Role of Cytokines in Transplantation Tolerance: Lessons Learned from Gene-Knockout Mice

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Transplantation tolerance may be defined as indefinite allograft survival in the absence of continuous immunosuppressive therapy (1). Implicit in this definition is that tolerant recipients are unresponsive to donor antigens but maintain reactivity to other (third party) antigens (1-3). In the clinical setting, a tolerant individual is someone who is capable of mounting an effective immune response against vaccines or pathogens but is incapable of rejecting the transplanted organ. Except for the occasional patient who stops taking immunosuppressive therapy yet maintains a functioning allograft, transplantation tolerance has not been achieved in humans. However, several immunomodulatory strategies have been shown to induce tolerance in experimental animals. These strategies involve short-term, perioperative administration of T cell antibodies (4,5), donor MHC-derived peptides (6), donor-specific transfusion of blood or bone marrow (7,8), and/or molecules that block antigen-presenting cell (APC)/T lymphocyte interactions (9,10).

The immunologic pathways that lead to tolerance are traditionally divided into central and peripheral (1-3). Central tolerance occurs in the thymus where antigen-specific lymphocytes are eliminated. Peripheral tolerance, on the other hand, occurs extrathymically and is crucial for achieving antigen-specific unresponsiveness in adult animals in whom the thymus has involuted. Mechanisms proposed to mediate peripheral tolerance include deletion, anergy, suppression, and immune deviation of alloantigen-specific, mature T lymphocytes (1-3).

The clonal deletion hypothesis presumes that tolerance results from apoptosis of the alloreactive T cell population. Alternatively, a tolerance-inducing strategy could inactivate alloreactive T lymphocytes without causing their death (anergy), generate suppressor cells that block T lymphocyte proliferation (suppression), or induce differentiation of alloreactive T lymphocytes into a nonharmful phenotype (immune deviation). These mechanisms are not mutually exclusive, and their relative contributions to tolerance depend on the organ being transplanted, the tolerance-inducing regimen used, and the degree of donor-recipient histo-incompatibility.

Cytokines are soluble proteins that regulate lymphocyte activation, proliferation, differentiation, and survival (11). Their role in allograft rejection and tolerance induction is largely inferred from their in vitro functions and from in vivo experiments in which cytokine expression is correlated with graft rejection or acceptance (12,13). These studies, however, have failed to pinpoint the contribution of individual cytokines to tolerance induction because of the complexity of cytokine cascades triggered after transplantation. Recently, gene-knockout mice have emerged as a tool to dissect cytokine functions in the context of the whole immune system. As will be reviewed here, unexpected findings in these mice provide new insights into the role of cytokines in transplantation tolerance.

The Th1/Th2 Paradigm

The discovery of T helper lymphocyte subsets (Th1 and Th2) that differ in their cytokine secretion patterns and effector functions has provided a model for understanding how cytokines regulate immune and inflammatory processes (14-16) (Figure 1). Studies in mice, rats, and humans indicate that Th1 lymphocytes produce interleukin-2 (IL-2), interferon-γ (IFNγ), and lymphotoxin, and Th2 lymphocytes produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Both cell types originate from the same precursor lymphocyte (Th0), whose differentiation is largely influenced by the cytokines present at the time of antigen recognition. IL-12 and IFNγ favor the Th1 phenotype, whereas IL-4 promotes generation of Th2 lymphocytes. Differentiation of Th0 cells into either a Th1 or Th2 phenotype is also governed by the antigen dose, the strength of interaction between the T cell receptor for antigen and the major histocompatibility molecule (MHC)/peptide complex, and the nature of costimulatory molecules expressed on APC (16). Th1 and Th2 subsets are reciprocally regulated. IFNγ inhibits the differentiation and proliferation of Th2 cells, resulting in a dominant Th1 response. On the other hand, IL-4 and IL-10 inhibit IFNγ production by Th1 lymphocytes.

Th1-derived cytokines are postulated to promote allograft rejection by mediating delayed-type hypersensitivity reactions, cytotoxic T lymphocyte (CTL) generation, macrophage activation, and production of antibodies that facilitate antibody-dependent cellular cytotoxicity (13,17) (Figure 1). In contrast, Th2-derived cytokines are postulated to protect against rejection by suppressing delayed-type hypersensitivity reactions and counteracting IFNγ’s actions on macrophages. They also deviate antibody production toward IgE and noncytotoxic subclasses of IgG. Intragraft expression of Th1-type cytokines is
often associated with acute rejection, whereas expression of Th2-type cytokines has been shown to correlate with allograft acceptance (18-21). This led to the proposal that a balance between Th1 and Th2 lymphocytes determines the fate of a transplanted organ (13,17,22) (Figure 1). Much of the evidence that supports this hypothesis is based on assessing intragraft cytokine expression by reverse transcription-PCR or by identifying graft-infiltrating cells that stain for cytokine protein by immunohistochemistry (23). Although important, these data do not establish cause–effect relationships between cytokines and allograft rejection or acceptance.

**Cytokine Gene-Knockout Mice as an Experimental Tool**

Four complementary methods are available to study the biological roles of cytokines in vivo. The first method relies on systemic administration of large quantities of recombinant cytokines into mice. Because cytokines act predominantly in a paracrine manner, systemic delivery of a cytokine may result in adverse rather than physiologic effects. This approach is further hampered by the short half-life of injected cytokines. The second method is based on studying transgenic mice in which a cytokine is overexpressed systemically or locally. Unlike transient cytokine expression observed in wild-type animals, cytokine production in transgenic mice is constant and could lead to nonphysiologic effects. The third method uses cytokine-neutralizing antibodies or cytokine receptor antagonists (24). The main disadvantage of this method is that one cannot be absolutely sure that these agents completely block the actions of cytokines in vivo. Furthermore, heterologous antibodies injected into the mouse may stimulate the immune system or may modulate the function of cells that express Fc receptors. The fourth method is based on gene-knockout technology in which a cytokine or cytokine receptor gene is disrupted by homologous recombination in embryonic stem cells (25,26). Although this strategy eliminates the problems associated with administering cytokine-neutralizing agents, two caveats should be kept in mind when interpreting results in gene-knockout mice (27). First, cytokine gene inactivation in the embryonic stem cell may alter lymphocyte ontogeny or lymphoid organ development leading to multiple abnormalities that affect immune responsiveness in the adult animal. Second, a “negative” result in a gene-knockout mouse does not necessarily prove that the cytokine does not participate in the process being studied, because other molecules may be compensating for its absence. Studies in cytokine gene-knockout mice should therefore be complemented with experimental approaches using recombinant cytokines or cytokine-neutralizing agents.

This review will summarize observations made in mice lacking IL-2 (28), IL-4 (29,30), or IFNγ (31). The validity of these observations is strengthened by the fact that lymphocyte ontogeny and lymphoid organ development are normal in these mice (26), and that the findings were duplicated in cytokine receptor gene-knockout animals and in wild-type mice injected with cytokine-neutralizing antibodies.

**Lessons Learned from Cytokine Gene-Knockout Mice**

**The Th1/Th2 Paradigm and Allograft Rejection: Cytokines Are Redundant**

A study by Strom and colleagues was the first to point out the redundancy of cytokines in the acute rejection process (32). They demonstrated that IL-2 gene-knockout (IL-2−/−) mice are capable of rejecting fully allogeneic pancreatic islet cells. This suggested that IL-2, a potent T cell mitogen in vitro, is not critical for allograft rejection. Dai et al. (33) confirmed these findings in a murine vascularized cardiac allograft model. Despite experimental evidence indicating that IL-2 promotes CTL generation in vitro, IL-2−/− mice were found to mount effective (CTL) responses to allogeneic splenocytes and tumor cells in vivo (32,33). These studies appear to contradict those in which allograft rejection was prevented by blocking IL-2 receptors with monoclonal antibodies, but it is important to remember that the mechanism of action of these antibodies is unclear (34,35). In addition to inhibiting the binding of IL-2 to its receptor, IL-2 receptor antibodies may induce T cell deletion or modulate T cell function (36).

Since it has been shown that IL-4 stimulates T cell proliferation in vitro and that administration of soluble IL-4 recep-
tors delays cardiac allograft rejection in vivo, it has been proposed that IL-4 mediates allograft rejection in the absence of IL-2 (37). However, IL-4-deficient mice (IL-4−/−) and double gene-knockout mice deficient in both IL-2 and IL-4 were found to reject pancreatic islet cell and cardiac allografts with the same vigor as wild-type recipients (38–40). These findings raise the possibility that other cytokines that signal through the γ-chain of the IL-2 receptor may compensate for the absence of IL-2 and/or IL-4. The γ-chain is shared by the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors and is responsible for signal transduction following binding of these cytokines to their receptors on T lymphocytes (41). In fact, gene-knockout mice that lack the γ-chain are severely immunodeficient and succumb to infections at a young age (42).

It is postulated that IFNγ is a crucial mediator of acute allograft rejection because it upregulates MHC class II expression on APC and activates natural killer cells, CTL, and macrophages in vitro (43). Despite the complete absence of IFNγ, however, fully allogeneic vascularized cardiac graft survival is not prolonged in untreated IFNγ gene-knockout (IFNγ−/−) mice (44,45). In fact, acute rejection occurs at a slightly faster rate in IFNγ−/− recipients than in wild-type mice (median survival time of 5 and 6 d, respectively). Furthermore, Steiger et al. demonstrated that acute cellular rejection of fully allogeneic pancreatic islet cells is not delayed in IFNγ receptor knockout (IFNγR−/−) recipients (46). A compensatory increase in the expression of proinflammatory cytokines such as tumor necrosis factor-α and IL-2 was not detected in IFNγ−/− or IFNγR−/− recipients (44–46). A study in which IFNγ- neutralizing antibodies failed to prolong skin allograft survival in rhesus monkeys also suggests that IFNγ activity is not critical for acute rejection of fully allogeneic transplants (47).

Unlike fully allogeneic or MHC class I-disparate skin, rejection of MHC class II-incompatible skin is markedly delayed in wild-type mice treated with IFNγ-neutralizing antibodies and does not occur in IFNγ−/− recipients (48,49). The mechanisms by which IFNγ mediates acute rejection of MHC class II-incompatible skin transplants are not known. Rosenberg et al. proposed that MHC class II alloantigen expression on keratinocytes, the principal target of rejection, is dependent on IFNγ (48).

In summary, these studies indicate that IL-2, IL-4, and IFNγ may contribute to acute rejection but that none of these cytokines is critical for rejecting fully allogeneic grafts. They also suggest that for a cytokine-based immunosuppressive strategy to be successful, it should target multiple cytokines or block a common pathway of cytokine action such as the IL-2 receptor γ-chain. Most importantly, the observations summarized here underscore that in vitro findings do not always predict the in vivo functions of cytokines.

The Th1/Th2 Paradigm and Transplantation Tolerance: An Oversimplification?

A direct correlation between intragraft Th2 cytokine expression and long-term allograft acceptance has been observed in experimental animals treated with tolerance-inducing agents (18,20,21) (reviewed in reference 23). Furthermore, IL-4-producing T lymphocytes were shown to transfer transplantation tolerance (infectious tolerance) from anti-CD4-treated animals to naive recipients (50,51). When the donor and recipient were mismatched at minor histocompatibility loci, IL-4-neutralizing antibody abrogated “infectious” tolerance (52). Similarly, administering IL-4-neutralizing antibodies or IFNγ prevented induction of neonatal tolerance to fully allogeneic skin grafts (53,54) (reviewed in reference 22). These data raise the possibility that IL-4-mediated deviation of the alloimmune response from a Th1 to a Th2 phenotype contributes to transplantation tolerance.

Several studies, particularly those performed in cytokine gene-knockout mice, raise questions about the applicability of the Th1/Th2 paradigm to induction of peripheral transplantation tolerance to fully allogeneic grafts transplanted into adult mice. First, unopposed action of IL-4 in IL-2−/− or IFNγ−/− recipients does not lead to spontaneous prolongation of allograft survival (32,33,44,45). Second, overexpression of IL-4 in pancreatic islet cells or in cardiac tissue does not significantly delay graft rejection (55,56). Third, long-term cardiac allograft survival induced by agents that block T cell costimulation by APC is readily achieved in IL-4−/− recipients despite the preponderance of Th1 cytokine expression (38,39). In fact, induction of long-term allograft survival in this model is facilitated by endogenous production of Th1 cytokines (IL-2 and IFNγ) (33,45) (discussed below). Fourth, administering IL-10 to mice does not prevent acute rejection and may, in some cases, accelerate graft loss by activating CTL (57). As shown by Suthanthiran and colleagues, intragraft IL-10 expression correlates with acute renal rejection in humans (58). Fifth, in vivo neutralization of IL-12 activity with soluble IL-12 receptor components does not prolong allograft survival as predicted by the Th1/Th2 paradigm, but instead accelerates acute rejection (59).

T Lymphocyte Activation in the Presence of IFNγ and IL-2 May Be a Prerequisite for Tolerance Induction

IFNγ and IL-2 are produced by T lymphocytes shortly after alloantigenic stimulation (45,60,61). On the basis of their immunostimulatory actions in vitro, it is postulated that IFNγ and IL-2 impede induction of transplantation tolerance. Recent studies in IFNγ−/− and IL-2−/− mice, however, raise doubts about this assumption and provide evidence that IFNγ and IL-2 have essential immunoregulatory functions in vivo. Konieczny et al. (45) found that the postoperative blockade of the CD28 and CD40 ligand (CD40L) T cell costimulation pathways induces long-term survival of fully allogeneic cardiac and skin grafts in wild-type mice, but fails to do so in IFNγ−/− recipients or in wild-type mice treated with IFNγ-neutralizing antibodies at the time of transplantation. Similarly, T cell costimulation blockade failed to induce long-term cardiac allograft acceptance in IL-2−/− mice or in wild-type recipients injected with IL-2-neutralizing antibodies (33). These findings are supported by studies which demonstrate that tolerance to protein antigens cannot be induced in IFNγ−/− or IL-2−/− mice (62–64).

What are the mechanisms by which IFNγ and IL-2 facilitate
induction of long-term allograft survival? Compared with wild-type T cells, IFNγ−/− T lymphocytes display exaggerated proliferation and CTL activity upon allostimulation in vitro (31,45). Addition of recombinant mouse IFNγ reduces proliferation of IFNγ−/− T cells, whereas addition of IFNγ-neutralizing antibody increases proliferation of wild-type T cells in mixed lymphocyte cultures (31,45). Exaggerated T lymphocyte proliferation is also observed in vivo after stimulating IFNγ−/− mice with either a bacterial superantigen or allogeneic splenocytes (45) (G. H. Ring, Z. Dai, B. T. Konieczny, and F.G. Lakkis, unpublished observations). IFNγ did not appear to regulate activation-induced T cell apoptosis in these studies. Taken together, these findings indicate that IFNγ is crucial for downregulating alloimmune responses by limiting the proliferation of activated T cells. Immunosuppressive properties of endogenous IFNγ have been demonstrated in other murine models such as collagen-induced arthritis (65,66), experimental allergic encephalitis (67,68), anti-CD3-induced cytokine release syndrome (69), and murine graft-versus-host disease (70).

Although IL-2 is a potent T cell mitogen in vitro, IL-2−/− mice or those that lack high-affinity IL-2 receptors (IL-2Rα−/− and IL-2Rβ−/−) are immunocompetent (71,72). When housed in a non-germ-free environment, these mice display lymphoid hyperplasia due to increased proliferation of mature T and B cells and develop severe autoimmunity characterized by hemolytic anemia or inflammatory bowel disease (73–76). Uncontrolled lymphocyte proliferation and autoimmunity in IL-2−/− and IL-2R−/− mice is in part attributed to impaired apoptosis of activated T lymphocytes. Lenardo and coworkers demonstrated that IL-2 predisposes mature CD4+ and CD8+ T lymphocytes to apoptosis after antigen restimulation and that antibody blockade of IL-2 inhibits antigen-induced deletion of T cells in vivo (77–79). Other studies have confirmed these findings by demonstrating that Fas-mediated, activation-induced T cell death is impaired in IL-2−/− and IL-2Rα−/− mice (63,64). Exaggerated T cell proliferation and impaired T cell apoptosis is also observed in IL-2−/− mice repeatedly stimulated with allogeneic splenocytes (33). These data strongly suggest that IL-2 plays a dual role in the immune system (63) (Figure 2). On one hand, it has a redundant mitogenic role that can be replaced by other lymphocyte growth factors such as IL-4, IL-7, IL-9, and IL-15. On the other hand, it is critical for terminating immune responses by predisposing activated T cells to apoptotic signals. IL-2 can also trigger additional feedback mechanisms that inhibit T lymphocyte proliferation. For example, CTLA4 expression on the cell membrane is upregulated after T cell activation (80). Subsequently, high-affinity APC-CTLA4 interactions inhibit cell cycle progression and contribute to tolerance induction (81–83).

Unlike the Th1/Th2 paradigm of transplantation tolerance, these studies indicate that IL-2 and IFNγ are essential for downregulating alloimmune responses (Figure 2). However, it remains to be determined whether these cytokines contribute to tolerance induction and whether they do so by promoting clonal anergy or deletion of alloreactive T lymphocytes. The answers to these questions could have important clinical implications, mainly that immunosuppressive agents which inhibit IL-2 and IFNγ production may interfere with achieving donor-specific unresponsiveness. This suggests that cyclosporine and tacrolimus, although highly effective in preventing organ rejection, may hinder tolerance induction. Until the immunologic mechanisms of transplantation tolerance are better understood, and experimental tolerance-inducing protocols are thoroughly tested in primates, conventional immunosuppression should remain the mainstay of treatment in transplanted patients.

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