

The article STEALTH on the preclinical path to tolerance, DOI 10.1177/1522162802005006001, by William J. Hubbard, Devin Eckhoff, Juan L. Contreras, Francis T. Thomas, Anne Hutchings, et al, has been retracted at the request of the authors due to their discovery that the study included flawed data. The published study of kidney allograft tolerance included some animals that had not undergone bilateral nephrectomies and were later proven to have retained an intrinsic kidney. This article has therefore been withdrawn from *Graft*, Volume 5, Issue 6, pages 322-330, September 2002.

# STEALTH on the Preclinical Path to Tolerance

William J. Hubbard, Devin Eckhoff, Juan L. Contreras, Francis T. Thomas, Anne Hutchings, and Judith M. Thomas

## ABBREVIATIONS:

APC	Antigen-presenting cell
CIT	Chronic immunosuppressive therapy
DC	Dendritic cell
DSG	Deoxyspergualin
IDDM	Insulin-dependent diabetes mellitus
IT	Immunotoxin
LN	Lymph node
MHC	Major histocompatibility complex
NHP	Nonhuman primates
STZ	Streptozotocin

## IMMUNOGENETICS:

Branch of genetics examining the inheritance of differences in antigens or antigenic response.

Judith M. Thomas, MD, PhD  
University of Alabama at Birmingham  
1808 Seventh Avenue South  
BDB 563  
Birmingham, Alabama, USA 35294-0012  
e-mail: jthomas@uab.edu

## Background Overview

STEALTH is one of 3 promising contemporary paradigms of immune tolerance induction in non-human primates (NHP), the penultimate step to clinical translation.<sup>1</sup> Unlike more familiar approaches and mechanisms that use chimerism<sup>2-5</sup> or costimulatory blockade,<sup>6</sup> the most recent approach to long-lasting immune tolerance to NHP organ and islet transplants, STEALTH, uses neither chimerism nor chronic immunosuppressive therapy (CIT). Yet this novel paradigm has enabled us to achieve seemingly permanent acceptance in a large majority of mismatched allografts in rhesus macaques (*Macaca mulatta*, *Mamu*). In this review of the STEALTH model, we have not included discussion of these other important and thoroughly reviewed primate tolerance strategies (chimerism, irradiation, costimulatory blockade, and pretransplant conditioning<sup>7-10</sup>) so that we can focus solely on the details of this emerging and intriguing model.

STEALTH exploits a concise peritransplant treatment strategy of a 2-week duration that involves a synergy between 2 novel immunosuppressive agents. These are CRM9 diphtheria-based anti-CD3 $\epsilon$  immunotoxin (IT), an unusually effective eradicator of T cells, and 15-Deoxyspergualin, a specific inhibitor of NF- $\kappa$ B/RelB nuclear translocation, a signaling pathway critical for myeloid dendritic cell (DC) maturation<sup>11-13</sup> (C. Asiedu, submitted for publication). A consistent property of long survivors in this NHP tolerance model is in vivo immunological donor specificity. It is also worth emphasizing that every aspect of the STEALTH model was engineered for application to unrelated cadaveric donor transplantation. Thus, no preconditioning period is used. Since most unrelated human

transplants are incompatible for major histocompatibility complex (MHC) alleles, all our rhesus donors and recipients are prescreened by PCR-based typing with *Mamu*-specific MHC primers,<sup>14,15</sup> ensuring both MHC class I and class II incompatibility to mimic the human situation. Thus, from an immunogenetic view, the peritransplant STEALTH tolerance induction model is rigorous.

## The Underlying Concept

Conceptually, we view tolerance induction by STEALTH as a maneuver to evade interactions between cells involved in immune activation, thereby enabling graft-borne MHC alloantigens to be introduced below the immunological "radar" screen. Outside this "immunological danger" zone, as Matzinger has described it, alloantigens would be virtually imperceptible.<sup>16</sup> Our working concept of STEALTH induction is that it represents a synergy between profound LN T cell depletion by IT with concomitant blockage of NF- $\kappa$ B (particularly RelB nuclear transcription). In effect, this allows the host to ignore early proinflammatory "danger" alarms normally elicited by necrotic donor cells in the ischemic reperfused organ or cell transplant. In contrast to CIT, which dampens already triggered proinflammatory activation, the STEALTH tolerance approach is designed to be proactive, diverting the earliest direct and indirect alloimmune responses away from proinflammatory signaling and toward immune downregulation.

## Assumptions Related to the Model

In evolving the STEALTH protocol, we incorporated 2 fundamental assumptions. The first is that steady-state NF- $\kappa$ B/RelB-dependent processes are

“on standby,” operationally poised to accelerate maturation of host or donor myeloid DCs that process fragments of necrotic donor cells.<sup>11,17,18</sup> After antigen processing, DCs home to LN, a process that hinges on DC upregulation of CCR7, the receptor for RelB-dependent LN ligands in the T cell area of the LN.<sup>19</sup> Within the immune epicenter of the LN,<sup>20</sup> the presence of antigen-bearing, mature DCs promotes activation of type 1 helper and cytotoxic T cells and Ig switching in B cells.<sup>17</sup> Since RelB expression is crucial for myeloid DC maturation and LN homing, we reasoned that activation of type 1 responses might be interrupted if RelB nuclear translocation could be prevented early during antigen exposure.

A second assumption in the development of this protocol was that it might be necessary to transiently remove memory T cells (putatively alloantigen cross-reactive) from the playing field since they can be activated without DC interaction. Parenthetically, a pan-T cell IT is used because the T<sup>memory</sup> cell phenotype is presently too complex to allow for construction of a T<sup>memory</sup>-specific immunotoxin at this time. T memory cell activation can proceed in a DC-independent fashion through NF-κB-driven costimulatory adhesion molecules expressed on nonprofessional antigen-presenting cells (APCs), such as endothelial cells.<sup>21</sup> Activation of T<sup>memory</sup> cells in the absence of DCs is also enhanced by common γ chain cytokines (IL-2, IL-15, etc.).<sup>22</sup> In this context, if T<sup>memory</sup> cell contributions could be blunted, induction of robust, enduring tolerance to multiple MHC incompatibilities in outbred NHP species might be achieved, without having to employ irradiation, chimerism, or CIT. To effect this outcome, we used anti-CD3IT to rigorously deplete both the circulating T cells and T cells in tissues, wherein the memory cells are sheltered. At the same time, we used deoxyspergualin (DSG) to uproot the NF-κB/RelB-dependent proinflammatory surge of cytokines and related sequelae.<sup>12,13,23-26</sup>

### Immunological Profiles after STEALTH Induction

To date, studies of early immunological changes during STEALTH induction in rhesus monkeys point to the involvement of 3 interrelated elements.<sup>1</sup> These are as follows:

1. IT-induced profound, transient depletion of recipient T cells in LNs, which are functionally devoid of T cells for 1 to 2 weeks after IT treatment;
2. DSG-induced blockade of antigen presentation in LN by maturation arrest of myeloid DCs that are crucially dependent on RelB-mediated signal transduction; and
3. striking deviation of cytokines to a type 2 pattern of immunological downregulation, with sustained production of IL-4 and IL-10, which reflects a unique synergy between IT and DSG. Perhaps a fourth element should be added, in that the STEALTH environment apparently preempts generation of IgG antibody responses to donor-specific alloantigens introduced during induction, suggesting development of both operational B and T cell tolerance.<sup>13</sup>

This final point may be pivotal in biasing the graft toward STEALTH tolerance.

### STEALTH Tolerance Outcomes

#### *Survival of Rhesus Monkey Organ and Cellular Grafts*

The high frequency of allograft survival in the STEALTH model, in the absence of any chronic immunosuppression, is remarkable. Moreover, induction of tolerance by the STEALTH strategy is not restricted to the type of graft. This suggests that mechanisms underlying tolerance induction to MHC-incompatible *Mamu* allografts are fundamental to different types of transplants. The peritransplant treatment with IT plus DSG promoted long-lasting acceptance of both kidney<sup>13</sup> and isolated pancreatic islet grafts<sup>1</sup> (in streptozotocin [STZ]-induced diabetics) for years. In current follow-up, some of the earliest members of the kidney allograft series are in excellent health > 6 years posttransplant. There are an unprecedented number (62.5%, 10/16) of survivors at > 3 years' follow-up without physiological or histological evidence of acute or chronic rejection (Fig. 1). The duration of islet allograft function after one single donor transplant is particularly significant, given the necessity for multiple transplantation in the most successful

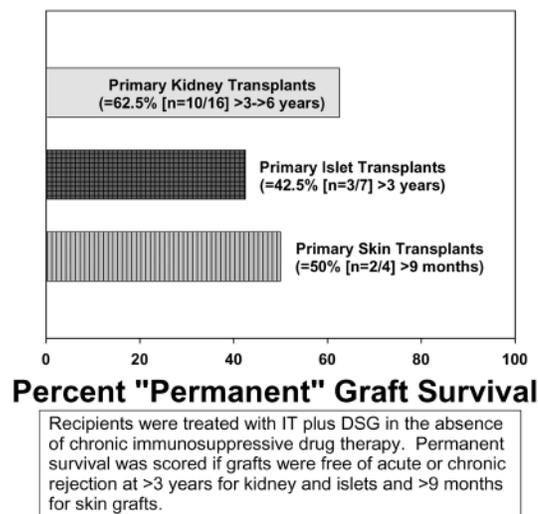


Figure 1. Actual long-term rejection-free graft survival.

current clinical protocol.<sup>27</sup> In these single-donor islet transplants, we have observed 42.9% (3/7) currently surviving > 3 years with stable euglycemia by several criteria. Finally, recent studies have extended tolerance induction to primary full-thickness rhesus skin grafts (J. Thomas et al., unpublished data), which exhibit an overall lower rate (50%) of indefinite survival (now > 9 months' follow-up) compared to kidney and islet grafts. Thus, by comparison with any current NHP tolerance protocol that has been reported, STEALTH-induced tolerance is unsurpassed in the duration and frequency of seemingly permanent tolerance.

With reference to islet transplantation, it is worth noting that operational tolerance has also been successfully induced to concordant rhesus islet xenografts in spontaneous insulin-dependent diabetes mellitus (IDDM) African green and cynomolgus recipients using a modification of the IT plus DSG strategy.<sup>28</sup> Those studies used cyclosporine in place of DSG. It is noteworthy that cyclosporine plus IT did not achieve long-term success in either the spontaneous diabetics or STZ-induced IDDM. We have not yet examined the IT plus DSG treatment strategy in spontaneous IDDM. The apparent discrepancy between results using either DSG or cyclosporine in the 2 models is intriguing, although the reasons are presently unclear. This is in large part related to the many

variables between the models. Among these is the etiology of diabetes, which was either spontaneous (putatively involving autoimmunity<sup>29</sup>) or high-dose STZ induced.<sup>30</sup> Other variables include age (aged vs. young recipients), type of donor islets (concordant xenograft vs. MHC incompatible allograft), and recipient species (*M. fascicularis* and *Cercopithecus aethiops* vs. *M. mulatta*). While we have not yet systematically examined the IT plus DSG model in concordant and discordant xenoislet transplants, it is conceivable that it will yield appropriate protocols to promote xenograft islet acceptance or tolerance.

#### *Challenge Grafts Reveal Specificity and Stability of STEALTH*

The studies of Murray et al. provided the original criteria to assess immunological tolerance in large animal species.<sup>31</sup> Arguably, the most critical of these is the acceptance of a second graft from the donor without any immunological intervention. We applied this test, using challenge donor skin and second kidney transplantation, to tolerant kidney allograft recipients. The results satisfied the conditions of Murray et al.—namely, that the second kidney graft was accepted without consequence and performed optimally.<sup>13</sup> Skin graft challenges in the same animals revealed an additional interesting pattern. First, third-party grafts were briskly rejected in a week, while donor skin transplanted at the same time was accepted without acute rejection for prolonged periods, confirming donor specificity in the immune-tolerant state. Within 2 months, however, the donor skin grafts succumbed to a slow, chronic-type rejection. This event had no effect on the continued function of the long-term kidney grafts, suggesting that the late rejection of donor-specific skin might be a response to skin-specific minor antigens. To further examine this notion, we transplanted a second donor-specific skin graft, which resulted in accelerated rejection of the skin but again without any change in renal allograft function, a trend that held for at least 2 years' follow-up. Overall, we interpret these results (with challenge primary and secondary specific donor skin grafts and secondary specific donor kidney grafts) to indicate an exquisite level of immune precision in the tolerance achieved to

## CHRONIC REJECTION:

A host versus graft reaction that occurs over a period of months to years, characterized histologically in most transplanted organs by arterial and arteriolar thickening (intimal layer), atrophy, and/or interstitial fibrosis.

the mismatched donor MHC antigens that were presented to the recipient during the induction phase of tolerance. In contrast, the tolerant recipients are able to respond to minor skin-specific donor antigens that were not introduced during tolerance induction.

*Freedom from Chronic Rejection*

Chronic rejection is the foremost problem confounding long-term success in conventional (i.e., chronic immunosuppressive drug-based) transplantation. In human transplantation, late graft loss due to chronic rejection has thus far resisted the best efforts to eradicate it. Both immunological and nonimmunological factors are implicated in the pathogenesis of chronic rejection. Because immunosuppressive drugs blunt (but do not eliminate) a smoldering host allograft response, immune tolerance induction may provide a means to discriminate between immune and nonimmune responses in the etiology of chronic rejection. With STEALTH tolerance induction, we have observed an apparent absence of chronic rejection (humoral and cellular) in long-term survivors.<sup>13</sup> The reasons for the lack of chronic rejection even at 6 years posttransplant are under study. It is possible that the use of DSG during the induction period ameliorates injuries of a nonimmunological nature that further compromise long-term graft integrity and function. A caveat regarding the chronic rejection-free status of the many long-term survivors in our NHP tolerance model is that we are transplanting living unrelated donor grafts rather than stored grafts, as is the case in most human cadaveric kidney donor transplants.<sup>26,32</sup> Thus, we have not assessed the impact of graft storage stresses. However, we are examining the hypothesis that DSG-induced blockade of the surge of NF- $\kappa$ B/RelB-dependent DC maturation and related "alarm signals" from necrotic donor cells during the healing-in period might also be applicable to stored organs. In the same context, if the final common pathway of risk for chronic rejection in cadaveric organ donors arises from donor cell apoptosis and necrosis due to organ storage and cumulative stresses related to brain death, it is not unreasonable that the STEALTH strategy may supplant chronic rejection with tolerance.

*Absence of Antidonator Alloantibody*

Once mounted, an IgG antibody response to the graft is extremely difficult to control and thus may be an important factor in both acute and chronic rejection.<sup>33</sup> With STEALTH, we have the ideal situation of a specific absence of alloantibody responses coexisting with the capacity to produce antibodies to new (e.g., vaccines) or ostensibly previously encountered antigens.<sup>13,30</sup> Once again, this alloantibody unresponsiveness arises from the synergy between peritransplant T cell depletion and DSG. Besides virtually eliminating T cell help for B cells, DSG is known to block antibody synthesis by 2 avenues. One is the direct inhibition of NF- $\kappa$ B-dependent immunoglobulin synthesis by DSG,<sup>34</sup> consistent with<sup>35</sup> the originally described role of NF- $\kappa$ B in the constitutive expression of Ig  $\kappa$  chain synthesis;<sup>36</sup> the other is that DSG blocks maturation of DCs in LN. Mature DCs express CD40L, as well as other costimulatory molecules, which can directly activate B cells without T cell help.<sup>37</sup> For these reasons and probably others, the presence of DSG in the immediate peritransplant period appears to be critical to enhance B cell tolerance induction to nominally recognized alloantigens. Finally, the evidence of specific in vivo T and B cell tolerance in our long-term survivors, who are uniformly free of chronic rejection, bolsters the notion that tolerance induction may resolve the nagging problem of chronic rejection.<sup>38</sup>

*The Tolerance Window*

The treatment "window" for tolerance induction in the STEALTH model encompasses a 2-week-long interval, characterized by a quiescent state with low numbers of mature T cells and a preponderance of immature APCs.<sup>13</sup> Broadly considered, this condition resembles that of the developing neonatal immune system, which is highly susceptible to induction of tolerance. Interestingly, the temporal window for neonatal tolerance induction in mice is within 1 to 2 weeks of birth. Both limited T helper cells<sup>39</sup> and immature APCs<sup>40</sup> have been postulated to enable neonatal tolerance induction. In the NHP STEALTH model, as grafted tissues "heal in" and resume normal function during the peritransplant grace period,<sup>41</sup> it is conceivable that there is sufficient time for necrotic cell debris,

generated by surgical reperfusion injury, to be cleared without eliciting alarm signals that would normally ensue from NF- $\kappa$ B-dependent immune response mechanisms. In many ways, this notion is consistent with the “danger model,” in that it predicts that the residual immune system and repopulating T cells will simply accept the graft as “non-dangerous.”<sup>16</sup> The repopulation of mature peripheral T cells, from both peripheral and central sources, accelerates around the end of the first month<sup>26,42</sup> (Z. Chen, personal communication, 2002) and proceeds steadily and without a loss of immune competence. Despite the profound T cell depletion, these recipients are apparently resistant to opportunistic infection and neoplasia. We speculate that this may be due to preservation of innate and non-T cell immunity and the relatively rapid recovery of T cell immunity with respect to cell numbers and repertoire.<sup>42</sup>

#### **Mechanisms Involved in STEALTH Tolerance Induction**

As noted above, induction of tolerance in this model depends on a novel synergy between immunotoxin-induced T cell depletion and DSG-induced NF- $\kappa$ B inhibition. Neither IT treatment alone nor DSG treatment alone can reproducibly yield durable or lasting tolerance to MHC disparate grafts.<sup>1,12,13,41</sup>

#### **Immunotoxin-Mediated T Cell Depletion**

The development of an FN18 CD3 $\epsilon$ -directed IT by Neville and his associates provided a high-performance T cell-killing reagent that produces remarkable T cell depletion (typically > 99.5%) in both blood and LN, with no demonstrable nonspecific toxicity.<sup>10,43</sup> This claim applies to rhesus recipients that express the FN18 epitope, which is not uniformly expressed in the overall rhesus population.<sup>24</sup> With proper management, the monkeys are physiologically stable, and both kidney and islet transplant recipients tolerate IT treatment well.<sup>32</sup> The current version of IT is based on a precise conjugation of anti-rhesus CD3 $\epsilon$  mAb<sup>24</sup> and genetically engineered diphtheria toxin.<sup>43</sup> We have evaluated both intact mAb- and F(Ab)<sub>2</sub>-based IT conjugates and have found both to be effective,<sup>12,26</sup> although the noninflammatory F(Ab)<sub>2</sub>IT appears to have an

advantage in promoting long-term, permanent rejection-free survival. Fully recombinant sFv-toxin fusion proteins are under development by Novartis to replace the chemically conjugated IT construct.<sup>10</sup> Practically, preclinical and clinical use of IT will be enabled by the continuity of supply and quality that are afforded by the recombinant product.

#### **Repopulation of T Cells, Including Those with Memory Phenotype**

Following acute depletion by IT, the T cell repopulation proceeds steadily after the first month with apparent contributions from residual peripheral T cell pools as well as from thymic origin<sup>42</sup> (Chen, unpublished TREC analyses). Measuring a “memory” T cell subset (CD3<sup>+</sup>CD45RO<sup>+</sup>CD62L<sup>-</sup> or CD11a<sup>+</sup>) by flow cytometry, we have observed an early reduction and subsequent burst of expansion in this population at approximately 2 to 4 months.<sup>42</sup> This is not without precedent, as studies in patients experiencing therapeutic ablation of lymphoid tissues reveal a similar pattern.<sup>44</sup> Presumably, this phenomenon reflects homeostatic repopulation. However, based on recent studies in mice, these findings may be subject to reinterpretation, as memory cell expansion could reflect a conversion of naive T cells into a memory phenotype.<sup>45,46</sup> This property of naive T cells to divide homeostatically has been demonstrated to be independent of antigen stimulation,<sup>47,48</sup> thus creating a population of antigen-inexperienced memory cells. Further studies are under way to examine whether such cells persist and the degree to which T cell memory is functionally retained or lost. Resolving these issues may have implications for STEALTH intervention in autoimmunity.

#### **Peritransplant Deoxyspergualin Institutes the “Quiet Revolution” through NF- $\kappa$ B Signal Blockade in the T Cell-Depleted Recipient**

The original impetus to use DSG in combination with IT in monkey kidney transplants was to ameliorate early complications of IT administration, specifically the vascular leak syndrome and attendant “proinflammatory cytokine storm.” Without DSG, IT treatment elicited substantial production of IL-12p70, INF- $\gamma$ , and TNF- $\alpha$ .<sup>32</sup> However, it soon became apparent that the DSG-mediated

## IMMUNOTOXIN:

Any toxin that is conjugated to either an immunoglobulin or Fab fragment directed against a specified antigen.

inhibition of NF- $\kappa$ B nuclear translocation had more far-reaching advantages for tolerance induction than we had initially expected.

At the outset, DSG-mediated inhibition of NF- $\kappa$ B-driven signal transduction virtually eliminates proinflammatory mediated response cascades, thereby minimizing “danger” in the early posttransplant immunological environment. In this context, it is worth noting that endotoxin (LPS) can preempt DSG inhibitory effects on the translocation of activated NF- $\kappa$ B and thus can circumvent DSG’s anti-proinflammatory effect.<sup>35</sup> Accordingly, antibiotic therapy is routinely administered during the first 1 to 2 weeks posttransplant to minimize exposure to gram-negative bacteria and/or endotoxin. Second, DSG interrupts NF- $\kappa$ B/RelB-dependent maturation of DCs that process antigen. The evidence for this claim derives from 3 sets of observations:

1. the unique paucity of mature CD83<sup>+</sup>CD86<sup>+</sup>DR<sup>+</sup>p55<sup>+</sup> DC in LN tissue of monkey recipients under DSG treatment;
2. negligible expression of TNF- $\alpha$  or anti-CD40-induced mature DC phenotype in cultures containing DSG,<sup>13,41</sup> and
3. DSG-induced upregulation of the TRAIL death receptor (DR4) and heightened sensitivity to TRAIL-induced apoptosis in DCs activated by anti-CD40 or TNF- $\alpha$ , both NF- $\kappa$ B-driven signal transduction pathways.<sup>49</sup>

By inference, these consequences would preclude indirect and direct alloantigen presentation, as we currently understand the processes.<sup>17</sup> Finally, the original pharmacological property of DSG—namely, blockade of antibody synthesis—may also contribute to a dramatic reduction in alloantibody production when coupled with the lack of mature DCs to stimulate B cells.<sup>35</sup>

#### *Immune Deviation toward a Type 2 Profile*

A remarkable aspect of STEALTH induction is the rapid deviation toward systemic type 2 cytokine production. Within 1 to 2 weeks posttransplant, there is a dramatic rise in plasma IL-4, IL-10, and (transiently) TGF- $\beta$ , which is protracted for months.<sup>13,41</sup> In seeking to identify the source of this outpouring of type 2 cytokines at a time when recipient blood and LN T cells were severely deplet-

ed, we have examined recipient LN sections. Immunohistochemical analysis has confirmed type 2 cytokine-producing cells and secreted cytokines in the LN within 4 days posttransplant. Notably, such cells are not seen in normal LN or when IT or DSG is administered alone.

In contrast to the abundance of type 2 cytokines following IT plus DSG treatment, the plasma and LN are virtually devoid of interferon- $\alpha$ .<sup>12,13,41</sup> These observations have profound implications for establishing an immunoregulatory milieu. T cells undergoing primary activation in such an environment become polarized toward a type 2 or “immunosuppressive” profile by the action of specific transcriptional regulatory signals (reviewed in ref. 50). Thus, it is probable that naive alloreactive cells that repopulate the periphery during and after the 2-week induction period will be diverted into a functional type 2 phenotype, contributing to a nonalloreactive environment.<sup>1,13</sup> Consistent with this notion is the observation of increasing numbers of CRTH2<sup>+</sup>CD3<sup>+</sup> (type 2 cytokine-secreting) cells in the circulation for months after STEALTH induction with IT and DSG.<sup>42,51</sup>

A definition of the cell type(s) responsible for the early and late production of type 2 cytokines in the STEALTH model is a current focus in our laboratory. For the early period of STEALTH induction (a time devoid of T cells capable of producing these cytokines), a likely prospect is the natural killer (NK) T cell. Rhesus monkey NKT cells express low levels of CD3 and appear to be resistant to IT-mediated killing (B. Gansuud, B. Hubbard, and J. Thomas, manuscript in preparation). NKT cells, which represent a minor lymphocyte population (~0.5% overall),<sup>52</sup> are normally absent in peripheral LN but highly represented in liver and bone marrow, where they comprise 30% to 50%, and 20% to 30% of lymphocytes, respectively. A prominent early presence of NKT cells in LN tissue is suggested from our studies of LN node biopsies taken during the T cell nadir (day 4 posttransplant). Immunohistochemical analysis of cytokine expression in these cells reveals prominent IL-4 but also IL-10. NKT cells are a major source of IL-4.<sup>52</sup> Although production of IL-10 by NKT cells is not well documented, IL-10 has been implicated in the suppressive action of NKT cells in several models of

experimental autoimmunity.<sup>53,54</sup> It is clear that activation of NKT cells in vivo elicits IL-4 and IL-10 production and promotes Th2 polarization.<sup>53,55,56</sup>

The exact cell source(s) of sustained IL-10 and IL-4 production in long-term tolerant recipients remains to be established. A role for T regulatory cells, rather than NKT, in late cytokine production is suggested by the exquisite immune specificity that characterizes the long-term tolerant recipients as described above. Thus, it seems likely that antigen-driven chronic activation of clonally specific T regulatory cells is involved, although some degree of nonspecific bystander effects of IL-4 and IL-10 deviation must prevail. In this context, it is puzzling why, in the face of systemic IL-10 and IL-4 production, there is not an increase in infection or malignancy in these monkeys. Likewise, the question is applicable to neonatally induced tolerance in which a similar type 2 cytokine profile exists. However, there is increasing evidence for the role of IL-10-producing T regulatory cells in allograft tolerance (reviewed in refs. 57-59). As a source of active immune suppression, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells have a cytokine secretion profile that would fit with our observed high levels of IL-10.<sup>60,62</sup> Although evidence for their role in allograft tolerance is compelling,<sup>60,63</sup> CD4<sup>+</sup>CD25<sup>+</sup> cells do not typically produce IL-4,<sup>61</sup> so it is possible that they may represent only a piece of the regulatory cell puzzle in the NHP STEALTH tolerance model. On the other hand, the recent studies of Asiedu et al. have shown that long-term allograft acceptance after treatment of mice with anti-T cell antibody and DSG requires IL-10 and not IL-4, as shown by a complete lack of prolongation in IL-10 knockouts (C. Asiedu, presented at AST 2002, manuscript in preparation).

Finally, it is important to reemphasize that the resolute shift to type 2 cytokines (IL-4 and IL-10) in the NHP STEALTH model is a manifestation of unique IT-DSG synergy. This change, not previously reported in other NHP studies, seems to be favored by a milieu devoid of both mature T cells and mature myeloid DCs. As noted earlier, the neonatal mouse shares the latter milieu. In this context, the recent observations of Pack et al. are relevant.<sup>64</sup> These authors reported that exposure to antigen in the neonatal period initiates type 2 im-

mune deviation with prominent IL-10- and IL-4-producing cells in lymphoid tissues. The cellular and molecular mechanisms responsible for these perturbations are not yet understood. It is tempting to speculate, however, that a deficiency of NF-κB/RelB-dependent chemokines in the lymphoid network may influence the unusual homing pattern of IL-10- and IL-4-producing cells. In support of this notion are the recent data of Weih et al. showing that RelB expression by lymphoid tissue stroma is essential for expression of homing chemokines in both the paracortex and germinal centers of the LN microarchitecture.<sup>19</sup> It is conceivable, therefore, that the so-called "tolerance window" created by a mere 2-week treatment in outbred primates reflects a unique coincidence in which alloantigens are introduced to a milieu that is at once deficient in mature T cells, mature myeloid DCs, T cell and DC homing signals, and B cell Ig transduction signals. The result is the acceptance of foreign antigens, which are introduced in that window and persist as self thereafter.

## Conclusions

The STEALTH tolerance induction strategy has satisfied preclinical test objectives in a challenging NHP model and is under active development for clinical translation. The induced tolerant state is both robust and long-lived, with immunosuppressive drug-free kidney allograft survivals in excess of 5 years and islet allografts in excess of 3 years. During this time, there appears to be no activation of specific antidonor cellular or humoral alloimmune responses, which occurs without known compromise to the host's functional immunity in general. Taken together, these findings seem to satisfy the accepted criteria for immune tolerance.

## REFERENCES

1. Thomas JM, Hubbard WJ, Sooudi S, Thomas FT. STEALTH matters: a novel paradigm of durable primate allograft tolerance. *Immunological Reviews* 2001;183:223-33.
2. Thomas JM, Carver FM, Kasten-Jolly J, Haisch CE, Rebellato LM, Gross U, et al. Further studies of veto activity in rhesus monkey bone marrow in relation to allograft tolerance and chimerism. *Transplantation* 1994;57:101-15.
3. Kawai T, Cosimi AB, Colvin RB, Powelson J, Eason J, Kozlowski M, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. *Transplantation* 1995;59:256-62.
4. Thomas JM, Thomas FT. A preclinical view of transplant tolerance and chimerism: tolerance induction by posttransplant lymphoid irradiation and

## ALLOANTIBODY:

An antibody specific for an alloantigen.

- donor bone marrow. In: Istad S, editor. Chimerism and tolerance. Heidelberg, Germany: Springer Verlag; 1995. p. 143-58.
5. Smith JP, Kasten-Jolly J, Rebellato L, Haish CE, Thomas JM. Use of allogeneic bone marrow labeled with neomycin resistance gene to examine bone marrow-derived chimerism in experimental organ transplantation. *Cell Transplantation* 1997;6:369-76.
  6. Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in non-human primates [see comments]. *Nat Med* 1999;5:686-93.
  7. Kirk AD. Transplantation tolerance: a look at the nonhuman primate literature in the light of modern tolerance theories. *Crit Rev Immunol* 1999;19:349-88.
  8. Knechtle SJ. Knowledge about transplantation tolerance gained in primates. *Curr Opin Immunol* 2000;12:552-6.
  9. Sachs DH. Immunologic tolerance to organ transplants. *J Gastrointest Surg* 1999;3(2):105-10.
  10. Hubbard WJ, Contreras JL, Eckhoff DE, Thomas FT, Neville DMJ. Immunotoxins and tolerance induction in primates. *Current Opinions in Organ Transplantation* 2000;5:29-39.
  11. Wu L, D'Amico A, Winkel KD, Suter M, Lo D, Shortman K. RelB is essential for the development of myeloid-related CD8alpha<sup>+</sup> dendritic cells but not of lymphoid-related CD8alpha<sup>+</sup> dendritic cells. *Immunity* 1998;9:839-47.
  12. Contreras JL, Wang PX, Eckhoff DE, Lobashevsky AL, Asiedu C, Frenette ML, et al. Peritransplant tolerance induction with anti-CD3-immunotoxin: a matter of proinflammatory cytokine control. *Transplantation* 1998;65:1159-69.
  13. Thomas JM, Eckhoff DE, Contreras JL, Lobashevsky AL, Hubbard WJ, Moore JK, et al. Durable donor specific T and B cell tolerance in rhesus macaques induced with peritransplantation anti-CD3 immunotoxin and deoxyspergualin: absence of chronic allograft nephropathy. *Transplantation* 2000;69:2497-503.
  14. Lobashevsky A, Smith JP, Kasten-Jolly J, Horton H, Knapp L, Bontrop RE, et al. Identification of DRB alleles in rhesus monkeys using polymerase chain reaction-sequence-specific primers (PCR-SSP) amplification. *Tissue Antigens* 1999;54:254-63.
  15. Lobashevsky AL, Wang PX, George JF, Contreras J, Townsend J, Thomas JM. DR non-B1 mismatches influence allogeneic MLR-induced TH1- or TH2-like cytokine responses in rhesus monkeys. *Hum Immunol* 1998;59:363-372.
  16. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* 1994;12:991-1045.
  17. Steinman RM, Inaba K, Turley S, Pierre P, Mellman I. Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies. *Hum Immunol* 1999;60:562-7.
  18. Inaba K, Turley S, Yamaide F, Iyoda K, Mahnke M, Inaba M, et al. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J Exp Med* 1998;188:2163-73.
  19. Weih DS, Yilmaz ZB, Weih F. Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. *J Immunol* 2001;167:1909-19.
  20. Zinkernagel RM. Localization dose and time of antigens determine immune reactivity. *Semin Immunol* 2000;12:163-71.
  21. Dengler TJ, Pober JS. Human vascular endothelial cells stimulate memory but not naïve CD8<sup>+</sup> T cells to differentiate into CTL retaining an early activation phenotype. *J Immunol* 2000;164:5146-55.
  22. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naïve, central memory, and effector memory CD4(+) T cells. *J Exp Med* 2001;194:1711-19.
  23. Neville DM Jr, Scharff J, Hu HZ, Rigaut K, Shilach J, Slingerland W, et al. A new reagent for the induction of T-cell depletion, anti-CD3- CRM9. *Journal of Immunotherapy with Emphasis on Tumor Immunology* 1996;19:85-92.
  24. Nooij FJ, Borst JG, Van Meurs GJ, Jonker M, Balner H. Differentiation antigens on rhesus monkey lymphocytes. I. Identification of T cells bearing CD3 and CD8, and of a subset of CD8-bearing cells. *Eur J Immunol* 1986;16:975-9.
  25. Knechtle SJ, Vargo D, Fechner J, Zhai Y, Wang J, Hanaway MJ, et al. FN18-CRM9 immunotoxin promotes tolerance in primate renal allografts. *Transplantation* 1997;63:1-6.
  26. Thomas J, Neville DM, Contreras JL, Eckhoff DE, Meng G, Lobashevsky AL, et al. Preclinical studies of allograft tolerance in rhesus monkeys: a novel anti-CD3-immunotoxin given peritransplant with donor bone marrow induces operational tolerance to kidney allografts. *Transplantation* 1997;64:124-35.
  27. Shapiro AM, Ryan EA, Lakey JR. Clinical islet transplant: state of the art. *Transplant Proc* 2001;33:3502-3.
  28. Thomas FT, Ricordi C, Contreras JL, Hubbard WJ, Jiang XL, Eckhoff DE, et al. Reversal of naturally occurring diabetes in primates by unmodified islet xenografts without chronic immunosuppression. *Transplantation* 1999;67:846-54.
  29. Sun Y, Ma X, Zhou D, Vacek IMA. Normalization of diabetes in spontaneously diabetic cynomolgus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. *J Clin Invest* 1996;98:1417-22.
  30. Thomas JM, Contreras JL, Smyth CA, Lobashevsky A, Jenkins S, Hubbard WJ, et al. Successful reversal of streptozotocin-induced diabetes with stable allogeneic islet function in a preclinical model of type 1 diabetes. *Diabetes* 2001;50:1227-36.
  31. Murray J, Sheil AG, Mosley R, Knight P, Dickinson J, Dammin J. Analysis of mechanism of immunosuppressive drugs in renal homotransplantation. *Ann Surg* 1964;160:449-73.
  32. Contreras JL, Eckhoff DE, Cartner S, Frenette L, Thomas FT, Robbin ML, Neville DM Jr, Thomas JM. Tolerability and side effects of anti-CD3-immunotoxin in preclinical testing in kidney and pancreatic islet transplant recipients. *Transplantation* 1999;68:215-9.
  33. McKenna RM, Takemoto SK, Terasaki PI. Anti-HLA antibodies after solid organ transplantation. *Transplantation* 2000;69:319-26.
  34. Tepper MA, Nadler SG, Esselstyn JM, Sterbenz KG. Deoxyspergualin inhibits kappa light chain expression in 70Z/3 pre-B cells by blocking lipopolysaccharide-induced NF-kappa B activation. *J Immunol* 1995;155:2427-36.
  35. Nadler SG, Eversole AC, Tepper MA, Cleaveland JS. Elucidating the mechanisms of action of the immunosuppressant 15-deoxyspergualin. *Therapeutic Drug Monitoring* 1995;17:700-3.
  36. Snapper CM, Zelazowski PF, Rosas FR, Tian, MF, Baltimore DF, Sha WC. B cells from p50-NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J Immunol* 1996;156:183-91.
  37. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998;392:245-52.
  38. Worner KL, Lee RS, Madsen JC, Sayegh MH. Tolerance and chronic rejection. *Philos Trans R Soc Lond B Biol Sci* 2001;356:727-38.
  39. Bretscher PA. A two-step, two-signal model for the primary activation of precursor helper T cells. *Proc Natl Acad Sci U S A* 1999;96:185-90.
  40. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells [see comments]. *Science* 1996;271:1723-6.
  41. Thomas JM, Contreras JL, Jiang XL, Eckhoff DE, Wang PX, Hubbard WJ, et al. Peritransplant tolerance induction in macaques: early events reflecting the unique synergy between immunotoxin and deoxyspergualin. *Transplantation* 1999;68:1660-73.
  42. Hubbard WJ, Moore JK, Contreras JL, Smyth CA, Chen ZW, Lobashevsky AL, et al. Phenotypic and functional analysis of T-cell recovery after anti-CD3 immunotoxin treatment for tolerance induction in rhesus macaques. *Hum Immunol* 2001;32:479-87.
  43. Neville DMJ, Scharff J, Hu HZ, Rigaut K, Shiloach J, Slingerland W, et al. A new reagent for the induction of T-cell depletion, anti-CD3-CRM9. *Journal of Immunotherapy with Emphasis on Tumor Immunology* 1996;19:85-92.
  44. Donnerberg AD, Margolick JB, Donnerberg VS. Lymphopenia, apoptosis, and immune amnesia. *Ann NY Acad Sci* 1995;770:213-26.
  45. Cho BK, Rao VP, Ge Q, Eisen HN, Chen J. Homeostasis-stimulated proliferation drives naïve T cells to differentiate directly into memory T cells. *J Exp Med* 2000;192:549-56.

46. Goldrath AW, Bogatzki LY, Bevan MJ. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J Exp Med* 2000;192:557-64.
47. Clarke SR, Rudensky AY. Survival and homeostatic proliferation of naive peripheral CD4<sup>+</sup> T cells in the absence of self peptide:MHC complexes. *J Immunol* 2000;165:2458-64.
48. Murali-Krishna K, Ahmed R. Cutting edge: naive T cells masquerading as memory cells. *J Immunol* 2000;165:1733-7.
49. Wu J, He J, Sooudi S, Jiang XL, Ray P, Thomas F, et al. Pretreatment of HL60 cells with deoxyspergualin enhances trail-induced apoptosis independent of caspase 3 activation. *Transplant Proc* 2001;33:278.
50. Rengarajan J, Szabo SJ, Glimcher LH. Transcriptional regulation of Th1/Th2 polarization. *Immunol Today* 2000;21:479-83.
51. Nagata K, Tanaka K, Ogawa K, Kimmotsu K, Imai T, Yoshie O, et al. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol* 1999;162:1278-86.
52. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. *Immunol Today* 2000;21:573-83.
53. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, et al. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 2001;194:1801-11.
54. Hammond KJ, Poulton LD, Palmisano LJ, Silveira PA, Godfrey D, Baxter AG. Alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* 1998;187:1047-56.
55. Burdin N, Brossay L, Kronenberg M. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur J Immunol* 1999;29:2014-25.
56. Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, et al. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* 1999;163:4647-50.
57. Zhai Y, Kupiec-Weglinski JW. What is the role of regulatory T cells in transplantation tolerance? *Curr Opin Immunol* 1999;11:497-503.
58. Cobbold S, Waldmann H. Infectious tolerance. *Cur Opin Immunol* 1998;10:518-24.
59. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* 2001;166:3789-96.
60. Levings MK, Roncarolo MG. T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. *J Allergy Clin Immunol* 2000;106:S109-12.
61. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal MR, Roncarolo MG. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* 2001;166:5530-9.
62. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 2001;31:1122-31.
63. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) Immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med* 2001;193:1311-8.
64. Pack C, Cestra AE, Min B, Legge KL, Li L, Caprio-Young JC, et al. Neonatal exposure to antigen primes the immune system to develop responses in various lymphoid organs and promotes bystander regulation of diverse T cell specificities. *J Immunol* 2001;167:4187-95.