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Alginate Encapsulant Incorporating CXCL12 Supports Long-Term Allo- and Xenoislet Transplantation Without Systemic Immune Suppression

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Islet transplantation represents a potentially curative approach for individuals with Type I Diabetes. The requirement for systemic immune suppression to control immune-mediated rejection of transplanted islets and the limited human islet supply represent significant roadblocks to progress for this approach. Islet microencapsulation in alginate offers limited protection in the absence of systemic immunosuppression, but does not support long-term islet survival. The chemokine, CXCL12, can repel effector T cells while recruiting immune-suppressive regulatory T cells (Tregs) to an anatomic site while providing a prosurvival signal for beta-cells. We proposed that coating or encapsulating donor islets with CXCL12 would induce local immune-isolation and protect and support the function of an allo- or xenograft without systemic immune suppression. This study investigated the effect of alginate microcapsules incorporating CXCL12 on islet function. Islet transplantation was performed in murine models of insulin-dependent diabetes. Coating of islets with CXCL12 or microencapsulation of islets with alginate incorporating the chemokine, resulted in long-term allo- and xenoislet survival and function, as well as a selective increase in intragraft Tregs. These data support the use of CXCL12 as a coating or a component of an alginate encapsulant to induce sustained local immune-isolation for allo- or xenoislet transplantation without systemic immunosuppression.

Abbreviations: CsA, Cyclosporin A; CXCL12, C-X-C motif chemokine 12; CXCR4, C-X-C chemokine receptor type 4; Tregs, T regulatory cells

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Introduction

Islet transplantation is a potential cure for type I diabetes mellitus (T1DM). However, its utility is limited by acute and chronic immune rejection of transplanted cells (1–6). Rejection is exacerbated when porcine islets, which are posited as a substitute for the limited supply of human alloislets, are used. Immune rejection is currently managed by continuous systemic immune suppression, an approach that has not shown significant long-term effectiveness, while exposing recipients to increased risks of infection and cancer (7,8). A number of alternative strategies are in development that attempt to overcome the need for systemic immunosuppression through the induction of local anatomic site specific immune modulation or isolation (1). These include the micro- or macroencapsulation of islets in alginate-based hydrogel materials to protect them from the anti-islet immune response and/or cell based therapeutic technologies that attempt to manipulate the immune environment surrounding the islet graft (9,10), including the co-transplantation with immune suppressive regulatory T cells (Tregs) (11), Sertoli cells (12,13) or mesenchymal stem cells (14,15). We have taken a novel approach that would be applicable potentially to both allo- and xenoislet transplantation and involves the simple modification of a clinical grade alginate encapsulant to contain the immune modulating chemokine, CXCL12, that also is known to serve as a prosurvival factor for insulin producing, beta-cells.

We previously demonstrated that the CXCR4-binding chemokine, CXCL12, when overexpressed at a specific

anatomic location in both transplantation and cancer models, repels effector T cells and selectively retains immune suppressive Tregs at that site (16,17). Additionally, CXCL12 has been shown by others to play a role in limiting immune and inflammatory responses at sites of injury (18,19), foster accelerated healing through the recruitment of endothelial progenitor cells (20–22), and serve as a prosurvival signal for beta-cells (23). Here we report for the first time, that the direct coating or microencapsulation of islets with an alginate incorporating CXCL12 generates a long term (>300 days), site-specific immune protective and pro-survival environment that protects allo- or xenoislet grafts without the need for concurrent systemic immune suppression.

Materials and Methods

Animals and induction of diabetes

Six-week-old female BALB/c ($H2^d$), 6-week-old female C57BL/6 ($H2^b$) and 4-week-old female NOD/LtJ ($H2^{g7}$) mice were used in this study. All animals were purchased from Jackson Laboratory (Bar Harbor, ME). All procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Hyperglycemia was spontaneous in 18–20-week-old NOD/LtJ mice and was induced in 4-week-old NOD/LtJ and 6-week-old C57BL/6 mice by intraperitoneal (IP) injection of 200 mg/kg streptozotocin (STZ) (Sigma–Aldrich, St Louis, MO). Mice with three consecutive blood glucose readings above 250 mg/dL were considered hyperglycemic. Diabetic STZ treated C57/B6 or NOD/LtJ mice were transplanted with coated or encapsulated islets at 8–10 weeks of age and spontaneously diabetic NOD/LtJ received islet transplants at 20–22 weeks of age.

Pancreatic islet isolation and CXCL12 coating of murine islets

Primary islets were isolated from donor mice as previously described (1). Briefly, islets were isolated from female 6-week-old BALB/c donors, female 6-week-old C57BL/6 donors, or female 4-week-old NOD/LtJ donors. Pancreata were infused via the common bile duct with Liberase TL (83 μg/mL) (Roche Diagnostics, Branchburg, MJ) and digested for 20 min at 37°C. Islets were purified on a polysucrose/glucose density gradient (Mediatech, Manassas, VA) and hand-picked under a microscope. The islets were cultured in RPMI 1640 (Mediatech, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine) for at least 2 days to allow for recovery. A minimum of 450 islets to be transplanted via the kidney capsule were incubated at 37°C for 3 h prior to transplantation in DPBS (Mediatech) with or without 10 ng/mL, 100 ng/mL or 1 μg/mL murine CXCL12α (PeproTech, Inc., Rocky Hill, NJ). Four batches of purified adult porcine islets were obtained from the pancreata of Landrace pigs under standard operating procedures from the University of Minnesota. Each batch of porcine islets was used in both control and experimental transplant conditions in each run of the experimental protocol.

Production of Ca-LVM alginate capsules incorporating murine or porcine islets

On average 1000 C57BL/6 islets or porcine islets were mixed in 0.75 mL of a 1.6% alginate (ultra-pure LVM, Novamatrix) in 300 mOsmo NaCl solution with or without 1 μg/mL CXCL12. The mixture was then run through a syringe driven encapsulator (Inotech Research Encapsulator, Livermore, CA, IE-50R) using a 300 μm nozzle charged to 1.21 kV vibrating at 1500 Hz. Alginate was cross-linked in 300 mOsmo (approximately 118 mM) $CaCl_2$ for 5 min, filtered, and washed with DMEM (Mediatech) to remove excess calcium.

Islet transplantation models, glucose monitoring posttransplant, intraperitoneal glucose tolerance tests and removal of the islet graft and tissue harvest for immunohistochemical studies

CXCL12 coated or uncoated islets were transplanted under the left renal capsule of recipient mice. Islets were also encapsulated in Ca-LVM alginate or alginate containing CXCL12 were transplanted into the peritoneal cavity of recipient diabetic mice. Five different transplant models were used using coated or encapsulated islets: (1) BALB/C islets coated with CXCL12 or exposed to DPBS alone were transplanted under the renal capsule of 8–10-week-old STZ-induced diabetic C57BL/6 mice (2). Islets from prediabetic NOD/LtJ islets coated with or without CXCL12 were transplanted under the renal capsule of 20–22-week-old spontaneously diabetic NOD/LtJ mice (3). Islets from NOD/LtJ mice coated with or without CXCL12 were transplanted under the renal capsule of 8–10-week-old STZ-induced diabetic NOD/LtJ mice (4). Islets from C57BL/6 mice were encapsulated in Ca-LVM alginate with or without CXCL12 and transplanted into the peritoneal cavity of 8–10-week-old STZ-induced diabetic NOD/LtJ mice (5). Alginate encapsulated adult porcine islets were transplanted into the intraperitoneal cavity of 8–10-week-old diabetic C57/B6 mice. Recipients' tail vein blood glucose level was monitored in all groups at least twice a week and was used to interpret islet graft function. Intraperitoneal glucose tolerance tests were performed at 100 days post islet transplantation for normoglycemic xenoislet recipients in group (5). Graft rejection was defined as a return to hyperglycemia (two consecutive blood glucose measurements above 250 mg/dL). Nephrectomies were performed for retrieval of coated grafts and laparotomies were performed to retrieve microencapsuled islet grafts for immunohistochemical studies.

Statistical analysis

Survival data were analyzed by using the GraphPad Prism 5 statistic software. Islet graft survival between control and experimental groups was compared using the Logrank test. Numerical variables were compared using Student's t-test. The Holm's–Sidak's multiple comparisons test was used to compare multiple numerical variables. A p-value less than 0.05 was considered to be statistically significant.

Results

CXCL12 coating prolongs alloislet function in vivo

First, we explored whether simply coating alloislets with CXCL12 prior to transplantation could result in prolonged islet survival and function. This approach exploits the fact that the islet capsule contains fibronectin, and that CXCL12 can both stably bind to and elute from this matrix protein (24). Islets from BALB/C mice were exposed to PBS or coated with CXCL12 at a concentration of 100 ng/mL or 1 μg/mL and transplanted under the left kidney capsule of streptozotocin (STZ)-treated diabetic C57BL/6 mice. Mice were sacrificed at the point when they returned to a diabetic state with three sequential blood glucose recordings of >250 mg/dL. Allogeneic islet grafts coated with CXCL12 at 1 μg/mL, but not 100 ng/mL, resulted in the maintenance of recipient mice in a non-diabetic state for a significantly longer time than alloislets that were exposed to PBS alone (Figure 1A, $p = 0.012$; Log-rank test). Islets coated with 100 ng/mL of CXCL12 were rejected at a similar rate to PBS-exposed islets ($p = 0.31$). Mononuclear cell infiltration into the grafts was subjectively reduced in the context of CXCL12 coating at 1 μg/mL compared to PBS controls

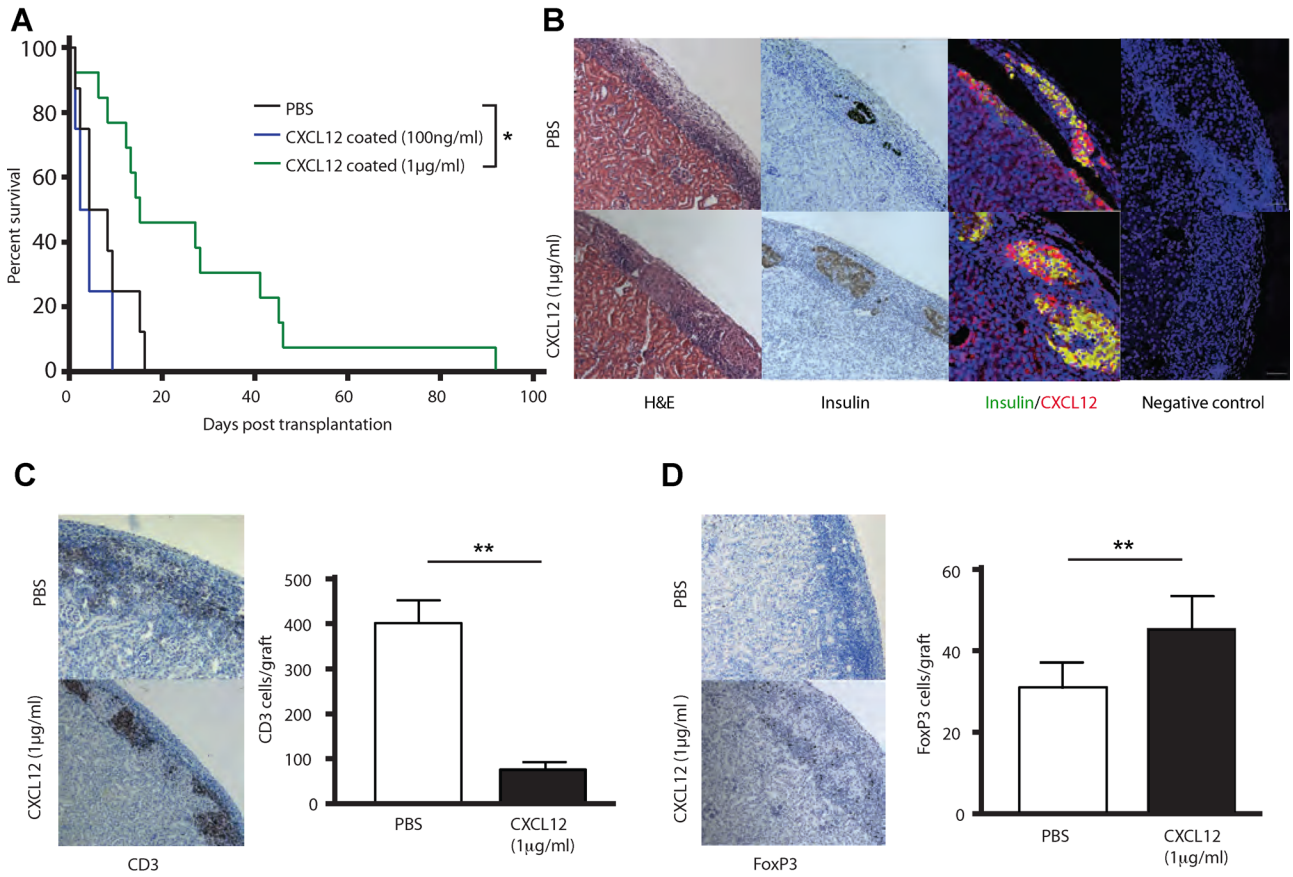

Figure 1: Coating of alloislets with a high concentration of CXCL12 delays rejection. (A) BALB/C islets were exposed to CXCL12 at concentrations of 100 ng/mL or 1 μg/mL or PBS alone and transplanted under the renal capsule of STZ-treated diabetic C57BL/6 recipients. Return to hyperglycemia was considered to be an indicator of graft rejection, while sustained normoglycemia was considered to be an indicator of allograft survival. Coating of islets with 1 μg/mL, but not 100 ng/mL, of CXCL12 significantly delayed graft rejection compared to PBS controls (*$p = 0.012$, Logrank test) (12 animals per group). (B) Representative hematoxylin and eosin (H&E) staining of subcapsular islet graft sites showed reduced mononuclear cell infiltration in 1 μg/mL CXCL12-coated islets compared to uncoated controls (left panels). Insulin staining (brown) of 1 μg/mL CXCL12 and PBS coated islet graft areas showed a greater number of functional islets and level of insulin secretion in CXCL12 coated islets compared to PBS controls (middle panels) at day 20 posttransplantation. Additionally, fluorescent staining showed evidence of CXCL12 staining (red) in CXCL12-coated islets compared to controls (right panels). Insulin staining appears green and co-staining of CXCL12 and insulin in yellow. (C) CD3 immunostaining of islet grafts demonstrated significantly decreased infiltration of CD3+ cells into the graft area in 1 μg/mL CXCL12-coated grafts ($p = 0.001$) compared to uncoated controls (6 animals per group). (D) FoxP3 staining of CXCL12 and PBS-coated islet grafts showed significantly greater FoxP3+ cell localization to 1 μg/mL CXCL12-coated grafts than to PBS controls ($p = 0.0016$) (**$p < 0.005$, Student's t-test) ($n = 6$).

(Figure 1B). Insulin expression and CXCL12 were also clearly evident in CXCL12-coated alloislets compared to PBS-treated allografts using immunohistochemistry (IHC) (Figure 1B). Interestingly, low levels of intrinsic CXCL12 expression were seen in non-coated islets. CXCL12 is known to be upregulated in the context of inflammation (25) and can act as an anti-inflammatory agent (26). CD3 and FoxP3 T cell infiltration into grafts were quantified. CD3+ T cell infiltration into islet grafts was significantly reduced in CXCL12 compared to PBS-exposed alloislets as determined by IHC, (Figure 1C, $p = 0.001$). CXCL12 coated grafts were observed to be surrounded by a cuff of CD3+ T cells which are under the renal capsule but do not infiltrate the graft. This is consistent with our prior study of CXCL12 as a T cell chemorepellent in which alloislets were engineered to express high levels of CXCL12 that resulted in T cell infiltration up to but not into the intact and functioning islet graft (16). Surprisingly, CXCL12 coating of islet allografts was associated with a significant increase in FoxP3+ T cell infiltration within and around the CXCL12-coated graft compared to PBS-exposed alloislets (Figure 1D, $p = 0.0016$) at day 20 posttransplantation.

CXCL12 coating of syngeneic islets does not delay rejection in NOD/LtJ mice

We investigated whether CXCL12 coating could play a role in reducing or preventing rejection in the context of

syngeneic islet transplantation into diabetic NOD/LtJ mice. In this model, syngeneic islets from prediabetic NOD/LtJ mice were transplanted into STZ-treated diabetic NOD/LtJ mice. CXCL12 coating of syngeneic islets did not lead to a prolonged period of normoglycemia compared to PBS exposed islets (Supplementary Figure S3A, $p = 0.299$). However, histopathological and immunohistochemical studies demonstrated heavy mononuclear cell infiltration into PBS-treated islets but not CXCL12-coated islets (Supplementary Figure S3B and C). Staining for CXCL12 and insulin in this context demonstrated insulin-producing and CXCL12-positive islets in comparison to PBS-exposed islets (Supplementary Figure S3B). CXCL12 coating of syngeneic islets also reduced CD3+ T cell infiltration into donor islets in this context compared to PBS-exposed syngeneic NOD/LtJ islets (Supplementary Figure S3C, $p = 0.0081$). CXCL12 coating of syngeneic NOD/LtJ islets also led to a significantly increased number of FoxP3+ cells in the islet graft site compared to PBS-treated controls (Supplementary Figure S3D, $p = 0.0019$). Similarly, CXCL12 coating of syngeneic islets did not reduce the rate of recurrence of diabetes when transplanted into spontaneously diabetic NOD/LtJ mice (Supplementary Figure S4A, $p = 0.24$). Despite this finding, hematoxylin and eosin and insulin/CXCL12 staining demonstrated reduced mononuclear cell infiltration and increased insulin expression in CXCL12-coated islets compared to controls (Supplementary Figure S4B). In addition, there was a consistent reduction in CD3+ T cell infiltration and increased FoxP3+ cell infiltration into the CXCL12 coated graft compared to PBS controls (Supplementary Figure S4C and D). The difference in protection that CXCL12 coating offers between the syngeneic NOD islet transplantation into STZ-treated or spontaneously diabetic NOD mice may be attributable to different humoral and cell-mediated immune mechanisms of islet immune attack that operate in these two models. The lack of a pro-survival effect of CXCL12 coating of syngeneic islets in spontaneously diabetic transplant setting may be attributable to a number of reasons including the fact that established humoral anti-islet immune responses were not blocked by CXCL12 alone and/or that the chemokine could not reverse the regulatory defect present in spontaneously diabetic NOD mice.

Alginate encapsulant incorporating CXCL12 supports the long-term function of alloislets

Early expression of insulin autoantibodies correlates with progression to diabetes and probably primarily reflects insulitis (29–31), and in view of the finding that the efficacy of CXCL12 coating of islets for transplantation was likely limited as an intervention in the setting of preformed anti-islet antibodies, we proposed that the incorporation of CXCL12 into alginate microcapsules might potentially protect transplanted islets by providing both a physical and a biological barrier to cell-mediated and humoral anti-islet immunity.

In view of the fact that CXCL12 has also been shown to be a pro-survival factor for islets, we examined the effect of the incorporation of CXCL12 into a Ca-LVM encapsulant on islet viability. We showed that incorporation of CXCL12 into the encapsulant significantly decreased the level of caspase-3 activity in encapsulated islets determined after 48 h of *in vitro* culture as compared to unmodified Ca-LVM alginate (100 ng/mL CXCL12, $p = 0.0019$) (1 μg/mL CXCL12, $p = 0.00028$) (Figure 2C). This *in vitro* study confirmed the previously demonstrated direct pro-survival effect of CXCL12 on beta cells which is specifically mediated via Akt signaling (23,34).

We then explored the use of the Ca-LVM-CXCL12 in a murine model of alloislet transplantation into spontaneously diabetic NOD/LtJ mice without systemic immune suppression. We found that incorporation of CXCL12 into Ca-LVM alginate significantly prolonged alloislet function and survival in the context of alloislet transplantation into the peritoneal cavity of diabetic NOD/LtJ mice in comparison to the unmodified Ca-LVM alginate (mean days in non-diabetic state posttransplantation, Ca-LVM-CXCL12 = 136; Ca-LVM = 62) (Figure 2D, $p = 0.0132$). Phase contrast and histopathological studies of retrieved Ca-LVM-CXCL12 capsules at 6 weeks posttransplant consistently revealed intact islet morphology in comparison to unmodified Ca-LVM capsules in which islets were necrotic or degenerative (Figure 2E). Alloislets from C57BL/6 mice were then transplanted into spontaneously diabetic NOD/LtJ mice that had previously received and rejected a skin transplant from C57/B6 mice. Alloislets encapsulated with Ca-LVM-CXCL12 survived and maintained a normoglycemic state in recipients significantly longer than islets encapsulated with Ca-LVM alone (Figure 2F). This demonstrated that CXCL12 incorporation into encapsulant protected islets from an immune memory response in recipient mice. Finally, alginate encapsulated islets with incorporated CXCL12 were carefully removed from the intraperitoneal cavity of non-diabetic recipient mice at 100 days posttransplantation. All non-diabetic mice that underwent this procedure reverted to a diabetic state with blood glucose measurements greater than 250 mg/dL (see Figure 2G). This demonstrated that the non-diabetic state was not induced by regeneration of native pancreatic islet tissue.

Alginate encapsulant incorporating CXCL12 supports the long-term function of xenoislets

We explored whether xenoislets could be protected by an alginate encapsulant that incorporated CXCL12. Porcine xenoislets were encapsulated in Ca-LVM or Ca-LVM with CXCL12 at a concentration of 10 ng/mL, 100 ng/mL or 1 μg/mL, and transplanted into the peritoneal cavity of diabetic C57BL/6 mice. Porcine islets encapsulated with Ca-LVM containing CXCL12 at a concentration of 1 μg/mL sustained normoglycemia in recipient mice for a significantly longer time period than either Ca-LVM ($p = 0.0003$) (Log Rank test), Ca-LVM-CXCL12 (10 ng/mL) or Ca-LVM-CXCL12 (100 ng/mL) encapsulated islets (Figure 2H). Swine C

peptide was detectable in the serum of non-diabetic recipients receiving Ca-LVM-CXCL12 encapsulated xenoislets at 100 days posttransplant at levels between 48 and 110 pM (mean = 59 pM) as determined by commercially available ELISA. Swine C-peptide was not detectable in diabetic mice that had received and rejected Ca-LVM encapsulated porcine islets or Ca-LVM islets containing low concentrations of CXCL12 (data not shown). Microencapsulated islets with incorporated CXCL12 were removed from the intraperitoneal cavity of transplanted non-diabetic mice at 100 days posttransplantation. All non-diabetic mice that had received islet transplanted reverted immediately and sustainedly to a diabetic state with blood glucose measurements greater than 250 mg/dL (data not shown) (mean glucose postexplant = 489 ± 61 mg/dL; n = 6). This demonstrated that the non-diabetic state was not induced by regeneration of native pancreatic islet tissue.

In view of the finding that early encapsulated islet loss of function during the first 2 weeks posttransplantation was reduced in the context of Ca-LVM encapsulant containing CXCL12 we examined the impact of the chemokine on inflammatory cellular infiltration around the microcapsules. We found that the inclusion of CXCL12 in Ca-LVM encapsulant resulted in a significant reduction in both macrophage ($p = 0.0005$) and fibroblast ($p = 0.00003$) infiltration of the surface of microcapsules at 2 weeks posttransplantation (Supplementary Figure S6). This is consistent with the finding that CXCL12 has anti-inflammatory activities including the recruitment of immune suppressive macrophages to and the reduction of fibrosis at sites of inflammation (26).

Treg and CD8+ T cell CXCR4 expression and migratory responses to CXCL12

We previously demonstrated that expression of CXCL12 by engineered islets or tumor cells resulted in the selective repulsion of effector T cells and the retention of Treg cells at the specific anatomic site (16,17). Therefore, we proposed that the mechanism by which CXCL12 coating or incorporation in the encapsulant sustains immune isolation of the xeno or alloislet graft is through the selective repulsion of CD8+ T cells and attraction of CD4+ Treg cells to the graft site. We proposed that this might be due to differential migratory responses of these two T cell subpopulations to the chemokine which itself might be associated with different levels of expression of CXCR4. To investigate this, the expression of CXCR4 on and migration of T cell subpopulations from NOD/LtJ mice were studied by flow cytometry and transmigration assays, respectively. T cells derived from NOD/LtJ mice in response to medium alone, recombinant CXCL12, CXCL12-coated or Ca-LVM-CXL12 encapsulated islets were studied in Boyden chamber based assays. Upper chambers were loaded with purified CD3+CD8+ (Figure 3A and C) or CD4+CD25hi+ Treg cells (Figure 3B and D) for each condition. Upper and lower wells were loaded with media, CXCL12 (1 μg/mL) or islets coated with CXCL12 (1 μg/mL) or encapsulated with Ca-LVM-CXCL12 (1 μg/mL). Both CD3+CD8+ T cells and CD4+CD25Hi Tregs underwent chemotaxis in response to CXCL12, CXCL12-coated or Ca-LVM-CXCL12-encapsulated islets. However, a significantly larger fugetactic response was observed when CD8+ cells were incubated with CXCL12, CXCL12-coated islets or Ca-LVM-CXCL12-encapsulated islets than Treg cells. In fact none of the conditions studied resulted in detectable levels of Treg cell fugetaxis. We then explored whether the observed difference in migratory responses to CXCL12 by these two T cell subpopulations was associated with differential expression of the chemokine's cognate receptor, CXCR4. CD8+ T cells from the spleen of NOD/LtJ mice expressed significantly lower levels of CXCR4 than CD4+CD25HiFoxP3+ Treg cells ($p < 0.001$) (Figure 3E and F). These data support our hypothesis that CXCL12 coating or CXCL12 incorporation in an encapsulant surrounding transplanted alloislets results in preferential recruitment of Tregs within the graft while repelling CD8+ T cells in this transplant model.

Discussion

We demonstrate that coating islets with CXCL12 delays islet rejection in the allotransplant setting but not in the context of syngeneic islet transplantation in STZ treated and spontaneously diabetic NOD/LtJ mice. These data support the view that while CXCL12 coating may lead to local and short acting immune isolation of the graft, including protection from acute and elements of chronic cell-mediated rejection, it cannot offer protection from humoral anti-islet antibodies. These data are consistent with previously published work that demonstrated that constitutive expression of CXCL12 by transgenic islets prolongs survival and induces a state of local immune isolation of alloislet grafts in the non-sensitized host (16). It should be noted that the efficacy of the CXCL12 containing alginate encapsulant may be limited in its ability to protect against an immune response against a pancreas related antigen (such as insulin) in the spontaneously T1D induced NOD mice despite its efficacy in the alloislet and xenoislet transplant models described above. In contrast, a Ca-LVM based alginate encapsulant incorporating CXCL12 induced long-term protection of alloislets in diabetic mice with preexistent humoral and cell-mediated anti-islet responses. Furthermore, an alginate-based microencapsulant incorporating CXCL12 was also capable of inducing long-term islet immune protection of porcine xenoislets. This is a striking finding that suggests a role of local expression of high levels of CXCL12 in the prevention of both early acute and long-term chronic rejection of a xenograft. This is supported by studies of the pleiotropic activities of CXCL12 both as a locally acting anti-inflammatory and immune suppressant protein potentially augmenting the activity of M2 macrophages as well as recruiting, polarizing and retaining Tregs to a specific anatomic site while repelling effector T cells from that

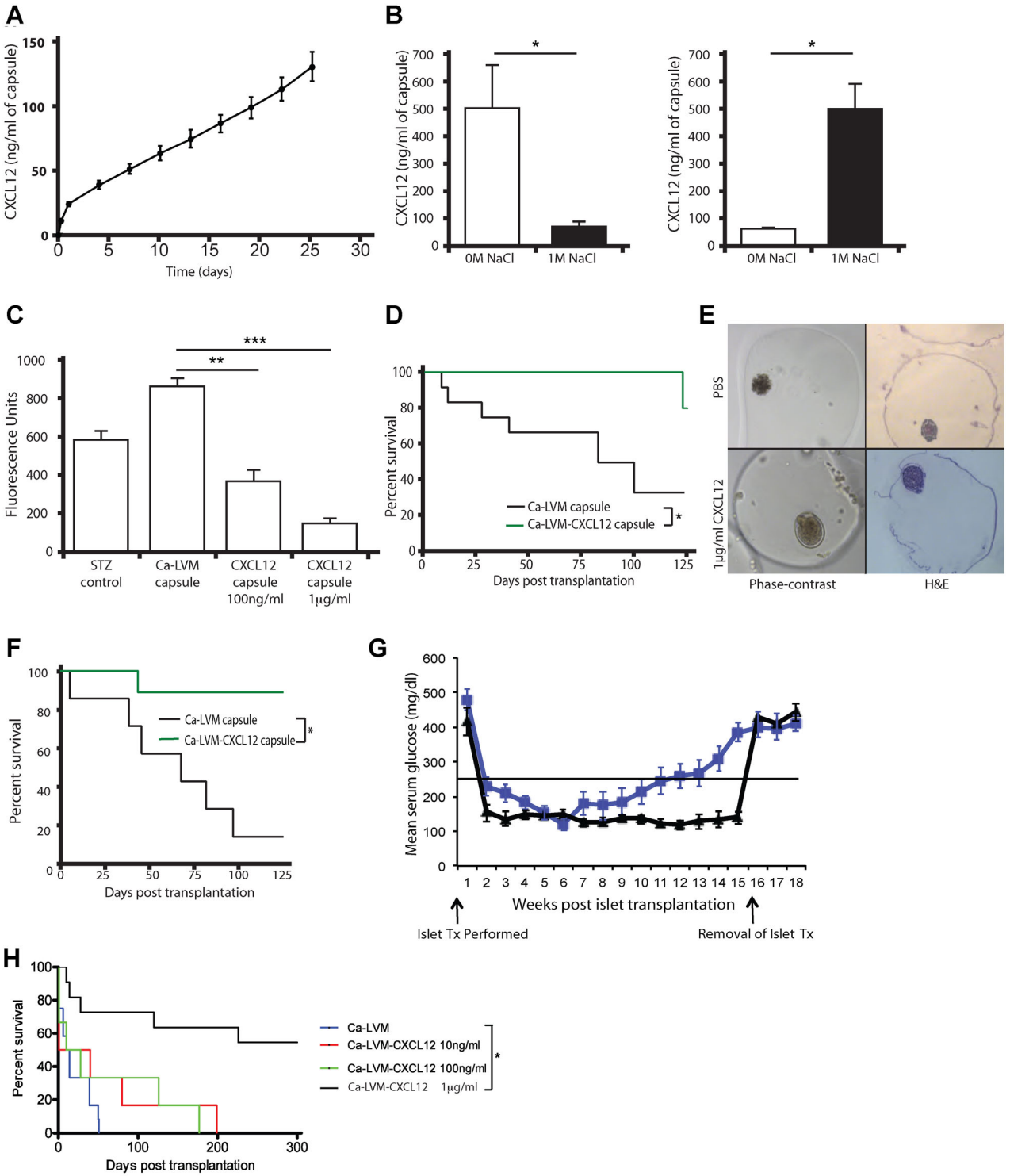

Figure 2: Continued.

location (26,27). Furthermore, mice receiving encapsulated islets with the highest concentration of CXCL12 (1 μg/mL), generated normal glucose tolerance test responses at 100 days posttransplant, following intravenous glucose challenge in comparison to lower concentrations of the chemokine (10 or 100 ng/mL) even though these mice had normal range random glucose recordings at this time. This supported the view that the alginate containing 1 μg/mL CXCL12 supported both islet survival and glucose responsiveness.

CXCL12 is a chemokine, which has been shown to have a multitude of effects on a variety of different tissues and cell types (32,33,35). These effects are elicited via its interaction with its receptor, CXCR4, on the cell surface. CXCL12 has been shown to direct T cell development and T cell migration (36–38). In addition, CXCL12 has been shown to be a pro-survival signal for islet cells (34) and an anti-inflammatory agent (26). Our data support the view that these activities of CXCL12 may be relevant to the protection and support of insulin producing cells in the context of islet transplantation. However, additional studies of other trophic and engraftment effects mediated by CXCL12 and related to inflammation but not to specific immunity are clearly warranted. It is of interest that the concurrent use of CXCL12 coating and low dose of CsA did not prolong islet function in the context of allotransplantation. The fact that CsA is known to negatively impact CXCL12/CXCR4 (28) signaling and in our model abrogates immune protective effects of the chemokine highlights the importance of understanding signaling pathways underlying immune suppression when combinatorial treatment approaches are used.

We consistently demonstrate in our transplant models that coating with CXCL12 results in accumulation of Tregs at the graft site. Natural Treg cells are a subset of CD4+ T cells originated from thymus which play a significant role in maintenance of tolerance. Tregs also play an active role in immune modulation, and suppress alloimmune responses of transplant rejection (39–42). Furthermore, Tregs can prevent murine autoimmune diabetes and control autoreactive destruction of transplanted islets (43,44). Many groups have shown that the generation of Tregs *in vivo* prolongs the survival of islet allografts (45–48). Effector T cells can be converted into graft-protective regulatory T cells and induce islet allograft tolerance (49). In addition, coating pancreatic islets with regulatory T cells has been shown to induce local immunoprotection and islets SA-FasL engineered pancreatic islets can establish localized tolerance of islet graft by inducing regulatory T cells (50). Our data adds to this literature by demonstrating that coating of an allogeneic islet graft with CXCL12 selectively recruits Treg cells to the islet graft and thereby prolong graft survival. Interestingly, murine and human tumors such as melanoma and ovarian cancer which constitutively express CXCL12 are also heavily infiltrated with immune suppressive Treg cells (17). We demonstrated in human cervical cancer, that levels of infiltrating FoxP3+ Treg cells were positively correlated with CXCL12 expression levels (51). Treg infiltration can be abrogated in the context of murine ovarian cancer by treatment with the highly selective CXCR4 antagonist, AMD3100. In tumor models, the retention of Tregs but repulsion of anti-tumor Teffector cells were found to be related to the differential expression of CXCR4 on the two T cell subpopulations and their migratory behaviors to the chemokine (17). The finding that

Figure 2: (*Overlraf*) Incorporation of CXCL12 into Ca-LVM alginate capsules delays rejection of allogeneic and xenogeneic islets transplanted into the peritoneal cavity of recipient diabetic C57/B6 mice. (A) Kinetics of CXCL12 release from cell-free calcium cross-linked 3.3% alginate encapsulant over time *in vitro*. The concentration of CXCL12 in un-cross-linked sodium alginate was 1 μg/mL; a significant amount of CXCL12 was lost in the $CaCl_2$ cross-linking solution ($n = 3$). There were no differences in CXCL12 release profiles for alginate concentrations of 1.5% to 3.3% (data not shown). Initial release rate of CXCL12 from 1.5% alginate capsules during the first 24 h was 1.75 ± 0.01 ng/mL/h and after 4 days stabilized at a release rate of 0.18 ± 0.002 ng/mL/h. (B) Electrostatic interaction between CXCL12 and barium cross linked alginate capsule. A significantly lower amount of CXCL12 remained in capsules following incubation with 1 M NaCl for 6 h compared to incubation in the absence of NaCl for the same time period (left panel). A significantly higher amount of CXCL12 was eluted in the medium following incubation with 1 M NaCl for 6 h (right panel) compared to incubation in NaCl-free medium ($^*p < 0.05$, $n = 3$). (C) CXCL12 incorporation significantly reduces caspase-3 activity in encapsulated murine islets ($^{**}p = 0.0019$ for 100 ng/mL CXCL12 and $^{***}p = 0.00028$ for 1 μg/mL CXCL12 vs. control). Murine islets were encapsulated with Ca-LVM or Ca-LVM incorporating either 100 ng/mL or 1 μg/mL CXCL12 (Ca-LVM-CXCL12) and then cultured *in vitro* for 48 h, after which caspase-3 activity was determined. (D) Incorporation of 1 μg/mL CXCL12 into a Ca-LVM encapsulant delays rejection of allogeneic islets ($^*p = 0.0132$, Logrank test, $n = 12$ for both groups). (E) CXCL12 incorporation improves islet health and decreases necrosis 6 weeks after transplant. Islets encapsulated with Ca-LVM-CXCL12 appeared viable and intact using phase contrast microscopy and H&E staining at 6 weeks posttransplantation in comparison to necrotic islets encapsulated in unmodified Ca-LVM. (F) Incorporation of 1 μg/mL CXCL12 also delays rejection of encapsulated allogeneic islets transplanted into allo-sensitized NOD/LtJ mice ($p = 0.0054$, Logrank test; Ca-LVM Capsule, $n = 7$; Ca-LVM-CXCL12 Capsule, $n = 9$). (G) Blood glucose levels were monitored in allosensitized NOD/LtJ mice receiving islet transplants as described in (F) and following encapsulated graft removal at 100 days postislet transplantation. Weekly mean blood glucose values are shown ± standard error of the mean. All mice ($n = 4$) that received allogeneic islets transplants encapsulated with Ca-LVM-CXCL12 returned to the hyperglycemic state immediately after removal of the islet graft. All mice receiving allogeneic islets encapsulated in Cal-LVM were hyperglycemic at 100 days postislet transplant ($n = 7$). (H) Incorporation of 1 μg/mL CXCL12 significantly delays rejection of encapsulated porcine xenogeneic islets transplanted into diabetic C57BL/6 mice ($^*p = 0.0003$, Logrank test). Control and experimental groups: Ca-LVM capsule ($n = 11$), Ca-LVM-10 ng/mL CXCL12 capsule ($n = 6$), Ca-LVM-100 ng/mL CXCL12 capsule ($n = 6$), Ca-LVM-1 μg/mL CXCL12 capsule ($n = 11$) ($^*p < 0.01$, Logrank test).

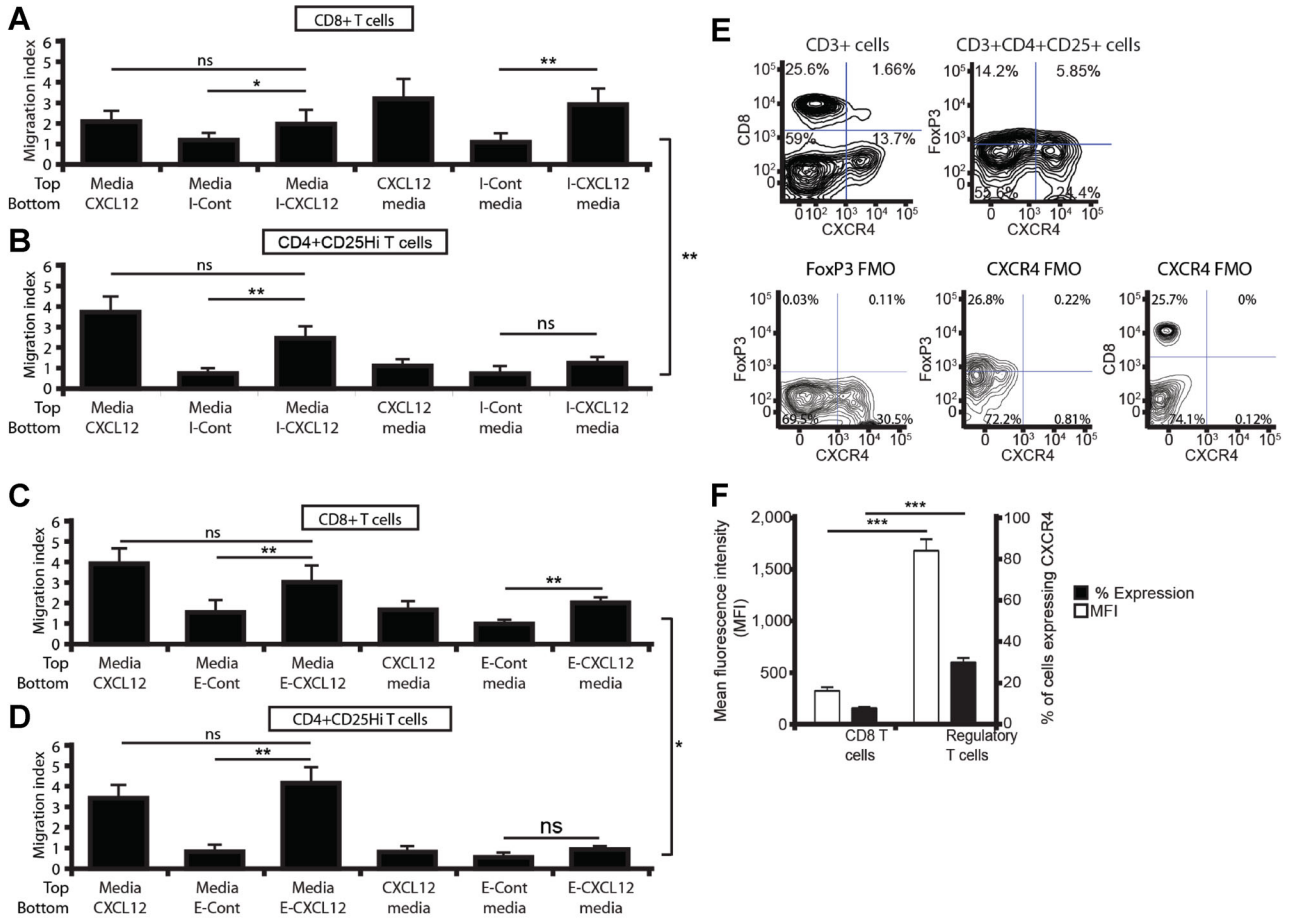

Figure 3: Migratory behaviors of T cell subpopulations to CXCL12 and associated CXCR4 expression. Migratory responses of CD3+CD8+ (A and C) and CD3+CD4+CD25hi (B and D) T cells were quantitated in response to CXCL12, islets coated with CXCL12 (I-CXCL12) and islets encapsulated with CXCL12 (E-CXCL12). CXCL12 was used at a concentration of 1 μg/mL in all three settings. CD8+ and CD4+CD25Hi T cells underwent low levels of chemotaxis to 1 μg/mL CXCL12 (M/CXCL12) and islets coated or encapsulated with CXCL12. CD8+, but not CD4+CD25Hi, T cells underwent fugetaxis or chemorepulsion in response to CXCL12 coated or encapsulated islets. Minimal levels of both chemotaxis and fugetaxis were seen for CD8+ or CD4+CD25Hi T cells exposed to islets that were not coated with CXCL12 (I-Cont) or encapsulated islets alone (E-Cont). $^{**}p < 0.005$, ns = non-significant, Holm–Sidak's multiple comparisons test) (n = 9). To explain these different migratory responses, CXCR4 expression on CD8+, and regulatory T cells was compared. (E and F) (E, upper panels: representative contour plots for CD3+CD8+CXCR4+ and CD3+CD4+CD25+FoxP3+CXCR4+ subpopulations in the spleen. E, lower panels: gating strategy for FoxP3 and CXCR4 staining using FMOs). Significantly higher expression of CXCR4 by Tregs was observed compared to CD8+ T cells ($^{***}p < 0.001$, Student's t-test) (F).

Tregs accumulate within and around a CXCL12 coated islet allo or syngeneic graft is consistent with the above findings and previous demonstration that the expression of this chemokine is associated with the retention of Tregs in the bone marrow (52). In both tumor and transplant settings the retention/accumulation of Tregs would be expected to be associated with the establishment of an immune suppressive microenvironment.

We demonstrate that coating or encapsulation of allo or xenoislets with CXCL12 can lead to delayed islet rejection and concomitant prolonged islet function via the induction of site specific immune isolation and the provision of a prosurvival signal to the beta cells. We believe that CXCL12 mediates this effect by its direct interaction with CXCR4 on Tregs and beta islet cells, respectively. The demonstration that CXCL12 incorporation into clinical grade alginate encapsulant surrounding transplanted allo- and xenoislets allows sustained islet function and protection from immune destruction in the sensitized host without systemic immune suppression is a striking and clinically translatable finding. This suggests that CXCL12, present at the time of islet transplantation, is capable of overcoming both acute and chronic immune destruction of the graft in a site

specific manner and abrogates the need for concurrent systemic immune suppression. This approach may also broadly support and provide site specific immune protection for retrievable devices that deliver alginate micro and macro-encapsulated islets or insulin producing cells.

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Authors' Contributions

T.C. and M.H. performed most of the experimental studies; J.Y., B.K., M.S. and T.C. performed flow cytometric analysis; B.K., M.L.H., H.L and J.C. carried out ELISAs; S.K., B.K., M.M., G.M. and H.L. performed IHC studies; T.C., D.S., J.M., J.L. and M.C.P. designed the preclinical studies; M.T. contributed to design and execution of the islet apoptosis studies. T.C., M.H. and M.C.P analyzed the data and wrote the manuscript with the assistance of J.Y., J.M., D.S., C.H. and A.S. S.D., A.S. and M.C.P. contributed to the development and characterization of Ca-LVM-CXCL12 encapsulant and edited the manuscript.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Survival curve of the proportion of C57/B6 mice remaining non-diabetic after transplantation of CXCL12-coated or uncoated islets with or without CsA treatment.

Figure S2: CXCL12 coating does not affect the number of BL/6-specific CD4 T cells.

Figure S3: CXCL12 coating or PBS exposure of NOD/LtJ mouse syngeneic islets transplanted under the renal capsule of STZ treated diabetic NOD/LtJ mice.

Figure S4: CXCL12 coating or PBS exposure of NOD/LtJ mouse syngeneic islets transplanted under the renal capsule of spontaneously diabetic NOD/LtJ mice.

Figure S5: Intraperitoneal glucose tolerance tests on mice receiving encapsulated xenoislets containing CXCL12.

Figure S6: Immunostaining and quantitation of macrophages and fibroblasts infiltrating the surface of capsules with and without CXCL12 and containing xenoislets at 100 days posttransplantation.