Prostanoids modulate inflammation and alloimmune responses during graft rejection

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Abstract

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Acute rejection of a transplanted organ is characterized by intense inflammation within the graft. Yet, for many years transplant researchers have overlooked the role of classic mediators of inflammation such as prostaglandins and thromboxane (prostanoids) in alloimmune responses. It has been demonstrated that local production of prostanoids within the allograft is increased during an episode of acute rejection and that these molecules are able to interfere with graft function by modulating vascular tone, capillary permeability, and platelet aggregation. Experimental data also suggest that prostanoids may participate in alloimmune responses by directly modulating T lymphocyte and antigen-presenting cell function. In the present paper, we provide a brief overview of the alloimmune response, of prostanoid biology, and discuss the available evidence for the role of prostaglandin E2 and thromboxane A2 in graft rejection.

Introduction

Alloimmune response is a term that describes the immune system's reaction to alloantigens (1). The main alloantigens in transplantation are the major histocompatibility complex (MHC) molecules present on the surface of graft cells. T lymphocytes of the host are capable of detecting foreign MHC through surface proteins called T cell receptors (TCRs). However, transplant immunologists have long recognized that the mere binding of foreign MHC to the TCR - the so-called signal 1 - is not sufficient to induce an immune response. A second signal, delivered by activated antigen-presenting cells (APCs), also known as co-stimulation, is required for the full activation of T cells (2).

In fact, in the absence of co-stimulation, alloantigen recognition by T lymphocytes promotes tolerance and not immunity. Since APCs are not antigen-specific and, thus, do not possess the capability of recognizing foreign MHC, other stimuli are required for APC activation.

Organ transplantation involves surgical procedures that invariably result in tissue damage and cell death; such damage results in inflammation and sends the "alarm signals" needed to activate host APCs (3). In cadaveric organ transplantation, additional factors such as hemodynamic instability and systemic release of cytokines, cold ischemia time, and reperfusion injury confer a pro-inflammatory state to the graft before transplant that enhances its immunogenicity. For

example, experimental evidence has shown that inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells (4). Inflammation also up-regulates the expression of co-stimulatory molecules such as B7 members and CD40 on the surface of APCs (5). On this basis, perhaps kidneys from living donors are accepted more easily than those from cadavers because of the lower extent of damage to the graft.

Once activated, APCs can then home to local lymph nodes and efficiently present foreign MHC to T cells. Proper T cell activation by alloantigens in the context of danger signals initiates a cascade of events culminating in the generation of effector elements that mediate graft damage (6). Activated CD8+ T cells injure the graft by way of perforin- or fas-induced apoptosis. CD4+ T cells activate B cells by way of cell-to-cell interactions (such as CD40-CD40L) and Th2 cytokines; activated B cells mature into plasma cells and secrete alloantibodies that harm the graft by activating the complement cascade or antibody-dependent cellular cytotoxicity (7). Finally, CD4+ T cells release several cytokines and chemokines that orchestrate a delayed hypersensitivity-type response, attracting inflammatory cells such as neutrophils, eosinophils and macrophages to the graft. Activated macrophages produce soluble mediators that can regulate graft function and modulate the alloimmune response. If left unchecked, the full-blown alloimmune response leads to acute graft rejection (Figure 1), which is characterized by intense inflammation within the graft. In the absence of immunosuppression, subjects with acute rejection present impaired graft function along with erythema, swelling, tenderness, and increased temperature at the graft site, the cardinal features of inflammation.

Therefore, inflammation is not only central to the initiation of the alloimmune response but also occurs as a result of the effector elements generated by this response. In this manuscript, we will focus on the role of the prostanoids, a group of classic soluble mediators of inflammation generated during acute allograft rejection (8). Prostanoids is the collective term used to describe the cyclooxygenase (COX) metabolites of arachidonic acid, prostaglandins and thromboxane A2 (TXA2). As we will show, these molecules have several functions that are relevant to their ability to modulate the intensity of graft rejection, such as regulation of platelet aggregation, vascular tone and permeability, as well as direct effects on T cells and APCs.
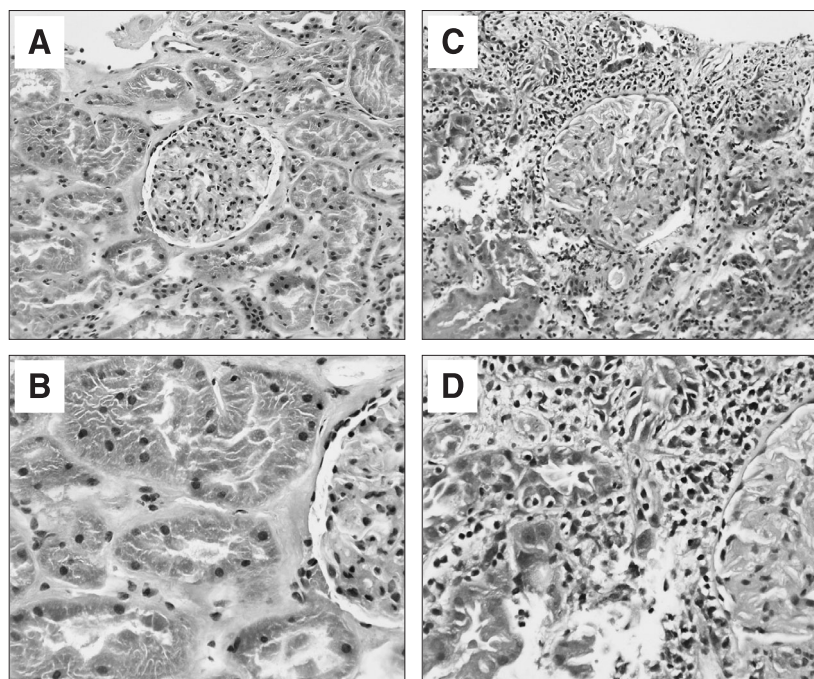

Figure 1. Inflammatory infiltrate in acute renal allograft rejection. *A* and *B*, A hematoxylin and eosin-stained renal allograft biopsy specimen obtained from a patient without rejection during a protocol biopsy. *C* and *D*, Intense interstitial inflammatory infiltrate characteristic of acute renal allograft rejection (Banff' 97 grade IB). These inflammatory cells are the source of the inflammatory mediators (cytokines, chemokines, prostanoids) that orchestrate the alloimmune response. Top figures (A and C), 50X magnification; bottom figures (B and D) 100X magnification.

Overview of prostanoid biology

The breakdown of cell membrane phospholipids into the 20-carbon fatty acid arachidonic acid, the initial rate-limiting step in prostanoid production, is mediated by a group of cytosolic enzymes called phospholipases

(Figure 2). Several noxious stimuli and cytokines can up-regulate the activity of these enzymes, which increases the availability of the substrate arachidonic acid during inflammatory states. Conversely, adrenal steroids inhibit phospholipases and, consequently, the production of prostanoids; this is one of the mechanisms behind the anti-inflammatory properties of corticosteroids.

Prostaglandin H (PGH) synthase, also known as COX, catalyzes the conversion of arachidonic acid into PGG2, an unstable prostaglandin that is peroxidized to PGH2. There are two known COX isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in virtually every nucleated cell and is responsible for the basal production of prostanoids involved in physiologic functions. Conversely, COX-2 activity is not detectable in most tissues during physiologic states but is characteristically up-regulated by inflammatory stimuli. Therefore, COX-2 is responsible for the increased output of prostanoids during pathologic conditions such as arthritis, glomerulonephritis, and acute allograft rejection.

PGH2 is the intermediate prostaglandin that is metabolized by tissue-specific isomerases to generate prostaglandins and TXA2. After synthesis, prostanoids exit the cell via poorly characterized prostaglandin transporters and are rapidly converted into inactive metabolites; therefore, these compounds have few systemic effects and must exert their actions on neighboring cells or on the very cells from which they were generated. The cellular actions of prostanoids are mediated via specific G-protein coupled receptors present in most tissues and particularly abundant in cells of the immune system. These receptors have been well characterized and the genes encoding them have been cloned. Mouse lines with targeted disruption of each prostanoid receptor have been subsequently generated, leading to a better understanding of the function of individual prostanoids in physiology and disease.

In transplantation, the function of two prostanoids, PGE2 and TXA2, has been best characterized. Since these molecules have very distinct roles in graft rejection, they will be analyzed separately.

PGE2 in transplantation

PGE2 is generated by the sequential metabolism of arachidonic acid by COX and PGE synthase. The protean biologic actions of this compound are mediated by four receptors: EP1, EP2, EP3, and EP4. Mice with targeted deletions of each receptor subtype have been generated, contributing to a detailed understanding of the role of these receptors in physiology and disease. On the immune system, the actions of PGE2 result in suppression of T cell and APC functions, effects that are mediated via EP2 and EP4 receptors (9). Both receptor subtypes share
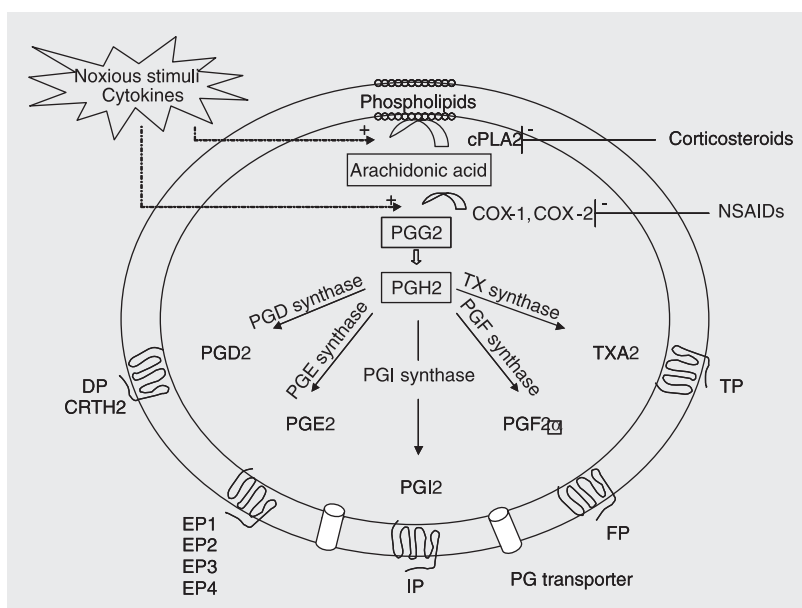

Figure 2. Biosynthesis of prostanoids. The biosynthesis of prostanoids begins when phospholipases break down cell membrane phospholipids into arachidonic acid, which is the substrate for the prostaglandin H (PGH) synthases (also known as cyclooxygenases). Prostanoids are synthesized by the metabolism of PGH by various isomerases such as PGE synthase and TX synthase. After synthesis, prostanoids exit the cell via prostaglandin transporters and bind to specific G protein coupled receptors present on the surface of neighboring cells. cPLA2 = cytosolic phospholipase A2; COX = cyclooxygenase; NSAIDs = non-steroidal anti-inflammatory drugs; PG = prostaglandin; TX = thromboxane; CRTH2 = chemoattractant receptor-homologous molecule expressed on Th2 cells.

similar intracellular signaling pathways that involve binding to Gαs, stimulation of adenylate cyclase and generation of increased intracellular levels of cyclic AMP, a phenomenon typically associated with suppression of immune cell function. In an elegant study, Nataraj et al. (9) demonstrated that the suppressive effects of PGE2 on T cells are mediated by EP2 receptors whereas in macrophages, both EP2 and EP4 receptors are involved.

In macrophages, PGE2 down-regulates the expression of MHC class II antigens (10), inhibits the production of IL-1 (11), IL-12 (12,13), TNF-α (14), and superoxide (15) and increases IL-10 production in response to LPS (12,16). This pattern of cytokine release by APCs favors the differentiation of naive T cells towards a Th2 phenotype and promotes B lymphocyte isotype switching to IgE. In addition, PGE2 has been shown to directly inhibit T cell proliferation in response to mitogens and alloantigens, an effect that has been attributed to inhibition of IL-2 production (17-21).

In concert with their properties of inhibiting cell-mediated immunity *in vitro*, PGE analogues have been shown to prolong the survival of renal (22), cardiac (23), and small-intestinal (24) allografts in rats, as well as skin allografts in mice (25). Human transplantation studies also indicate a favorable effect of PGE analogues. In a double-blind, placebo-controlled study of 77 renal allograft recipients receiving a cyclosporine- and prednisone-based regimen, Moran et al. (26) randomized patients to receive additional treatment with misoprostol (N = 38) or placebo (N = 39) for the first 12 weeks; the drug or placebo were then discontinued and the patients followed for an additional 4 weeks. At the end of follow-up, patients receiving misoprostol had significantly better renal function, as judged by serum creatinine and creatinine clearance. Moreover, misoprostol-treated patients experienced a 50% reduction in the incidence of acute rejection (10/

38 *vs* 20/39, P = 002). Beneficial effects of PGE have also been demonstrated in liver transplantation. Henley and co-workers (27) showed that, compared to placebo (N = 82), the intravenous infusion of a PGE1 analogue (N = 78) for the first 21 days after liver transplantation reduced the intensive care unit stay, the overall duration of hospitalization, and the need for renal support.

Thromboxane A2 in transplantation

TXA2 is generated by the metabolism of arachidonic acid by COX and TX synthase. TXA2 is widely known for its effects on blood vessels (vasoconstriction) and platelets (aggregation) but an increasing body of evidence indicates that TXA2 also has important actions on the immune system. The cellular actions of TXA2 are mediated by a single thromboxane prostanoid (TP) receptor, which is highly expressed in immune cells; indeed, the mouse organs with the highest concentrations of TP receptors are the thymus and the spleen (28). The TP receptor signals through Gαq and promotes an increase in intracellular calcium concentration, a phenomenon associated with activation of immune cells.

Leung and Mihich (29) were the first to suggest that TXA2 might have immuno-stimulatory functions. These authors showed that inhibition of TXA2 synthesis with imidazole in cell culture experiments caused a significant reduction in splenocyte proliferation and cytotoxicity. These results suggested that TXA2 might be required for adequate splenocyte proliferation. Alternatively, pharmacologic inhibition of TX synthase could have resulted in increased substrate availability and led to increased production of suppressor prostanoids such as PGE2, a phenomenon known as endoperoxide shunting. To clarify this issue, Ruiz et al. (30) compared the effects of a TX synthase inhibitor, a TP receptor antagonist, or both in combination on the proliferation of un-

primed mouse spleen cells in the mixed lymphocyte reaction. They demonstrated that the synthase inhibitor and receptor antagonist similarly inhibited splenocyte proliferation and that the use of both drugs in combination did not promote any additional effect, indicating that TXA2 directly influences lymphoproliferative responses in the mixed lymphocyte reaction. Recently, researchers in Dr. Thomas Coffman's (31) laboratory generated mice with a targeted deletion of TP receptors (TP–/–). *In vitro* assays demonstrated that, compared to wild-type controls, both mitogen- and alloantigen-induced proliferative responses are substantially reduced in TP–/– spleen cells (32). Taken together, these results unequivocally confirm direct pro-inflammatory actions of TXA2 enhancing cellular immune responses.

Additional studies have shown that TXA2 enhances cell-mediated immunity through distinct actions on T cells and APCs. In macrophages, TXA2 induces MHC class II molecule expression (10) and regulates TNF-α and IL-1 synthesis (33). Moreover, preliminary data from our laboratory have revealed that, compared to wild-type cells, TP–/– macrophages stimulated with IFN-γ express lower levels of the co-stimulatory molecules CD40 and CD86 (Rocha PN and Coffman TC, unpublished observations). TXA2 also has direct effects on T cells. We have shown that a population of highly purified TP–/– T cells (>97% purity) exhibits a decreased proliferative response to plate-bound anti-CD3 antibody (32). In our studies, the decreased proliferation observed in TP–/– lymphocytes was not associated with altered cytokine production or expression of co-stimulatory molecules. Rather, we found that when TP–/– cells were exposed to stimuli that triggered an exaggerated, antigen-independent calcium signal (i.e., phorbol 12-myristate 13-acetate + ionomycin), the proliferative defect was completely reversed (32). Our interpretation of these results is that TP receptors might contribute to the

intensity of the calcium signal set off by antigen engagement of the TCR.

A possible role for TXA2 in human transplant rejection was first proposed by the studies of Foegh et al. (34), who measured the levels of TXB2 (the stable metabolite of TXA2) in daily urine samples from 12 patients after renal transplantation and demonstrated a several-fold increase in urinary TXB2 excretion during episodes of acute rejection. In the vast majority of cases, the increase in TXB2 excretion preceded the increase in serum creatinine and the clinical diagnosis of rejection. Because of this, several investigators have since advocated the use of urinary TXB2 levels as a noninvasive tool to aid in the diagnosis of acute renal allograft rejection (35-38). The sources of TX in this setting are the inflammatory cells infiltrating the graft, as the intensity of the infiltrate seems to correlate with the amount of prostanoid produced. Moreover, treatment of acute renal transplant rejection with cyclophosphamide has been shown to decrease the urinary excretion of TXB2 (39); as this drug does not interfere with any of the steps involved in TXA2 synthesis, the likely explanation for the reduction in TXB2 excretion in these studies is the resolution of the inflammatory infiltrate. Of the mononuclear cells typically encountered in the rejecting allograft, monocytes and macrophages are known to produce prostanoids in response to a variety of inflammatory stimuli, such as cytokines, bacterial products, and complement components.

Given the *in vitro* actions of TXA2 on immune cells discussed above, we may speculate that TXA2 produced during acute allograft rejection might act to enhance cell-mediated immunity and thereby boost the rejection process. In agreement with this hypothesis, Coffman et al. (39,40) showed that inhibition of TXA2 synthesis led to improved outcomes of renal allografts in rats. In our studies with TP–/– mice, we also observed a modest prolongation in cardiac

allograft survival compared to wild-type controls; allografts harvested from TP knockout animals exhibited a marked attenuation in acute rejection scores (32). These effects were only seen when recipients were treated with small doses of cyclosporin A (CsA) during the initial week of transplantation. In a kidney transplantation model, however, we observed attenuation in acute rejection in TP–/– animals even in the absence of pharmacologic manipulation (Rocha PN and Coffman TC, unpublished observations).

The detrimental effects of TXA2 during acute rejection may extend beyond its effects on immune cells. For example, the initial fall in glomerular filtration rate (GFR) during acute renal allograft rejection is much greater than expected based on the observed morphologic changes in the kidney (41-43). The rapid fall in GFR in this setting is likely to represent a vasoactive phenomenon mediated by molecules such as TXA2 that are produced within the allograft being rejected. Similarly, the quick (and sometimes dramatic) improvement in GFR observed after steroid treatment for acute rejection is due, at least in part, to reversal of TXA2-induced renal allograft vasoconstriction. The same rationale was used by Pierucci et al. (44) to explain the improvement in renal function observed after the acute infusion of a TXA2 antagonist in humans with lupus nephritis. It was later shown that chronic TXA2 blockade profoundly altered the course of renal disease in lupus-prone mice, effects that go beyond vasodilation (45).

The role of TXA2 in vascular thrombosis is well established and has been elegantly redemonstrated by Cheng et al. (46). Local production of this molecule during acute rejection may lead to intragraft thrombosis and irreversible dysfunction. TXA2 blockade is also essential for the prevention of coronary events in high-risk patients. Since a large number of renal transplant recipients are at increased risk for coronary disease, prevention of myocardial infarction constitutes another advantage of TXA2 blockade in this setting (47).

In summary, TXA2 produced by immune cells infiltrating the graft during acute rejection might contribute to graft dysfunction and injury by: 1) exerting direct pro-inflammatory effects on T cells and APCs that enhance cell-mediated immunity and, consequently, the intensity of allograft rejection; 2) inducing vasoconstriction and reducing graft perfusion; 3) promoting platelet aggregation and intragraft thrombosis. Finally, TXA2 has also been implicated as a potential mediator of CsA nephrotoxicity. Animal studies by Spurney et al. (48) showed that the production of TXA2 is increased during experimental chronic CsA toxicity and that TXA2 blockade is beneficial in this setting. To investigate the significance of these findings in humans, Smith et al. (49) administered the TX synthase inhibitor CGS 13080 in a 48-h intravenous infusion to kidney transplant recipients with renal impairment due to CsA toxicity. Renal function and plasma flow were measured before and 1 week after the infusion. At the end of the observation, there was a 9% improvement in GFR and a 33% increase in renal plasma flow compared to baseline values. In a subsequent study, however, the oral administration of the TX synthase inhibitor CGS 12970 for 4 weeks did not improve renal function or plasma flow in patients with CsA nephrotoxicity (50).

These data suggest that, in the transplant setting, there might be several potential advantages to blocking TXA2 generation or signaling. A potential concern is that TXA2 inhibition at the time of transplantation might impair the development of thymic allospecific tolerance. It is known that the enzyme responsible for TXA2 generation, TX synthase, is highly expressed in the thymus (51). It is also known that CD4+CD8+ thymocytes - the cell population that undergoes negative selection - express high concentrations of TP receptors (52). When exposed to a synthetic TXA2 agonist *in vitro*, these cells quickly undergo apoptosis (52). In concert

with these findings, we have recently shown that TXA2 mediates CD4+CD8+ thymocyte apoptosis *in vivo* (53). Finally, Remuzzi and colleagues (54) demonstrated that administration of a TP receptor antagonist during the peritransplant period completely abrogated the unresponsive state induced by intra-thymic injection of synthetic class II MHC allopeptides in the Wistar-Furth x Lewis rat strain combination. In the same study, inhibition of TXA2 generation by aspirin or dexamethasone also abolished the induction of acquired thymic tolerance. These findings, if confirmed, indicate that future immunosuppressive regimens aiming to achieve acquired central tolerance to alloantigens should not include drugs that promote TXA2 blockade in the peritransplant period.

Effect of cyclooxygenase inhibition on the alloimmune responses

Prostanoids with opposing actions on immune cells are generated during an episode of acute rejection (Figure 3). Whereas inhibition of TXA2 is at least theoretically advantageous, the same is not true for PGE2, which has well-described immunosuppressive properties. The COX inhibitors (selective or non-selective) are capable of globally inhibiting the synthesis of all prostanoids. Despite the clinically relevant anti-inflammatory properties of these agents, *in vitro* studies indicate that COX blockade with indomethacin actually enhances cellular immune responses, as evidenced by proliferation and cytotoxicity assays (29,32). One can speculate that these experiments reveal the dominant effects of immunosuppressive prostanoids such as PGE2 in this model. Alternatively, global COX blockade might shift eicosanoid production towards the lipoxygenase pathway and lead to increased production of pro-inflammatory leukotrienes. Regardless of the exact mechanism behind these apparent pro-inflammatory actions of indomethacin on immune cells, it is reason-

able to theorize that global COX inhibition could exert deleterious effects on allograft function. In kidney allografts, nonsteroidal anti-inflammatory drugs (NSAIDs) could also promote worsening renal function by blocking vasodilator prostanoids. Unfortunately, the effects of traditional NSAIDs on allograft function have not been well studied *in vivo*. The availability of COX-deficient mice and specific pharmacologic inhibitors will certainly facilitate this project (55,56). Such studies are urgently needed given that NSAIDs are amongst the most commonly used drugs on the market and transplantation has become the preferred form of therapy for many heart, kidney, and liver diseases.

A recent study by Ma et al. (57) showed that the administration of a selective COX-2 inhibitor doubled the survival of cardiac allografts in rats. This prolongation in allograft survival was associated with significant attenuation of histological changes at days 3 and 5, as well as a reduction in the number of
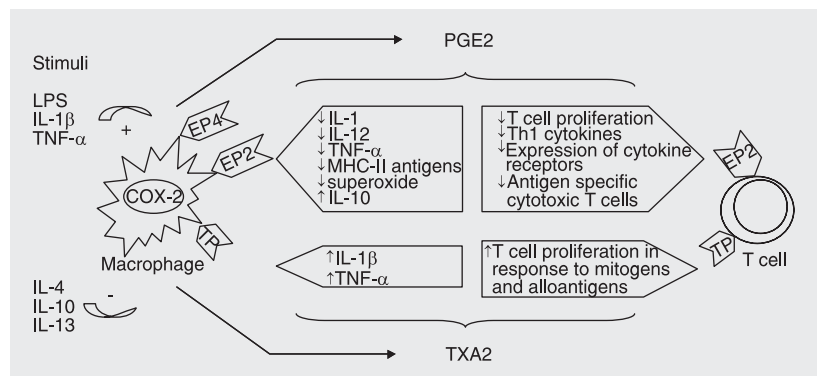

Figure 3. Effects of prostanoids on immune cells. Schematic drawing depicting the generation of prostanoids by antigen-presenting cells (APCs), represented here by a macrophage, and the subsequent actions of these prostanoids on APCs and T cells. Inflammatory cytokines such as IL-1 and TNF-α up-regulate COX-2 in macrophages and thereby promote an increase in the output of prostanoids. The actions of PGE2 are mainly immunosuppressive. In macrophages, PGE2 causes an increase in the production of the anti-inflammatory cytokine IL-10 and a reduction in the production of pro-inflammatory cytokines and superoxide, as well as a reduction in MHC class II antigen expression. These actions are mediated by EP2 and EP4 receptors. In T cells, PGE2 binds EP2 receptors and suppresses proliferation, cytokine production and antigen-specific cytotoxicity. Conversely, TXA2 appears to stimulate both macrophage and T cell functions, effects that are mediated via a single TP receptor. In macrophages, TXA2 is known to increase the production of pro-inflammatory cytokines IL-1 and TNF-α. In T cells, TXA2 increases the proliferative responses to mitogens and alloantigens. LPS = lipopolysaccharide; IL = interleukin; TNF-α = tumor necrosis factor α; COX = cyclooxygenase; MHC = major histocompatibility complex; Th = T helper; PG = prostaglandin; TX = thromboxane.

apoptotic cardiomyocytes. Taken together, these findings suggest that COX-2-derived prostanoids play a dominant pro-inflammatory role during allograft rejection.

Final considerations

We have discussed the intricate relationship between inflammation and the alloimmune response. In the perioperative stages of transplantation, tissue damage and inflammation set the stage for allorecognition to occur in the presence of danger signals; these signals activate quiescent APCs that, in turn, provide the critical co-stimulatory stimuli required to activate host's T cells. During episodes of acute rejection, inflammation occurs because of the effector elements generated by the alloimmune response. Prostanoid inflammatory mediators produced by activated mononuclear cells infiltrating the graft can modulate the intensity of the alloimmune response and directly regulate graft function by changes in blood flow (Summary box). TXA2 promotes vasoconstriction and platelet aggregation and enhances cell-mediated immunity via direct actions on both APCs and T cells. These effects are deleterious to the allograft and acute rejec-

tion has been attenuated in animal models by inhibiting the actions of TXA2. In contrast, PGE2 is a vasodilator and powerful immunosuppressant and PGE analogues have been shown to ameliorate acute rejection in animal and human transplantation. Global blockade of prostanoid production with COX inhibitors enhances alloimmune responses *in vitro*, revealing a dominant effect of PGE2, but at present the relevance of COX inhibition in animal models of transplantation is poorly understood. Steroids are the only drugs in the current immunosuppressive armamentarium that possess anti-inflammatory properties and are capable of inhibiting the synthesis of prostanoids. Recently, there has been growing interest in steroid-free protocols. In most of these protocols, however, steroids are still used during the times of inflammation described herein: the perioperative stage of transplantation and episodes of acute rejection. Given the data on TXA2 and thymic tolerance, one might question if steroids are the ideal anti-inflammatory drugs for the perioperative period. Perhaps, in the future, we will have better drugs to down-regulate innate immunity at the time of transplantation to efficiently shut off the innate immune system and transform allorecognition into an event that generates tolerance instead of immunity. In addition, targeted anti-inflammatory agents that can inhibit only the pro-inflammatory prostanoids (such as TXA2) and leave the anti-inflammatory agents (such as PGE2) untouched, might be of great value for the treatment of acute rejection.

Acknowledgments

Summary Box
Prostanoids and Graft Rejection

· Prostanoids are produced by inflammatory cells within the rejecting allograft

· The vasoactive properties of these compounds enable them to rapidly influence graft function by altering blood flow

· Prostanoid-induced changes in vascular permeability promote local swelling and facilitate the leakage of inflammatory cells into the interstitium of the graft

· Platelet function is also regulated by prostanoids: TXA2 promotes whereas PGI2 inhibits aggregation

· Prostanoids modulate the intensity of allograft rejection by direct effects on T cells and APCs

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