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EDITORIAL

Common and small molecules as the ultimate regulatory and effector mediators of antigen-specific transplantation reactions

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Abstract

In spite of intensive research, the molecular basis of allograft and xenograft rejection still remains not fully understood. The acute rejection of an allograft is associated with the intragraft Th1 cytokine response, while tolerance of an allograft or xenograft rejection is accompanied by a higher production of the Th2 cytokines interleukin (IL)-4 and IL-10. Nevertheless, these cytokines are not the final regulatory and effector molecules mediating transplantation reactions. Data indicate that the functioning of common molecules with enzymatic activities, such are inducible nitric oxide synthase (iNOS), arginase, heme oxygenase-1 (HO-1) or indoleamine-2,3-dioxygenase (IDO), the bioavailability of their substrates (L-arginine, tryptophan, heme) and the cytotoxic and regulatory actions of their small gaseous products (NO, CO) can be the ultimate mechanisms responsible for effector or regulatory reactions. Using models of transplantation immunity and tolerance we show that T cell receptor-mediated recognition of allogeneic or xenogeneic antigens as well as the balance between immunity/tolerance induces distinct cytokine production profiles. The ratio between Th1 and Th2 cytokines efficiently regulates the expression of genes for common enzymes, such as iNOS, arginase, HO-1 and IDO. These enzymes may compete for substrates, such as L-arginine or tryptophan, and the final product of their activity are small molecules (NO, CO) displaying effector or regulatory functions of the immune system. Thus, it is suggested that in spite of the high immunological specificity of transplantation reaction, the ultimate players in regulatory and effector functions could be small and common molecules.

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Key words: Immunoregulation; Graft rejection; Tolerance; Th1/Th2 balance; Macrophages; Nitric oxide; Arginase

Core tip: The paper discusses the role of small and common molecules, such are inducible nitric oxide synthase, arginase, heme oxygenase-1 or indoleamine-2,3-dioxygenase, the bioavailability of their substrates (L-arginine, tryptophan, heme) and the cytotoxic and regulatory actions of their small gaseous products (NO, CO), in regulation of transplantation reactions.

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INTRODUCTION

The recognition of graft donor antigens, either by a direct or indirect route, induces an immune response that includes the participation of phenotypically and functionally distinct cell populations. The activity and mutual cooperation of these cells result in the stimulation of effector cytotoxic cells and graft rejection on one side or in the activation of regulatory Tregs (T) cells and regulatory Bregs (B) cells and the induction of transplantation tolerance on the other side. Both effector cytotoxic reactions and transplantation tolerance are strictly haplotype specific.

It is now well recognized that due to the abundance of immunological mechanisms, more different cell populations and a number of different mechanisms are involved in the regulation of the immune reaction and contribute to graft rejection or tolerance induction. Regulatory activity is not restricted to the best characterized $CD4^+CD25^+$ $Foxp3^+$ T cells, as $CD8^+$, $CD8^+CD28^-$, $CD4^-CD8^-$ and NKT cells have been shown to inhibit immune reactions in some models of transplantation tolerance[1-3]. Recently, a regulatory activity, independent of antibody production, has been attributed to a B cell population called B cells[4,5]. These cells inhibit immune reactions, including transplantation immunity[6,7]. Similarly, effector cytotoxic reactions are not confined only to the activity of the originally described cytotoxic $CD8^+$ T cells, but cytotoxic $CD4^+$ T cells, NK cells and especially activated macrophages can kill allogeneic and xenogeneic cells of graft donor origin. Recent data suggest that the specificity and type of transplantation reaction are ensured during the recognition of antigens by the antigen-specific T cell receptor and by the cytokine environment. Different types of transplantation antigens and/or different immunization/tolerization conditions induce distinct patterns of cytokine production (Table 1). The published data indicate that individual cytokines stimulate the expression of functionally different, but in the organism common genes, that are responsible for the generation of small effector molecules representing the ultimate regulatory and effector elements of the immune system. Taking into account the recognized mechanisms of $CD4^+CD25^+$ $Foxp3^+$ T and B cell action and the mechanism of the cytotoxic activity of activated macrophages we suggest that at least some regulatory and effector functions of the immune system are mediated by "common small" molecules that are functionally not confined only to the immune system.

MACROPHAGES AS IMPORTANT EFFECTOR CELLS INVOLVED IN GRAFT REJECTION

For many years, cytotoxic $CD8^+$ T lymphocytes which kill cells of the graft donor haplotype *in vitro*, had been considered as the main effector cells responsible for graft rejection. However, experiments have shown that the depletion of $CD8^+$ T cells does not prevent graft rejection[8]. A more important role in the rejection reaction has been attributed to $CD4^+$ T cells. Elimination of $CD4^+$ T cells results in the prolonged survival of both allografts and xenografts or even in a permanent allograft tolerance[9-11]. An important role in allograft rejection has been attributed to two $CD4^+$ T cell subsets - to proinflammatory Th1 cells producing interleukin (IL)-2 and interferon (IFN)-γ and to Th17 cells producing IL-17[12,13]. In addition to $CD4^+$ T cells, a significant role in graft rejection is played by macrophages, which represent an abundant cell population infiltrating rejected allografts and xenografts[14,15]. The involvement of macrophages in both the recognition and rejection of grafted cells has been described[16,17]. It has been shown in a kidney allograft model that the greatest accumulation of macrophages producing nitric oxide (NO) occurs in those sites in the graft where the greatest degree of damage and the highest occurence of apoptotic graft cells are seen[17].

Table 1 Polarization of cytokine production in response to transplantation antigens

Model	Type of cytokine response[1]
Acute rejection of allograft	Th1 and Th17
MLC to xenoantigens	Th2
Rejection of xenograft	Predominantly Th2
Neonatally induced tolerance of allografts	Th2
Anti-CD4 induced tolerance in adulthood	Th2
Immunosuppressive drug induced tolerance to alloantigens	Th2
Acute graft-versus-host reaction	Th1
Chronic graft-versus-host reaction	Th2
Mucosal tolerance to alloantigens	Th2 (or Th3)

[1]The Th1 type of cytokine response is characterized by the predominant production of interleukin (IL)-2 and interferon γ. For the Th2 type of response, the higher production of IL-4, IL-5, IL-10 and IL-13 is typical. The production of the proinflammatory cytokine IL-17 is characteristic of the Th17 response. The Th3 type of response is characterized by the production of IL-4 and IL-10 and by the high production of the inhibitory cytokine transforming growth factor β.

Macrophages require for their activation a signal from stimulated T cells. It has been demonstrated in various models of allotransplantation that alloantigen-stimulated $CD4^+$ T cells are the main activators of graft infiltrating macrophages and that IFN-γ is the principal cytokine responsible for their activation[18,19]. According to the type of activation signal, two distinct populations of macrophages have been described[20,21]. The so-called classically activated or "killer" macrophages (M1) are activated by IFN-γ (or other Th1 or Th17 cytokines) and produce reactive oxygen species, proinflammatory cytokines and drive an inflammatory/rejection reaction. In contrast, alternatively activated or "healer" macrophages (M2) are stimulated by the Th2 cytokines IL-4 and IL-13 and contribute to debris scavenging, angiogenesis and the wound healing process. Their phenotype and activity can be enhanced by another Th2 cytokine IL-10[22].

Since individual T cell subpopulations differ in their ability to produce different patterns of cytokines and to activate M1 or M2 macrophage subpopulations, the

expression of effector mechanisms of the rejection reaction will depend on the cytokine spectrum at the site of rejection and subsequently on the activity of graft infiltrating macrophages. The classically activated macrophages produce NO as one of the toxic effector molecules involved in graft rejection.

NITRIC OXIDE IN ALLOGRAFT REJECTION

NO is an ubiquious molecule that is toxic for a variety of pathogens and foreign cells. The production of NO is catalyzed in the body by the enzyme nitric oxide synthase (NOS) which occurs in three isoforms: endothelial NOS, neural NOS and inducible NOS (iNOS). Especially iNOS which is expressed in a variety of cells of the immune system and mainly in macrophages, can inducibly produce large quantities of NO. Elevated levels of NO have been detected during the rejection of skin, kidney, heart, liver, lung and corneal allografts[23-25]. The production of NO after allotransplantation correlates with the kinetics of graft rejection and with the fate of the graft[18] and the highest iNOS expression is seen in those sites in an allograft where the highest level of apoptosis of the grafted cells occurs[17]. The observations that the inhibition of NO production by means of specific iNOS inhibitors[18,26,27] or by NO scavenging[28] prevents graft rejection and prolongs allograft survival can be considered as direct evidence for involvement of NO in allograft rejection.

THE RELATIONSHIP BETWEEN INOS/ARGINASE AND NO PRODUCTION

iNOS is expressed in a variety of immunologically active cells, and among them activated macrophages are the main producers of NO. Once induced, iNOS oxidizes L-arginine as a substrate to form NO and citrulline. However, iNOS has to compete for L-arginine with arginase, another intracellular enzyme that utilizes L-arginine. Arginase which converts L-arginine into urea and L-ornithine, is produced in two molecular forms, arginase Ⅰ and arginase Ⅱ. Both isoforms differ in their cellular sublocalization and their tissue distribution. Arginase Ⅰ, the cytosolic isoform, is mainly found in the liver and less so in other tissues, whereas arginase Ⅱ, the mitochondrial isoform, is found predominantly in the kidney, prostate, small intestine, and breast[29]. Significant differences in the tissue expression of arginase isoforms also exist among various species. For example, while mouse macrophages express both isoforms, only arginase Ⅰ was found in rat macrophages[30]. Human arginase Ⅰ can be found among myeloid cells only in granulocytes, and its expression is not modulated by a variety of proinflammatory or anti-inflammatory stimuli[31]. It seems that the genes for both isoforms are regulated differentially and have different kinetics of expression in stimulated cells[30].

Both iNOS and arginase compete for L-arginine as a common substrate and thus affect each other. Biochemical data showed that while Km for L-arginine is in the 2-20 mmol/L range for arginase compared with the 2-20 μmol/L range for various NO synthases, the *V*max of arginase is 1000-fold higher than for NOS[32]. Furthermore, the NOS product hydroxyarginine is an inhibitor of arginase while conversely, polyamines inhibit the NOS enzymes[33]. The amount of NO formed thus depends critically on the bioavailability of the substrate[34]. In other words, the increased formation of arginase decreases the bioavailability of L-arginine for iNOS and thus reduces or even attenuates the production of NO. These biochemical properties are likely to have functional significance since it has been demonstrated that arginase activity in macrophages limits NO production[35,36].

CYTOKINE-INDUCED REGULATION OF THE INOS/ARGINASE RATIO

The expression of both L-arginine utilizing enzymes, iNOS and arginase, is reciprocally regulated by cytokines. While Th1 cytokines stimulate the production of iNOS and rather inhibit the expression of the genes for arginase, Th2 cytokines activate arginase and suppress iNOS formation[35-37]. This dichotomy in the cytokine regulation of the iNOS/arginase ratio is demonstrated in Figure 1. The main cytokine activating iNOS expression and NO production in macrophages is IFN-γ, but other proinflammatory cytokines, such as TNF, IL-1 and IL-17, can also stimulate NO production. The production of arginase is stimulated by Th2 cytokines[35], mainly by IL-4, IL-10, IL-13 and transforming growth factor β. The cytokines that stimulate arginase, suppress the cytotoxic functions of macrophages and inhibit NO production. Thus, it is obvious that the activity of the macrophages participating in an immune response is regulated by the ratio between Th1/Th2 cytokines in the environment. While Th1 cytokines stimulate NO production, the presence or an excess of Th2 cytokines inhibits NO formation through the upregulation of arginase and subsequently by the exhaustion of L-arginine. This differential activation of the enzymes iNOS/arginase is further complicated by the recent discovery of the additional $CD4^+$ proinflammatory T cell subsets Th17 and Th22 which modulate iNOS activity by the production of IL-17 and IL-22[38]. The dichotomy in the upregulation of iNOS or arginase production correlates with the above mentioned M1 or M2 macrophage phenotype[20,21]. M1 macrophages produce iNOS which uses L-arginine as a substrate to produce NO. In contrast, M2 macrophages constitutively produce the enzyme arginase Ⅰ, which sequesters L-arginine from iNOS and results in the production of ornithine and downstream polyamines and L-proline[20].

THE INOS/ARGINASE RATIO DURING GRAFT REJECTION

Macrophages represent an abundant cell population infil-

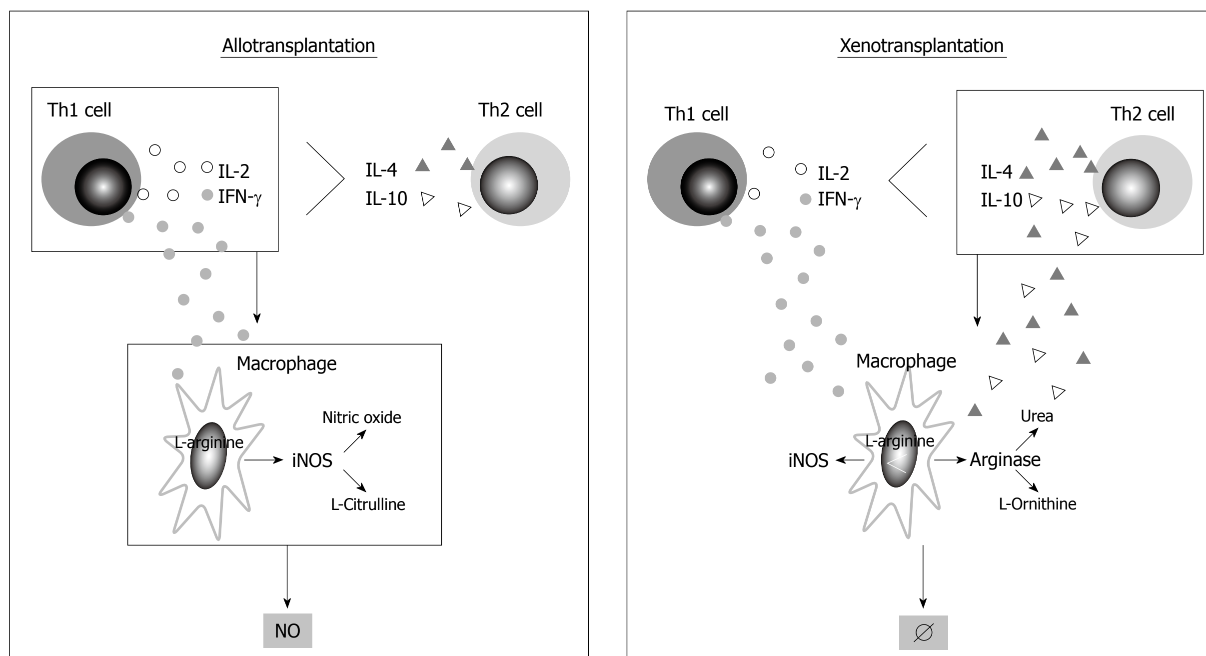

Figure 1 The distinct cytokine production profiles that are induced during allograft or xenograft rejection differentially regulate the expression of the genes for the enzymes inducible nitric oxide synthase and arginase. During allograft rejection, high levels of interleukin (IL)-2 and interferon (IFN)-γ and very low amounts of IL-4 and IL-10 are produced. The proinflammatory cytokine IFN-γ stimulates the expression of the gene for inducible nitric oxide synthase (iNOS), and significant NO generation can be observed in rejected allografts. In contrast, the rejection of xenografts (or allograft tolerance) is associated with the high expression of the genes for the Th2 cytokines IL-4 and IL-10, in addition to the production of Th1 cytokines. Both iNOS and arginase are formed during xenograft rejection. Arginase successfully competes with iNOS for L-arginine as a common substrate. As a consequence, the availability of L-arginine for iNOS becomes limited, and little or no NO generation can be detected in rejected xenografts.

trating rejected allografts and xenografts[39]. Other cell types, such as activated $CD4^+$ or $CD8^+$ T cells, also occur regularly at the site of graft rejection and are a potent source of various cytokines. Therefore, the local cytokine milieu created by various graft-infiltrating T cell subsets regulates the iNOS/arginase ratio and the production of NO by macrophages. Since a strong Th1 cytokine response is regularly observed during allograft rejection[40,41], overexpression of the *iNOS* gene and enhanced NO production can be expected during allograft rejection. Numerous studies have confirmed that increased levels of NO are, in fact, produced during allograft rejection[17-19,42]. Conversely, the higher production of IL-4 and IL-10, *i.e.*, cytokines stimulating arginase, dominates during xenograft rejection or in the state of transplantation tolerance[43-45]. Since arginase utilizes L-arginine with a high affinity, which then becomes less available for iNOS, NO production can be expected to be attenuated. Indeed, we found a lack of NO formation in rejected rat skin xenografts, in spite of abundant *iNOS* gene expression and iNOS protein accumulation in the xenografts[46]. Using selective inhibition of arginase activity with the specific inhibitor N^{ω}-hydroxy-L-arginine, the production of NO in the rejected skin xenografts was restored[47]. Similarly, the production of NO in xenograft explants was restored by adding an excess of L-arginine to the cultures[47]. Furthermore, we demonstrated that the activation of arginase was inhibited or decreased when xenograft recipients were treated with an anti-CD4 mAb, eliminating $CD4^+$ T cells as the principal source of Th2 cytokines after xenotransplantation, or with anti-IL-4 mAb, the antibody neutralizing the main cytokine that activates the expression of the arginase genes. Both of these treatments restored, at least partially, NO production after xenotransplantation. Taken together, these results suggest that the Th1/Th2 ratio during allograft or xenograft rejection regulates NO production through its influence on the iNOS/arginase balance and that $CD4^+$ T cells are the main players regulating this pathway.

GENERAL CONCLUSIONS CONCERNING INOS/ARGINASE REGULATION

The production of NO by graft infiltrating macrophages is effectively regulated by the cytokine milieu at the site of graft rejection. Th1 cytokines which predominate during acute allograft rejection support the development of M1 macrophages, and stimulate iNOS expression and NO production. Conversely, Th2 cytokines which are abundantly produced during the state of allograft tolerance or during the rejection of xenografts, stimulate the activation of M2 macrophages as well as arginase formation and thus cause a decrease in bioavailability of L-arginine for iNOS. As a consequence of this pathway, NO production is attenuated. This regulatory pathway may ensure the absence of NO production as a cytotoxic effector molecule during allograft tolerance. The production of IL-10, a typical Th2 cytokine, is also a main mechanism of Breg-mediated immunosuppression. As

evidence, neutralization of IL-10 abrogates B-cell mediated suppression in a majority of systems[5,48]. The role of B cells in transplantation tolerance has been shown[6,7]. As mentioned above, IL-10 is one of the cytokines that stimulates in macrophages the expression of arginase, which successfully competes with iNOS for the common substrate L-arginin and thus attenuates NO production by iNOS. The absence of NO decreases rejection reaction and supports graft tolerance, Similarly, NO generation is also very low or absent during xenograft rejection which is associated with the elevated production of the Th2 cytokines IL-4 and IL-10. The participation of other cell populations, such as NK cells, eosinophils and cytotoxic $CD8^+$ T cells, which are not so frequent in rejected allografts, or the production of cytotoxic anti-xenograft antibodies can overcome the absence of NO during xenograft rejection.

From a more general point of view, the ability of arginase to inhibit NO generation by competing for L-arginine may have an important physiological significance. High levels of Th2 cytokines and strong arginase activity are regularly induced in the host by different parasite and pathogen infections. It has been demonstrated that the level of host arginase represents a marker of resistence or susceptibility to trypanosome infections[49]. Other studies have suggested that the induction of arginase may represent an evolutionary escape mechanism ensuring the survival of the pathogen[50,51]. The production of arginase by pathogens themselves can represent another mechanism representing a strategy for bacterial survival[52]. Conversely, high NO production during a strong immune response would damage the cells and tissues of the host. In this context, arginase can be considered a protective factor for the host by its ability to lower NO production, which can limit tissue damage or immunosuppression[53]. This may also be the case with the down-regulation of NO production during a strong xenograft reaction, when arginase can limit NO production and thus protect the host tissues from damage by high NO secretion. Therefore, Th2 cytokines stimulating arginase activity in these situations may represent a self-protective mechanism saving the body's own cells from harmful effects of high concentrations of NO.

IMMUNOREGULATORY EFFECTS OF INDOLEAMINE-2,3-DIOXYGENASE

Indoleamine-2,3-dioxygenase (IDO) is an intracellular enzyme that regulates the initial rate-limiting step in tryptophan degradation along the kynurenine pathway[54]. IDO is expressed in various tissues and its expression is induced by IFN-γ and other proinflammatory cytokines[55]. The enzymatic activity of IDO regulates the bioavailability of tryptophan for a cell, and the starvation of tryptophan by its consumption by IDO results in an inhibition of T cell proliferation and activation. In addition, the low molecular weight products of tryptophan metabolism, such as kynurenine derivates and O_2 free radicales, inhibit T cell proliferation and functions[56,57]. The activity of IDO was suggested as a mechanism of the immunosuppressive action of tolerogenic dendritic cells and the suppression mediated by bone marrow-derived mesenchymal stem cells[58,59]. The inhibition of T cell function through tryptophan metabolism and the effects of tryptophan starvation by IDO consumption thus appear as another mechanism involved in the suppression, in a cytokine-dependent manner, of transplantation and other immune reactions[60]. The results indicate that tryptophan is another substrate, similarly as L-arginin, whose concentrations and bioavailability regulate immune reactions and thus can be one of the molecular mechanisms participating in the state of transplantation tolerance.

FINAL CONSIDERATION: ARE "COMMON SMALL" MOLECULES THE ULTIMATE PLAYERS IN THE EFFECTOR AND REGULATORY FUNCTIONS IN THE IMMUNE SYSTEM?

This review suggests that cytokine-induced enzymes, such as NOS, arginase and IDO, and their substrates and products (L-arginine, tryptophan, NO) strongly influence the expression of the cytotoxic effector functions of the immune system. This suggestion is supported by the elucidation of the molecular mechanisms of immunoregulation. An important role in the downregulation of the immune system is played by $CD4^+CD25^+$ T cells, which inhibit the proliferation and cytokine production of other immunocompetent cells[61]. The development and functioning of these T cells are associated with the expression of the forkhead box P3 transcritional factor (Foxp3)[62,63]. It has been suggested that Foxp3 activates the expression of the gene for heme oxygenase-1 (HO-1)[64]. HO-1 catalyzes the degradation of heme and this reaction results in the liberation of equimolar amounts of iron, CO and biliverdin. Since CO has been shown to exert antiproliferative effects[65] and can block IL-2 production[66], this small molecule can be the ultimate effector of T cell-mediated immunosuppression[64]. Indeed, blocking HO-1 in $CD25^+CD4^+$ T cells abrogated their suppressor function[67]. In addition, Oh *et al*[68] demonstrated that the upregulation of HO-1 expression can block the expression of iNOS and NO production, and that CO was responsible for this suppression. Thus, CO produced by the activity of HO-1 expressed in T cells at the site of a tolerated graft can contribute to the suppression of iNOS expression, silencing NO production and to the protection of the graft from the toxic effects of NO.

In summary, the recent data suggest that common molecules, such as NOS, arginase, IDO and HO-1, and their substrates or products, such as L-arginine, tryptophan, NO and CO, are the ultimate players mediating immunoregulatory and effector functions of the immune system.

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