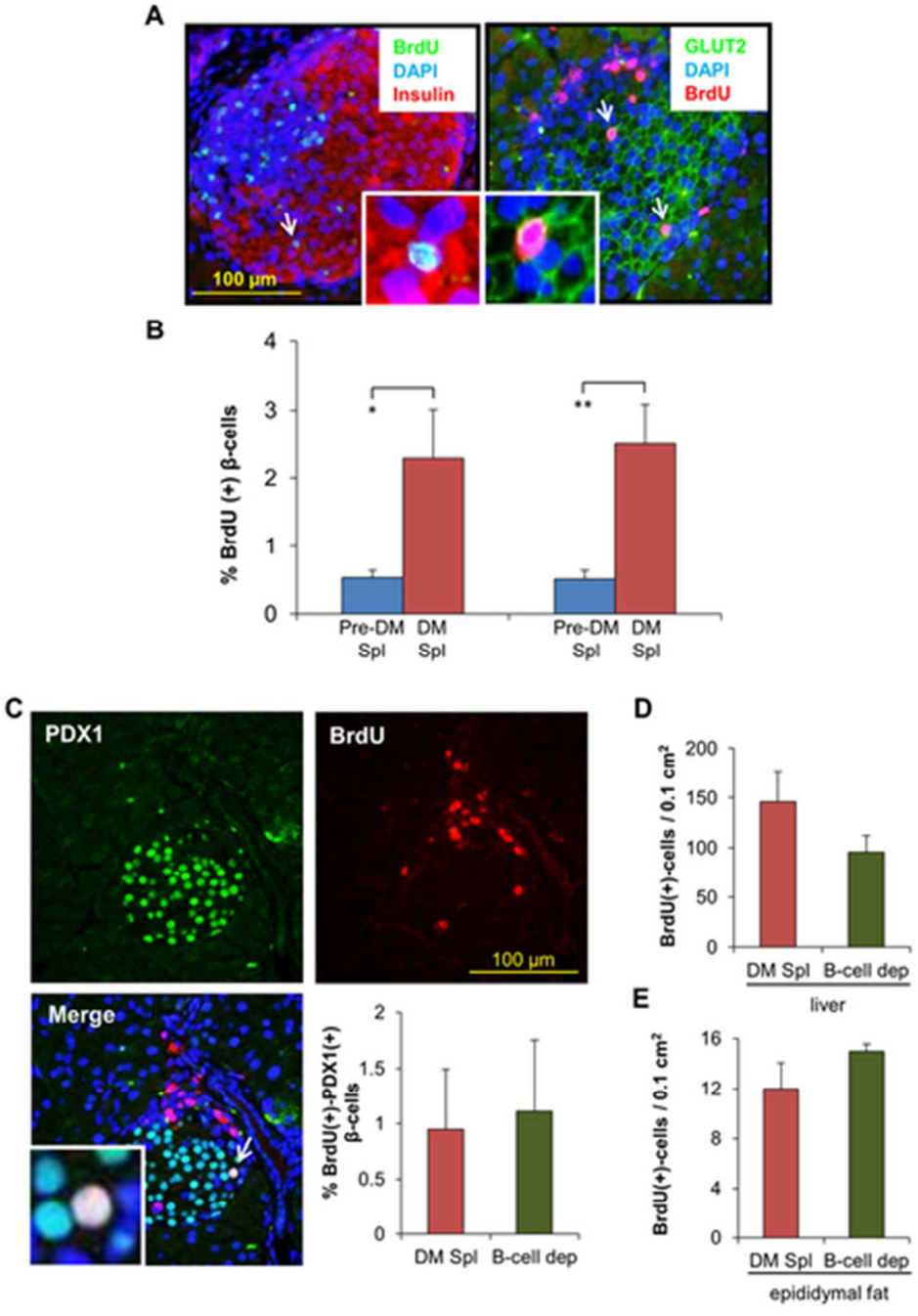
Supplemental Figure 1. Immune infiltration in NOD mice. (**A**) H&E staining of pancreas sections from 7-8 week old Pre-DM and 20 week old hyperglycemic DM mice. (**B**) Pancreatic islet sections harvested from female NOD mice 3 weeks after injection of female NOD.Raspberry mice splenocytes. Images represents control (top panel) and infiltrated (bottom panel) islets stained for H&E, insulin (red), mRaspberry (DSRed-green) and DAPI (blue). (**C**) Sorted β-cells (Fig. 2Q), stained positive for CD45, were analyzed for BrdU incorporation as an internal control. (**D,E**) Dot-plot showing CD45+, BrdU + cells from Pre-DM (**D**) or DM (**E**) splenocyte transferred mice. (**F**) Quantification of data in **D** and **E**. (n=3 each group). * $P < 0.05$. Experiment was performed in triplicate. Data are expressed as means ±SEM.



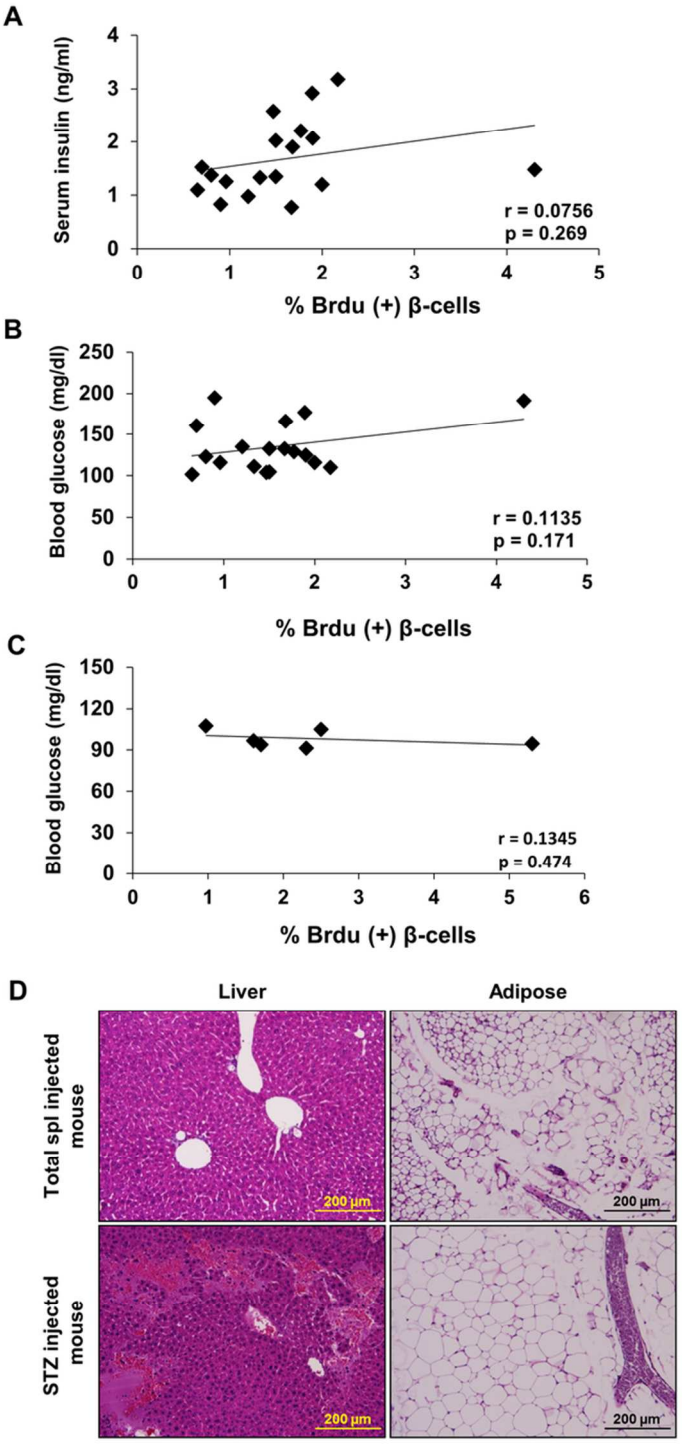




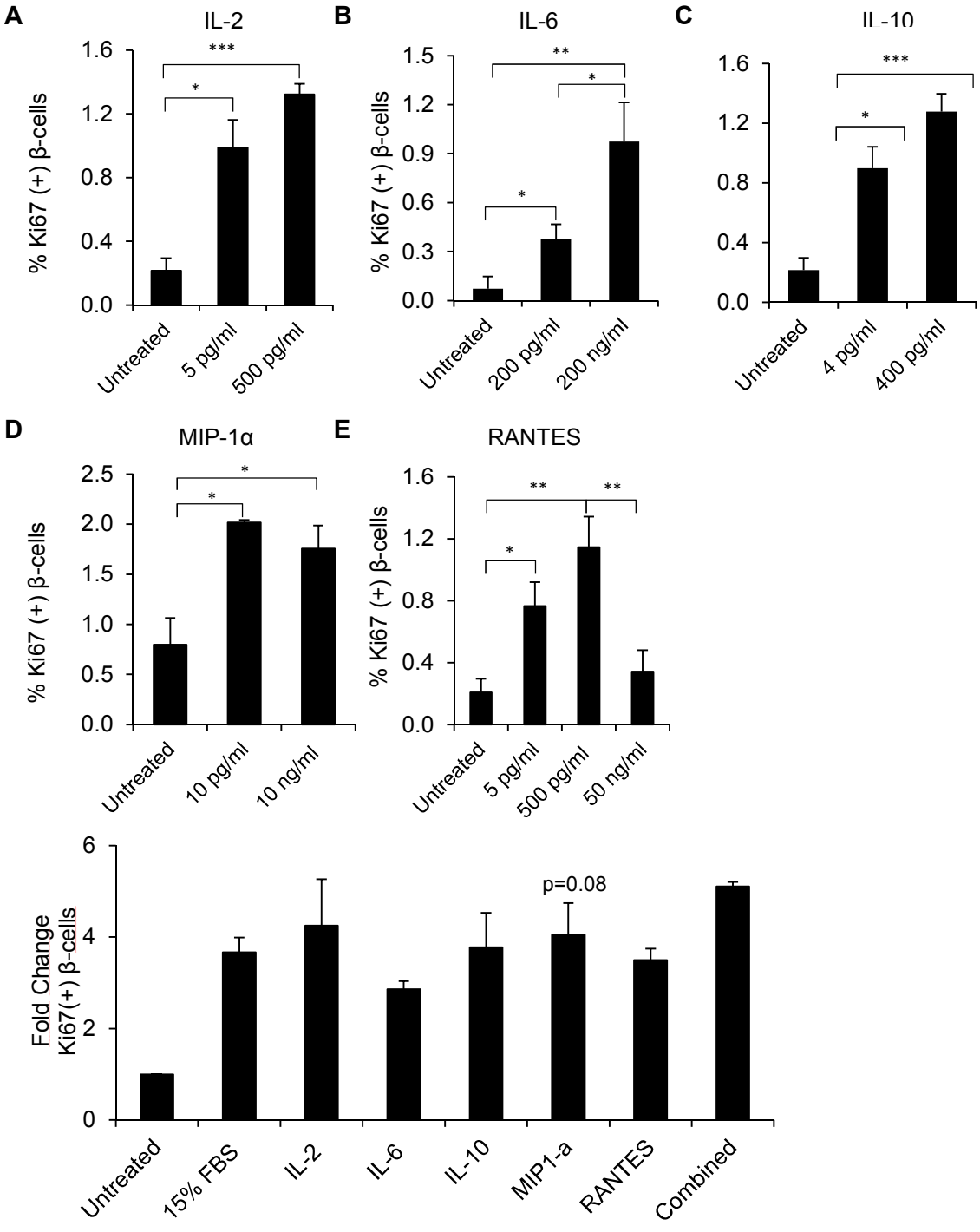
Supplemental Figure 2. T-cells, but not B-cells are involved in the β-cell proliferation process (**A**) Paraffin-embedded sections of pancreatic tissues derived from recipient mice three weeks after receiving total Pre-DM or DM mouse splenocytes, stained with proliferation marker BrdU and β-cell marker insulin or GLUT2. Original magnification x200. Arrows indicate proliferating β-cells. Inset shows magnified image of a representative BrdU(+) β-cell. (**B**) Quantification of proliferating β-cells in pancreatic sections described in **A**. (n=4-6). (**C**) Co-immunostaining (PDX1, BrdU and DAPI) of pancreatic sections harvested from NOD.RAG1$^{-/-}$ animals injected with total or B-cell depleted DM splenocytes. Arrow in merged image shows proliferating β-cell (x200). Inset is the magnified image of the same picture. Bar graph in **C** shows BrdU(+)/PDX1(+) β-cells for two groups (n=6) (**D,E**) Proliferating BrdU + cell number per 0.1 cm^2 in liver (**D**) and epididymal fat (**E**) tissues from the same animal cohorts in **a*** $P < 0.05$ and ** $P < 0.01$ (student's *t*-test).



Supplemental Figure 3. β-cell proliferation in T1D progression is independent of the effects of blood glucose and insulin and inflammatory response is specific to pancreas (**A**) Linear regression of serum insulin and BrdU(+) β-cells in pancreatic islets harvested from STZ-injected NOD mice. Each square represents an animal (n=18, 3 animals/day for 6 days). *r = 0.07*. (**B,C**) Regression analysis between blood glucose and BrdU(+) β-cells from STZ-injected NOD mice (n=18, *r =0.11*) (**B**) and total DM splenocyte injected NOD.RAG1$^{-/-}$ animals (n=6, *r =0.13*) (**C**). (**D**) Representative images of liver and adipose tissues harvested from total DM splenocyte-injected NOD.RAG1$^{-/-}$ mouse (upper panel) and STZ-injected NOD mouse (lower panel).



Supplemental Figure 4. Cytokines increase pancreatic β-cell proliferation *in vitro*. (**A-E**) Qantification of β-cell proliferation in agar-embedded pancreatic islets from C57BL/BJ mouse treated in the absence (control) or presence of low or high dose IL-2 (**A**), IL-6 (**B**), IL-10 (**C**), MIP-1α (**D**) and RANTES (**E**) for 48h (n=3-4 each condition). (**F**) Quantification of β-cell proliferation in a separate set of experiments performed on pancreatic islets treated in the absence (control) or presence of 15% FBS (positive control), low dose or a combination of low dose recombinant proteins (n=3-4 each condition). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (student's *t*-test).



Supplemental Figure 5. β-cell apoptosis levels are similar in both *in vivo* and *in vitro* splenocyte depletion studies. (**A**) Quantification of beta cell death in NOD.RAG1$^{-/-}$ mice transferred with total (Pre-DM and DM) or B-cell, CD4, CD8 and CD4/CD8 double depleted splenocytes. (**B**) Isolated NOD.RAG1$^{-/-}$ islets co-cultured with total (Pre-DM and DM), negatively or positively selected diabetic mice splenocytes at a 1-10 ratio with transwell conditions. Apoptosis was determined by counting TUNEL(+)/insulin(+) β-cells (n=3-6 each condition).