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Review Article

Implications of Calcineurin/NFAT Inhibitors' Regulation of Dendritic Cells and Innate Immune Cells in Islet Xenotransplantation

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Calcineurin inhibitors, such as cyclosporine and tacrolimus (FK506) are broadly used in organ transplantations as immune suppressants. As the calcineurin/NFAT signaling pathway has been identified as critical pathway in the interleukin-2 (IL-2) production of T cells, inhibition of T-cell derived IL-2 has been considered the major mechanism of calcineurin inhibitors. However, there is increasing evidence that NFAT transcription factor is involved in multiple functions of dendritic cells and innate immune cells as well. NFAT expression is not restricted to T cells, and IL-2 can be produced in dendritic cells and macrophages through the calcineurin/NFAT pathway. Furthermore, it has been discovered that NFAT regulates expressions of several inflammatory mediators, including TNF-α and cyclooxygenase-2 in innate immune cells. Therefore, calcineurin inhibitors may have much broader effects in the transplant recipients than previously being considered. In this review, we reviewed recently discovered roles of NFAT pathway in dendritic cells and innate immune cells, and discussed positive and negative implications of calcineurin inhibitors' broader effects with a focus on islet xenotransplantation.

Key Words: Calcineurin inhibitors, Innate immunity, Islet transplantation, Xenotransplantation

I. INTRODUCTION

Nuclear factor of activated T cells (NFAT) was originally identified in nuclear extracts of activated T cells as a DNA-binding factor which binds to the interleukin-2 (*Il2*) promoter (1). Later, it was shown that inhibition of NFAT is the primary mechanism of calcineurin inhibitors, including cyclosporine and FK506 (tacrolimus) (2, 3). These potent immune suppressants revolutionized allogeneic organ transplantations, dramatically raising 5-year survival rates of allografts since the introduction of cyclosporine into clinical practice. Before recent discoveries, inhibition of NFAT-mediated IL-2 production in T cells had been considered the principal mechanism of action for the calcineurin inhibitors. However, despite its name, the expression of NFAT is not limited to T cells. It has been shown that NFAT is expressed by almost every cell type, including other cells of the immune system (4~8). Due to the recent discoveries, it is now clear that NFAT has important functions in cells

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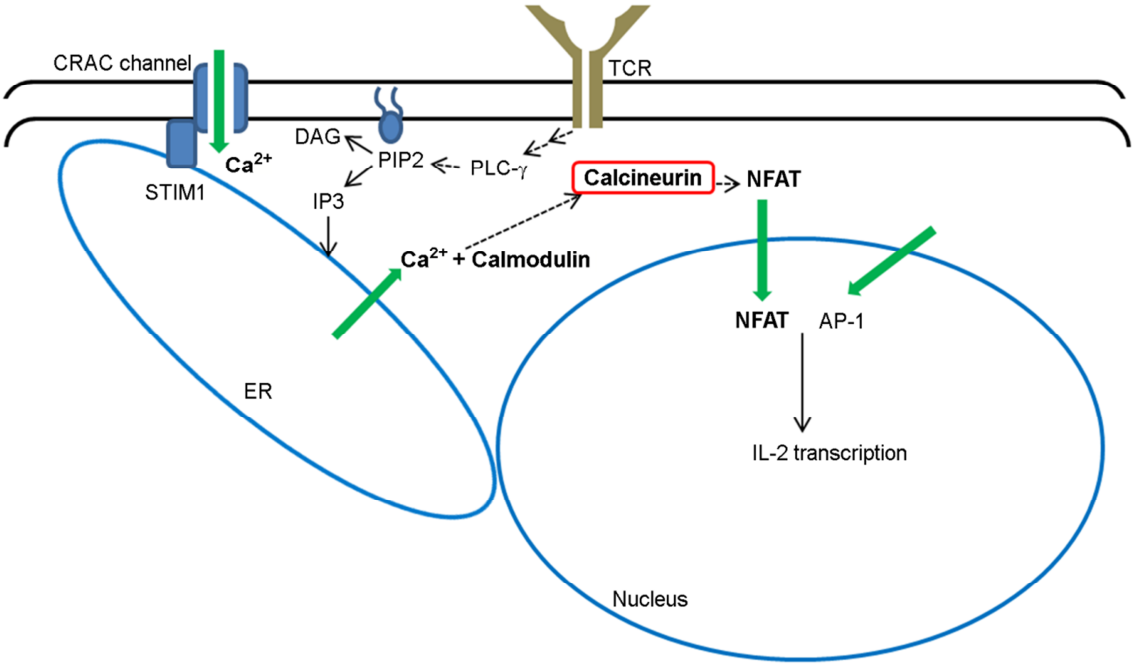

Figure 1. The classical calcienruin/NFAT signaling pathway in T cells

of the innate immune system (9).

During the past two decades, significant progress has been made in the field of xenotransplantation. Especially, successful long-term survival results have been achieved in pre-clinical pig-to-non-human primate islet xenotransplantation (10). Due to successful results in pre-clinical studies, it seems that clinical trials of islet xenotransplantation may take place within a few years (11, 12). Therefore, transplantation of islets is likely to be the first introduction of xenotransplantation into clinic, thereby providing an ultimate solution to the problem of donor organ supply for curing Type I diabetes.

Though the acute rejection of xenogeneic islets is primarily a T cell-dependent process (13), regulations of the innate immunity may bring beneficial effects for achieving long-term graft acceptance in islet xenotransplantation. In this review we overview the actions of calcineurin/NFAT inhibitors in T cells and innate immune cells, and discuss its implications in islet xenotransplantation.

II. The Classical Calcineurin/NFAT Signaling Pathway in T Cells

The classical calcineurin/NFAT signaling pathway in T cells and its inhibition by calcineurin inhibitors can be summarized as follows (Fig. 1). Upon antigen binding to a T cell receptor (TCR), cytosolic phospholipase C-γ (PLC-γ) is recruited to the plasma membrane and becomes activated. Activated PLC-γ catalyzes the hydrolysis of the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 then binds to specific IP3 receptors on endoplasmic reticulum and results in Ca^{2+} release from endoplasmic reticulum Ca^{2+} stores into the cytoplasm. The depletion of endoplasmic reticulum Ca^{2+} is sensed by an endoplasmic reticulum membrane protein called stromal interacting molecule 1 (STIM1), which triggers opening of plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Subsequently, influx of extracellular Ca^{2+}

further increases the cytosolic Ca^{2+} concentration. Cytosolic free Ca^{2+} then binds to a Ca^{2+}-dependent regulatory protein called calmodulin, and these Ca^{2+}-calmodulin complexes can activate a protein serine/threonine phosphatase called calcineurin. The activated calcineurin dephoshorylates phoshoserines in NFAT, thereby exposing a nuclear localization signal that permits NFAT to translocate from the cytoplasm into the nucleus. Inside the nucleus, NFAT proteins interact with multiple transcriptional partners to assemble active transcription complexes. In TCR signaling, NFAT cooperates with AP-1 and induces transcription of IL-2 and other cytokine genes (7, 8).

In fact, the mechanism of NFAT activation was discovered indirectly by mechanism studies of the calcineurin inhibitors, cyclosporine and tacrolimus (2, 3). Cyclosporine binds to a cellular protein called cyclophilin. The cyclosporine-cyclophilin complex binds to inhibit the enzymatic activity of the calcineurin, thereby disrupting the dephosphorylation and nuclear translocation of NFAT. Tacrolimus (FK506) is a widely used calcineurin inhibitor, which binds to its binding protein (FKBP), and then the tacrolimus-FKBP complex binds calcineurin and inhibits its activities, including the NFAT-mediated transcription of IL-2 in T cells, thereby inhibiting T cell proliferation.

The NFAT family consists of five members: NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5 (8). Four of these (NFAT1~4) are regulated by intracellular Ca^{2+} signaling and calcineurin. On the other hand, NFAT5 is a tonicity-responsive protein, and is activated in response to osmotic stress (9).

III. Calcineurin/NFAT Signaling Pathway in Dendritic Cells and Innate Immune Cells

1. NFAT-mediated IL-2 production in dendritic cells and innate immune cells

As described above, calcineurin/NFAT signaling induces IL-2 production in T cells. Recently, this pathway has also been reported to induce IL-2 production in dendritic cells (DCs) and innate immune cells. Similar to the pathway downstream of the TCR, two signal transduction pathways downstream of pattern recognition receptors (PRRs) have been characterized in terms of their capacity to induce NFAT-mediated IL-2 production in innate immune cells. These are the pathways initiated by dectin-1 and TLR4/CD14 in DCs and macrophages.

Dectin-1 is a carbohydrate receptor which belongs to the C-type lectin family, and recognizes β-glucan. Since β-glucan is a fungal cell wall component, dectin-1 mediates the anti-fungal immune response. Goodridge *et al*. (14) had reported the first evidence for NFAT activation, with dectin-1 stimulation, which is in addition to the previously known function of activating NF-κB. Upon β-glucan binding, Src-family kinase phosphorylates immunoreceptor tyrosin-based activation motif (ITAM)-like motif at the intracellular tail of dectin-1, thereby creating a docking site for Syk. Recruited tyrosine kinase Syk phosphorylates and activates PLC-γ. Activated PLC-γ then hydrolyzes PIP2 into IP3 and DAG. IP3 induces the endoplasmic reticulum Ca^{2+} release and subsequent calcineurin/NFAT activation, which results in IL-2 production in DCs and macrophages (Fig. 2).

Besides dectin-1, Toll-like receptor 4 (TLR4) and its co-receptor CD14 had been reported to induce NFAT-mediated IL-2 production independently to the canonical MyD88/TRIF pathway (15, 16). Lipopolysaccharide (LPS) engagement of TLR4/CD14 activates Src-family kinase and PLC-γ. Activated PLC-γ then hydrolyzes PIP2 into IP3 and DAG. IP3 induces influx of extracellular Ca^{2+} and subsequent calcineurin/NFAT activation, which results in IL-2 production in DCs (Fig. 2).

Although adjacent T cells of adaptive immunity can utilize the DC-derived IL-2 as a growth factor, interferon-γ-producing activity of natural killer (NK) cells and NKT cells can also be enhanced by DC-derived IL-2 (17~19). Therefore, when calcineurin inhibitors are administered, inhibition of NFAT-mediated IL-2 production not only affects T-cell mediated adaptive immunity, but also downregulates innate immune responses.

2. NFAT-mediated production of inflammatory mediators and prolongation of innate immune cells' survival

There have been reports revealing the role of NFAT in

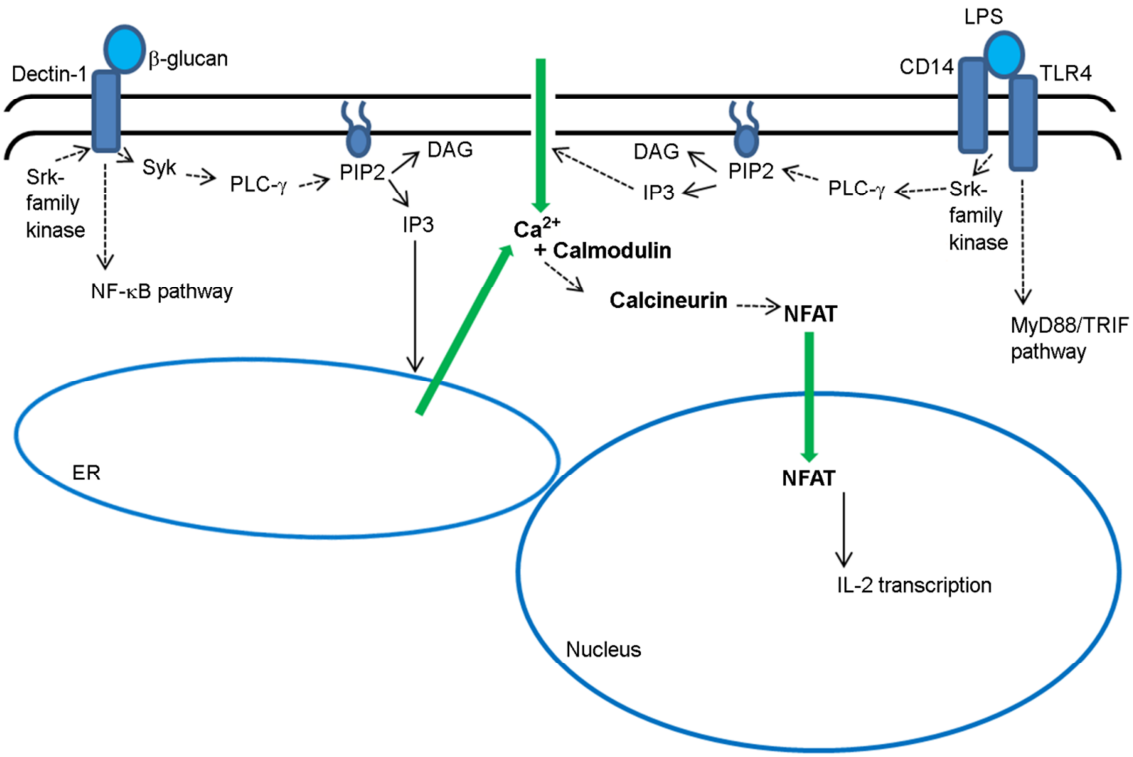

Figure 2. NFAT-mediated IL-2 production in dendritic cells and macrophages

regulating several key modulators of innate immunity, and calcineurin inhibitors may suppress innate immune responses with IL-2-independent mechanisms.

Tumor necrosis factor-α (TNF-α) is a prototypic pro-inflammatory cytokine, and NFAT1 and NFAT2 are critically involved in the expression of TNF-α in mast cells and NK cells (20, 21). IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are required for differentiations of myeloid lineage innate immune cells. Furthermore, GM-CSF regulates the release of neutrophils from the bone marrow (22). It had been revealed that NFAT contribute to the transcriptions of IL-3 and GM-CSF (21, 23, 24). In addition, it has been discovered that NFAT activation regulates the induction of cyclooxygenase-2 (COX-2) (14), which is required for the production of key inflammatory mediators including prostaglandins. Besides, NFAT transcriptionally controls *Ptges1* that codes a protein called microsomal prostaglandin E synthase 1 (mPGES-1), a key enzyme in PGE2 biosynthesis (25). Indeed, it had been proven that cyclosporine abolished COX-2 upregulation and PGE2 release in human neutrophils (26). Taken as a whole, inflammatory response can be profoundly decreased by the calcineurin inhibitor, and thus has considerable implications for the clinic (27).

Recently, the mechanism of mast cells' prolonged survival had been proven to be linked to the NFAT-dependent transcriptional induction of anti-apoptotic Bcl-2 family protein A1 (28). Previously reported A1-mediated prolonged survival of mast cells on IgE crosslinking (29) was abrogated by inhibition of calcineurin/NFAT by cyclosporine (28). In addition, anti-apoptotic functions of A1 had previously been reported in macrophages and neutrophils (30~32). Therefore, additional immune suppressive function of calcineurin inhibitors by inhibiting prolonged survival of innate immune cells is anticipated in organ transplant recipients.

IV. Positive Implications in Clinical Islet Transplantation

1. NFAT-mediated cytokines targeted in clinical islet transplantation (IL-2 and TNF-α)

In 2000, Shapiro *et al*. (33) reported successful clinical islet allotransplantation with the Edmonton protocol, and it became a standard immune suppression regimen. Tacrolimus was included as the calcineurin inhibitor, and daclizumab was used as an IL-2 receptor antagonist. In addition to the well-known suppression of T cell-derived IL-2, suppression of DC-mediated IL-2 by tacrolimus might have contributed to the potent immune suppression. Recently, several groups are conducting clinical trials of islet transplantation to improve the immune suppression regimen (34~36). They additionally used etanercept, which is a recombinant fusion protein of TNF receptor and IgG1 Fc domain (37), and functions as a decoy receptor that binds to and sequesters TNF-α (38). In addition, most of successful pre-clinical islet xenotransplantations targeted TNF-α with etanercept or adalimumab, an anti-TNF-α monoclonal antibody (39~45). Therefore, aforementioned NFAT-mediated expression of TNF-α and its inhibition by calcineurin inhibitors have additional positive implication to the importance of TNF-α blockade in clinical islet transplantation.

2. Alleviation of Instant Blood-Mediated Inflammatory Reaction (IBMIR)

Clinical islet transplantation is performed to the liver through the portal vein. In this situation, the infused islets have direct contact with the blood stream, and results in Instant Blood-Mediated Inflammatory Reaction (IBMIR) which causes a considerable amount of early islet loss. The IBMIR is a multifaceted phenomenon comprising activation of the coagulation pathway, complement system, and platelets quickly followed by the recruitment and infiltration of neutrophils and monocytes (46~48) (Fig. 3). Since calcineurin inhibitors suppress NFAT-mediated development and release of inflammatory cells, they can reduce the recruitment of neutrophils and monocytes and alleviate the IBMIR. In addition, suppression of NFAT-mediated TNF-α can further decrease the recruitment and activation of neutrophils and monocytes. In other arms of IBMIR, activated platelets release a soluble form of CD40 ligand (sCD40L), and activate CD40-expressing neutrophils (49). Crist *et al*. had reported that NFAT1 is a key transcriptional regulator of CD40L expression in megakaryocytes, the precursors of

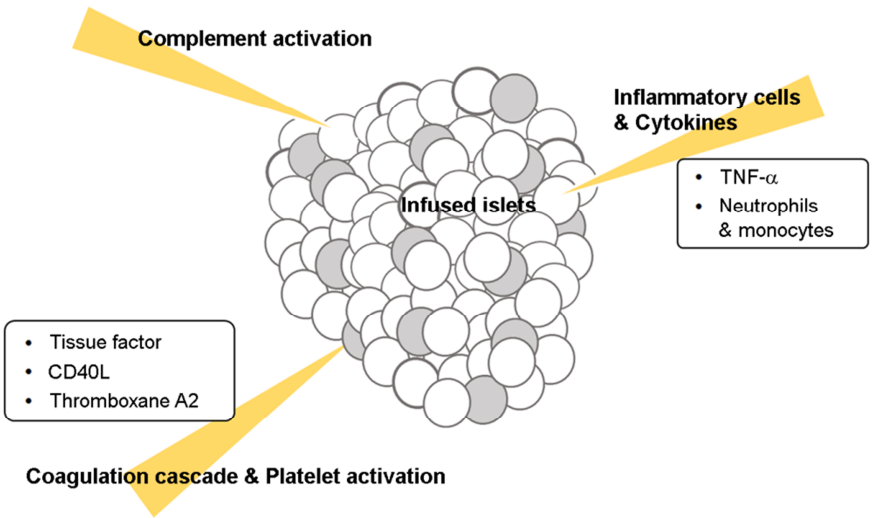

Figure 3. Instant Blood-Mediated Inflammatory Reaction (IBMIR) and NFAT-mediated targets

blood platelets, and that biochemical inhibition of NFAT activity in megakaryocytes diminishes platelet CD40L (50, 51). Additionally, since calcineurin inhibitors suppress NFAT-mediated expression of COX-2, thromboxane A2 production and subsequent platelet aggregation can be diminished. Furthermore, calcineurin inhibitors including cyclosporine inhibit NFAT-mediated expression of tissue factor (52), which interacts with coagulation factor VII and initiates the coagulation pathway (53). To summarize, calcineurin inhibitors may contribute to alleviation of IBMIR by suppressing activations of coagulation pathway, platelets, neutrophils and monocytes, thereby minimizing early islet loss in clinical islet transplantation.

V. Negative implications of Innate Immune Regulation by calcineurin inhibitors

1. Increased susceptibility to opportunistic infections

Since calcineurin inhibitors suppress not only T cells but also dendritic cells and innate immune cells, potent suppression of immune rejection in transplantation could be anticipated. However, additional suppression of innate immune cells may elevate susceptibility to opportunistic infections in the recipients. Indeed, correlation between intense NFAT suppression and recurrent infections in cyclosporine-treated patients has been reported (54). Recent discoveries of calicineurin/NFAT signaling pathway activation downstream of pattern recognition receptors (PRRs) may explain the greater susceptibility of opportunistic infections (27, 55). The fact that conditional deletion of calcineurin in neutrophils decreased resistance to infection with *Candida albicans* in mice suggests the importance of NFAT-mediated innate immune responses (56). In addition to aforementioned dectin-1 and CD14/TLR4, expression of another PRR nucleotide-binding oligomerization domain 1 (Nod1) and neutrophil phagocytic killing activity was significantly reduced in cyclosporine-treated mice (57). These interactions between calcineurin/NFAT signaling pathway and PRRs may explain the increased susceptibility to opportunistic fungal or bacterial infections in transplant recipients treated with calicneurin inhibitors.

2. Homeostasis dysregulation with interruption of innate immunity

It is well-known that NFAT is involved in the development of T cell and B cells (58~61). Though the role of NFAT in the regulation of hematopoiesis of innate immune cells is largely unknown, members of the NFAT family are expressed in $CD34^+$ hematopoietic stem cells and their differentially regulated expression during the lineage-specific differentiation of myeloid cells have been reported (62, 63). Recently, Fric *et al*. revealed that NFAT is a potent negative regulator of myeloid cell development (64). Therefore, calcineurin inhibitors may disrupt the hematopoiesis and homeostasis of the innate immune cells.

Since soluble mediators of innate immunity do not discriminate between the host and the graft, immune responses against transplanted grafts may provoke host tissue damage. Anti-inflammatory cytokine IL-10 has a role in regenerative healing, and its expression can be upregulated through calcineurin/NFAT pathway (14, 56, 65). Therefore, calcineurin inhibitors may interrupt homeostatic healing of host tissue damage in the graft recipients.

3. Unfavorable effects of calcineurin inhibitors on regulatory T cells

Recently, Shin *et al*. reported long term survival (167 ~ >603 days) of pig islet xenografts in non-human primates with infusion of autologous regulatory T cells in conjunction with other immune suppressants (44). Regulatory T cells can suppress diverse immune responses, and have physiological functions in self-tolerance. Notably, the induction of regulatory T cells may induce graft-specific tolerance in transplant recipients (66).

Regulatory T cells have higher dependence on IL-2 than effector T cells for their maintenance (67~69). As mentioned earlier, calcineurin/NFAT signaling is required for the expression of not only T cell-derived IL-2 but also DC-derived IL-2. Therefore, calcineurin inhibitors may not be beneficial in terms of regulatory T cell maintenance. Foxp3 is a lineage-defining transcription factor of $CD4^+$ regulatory T cells, and has crucial roles for the suppressive function of

these cells. NFAT interact with Foxp3 as a transcriptional partner (70~73), and NFAT-Foxp3 transcriptional complex induces the expression of IL-2 receptor α chain (CD25) and cytotoxic T lymphocyte antigen 4 (CTLA-4) (71), which are involved in the suppressive function. In addition, it has been reported that NFAT was essential for the peripheral conversion of $CD4^{+}Foxp3^{-}$ T cells to $CD4^{+}Foxp3^{+}$ regulatory T cells (74), and the Foxp3 induction was completely blocked by cyclosporine (75).

4. Unfavorable effects of calcineurin inhibitors on neovascularization

In contrast to transplantation of vascularized solid organs, transplantation of islets requires neovascularization to the islet cells for engraftment. It has recently been shown that NFAT regulates the expression of hypoxia-inducible factor 1α (HIF-1α) in mast cells (76). HIF-1α is critical for adaptation to oxygen deficit and it regulates angiogenesis (77). PGE2 and vascular endothelial growth factor (VEGF) stimulate endothelial cell proliferation, migration and, eventually, neovascularization. However, this angiogenesis had been shown to be inhibited by cyclosporine (78). As described earlier, NFAT regulates PGE2 biosynthesis. In addition, engagement of VEGF receptors on endothelial cell by VEGF induces the expression of additional VEGF and VEGF receptors through the calcineurin/NFAT signaling pathway (79). In other words, calcineurin inhibitors may have unfavorable effects on the neovascularization and engraftment of the transplanted islets.

VI. Effects on Beta-cell Function

Islet transplantation aims to supply insulin-producing β-cells and normalize blood glucose levels in Type 1 diabetes patients. To achieve this goal, transplanted β-cells have to maintain viability, and produce and secrete insulin. In addition, insulin has to be utilized by the target cells, including muscle cells. Although the molecular mechanisms are not completely understood, calcineurin is involved in regulation of replication and survival of β-cells (80), and production and secretion of insulin (81, 82). Skeletal muscle is the primary site for glucose uptake in response to insulin (83), and is composed of a mixture of three myofiber types which have variable insulin sensitivity. NFAT has been reported to be responsible for the transcriptional activation and repression of distinct myosin fibers, thereby increasing insulin-sensitive myofibers and decreasing insulin-resistant myofibers in the skeletal muscle (84~86).

New-onset diabetes mellitus after transplantation (NODAT) occurs in 15~30% of recipients after renal transplantation with immunosuppressive drugs (87~89), and use of calcineurin inhibitor is one of its risk factors (90). Since calcineurin /NFAT is involved in above-mentioned functions of β-cells, calcineurin inhibitors may induce β-cell death (91), diminished insulin production and secretion (81, 92), and impaired insulin sensitivity (93, 94). Considering the reversibility after withdrawal of the drug (95), impaired insulin secretion and insulin resistance seems to be the major mechanisms.

VII. CONCLUSION

Activation of adaptive immunity with T cell-derived IL-2 production had been thought as the principal role of calcineurin/NFAT. However, it has become evident that calcineurin/NFAT has multiple roles in the regulation of dendritic cells and innate immune cells. Although calcineurin inhibitors are widely used in clinical transplantations, our attention on calcineurin inhibitors has not been extended to the recently discovered roles in innate immune system. In islet xenotransplantation, intense immune suppression covering innate immunity and possible alleviation of IBMIR may be beneficial. However, long-term use of calcineurin inhibitors may not be favorable due to the possible effects on opportunistic infections, disruption of homeostasis, regulatory T cells, neovascularization, and β-cell functions.

The complicated functions of NFAT in various cell types are not fully understood. Various cell types do not homogenously express the five NFAT isomers, thereby varying the effects of calcineurin inhibitors. In addition, the NFAT's transcriptional partners such as AP-1 and Foxp3 are differentially expressed in various cell types, and the balance of these cofactors present may result in the different

outcomes (27). Although it is complicated, thorough research and understanding of the roles of NFAT in diverse immune cells is required. Through the improvement in the understanding on the roles of calcineurin/NFAT, calcineurin inhibitors can be utilized more effectively and safely in the transplant recipients.

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