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In Vivo Monitoring of Inflammation and Regulation in Type 1 Diabetes

Mi-Heon Lee[1,3], Sonya Liu[2], Wen-Hui Lee[1] and Chih-Pin Liu[1]
[1]*Department of Immunology, Department of Diabetes and Metabolic Diseases Research, Beckman Research Institute, City of Hope, Duarte, CA,*
[2]*Department of Biochemistry, University of California, Los Angeles, Los Angeles, CA,*
[3]*Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

1. Introduction

T1D is a tissue specific and T cell–mediated autoimmune disease characterized by the inflammation of pancreatic islets, namely insulitis, resulting in selective destruction of insulin-producing beta islet cells and development of overt diabetes (Atkinson & Leiter, 1999, Bach, 1994, Mathis et al., 2001, McDevitt, 2001, Nepom & Kwok, 1998, Quinn et al., 2001). The onset of T1D is preceded by at least two main inflammatory stages during insulitis development (Bach, 1994, Robles et al., 2003). In the first stage, termed peri-insulitis, a mixed population of leukocytes, including macrophages, dendritic cells, and T cells, migrate from draining lymph nodes to the peripheral space to the islets. In the second stage, the leukocytes further migrate and infiltrate into the islets, resulting in invasive insulitis. Overt T1D will develop when the majority of insulin-producing β cells in the islets are destroyed by the invading leukocytes and insufficient insulin is produced to control blood glucose levels in the body. Among leukocytes that infiltrate the islets, the autoantigen-specific diabetogenic T cells play a critical role in development of T1D. Recruitment or homing of these diabetogenic T cells into the islets is a critical component of insulitis leading to T1D. Thus inhibition of their homing to the islets would prevent the development of insulitis and T1D. Consequently, it is necessary to understand the homing or trafficking behavior of diabetogenic T cells during the formation of peri-insulitis and invasive insulitis. The knowledge gained from studies addressing those questions is imperative to the development of early diagnosis methods and immune modulatory approaches to treat T1D.
Due to the low number and frequency of T cells specific for an autoantigenic peptide, it has been difficult to identify and trace autoantigen-specific T cells in animals. We have previously addressed this problem by generating novel MHC class II tetramers that can stain T cells specific for a self peptide recognized presented by the MHC class II molecules such as the I-Ag7 in NOD mice (Liu et al., 2000, Chen et al., 2003, You et al., 2003). Using this approach, we have been able to successfully identify and trace autoantigen-specific T cells, such as the $CD4^+$ BDC2.5 (BDC) T cells. The tissue localization of various T cell populations at any given time point can be identified using techniques such as flow cytometry and immunofluorescence. However, these methods involve an invasive approach requiring tissue removal from animals, and therefore only provide terminal data. As such, these

methods cannot help us assess real-time dynamic cell migration patterns *in vivo* as a longitudinal study. Therefore, it is desirable to address the above-mentioned questions using non-invasive *in vivo* imaging approaches.

Previous studies have shown that it is possible to monitor T cells and other immune cells in deep organs in small animals (Weissleder, 1999). In particular, positron-emission tomography imaging (PET) of radio-labeled cells (Oku et al., 1994, Koike et al., 1997, Kikkawa et al., 2000, Melder et al., 2002, Adonai et al., 2002) and magnetic resonance imaging (MRI) of probe-labeled cells (Lewin et al., 2000, Dodd et al., 2001, Moore et al., 2002) have been used to image cell trafficking in small animals. In addition, *in vivo* imaging of pancreatic infiltration of $CD8^+$ T cells labeled with nanoparticles using MRI (Moore et al., 2004) has been demonstrated. These techniques offer high resolution images. However, the intensity of the probes or radio-isotopes used for labeling cells is rapidly decreased over time. This is inappropriate for *in vivo* imaging that requires long-term monitoring and evaluation of T cell trafficking in various animal tissues. Therefore, the need for a non-invasive longitudinal imaging method for use in living animals is especially relevant to studies on a chronic autoimmune disease like T1D. Such methods may be used to reveal the T cell *in vivo* behavior, the kinetics of their appearance, and their persistence in various tissues over the course of the disease and in response to therapy. Using whole body *in vivo* imaging, one can develop imaging-guided assays that help investigators to determine the timing for tissue sampling, and ex vivo assays for functional and pathological assays. Whole body *in vivo* imaging assays help investigators assess whether novel T1D treatments may be effective in preventing or intervening T1D by modulating T cell trafficking in living animals. Therefore, other than PET and MRI, alternative imaging approaches need to be developed to better understand the trafficking behavior of diabetogenic T cells in pre-clinical animal studies and to evaluate the effects of various diabetes treatment regimens on the trafficking of these T cells.

Over the past decade, new imaging technologies for monitoring molecular and cellular changes in living animals have been developed. These methods were developed to help reveal the *in vivo* migratory behavior and/or paths of vitally important lymphocytes in response to altered immune conditions. In particular, bioluminescence imaging (BLI) technology has been a promising noninvasive imaging method that can be employed not only to image lymphocyte migration *in vivo* but also longitudinally monitor their behavior under various disease conditions (Hardy et al., 2001, Costa et al., 2001, Bhaumik and Gambhir, 2002, Scheffold et al., 2002, Edinger et al., 2003). These unique features are important for studies that need to investigate the *in vivo* roles of lymphocytes in chronic autoimmune diseases such as T1D. In our studies, we have used BLI to longitudinally monitor T cell trafficking in real time in living animals. To understand the *in vivo* behavior and tissue localization of T cells involved in T1D, we have established the experimental condition in which the trafficking of autoantigen-specific diabetogenic T cells in living animals can be successfully visualized using BLI. More importantly, aided by using the BLI approach, we have been able to examine the *in vivo* roles of nTreg cells on autoantigen-specific diabetogenic T cells during the pathogenesis of T1D. These studies also have helped us to uncover the tissue localization and potential site of action of nTreg cells during the induction of immune tolerance that prevents T1D development.

In the following sections, we will review some of the results obtained from *in vivo* BLI analyses of dynamic trafficking patterns of luciferase-expressing diabetogenic T cells under

various disease conditions. We will focus our discussion on the findings from studies that have examined the *in vivo* behavior of autoantigen-specific diabetogenic T cells in pre-diabetic and newly diabetic animals, under conditions that either have or have no nTreg cells present in animals. We will also review the effect of nTreg cells on trafficking of pathogenic T cells *in vivo*, and the potential role and site of action of nTreg cells during the development of T cell-mediated insulitis leading to T1D.

2. *In vivo* imaging of pathogenic T cell-mediated insulitis during development of overt type 1 diabetes

In vivo tracking of autoreactive lymphocytes in intact micro-environment and macro-environment *in vivo* in real time has been a long sought-after goal of investigators who are developing imaging techniques in the context of autoimmunity. T1D is an organ-specific autoimmune disease in which both autoreactive $CD4^+$ and $CD8^+$ T cells participate in the destruction of pancreatic insulin-producing islet β cells. Development of clinical symptoms of overt diabetes is preceded by a prolonged period of chronic inflammation in the islets. This is characterized by mononuclear cell infiltration of the islets that involves the recruitment and differentiation of autoreactive T cells (Liblau et al., 2002). A study aiming at understanding the role of $CD8^+$ T cells in T1D has demonstrated pancreatic infiltration of $CD8^+$ T cells using MRI by labeling the T cells with nanoparticles (Moore et al., 2004). In addition, the small animal imaging systems using PET or MRI have made it possible to monitor not only T cells but also other immune cells in deep organs in rodents (Weissleder, 1999, Oku et al., 1994, Koike et al., 1997, Kikkawa et al., 2000, Melder et al., 2002, Adonai et al., 2002). Although considered as sensitive techniques, these approaches are not appropriate for a longitudinal study of chronic diseases such as T1D. In addition, although intravital microscopy provides greater spatial resolution than the other imaging approaches (Jain et al., 2002), this method involves invasive procedures. In order to better image ongoing immune response and T cell trafficking in living animals, several noninvasive imaging techniques have been employed to monitor leukocytes in various disease conditions using animal models (Moore et al., 2004, Denis et al., 2004, Hardy et al., 2001, Contag et al., 1998).

Despite extensive studies on the role of T cells in T1D, their *in vivo* behavior, trafficking pattern and tissue localization over the course of chronic insulitis development leading to T1D remain unclear. In order to address these important questions, we have performed longitudinal studies using *in vivo* BLI analyses in real time to noninvasively visualize autoantigen-specific diabetogenic T cell trafficking in mice under normal and diseased conditions. These studies were performed prior to and after the development of insulitis and overt diabetes in animal models. This approach of using BLI to image T cell trafficking offers the advantages of excellent temporal analyses, cell-labeling versatility, easily accessible instrumentation, and high sensitivity of signal detection (Cao et al., 2005, Cao et al., 2004, Wetterwald et al., 2002, Hardy et al., 2001, Contag et al., 1998). It is well known that the trafficking of diabetogenic $CD4^+$ T cells such as BDC cells to the pancreas is necessary in order to induce insulitis and cause diabetes. Diabetogenic $CD4^+$ T cells can also facilitate the homing of $CD8^+$ T effector cells to the islets during insulitis and diabetes development (Thivolet et al., 1991). Therefore, these T cells play an important role during T1D development. However, the longitudinal trafficking behavior of diabetogenic $CD4^+$ T cells to the pancreas during islet inflammation and diabetes development still remains largely

unclear. Although we have been able to trace T cells such as BDC cells using antigen-specific tetramers (Liu et al., 2000, You et al., 2003), these methods are invasive, requiring tissue removal from animals; hence, only terminal data can be obtained. The data obtained from non-invasive imaging analyses can help guide investigators in making decisions on tissue sampling for the invasive assays. Whole-body *in vivo* imaging such as BLI will help the investigators perform longitudinal studies that examine the *in vivo* behavior of diabetogenic T cells. Additionally, they will be able to assess whether treatments to prevent diabetes or islet graft rejection can be mediated through modulating the systemic trafficking T cells in living animals, in addition to other cellular functions.

2.1 Real time bioluminescent imaging analyses

Previous studies have shown that BLI analyses can be used to view cell trafficking in small animals, using luciferase (Luc) as an *in vivo* reporter (Hardy et al., 2001, Costa et al., 2001, Bhaumik and Gambhir, 2002, Scheffold et al., 2002, Edinger et al., 2003). Luciferase provides excellent signal to noise ratios owing to the fact that there is almost no background luminescence from mammalian tissues. The photons emitted from Luc-expressing cells that are transmitted through tissues can be detected using charge-coupled device (CCD) cameras designed for lowlight imaging. BLI allows for long-term longitudinal studies of cell-trafficking patterns because the reporter is encoded in the genome of the cells and the signal is therefore replicated during cell division, and is not diluted (Nakajima et al., 2001, Costa et al., 2001). BLI modalities that use light-generating enzymes (e.g. luciferase) and low-light imaging devices based on exquisitely sensitive CCD cameras have been developed and refined during the past decade. This system is capable of noninvasively revealing the cellular and molecular features of normal and diseased conditions *in vivo*. This approach offers the advantages of excellent temporal analyses, cell-labeling versatility, easily accessible instrumentation, and high sensitivity of signal detection (Contag et al., 1998, Hardy et al., 2001, Cao et al., 2005, Cao et al., 2004, Wetterwald et al., 2002). This approach has also been proven to be very useful for longitudinally monitoring the *in vivo* behavior of the lymphocytes that cause chronic inflammation prior to development of autoimmune or inflammatory diseases.

2.2 Noninvasive real-time *in vivo* bioluminescence imaging of diabetogenic T cell trafficking patterns

The $CD4^+$ BDC2.5 (BDC) cells are islet antigen–specific and highly diabetogenic T cells (Haskins and McDuffie, 1990, Haskins et al., 1989). Activated BDC cells from BDC TCR transgenic NOD mice (BDC mice) induce an aggressive form of diabetes (Chen et al., 2006, Katz et al., 1995). To label T cells from these animals for trafficking studies, we crossed a transgenic reporter mouse line that expresses luciferase by a strong, ubiquitous, constitutive promoter with BDC mice for more than 8 generations (luc-BDC/NOD mice) (Cao et al., 2004). This synthetic hybrid promoter is composed of the chicken β-actin promoter and the immediate early enhancer from cytomegalovirus, and thus drives expression of Luc into virtually all cells in the mice (Cao et al., 2004). Therefore, transgenic expression of the Luc transgene in BDC mice results in stable expression of Luc in all BDC cells. Using these Luc-expressing BDC/NOD mice allows for long-term longitudinal *in vivo* imaging studies of Luc^+ BDC cell trafficking to the pancreas and the islets, as well as other lymphoid and nonlymphoid tissues relative to varied degrees of disease states in animals.

It is known that transferring CD4+ BDC cells alone into NOD/scid mice failed to induce diabetes. However, cotransfer of CD4+ cell–depleted splenocytes isolated from diabetic NOD mice with CD4+ BDC cells induced accelerated diabetes in recipient mice. It has also been observed that there can be a respectful degree of peri-insulitis or invasive insulitis (Katz et al., 1995, Katz et al., 1993, Peterson & Haskins, 1996). However, the mechanisms responsible for these novel observations were not clear. At least two non-mutually exclusive possibilities exist that may explain the effect of the non-CD4 splenocytes on CD4+ BDC cells during induction of expedited diabetes: (1) The non-CD4 splenocytes may cause an altered systemic trafficking pattern of BDC cells that prevents their homing to the islets, or (2) they may exert their effect on BDC cells locally at tissue levels. We have addressed these questions in our studies which will be discussed in more detail below.

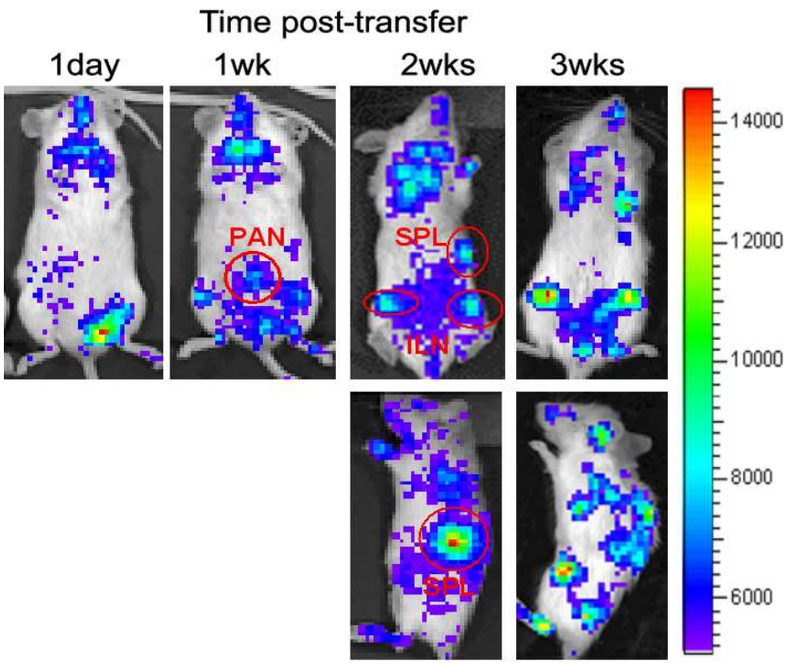

Fig. 1. Real-time *in vivo* bioluminescence imaging of luciferase-expressing primary CD4+ BDC cells. The CD4+ T cells were isolated from luc-BDC/NOD mice and i.v. injected into 9-wk old female NOD/scid mice ($10x10^6$ cells / mouse). The images were taken at different time points after cell transfer by a CCD camera. SPL: spleen, ILN: inguinal lymph nodes, PAN; pancreas.

We imaged BDC cells *in vivo* using BLI and compared their trafficking patterns in nondiabetic mice that received BDC cells alone with the trafficking patterns in prediabetic and newly diabetic recipients that were cotransferred with BDC cells plus CD4- splenocytes isolated from diabetic NOD mice. We also used these data to determine the best timing and the target tissues for further analyses using methods that would enable us to assess the effects of CD4+ cell–depleted splenocytes on the local patterns of BDC cells.

Using an adoptive transfer model, we have imaged Luc+ CD4+ BDC cells and examined their trafficking patterns in real time in NOD/scid mice recipients (Fig. 1). In order to monitor diabetogenic T cell trafficking patterns in real-time *in vivo* using noninvasive BLI, we first isolated CD4+ Luc+ BDC cells from Luc-BDC/NOD mice and adoptively transferred them

into NOD/scid recipient mice for BLI analyses. Using this approach, we were able to visualize just the BDC cells that expressed luciferase. To perform longitudinal studies on the trafficking behavior of the $CD4^+$ BDC cells in recipient mice, the same living recipient mice were imaged at selected times over a period of 16 weeks following cell transfer. Initial signals from the transferred Luc^+ BDC cells in recipient mice were detected in their lungs on the first day after transfer. The BLI signals were then detected in other organs including the pancreas and the liver by day 3 (data not shown). We have also detected the BLI signals from Luc^+ BDC cells in lymphoid organs such as the spleen and lymph nodes within 1 week after the cell transfer (Fig. 1).

To quantify the bioluminescent signals detected from various tissues in the recipient mice, the total flux (photons/sec) was determined by drawing regions of interest (ROI) over the body corresponding to the designated organs. Based on these results, we have determined the total photon output per ROI to calculate the bioluminescence signal intensity. Each ROI was visually defined to be specific areas that correspond to selected organs from both recipient animals and control mice. The rates of increase in the *in vivo* bioluminescence signals detected in the ROI corresponding to the pancreas, spleen, and inguinal lymph nodes closely correlated with each other. These results indicate that the transferred BDC cells eventually accumulated and may also have proliferated in these tissues at the later time points, perhaps due to homeostatic expansion (Lee et al., 2007). Owing to the relatively low level of photon flux (photon/sec), especially in the pancreas at early time points, different colored bar scales were used for day 1 through day 15 (2,000–50,000) and for day 20 through day 113 (5,000–50,000). The data shown are from a representative mouse from at least 20 mice of four different experiments. The total photon flux was greatest in the spleen and lungs; the pancreas and mesenteric lymph nodes also showed significantly higher photon flux than the other tissues analyzed. The BLI signals from control mice were negligible. Quantification of luciferase activity in the dissected tissues is subject to oxygen availability in addition to levels of substrate and adenosine triphosphate. As such, it may not constitute a highly quantitative measurement. Additionally, although we cannot directly compare these *in vivo* BLI signals among different tissues without normalizing the tissue volume, we have also performed ex vivo BLI analyses by excising the tissues from the mice for BLI. The results from ex vivo BLI analyses on each tissue were confirmed by using the more quantitative FACS analyses by staining the BDC cells with BDC cell-specific I-Ag7-tetramers.

In summary, these noninvasive *in vivo* imaging studies revealed that, although BDC cells migrated to the pancreas rapidly after cell transfer, and the BLI signals of BDC cells detected in the pancreas ROI increased significantly with time, the recipient mice did not develop diabetes. Other than revealing in real time the rapid appearance of a large number of BDC cells in the pancreas without causing diabetes, these imaging results are also consistent with the idea that islet-antigen-specific diabetogenic T cells are able to traffic to the pancreas regardless of whether they induce diabetes. Therefore, these results support the notion that the recruitment and accumulation alone of a large number of diabetogenic T cells, like the BDC cells, are insufficient to induce overt diabetes.

2.3 Real time bioluminescent imaging of diabetogenic T cells during the development of insulitis leading to diabetes

The fact that a large number of Luc^+ $CD4^+$ BDC cells present in the islets did not induce diabetes in the recipient mice was not due to the introduction of luciferase transgene into

BDC mice. This is because purified Luc$^-$ CD4$^+$ BDC cells obtained from BDC mice of the same age in our mouse colony also did not induce diabetes in recipient mice (unpublished data). One possible explanation of these results is that the BLI analyses study was terminated before overt diabetes developed; however, the control mice receiving whole BDC mouse splenocytes develop diabetes within 6 weeks after cell transfer (Chen et al., 2006). This explanation seems unlikely because our additional studies have shown that recipient mice did not develop diabetes even at 23 weeks following the transfer of BDC cells alone (unpublished data). Alternatively, it is possible that BDC cells require the presence of other types of cells in the spleen in order to destroy the islets and induce diabetes.

It has been shown that transfer of T cells alone did not induce diabetes, and the presence of non-CD4$^+$ cells such as CD8$^+$ T cells are necessary (Bendelac et al., 1987, Christianson et al., 1993, Miller et al., 1988). Previous studies have also shown that cotransfer of the CD4$^+$ BDC cells with CD4$^+$ cell–depleted splenocytes from newly diabetic NOD mice induced accelerated diabetes in the recipient animals (Peterson and Haskins, 1996). In order to understand the trafficking behavior of BDC cells during islet inflammation that leads to diabetes, we co-transferred purified CD4$^+$ Luc$^+$ BDC cells and CD4$^+$ cell–depleted Luc$^-$ splenocytes from newly diabetic NOD mice into NOD/scid recipient mice. Our results showed that the recipient mice receiving non-CD4$^+$ splenocytes alone did not develop diabetes. However, the mice that received both populations of cells developed diabetes as early as 9 days following cell transfer, and all recipient mice developed accelerated diabetes within 3 weeks.

In order to understand the behavior of BDC cells in these mice, BLI studies showed that the kinetics of Luc$^+$ BDC cells' trafficking in mice that were cotransferred with both CD4$^+$ Luc$^+$ BDC cells and CD4$^+$ cell–depleted Luc$^-$ splenocytes from newly diabetic NOD mice were similar, at the level of the whole body and organs, to the kinetics in recipient mice that have BDC cells alone. In both types of recipients, the BDC cells migrated first to the lung in one day and then to the pancreas, inguinal lymph nodes, and spleen within one week. To further quantify and compare the changes in BLI signal intensity (total flux) in various tissues of the two different groups of recipient mice at the same time points, we imaged BDC cells and analyzed the ROI drawn on various tissues including the pancreas. The ROI analyses showed that the total BLI signal intensity gradually increased over time. A similar trend of signal intensity increase was also detected in the pancreas ROI in both cohorts, although the transferred Luc$^+$ BDC cells continued proliferating in the lymph organs in mice that received both cell types. Comparable levels of BLI intensity over time were detected in the lymphoid organs in both groups of mice before all the mice that received both types of cells became diabetic following a cotransfer of the cells. However, the BLI intensity in lymphoid organs increased at a faster rate in co-transferred mice during the post-diabetic period than that detected in nondiabetic mice that received BDC cells alone. Therefore, cotransfer of CD4$^-$ splenocytes led to diabetes but did not otherwise change the overall trafficking patterns of BDC cells to these tissues during the pre-diabetic stage. These results suggest that the co-transferred splenocytes may not help BDC cells to induce accelerated diabetes by modulating the trafficking and the total number of BDC cells in the pancreas. Instead, they may cause an increased proliferation of BDC cells in tissues other than the pancreas. BDC cells were able to induce insulitis in both groups of recipients, but only the co-transferred recipients developed diabetes. Therefore, it is likely that the total number of BDC cells present in the pancreas is not responsible for the development of diabetes. It may be that the cellular function of immune cells present within the islets and the microscopic localization of the cells within the tissue were the contributors to diabetes development in our studies.

Different degrees and types of insulitis may occur in the recipient animals after transfer of varied cell populations. Peri-insulitis does not lead to islet destruction, whereas invasive insulitis destroys islets and leads to diabetes. To further evaluate why the cotransferred $CD4^-$ splenocytes may help BDC cells induce accelerated diabetes, we examined whether these splenocytes promote infiltration of BDC cells into the islets and induce invasive insulitis. To make these distinctions, we performed microscopic imaging of the islets (since there is no macroscopic imaging method able to distinguish cell distribution differences at this resolution). Initial ex vivo BLI analyses of excised pancreases showed that comparable bioluminescent signals were detected in the pancreas of both groups of recipients (Lee et al., 2007). These results demonstrated that BDC cells can traffic to the pancreas independent on the presence of other splenocytes. Additonal histological analyses showed that mice co-transferred with both cell types developed more severe invasive insulitis than mice that received BDC cells alone. The islets in the pancreatic sections obtained from co-transferred mice on day 20 following cell transfer showed invasive insulitis and were severely damaged. Only peri-insulitis were detected in the islets from mice transferred with BDC cells alone and their islets remained intact. Therefore, the co-transferred splenocytes may help induce diabetes by enhancing the local infiltration of BDC cells into the islets.

We also performed FACS analyses to better understand the cellular component of the invasive insulitis found in the co-transferred mice. We found that the percentage of both $CD11b^+$ cells and $CD11c^+$ cells showed increase only in the islets of co-transferred mice (Lee et al., 2007). These results suggest that these innate immune cells are involved in promoting invasiveness of BDC cells into the islets. One possibility is that these cells help activation of BDC cells, leading to more activated BDC cells in the islets of co-transferred mice and resulting in accelerated diabetes. Investigation on the activation status of BDC cell present in the islets show that there was no difference in expression of activation markers, CD69 and CD62L, on BDC cells isolated from the islets of both cohorts. Overall, our results suggest that efficient trafficking of BDC cells alone to the pancreas is unable to induce T1D alone. The presence of $CD4^-$ splenocytes is necessary to help these pathogenic T cells locally infiltrate the islets without altering their activation status and systemic trafficking patterns.

In summary, these findings are possible because investigators have the ability to image T cell trafficking and tissue localization over a range of varied scales of techniques, from macroscopic imaging and whole-body BLI, to microscopic imaging via light microscopy of tissue sections and single cell analyses using FACS.

2.4 Application of BLI to understand the role of nTreg cells in regulating the *in vivo* behavior of diabetogenic T cells during T1D

The naturally-arised $Foxp3^+$ $CD4^+$ $CD25^+$ nTreg cells play a critical role in immune tolerance induction and autoimmune disease prevention (Sakaguchi and Powrie, 2007). It is well known that a deficiency in nTreg cells may contribute to development of insulitis and T1D in both humans and mice. Rebuilding a sufficient number of nTreg cells can re-induce immune tolerance, leading to inhibition of T1D (Herman et al., 2004, Tang et al., 2004, Tarbell et al., 2004, Brusko et al., 2005, Lindley et al., 2005, Tritt et al., 2008). Extensive studies have been performed to understand the role of nTreg cells during T1D. However, it is still largely unclear as to how these potent nTreg cells prevent destruction of the insulin-producing beta islet cells by pathogenic T cell-mediated immune responses.

As discussed in previous sections, the development of insulitis precedes development of overt T1D, and immune tolerance induced by nTreg cells often leads to prevention of insulitis in diabetes-free mice. It is conceivable that the development of insulitis involves at least four stages as outlined and depicted in Figure. 2. These include: (1) migration of antigen-presenting cells (APCs), such as macrophages and dendritic cells, to islet draining lymph nodes where the APCs can pick up islet antigens and present them to T cells; this leads to activation of autoreactive pathogenic T cells such as BDC cells in the lymph nodes, (2) expansion of activated pathogenic T cells, (3) migration of activated pathogenic T cells to the islets, and (4) infiltration of the pathogenic T cells into the islets, causing invasive insulitis and destruction of the islets. It is possible that in order to inhibit insulitis and T1D, nTreg cells may function by blocking pathogenic T cells at one of these four stages. For example, nTreg cells may modulate systemic trafficking behavior of pathogenic T effector cells such as BDC cells as well as APCs during the pre-diabetic stage. In addition to suppressing pathogenic T effector cell proliferation and expansion, nTreg cells may block

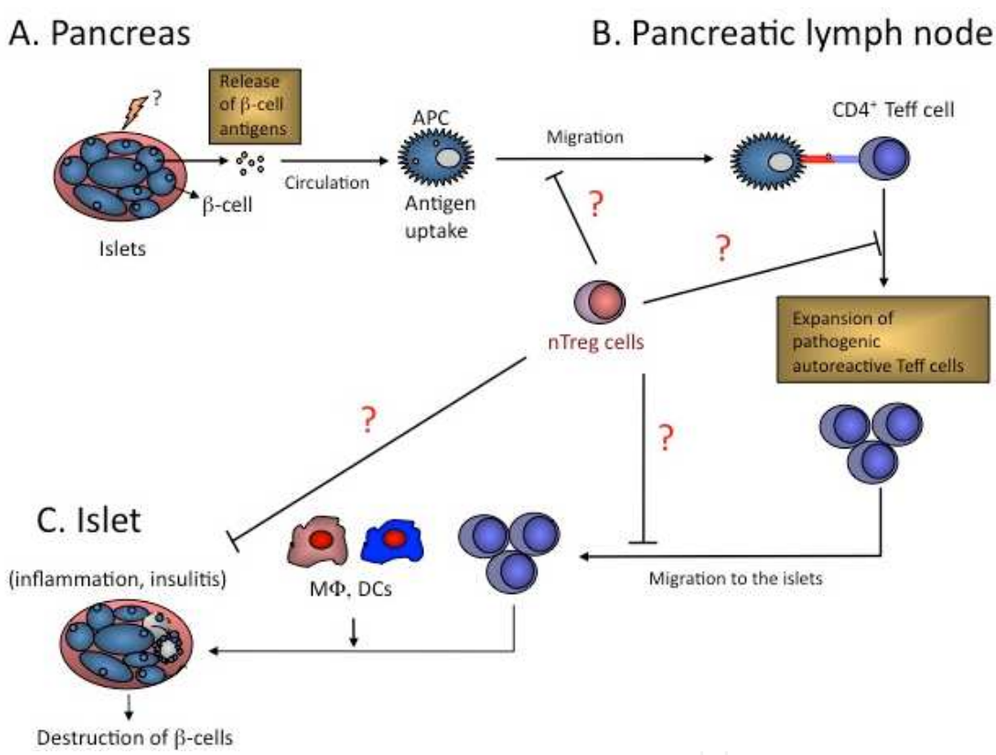

Fig. 2. Potential roles of nTreg cells in controlling T1D pathogenesis. Beta cell antigens are released from the islets into the circulation system. The islet antigens are taken up by the antigen presenting cells (APCs) that have migrated from the circulation to pancreatic draining lymph nodes. In the draining lymph nodes, islet antigen-specific pathogenic T effector cells are activated by islet antigen-loaded APCs, leading to expansion of pathogenic T cells. The expanded pathogenic T cells then traffic to the islets and induce insulitis. Another causative factor in insulitis development is the recruitment of innate immune cells such as macrophages and dendritic cells. This process eventually leads to destruction of islet beta cells and onset of overt diabetes. It is hypothesized that nTreg cells may prevent insulitis and T1D development at one of the four indicated stages.

the trafficking and homing of such T cells to the islets, resulting in inhibition of invasive insulitis that causes overt T1D. It is also possible that, in the absence of nTreg cells, the pathogenic T cells such as BDC cells may traffic more rapidly to and accumulate faster in the islets during pre-diabetic stage. This eventually leads to destruction of the insulin-producing islet beta cells and onset of diabetes.

In our studies to test these different hypotheses, we have performed several experiments, including real time imaging studies, to investigate whether BDC cells showed an altered trafficking pattern in nTreg cell-deficient mice compared to that in nTreg cell-sufficient mice. In order to examine the role of nTreg cells *in vivo* in T1D and distinguish their effects on T1D onset during the four stages of insulitis development, we have used the novel animal models described in the previous sections for imaging analyses. In these studies, as part of our approaches, we have performed noninvasive BLI-guided *in vivo* analyses of BDC cell trafficking in real-time under varied conditions in the presence or absence of nTreg cells (Lee et al., 2010). In particular, in these studies we performed experiments to address the following questions: (1) Compared to nTreg cell-deficient mice, can the trafficking and systemic tissue localization of BDC cells be modulated by nTreg cells during development of T1D? (2) Does the presence of nTreg cells affect the *in vivo* priming, activation, and expansion of BDC cells during the development of T1D? and (3) How does nTreg cells modulate BDC cell-mediated invasive insulitis during T1D?

As part of our studies to address these questions, we used a cell adoptive transfer model by transferring $CD4^+$ BDC cells into NOD/scid recipient mice. It is known that the transfer of $CD4^+$ $CD25^-$ (nTreg-deficient) BDC cells into NOD/scid mice induces aggressive T1D, whereas the transfer of $CD4^+$ (nTreg cell-sufficient) BDC cells does not cause onset of T1D in recipient mice. Therefore, to longitudinally monitor trafficking of the adoptively transferred BDC cells in mice with or without nTreg cells, we have isolated Luc^+ $CD4^+$ $CD25^-$ (nTreg-deficient) or Luc^+ $CD4^+$ (nTreg cell-sufficient) BDC cells from Luc-BDC/NOD mice. We then adoptively transferred these cells separately into NOD/scid recipient mice. Trafficking and tissue localization of the transferred Luc^+ BDC cells in the two cohorts were monitored until after the recipient mice that received Luc^+ $CD4^+$ $CD25^-$ (nTreg-deficient) BDC cells developed T1D (Lee et al., 2010).

We have performed initial macro imaging analyses using BLI on recipient mice during the pre-diabetic stages. Our *in vivo* BLI analyses results showed that, during the pre-diabetic stage on and before the fifth day after cell transfer, there was no significant difference in bioluminescence signal noted in any of the tissues examined between the two cohorts. These results suggest that the presence or absence of nTreg cells may not significantly affect the trafficking of BDC cells prior to development of T1D. On the other hand, an increased BLI signal was detected in several tissues in nTreg cell-deficient, but not control nTreg cell-sufficient mice, only at the postdiabetic stage after development of T1D on or after day 7. The reasons for this difference were unclear, but it could have been a result of a more rapid local expansion of BDC cells that migrated to and resided in these tissues in the absence of nTreg cells. These results demonstrated comparable trafficking and tissue localization of BDC cells during the prediabetic stage in both cohorts of mice. The imaging study results also suggest that nTreg cells may not prevent T1D by modulating the systemic trafficking behavior of pathogenic T cells or the expansion of such cells during the pre-diabetic stages.

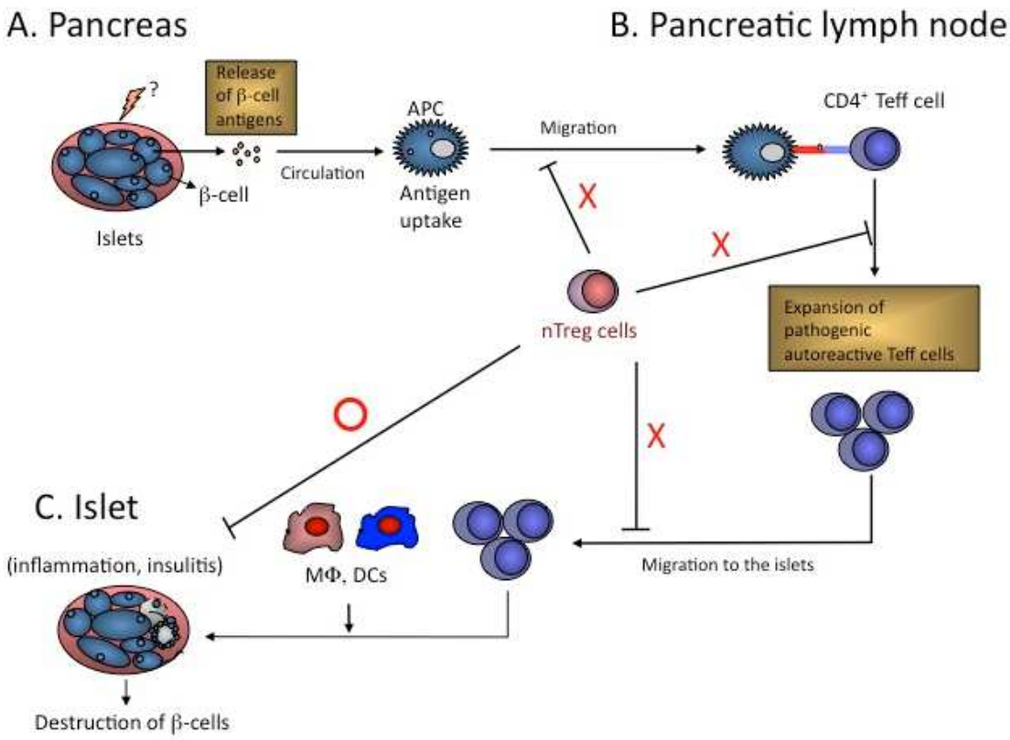

Fig. 3. The nTreg cells may prevent inulitis and T1D by functioning locally in the islets. They may negatively affect not only pathogenic T cells but also innate immune cells recruited to the islets.

In addition to macro imaging studies on systemic *in vivo* trafficking of T cells, we also determined whether nTreg cells may prevent T1D by modulating inflammatory processes locally in the islets. In particular, we performed micro imaging analyses and histological analyses of the islets. These studies showed that depletion of nTreg cells promoted progression and increased severity of insulitis in nTreg cell-deficient mice. Therefore, nTreg cell-deficient mice developed more severe invasive insulitis, compared to mostly peri-insulitis found in control nTreg cell-sufficient mice (Lee et al., 2010). However, despite these differences, the nTreg cell-deficiency did not affect the number and activation status of BDC cells during the pre-diabetic stage. These results suggested that cellular components other than or in addition to BDC T cells may be involved in the development from peri-insulitis to invasive insulitis leading to T1D. It is possible that nTreg cells helped maintain the steady-state of peri-insulitis in nTreg-sufficient mice. However, the steady-state of peri-insulitis in nTreg cell deficient mice may have been broken in the absence of nTreg cells. This in turn led to aggressive infiltration of immune cells from periphery space into the islets, resulting in invasive insulitis. Altogether, these results provide strong evidence supporting the notion that nTreg cells function locally in the islets.

Based on the results discussed above, one can hypothesize that nTreg cells can exert their function by preventing the infiltration of immune cells from the periphery space into the islets. In the absence of nTreg cells, one would expect that the cellular components of invasive insulitis would be altered in comparison to those found in peri-insulitis that are maintained by the presence of nTreg cells. In addition, the change in cellular components in

the islets may be due to the nTreg cells' ability to regulate the local migration of immune cells. In studies to address these questions, we found that, compared to that in control nTreg cell-sufficient mice, the accelerated invasive insulitis in nTreg cell-deficient recipient NOD/scid mice was predominated by a population of $CD11c^+$ dendritic cells, instead of by the adoptively transferred $CD4^+$ BDC T cells (Lee et al., 2010). These results suggest that the presence of nTreg cells may inhibit islet-infiltration of $CD11c^+$ dendritic cells. Indeed, using an *in vitro* chemotaxis assay, we have found that the presence of nTreg cells were able to negatively regulate the chemotaxis of $CD11c^+$ dendritic cells isolated from the islets in NOD mice. Therefore, these novel results suggest that nTreg cells may function locally in the islets by preventing invasive islet-infiltration of innate immune cells such as $CD11c^+$ dendritic cells during development of overt T1D. In animals lacking nTreg cells, the deficiency may lead to enhanced islet infiltration of disease-promoting cells such as $CD11c^+$ dendritic cells, perhaps due to an altered response to chemokines.

In summary, using *in vivo* BLI-guided analyses, we have found that nTreg cells do not affect systemic localization of pathogenic T cells. Our results support the conclusion that nTreg cells can function locally in the islets to prevent inflammatory responses that cause invasive infiltration of innate immune cells, such as $CD11c^+$ dendritic cells, into the islets. The nTreg cells may exert such function, at least in part, by regulating their chemotaxis in the islets. Altogether, our study demonstrated that dendritic cell-dominated invasive insulitis may precede T1D onset, and nTreg cells can prevent T1D by inhibiting local invasiveness of DCs, not $CD4^+$ T cells, into the islets.

3. Conclusions and therapeutic implications

We have developed novel animal models that help investigators to perform *in vivo* BLI to visualize, in real time, the trafficking behavior of diabetogenic T cells in living animals under conditions that may or may not lead to diabetes. Based on the results obtained from these studies, we now have a better understanding of the kinetics, tissue localization, and relative cell numbers in various tissues in living animals during the development of islet inflammation and T1D. This approach is also used as an excellent initial assessment to understand the potential *in vivo* roles and site of action of nTreg cells during the development of T1D. By combining the sensitive imaging studies through performance of BLI analyses with other approaches, our studies obtained from experiments on *in vivo* nTreg cell function in adoptive transfer animal models have helped us to uncover the structural and kinetic features associated with islet destruction and T1D pathogenesis in mice. These results have demonstrated that non-invasive whole-body *in vivo* imaging-guided analyses, such as the use of BLI, is a useful and reliable tool for examining cell-trafficking patterns in animals during the onset of islet inflammation and diabetes. These methods are also useful for monitoring the potential outcomes or effects of various disease treatment regimens. For example, following treatment of chronic diseases like T1D, these approaches can help investigators to monitor the effects of the treatment on *in vivo* behavior of T cells in long-term longitudinal studies.

More importantly, these novel results demonstrate that, by combining micro and macro imaging analyses methods, the non-invasive *in vivo* imaging analyses can help guide the investigators to the times and tissues where the unique biology is taking place prior to and after development of inflammatory responses. These techniques will be a useful approach for monitoring the effects of various treatments that may regulate immune cell trafficking

and inhibit diabetes. In summary, *in vivo* imaging approaches can help elucidate the temporal patterns, spatial distribution, and site of action of various immune cells in the body during the development of T1D. In addition, based on the results obtained from these *in vitro* and longitudinal *in vivo* assays, it is possible to better understand the important roles of nTreg cells during the regulation of autoimmunity and restoring of self-tolerance in the treatment for autoimmune diseases like T1D. Cell-based immunotherapy, such as the ones involving nTreg cells, has provided promising hope for treating diseases such as T1D and graft-vs-host-diseases (Roncarolo and Battaglia, 2007). The information gained from the combination of various imaging approaches, including the ones described in this chapter, should aid in the design of Treg-based novel immune therapies to treat these life-threatening diseases.

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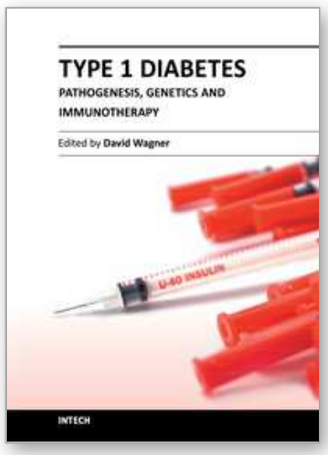

Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy
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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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