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Promoting Autoimmune Diabetes in Non-Human Primates

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Even if an enormous number of therapeutic approaches to cure diabetes have been successfully tested in the NOD mouse -- the genetically diabetes-prone non-obese diabetic mouse strain, whose etio-pathogenesis is widely-held to parallel the one that occurs in humans -- the majority of them simply didn't work in humans. The gap between mice and humans seems to be too large to justify the translation of therapies efficacious in mice directly to human individuals. But, even if the non-human primates (NHP) seem to be the best animal model available for testing new therapeutic protocols of this kind, on the basis of the phylo-genetic similarities between monkeys and humans, we have to consider that only chemically-induced (e.g., via Streptozotocin injections) diabetic recipients can be used to this aim since NHP do not spontaneously develop an autoimmune diabetes. The absence of an autoimmune diabetic monkey model is a major hurdle to properly validate diabetes-specific therapies for a realistic translation to human patients. Given these considerations we thought it necessary to find the means to promote autoimmune diabetes in NHP.					
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**W81XWH-11-1-0417**  
**Final Report (07/12/2011 – 01/11/2014)**  
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## INTRODUCTION/ORIGINAL STATEMENT OF WORK

### ORIGINAL STATEMENT OF WORK

Diabetes mellitus describes a syndrome underlined by the absence or insufficiency of appropriate gluco-regulation. The syndrome can be broadly grouped into two subtypes: an autoimmune-mediated impairment and chronic destruction of insulin-producing beta cells (type 1 diabetes, T1D) and a heterogeneous set of syndromes which share insulin production insufficiency with or without peripheral insulin resistance (type 2 diabetes, T2D). For all patients that already exhibit T1D or T2D, insulin replacement offers at the moment imperfect, but acceptable means of gluco-regulation and lifestyle. However, insulin replacement is inadequate to control the homeostatic dysregulation that results in the complications of diabetes; mainly cardiovascular diseases, nephropathy, retinopathy and neuropathy. Currently, the most actively pursued therapeutic approaches able to substitute insulin treatments are islet allo-transplantation, stem cell-based islet replacement, beta cell regeneration attempts, and viral vector-based gene therapies. Islet allo-transplantation was for decades sought as a possible alternative to recombinant insulin administrations and, in year 2000, indeed its clinical utility has been formally demonstrated.

Islet allo-transplantation has achieved excellent short-term results in normalizing glucose homeostasis in T1D patients in which insulin therapy was unable to achieve tight gluco-regulation. However, the shortage of islet donors, poor long-term functional outcomes (in part due to strong immunosuppression with drugs toxic for the cells in the islets), and the risk of allo-sensitization (associated with the need of multiple different donors to reach a minimally sufficient islet cell mass in each recipient) jeopardizing the future possibilities of a necessary kidney transplantation, have led to active discussions about future directions of this field. The unlimited availability of pigs as a source of islets could be used to solve the first obstacle (i.e., the shortage of human islet donors) and eventually make islet transplantation available to all the patients in need for it. Porcine insulin has been successfully used for decades to treat diabetic patients. Furthermore, the availability of genetically engineered (GE) pigs that are resilient to hyper-acute rejection and early vascular rejection, should solve the major impediment to xenogeneic islet transplantation. Also, successful xeno-transplantation of porcine insulin producing cells into diabetic patients could restore physiologic islet function, without the risk of allosensitization.

Although significant advances have been made in both stem cell-based islet replacement or gene therapy, and islet allo- or xeno-transplantation are only examples of possible alternative approaches to insulin administrations, all these possible remedies need to be tested in animal models before being proposed for clinical trials. Even if an enormous number of therapeutic approaches to cure diabetes have been successfully tested in the NOD mouse -- the genetically diabetes-prone nonobese diabetic mouse strain, whose etio-pathogenesis is widely-held to parallel the one that occurs in humans -- the majority of them simply didn't work in humans. The gap between mice and humans seems to be too large to justify the translation of therapies efficacious in mice directly to human individuals. But, even if the non-human primates (NHP) seem to be the best animal model available for testing new therapeutic protocols of this kind on the basis of the phylo-genetic similarities between monkeys and humans, we have to consider that only chemically-induced (e.g., via Streptozotocin injections) diabetic recipients can be used to this aim since NHP do not spontaneously develop an autoimmune diabetes. Given these considerations we thought it necessary to find the means **to promote autoimmune diabetes in NHPs**.

Towards this objective, we are motivated by the genetic evidence proving that the level of thymic, diabetes-relevant, auto-antigens determines the generation of T cells that target the insulin-producing beta cells, the hallmark immunological feature of T1D. Insulin expression in the thymus has been implicated in regulating the negative selection of auto-reactive T cells and in mediating the central immune tolerance towards pancreatic beta cells. To gain the functional insights of this ectopic insulin expression, we took advantage of the Cre-lox system to knockout the mouse *Ins2* gene specifically in *Aire*-expressing medullary thymic epithelial cells (mTECs), without affecting its production in the pancreatic beta cells. These animals (designated as ID-TEC mice for insulin-deleted mTEC), that were previously also crossed to *Ins1* knockout background, spontaneously developed diabetes around three

weeks after birth. Beta cell specific autoimmune destruction was observed and documented by the presence in the islets of effector T-cells, directed specifically against insulin epitopes. More pertinent to this application were, however, the results from ID-TEC thymus transplantation experiments that proved how the Ins2-depleted thymus is sufficient, in a nude (thymusdeprived) mouse, to a) successfully reconstitute the T cell repertoire, b) break central tolerance and c) induce anti-insulin autoimmunity (see Background & Preliminary Studies). On this basis we now intend to engineer autoimmune diabetes in monkeys by reaching three specific objectives:

**Objective 1: A) To safely harvest the thymus from Cynomolgus monkeys (i.e., *Macaca Fascicularis*), which, once exposed *in vitro* to an insulin-specific short-hairpin RNA (shRNA), will eventually be transplanted back into the donor monkey. B) To select the shRNA most effective in specifically blocking the expression of insulin in the thymic medullary cells of the monkeys.**

**Objective 2: To prove that the shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of an autoimmune reaction, specifically directed against the beta cells of the pancreas.**

**Objective 3: To determine whether the insulin-specific autoimmune reaction can be promoted by directly injecting a lentivirus, carrying the specific anti-insulin shRNA, into the thymus of the immunocompetent-cell-depleted monkey.**

Although the virus certainly cannot transfect all the cells of the thymic medulla, either *in vitro* or *in vivo*, a drastic reduction in their self-antigen expression/presentation should be sufficient to favor the development of a specific autoimmune reaction, as convincingly demonstrated by Chentoufi & Polychronakos in 2002. In their study they established a mouse model in which there is graded thymic insulin deficiency in linear correlation with insulin gene copy numbers, while pancreatic insulin remained unaltered. Mice expressing low thymic insulin levels exhibited detectable peripheral reactivity to insulin, whereas mice with normal levels showed no significant increases in anti-beta cell reactivity compared to non-autoimmune controls.

**Our first quarterly scientific progress report for the initial year of our project (07/12/2011 – 10/11/2011) described the following:**

In the first quarterly we focused our efforts on optimizing the surgical technique to safely harvest the thymus from a monkey, as originally proposed in Objective 1:

**A) To safely harvest the thymus from Cynomolgus monkeys (i.e., *Macaca Fascicularis*), which, once exposed *in vitro* to an insulin-specific short-hairpin RNA (shRNA), will eventually be transplanted back into the donor monkey.**

**B) To select the shRNA most effective in specifically blocking the expression of insulin in the thymic medullary cells of the monkeys.**

To fulfill the A part of this aim, we selected the perhaps least invasive approach of thymectomy, the so-called **infrasternal approach**. This has been the surgical approach preferred by our pediatric surgeons to treat their patients affected by Myasthenia Gravis (1).

Briefly, anesthesia is performed in the primate by intramuscular ketamine (10 mg/kg) and maintained with isoflurane (<2%) and oxygen, using endo-tracheal intubation and positive mechanical ventilation. Arterial filling, heart rate and SpO<sub>2</sub> is continuously monitored during the anesthesia. A peripheral i.v. line(s) is placed for intra-operative infusion of normal saline. The chest and upper abdomen of the animal is shaved and sterilized. The animal is then placed in a straddled, supine and in cervically extended position. A transverse incision is made about 4 to 5 cm above the xiphoid process. If necessary, the xiphoid process is excised after the rectus abdominis muscles are detached. The posterior table of the sternum is then exposed to insert the Laparofan (Origin, Co, Ltd, Menlo Park, CA) retractor (or equivalent) into the lower part of the sternum and the inferior thoracic wall lifted with the Laparolift (**Figure 1**).

After lifting the anterior inferior thoracic wall and creating adequate space, the mediastinoscope, Harmonic Scalpel (Ethicon Endo-Surgery, Inc, Cincinnati, OH) (or equivalent) and graspers are inserted into the wound. A monitor is placed at the side of the animal, and resection of thymus is performed with an Olympus laparoscope. The exposure through mini-incision on xiphoid process and lifting of the inferior thoracic wall is generally sufficient to perform a total thymectomy with a laparoscope. However, if necessary, upper mini-incision (3-5 cm) on the jugulum of the sternum can be made to guarantee the safe dissection of upper thymus without damaging adjacent main vessels in the mediastinum. After the completion of total thymectomy, hemostasis is obtained by electrocautery. If necessary, one or 2 small drains (Jackson-pratt or equivalent) are placed for 1 or 2 days to check post-operative bleeding and to avoid fluid collection in the mediastinum. Wound closure is achieved by apposing the muscle layers and subcutaneous tissues with interrupted non-absorbable 3-0 sutures (Prolene). The skin incision(s) are then closed with 4.0 PDS using a sub-cuticular or intra-dermal pattern. Animals may wear a jacket for 2 days if the drains were placed.



**Figure 1**

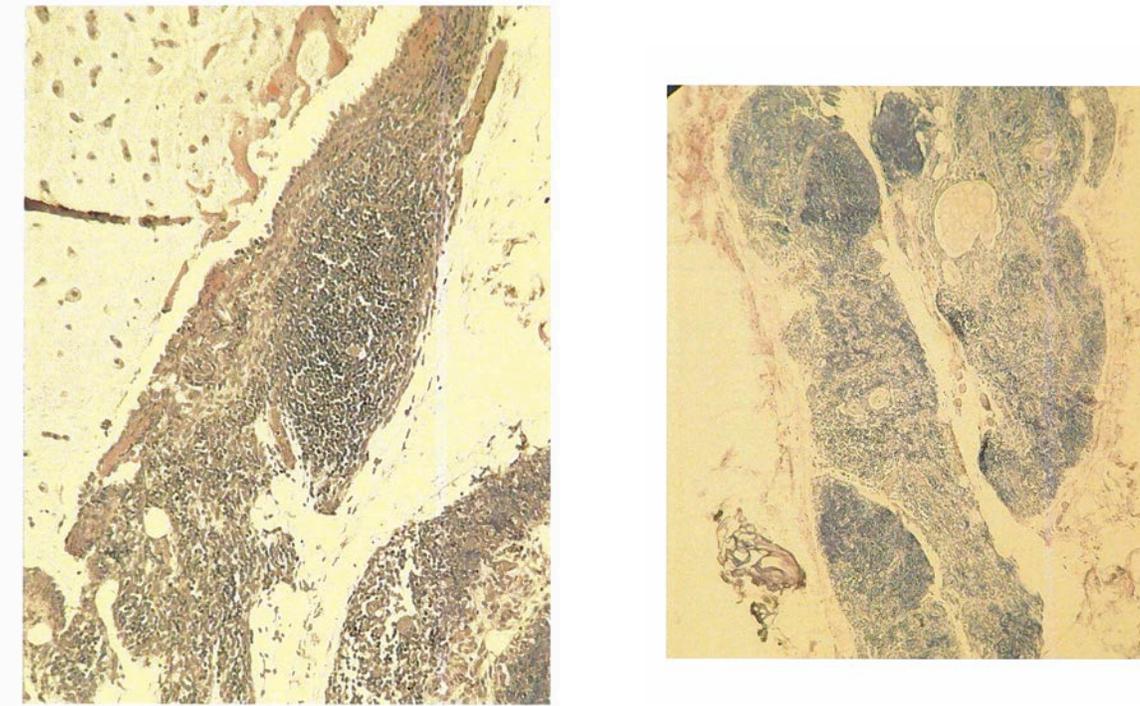
The above-mentioned procedure is safe in human patients and gives excellent results. There are already several series containing large numbers of operations (2).

Our pediatric surgeons performed this procedure on three aged monkeys – already used for unrelated protocols -- to receive the green light from the veterinarians to proceed with the entire protocol.

These mock interventions were useful to adapt the tools, we borrowed from the pediatric surgery room, to our relatively small monkeys.

In all three cases the thymus was recovered successfully, even if in these not too young monkeys the thymus resulted to be already dramatically reduced in size (**Figure 2**)

Problems of excessive bleeding encountered in the first intervention were solved in the last two performed interventions to the satisfaction of our veterinarians. The monkeys recovered quickly and completely after the intervention as requested by our protocol.



**Figure 2.** H&E staining of the thymus harvested from the monkeys.

In order to deplete the thymectomized monkey from pre-existing, already matured, T cells, we will use the anti-CD52 antibody (i.e., Campath) as originally proposed, starting from day 3 after thymectomy. Campath is currently used in clinic for the preconditioning of transplantation patients and we have already matured a good experience in the use of Campath in monkeys as well.

The administration of immunosuppressants in this study will be used initially to deplete the number of T lymphocyte and consequently stimulate their production in the bone marrow, but not to chronically immunosuppress the animal. Given Campath only once, we may observe the initial drug-associated undesired side effects but not the most common secondary problems typical of chronic immunosuppression.

In addition, thymectomy may induce a temporary immune impairment with increased risk for infections. Thymus implants should re-establish full immune competence as it is observed in children treated with thymus transplantation for Di George disease. During athymia, more intense monitoring for possible infections will be maintained and antibiotic prophylaxis will be carried out after thymectomy to prevent infectious events.

Once the thymic explant slices have been exposed to the shRNA for a sufficient number of days, and before they are transplanted back into the donor monkey, a treatment with the anti-CD52 antibody (Campath-1H) of the recipient animal will be performed so to allow a fresh start for the immature T cells coming from the bone marrow to reconstitute the monkey T cell repertoire through the insulin-deprived thymus.

We have a well established expertise relative to the use of Campath in both monkeys and humans (93). As the target CD52 molecule is expressed on erythrocytes of most NHP strains, using Alemtuzumab (commercial name for the anti CD20 mAb) in these species would cause massive

hemolysis. Cynomolgus monkeys of Indonesian origin will then be screened by agglutination assay for absence of CD52 on erythrocytes, before being administered Alemtuzumab. A cumulative dose to a maximum of 60 mg/kg has been determined as appropriate. Mycophenolatemofetil (MMF) can be added as maintenance therapy. Complete depletion of T and B lymphocytes (>99.5%) is achieved with 20 mg/kg Alemtuzumab and is more profound than in monkeys treated with anti-thymocyte globulin as quantified by flow cytometry (Figure 3). Without MMF, repopulation of CD20<sup>+</sup>B cells and CD8<sup>+</sup>T cells is complete within 2 and 3 months, respectively, and repopulation of CD4<sup>+</sup> T cells is 67% after 1 year. MMF significantly delay CD4<sup>+</sup> T-cell repopulation. Among repopulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells, a phenotypic shift is observed from naive toward effector memory cells. In lymph nodes, the depletion of naive cells is more profound than of memory cells (3).

This preclinical transplantation treatment offers the opportunity to obtain a lymphocyte depletion/repopulation process suitable for our main specific aim.

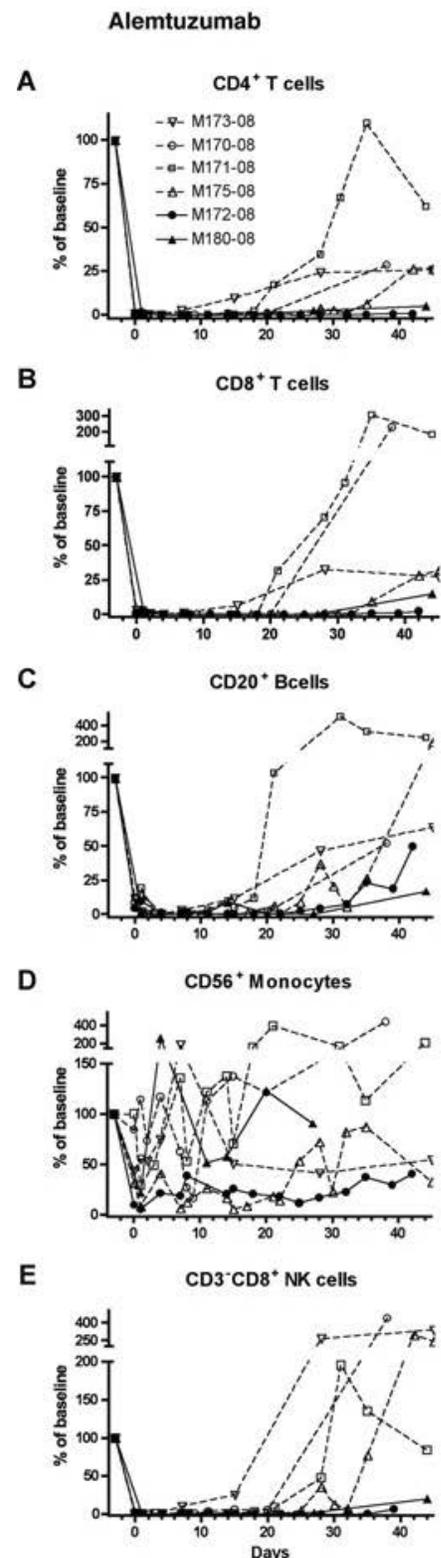
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3. van der Windt DJ, Smetanka C, Macedo C, He J, Lakomy R, Bottino R, Ekser B, Echeverri GH, Metes D, Ijzermans JNM, Trucco M, Cooper DKC, Lakkis FG: Investigation of lymphocyte depletion and repopulation using Alemtuzumab (Campath-1H) in cynomolgous monkeys. *Am J Transplantation* 10:773, 2010.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter. In the next quarter we will focus our efforts to fulfill the B part of the originally proposed Aim 1:

**To select the shRNA most effective in specifically blocking the expression of insulin in the thymic medullary cells of the monkeys.**

Since little is known about the insulin gene from cynomolgus monkey, we cloned and sequenced the monkey insulin gene (mINS) and compared this sequence with that of the human insulin gene (hINS). Of all the five shRNA sequences that were proven to be effective in knocking down hINS expression, only one had the perfect match with the correlating monkey sequence (#49). As a perfect match of sequence is not always required to efficiently knock down target gene expression, all five shRNA sequences were tested *in vitro* with a genetically modified HEK 293 cell line (designated as ml-293) stably transfected with a YFP-IRES-mINS bi-cistronic expression construct. If



**Figure 3:** Depletion of lymphocytes after the in vivo administration of Alemtuzumab (Campath) in cynomolgus monkeys (3).

the shRNAs are able to recognize the mRNA sequence of the monkey insulin, both YFP and the monkey insulin will be inhibited. After transduced with lentiviruses expressing the hINS shRNAs, ml-293 cells were selected with puromycin, expanded, and analyzed by flow cytometry to examine the levels of YFP expression. Both lentiviruses expressing shRNA #49 and #51 were able to effectively knock down YFP expression, whereas no effect was observed with shRNA #48, #50 and #52. Direct examination of mINS transcripts by RT-qPCR also showed that shRNA #49 and #51 can effectively knock down levels of mINS expression to 20-30% of controls. For our future work, lentivirus-expressing shRNA #49 will be chosen to knock down thymus mINS expression as it shared the same sequence as mINS and displayed the highest levels of mINS knockdown.

**To this aim we will:**

- Slice the harvested thymus into thin sections (about 0.5mm) with a tissue slicer and cultured for 7 days in DMEM medium supplemented with 2- deoxyguanosine.
- Starting from day 8 in the culture, monkey insulin-specific shRNA lentiviral particles will be added to the culture to transduce the stromal cell population.
- On day 11, thymic slices will be washed with PBS 6 times prior to intermuscular transplantation.

We envisioned a number of obstacles for the lentivirus-mediated mINS knock-down approach: 1) The large size of the thymus would prevent the lentiviruses as well as nutrients from gaining access to stromal cells inside the organ; 2) The large number of thymocytes present in the thymus (~100-1000 times of the stromal cells) might affect the efficiency of lentivirus transduction of stromal cells.

It is to overcome these obstacles that we will employ the tissue slicers to cut the thymus into 0.5mm sections and culture them in 6-well transwells in the presence of 2-deoxyguanosine to eliminate proliferating thymocytes. Under such *ex vivo* culture conditions, stromal cells within thymus sections are viable and are sufficient to support thymocyte development when transplanted under the kidney capsules of athymic nude mice. The generation of thin sections is also useful for directly implementing the transplantation approach previously described for treating complete Di George syndrome and selected by our surgeons for reaching the final aim of this application.

**Our second quarterly scientific progress report for the initial year of our project (10/12/11 – 01/11/12) described the following:**

To fulfill the Objective 1 of our original proposal, in the second quarter of our program we completed the first experiment.

**Objective 1: A) To safely harvest the thymus from Cynomolgus monkeys (i.e., *Macaca Fascicularis*), which, once exposed *in vitro* to an insulin-specific short-hairpin RNA (shRNA), will eventually be transplanted back into the donor monkey.**

Perhaps the least invasive approach among all methods of thymectomy is the so-called **infrasternal approach**. This procedure is safe in human patients and gives excellent results as proven in several series containing large numbers of operations (*Uchiyama et al. Intrasternal mediastionoscopic thymectomy in myasthenia gravis: surgical results in 23 patients. Ann Thorac Surg* 72:1902, 2001; *Kido et al. Resection of anterior mediastinal masses through an infrasternal approach. Ann Thorac Surg* 67:263, 1999). This procedure, first tested by our pediatric surgeons on three monkeys successfully (see previous Quarterly Report), was selected as the choice procedure of thymectomy in our first complete experiment (**Figure 1**).

Anesthesia was introduced in the primate with intramuscular ketamine (10 mg/kg) and maintained with isoflurane (<2%) and oxygen, using endo-tracheal intubation and positive mechanical ventilation. Arterial filling, heart rate and SpO<sub>2</sub> were continuously monitored during the anesthesia. Peripheral lines were placed for intra-operative infusion of normal saline. The chest and upper abdomen of the animal was shaved and sterilized. The animal was then placed in a straddled, supine and in cervically extended position. A transverse incision was made about 4 to 5 cm above the xiphoid process. The posterior table of the sternum was then exposed to insert the Laparofan (Origin, Co, Ltd, Menlo Park, CA) retractor into the lower part of the sternum and the inferior thoracic wall lifted with the Laparolift.



**Figure 1**

After lifting the anterior inferior thoracic wall and creating adequate space, the mediastinoscope, Harmonic Scalpel (Ethicon Endo-Surgery, Inc, Cincinnati, OH) and graspers were inserted into the wound. A monitor was placed at the side of the animal, and resection of thymus performed with an Olympus 30-degree-angled laparoscope. The exposure through mini-incision on xiphoid process and lifting of the inferior thoracic wall was sufficient to perform a quasi-total thymectomy with the laparoscope. After the completion of thymectomy, hemostasis was obtained by pressure. Small drains (Jackson-pratt or equivalent) were not considered necessary for checking post-operative bleeding since no fluid collection in the mediastinum was observed. Wound closure was achieved by apposing the muscle layers and subcutaneous tissues with

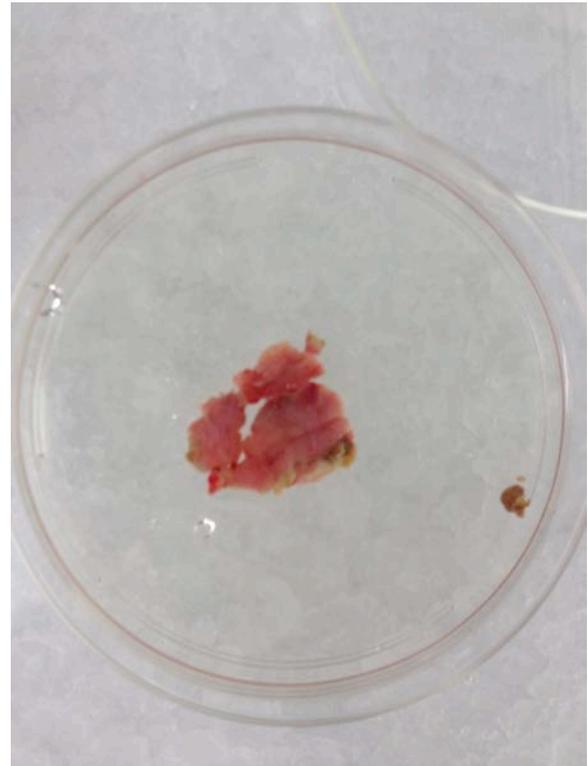


**Figure 2.** The first portion (~30%) of the harvested thymic tissue.

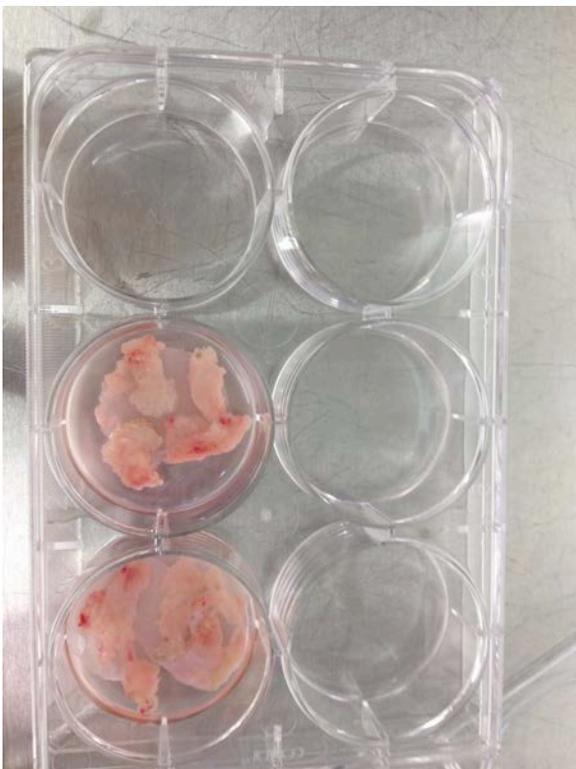
interrupted non-absorbable 3-0 sutures (Prolene). The skin incision was then closed with 4.0 PDS using a sub-cuticular or intra-dermal pattern (**Figure 2 and 3**).

Postnatal thymus allo-transplantation after treatment with immunosuppression (e.g., Thymoglobulin) is the therapy of choice for infants with complete DiGeorge syndrome. Complete DiGeorge syndrome is a fatal congenital disorder characterized by athymia, hypoparathyroidism, and heart defects. The success of allo-transplant is certified by a restored, polyclonal T cell function. Results from many different groups support both the safety of thymus transplantation and efficacy reflected in a pronounced improvement in T cell function and reduced rates of infection.

This approach was selected for transplanting the removed thymus back into the leg's mussel of the donor monkey, after being exposed for 7 days *in vitro* to the short-hairpin RNA specific for the monkey insulin (**Figure 4**). Once the thymic explant slices have been exposed to the shRNA for a sufficient number of days, and before they are transplanted back into the donor monkey, a treatment with the anti-CD52 antibody (Campath) of the recipient animal must be performed so to allow a fresh start for the immature T cells coming from the bone marrow to reconstitute the monkey T cell repertoire through the insulin-deprived thymus.



**Figure 3.** All harvested thymic tissue (~70% of the intere gland).



**Figure 4.** Slices of thymic tissue were exposed *in vitro* to monkey insulin-specific shRNA.

We have a well-established expertise relative to the use of Campath in both monkeys and humans. (*van der Windt et al. Investigation of lymphocyte depletion and repopulation using Alemtuzumab (Campath-1H) in cynomolgous monkeys. Am J Transplantation 10:773, 2010*). As the target CD52 molecule is expressed on erythrocytes of most NHP strains, using Alemtuzumab (commercial name for the anti CD20 mAb) in these species would cause massive hemolysis. Cynomolgus monkeys of Indonesian origin will then be screened by agglutination assay for absence of CD52 on erythrocytes, before being administered Alemtuzumab. A cumulative dose to a maximum of 60mg/animal has been determined as appropriate. Mycophenolatemofetil (MMF) can be added as maintenance therapy. Complete depletion of T and B lymphocytes (>99.5%) is achieved with 20 mg/kg Alemtuzumab and is more profound than in monkeys treated with anti-thymocyte globulin as quantified by flow cytometry. Without MMF, repopulation of CD20<sup>+</sup>B cells and CD8<sup>+</sup>T cells is complete within 2 and 3 months, respectively, and repopulation of CD4<sup>+</sup> T cells is 67% after 1 year. MMF significantly delays CD4<sup>+</sup> T cell repopulation. Among repopulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells, a phenotypic shift is observed from naive toward effector memory cells. In

lymph nodes, the depletion of naive cells is more profound than of memory cells. This preclinical transplantation treatment offers the opportunity to obtain a lymphocyte depletion/repopulation process suitable for our main specific aim.

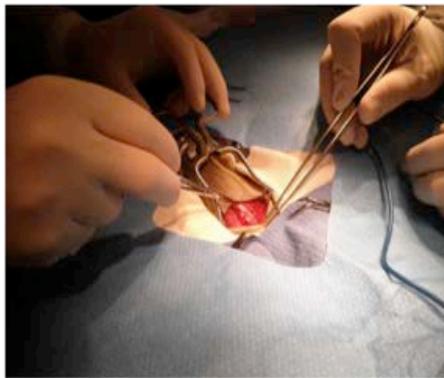
Accordingly, in order to deplete the thymectomized monkey from pre-existing, already matured, T cells, we used the anti-CD52 antibody (i.e., Campath), starting the day of thymectomy. The administration of immunosuppressants in this study was used initially to deplete the number of T lymphocyte and consequently stimulate their production in the bone marrow, but not to chronically immunosuppress the animal. Given Campath only once, we may observe the initial drug-associated undesired side effects but not the most common secondary problems typical of chronic immunosuppression. More specifically, after the thymectomy, while maintaining the animal under general anesthesia, Campath was administered after a pre-medication which included: Benadryl (Dyphenidramine) 2.5mg/Kg body weight, Reglan (Metoclopramide) 0.5mg/Kg, and Solumedrol 5mg/Kg i.v., to prevent the potentially harmful effects of massive cytokine release secondary to Campath. Campath was then administered at the dose of 20mg/Kg i.v. in a saline solution over a period of 4 hours.

Since thymectomy may induce a temporary immune impairment with increased risk for infections, during athymia, more intense monitoring for possible infections will be maintained and antibiotic prophylaxis has been started to prevent infectious events.

Seven slices of thymus tissue were inserted into the quadriceps muscle in an open procedure in the operating room. After induction of general anesthesia, a vertical skin incision is made over the anterior thigh compartment and the fascia was opened. Furrows were created between muscle fibers throughout the quadriceps muscle and individual slices of thymus tissue implanted (**Figure 5**). Thymus implants should re-establish full immune competence as it is observed in children treated with thymus transplantation for Di George disease.



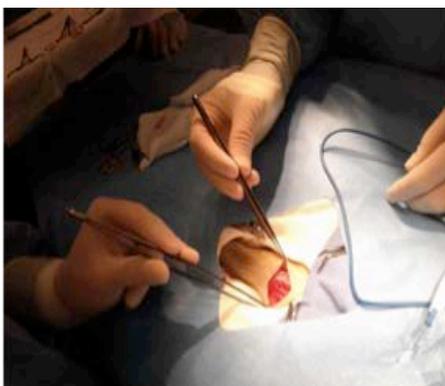
One of seven pieces after culture ready for tx.



Tight. Making space for the tx.



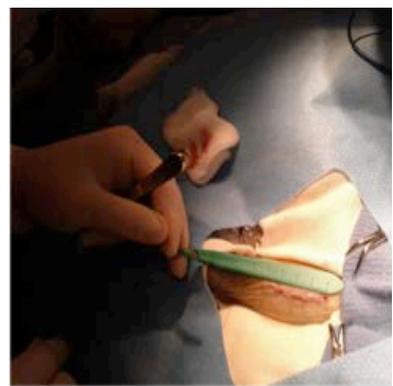
Piece (white) tx in pocket



Getting ready to close.



Closing.



Cut of approx. 4 cm.

After the surgical procedure was completed the recipient was treated with Cefazolin (10mg/Kg) for three days to prevent infections. Also the recipient was treated with Buprenex (0.01-0.03mg/Kg body weight i.m.) for three days to limit the possible pain and consequently also avoid the scratching of the wound by the animal. So far, no complications were recorded. One week after Campath treatment the number of T lymphocytes had dropped from 5100/ $\mu$ l to 11/ $\mu$ l as expected. T cell number recovery should be achieved in approximately 4-8 weeks post-treatment.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter we will focus our efforts to fulfill the Objective 2 of the original proposal:

**Objective 2: To prove that the shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of an autoimmune reaction, specifically directed against the beta cells of the pancreas.**

by testing periodically: a) the number of T lymphocytes, and b) the glycemia of the recipient.

Once the lymphocytes start to recover a sufficient number, ELISPOT will be used to determine whether insulin-specific effector T cell clones are matured into the transplanted thymus and eventually if they started to infiltrate the islets in the pancreas of the treated monkey.

Also, a second complete experiment will be performed to confirm the results obtained with the just-described, first one, so to more strongly fulfilling the aim of the first, original, Objective 1:

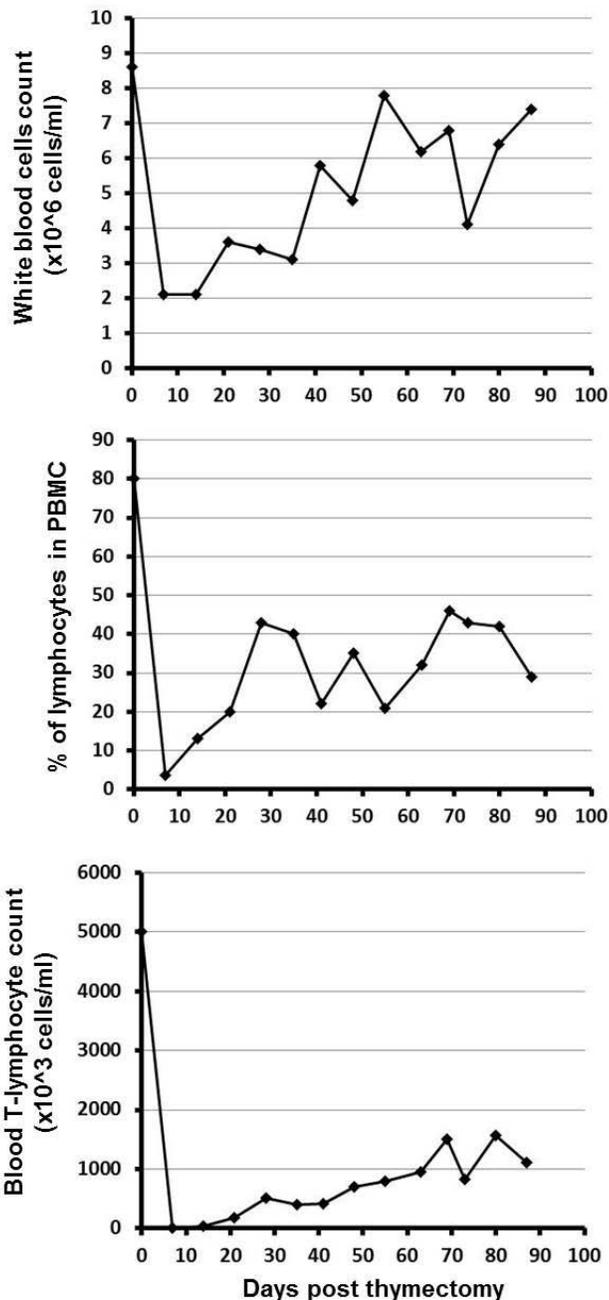
**Objective 1: A) To safely harvest the thymus from Cynomolgus monkeys (i.e., *Macaca Fascicularis*), which, once exposed *in vitro* to an insulin-specific short-hairpin RNA (shRNA), will eventually be transplanted back into the donor monkey.**

**In the third quarterly scientific progress report (01/12/12 - 04/11/12) of year 01, we now report on our cumulative results.**

In the third quarter of our program, we focused on the Objective 2 of our original proposal:

**Objective 2: To prove that the shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of autoimmune reaction, specifically directed against the beta cells of the pancreas.**

As the first step, we set to validate that shRNA-treated thymus retained its capability to attract the homing of T-cell progenitors and facilitate the development of thymocytes at extrathymic anatomical site – in between the skeleton muscle fibers of the monkey leg. Starting from one week post transplantation of the shRNA-treated thymus, 3-4 ml of blood was sampled on a weekly basis. To evaluate the efficacy of Campath-1H (Alemtuzumab) treatment for leukocyte depletion, as well as the monkey’s capability to recover from leukopenia, we followed

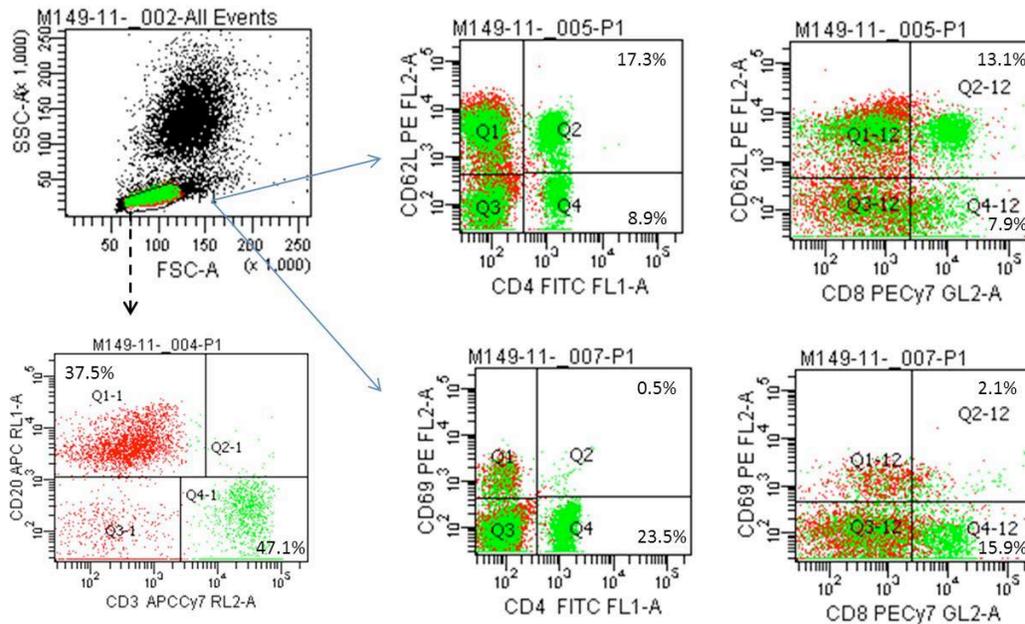


the whole blood leukocyte count to date. At 8 weeks after the Campath treatment (Figure 1, top panel), the whole blood cell count was 90% of the pre-treatment level, indicating successful hematogenesis. Similarly, the number of circulating lymphocytes was recovered to about 40% of pre-Campath treatment levels in approximately 4 weeks (Figure 1, middle panel).

To examine the number of T-cells in the blood, flow cytometry (FCM) analysis was performed with antibodies specific to monkey CD4 and CD8 antigens. As shown in Figure 1 (lower panel), a slow but steady recovery of circulating T-lymphocytes was observed. 10-weeks after the transplantation of shRNA treated thymic tissues, blood T-cell count increased from the initial negligent level (11/ul) to 1570/ul, which is above 30% of the pre-treatment count (5000/ul). Of note, the kinetics of T-cell recovery is line with our previous observation on mouse thymus-transplantation models.

**Figure 1.** Recovery of T-lymphocytes in thymectomized monkey after intramuscular transplantation of shRNA-treated thymic sections. Thymic reconstitution was performed at day 7.

As the T-cells in circulation could arise from the transplanted shRNA-treated thymic sections, from the residue thymus (the thymectomy is about 70% complete), or from the proliferation of residue T-cells residing in the secondary lymph organs in response to lymphopenic environmental stimulation. At current stage, we were unable to distinguish T-cells derived from the first two origins; however, T-cell expanded from the secondary lymphoid organs will carry memory T-cell markers, such as CD69, while naïve cell marker, CD62L, expression will be down-regulated. Expression of surface markers on circulating T-cells were characterized by FCM analyses of blood samples harvested from the experimental monkey. As shown in **Figure 2**, 10 weeks after thymic tissue transplantation, distinct populations of lymphocytes (37.5% CD20+ B-cells and 47.1% CD3+ T-cells) are present in the white blood cells. Further characterization of CD3+ T-cell population revealed that the majorities of CD4+ T-helper cells and CD8+ cytotoxic T-cells are expressing CD62L, suggesting that they are newly generated T-cells from the thymic tissues (**Figure 2**, right panels on top). Consistently, neither CD4+ nor CD8+ T-cells are expressing the surface marker, CD69, of activating T-cells, suggesting further that the majority of the circulating T-cells remains at the naive stage (**Figure 2**, right panels at the bottom).



**Figure 2.** Characterization of circulating T-cells 10 weeks post reconstitution with shRNA-treated thymic sections.

Of note, similar FCM results were obtained from white blood cells harvested prior to Campath-L treatment and thymectomy, as well as other time points (Summarized in **Table 1**). Taken together, these data highly suggest that intramuscular transplantation of the shRNA-treated thymic tissues is sufficient to support thymocyte development and maturation.

**Table 1. Summary of white blood cells in thymus reconstituted monkey**

Post Thymectomy Days	# of T-cells	WBC	Lympho %	T-Cell %	CD62L:CD3%	CD69:CD3%	CD4:CD62L%	CD69:CD4%
0	5000	8.6	80	74.3	n/a	n/a	33.2	0.7
7	11	2.1	3.7	15.1	13	0.4	31.7	0.9
14	35	2.1	13	12	5.4	0.5	23.9	0.6
21	183	3.6	20	25.4	7.3	6	n.d.	n.d.
28	510	3.4	43	34.9	8.3	6	n.d.	n.d.
35	406	3.1	40	32.8	10.2	7.8	13.2	2.1
41	411	5.8	22	32.2	14.5	0.9	14.7	0.9
48	692	4.8	35	41.2	22.9	6	21.9	1.4
55	790	7.8	21	48.2	23.5	5.7	19.4	0.7
63	946	6.2	32	47.7	35.8	6.1	33.1	1.8
69	1498	6.8	46	47.9	24.8	7.8	24.7	0.8
73	830	4.1	43	47.1	31.4	2.2	32.1	0.9
80	1570	6.4	42	58.4	37.8	7.8	34.8	0.6
87	1105	7.4	29	51.5	29.9	3.3	31	0.4

**Note:** WBC, white blood cells; Lympho, lymphocytes (including both T and B cells); units for T-cell # and WBC are  $10^3/\text{ml}$  and  $10^9/\text{ml}$ , respectively.

Our success in reconstituting the monkey T-cell repertoire after Campath treatment and thymus transplantation prompted us to examine the presence of insulin-specific autoreactive T-cells in peripheral blood. Enzyme-linked immunosorbent spot (ELISPOT) assays were performed, in which human insulin molecules were used as stimulating autoantigens. Specifically, peripheral blood mononuclear cells (PBMCs) were harvested from the monkey 10 weeks after thymic transplantation and cultured in 96-well tissue culture dishes coated with anti-IFN $\gamma$  antibody overnight, in the presence or absence of insulin (100ug/ml). As positive control of T-cell function under stimulus conditions,  $10^4$  PBMCs were cultured with PMA+ionomycin, which activate T-cells in an antigen independent manner. As shown in **Figure 3**, robust T-cell activation and IFN $\gamma$  secretion were observed upon PMA+ionomycin stimulation, indicating that the T-cells derived from the reconstituted thymus are functional capable to mount TH1-type responses under proinflammatory conditions, an essential prerequisite for successful adaptive immunity. At present, we did not observe any elevated number of insulin-reactive T-cells in peripheral circulation, in comparison to pre-treatment blood samples.



**Figure 3.** ELISPOT analysis of PBMCs isolated from monkey blood 10-weeks after thymus reconstitution. PMA+Ionomycin,  $10^4$  cells; all other conditions,  $3 \times 10^6$  cells.

**12.** Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

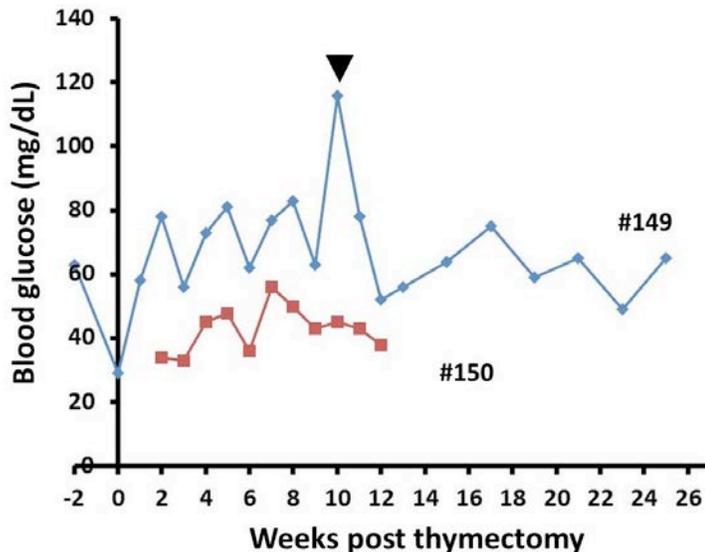
In the next quarter, we will apply the procedure on more monkeys to confirm the results described. In addition, ELISPOT will be performed on more blood samples to monitor the appearance of circulating insulin-specific autoreactive T-cells. Blood glucose levels will also be closely monitored to detect any sign of abnormal islet function.

**In the fourth quarterly scientific progress report (04/12/12 - 07/11/12) of year 01, we now report on our new and year 01 cumulative results.**

In this quarter, we continued our focus on the Objective 2 of our original proposal to prove that “the shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of autoimmune reaction, specifically directed against the beta cells of the pancreas”. We experimented on the second monkey to perfect the surgical procedures of thymectomy and to improve the ex vivo modification and culture conditions of the thymus sections. In addition, we continued our follow up on the first monkey to capture any sign of abnormalities in blood glucose control.

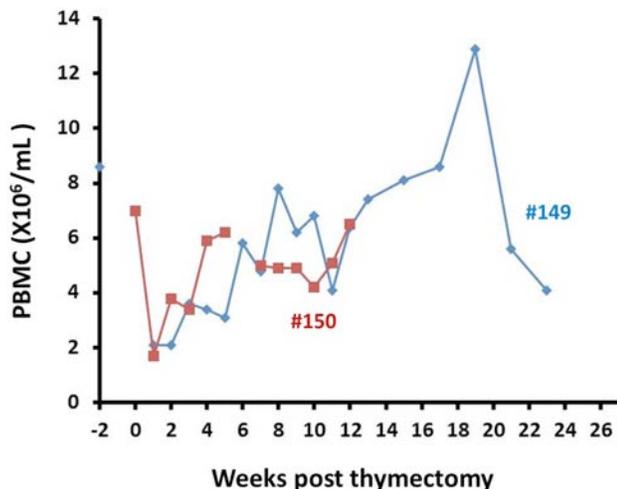
The first improvement we achieved in this quarter is the thymectomy procedure. We have successfully removed more than 99% of the thymus from the second monkey (Percy, #150-11), in contrast to the 70% removal in the first one (Leo, #149-11). The close to complete removal of the thymus enable us to evaluate the effectiveness of the ex vivo modified thymic sections in reconstruction of the monkey immune system. To increase the lentiviral transduction efficiency, we injected the insulin-specific shRNA lentiviral particles at multiple sites (5-10 uL per injection) before subjecting the thymus to tissue slicer and spin infection protocol. After culture for 7-days, the resulting thymic sections were transplanted intramuscularly into the thymectomized monkey (#150-11).

**Figure 1. Blood Glucose of the Treated Monkeys.**



Similar to the first treated monkey (#149), no obvious physical side effect was observed in #150. After a mild drop at two weeks post-surgery, its body weight was stabilized around 3 kilograms (Table 1). No major changes of blood glucose levels were observed, which ranged from 30-60 mg/dL, typical for this particular monkey species (Figure 1). Of note, a spike of blood glucose read was observed at week 10 after thymectomy (Figure 1, arrow head). This could suggest the existence of factors that caused a small disturbance in glycemic control. However, the data should be interpreted with caution, as the elevation of blood glucose was transient, and occurred only once. To evaluate both monkeys' capabilities to maintain glucose homeostasis

upon glucose tolerance, we will perform oral glucose tolerance test (OGTT). We will also measure the total amounts of insulin secreted upon glucose oral intake, to further evaluate their pancreatic islet function.



A drastic drop of peripheral blood mononuclear cell (PBMC) count was also found in monkey #150 after thymectomy and Campath-1H (Alemtuzumab) treatment (Figure 2). A slow but steady increase of PBMC count was observed, indicating that the Campath-1H treatment will not cause permanent damage to hematopoiesis in the bone marrow. Of note, the kinetics of PBMC recovery was similar in both monkeys #149 and #150. To further evaluate the general health status of the treated monkeys, we followed closely their complete blood count (CBC), a panel of tests that evaluates the three types of cells, including the red blood cells, the white blood cells and platelets (Tables 1-4). As shown in Tables 1 & 2, a transit Hemocrit's

decrease was observed in a period of 6-7 weeks after the Campath-1 treatment. No prominent changes were detected in most of the parameters of red blood cell (RBC) indices. Only a mild and transit increase of RBC distribution width (RDW), an indicator of variation of sizes of RBCs, was observed, indicating a slight increase of immature RBCs in the blood after the Campath-1 treatment. Nevertheless, all the RBC indices were fully recovered to pretreatment levels after 10 weeks.

**Table 1. RBC Evaluation of Monkey #150**

Weeks post Thymectomy	Body Weight (kg)	Hematocrit (HCT, %)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
0	3.2	36.6	57.2	18.1	31.6	13
1	n.d.	28.4	57.3	17.8	31.1	13.7
2	3.1	30.4	57.4	18.1	31.6	14.8
3	2.8	31.5	56.9	17.5	30.8	14.8
4	n.d.	32.6	57.1	18.3	32	14.6
5	3.1	33.5	59.3	17.7	29.8	16.2
6	3	n.d.	n.d.	n.d.	n.d.	n.d.
7	3.1	37.9	58.7	18.1	30.8	15.9
8	3	38.6	57.8	17.8	30.8	15
9	3	38.6	57.3	17.9	31.2	14.2
10	2.9	39.9	56.9	17.7	31.1	14.1
11	3	39	57.3	17.7	31	13.8
12	3	37.7	58.4	18	30.8	12.7

**MCV**, Mean Corpuscular Volume; **MCH**, Mean Corpuscular Hemoglobin; **MCHC**, Mean Corpuscular Hemoglobin Concentration; **RDW**, RBC Distribution Width; **n.d.**, not done.

Blood count of platelets (thrombocytes), which play a very important role in healing process and the formation of blood clots at the time of injury, was maintained in the normal range (150 – 400, 000 per microliter) (Tables 3 and 4). Consistently, little changes of mean platelet volume (MPV) were found in both monkeys before and after the treatments (Tables 3 and 4).

**Table 2. RBC Evaluation of Monkey #149**

Weeks post Thymectomy	Body Weight (kg)	Hematocrit (HCT, %)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
-2	3.3	35.8	56.7	18.1	32	16
0	3.3	n.d.	n.d.	n.d.	n.d.	n.d.
1	4.3*	31.2	54.6	17.8	32.6	17.6
2	3.2	30.6	55.7	18	32.4	17.4
3	3.3	31.8	56.4	17.5	31	17.5
4	3.2	34.6	57	17.4	30.5	17.5
5	3.2	34.4	57	17.5	30.7	17
6	3.3	37	58.1	17.9	30.7	17
7	3.3	36.9	58.2	17.8	30.5	16.2
8	3.5	38.6	58.6	17.6	30	15.2
9	3.4	37.6	57.8	17.7	30.7	15.8
10	3.4	41.4	57.7	17.8	30.9	14.5
11	3.5	37.1	57.3	17.6	30.8	15.4
12	3.6	37.8	56.8	17.8	31.4	15.5
13	3.5	38.6	57	17.7	31	15.9
15	3.5	36.4	57	17.8	31.3	17.1
17	3.6	37.1	57.2	17.7	30.9	17.6
19	3.8	35.3	58.2	17.6	30.2	18.4
21	3.8	36.8	58.5	18	30.7	16.9
23	3.7	38.9	57.6	18	31.3	15.3
25	3.8	35.4	59.6	18.4	30.9	14.3

**Note:** MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, RBC Distribution Width; n.d., not done; \*, weight with jacket.

WBC differential tests revealed a sharp increase of the percentage of neutrophils in both monkeys after the Campath-1 treatment (Tables 3 and 4). Similarly, an increase of percentage of monocytes was also observed. For example, the monocyte percentage in monkey #150 jumped from 12% in week 0 to 18% in week 1 (Table 4). In contrast, a significant decrease of percentage of lymphocytes was detected in both monkeys, dropping from 60-80% before the treatment to 3-5% one week after Campath-1. These data suggested that although Campath-1 antibody treatment can effectively target all white blood cells, T- and B-lymphocytes were the most affected cell types. Nevertheless, the percentage of lymphocytes in monkey #150 rebounded to pretreated level 10-week after the treatment (Table 3). In monkey #149, which displayed abnormal high levels blood lymphocyte prior to thymectomy, the lymphocyte percentage recovered to about 45% within the same period of time (Table 4).

**Table 3. Evaluation of Platelets and WBCs of Monkey #150**

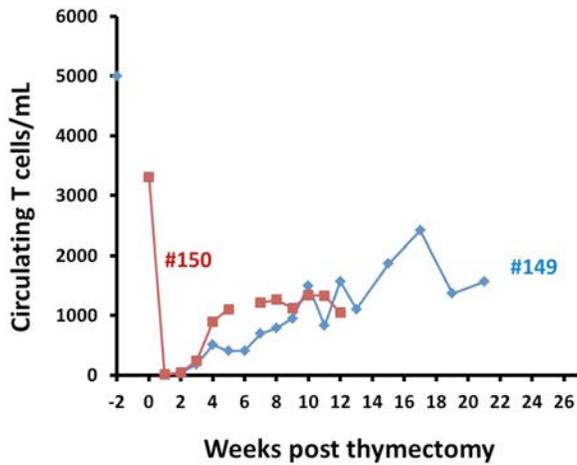
<b>Weeks post Thymectomy</b>	<b>Platelet (1x10<sup>3</sup>/μL)</b>	<b>Mean Platelete Volume (fL)</b>	<b>Differential Neutrophils (%)</b>	<b>Lymphocytes (%)</b>	<b>Monocytes (%)</b>
0	231	12.5	18	63	12
1	230	8.4	67	7	18
2	205	8.6	74	4	19
3	197	9.1	66	26	4
4	265	8.8	49	32	6
5	229	9.4	48	40	8
7	225	11.5	40	51	8
8	218	9.9	38	50	11
9	241	10.1	39	49	9
10	260	10.1	12	71	9
11	214	10.1	37	50	11
12	197	9.7	56	34	9

**Table 4. Evaluation of Platelets and WBCs of Monkey #149**

<b>Weeks post Thymectomy</b>	<b>Platelet (1x10<sup>3</sup>/μL)</b>	<b>Mean Platelete Volume (fL)</b>	<b>Differential Neutrophils (%)</b>	<b>Lymphocytes (%)</b>	<b>Monocytes (%)</b>
-2	260	9.1	14	80	5
1	307	8.6	78.3	3.7	15.2
2	228	8.9	75	13	9
3	217	9.4	63	20	14
4	296	9	46	43	7
5	221	9.4	52	40	7
6	283	10.9	74	22	3
7	293	9.1	60	35	4
8	213	9.3	72	21	6
9	268	9.2	63	32	4
10	294	9.4	49	46	5
11	248	9	51	43	5
12	209	9.1	53	42	2
13	71	9.5	68	29	3
15	309	8.9	59	37	4
17	323	9.2	50	44	5
19	280	9.1	80	16	4
21	272	8.9	50	43	5
23	283	9.1	36	58	5
25	332	8.5	68	28	4

The recoveries of T-lymphocytes of both monkeys were examined with flow cytometry (FCM) analysis of PBMCs (Figure 3). 10-weeks after the thymectomy and the Campath-1

**Figure 3. Recovery of T-cells after thymus transplantation**



treatment, the circulating T-cell counts were above 1000 cells/mL in both monkeys, which were more than 1/3 of the pretreatment levels. Of note, T-cell recovery was accelerated in monkey #150, in comparison to that of #149, suggesting that the transplanted thymic sections functioned better to reconstitute the T-lymphocytes in the Campath-1 treatment lymphopenic monkey. Nevertheless, both monkeys displayed an encouraging trend to gain fully recovery from the thymectomy.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter, we will focus on developing protocols to increase the transduction efficiency of insulin-specific shRNA lentiviral particles. Specifically, we will explore the possibilities of reconstructing the monkey thymus from separated stromal cells to maximize the possibility of reconstituting the T-cell repertoire with an insulin-inactivated thymus.

## KEY RESEARCH ACCOMPLISHMENTS

Our previous study in the mouse (*Fan Y, Rudert WA, Grupillo M, He J, Sisino G, Trucco M: Thymus-specific deletion of insulin induces autoimmune diabetes. The EMBO Journal 28:2812, 2009*) suggested that also in a monkey, a drastic reduction of expression of self-antigens (e.g., insulin) in the epithelial cells of the thymic medulla (mTECs) might favor the generation of an autoimmune reaction specifically directed against the insulin-producing cells of the pancreas, eventually bringing the animal to clinically overt T1D.

On this basis we were able to:

- Safely remove the thymus from *Cynomolgus* monkeys (i.e., *Macaca Fascicularis*);
- Transducing the harvested thymus *in vitro* with a lentivirus expressing monkey-insulin-specific short-hairpin RNA (shRNA) to knock-down insulin expression;
- Returning the transduced thymus back into the donor monkey (whose mature B- and T-cells had been concomitantly depleted using Campath), to facilitate the onset of autoimmunity against pancreatic beta cells. Following depletion of all immuno-competent cells in the thymectomized monkey and transplantation of the transduced thymus back to it, we observed a successful repopulation of its immune cells (mature and functional T- and B-lymphocytes) after roughly three months from transplantation.
- The virus might not have transfected all the cells of the thymic medulla, so that a drastic reduction in insulin expression/presentation resulted to be insufficient to favor the development of a specific autoimmune reaction. No more than ~5% of insulin reduction was achieved in the thymus, by the process of transducing *ex vivo* the sliced thymic tissue by exposing it *in vitro* to the monkey insulin-specific shRNA expressing vector. This reduction was insufficient to break central tolerance and failed to promote clinically-overt diabetes in the recipient monkey.
- We then concluded that the only way to achieve better transduction with the vector -- similar to the outcome *in vitro* using a cell line carrying a monkey-insulin expression vector -- was to decompose the thymic tissue so to obtain a single cell population, which could be more efficiently exposed to the shRNA carrying vector. With this approach ~ 25-45% of the mTECs were transduced.
- We remained then with the problem of how to reconstruct the thymus in order to transplant it back to the donor monkey. In the mouse we solved this final problem utilizing a process that successfully repopulates a thymic scaffold with enriched mTECs. Transplanting the reconstructed thymus, we successfully reconstituted mature and functional T- and B-cells in nude mice.

## REPORTABLE OUTCOMES:

None, to date.

## CONCLUSION:

Based on the promising results obtained so far in our laboratory, we concluded that, by a sequential approach of thymic resection, its de-cellularization, harvesting of an mTEC-containing cell population, *ex vivo* insulin-gene transduction of the population enriched in mTECs, re-population of thymic scaffold with this transduced, donor mTEC population, and transplantation of the re-constructed thymus back into the donor, we should become able to make monkeys diabetic via an autoimmune process. Furthermore, by combining all the pieces of this exciting and certainly innovative mosaic into an efficient functional continuum, we should also be able to lay the basis for a possible innovative and safe (i.e., no need for chronic immuno-suppression) treatment of T1D in humans as well as a preventive method utilizable in the highest-risk individuals. In fact, thymectomy has been successfully used to treat Myasthenia Gravis (1,2) and postnatal thymus allo-transplantation is the therapy of choice for infants born with complete DiGeorge syndrome. DiGeorge is a fatal congenital disorder characterized by athymia, hypoparathyroidism, and heart defects. Results from many different groups support both the safety of thymus transplantation and its efficacy, reflected by pronounced improvement of T-cell function and reduced rates of infection (3,4).

1. Uchiyama A, Shimizu S, Murai H, Kuroki S, Okido M, Tanaka M: Intrasternal mediastinoscopic thymectomy in myasthenia gravis: surgical results in 23 patients. *Ann Thorac Surg* 72:1902, 2001.
2. Shrager JB, Nathan D, Brinster CJ, Yousuf O, Spence A, Chen Z, Kaiser LR: Outcomes after 151 extended transcervical thymectomies for myasthenia gravis. *Ann Thorac Surg* 82:1863, 2006.
3. Rice HE, Skinner MA, Mahaffey SM, Oldham KT, Ing RJ, Hale LP, Markert ML: Thymic transplantation for complete DiGeorge syndrome: medical and surgical considerations. *J Pediatric Surgery* 39:1607, 2004.
4. Markert ML, Devlin BH, Alexieff MJ, Li J, McCarthy EA, Gupton SE, Chinn IK, Hale LP, Kepler TB, He M, Sarzotti M, Skinner MA, Rice HE, Hoehner JC: Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: outcome of 44 consecutive transplants. *Blood* 109:4539, 2007.

## The So What Section:

### What are the implication of this research?

Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are type 1 DM. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. Type 1 diabetes is associated with a high morbidity and premature mortality due to complications. The annual cost from diabetes overall exceeds \$100 billion, almost \$1 of every \$7 dollars of US health expenditures in terms of medical care and loss of productivity. C-peptide administered regularly in physiological quantities might be an additive to insulin able to reduce those complications.

## **What are the military significance and public purpose of this research?**

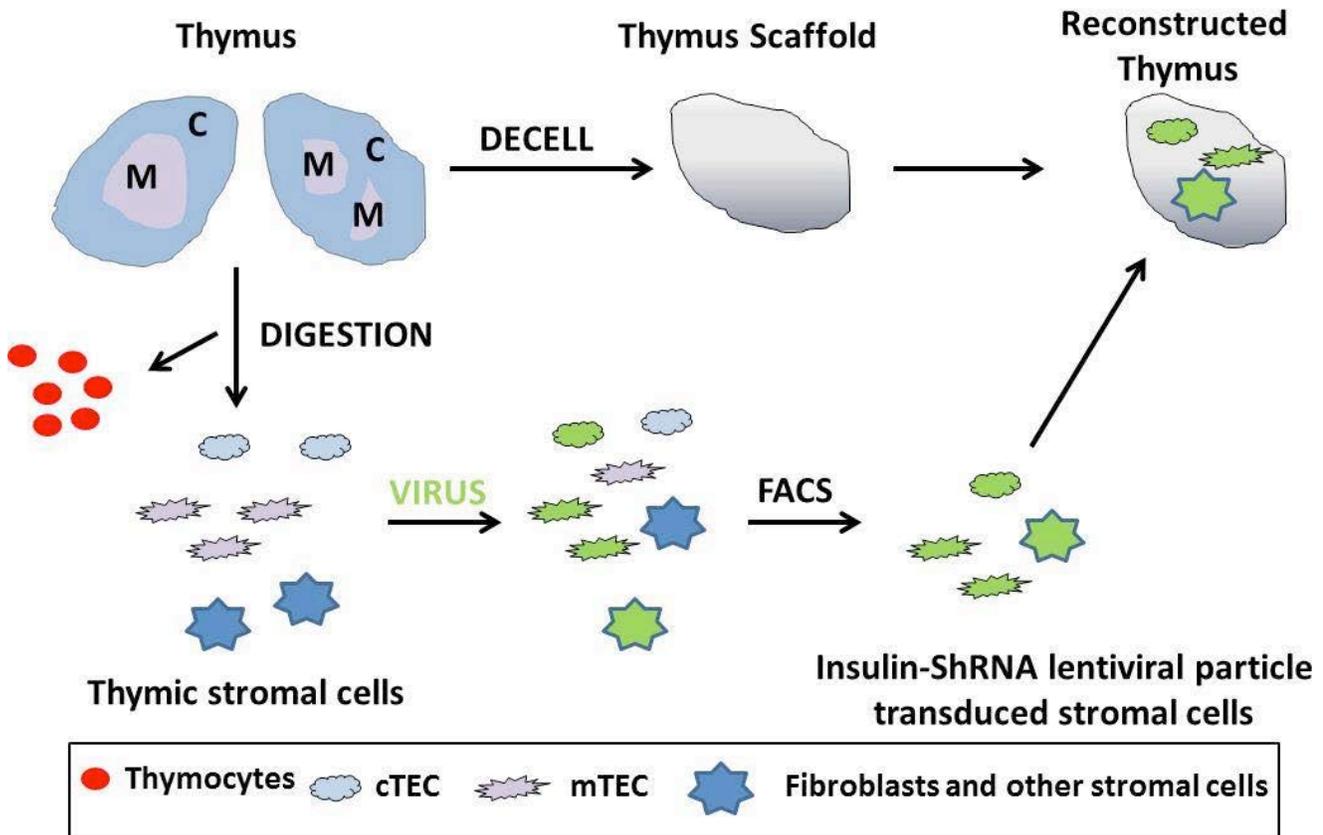
As the military is a reflection of the U.S. population, improved understanding of the underlying etiopathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patients' well being.

**Our first quarterly scientific progress report for the second year of our project (07/12/2012 - 10/11/2012) described the following:**

As described in the previous quarterly reports, we have performed experiments on two monkeys to test the hypothesis that *“the insulin-shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of autoimmune reaction, specifically directed against the beta cells of the pancreas”* (Objective 2). We have been following these two monkeys closely for the past couples of months. All the red blood cell (RBC) indices, including hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and RBC distribution width were fully recovered to pretreatment levels. In addition, both monkeys displayed a slow but steady increase of PBMC counts, indicating that the Campath-1H treatment will not cause permanent damage to hematopoiesis in the bone marrow. In both monkeys, the circulating T-lymphocyte counts reached to above 70% of their pretreated levels, indicating that transplantation of the shRNA treated thymus sections is sufficient to support lymphopoiesis and generate a new T-cell repertoire. Taken together, results from these two monkeys validate the feasibility of our approach and indicate that we can indeed modify the thymus genetically in nonhuman primates.

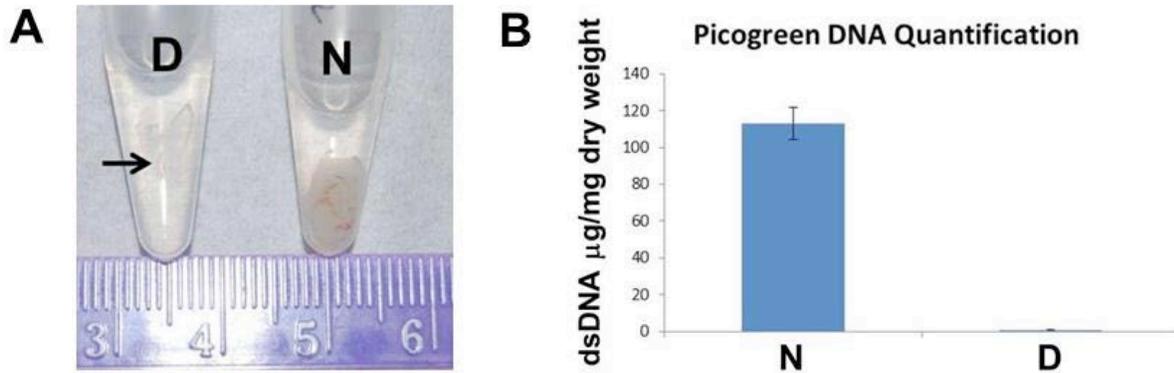
However, we did not observe any incidence of hyperglycemia or abnormality of glucose control in either monkey. This prompted us to re-evaluate the method used to achieve thymic insulin expression knockdown. Since the thymus sections subjected to insulin shRNA lentivirus transduction were about 500mm thick, whereas the average diameter of a cell is about 10-20mm, the average number of cells at the cross-section was about 25-50. Since only cells at the surface layer of the thymus section are exposed to the lentiviral particles, less than 5% of cells will be transfected when the spin-transfection is the only method used. To increase the transduction efficiency, we added an intrathymic injection step to the spin-transfection protocol. Still, it is rather unlikely that the current protocol will enable us to achieve the transfection target of >90% thymic stromal cells. Thus, insulin expression in the treated monkeys might not be knocked down to the level that will result in impairment in the negative selection process of insulin-reactive thymocytes.

In this quarter, we explored the possibility of using an alternative approach to improve the efficacy of lentiviral transduction of thymic medullary epithelial cells (mTECs), and thus to achieve more efficient knock-down of thymic insulin expression. The strategy is based on the recent findings that the 3D matrix structure of a decellularized organ can facilitate the homing and differentiation of the seeded progenitor cells. Such organ-reconstructing strategies using decellularized organ scaffolds have previously been attempted on the liver, the lung and the heart; and all showed promising outcomes, including success of repopulating the scaffolds, as well as partially recovery of the function. In term of the thymus, previous studies have shown that the 3D organization of the thymic epithelial cells is a unique and essential property of the thymus for the successful development of thymocytes. As the efficiency of lentiviral transduction of single cells, which can reach as high as 20-40% in our hands, is significantly higher than that of tissue sections, we modify the gene therapy protocol of the thymus by combining the two approaches: introducing the FACS sorted lentiviral transduced cells into the decellularized thymus scaffolds to reconstruct the monkey thymus (Figure 1).



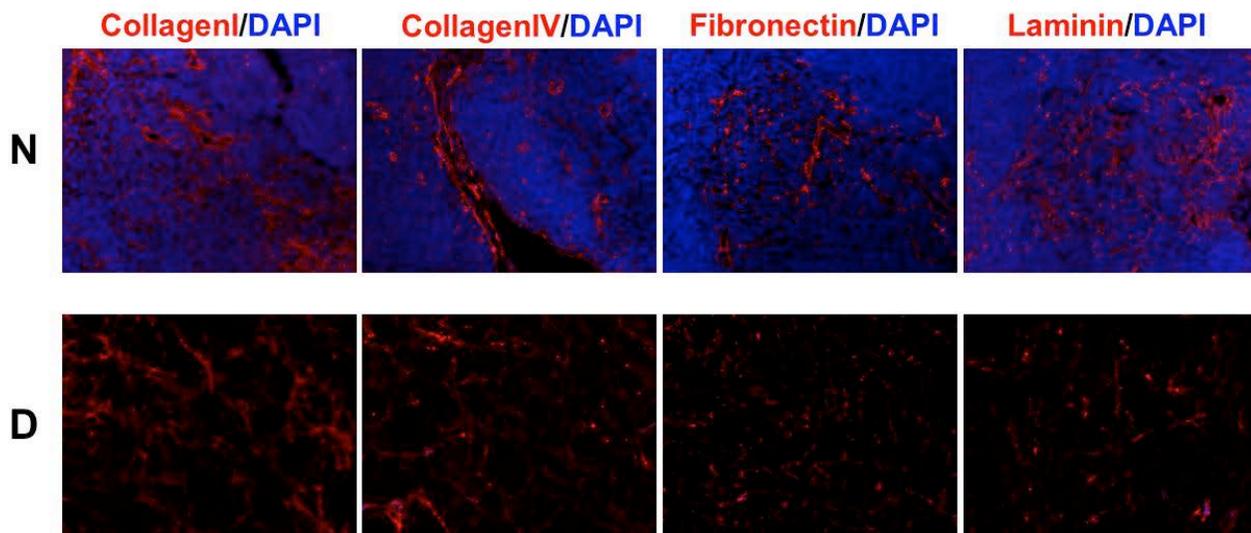
**Figure 1. Schematic view of thymus reconstruction strategies to genetically modify the thymic stroma.** CD45<sup>+</sup> thymocytes isolated from the thymus were depleted with CD45 microbeads (Miltenyi Biotec). Thymic stromal cells were transduced with insulin shRNA lentiviral particles (MOI 5:1), which co-express EGFP under the CMV promoter (light green). Cells transduced with the virus (EGFP<sup>+</sup>) were isolated with fluorescent activated cell sorting (FACS). Thymic lobes from other animals were decellularized (DECELL) with detergent (0.1%SDS overnight, followed by 1% Triton X-100 for 2 hours) to generate the 3D scaffolds. To reconstruct thymus, EGFP<sup>+</sup> cells were injected into the decell-ed thymus scaffold and cultured in vitro before for 3-7 days before transplanted into either genetically athymic or thyectomized animals. M, the medullary region; C, the cortical region.

To corroborate our approach to genetically modify the monkey thymus, we first tested the thymus reconstruction protocol in the mouse model. While the protocol to isolate CD45<sup>-</sup> thymic stromal cells is well-established in the lab, the method to prepare the 3D thymus scaffold is new to us. With the expertise assistance from Dr. Ipsita Banerjee and colleagues at the Department of Chemical and Petroleum Engineering, University of Pittsburgh, we successfully developed a protocol that enabled us to prepare the decellularized, 3D thymus scaffold from the mouse thymus (Figure 2A). Quantification of double strand DNA (dsDNA) with Picogreen kit (Invitrogen) revealed significant decrease of DNA contents in the decellularized thymic scaffolds, indicating that our procedures can not only effectively lyse the cells, but also are able to remove debris. To further characterize the structure of the decellularized scaffolds, we performed immunohistochemical analysis. As shown in Figure 3, extracellular matrix proteins within the thymus, including collagens (I and IV), fibronectin and laminin, of the thymus are well preserved and also organized in a similar pattern as the untreated naïve thymus, suggesting that the 3D structure of the extracellular matrix of thymus is largely intact.



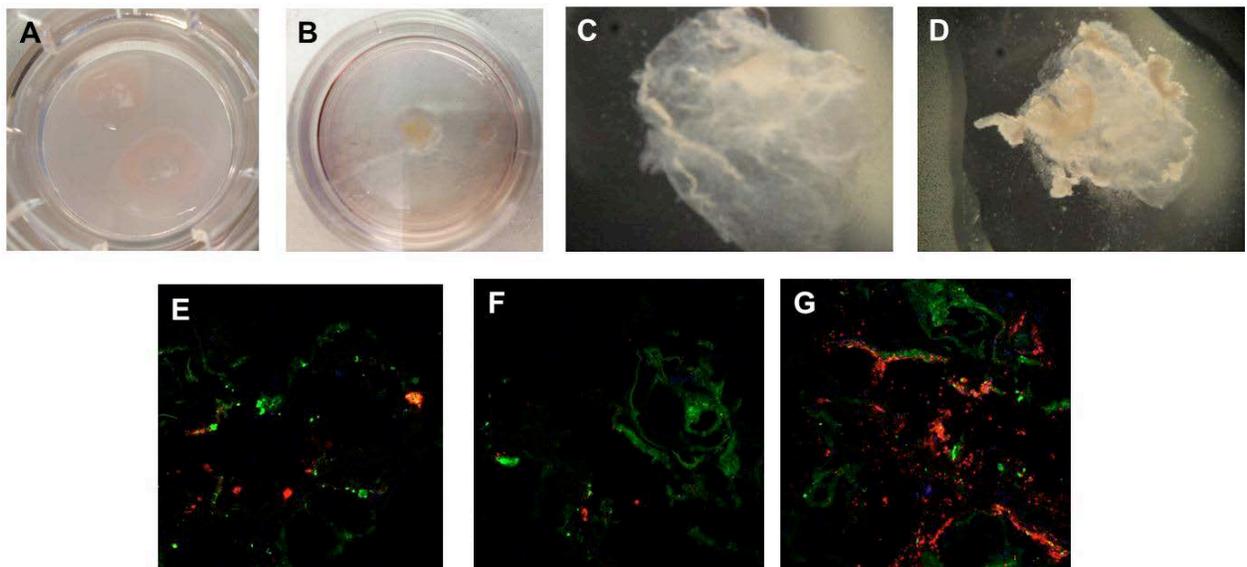
**Figure 2. Decellularized mouse thymus.** A. Representative photography of the decellularized thymic lobe of a 4-week old mouse (left tube, black arrow), comparing to the untreated, naïve thymic lobe from the same mouse. D, decellularized; N, naïve. B. Quantification of dsDNA in the decellularized thymic scaffolds with Picogreen kit (Invitrogen).

In our first attempt to reconstruct the thymus, we injected all the cells ( $\sim 1 \times 10^8$ ) from the whole thymus into the thymus scaffold, and culture it in transwells (Figure 4A). While the injected cells were retained in the scaffold, the 3D matrix structure collapsed after 3-days, probably due to the hydrolysis of the collagens by the injected cells. To overcome this obstacle, we subjected the digested thymic cells to CD90 microbeads to deplete the thymocytes, which comprised of more than 90% of the cells in the thymus.



**Figure 3. Preservation of extracellular matrix proteins in thymic scaffolds.** Representative cryo-sections of thymic scaffolds stained with antibodies against collagen I and IV, fibronectin and laminin, and counter-stained with DAPI for DNA. N, untreated naïve thymus; D, decellularized thymus.

The isolated stromal cells were introduced in a small volume (~20mL) into the scaffold which was soaked in RPMI-10 medium overnight. The reconstructed thymus was cultured as a hanging drop, on the cover of the tissue culture dish filled with PBS to maintain moisture (Figure 4B). After 7-days of culture, a more dense appearance was observed in the matrix scaffold injected with thymic stromal cells (Figure 4D), in comparison to the scaffold cultured alone (Figure 4C), suggesting the successful colonization of the injected cells. Indeed, immunohistochemical analysis of the reconstructed scaffold revealed that thymic epithelial cells, endothelial cells and stromal cells of hematopoietic lineage were all seeded and effectively formed clusters (Figure 4, E-G).



**Figure 4. In vitro reconstruction of mouse thymus.** **A.** Culture of thymus scaffold injected with entire population of thymic cells in 6-well transwell. **B.** Hanging drop culture of reconstructed thymic scaffold. **C** and **D.** Thymus scaffold cultured as hanging drop for 7 days. **C,** scaffold alone; **D,** reconstructed with stromal cells. **E, F** and **G.** Immunohistochemical analysis of reconstructed thymus. Representative cryosections of the reconstructed thymus were stained with anti-EpCAM (**E**, red, for thymic epithelial cells), anti-CD31 (**F**, red, for endothelial cells of blood vessels) and anti-CD45 (**G**, red, for stromal cells of hematopoietic lineages) antibodies, and counter-stained with UEA (green in **E, F** and **G**, for medullary thymic epithelial cells).

In summary, we have successfully developed in this quarter a novel protocol that enables us to generate the 3D scaffold of the thymus which retains all the extracellular matrix structure, but is in absence of any cellular component. We have also shown *in vitro* that thymic stromal cells can be introduced into and successfully colonize in the thymus scaffolds. We are currently testing whether these reconstructed thymus can effectively support lymphopoiesis *in vivo* by transplanting them into athymic nude mice.

2. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter, we will focus on investigating the development of thymocytes in the reconstructed thymus *in vivo*. Circulating bloods of the athymic nude mice transplanted with the reconstructed thymus will be sampled and examined for the presence of T-lymphocytes every week. 8-weeks after the thymus transplantation, the T cell subsets present in the secondary lymphoid organs will be characterized and their functions will be examined. In addition, we will also try to test the protocol in monkey thymus.

**Our second quarterly scientific progress report for the second year of our project (10/12/12 – 01/11/13) described the following:**

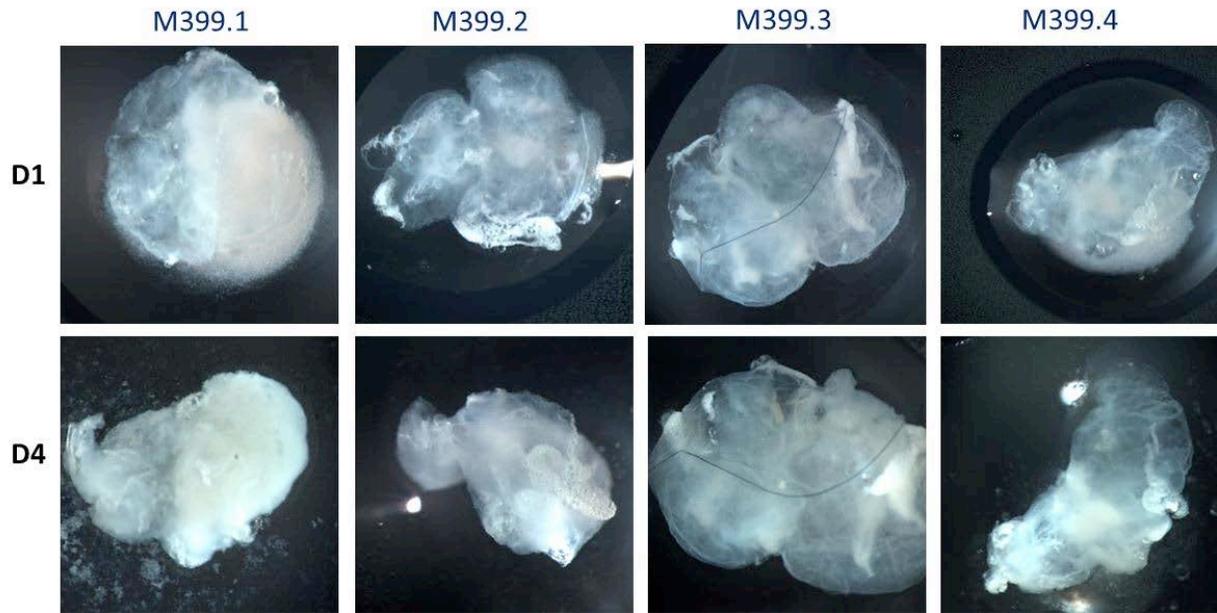
In the previous quarterly report, we focused our effort to examine the feasibility of using decellularized thymus scaffolds to reconstruct the thymus organ with genetically modified mouse thymic stromal cells *in vitro*. Such a novel approach, if successful, will allow us to achieve higher efficiency of thymic insulin expression knock-down, an essential prerequisite to test the hypothesis that “*the insulin-shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of autoimmune reaction, specifically directed against the beta cells of the pancreas*” (Objective 2).

Our results from the last quarter showed that we are able to develop a protocol to generate 3D thymic scaffolds that can retain all the extracellular matrix structure, but are in absence of any cellular component. Briefly, thymic lobes harvested from 3-8 week old mice were treated with 0.1%SDS overnight, followed by 1% Triton X-100 for 2 hours and stored in PBS at 4°C before use. To reconstruct the thymus, CD45 negative thymic stromal cells (i.e. thymic epithelial cells, fibroblasts and endothelial cells) harvested from with 3-4 week old mice, were injected into the decellularized thymus scaffolds and cultured *in vitro* as hanging drops in RPMI-10 medium for 3-7 days. The injected thymic stromal cells can successfully colonize the decellularized scaffolds and express cell-specific markers, such as EpCAM for TEC cells and CD31 for vascular endothelial cells, indicating that these cells can survive the 3D scaffold environment and maintain their identities.

In this quarter, we refined our protocol to achieve more efficient thymus reconstitution and examined the capability of these reconstructed thymi to support thymopoiesis *in vitro*. In addition, we performed thymus transplantation experiments, in which the reconstructed thymi were transplanted into athymic nude mice, to examine their potential to support T-cell development *in vivo*.

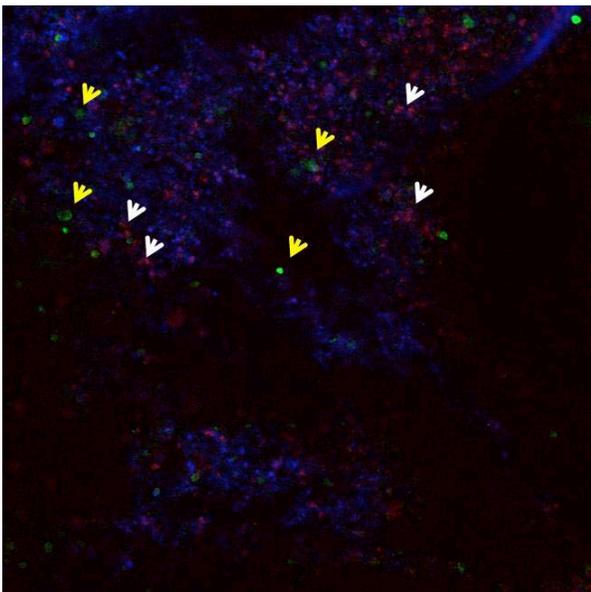
### **Reconstructed thymus supports thymocyte development *in vitro*.**

We refined both the thymus scaffold preparation protocol and the thymic stromal cell injection procedure to achieve better efficiency of thymus reconstitution. An example of one of the thymus reconstruction experiments was shown in Figure 1 (*top panels*). One day after the thymic cell injection, the reconstructed 3D scaffolds remained densely populated by the thymic cells, indicating success of cell colonization. Of note, an increase of density, comparing to that of day1, was observed on day 4 of the *in vitro* culture, suggesting an increase of cellularity within the reconstructed scaffold (**Figure 1**, lower panels).



**Figure 1. Effective reconstruction of the thymus with thymic stromal cells.** Decellularized thymus scaffolds were injected with CD45 negative thymic stromal cells and cultured as hanging drops. D1, one day after the reconstitution. D4, 4 days after *in vitro* culture.

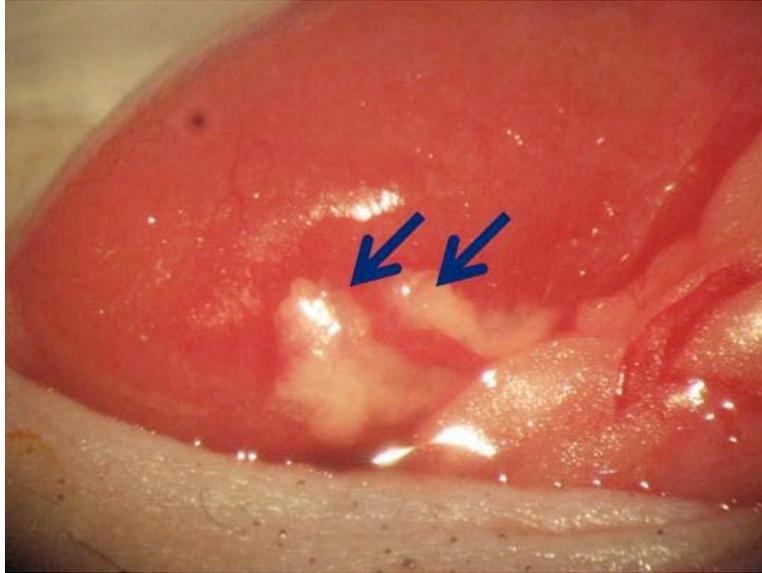
To investigate whether the thymic stromal cells seeded in the 3D scaffolds can support thymocyte differentiation *in vitro*, we injected the lineage negative bone marrow progenitor cells into the reconstituted thymic scaffolds, and cultured them for 4 days. As demonstrated with immunohistochemical analysis (**Figure 2**), CD4+ and CD8+ cells beyond DP stage of thymocyte development were readily detected, in conjunction with UEA1+ medullary thymic epithelial cells, indicating that thymic scaffolds reconstituted with lineage-minus bone marrow progenitor cells can support the development and differentiation of thymocytes.



**Figure 2. Thymocyte development in reconstructed thymus.** Representative cryosections of thymic scaffolds reconstructed with thymic stromal cells and bone marrow progenitor cells were stained with anti-CD4 and -CD8 antibodies (Red) for DP and SP thymocytes (White arrow heads), UEA-1 (Green) for mTECs (Yellow arrow heads), and counter-stained with DAPI (Blue). Of note, many cells were stained with neither DP/SP thymocyte marker, nor mTEC marker, which were comprised of developing thymocytes of early stage, uncommitted bone marrow progenitor cells, fibroblasts as well as other thymic stromal cell types.

### Reconstructed thymus supports T-cell development *in vivo*.

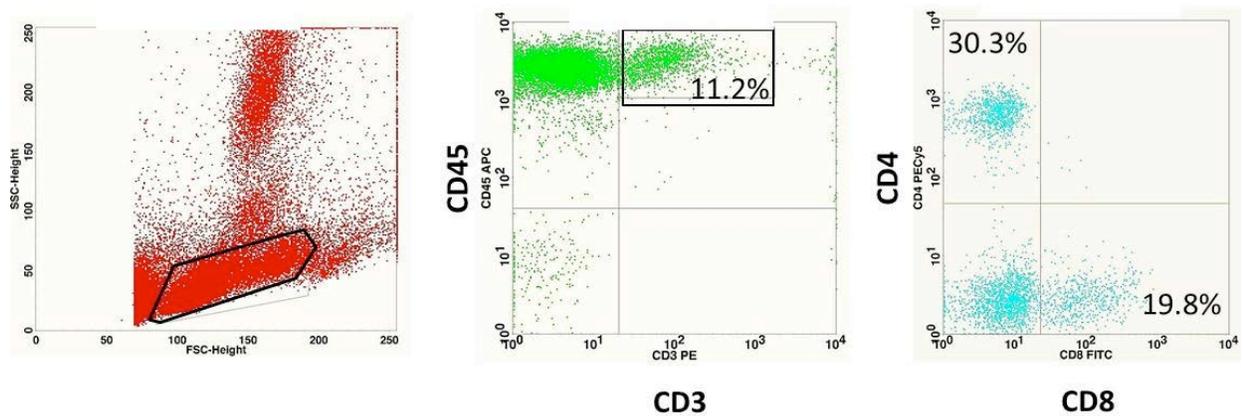
To demonstrate that the reconstructed thymi can support T-cell development *in vivo*, we performed thymus transplantation experiments, in which the reconstructed thymi were surgically transplanted underneath the kidney capsules of athymic nude mice (**Figure 3**).



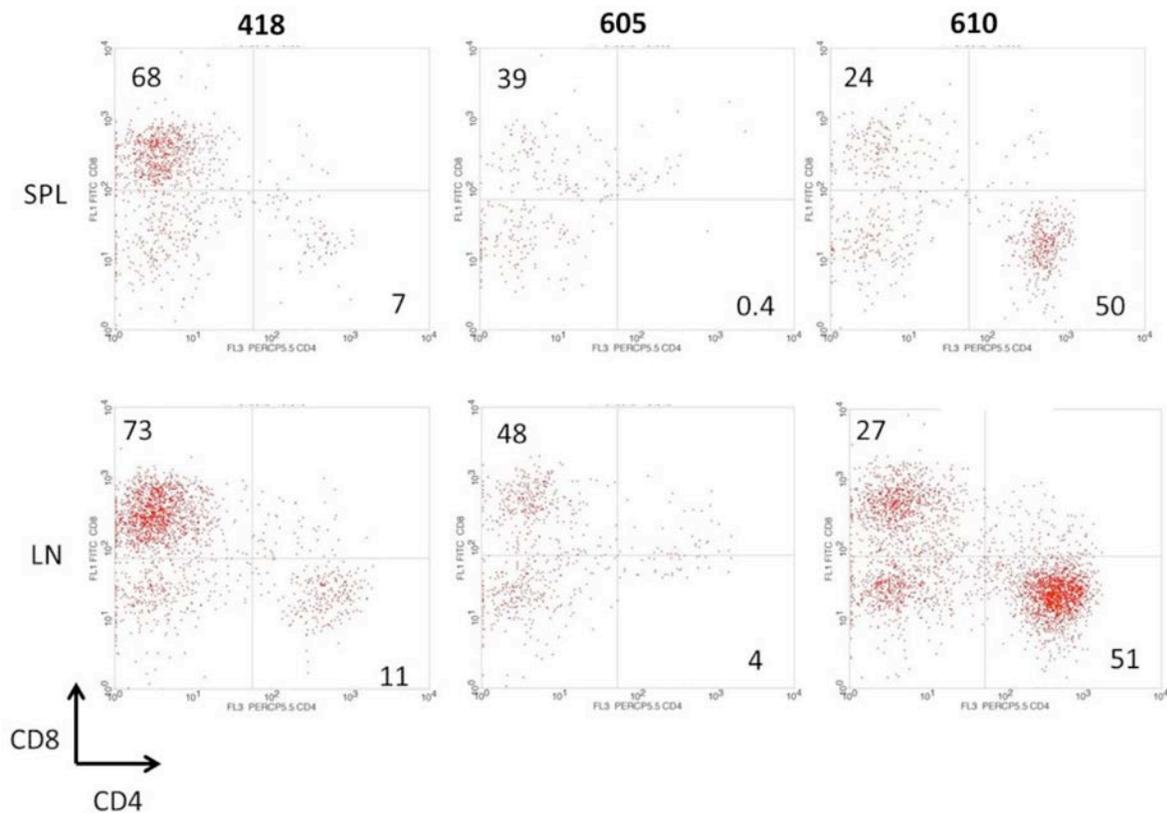
**Figure 3.** Transplantation of the reconstituted thymus (Blue arrows) underneath the kidney capsule.

To monitor the development of T-cells in circulation, peripheral blood samples were drawn 4 weeks post thymus transplantation, and analyzed by flow cytometry (FCM). As shown in **Figure 4**, both CD3+CD4+ T-helper cells and CD3+CD8+ cytotoxic T-cells were present in the circulation, at levels similar to normal B6 mice, suggesting that the reconstructed thymi are sufficient to support the maturation of developing T-cells.

To further evaluate the status of T-cell development in the nude mice transplanted with the reconstructed thymi, the mice were sacrificed and the presence of T-cells in the secondary lymphoid organs, their primary resident sites, was examined with FCM analysis. As shown in **Figure 5**, all three animals examined showed success of T-cell development, even though the degrees of success varied between animals, presumably due to the numbers of thymic stromal cells injected into the scaffolds. Nevertheless, these results further demonstrated that the reconstructed thymi transplanted were sufficient to support *de novo* thymopoiesis in the recipients.



**Figure 4. Presence of circulating T-cells in thymus reconstituted athymic nude mice.** 4-weeks after the surgical transplantation of the reconstituted thymus, blood samples were harvested from nude mice and stained with anti-CD45, anti-CD3, anti-CD4 and anti-CD8 antibodies. Representative FCM images were shown, which were first gated on the lymphocytes (left panel), followed by CD3 gating (middle panel), and analyzed for CD4+ and CD8+ T-cells (right panel).



**Figure 5. Presence of T-cells in secondary lymphoid organs in thymus reconstituted athymic nude mice.** Representative FCM images, from three athymic nude mice, 418, 605 and 618, were gated on the CD45+CD3+ cells and shown for CD4+ and CD8+ expression. SP, spleen; LN, lymph nodes.

In summary, using mouse models, we have demonstrated, both *in vitro* and *in vivo*, the feasibility of applying our novel thymus reconstruction protocol to genetically modify thymus for gene therapy purpose.

**12.** Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter, we will apply this novel approach to genetically modify insulin expression in the monkeys. To achieve such goals, we will need to first refine the protocol further so that thymic stromal cells can be cultured alive *in vitro* for 3-4 days, as it takes 48-72 hours for EGFP proteins expressed by the transduced lentiviruses to mature for FACS isolation.

**Our third quarterly scientific progress report for the second year of our project (01/12/2013 - 04/11/2013) described the following:**

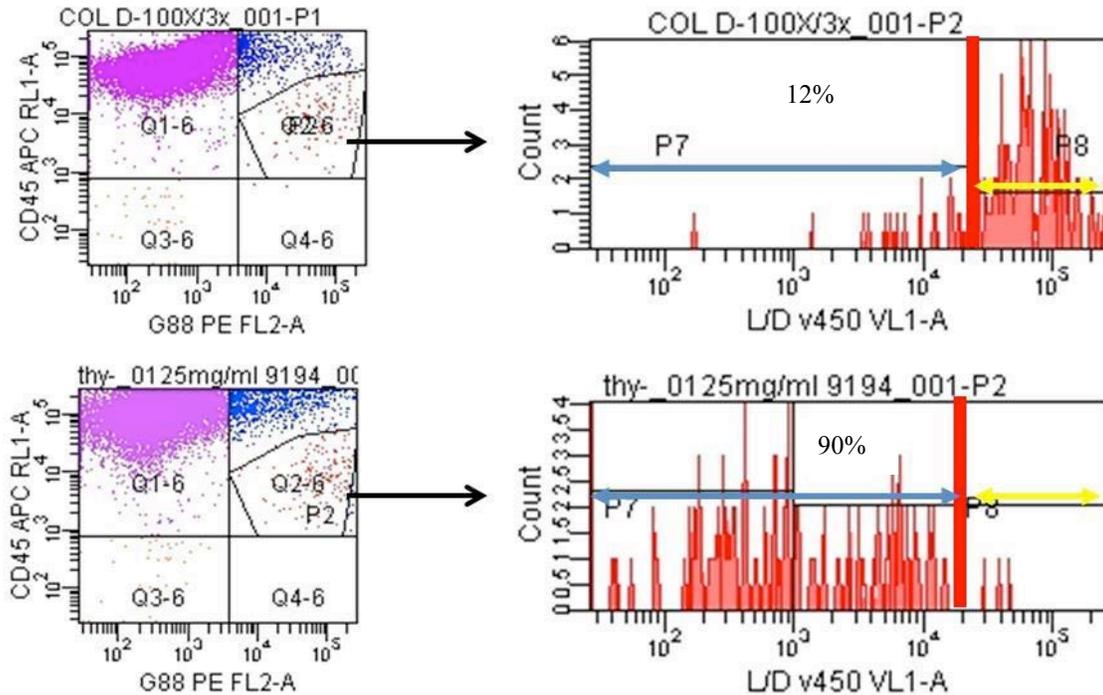
We have showed in the previous quarter that we were able to reconstruct the decellularized thymus scaffolds with isolated thymic stromal cells, and that these reconstituted thymi can support the development and maturation of thymocytes both *in vitro* and *in vivo*. While our results clearly demonstrated the technical feasibility of using this novel approach to generate a functional thymus from isolated thymic stromal cells, there are still a number of steps in the procedure that need to be further refined if we want to implement the protocol on large animals, such as nonhuman primates. First, it is notoriously difficult to isolate the thymic epithelial cells (TECs), as we routinely lose more than 50% of TECs with the widely used collagenase D-based isolation protocol. In addition, the purity of the crude collagenase D varies from batch to batch, and it is hard to get consistent results. Second, to genetically modify the TECs with lentiviral particles expressing the gene of interest, it is necessary to culture the cells *in vitro* for at least 48-72 hours. This will not only increase the transduction efficiency of the lentiviruses, but also allow the transgene to produce enough reporter molecules (EGFP in our case) so that the virus-transduced cells can be enriched with FACS. However, most of the TECs undergo apoptosis within the first 24 hours when cultured *in vitro* in absence of the unique thymic 3D environment. For those that survive the first 24 hours, they tend to lose their unique capability to support the survival and selection of developing thymocytes and express genes that are specific to terminally differentiated 2D epithelial cells such as the skin epithelial cells. Overcoming these technical bottlenecks is pivotal to the success of Objective 2 of the project: "*the insulin-shRNA-treated thymus, once transplanted back into the immunocompetent-cell-depleted donor monkey, will facilitate the onset of autoimmune reaction, specifically directed against the beta cells of the pancreas*".

In this quarter, we focused on our efforts to solve these technical challenges. First, we developed a novel TEC isolation protocol, with which we were able to isolate twice as many thymic stromal cells as before. In addition, we developed a fluorescent microscopy-based method to monitor the viability of cells within the scaffolds and found that these cells were able to survive more than 7-days *in vitro*. Furthermore, we tested a number of protocols and identified a method that will enable us to achieve high efficient lentiviral transduction of mTECs. By solving these technical bottlenecks in this quarter, we are at the stage to perform our first monkey thymus reconstruction experiment, in which decellularized monkey thymus scaffolds will be reconstructed with TECs transduced with insulin-specific shRNA lentiviral particles, and be transplanted into the thymectomized monkey.

#### **Refinement of the TEC isolation protocol.**

To improve the TEC isolation protocol, we experimented with various types of collagenases (i.e., collagenase D, collagenase P, dispase and liberase TM) and compared their impacts on the yield and viability of the isolated TECs. Replacing collagenase D with either collagenase P or dispase failed to improve the efficacy of TEC isolation (data not shown). In contrast, we were able to

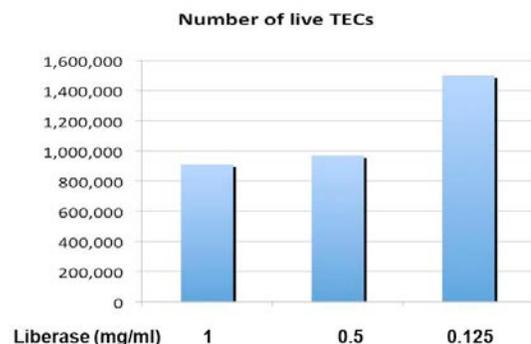
obtain twice as much of TECs per thymus using a blend of highly purified collagenase and neutral protease enzymes (liberase TM), in consistent with recent publications from Chidgey's group from Australia (Figure 1).



**Figure 1. Liberase TM digestion significantly improves the viability of isolated TEC cells.** Thymus was treated with either collagenase D (3 rounds of 15 minutes each), followed by one round of dispase digestion (15 minutes, *upper panels*), or liberase TM (3 rounds of 12 minutes each, *lower panels*). Isolated thymic cells were first stained with nucleus binding violet dye (v450) for dead cells, followed by staining with antibodies specific for TECs (g8.8) and hematopoietic lineage cells (CD45). Right panels: representative flow cytometry (FCM) quadrant plots from whole individual thymus are shown. Right panels: histograms show the percentage of viable CD45<sup>-</sup>g8.8<sup>+</sup> TECs based on the intensities of violet dye staining. Blue lines, live cells with low violet signal; yellow lines, dead cells.

To further refine the TEC isolation protocol, we experimented further with different concentrations of liberase TM, and were able to identify the optimal condition with which we can minimize the loss of viability and increase recovery of the TECs (Figure 2). Taken together, with the newly established protocol with 0.125mg/ml of liberase TM, we were able to obtain consistently twice as many live TECs per thymus, comparing to the traditional collagenase D protocol.

**Figure 2. Numbers of live TECs isolated per thymus with different concentration of liberase.**

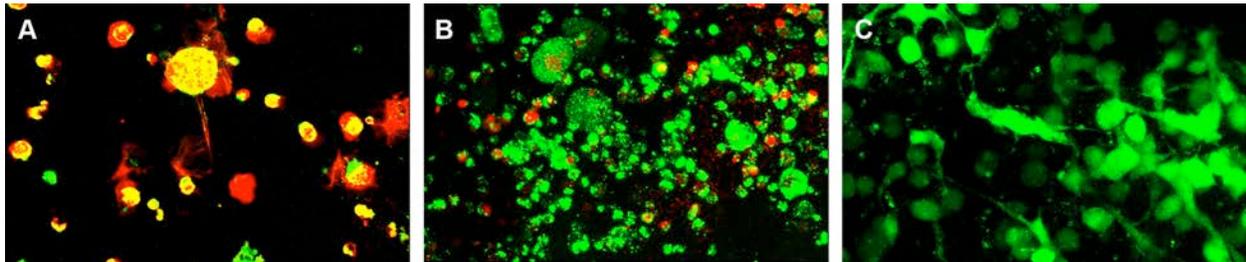


### Detection of live TECs in the reconstructed thymus

It is difficult to keep TECs alive *in vitro* in 2D culture, as they tend to undergo apoptosis and change their molecular property. To monitor the viability of TECs in the decellularized 3D thymus scaffolds, we experimented with the LIVE/DEAD® Viability/Cytotoxicity Kit from Invitrogen, which takes advantage of two distinguishing characteristics of live cells: the ubiquitous intracellular esterase activity and an intact plasma membrane. Live cells with intracellular esterase activity are stained with green-fluorescent calcein-AM, whereas dead cells with compromised plasma membrane integrity are stained with red-fluorescent ethidium homodimer-1.

We optimized the conditions for staining TECs in the 3D scaffolds. For comparison, we also stained the TECs cultured as hanging drops for two days. As shown in **Figure 3A**, most of the cells were stained with red-fluorescent ethidium homodimer-1, indicating that they are either dead cells (red) or cells undergoing apoptosis (yellow). In contrast, most of the cells remained positive for calcein-AM staining at 4 days after 3D scaffold culture (**Figure 3B**). At day 7, most of the ethidium homodimer-1 staining cells disintegrated and only live cells stained for calcein-AM were detectable (**Figure 3C**). Of note, these cells adopted various shapes similar to TECs and fibroblasts in the thymic stroma, suggesting that the TECs have successfully colonized the 3D scaffolds. Taken together, these results showed that we have successfully developed a method, which enabled us to maintain TECs alive for up to 7 days *in vitro*.

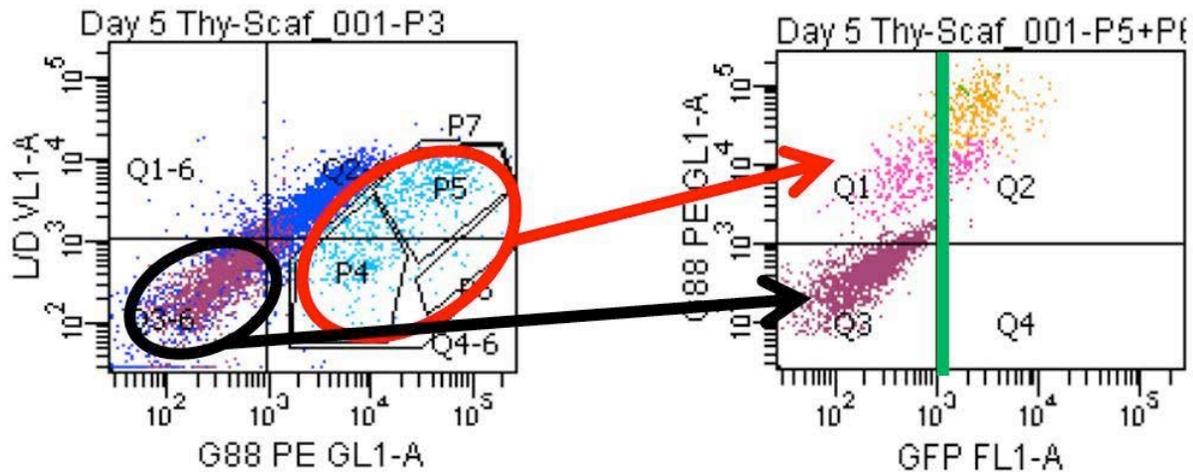
### Efficient lentivirus transduction of TECs.



**Figure 3. Detection of live TECs in the reconstructed 3D scaffolds cultured *in vitro*.**

To transduce the isolated TECs with lentiviral particles, we first experimented with TECs cultured in hanging drops supplemented with EGFP-expressing lentiviral particles at MOI 4:1. As shown previously in Figure 3A, most of the cells underwent apoptosis, and the transduction efficiency was extremely low (data not shown).

To address this bottleneck issue for genetic manipulation of TECs, we decided to take advantage of the established TEC culture methods in 3D scaffolds and transduced the TECs directly in the reconstructed thymus. Isolated TECs were resuspended in small volumes with medium supplemented with lentiviral particles at MOI 4:1 to reconstruct the decellularized 3D thymus scaffolds. The reconstructed thymus was cultured as hanging drops for 2 days, and was transferred to the top chamber of a transwell culture dish for 3 more days. At day 5, the scaffolds were harvested, digested and the transduction efficiency was evaluated with FCM. As shown in **Figure 4**, more than 40% of CD45-g8.8+ TECs isolated from the reconstructed scaffolds were positive for EGFP. Interestingly, lentiviral particles were found to preferably transduce TECs, as most of the CD45-g8.8- thymic stromal cells (e.g. fibroblasts and endothelial cells) were negative for EGFP under the same conditions. Thus, with these technical manipulations, we were able to transduce the TECs at higher efficiency than before, and were able to keep them alive at the same time.



**Figure 4. Lentiviral transduction of TECs in reconstructed thymus scaffolds.** Representative FCM plots of thymic stromal cells harvested from the reconstructed scaffolds, cultured for 5 days in the presence of EGFP-expressing lentiviral particles (MOI, 4). The cells were gated on the CD45- stromal cell population. Red circle, g8.8+ TECs; black circle, g8.8- thymic stromal cells.

In conclusion, we were able to solve three major technical challenges in the quarter. Specifically, we developed: 1) a protocol which significantly increased the yield and viability of the isolated TECs; 2) a method that enabled us to monitor the viability of TECs in real-time in vitro; 3) the protocol to transduce TECs with lentiviruses at high efficiency. These technical improvements are essential for the success of this project.

**12.** Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter, we will take advantage of the technical improvement developed in this quarter and perform the thymus reconstruction in monkeys.

**In the fourth quarterly scientific progress report (04/12/13 - 07/11/13) of year 02, we now report on our new and year 02 cumulative results.**

In type 1 diabetes (T1D), self-reactive T-cells kill the pancreatic, insulin-producing, beta cells. These T-cells, generated in the bone marrow, enter into the circulation due to faulty presentation of self-antigens (initially insulin) by the medullary TEC, an event that hinders their negative selection. Once the first few beta cells are damaged by insulin-specific effector T-cells, cellular debris are brought by scavenger cells (e.g., dendritic cells) to the proximal lymph nodes. Here, “new” (i.e., previously not exposed) antigens, originally lodged on these beta cell components, are presented to naïve T-cells that in turn get activated towards them. The newly activated effector T-cells eventually go back to the islets of the pancreas and kill additional beta cells targeting antigens other than insulin. This phenomenon, referred to as “epitope-spreading”, results in a vicious circle that brings the surviving pancreatic beta cells to a number too small to maintain the body’s insulin needs. At this juncture, clinical diabetes manifests. Pharmacologic

insulin replacement offers a quasi-normal lifestyle favored by an acceptable, yet imperfect gluco-regulation. In the long run, however, insulin replacement cannot adequately maintain glucose homeostasis to completely prevent diabetic complications like cardiovascular diseases, nephropathy, retinopathy and neuropathy (1).

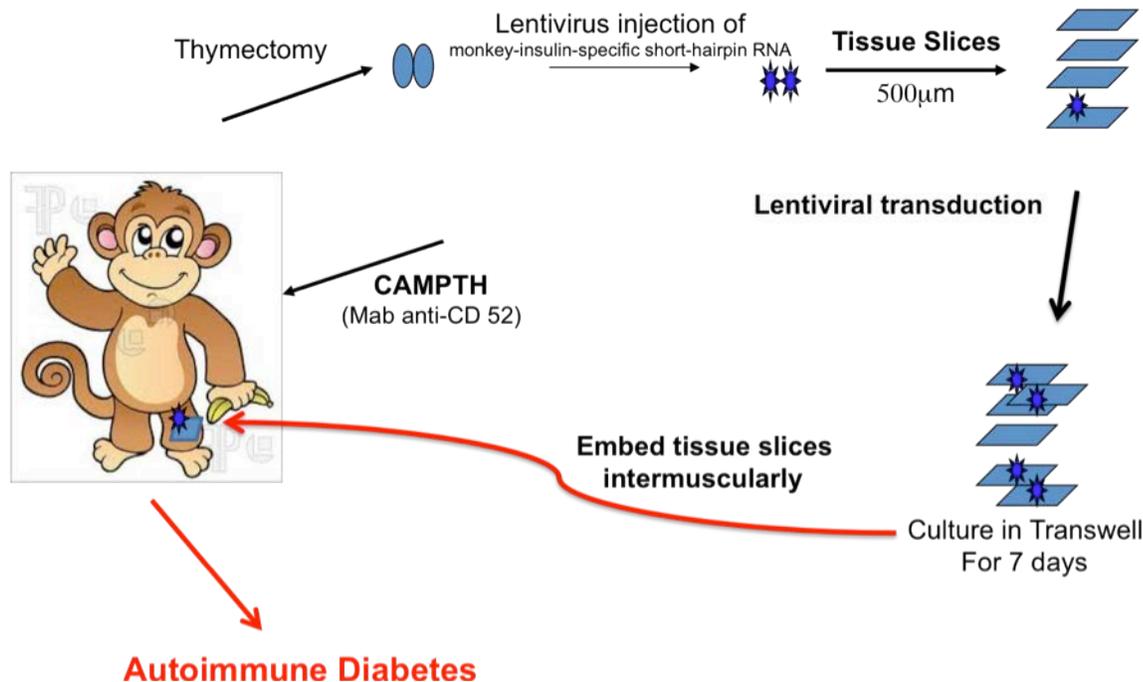
Currently, the most actively pursued therapeutic approaches to avoid these daunting complications include, together with more conventional immunosuppressive therapies, islet allo-transplantation, stem cell-based islet replacement, and beta cell regeneration attempts. Although significant advances have been made in all these areas, and an enormous number of new approaches to cure diabetes have been successfully tested in the NOD mouse -- the genetically diabetes-prone non-obese diabetic mouse strain, whose etiopathogenesis is widely-held to parallel the one that occurs in humans -- they simply did not work as well in humans (2). The gap between mice and humans seems to be too large to justify the translation of many immunomodulatory therapies efficacious in mice directly to human individuals. But, even if the non-human primate (NHP) seems to be the best species available for testing new therapeutics on the basis of the phylogenetic similarities between monkeys and humans, we have to consider that NHPs do not spontaneously develop autoimmune diabetes.

Given these considerations, we thought it useful to find the means to promote autoimmune diabetes in NHP. Towards this objective, we were motivated by the evidence proving that insulin expression in the thymus can regulate the negative selection of autoreactive T-cells, being the first self-antigen whose lack of expression in the thymus is sufficient to break central immune tolerance towards pancreatic beta cells (3-5). To gain the functional insights of this ectopic insulin expression, we also tested this tenet by taking advantage of the Cre-lox system to knockout the mouse *Ins2* gene specifically in *Aire*-expressing medullary TECs, without affecting insulin production in the pancreatic beta cells (6). These ID-TEC (i.e., carrying insulin-deficient TECs) mice - previously also crossed to an *Ins1* knockout background - spontaneously developed diabetes around three weeks after birth, even in a genetic background expressing diabetes-resistant H-2b MHC molecules.

Beta cell-specific, autoimmune destruction was observed and documented by the presence in the islets of effector T-cells, directed specifically towards insulin epitopes. More pertinent are the results obtained from ID-TEC-thymus transplantation experiments in a nude (thymus-deprived) mouse, that proved how the transplanted *Ins2*-depleted thymus is sufficient, to a) successfully reconstitute the animal's T cell repertoire, b) break its central tolerance, and c) induce anti-insulin autoimmunity.

This study suggested that in a monkey, like in the mouse, a drastic reduction of expression of self-antigens (e.g., insulin) in the medullary TECs might favor the generation of an autoimmune reaction specifically directed against the insulin-producing cells of the pancreas, eventually bringing the animal to clinically overt T1D. On this basis we intended to engineer autoimmune diabetes in *Cynomolgus* monkeys (i.e., *Macaca Fascicularis*) by: a) safely removing the thymus; b) transducing its tissue *in vitro* with a lentivirus expressing monkey-insulin-specific short-hairpin RNA (shRNA) able to knock-down insulin expression; and c) returning the transduced thymus back into the original donor monkey (whose mature B- and T-cells had been concomitantly depleted), to facilitate the onset of autoimmunity against pancreatic beta cells.

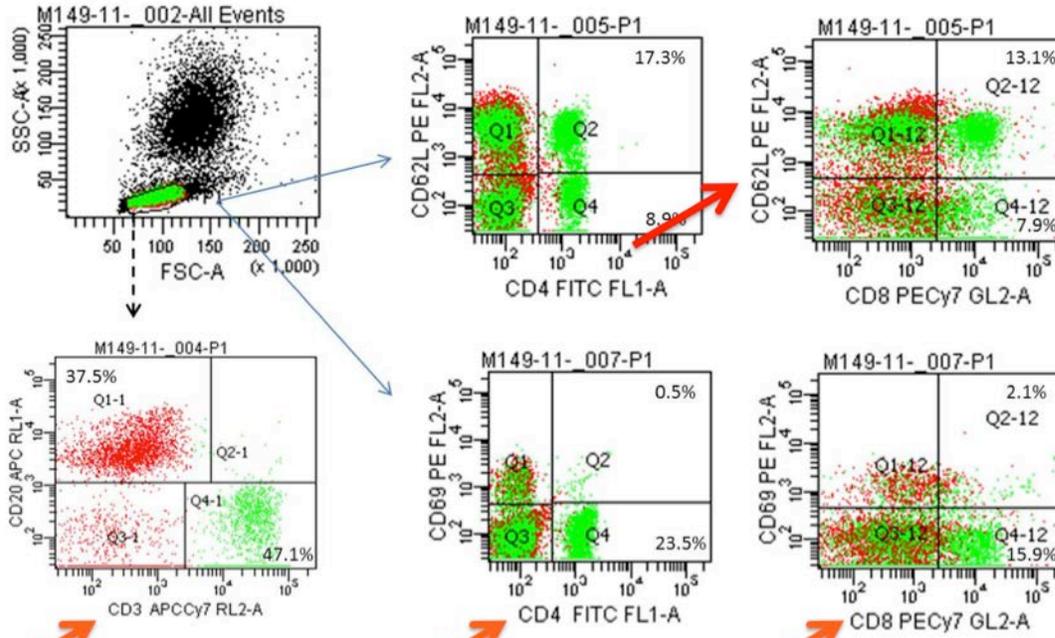
## Experimental Procedure



Even if the transduction with the viral vector did not result in 100% of the thymic cells transduced, a drastical reduction in insulin expression & presentation was expected to be sufficient to favor the development of a specific autoimmune reaction, as convincingly demonstrated by Chentoufi & Polychronakos in 2002 (7). Mice expressing low thymic insulin levels exhibited detectable peripheral T-cell reactivity to insulin, whereas mice with normal levels showed no significant increases in anti-beta cell T-cell reactivity compared to non-autoimmune controls. The different levels of thymic insulin expression seem to have the same consequences in humans (8).

Following Alemtuzumab depletion of all immuno-competent cells in the thymectomized monkey and transplantation of the transduced thymus back into the donor animal, we observed a successful repopulation of its immune cells (mature and functional T- and B-lymphocytes) after roughly three months after transplantation. In response to lymphopenic environmental stimulation, the T-cells in circulation could arise from the transplanted thymic sections, from the residual thymus (the thymectomy could be sometimes incomplete), or from the proliferation of residual T-cells residing in the secondary lymph organs that might not be killed by Campath. So far we were unable to distinguish T-cells derived from the first two origins; however, T-cell expanded from the secondary lymphoid organs will carry memory T-cell markers, such as CD69, while naïve cell marker, e.g., CD62L, expression will be down-regulated. Expression of surface markers on circulating T-cells were characterized by FCM analyses of blood samples harvested from the experimental monkey. Ten 10 weeks after thymic tissue transplantation, distinct populations of lymphocytes (37.5% CD20+ B-cells and 47.1% CD3+ T-cells) were present, practically overlapping the percentages of a non-treated, naïve monkey. Further characterization of CD3+ T-cell population revealed that the majorities of CD4+ T-helper cells and CD8+ cytotoxic T-cells are expressing CD62L, suggesting that they are newly generated T-cells from the thymic tissues. Consistently, neither CD4+ nor CD8+ T-cells are expressing the surface marker, CD69, of activating T-cells, further suggesting that the majority of the circulating T-cells remained at the naïve stage. Furthermore, the isolated T-cells were proven "functional" once proliferated in an MLR against irradiated allo-stimulators.

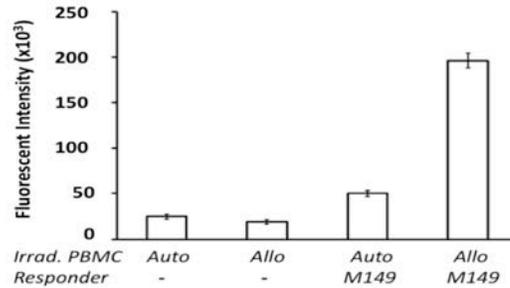
## Recovery of CD4 & CD8 T cells in the blood of a Monkey after Thymic Auto-Transplantation



## Summary of White Blood Cells in a Monkey after Thymic Auto-Transplantation

Post Thymectomy Days	# of T-cells	WBC	Lympho %	T-Cell %	CD62L:CD3%	CD69:CD3%	CD4:CD62L%	CD69:CD4%
0	5000	8.6	80	74.3	n/a	n/a	33.2	0.7
7	11	2.1	3.7	15.1	13	0.4	31.7	0.9
14	35	2.1	13	12	5.4	0.5	23.9	0.6
21	183	3.6	20	25.4	7.3	6	n.d.	n.d.
28	510	3.4	43	34.9	8.3	6	n.d.	n.d.
35	406	3.1	40	32.8	10.2	7.8	13.2	2.1
41	411	5.8	22	32.2	14.5	0.9	14.7	0.9
48	692	4.8	35	41.2	22.9	6	21.9	1.4
55	790	7.8	21	48.2	23.5	5.7	19.4	0.7
63	946	6.2	32	47.7	35.8	6.1	33.1	1.8
69	1498	6.8	46	47.9	24.8	7.8	24.7	0.8
73	830	4.1	43	47.1	31.4	2.2	32.1	0.9
80	1570	6.4	42	58.4	37.8	7.8	34.8	0.6
87	1105	7.4	29	51.5	29.9	3.3	31	0.4

## Isolated T-cells were proven “functional” in MLR test



Mix lymphocyte reaction (MLR) responses in monkeys with thymus transplantation.  $2 \times 10^5$  lymphocytes isolated from thymectomized and thymic section-transplanted monkey M149 (*Responder*) were cultured with equal amounts of X-ray irradiated (2000 rad) autologous (*Auto*) or allogeneic (*Allo*) monkey PBMCs. After 5-days of culture, the number of living lymphocytes was measured with CyQUANT direct cell proliferation kit (Invitrogen). All the assays were run in triplicate.

However, we quickly envisioned a number of obstacles for the lentivirus-mediated mINS knock-down approach: 1) The large size of the thymus prevented the lentiviruses as well as nutrients from gaining access to stromal cells inside the organ; 2) The large number of thymocytes present in the thymus (~100-1000 times of the stromal cells) affected the efficiency of lentivirus transduction of stromal cells, even if we employed tissue slicers to cut the thymus into 0.5mm sections and cultured them in 6-well trans-wells in the presence of 2-deoxyguanosine to eliminate proliferating thymocytes. Under such *ex vivo* culture conditions, stromal cells within thymus sections were viable and sufficient to support thymocyte development when transplanted under the kidney capsules of athymic nude mice. The generation of thin sections was also useful for directly implementing the transplantation approach previously described for treating complete DiGeorge syndrome and selected by our surgeons for reaching the final aim of our project. The reconstitution of the monkey T-cell repertoire after Campath treatment and thymus transplantation was a success. However, the exam of the presence of insulin-specific autoreactive T-cells in peripheral blood gave negative results. Enzyme-linked immuno-sorbent spot (ELISPOT) assays were performed, in which human insulin molecules were used as stimulating autoantigens. Specifically, peripheral blood mononuclear cells (PBMCs) were harvested from the monkey 10 weeks after thymic transplantation and cultured in 96-well tissue culture dishes coated with anti-IFN $\gamma$  antibody overnight, in the presence or absence of insulin (100ug/ml). As positive control of T-cell function under stimulus conditions,  $10^4$  PBMCs were cultured with PMA & ionomycin, which activate T-cells in an antigen independent manner. Robust T-cell activation and IFN $\gamma$  secretion were observed upon PMA & ionomycin stimulation, indicating that the T-cells derived from the reconstituted thymus are functional and capable to mount TH1-type responses under pro-inflammatory conditions, an essential prerequisite for successful adaptive immunity. At that time, however, we didn't observe any elevated number of insulin-reactive T-cells in peripheral circulation, in comparison to pre-treatment blood samples. Thus the important issues of the efficiency of the lentivirus transduction remained unsolved.

We concluded that no more than ~5% of insulin reduction in the transplanted thymus, obtained by the process of transducing *ex vivo* the sliced thymic tissue, exposing them *in vitro* to the monkey insulin-specific shRNA expressing vector was insufficient to break central tolerance and failed to promote clinically-overt diabetes in the recipient monkey.

**ELISpot**  
of  
**IFN- $\gamma$  Expressing T-cells in Monkey's PBMCs**  
(10 Weeks After Thymus Auto-transplant)

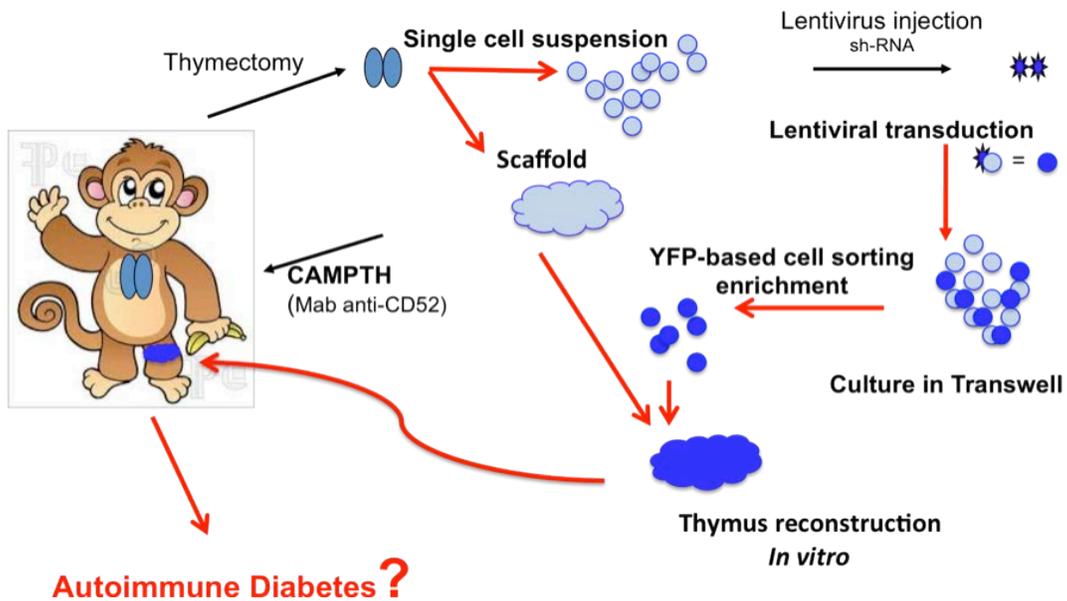


PMA+Ionomycin,  $10^4$  cells; all other conditions,  $3 \times 10^6$  cells.

The only way to achieve better transduction with the vector -- similar to the outcome obtained *in vitro* using a cell line carrying a monkey-insulin expression vector -- was then to decompose the thymic tissue so to obtain a single cell population, which could be more efficiently exposed to the shRNA carrying vector.

The problem remained as to reconstruct the thymic matrix in order to transplant it back to the donor monkey. Thymic slices were used in the first attempts specifically to facilitate this step. We solved this problem utilizing the process previously described that, in the mouse, successfully repopulates a thymic scaffold with enriched TECs/TEC precursors. Transplanting the reconstructed thymus, we successfully reconstituted mature and functional T- and B-cells in nude mice.

**Improved Experimental Procedure**



Recently we successfully accomplished the same task in thymectomized monkeys transplanted with reconstructed thymi following the same protocol. Also, after abrogation of insulin expression in TECs/TEC precursors, by exposing them, in a single cell suspension, to monkey-insulin-specific short-hairpin RNA, we generated insulin-specific autoimmunity in other recipient monkeys.

Thymus scaffold preparation: On the basis of results in mice, thymi were collected from monkey donors and stored at  $-80^{\circ}\text{C}$  until “de-cellularization” was initiated. For de-cellularization, thymi were thawed in a  $37^{\circ}\text{C}$  water bath. The process of freezing and thawing was repeated three times to enhance cell damage and facilitate cell loss. Next, ionic detergent, 0.1% SDS (Invitrogen) in deionized water was added to the thymi that were placed on a shaker for constant rotational speed of 30 rpm. After the tissues became translucent and white in color (24 hours), subsequent steps were: 15 min each of three PBS washes and 30 min of 1% TritonX-100 (Sigma Aldrich) in deionized water. This is followed by three more PBS washes of 15 min each. A final rinse step of PBS with Pen/Strep (100U/ml) was added and rotated for additional 48 hours to clear out all cellular debris. Once the clean (i.e., completely de-cellularized) scaffold was obtained, it was kept at  $4^{\circ}\text{C}$  in tissue culture medium until the TECs/TEC precursors from another thymus were collected in sufficient numbers. De-cellularized thymus scaffolds, stored at  $4^{\circ}\text{C}$  in PBS containing Pen/Strep were usable for up to one month.

### Monkey Thymus De-cellularization

1 hr

12 hr

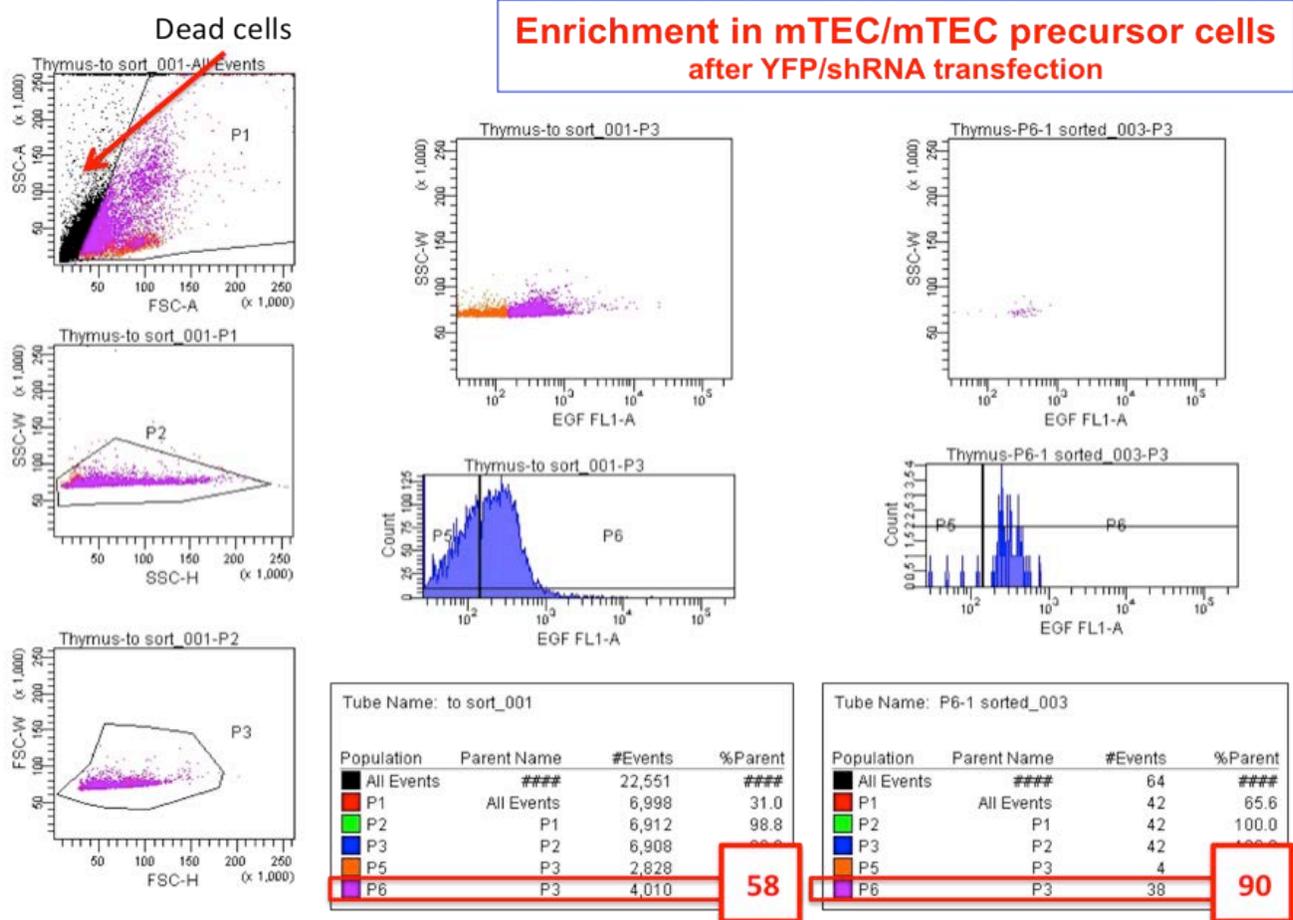
32 hr



It is perhaps useful to stress that a scaffold prepared this way is by definition **acellular** in nature so that allogeneic or even xenogeneic scaffolds can be used instead of syngeneic ones to reconstruct a thymus. In fact, should the procedure be translated in monkeys or in humans, the individual's own TECs/TEC precursors cannot be obtained from their thymus de-cellularized in the manner shown above to obtain a suitable scaffold. In monkeys and eventually in humans, TECs/TEC precursors will be harvested from the donor thymus with a different and gentler approach (see below) so to be successfully used to repopulate a genetically-heterologous, de-cellularized scaffold. The reason why we propose to first use the “thymic” scaffold instead of the scaffold of unrelated tissues or organs, or even synthetic scaffolds, is based on studies with other types of tissue and organ reconstruction which seem to suggest that the native scaffold is critical for the phenotypic and functional potency and maintenance of the original populating cells. In other words, at this time, we believe that “like attracts like” and thus TECs/TEC

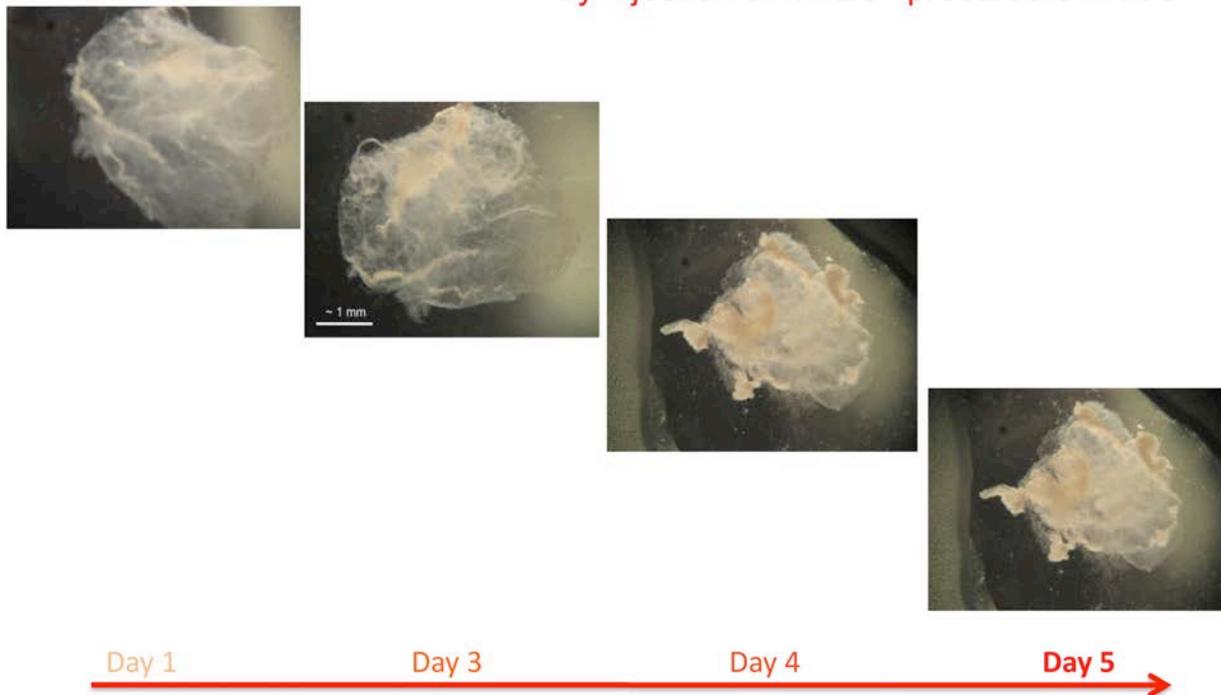
precursors will more efficiently remain phenotypically and functionally TECs when repopulating their natural scaffold niche.

Thymic stromal cells isolation: Thymi were harvested from 3-4 year old mice and needle-dissected into 2-3mm pieces. Thymic fragments were then digested in a collagenase solution (collagenase D, 1.25 mg/ml and DNase I, 0.2 mg/ml in RPMI-10 medium) for 15 minutes at 37°C. The supernatant was then collected and the remaining tissue fragments subjected to another round of digestion. The cycle was repeated 3 more times. In the final digestion step, the enzyme mixture was replaced with Collagenase/Dispase (0.2 mg/ml) and DNase I (0.2 mg/ml) in RPMI. All the supernatants were then pooled and passed through the 100µm cell strainer. Thymic stromal cells were negatively selected removing, with anti-CD45 magnetic beads (Miltenyi Biotec), other unwanted cells.



Thymus reconstruction: The day before thymic cellular reconstruction, the thymus scaffold was transferred to complete DMEM medium with 10% FBS and kept at 4°C. On the day of reconstruction, the thymus scaffold was placed on the cover of 6 cm petri dish. Enriched thymic stromal cells were then re-suspended in complete DMEM medium at a concentration of  $2 \times 10^7$ /ml. Approximately a volume of 10-20uL of thymic stromal cells (i.e., a single-cell suspension of ~200,000 cells) was injected into the thymus scaffold under the dissection microscope. Another 50uL of complete DMEM were added on top of the injected thymus scaffold. The reconstructed thymus was cultured as hanging drop on the petri-dish cover for 5 days, followed by culturing in the 6-well trans-well tissue culture dish for another week. The fully reconstructed thymus was finally transplanted into the muscle of the monkey's leg to support ectopic thymopoiesis *in vivo*.

## Thymus reconstruction by injection of mTEC\* precursors *in vitro*



\* mTEC = medullary thymic epithelial cells

Testing *in vivo* thymopoiesis and immune reconstitution: In the transplanted recipient, three months after transplant, the demonstrated presence of mature, circulating, CD4+ and CD8+ T cells testified to the regeneration of an efficient immune system as in the case of the monkeys transplanted with sliced thymi. MLR testing confirmed normal T cell functionality.

While the thin sections definitely improved the exposure of the thymic cells to the monkey-insulin-specific shRNA-carrying lentivirus, this strategy didn't reach the number of transfected TECs sufficient to obtain the wanted breaking of tolerance. A single cell suspension of enriched TECs/TEC precursors, considered the pre-requisite for a satisfactory efficiency in

transduction, was expected to promote not only a significant improvement of transduction efficiency but also of the number of transduced particles per cell. This should help us to obtain, in a reconstructed thymus, a level of insulin expression low enough to promote the breaking of central tolerance and consequently to generate an autoimmune reaction in the NHP as well. The generation of thin sections was also useful for directly implementing the transplantation approach previously described for treating complete DiGeorge syndrome and selected by our surgeons for reaching the final aim of our project.

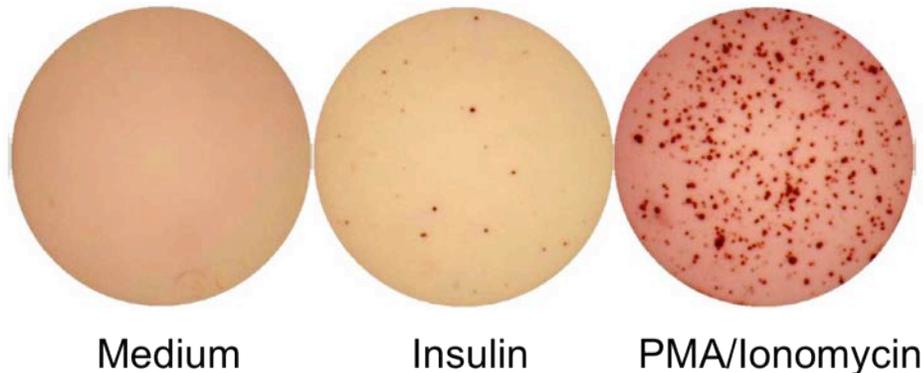
To down-regulate insulin expression we will use the most effective shRNA: As low levels of insulin transcripts are detected in the thymus of both human and rodents, we first examined whether similar levels of thymic insulin expression were present in the monkey. Thymi were harvested from four monkeys and insulin expression was examined by RT-PCR analysis. Insulin transcripts were readily detectable in all the monkey thymus samples examined. In addition, immuno-histochemical analyses revealed that insulin-positive cells are largely restricted to the Hassall's capsule structure within the medulla, as previously described in human thymus. Then, since little was known about the insulin gene from *Cynomolgus* monkey, we cloned and sequenced the monkey insulin gene (mINS) and compared this sequence with that of the human insulin gene (hINS). Of all the five shRNA sequences that were proven to be effective in knocking down hINS expression, only one had the perfect match with the correlating monkey sequence (#49). As a perfect match of sequence is not always required to efficiently knock down target gene expression, all five shRNA sequences were tested *in vitro* with a genetically modified HEK 293 cell line (designated as ml-293) stably transfected with a YFP-IRES-mINS bicistronic expression construct. If the shRNAs are able to recognize the mRNA sequence of the monkey insulin, both YFP and the monkey insulin expression are inhibited. After transduced with lentiviruses expressing the shRNAs, ml-293 cells were selected with puromycin, expanded, and analyzed by flow cytometry to examine the levels of YFP expression. Both lentiviruses #49 and #51 expressing shRNA effectively knocked down YFP expression, whereas no effect was observed with shRNA #48, #50 and #52. Direct examination of mINS transcripts by RT-qPCR also showed that shRNA #49 and #51 can effectively knock down levels of mINS expression to 20-30% of controls. Lentivirus expressing shRNA #49 was then chosen to knock down thymus mINS expression as it shared the same sequence as mINS and displayed the highest levels of mINS knockdown.

A single cell suspension of TECs/TEC precursors should be utilized for obtaining sufficient transduction efficiency with the lentiviruses expressing the shRNAs. Also, before re-population, cell sorting would further enrich YFP<sup>+</sup>, transfected TEC population.

The reconstitution of the monkey T-cell repertoire after Campath treatment and thymus transplantation was a success. The exam of the presence of insulin-specific autoreactive T-cells in peripheral blood finally gave positive results. Enzyme-linked immuno-sorbent spot (ELISPOT) assays were performed, in which human insulin molecules were used as stimulating autoantigens. Specifically, peripheral blood mononuclear cells (PBMCs) were harvested from the monkey 10 weeks after thymic transplantation and cultured in 96-well tissue culture dishes coated with anti-IFN $\gamma$  antibody overnight, in the presence or absence of insulin (100ug/ml). As positive control of T-cell function under stimulus conditions, 10<sup>4</sup> PBMCs were cultured with PMA & ionomycin, which activate T-cells in an antigen independent manner. Robust T-cell activation and IFN  $\gamma$  secretion were observed upon PMA & ionomycin stimulation, indicating that the T-cells derived from the reconstituted thymus are functional and capable to mount TH1-type responses under pro-inflammatory conditions, an essential prerequisite for successful adaptive immunity. Interestingly we have also observed a not

enormous yet clearly detectable number of insulin-reactive T-cells in peripheral circulation, in comparison to pre-treatment blood samples.

### **ELISpot: Presence of insulin-reactive T-cells in PBMCs harvested from the thymus-reconstructed monkey**



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## KEY RESEARCH ACCOMPLISHMENTS

Our previous study in the mouse (*Fan Y, Rudert WA, Grupillo M, He J, Sisino G, Trucco M: Thymus-specific deletion of insulin induces autoimmune diabetes. The EMBO Journal 28:2812, 2009; & Grupillo M, Gualtierotti G, He J, Sisino G, Bottino R, Rudert WA, Trucco M, Fan Y: Essential roles of insulin expression in Aire<sup>+</sup> tolerogenic dendritic cells in maintaining peripheral self-tolerance of islet b-cells. Cellular Immunology 273:115, 2012.*) suggested that also in a monkey, a drastic reduction of expression of self-antigens (e.g., insulin) in the epithelial cells of the thymic medulla (mTECs) might favor the generation of an autoimmune reaction specifically directed against the insulin-producing cells of the pancreas, eventually bringing the animal to clinically overt T1D.

On this basis we were able to:

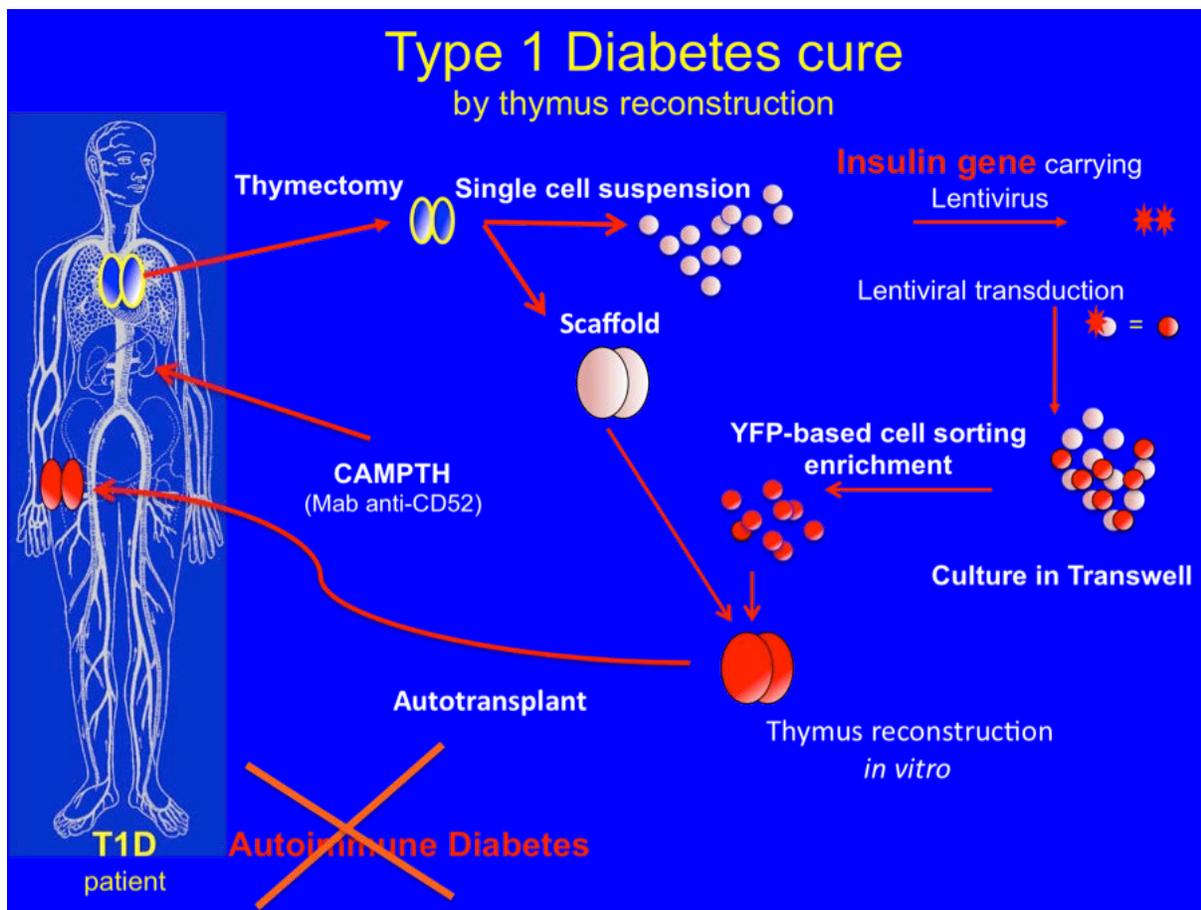
- Safely remove the thymus from Cynomolgus monkeys (i.e., *Macaca Fascicularis*);
- Transducing the harvested thymus *in vitro* with a lentivirus expressing monkey-insulin-specific short-hairpin RNA (shRNA) to knock-down insulin expression;
- Returning the transduced thymus back into the thymectomized donor monkey (whose mature B- and T-cells had been concomitantly depleted using Campath), we observed a successful repopulation of its immune cells (mature and functional T- and B-lymphocytes) after roughly three months from transplantation.
- The lentivirus have only achieved no more than ~5% of insulin reduction in the thymus, transducing *ex vivo* the sliced thymic tissue by exposing it *in vitro* to the monkey insulin-specific shRNA expressing vector. This reduction was insufficient to break central tolerance and failed to promote clinically-overt diabetes in the recipient monkey.
- We then concluded that the only way to achieve better transduction with the vector was to decompose the thymic tissue so to obtain a single cell population, which could be more efficiently exposed to the shRNA carrying vector. With this approach ~ 25-45% of the mTECs were transduced.
- The problem of how to reconstruct the thymus in order to transplant it back to the donor monkey was solved by utilizing a process that successfully repopulates a thymic scaffold with enriched mTECs.
- Using the approach previously successfully tested in the mouse (i.e., repopulating a thymic scaffold with enriched mTECs), we were able to regenerate a normal immune system in the thymectomized donor monkey.
- Using the approach successfully tested in the mouse -- yet modified by substituting the enriched mTECs with insulin-deprived mTECs -- we were able to reconstitute a thymus able to generate autoimmunity in a previously thymectomized monkey. This experiment can be considered a valid prove of principle which might facilitate its potential, clinical translation.

## REPORTABLE OUTCOMES:

Yong Fan, Asako Tajima, Giulio Gualtierotti, Saik Kia Goh, Nina Coppola, Paul Descani, Suzanne Bertera, William Rudert, Ipsita Banerjee, Rita Bottino, Massimo Trucco: **“Regenerating a functional thymus by populating acellular matrix with thymic stromal cells”** Submitted 2013.

## CONCLUSION:

Based on the promising results obtained so far in our laboratory, we concluded that, a completely novel approach to prevent and cure type 1 diabetes (T1D) can be proposed. It entails the reconstruction/reprogramming of the patient’s thymus in a manner that will avoid the generation of autoreactive cells. The thymus is harvested using techniques already in use clinically to treat Myasthenia Gravis (1,2) and the reconstructed/reprogrammed thymus is transplanted at readily-accessible sites also by methods in clinical use to treat infants born with complete DiGeorge syndrome (3,4). In brief, our approach consists in a sequential process of: **a)** thymic resection; **b)** selective collection of the thymic epithelial cells (TEC)-containing cell population; **c)** harvest of the de-cellularized structural thymic remnants, i.e., the “scaffold” **d)** *ex vivo* insulin-gene transduction of the population enriched in TECs/TEC precursors; **e)** repopulation of thymic scaffold with this cell-sorter-enriched, transduced, donor-TEC population; and **f)** transplantation of the reconstructed & reprogrammed thymus back into the donor, once the previously committed immunocompetent cells are pharmacologically eliminated.



This seemingly revolutionary yet clinically feasible approach lays the basis for a safe (i.e., no need for chronic immuno-suppression) treatment of T1D in humans, as well as a means to prevent T1D in the highest-risk individuals. The elimination of what are believed to be the initiating, diabetogenic, insulin-specific T-cells, should also obviate epitope spreading and the generation of secondary autoimmune T-cells in the transplanted recipient, whose immune system will be *de novo* rebuilt.

Other than the revolutionary concept and the strong enabling data in mice and monkeys, the other attractive aspect of this approach is that it will need to only be performed once and the treated patient will not need chronic immuno-suppression, given that the transplanted so-reconstituted, insulin-expressing, thymic cells will be the patient's own cells, able to enforce central tolerance through proper and physiologic negative selection of any, new, possibly insulin-reactive T-cells, eventually coming from the bone marrow afterwards, thus providing a **real cure for T1D**.

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### **The So What Section:**

#### **What are the implication of this research?**

Diabetes affects 16 million Americans and roughly 5-15% of all cases of diabetes are type 1 DM. It is the most common metabolic disease of childhood, and physicians diagnose approximately 10,000 new cases every year. Type 1 diabetes is associated with a high morbidity and premature mortality due to complications. The annual cost from diabetes overall exceeds \$100 billion, almost \$1 of every \$7 dollars of US health expenditures in terms of medical care and loss of productivity. C-peptide administered regularly in physiological quantities might be an additive to insulin able to reduce those complications.

#### **What are the military significance and public purpose of this research?**

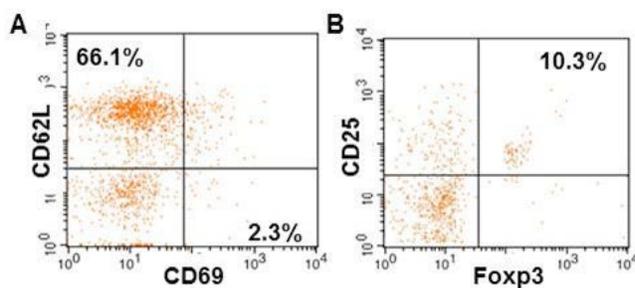
As the military is a reflection of the U.S. population, improved understanding of the underlying etiopathology of T1D will facilitate the development of potential therapeutics to prevent the onset of the disease or the development of diabetic complications among active duty members of the military, their families, and retired military personnel. Finding a cure to T1D will provide significant healthcare savings and improved patients' well being.

**Our first quarterly scientific progress report for the No-Cost Extension period of our project (07/12/2013 - 10/11/2013) described the following:**

We have shown in our previous Quarterly Reports and the last Annual Summary Report that we were able to reconstruct a functional thymus organoid by reconstructing a decellularized thymus scaffold with genetically modified thymic stromal cells. Moreover, we have demonstrated that the 3-D extracellular matrix (ECM) environment of the acellular thymus scaffold can support the survival of thymic stromal cells, comprised predominantly of thymic epithelial cells (TECs), for more than 2-weeks, and thus, provides an essential time window for the genetic manipulation of TECs *in vitro*. In the past quarter, we have primarily focused our efforts on the functionalities of bioengineered thymus organoids *in vivo*, using athymic nude mouse models. In addition, to improve the efficiency of the reconstructed thymus to support T lymphopoiesis, we experimented with a self-assembling peptide complex to obtain certain level of TEC organization. Here, we reported our preliminary findings.

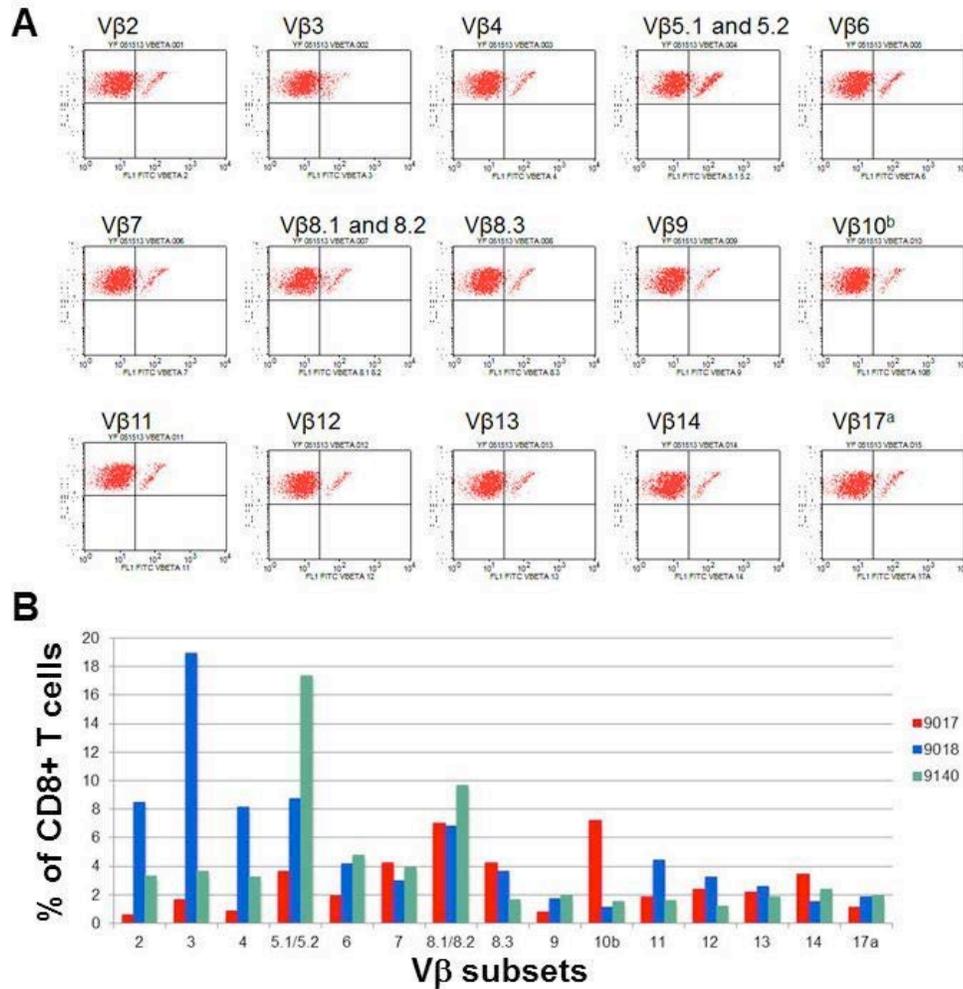
**Reconstructed thymus supports T-cell development *in vivo*.**

To investigate whether the reconstructed thymus organoids can effectively support T lymphopoiesis *in vivo*, we transplanted these artificial thymi under the kidney capsule of athymic nude mice. We have shown previously that about 10-12 weeks after the thymus transfer, we were able to detect both CD4<sup>+</sup> and CD8<sup>+</sup> mature T-cells in circulation with Flow Cytometry (FCM). To further characterize T-cells derived from the transplanted thymus, we sacrificed the nude mice 16 weeks post transplantation and examined the expression of surface markers. We found that most of the CD8<sup>+</sup> T cells belong to the CD69<sup>low</sup>CD62L<sup>high</sup> naïve T cell population (Fig. 1A). Moreover, CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs), the population of T cells that actively suppress the unchecked expansion of autoreactive T cells and are essential for maintaining self-tolerance, are present in percentages similar to those of naïve B6 mice, indicating that the reconstructed thymus can maintain homeostasis of T cell development (Fig. 1B).



**Figure 1. T-cell development in athymic nude mice transplanted with the artificial thymus organoid.** Representative graphs of FCM analyses of thymic recipients (16-weeks post-transplantation): A) splenocytes gated on CD3+CD8+ T-cells; B) splenocytes gated on CD3+CD4+ T-cells. Shown in each quadrant are percentages of gated cells in a representative recipient (n=3).

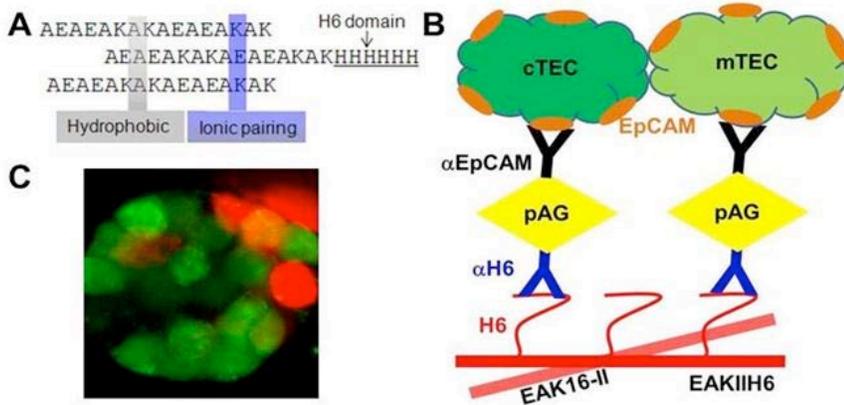
To further demonstrate that the bioengineered thymus organoid can support the development of a diverse and complexity T cell repertoire, we performed FCM-based analysis of the distribution of TCR V $\beta$  chain subsets in both the CD4<sup>+</sup> and the CD8<sup>+</sup> T-cell populations in thymus transplanted nude mice. V $\beta$  chain usage is a good indicator of the clonal diversity of the T-cell repertoire. Lymphocytes harvested from lymph nodes of the thymus-transplanted mice were stained with a panel of antibodies targeting specific subsets of mouse V $\beta$ -chains (BD Biosciences). As shown in Figure 2, a diverse T-cell repertoire, similar to naïve B6 mice, was observed in thymus reconstructed nude mice, showing further that the artificial bioengineered thymus can effectively support the development of T lymphocytes *in vivo*.



**Figure 2. Reconstructed thymus organoid can support the generation of a diverse and complex T-cell repertoire.** A. Representative FCM graphs showing the presence of population of CD4+ T-cells expressing subsets of TCR Vβ-chain. B. Summary of Vβ-chain usage of the CD8+ T-cells in three nude mice transplanted with the reconstructed thymus.

**Generate thymus-mimetic TECs clusters with a biocompatible, self-assembling peptide system.**

A potential drawback of in our original top-down approach is the lack of mechanistic control to ensure that the dissociated TECs will re-organize into functional units found in natural thymi. Spatial compartmentalization of TEC subsets will presumably provide better support for T lymphopoiesis. As a step towards engineering TECs to reprogram naïve T cell pools, we established collaboration with Dr. Wilson Meng, an expert in biomaterial engineering at Duquesne University, and have developed a peptide-based, injectable synthetic system by which small clusters of TECs, like a “mini” thymus, can be generated consistently. This bottom-up approach will provide a means to compartmentalize TEC subsets in the replenished natural thymic scaffold. It also has the potential to transform the TEC concept into clinically deployable strategies.



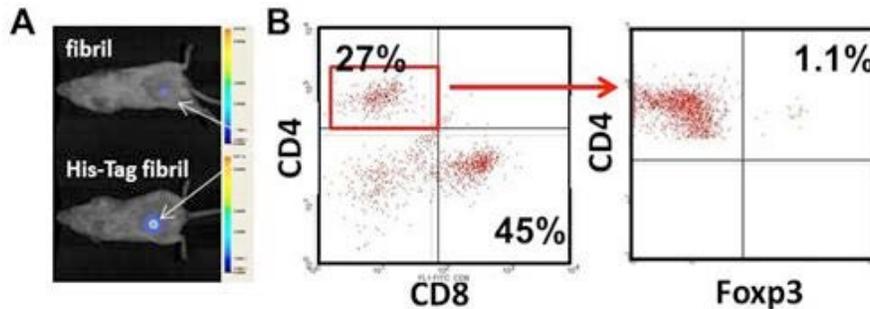
**Figure 3. The EAK/H6-TEC composites.** A) Co-assembly of EAK16-II with its histidinylated analogue EAKIIIH6. H6, tag with 6 Histidines. B) Schematics illustrate the composites of the TEC trapping EAK/H6 system. C) Typical fluorescent images of a TEC cluster assayed with LIVE/DEAD kit. Green, live cells; Red, dead cells.

This innovative idea lies in tailoring of an environmentally responsive biomaterial system to steer TECs into clusters. The strategy builds upon Dr. Meng’s previous work with the fibrils/anti-T cell antibody composites which can trap T cells both *in vitro* and *in vivo*. The building blocks of the fibrils, EAK16-II and its histidinylated analogue EAKIIIH6, are soluble in deionized water, but undergo gelation when exposed to ionic strength higher than 20mM NaCl (normal salt concentration in human body fluid is 154mM) (Fig. 3A). The resultant entangled fibrils formed stacked  $\beta$ -sheets, with the His-tags exposed to interact with the anti-His antibody ( $\alpha$ H6) which, together with the Fc-binding, recombinant protein A/G (pAG), serve as a multivalent linker for antibody-mediated cell trapping (Fig. 3B). The two polypeptide co-assembling system (hereafter referred to “EAK/H6 complex” in this proposal) allows us to control various properties of the composites (e.g. His-tag density, porosity and viscosity) to optimize performance.

In a pilot study, we used an anti-EpCAM antibody ( $\alpha$ EpCAM) to generate mini clusters of TECs with the EAK/H6 complex (Fig. 3B). In this prototype, pAG was complexed with  $\alpha$ H6 and  $\alpha$ EpCAM at a molar ratio 1:3:3 such that all Fc-binding sites were saturated. The EAK/H6 complexes were mixed with TECs and placed on transwell inserts for 2 hours, washed and cultured in complete RPMI-10 *in vitro* for 3 days. LIVE/DEAD analysis revealed mini-clusters of 20-30 TECs, with majority of them alive (Fig. 3C). Similar cell clustering was observed when the EAK/H6/TECs composites were injected into a “de-celled” thymus scaffold and cultured *in*

*vitro* (data not shown). These results, though preliminary, demonstrated the feasibility of generating TEC clusters with the antibody-functionalized EAK/H6 complex.

In an effort to determine the extent to which the “de-celled” thymic bioscaffolds containing TEC clusters support T lymphopoiesis *in vivo*, we first demonstrated that the EAK/H6 complex can maintain its physical and functional integrities *in vivo* for at least 12 days (Fig. 4A). To examine whether the TEC clusters in the EAK/H6 complex without the scaffold can still support thymocyte development, we injected them under the kidney capsules of athymic B6.nude mice. 16-weeks post operation, we were able to detect both CD4<sup>+</sup> and CD8<sup>+</sup> cells in the spleens of these mice (Fig. 4B), suggesting that these TEC clusters can function, at least partially, as “mini-thymi” to generate T cells.



**Figure 4. TEC clusters function *in vivo*.** **A)** Images of mice injected (s.c.) with EAKII-16 (top) or EAK16-II/EAKIIIH6 (bottom), probed with  $\alpha$ H6 labeled with a dye emitting at 800nm (arrows). **B)** Representative FCM graph of splenocytes of nude mice implanted with TEC clusters (gated on CD3<sup>+</sup> cells.).

While our pilot experiments with the anti-TEC antibody system resulted in successful thymocyte maturation, disproportion of CD4/CD8 ratios was observed in some animals [Fig. 4B, 0.1-0.6/1 in TEC clusters transferred nude (n=3) vs. 1.5-2/1 (n=15) in wild-type B6]. Moreover, a significant defect in regulatory T-cell (Foxp3<sup>+</sup>CD4<sup>+</sup>) development was observed, indicating that the EAK/H6/TECs composites alone cannot fully support the CD4 lineage-specification within the kidney capsule environment *in vivo*. These results further highlight the essential roles of the 3-D extracellular matrix environment within the thymus scaffold in supporting T-lymphopoiesis.

**12.** Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

In the next quarter, we will combine the scaffold technology with the EAK/H6 composite technology and examine whether can correct these thymocyte development defects and achieve greater T lymphopoiesis in nude mice with bioengineered thymus made from the EAK/H6-based TEC clusters. Moreover, we will examine the functions of T-cells derived from the reconstructed thymus, both *in vitro* and *in vivo*.

**In the second and final quarterly scientific progress report (10/12/13 – 01/11/14) for the No-Cost Extension period of our project, we now report on our cumulative findings for this entire project.**

In this final report of the grant, we can summarize the key research accomplishments of the past two-and-a-half years.

Mice with insulin gene specifically deleted in thymic epithelial cells develop autoimmune diabetes spontaneously, due to defective negative selection of insulin-reactive thymocytes. Based on these previous findings, we proposed to develop an autoimmune diabetes monkey model by reducing thymic insulin expression with lentiviral particles expressing insulin mRNA reactive shRNAs. As detailed in our previous quarterly reports, and also as summarized below, we were able to knock down insulin expression in isolated TECs with lentivirus, introduce the genetically engineered TECs into the decellularized thymus scaffolds, and transplant the reconstructed bioengineered thymus organoids into thymectomized monkeys. Six months after the treatment, insulin-reactive T-cells were detected in the blood circulation. While the treated monkeys have yet to develop diabetes, presumably due to the tolerogenic mechanisms which is largely intact in the periphery, our success is an important first step towards developing a nonhuman primate model for type 1 diabetes research.

Moreover, we have shown in both rodent and nonhuman primate models that transplantation of such a bioengineered thymus organoid can replenish the function of thymus both *in vitro* and *in vivo*. When transplanted into athymic nude mice, thymus organoids reconstructed from isolated TECs can support T lymphopoiesis and help to establish both humoral and cellular adaptive immunity *in vivo*. Nude mice engrafted with the bioengineered thymus were able to prompt acute rejection of skin allografts, and displayed strong seroreactivity against ovalbumin after vaccination. Conversely, tolerance to skin grafts of allogeneic donors was achieved by transplanting thymus organoids expressing both syngeneic and allogenic MHCs. Thus, our study demonstrated the technical feasibility of modulating thymic central selection with this bioengineering approach.

Despite the findings that the bioengineered thymus organoids can effectively support T lymphopoiesis and restore thymus functions for both cellular and humoral immunity, the technique is far from perfection at the present stage. The total numbers of T-cells in the spleens of transplanted thymic nude mice were about 10-20% of those of naïve B6 mice. Moreover, the T-cell repertoire in the thymus recipients was more restricted than that of naïve mice. Clearly, more work needs to be done to achieve a reconstructed thymus organoid that can fully recapitulate the potential of a native thymus. Many factors might contribute to this inefficiency. For example, the success of T lymphopoiesis depends on cross-talks between the developing thymocytes and subsets of TECs in a spatial and temporal manner. Our current top-down approach (breaking up and assembling) is limited in part by the mechanistic control that facilitates the dissociated TECs to re-organize into functional units found in a natural thymus. Second, the engraftment sites (kidney capsules and upper leg muscles for mouse and monkey, respectively) might not be optimal for the survival and function of the thymus organoids. The space between the kidney capsule and its cortex might not be sufficient to sustain the expansion of the thymus organoids due to the proliferation of developing thymocytes. Indeed, intact thymi of 2-week old mice had largely degenerated 16-weeks after transplantation underneath the kidney capsules (data not shown). Identification of an alternative engraftment site might be necessary to improve the efficacy the transplanted thymus organoids to support T lymphopoiesis.

Nevertheless, it is conceivable that in conjunction with gene therapy technology, the novel bioengineering technique for thymus reconstruction, developed with the support of the current grant, will have broad translational impacts in transplantation medicine as well as treatment of various autoimmune disorders.

## KEY RESEARCH ACCOMPLISHMENTS

Year 1 (07/12/2011 – 07/11/2012):

- We have demonstrated the success of safely removing the thymus from Cynomolgus monkeys. With the infrasternal approach for thymectomy, perhaps the least invasive procedure at present, we were able to remove >95% of the thymus for *in vitro* assays.
- We have successfully identified the sequences within monkey-insulin gene that can be targeted by specific short-hairpin RNA (shRNA) for insulin expression knock-down.
- We were able to deplete both mature T- and B-lymphocytes in the thymectomized monkey with Campth-1.
- We were able to make thin sections of the thymectomized thymus (at 0.5mm thickness), culture them *in vitro* for 7-days, and transduce them with lentiviral particles.
- We were able to transplant back thymus sections treated with shRNA expressing lentiviral particles back into the thymectomized monkeys. A successful repopulation of immune cells was observed about three months post thymus transplantation.
- We were unable to detect any insulin-specific T-lymphocytes in the treated monkeys.
- We found that low efficiency of lentiviral transduction under these settings. No significant reduction of insulin expression was achieved. We argued that the reduction would be insufficient to break central tolerance against insulin, and therefore, began to explore alternative avenues to achieve higher efficiency of lentiviral transduction.
- We were able to transduce 25-40% of mTECs when thymic epithelial cells (TECs) were harvested from mouse, and treated with lentiviral particles. We concluded that to achieve higher lentiviral transduction efficiency, we need to isolate TECs by dissociating the thymus organ with enzymatic digestion. This led us to develop a bioengineering approach to reconstruct the thymus organoids from isolated TECs.

Year 2 (07/12/2012 – 07/11/2013):

- We successfully developed a novel protocol that enabled us to generate 3-dimensional acellular scaffolds from both the mouse and the monkey thymus.
- We were able to demonstrate that TECs injected into the bioengineered thymus scaffolds can survive for up to 8-weeks *in vitro*.
- Reconstructed thymus organoids can support T-lymphocyte development when transplanted under the kidney capsules of the athymic nude mice. Both CD4+ and CD8+ T-cells were detected in blood circulation 4-10 weeks post-transplantation.
- We were able to demonstrate that T-cells developed in the nude mice are of recipient origin, indicating that the transplanted thymus organoids can successfully induce the homing of lymphocyte progenitors from the bone marrow, and differentiate them into mature T-cells.
- We were able to demonstrate that T-cells matured in the thymus transplanted nude mice can undergo proliferation response to stimuli (e.g., anti-CD3 and -CD28 antibodies), at similar fashion to wildtype B6 mice. In addition, we were able to demonstrate that

thymus reconstructed nude mice can reject skin allografts rapidly, in a similar kinetics as the wildtype controls. From these results, we concluded that the T-cells matured in the thymus transplanted nude mice are functional, and that the reconstructed thymus organoids can help to establish cellular immune response in the recipients.

- We were able to demonstrate that T-cells matured in the thymus transplanted nude mice can help to mediate Ig-switch in B-cells and establish humoral response in the recipient nude mice, when immunized with model antigens, such as ovalbumin.
- We were also able to improve our TEC isolation protocol which allowed us to double the numbers of TECs obtained.
- We were able to prepare decellularized monkey thymus scaffolds from monkey thymus.
- We were able to transduce isolated monkey TECs with insulin-specific shRNA lentiviral particles, enrich the transduced TECs with FACS, and inject them to the acellular monkey scaffolds.
- We were able to transplant the bioengineered thymus organoids reconstructed with genetically modified monkey TECs back into the Campth-treated, thymectomized monkeys.
- Most importantly, we were able to detect insulin-reactive T-cells in the circulating blood of the treated monkey, suggesting that reduction of insulin-expression in the bioengineered monkey thymus will result in faulty central negative selection of insulin-reactive thymocytes.

Year 3 (07/12/2013 – 01/11/2014):

- We further demonstrated that the transplanted bioengineered thymus can support the development of a diverse T-cell repertoire in athymic nude mice. However, the complexity of the repertoire is not up to par of that of the naïve B6 mice.
- We explored the possibility of generating thymus-mimetic TEC clusters with a biocompatible, self-assembling peptide system. These “mini-thymus” can support the development of T-cells in athymic nude mice when transplanted underneath the kidney capsules. Similar to T-cells matured in the bioengineered thymus organoids, T-cells developed from these mini-thymi can support both cellular and humoral immune responses in the recipient nude mice.

## REPORTABLE OUTCOMES:

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3. Bonner SM, Pietropaolo SL, **Fan Y**, Chang Y, Sethupathy P, Morran MP, Beems M, Giannoukakis N, Trucco G, Palumbo MO, Solimena M, Pugliese A, Polychronakos C, **Trucco M**, Pietropaolo M (2012). Sequence Variation In the Promoter of the Ica1 gene, which encodes a protein implicated in type 1 diabetes, causes the Transcription Factor AIRE to Increase Its Binding and downregulate Expression. *Journal of Biological Chemistry* 287: 17882-17893.
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## CONCLUSIONS

We have achieved two major goals in this study: 1) through genetic engineering, we were able to break central immune tolerance of insulin, and successfully generated monkeys with circulating insulin-reactive T-lymphocytes. Results from this proof-of-principle study will help us to further advance our quest to generate an autoimmune diabetes nonhuman primate model for type 1 diabetes. 2) We have developed a novel bioengineering approach to reconstruct functional thymus organoids by populating decellularized thymus scaffolds with isolated TECs. In conjunction with further advances of TEC stem cell technologies and gene therapy technologies, we would be able to bioengineer individualized thymus organoids to treat thymus defects, as well as other clinical conditions, such as organ-specific autoimmune disorders and solid organ transplantation.

Personnel receiving pay from the research effort, W81XWH-11-1-0417

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