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Monitoring Inflammation, Humoral and Cell-mediated Immunity in Pancreas and Islet Transplants

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Abstract: Type 1 diabetes (T1D) is caused by the chronic autoimmune destruction of insulin producing beta cells. Beta cell replacement therapy through whole pancreas or islet transplantation is a therapeutic option for patients in which a stable glucose control is not achievable with exogenous insulin therapy. Long-term insulin independence is, however, hampered by the recipient immune response that includes activation of inflammatory pathways and specific allo- and autoimmunity. The identification and monitoring of soluble and cellular biomarkers are of critical relevance for the prediction of graft damage, for the evaluation of responses to immune-modulating therapy, and for target pathways identification to generate novel drugs or therapeutic approaches. The final objective of immune monitoring is to find ways to improve the outcome of pancreas and islet transplantation. In this review, we discuss the available tools to monitor the innate, humoral and cellular responses after islet and pancreas transplantation, and the most relevant findings generated by these measurements.

Keywords: Autoantibodies, autoreactive T cells, diabetes, homeostatic proliferation, immune monitoring, islet transplantation, pancreas transplantation.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease in which the insulin-producing beta cells are selectively destroyed. Exogenous insulin administration is the most common therapeutic approach for patients who developed T1D, and in the majority of cases insulin therapy is sufficient to maintain a good glycaemic control. The improvements in insulin therapy with new available drugs and intensive treatment regimens substantially improved glycaemic homeostasis in recent years. Unfortunately, exogenous insulin therapy is still not sufficient to prevent long-term complications, and the life expectancy of patients with T1D is still shorter than that of the general population [1]. Moreover, in a significant number of patients, insulin therapy alone is not sufficient to achieve stable glucose levels [2]. Consequently, these patients can suffer of frequent hypo- and hyperglycaemic episodes and have an increased risk of developing long-term diabetes related complications. For these patients, beta cell replacement through islet or pancreas transplantation is clinically indicated to restore, at least temporarily, normoglycaemia and is therefore a potential cure for the disease.

The major obstacle to successful islet and pancreas transplantation is the recipient's immune response to the grafts. Both the innate and specific immune responses are involved in the immune-mediated damage of islet and pancreas allografts. The innate immune response occurs within few hours from transplantation through the release of inflammatory mediators and recruitment of cells such as monocytes/macrophages and neutrophils. This acute inflammatory reaction spontaneously exhausts within few days but appears to substantially impair beta cell engraftments. In the clinical setting of islet transplantation, it was estimated that up to 80% of transplanted islets are lost during the early inflammatory response [3].

The adaptive immune response occurs later and is responsible for the long-term reduction of the functional beta cell mass, resulting in the loss of insulin independence in a significant proportion of cases. Allogeneic islet or pancreas transplantation into a recipient with T1D triggers a complex adaptive B cell and T cell response. Donor islets and pancreases express allogeneic major and minor histocompatibility antigens that elicit host humoral and T cell responses, and can lead to a classical allograft rejection. In addition, donor beta cells express beta cell specific antigens that were targeted by T cells and B cells during the autoimmune process [4-7]. These include (pro)insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma associated protein 2 (IA2) and zinc transporter 8 (ZnT8) that were shown to be highly antigenic in humans both for B cells and T cells [8]. Therefore, transplanting islets or pancreas into a recipient who developed T1D can result in autoimmunity recurrence. Beta cell replacement into a recipient with pre-existing autoreactive T cells and B cells is essentially a re-challenge of an adaptive memory response. As a consequence, while the alloreactive response can be efficiently controlled with immunosuppression, autoimmunity recurrence appears to be highly resistant to standard immunosuppressive drugs, therefore posing an additional set of therapeutic obstacles for the success of beta cell transplantation. In this complex context, the improvement of the outcome of pancreas and islet transplantation

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depends on the capacity to design novel immunomodulating therapies taking in account not only the alloreaction, for which standard immunosuppression was designed, but also strategies to control early inflammation and autoimmunity recurrence. Finally, immune monitoring represents the tool to identify pathways and mechanisms to target and, importantly, to evaluate the efficacy of novel treatments tested in clinical trials. Ideally, immune monitoring should provide information about a single patient needs in terms of immunomodulation, helping to find personalized therapies to increase the chances of the transplantation to succeed.

By measuring functional parameters, loss of graft function and rejection are suspected when organ damage has been established, and potential intervention therapy would start relatively late in terms of initiation of the rejection process. Ideally, monitoring of relevant immunological biomarkers should anticipate and predict organ damage allowing a timely and early intervention. Monitoring the immune response by direct tissue biopsy is impractical both for pancreas and islet transplantation. Insulitis characterized by inflammatory T cell infiltration targeting beta-cells and sparing exocrine tissue was described after pancreas graft biopsy [9] but it is hard to propose such an invasive procedure in the absence of a misfunctioning graft or a biomarker favouring the suspect of immune rejection. On the other hand, in islet transplantation biopsy is impractical for a low-volume and widely dispersed endocrine tissue [10].

Cellular and antibody-based assays in peripheral blood have therefore been proposed for surrogate monitoring to evaluate the contributions of auto- and alloreactivity. From a technological point of view, there was a great improvement in our capacity to detect and follow changes in immune biomarkers, mainly due to the use of multiplexed platforms and the introduction of novel specific reagents such as MHC multimers, able to detect rare but fundamental cell populations. With the exception of autoantibody and alloantibody measurements, most of these techniques are not entirely validated and standardized among different laboratories. This represents a major challenge for the future in order to compare results from different treatments and different patients cohorts.

In this review, we discuss which information we can obtain from immune monitoring, what we need to monitor in order to collect clinically relevant information and when, during the clinical history of the transplanted patient, this information is of particular interest in terms of prediction and intervention. Finally, we will discuss recent technical improvements, and how immune monitoring has led to the discovery of relevant mechanisms of immune-mediated beta cell destruction and the development of innovative treatments.

MONITORING THE EARLY INFLAMMATORY RESPONSE

Early inflammatory events heavily affect islet and pancreas engraftment. It is now possible to perform simultaneous measurement of more than 50 inflammatory mediators, cytokines and chemokines on the Luminex platform, providing the instrument to monitor key inflammatory pathways and to identify the important ones. Measurements of inflammatory biomarkers both in the donor pre-transplant, and in the recipient early post-transplant can provide useful information with respect to the early adverse effects of transplantation. The inferiority of organs from brain dead donors is the result of hemodynamic stability, hormonal changes and neurological effects unleashing a cascade of inflammatory events. Brain dead donors are affected by activation of vascular endothelium, complement, coagulation, and the innate immune response, resulting in the systemic release of proinflammatory cytokines and chemokines such as IL-1, IL-6, TNFalpha, IL-8 and CCL2/MCP-1. A clear link between brain death and impaired graft survival was reported in kidney, heart, liver and lung transplantation [11].

Although the mechanism was not entirely elucidated, donor CCL2/MCP-1 level was shown to be an important predictive inflammatory biomarker of pancreas transplantation outcome. In fact, high circulating levels of CCL2/MCP-1 in the donor were negatively associated with graft survival after simultaneous kidney-pancreas transplantation [12]. Donor circulating CCL-2/MCP-1 was predictive of the levels of CCL2/MCP-1 released by the graft after revascularization and was associated with increased frequency of graft loss for thrombosis.

In islet transplantation, inflammatory mediators and chemokines released by islets in culture can be predictive of the transplantation outcome. The isolation and purification procedures of islets for transplantation provides stress signals that induce islets to produce and secrete "danger signals" including tissue factor (TF), high mobility group protein B1 (HMGB1), cytokines and chemokines such as CCL-2/MCP-1, CXCL12/SDF-1, and CXCL8/IL-8.

TF is a master mediator of platelet activation and aggregation and cause the instant blood mediated inflammatory injury (IBMIR) when islet preparations are injected into the portal vein. IBMIR consists of a thrombotic reaction characterized by activation of the coagulation and complement cascades [13], cloth formation and leukocytes recruitment into the islets. It is estimated that 60-80% of transplanted islets can be destroyed by IBMIR within days when islets are transplanted into the liver. Islet preparations supplemented with low-molecular-weight dextran sulphate instead of heparin, were shown to significantly prolong survival, due to its superior capacity to inhibit IBMIR [14].

HMGB1 is released by dying cells, and the concentration of HMGB-1 reflected the degree of islet damage and correlated with poor islet transplantation outcome in animal models and in autotransplantation [15]. Recently we showed a correlation between the release of HMGB1 and the release of pro-inflammatory factors (CXCL8, CXCL10, CXCL-9 and IFN-gamma). However, we were unable to confirm a predictive correlation with islet function in allotransplantation setting.

Islets produce and secrete chemokines with the potential to directly recruit cells of the innate immune system [16]. Secretion of CCL2/MCP-1 by islets has been largely investigated both in human and mouse models of islet transplantation showing its important role in islet damage mediated by monocytes and macrophages. CCL2/MCP-1 concentration in islet preparation was highly predictive of the local inflamma-

tory response and short-term human islet function in the intrahepatic transplantation model [17].

To reduce the impact of donor derived inflammatory mediators pre-transplant, ex-vivo treatment or organs has been suggested. This is particularly feasible for isolated islets to prevent local inflammation once transplanted. Specific anti-inflammatory strategies on cultured islet have shown the potential of this approach in animal models. These include inhibition of the master regulator of inflammation nuclear factor kappa-light chain-enhancer of activated B cells (NF-kB) in beta cells with *ex-vivo* gene therapy [18] and drugs [19,20].

The recipient's innate immune response includes the production of soluble mediators, often elicited by inflammatory molecules produced by the grafts, and the recruitment of innate cells at the graft site. Important specific pro-inflammatory molecules such as TNF-alpha and IL-1beta are released by isolated islets and by activated leukocytes during graft rejection. Biologicals specifically targeting these molecules have been included in induction protocols in clinical islet transplantation. These include Etanercept, a fusion protein that binds the TNF receptor 2 to the constant end of a human IgG1 antibody and acts as a TNF-alpha inhibitor, and Infliximab, a chimeric monoclonal antibody targeting TNF-alpha [21, 22]. The IL-1 receptor antagonist Anakirna is also used as induction anti-inflammatory strategy in the first phases of islet transplantation protocols [23].

Polymorphonuclear leukocytes (PMN) are rapidly and massively recruited to transplanted islet and pancreas grafts within hours and represent the predominant infiltrating population [24,25,26]. A master regulator of PMN infiltration in transplanted patients and in animal models is CXCL8/IL-8. CXCL8/IL-8 engages CXCR1/2 receptors and recruit neutrophils at the graft site. Specific targeting of CXCR1/2 with Reparixin (an allosteric non-competitive inhibitor) was shown to enhance pancreatic islet survival after transplantation [27]. On the basis of these pre-clinical results, Reparixin is currently in an ongoing phase 3, multicentre, randomized, double blind clinical trial for islet transplantation.

Immune monitoring of the innate immune response have had two important implications. The first is that the donor inflammatory status is predictive of the transplantation outcome. This is relevant to the selection of organ donors and the possibility (at least for islet transplantation) to use ex-vivo specific anti-inflammatory molecules in order to reduce the pro-inflammatory activity. The second is that an anti-inflammatory therapy could protect transplanted grafts by the innate response early post transplantation, and substantially improve the long-term transplantation outcome. Both selectivity and timing of the treatment are critical to determine the beneficial effect of anti-inflammatory therapy, and ongoing clinical trials will reveal the real impact of this therapeutic approach.

HUMORAL IMMUNITY

Islet or pancreas grafts can elicit a humoral response with autoantibodies and alloantibodies or both. Even though a direct pathogenic role of circulating auto and alloantibodies remains controversial, the information from immune monitoring of the humoral response has a high value as predictive biomarkers of the transplantation outcome. Measurements of circulating antibodies are also relatively easy and more standardized as compared to measurement of autoreactive T cells, making monitoring of the humoral response more reliable and suitable to perform in clinical trials. Many labs are specialized in measuring auto- and alloantibodies, and serum samples are easier to collect, preserve and ship for studies than frozen PBMC samples required for T cell measurements. Here we report the results of studies monitoring the humoral response pre- and post-transplant in terms of prediction of the transplantation outcome.

Monitoring Autoantibodies

Autoantibodies against (pro)insulin, GAD65, IA-2, and ZnT8, appeared in the pre-clinical phase of type I diabetes, in which they have a clear and important prognostic and diagnostic value [28]. Autoantibody measurements have undergone to an intensive effort for standardization among different laboratories making humoral immune monitoring highly reliable also between different patient cohorts. Autoantibodies can persist for years after the onset of diabetes and islet or pancreas transplantation is occasionally performed in patients who still have circulating autoantibodies. The presence of pre-transplant autoantibodies can only weakly predict the outcome of transplanted pancreata [7]. With respect to islet transplantation, it was early reported that autoantibody positive recipients pre-transplant showed an earlier loss of function than autoantibody negative patients [29]. Later reports, however, could not confirm the predictive value of pre-transplant autoantibodies in the islet transplantation outcome [30,31,[32].

Appearance or rise of autoantibodies after transplantation, despite immunosuppression and T cell depletion, indicates the presence of autoimmunity recurrence, even though the correlation with the transplantation outcome was not always clear. A correlation between changes in GAD65 and IA-2 autoantibody titres post pancreas transplantation was early reported [33,34]. The combination of autoantibody titers with measurements of autoreactive T cells have directly shown that autoimmunity recurrence, in the absence of any evidence of allorejection, may be a cause of loss of graft function and, importantly, that the number of cases of autoimmunity recurrence after pancreas transplantation are probably underestimated [7] . In a group of 25 pancreas recipients, the addition of ZnT8 autoantibody to the traditional autoantibody screening panel (GADA, IA and IA2A), increased capacity to predict graft failure to 80% with a sensitivity of 95%, while the autoantibody status before transplant did not influence the clinical outcome [35]. Similarly in islet transplantation, measurements of post-transplant autoantibody changes, including serum conversion, spreading and increasing titres were predictive of the islet transplantation outcome using simultaneous measurements of ZnT8A, GADA and IA2A [32]. These observations about the dynamics and the predictive role of autoantibodies clearly confirmed the important contribution of autoimmunity recurrence in the loss of function of pancreas or islet grafts. Several factors can influence autoantibody changes post islet transplant. In the allogeneic context, it important to note that MHC class I mismatch was shown to be protective for post-

transplant autoantibody changes and therefore for autoimmunity recurrence [32]. Post-transplant autoantibody changes are also influenced by immunosuppression. While anti-thymocyte globulin (ATG) and micophenolate mofetil (MMF) treatment were positively associated with the risk for autoantibody increases, the anti CD25 monoclonal antibody and rapamycin were shown to be protective [32].

Monitoring Alloantibodies

Patients undergoing to allogeneic transplantation can have preformed alloantibodies (PRA) and develop donor specific alloantibodies (DSA) post transplant. PRA may derive from previous sensitization to transplant, blood transfusion and pregnancy. These patients are considered "sensitized" and this represent a risk factor for development of allorejection post transplantation. Alloantibodies are directed against MHC class I and class II molecules. Recent improvements in alloantibody measurements have been recently introduced with the use of the Luminex platform, which substantially increased the sensitivity as compared to the classical complement fixing lymphocytes cross match (CDC) method [36]. With the Luminex method, serum samples are screened for HLA IgG and IgM antibodies, through HLA-A, -B, -C, Cw, -DR, -DQ, -DP beads and HLA-A, -B, -Cw, -DRB1, -DRB3, -B4, -B5, -DQA1, -DQB1, -DPA1, -DPB1 single beads [37]. This method can detect PRA and DSA positive subjects that failed to be detected with the CDC.

Initial reports showed a negative correlation between the presence of alloantibodies pre-transplant and the graft survival [38]. HLA antibodies detected by solid phase techniques with PRA>15% prior to transplantation were associated with reduced graft survival [39] suggesting that the immunosuppressive therapy was not sufficient to control allogeneic immune response in pre-sensitized patients. Subsequent studies, however did not confirmed these indications. In these studies, the presence of pre-transplant PRA was not predictive of subsequent islet transplantation outcome [40,32]. The presence of DSA antibodies was reported in patients with or without subsequent graft rejection after islet transplantation. DSA were present in around 50% of the patients before transplantation, and the outcome was improved in patients with pre-transplant DSA. Post islet transplant, around 30% of patients had an increase in DSA, which was associated with a reduction of the duration of insulin independence. Reduced graft survival was observed both in patients with concomitant increase of DSA and autoantibodies, but also in patients with increase in DSA only. MHC class I and class II mismatch was a risk factor for development of DSA post-transplant. Similar to autoantibodies, treatment with ATG and MMF was a risk factor for post-transplant DSA development [32].

The role of pre-transplant and post-transplant alloantibodies has been evaluated also in pancreas and simultaneous pancreas-kidney transplantation using Luminex technology. Around 25% of pancreas and pancreas-kidney recipients were found to have pre-transplant HLA antibodies and therefore were considered as sensitized. However, comparison showed similar outcome in sensitized and unsensitised patients [41,42]. De novo alloantibodies appeared in 38% of transplant recipients (with no differences between pancreas and pancreas-kidney sub-groups) and were significantly associated with poorer pancreas graft survival [41,42]. These data substantially confirmed previous similar observation in pre and post-transplant alloantibodies measured by flow-PRA [43].

Monitoring of auto and alloantibodies has provided insight into the mechanisms of graft loss mediated by autoimmunity recurrence and allorejection. It is clear that both processes are a fundamental cause of graft loss, that they can arise as single events, and that they can co-exist in a single transplantation. This was important to understand that the adaptive immune response can be of different nature in different patients and to define novel immunotherapeutic approaches selectively targeting autoimmunity or alloimmunity. In this context, simultaneous measurement of autoreactive and alloreactive T cell can substantially corroborate information provided by monitoring humoral immunity.

CELLULAR IMMUNITY

There is a large consensus that both CD4 and CD8+ T cells play a major role in autoimmunity recurrence and allorejection of transplanted pancreas and islet grafts. Monitoring cellular immunity is considered the most potential in predicting the clinical outcome in pancreas and islet transplantation. However, T cell responses can be detected with different methods and still suffer of low standardization despite a great effort has been made in this direction [44]. Important informations about the T cell response post islet and pancreas transplantation came from studies of the natural history of T1D. These studies showed that the presence of T cells reacting to beta cell antigens GAD65 and (pro)insulin were commonly found also in subjects with no other sign of autoimmunity. However, in contrast to control subjects in which autoreactive T cells display a largely naïve phenotype, in patients with T1D the phenotype of autoreactive T cells showed characteristics of antigen-experienced T cells [45,46,47]. These include response to lower antigen concentration, no requirements of co-stimulation, longer telomeres and the surface expression of memory markers such as CD45RO. The concept that patients with T1D harbour memory autoreactive T cells at the time of islet or pancreas transplantation has important implications with respect to the efficacy of immunosuppression and the reactivity of these cells to antigen re-exposure.

MEASUREMENT OF AUTOREACTIVE AND ALLOREACTIVE CD4+ T CELLS

The presence of CD4+ T cells specific for beta cell associated antigens has been traditionally determined by in vitro stimulation of PBMC with antigens and measurement of their proliferative response. Proliferation determined as incorporation of ^{3}H-thymidine is very sensitive and requires a small number of cells (<100,000 PBMC), but does not provide any additional information. Proliferation determined as dilution of the CFSE dye by flow cytometry allows measurement of additional parameters in the same sample, including the phenotype of the cells and cytokine production and provides the possibility to FACS-sort proliferating clones for further studies. One recent application is to determine the

sequence of the TCR alpha and beta by single cell PCR to study clonal expansion of the identified TCR in follow-up samples by deep-sequencing [48]. A limitation of both methods is that they do not provide information with respect to the precursor frequency and changes post transplantation but rather they determine whether or not CD4 T cell autoreactivity is present in a patient.

Early studies showed an association between the *in vitro* proliferative response to autoantigens and the loss of function of islet grafts [49]. Later evidences showed that the presence of autoreactive T cell responses pre-transplant were strongly associated with the outcome of islet transplantation and that alloreactivity to the allogeneic islets proved secondary to autoimmunity recurrence [31]. The rate of 1 year insulin independence was high in patients without GAD65 and IA-2 T cell autoreactivity pre-transplant, while all patients with pre-transplant autoreactivity became insulin independent. The use of *ex vivo* proliferation test proved to be reliable in pre-transplant samples. However, in post-transplant samples T cell proliferation tests can be impaired by the use of immunosuppressive drugs and lymphopenia. In islet transplantation performed according to the Edmonton protocol, a condition of mild and chronic lymphopenia is secondary to immunosuppression. The immune system reacted by increasing levels of the homeostatic cytokines IL-7 and IL-15 causing homeostatic proliferation of T cells. In this setting, *ex vivo* proliferation tests with GAD65 were impaired by the presence of a strong spontaneous *ex vivo* proliferation of both CD4+ and CD8+ T cells, in which it was not possible to discriminate proliferation in response to GAD65 from the homeostatic proliferation induced by IL-7 [50].

To measure the actual frequency of autoreactive T cell clones and changes after transplantation became possible with the development of MHC class II tetramers. GAD65-specific tetramers were used to detect and follow changes in GAD65 specific T cell clones both in islet [50] or pancreas transplantation [51] and showed how the presence and post-transplant expansion of autoreactive clones can be associated with graft loss. Even though not conclusive, these evidences are direct proofs of autoimmunity recurrence post islet and pancreas transplantation 30 years after the phenomenon was described. Seminal were identical twin transplants performed by David Sutherland in which transplant of pancreas segment from an unaffected twin to the twin with long term T1D in the absence of immune suppression resulted in the loss of graft beta cell function and insulitis reminiscent of what is seen at diabetes onset [52,53].

Measurement of alloreactive CD4+ T cells has been recently reported using a method that combine a proliferation test with the production of cytokines. In the method, PBMC from the islet recipient were stimulated with irradiated PBMC bearing the same MHC class II molecules of the donor. Proliferation was assessed as ^{3}H-thymidine uptake and the cytokine profile was determined in the supernatant. Patients achieving stable insulin independence showed a cytokine profile skewed toward IL-10+ regulatory T cells, whereas patients with poor transplantation outcome showed a cytokine profile with low IL-10 and high IL-2 production [54].

MEASUREMENT OF AUTOREACTIVE CD8+ T CELLS

Autoreactive CD8+ T cell clones can directly kill target beta cells and therefore are considered the most important cell type to be monitored post islet and pancreas transplantation. Monitoring the CD8 cellular response is largely based on the use of fluorescent peptide-MHC multimers. Peptide-MHC multimers can bind to autoreactive T cells through the affinity of the T cell receptor for the peptide-MHC complex. The use of peptide-MHC multimers to visualize autoreactive T cells by flow cytometry was in part limited by the low-medium affinity of T cell receptor for autoantigens. The increased avidity of multimers with a high number of peptide-MHC molecules, such as pentamers, and the use of bright fluorochromes such as phycoerythrin (PE) and allophycocyanin (APC) has substantially overcome these limitations. Using the currently available reagents it is now possible to evaluate the precursor frequency of single CD8+ T cell clones by direct staining of a PBMC sample, avoiding *in vitro* culture and expansion. This is of importance for studies using stored PBMC, in which peptide-MHC multimer staining is performed on all samples at the same time at the end of the follow-up. Still the low frequency of antigen specific T cell precursors remains a challenge, however the acquisition of a large number of CD8+ events (>500,000/sample) is usually sufficient to detect autoreactive CD8+ T cells in a PBMC sample. Several MHC class I restricted epitopes of beta cell antigens have been identified as potential targets for autoimmunity and fluorescent MHC multimers are available for immune monitoring. These include $GAD65_{114-123}$, Insulin B_{10-18}, $IA\text{-}2_{797-805}$, $IGRP_{265-273}$, $ppIAPP_{5-13}$ [55].

A number of studies have been conducted in islet and pancreas transplanted patients to address whether the presence of circulating autoreactive CD8+ T cells could reflect the autoimmune reaction to islet and pancreas grafts. In islet transplanted patients, circulating autoreactive CD8+ T cells specific for preproinsulin and insulin were associated with autoimmune mediated beta cell destruction. An important issue is the role of the donor/recipient HLA mismatch. Autoreactive T cells generated during the autoimmune process that lead to T1D, were selected for their specificity for a beta cell antigen peptide in an autologous MHC context. Data obtained with MHC multimers clearly showed that pre-existing autoreactive T cell clones maintained the MHC-peptide complex specificity post-transplant. In addition, in pancreas-transplanted patients, Vbeta sequences from MHC multimers binding cells were conserved between pre and post-transplant samples. Activation of these clones can be achieved by presentation of beta cell antigens by autologous antigen presenting cells; however how these CD8+ T cells can recognize and exert a cytotoxic activity on mismatched beta cells remains unclear. Transplantation of islets from an MHC class I mismatched donor was shown to delay or prevent destruction of islets mediated by CD8+ autoreactive T cells [55]. Comparison of CD8+ T cells specificities (insulin B_{10-18}) in the peripheral blood and in the transplanted pancreas removed due to pancreatitis, showed that measurements of circulating autoreactive CD8+ T cells parallel the specificity of CD8+ T cells found in the infiltrated pancreas [56]. This was important to validate the use of peripheral

blood samples for immune monitoring of autoimmunity occurring in transplanted islet and pancreas grafts.

An important issue in immune monitoring autoreactive CD8+ T cell responses was the identification of mechanisms of expansion post transplantation. Activation of autoreactive naïve T cells specific for beta cell antigens precedes the clinical onset of T1D [46]. Autoreactive T cells with a memory phenotype survive in the immune system for years of decades and can be reactivated by antigen re-exposure post islet or pancreas transplantation. How these cells can undergo to expansion under immunosuppressive regimen was unclear. The majority of clinically available immunosuppressive drugs are designed to target activation of T cells via antigen recognition and autocrine production of IL-2. These include rapamycin, calcineurin inhibitors such as FK506, and monoclonal antibodies to the IL-2 receptor alpha CD25. Other drugs act on more aspecific mechanisms such as the inhibitor of purine biosynthesis MMF. In a lymphopenic environment T cell proliferation is influenced by the increase of circulating homeostatic cytokines IL-7 and IL-15 [57]. Memory T cells proliferating in response to IL-7 develop properties that are remarkably similar to those of antigen expanded T cells, such as the production of IFN-gamma [58]. The presence of beta cell antigens from transplanted beta cells can provide a selective advantage to the expansion of autoreactive T cell clones. In islet transplantation with the Edmonton protocol (induction with CD25 and maintenance with rapamycin and FK506) a relative lymphodepletion is associated with the presence of high circulating Ki67+ T cells, including GAD65 specific CD8+ T cell clones [50]. Homeostatic T proliferation was resistant to rapamycin and FK506. Intriguingly, the use of anti CD25 Zenapax appeared to promote homeostatic proliferation [59]. By preventing the interaction of CD25 with the common gamma chain, Zenapax increased the availability of the common gamma chain for interaction with the alpha chain of the IL-7 receptor (CD127), thus increasing the T cell response to IL-7. On the other hand, the use of MMF suppressed homeostatic proliferation as shown *in vitro* and in islet transplanted patients substituting rapamycin (for adverse side effects) with MMF therapy. Another important effect of high circulating levels of IL-7 was the abrogation of suppressive activity of CD4+CD25+FOXP3+ regulatory T cells (Treg) [60]. Treg typically express low levels of CD127 and they respond to IL-7 only at pathological circulating concentration. In this condition IL-7 can trigger homeostatic proliferation of Tregs and the suppressive activity is severely impaired, thus allowing autoreactive T cells to expand. The IL-7/IL-7R axis represents a potentially novel target pathway to inhibit autoreactive T cell expansion post transplantation. IL-7R blockade was shown to prevent and reverse diabetes onset in the non-obese diabetic (NOD) mouse model [61,62]. However, so far no molecules specifically targeting the IL-7/IL-7R axis have been developed. We have recently identified a circulating soluble form of the IL-7 receptor alpha (sCD127) which act as an endogenous modulator of the IL-7 activity and can be potentially used to keep homeostatic T cell proliferation under control [63].

The role of alloreactive CD8+ T cells in islet and pancreas graft loss has been reported in few studies. Immune monitoring of the alloreactive CD8+ response can be performed using the cytotoxic lymphocyte precursors assay. In the assay, PBMC from the recipient are co-cultured with irradiated PBMC expressing the same MHC class I molecules from the donor, and using a limiting dilution assay to calculate the precursor frequency of cytotoxic T cells [49]. An increased cytotoxic T cell frequency was associated with rapid loss of islet allografts. [49,64]. Interestingly, tolerance to kidney allografts protect subsequent islet allograft–if the kidney donor express the same MHC class I molecules of the islet donor [65]. A similar phenomenon has been described also in patients receiving subsequential islet infusions from different donors expressing the same MHC class I molecules [65].

ADVANCES IN T CELL IMMUNE MONITORING

Among the available techniques for T cell immune monitoring, MHC multimers are rapidly developing into more sophisticated and informative methods for detection of autoreactive T cell responses. A novel combinatorial approach for the use of MHC multimers has been recently introduced by Velthius at al [55]. Using quantum dot conjugated HLA-A2 multimers bearing the beta cell epitopes $GAD65_{114-123}$, Insulin B_{10-18}, $IA\text{-}2_{797-805}$, $IGRP_{265-273}$, $ppIAPP_{5-13}$ as well as positive and negative control peptides, the authors showed simulatenous detection of multiple CD8 specificities in a single blood sample. The antigen specific clones were defined by the simultaneous binding of two MHC multimer bearing the same peptide but labelled with two different quantum dots to reduce aspecific binding and false positives (cells binding only one peptide-MHC multimer are excluded from the analysis). This assay is called Diab-Q-kit and was successfully used to determine the frequency and changes in CD8 autoreactivity both in the natural history of T1D and for immune-monitoring in patients receiving islet transplants, showing an association with the disease activity and transplantation outcome.

One important issue in the detection of autoreactive T cells with MHC multimers is that autoreactive TCR have usually a low affinity for peptide-MHC complexes as compared to TCR recognising viral peptides. This can result in a weak staining of autoreactive clones. To overcome this problem MHC dextramers have been recently introduced [66,67]. MHC dextramers are MHC-peptide complexes bound to a dextran polymer backbone. MHC dextramers carry more MHC-peptide and fluorochrome molecules than tetramers and pentamers. This increases their avidity for the specific T cell and enhances their staining intensity, thereby increasing resolution and the signal to noise ratio. MHC dextramers are particularly efficient in the detection of T cells carrying T-cell receptors with low affinity for MHC-peptide, such as autoantigens.

CONCLUDING REMARKS

The improvement of immune monitoring strategies (summarized in Table **1**) in recent years has highlighted that, in addition to the classical rejection of allogeneic grafts, islet or pancreas transplantation outcome can be severely affected by an intense early inflammatory response and by the reactivation of autoimmunity. Islet or pancreas transplantation into a recipient who developed T1D is a re-challenge of

Table 1. Immune biomarkers in pancreas and islet transplantation

	Biomarker	Test	Function	Application	Ref
Early inflammatory response	**CCL2/MCP-1**	**ELISA**	Donor serum levels negatively associated with graft survival for thrombosis	Simultaneous kidney-pancreas transplantation	[12]
	HMGB1	**ELISA**	Recipient serum levels indicate islet damage and poor outcome	Islet autotransplantation	[15]
Humoral immunity	**GADA, IA2A and ZnT8A**	**Radiobinding and Immunoprecipitation assay**	Post transplant changes predictive of the outcome	Islet allotransplantation Pancreas transplantation	[33] [34; 35]
	Class I and II PRA	**FlowPRA beads + single antigen beads for cytometry**	Sensitized patients showed inferior outcome	Islet allotransplantation	[39]
	PRA and DSA	**Single antigen beads for Luminex**	Concomitant PRA and DSA increase, or DSA alone, reduce graft survival De novo alloantibodies increment is associated with poorer pancreas graft survival	Islet allotransplantation Pancreas transplantation Pancreas-kidney transplantation	[37] [41;42]
Cellular immunity	**IA-2, GAD65, insulin reactive T cells**	**^{3}H thymidine proliferation**	Pre-transplant autoreactivity associated to loss of function	Islet allotransplantation	[49] [31]
	GAD65 reactive T cells	**MHC I and II multimers**	Post-transplant expansion indicate autoimmunity recurrence	Islet allotransplantation Pancreas Transplantation	[50] [51]
	Alloreactive lymphocytes cytokine profiles	**Luminex of cytokines secreted by MLC**	IL-10+ regulatory T cells associated with insulin independence	Islet allotransplantation	[54]
	GAD65, InsulinB, IA2, PPI, IGRP, ppIAPP autoreactive CD8+ T cells	**Quantum dot conjugated HLA2 multimer staining**	Preproinsulin autoreactive CD8+ T circulating cells are associated with autoimmune mediated beta-cell destruction Association between autoreactivity and transplantation outcome Circulating CD8+ T cells parallel the specificity of intrapancreatic CD8+ T cells	Islet allotransplantation Pancreas transplantation	[55] [56]

Abbreviations: CCL2, chemokine (C-C motif) ligand 2; MCP-1, monocytes chemotactic protein 1; ELISA Enzime-linked immunosorbent assay; HMGB1, high mobility group box 1; GADA, glutamic acid decarboxylase 65 autoantibodies; IA2A, insulinoma-associated protein 2 autoantibodies; ZnT8A, zinc transporter 8 antigen autoantibodies; PRA, panel reactive antibodies; DSA, donor-specific antibodies; GAD65, glutamic acid decarboxylase 65; MHC, major histocompatibility complex; MLC, mixed lymphocyte cultures; IL-10, interleukin 10; IL-2, interleukin 2; PPI, preproinsulin; IGRP, islet specific glucose-6-phospatase catalytic subunit-related protein; ppIAPP, prepro-islet amyloid polypeptide; HLA2, human leukocyte antigen 2

an autoreactive memory response, which can result in autoimmunity recurrence after transplantation. It is now clear that the classical immunosuppressive therapy to prevent allorejection is not sufficient to keep the early inflammatory response and autoimmunity recurrence under control. Immune monitoring has been of great importance to better define the role of these events in graft loss and transplantation outcome. Moreover, it helped to identify novel pathways for specific targeting of specific events. Further studies are needed to define whether immune monitoring can be used to predict the outcome before a patient is transplanted and therefore, to select patients who would benefit from a beta cell replacement therapy though islet or pancreas transplantation. Ideally, the future goal of immune monitoring is to define biomarkers and immune parameters to adapt the immonomodulatory therapy to the single patient needs, in order to perform a personalized therapy according to the nature and intensity of the immune reaction that each single patient can developed after transplantation.

CONFLICT OF INTEREST

Paolo Monti, Debora Vignali, and Lorenzo Piemonti declare that they have no conflict of interest.

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