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REGULATORY CD4$^+$CD25$^+$T CELLS IN PREVENTION OF ALLOGRAFT REJECTION

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1. ABSTRACT

Long-term survival of transplanted organs currently requires chronic immunosuppressive treatment of recipients. While the efficacy of these therapies is satisfactory, their toxicity to host tissues and non-specific inhibition of the immune response are disadvantageous. The ideal in transplantation is a situation of donor-specific unresponsiveness, but agents capable of effecting specific tolerance to transplanted tissues have been elusive.

Accumulating evidence suggests that immunoregulatory CD4$^+$CD25$^+$T cells are essential in regulating the immune response to self and foreign antigen. As these cells are capable of suppressing the alloresponse, they represent a potentially invaluable tool for prolonging survival of allografts. In this report, we summarize studies characterizing regulatory T cells and addressing their ability to extend allograft survival. While the capacity of this population to promote allograft tolerance has been demonstrated, many questions remain to be answered before their potential for clinical applicability can be fully defined. Despite this, it is clear from initial studies that regulatory T cells represent an exciting avenue for further investigation in the quest to induce donor-specific unresponsiveness.

2. INTRODUCTION

Currently, organ transplantation represents the only curative therapy for a diverse range of devastating disease processes including hepatic failure, renal failure, and diabetes. While great strides have been made in surmounting the technical difficulties in these procedures, transplantation is largely limited by our inability to exert long-lasting, specific control over the immune response. Immunosuppressants preventing allograft rejection are problematic in that they typically require lifelong administration and inhibit the immune response both non-specifically and globally. These alterations leave the recipient vulnerable to opportunistic infection and malignancy, and many therapies are also toxic to the transplanted graft or other organs.

Ideally, use of adjunct immunosuppression could be avoided altogether by induction of donor-specific unresponsiveness. As each individual already maintains a state of self-tolerance, understanding the mechanisms of this regulation may enable us to adapt these strategies to tolerize the immune system to specific allograft antigens. Our current understanding of self-tolerance identifies four modalities necessary for its maintenance: deletion of self-reactive T cells, induced non-responsiveness of these cells (anergy), sequestration of target antigens away from immune surveillance (ignorance), and active suppression of auto-aggressive cells by immune cells with self-protective function.

From the perspective of the transplant immunologist, anergy, ignorance, and deletion are limited by the requirement of these processes to act on a majority of donor-reactive cells in order to protect transplanted

tissue. In this regard, suppression represents an attractive alternate avenue, especially when many of the features reviewed here are considered. Regulatory cells, as they have been described, compose only a small percentage of recirculating lymphocytes. In addition, the activity of one such cell may inhibit multiple donor-reactive cells. This feature stands in marked contrast to other modalities in which each cell must be individually tolerized.

While a highly specific surface marker distinguishing regulatory T cells is currently lacking, the IL-2 receptor alpha chain (CD25) has thus far proven to be the foremost identifier. Herein, we summarize recent findings on the capacity of $CD25^+$T cells to regulate the immune response to allografts. We detail relevant findings on the formation and function of $CD4^+CD25^+$T cells before examining studies demonstrating a role for this regulatory population in prolonging allograft survival. It is clear that $CD4^+CD25^+$T cells represent a powerful tool in regulating the immune response to foreign antigen, and increased understanding of their generation and function may foster our ability to selectively downregulate the immune response.

3. $CD25^+$T CELLS: DISCOVERY AND SIGNIFICANCE

Discovery of immunoregulatory $CD4^+CD25^+$T cells stemmed from accumulating evidence indicating that specific, identifiable T cell lineages may be specialized for a regulatory role. Early studies attempting to recognize and characterize such regulatory lineages demonstrated that several markers identify regulatory T cells non-specifically. In an initial report, Sakaguchi, et. al. found that transfer of Lyt^- cells into immunodeficient nude mice resulted in organ-specific autoimmune disease (1). Co-transfer of Lyt-1 T cells prevented development of autoimmunity, supporting the concept of identifiable T cell groups specialized to prevent self-destructive responses by other cells. Initial data from the Sakaguchi group were extended when others demonstrated the presence of putatively regulatory $CD4^+$ T cell subsets. For instance, several studies showed that transfer of $CD45RB^{hi}$ (or the rat counterpart $CD45RC^{hi}$) cells into mice (rats) induced organ-specific autoimmune disease, while co-transfer of $CD45RB^{lo}$ (RC^{lo}) cells restored self-tolerance (2-6). Similarly, studies demonstrated that transfer of $CD5^{lo}$ cells prevented autoimmunity induced either by day 3 thymectomy or by transfer of $CD5^{hi}$ populations into immunodeficient mice (7-8). Collectively these data suggested that a specific lineage of cells designed to prevent autoimmune reactions existed and could be isolated. Because expression of CD45RB and CD5 is not limited to regulatory T cells, a T cell marker capable of distinguishing suppressor T cells more precisely was coveted. In considering T cell markers upregulated on $CD5^{hi}$ and $CD45RB^{lo}$ T cells, Sakaguchi, et. al. first uncovered the IL-2 receptor alpha chain molecule (CD25) as a more specific marker for a $CD4^+$ T cell population with specialized regulatory function (9). Though CD25 is also non-specific, it remains the surface marker most commonly used in distinguishing regulatory T cells.

The significance of $CD4^+CD25^+$T cells in maintenance of self-tolerance has now been demonstrated in several different models of autoimmunity. In the initial report on regulatory $CD4^+CD25^+$T cells, Sakaguchi, et. al. demonstrated that transfer of lymphocyte suspensions depleted of $CD25^+$cells into athymic nude mice resulted in development of organ-specific autoimmunity (9). Co-transfer of $CD4^+CD25^+$T cells maintained self-tolerance, illustrating a role for these cells in prevention of spontaneous autoimmunity. This group and another then reported that autoimmunity induced by thymectomy at day 3 of life in susceptible mouse strains develops largely because of the absence of this regulatory population (10,11).

In addition to documenting the importance of $CD25^+$cells in self-tolerance, autoimmune models have also proven consequent in elucidating the mechanism of $CD25^+$T cell-mediated suppression, as will be discussed later. Two models have been especially significant in this capacity. First, Powrie, et. al. have characterized an autoimmune inflammatory bowel disease-like syndrome induced by transfer of $CD45RB^{hi}$ cells into immunodeficient SCID mice. Disease is prevented by co-transfer of $CD45RB^{lo}$ cells, and recent studies indicate that the suppressive subset within the $CD45RB^{lo}$ population is the $CD25^+$fraction (2,5,12,13). This model has served to clarify the involvement of $CD25^+$T cells in prevention of inflammatory immune responses to self or foreign antigens. More recently, Shevach et. al. found that co-transfer of $CD4^+CD25^+$T cells prevents autoimmune gastritis induced by transfer of a cloned line of self-reactive T cells specific for the H/K ATPase (11). These autoimmune models have been principal systems used in analyzing the involvement of particular molecules in $CD25^+$T cell function.

In summary, attempts to identify a T cell subset specialized to a regulatory capacity has uncovered the existence of regulatory $CD4^+CD25^+$T cells. The importance of these cells is evident in the variety of autoimmune reactions developing in their absence. Autoimmune disease models are now being used to assess the *in vivo* mechanism of $CD4^+CD25^+$T cells.

4. REGULATORY $CD4^+CD25^+$T CELLS ARE A DISTINCT IMMUNOREGULATORY LINEAGE THAT IS BOTH ANERGIC AND SUPPRESSIVE

Though regulatory cells were initially signified by their hypoproliferative phenotype (13-18) and expression of CD25, these qualities characterize a heterogeneous group of cells. Activated T cells are known to express the CD25 molecule and are often refractory to restimulation. Furthermore, anergy can be induced in a variety of ways (reviewed in 19) and is not specific to the regulatory population. Because of this, it was necessary to address whether the anergic $CD25^+$population contains a distinct regulatory lineage or whether all anergic and/or $CD25^+$T cells possess suppressive capabilities. The characteristics of immunoregulatory $CD4^+CD25^+$T cells, activated T cells expressing CD25, and anergic T cells are detailed below and comparatively summarized in Table 1.

Table 1. Comparative summary of the characteristics of immunoregulatory CD4$^+$CD25$^+$T cells, activated T cells expressing CD25, and anergic T cells

Population	Anergic	Suppressive	CD25 expression
Immunoregulatory CD4$^+$CD25$^+$T cells	Yes; anergy is default state	Yes; suppressive capacity is default state	Constitutive and further upregulated after activation
Activated T cells expressing CD25	Yes	No	No expression while resting; transient upregulation of CD25 after activation
Anergic T cells[1]	Yes, but will not revert to the anergic state if anergy is broken	Yes, but will not revert to the suppressive state if anergy is broken	Express CD25 with induction of anergy; expression is not as robust or stable as that of the naturally-occurring immunoregulatory lineage

[1]The properties of anergic T cells do vary depending on the method of anergy induction.

In differentiating naturally-occurring regulatory CD25$^+$T cells from T cells expressing CD25 after activation, several studies have shown that the latter are not suppressive *in vitro* or *in vivo* (9,11,15,16,20). For instance, if a TcR transgenic T cell population containing few CD25$^+$cells is transferred to a day 3 thymectomized mouse, activation of those T cells with cognate antigen does not prevent onset of autoimmunity (11). To further differentiate these groups, activated T cells entirely lose CD25 expression upon cessation of stimulation, while the regulatory population reverts to its basal level of expression (20). Finally, immunoregulatory T cells increase CD25 expression more rapidly and to higher levels after activation than non-suppressor cells (20). These findings indicates that T cells activated to express the CD25 molecule are separable from the immunoregulatory lineage.

In comparing CD25$^+$T cells to anergic cells, Kuniyasu, et. al. found that expression of CD25 is higher and more stable in the CD25$^+$regulatory population than in other anergic cells (20). Furthermore, the anergic, suppressive state appears to be the default state of regulatory CD25$^+$T cells, as these cells but not other anergic cells revert to this condition after anergy is broken. A difference between naturally-occurring regulatory CD4$^+$CD25$^+$T cells and other anergic or activated cells is also implied by the thymic origin of the former, which is discussed in greater detail below.

In summary, though activated T cells and anergic cells share some phenotypic properties with the regulatory CD4$^+$CD25$^+$T cell lineage, it is clear that the latter is a distinct immunoregulatory population. Despite this, CD25 is also imperfect in distinguishing regulatory T cells, and markers which identify the regulatory subset more specifically remain sought after. Recent data indicate that a gene with increased expression in CD4$^+$CD25$^+$T cells (Foxp3) may be a more specific marker for regulatory function (discussed later).

5. MECHANISMS BY WHICH CD4$^+$CD25$^+$T CELLS EXERT SUPPRESSION

Because CD4$^+$CD25$^+$T cells are critical in maintenance of tolerance to self and foreign antigens, many groups have focused efforts in elucidating their mechanism of action. At this time, the mechanism remains largely unknown. CD25$^+$T cells have been observed to function differently *in vivo* than *in vitro*, and *in vivo* models have suggested diverse mechanisms of action. Whether these cells are simply heterogeneous in function or we are in fact studying separate lineages of regulatory CD25$^+$T cells remains fertile ground for investigation.

Despite the complexity of these issues, some general aspects of CD25$^+$T cell function are consistently demonstrated. Evidence indicates that CD25$^+$T cells require TCR stimulation to function (13,15,21-23). After TCR activation, these cells suppress in a non-specific fashion, as they are able to inhibit CD4$^+$ cells with alternate antigen and MHC specificities (13,20,22,23). In addition, the population appears to suppress through short range signaling, as several groups have shown that CD25$^+$T cells cannot suppress across a semi-permeable membrane (13,15,16). This data has been interpreted as indicating the requirement for cell/cell contact in CD25$^+$T cell suppression but does not necessitate actual contact. Paracrine signaling over a very short distance could mediate suppression, requiring the cells to be juxtaposed but not necessarily contiguous. Lastly, the function of this population appears to be dependent on immunoregulatory molecules. Which cytokine or surface molecule is critical in suppression is not fully established, but evidence has thus far suggested the involvement of three such factors: CTLA-4, IL-10, and TGF-beta.

5.1. CTLA-4

Cytotoxic T lymphocyte-associated antigen 4 (reviewed in 24) is a close homolog of the costimulatory CD28 molecule. It binds the same ligands (B7-1 and B7-2) as CD28 but with higher affinity. Unlike CD28, which provides a costimulatory signal needed for T cell activation, CTLA-4 transduces an inhibitory signal which downregulates the immune response. This negative regulatory function is confirmed by the fatal lymphoproliferative disorder arising in CTLA-4$^{-/-}$ mice (25,26). The exact mechanisms by which the inhibitory signal is delivered, however, remain unclear. Evidence indicates that CTLA-4 may function to raise the threshold for full T cell activation and limit T cell proliferation by blocking cell cycle progression (27). The role for CTLA-4 in preventing T cell activation and division suggests potential involvement in the function of CD4$^+$CD25$^+$T cells.

The balance of evidence suggests that CTLA-4 is involved in CD25$^+$T cell-mediated suppression. To explain how CTLA-4 could be involved in a short range signaling-

dependent mechanism, Read, et. al. and Takahashi, et. al. initially showed that CTLA-4 is constitutively expressed at the surface of regulatory $CD25^+$cells, and further upregulated after activation (12,28). CTLA-4 at the surface of the regulatory cell may deliver an inhibitory signal to an adjacent responding cell and/or the antigen presenting cell, effecting close range suppression. To further establish involvement of CTLA-4 in the function of $CD4^+CD25^+$T cells, these groups and Nakamura, et. al. all showed that high doses of an anti-CTLA-4 antibody increased proliferation of CD25- populations in the presence of $CD25^+$T cells (12,28,29). Furthermore, $CD4^+CD25^+$T cells from CTLA-$4^{-/-}$ mice effected less potent suppression of responding cells *in vitro* (28). *In vivo* evidence implicating the molecule was found in the inflammatory bowel disease model mentioned above (section 3), as suppression by $CD4^+CD45RB^{lo}$ cells was found to be dependent on CTLA-4 (12). Lastly, CTLA-4 was also shown to be involved in the capacity of $CD25^+$T cells to promote tolerance to alloantigen, as Kingsley, et. al. found that allograft tolerance effected by $CD25^+$T cells could be broken by an anti-CTLA-4 antibody (30). Collectively, these findings suggest that $CD4^+CD25^+$T cells may require CTLA-4 for suppressive function, and that surface expression of CTLA-4 may promote short range inhibition of responding T cells.

The requirement for CTLA-4 in $CD4^+CD25^+$T cell function has not been universal, however. In an alternative system, Shevach, et. al. found that both low and high doses of anti-CTLA-4 antibody failed to break suppression by $CD25^+$T cells *in vitro*, a finding recently corroborated by Chai, et. al. (15,21,31) Furthermore, anti-CTLA-4 also failed to break suppression *in vivo* in the aforementioned autoimmune gastritis model (31), opposing involvement of CTLA-4 in $CD25^+$T cell-mediated suppression in this particular system.

Overall, evidence supports the notion that blocking or activating CTLA-4 signaling impacts suppression, but it is difficult to assert a requisite role. Surface expression of CTLA-4 suggests a direct effect, but we cannot exclude the possibility that this molecule serves as one of many means to activate the final pathway of suppression. For instance, CTLA-4 ligation has been shown to promote secretion of TGF-beta (32), which has also been implicated in $CD25^+$T cell-mediated suppression (detailed in section 5.3). One further confounding issue is that the studies detailed cannot exclude the possibility that antibodies to CTLA-4 hinder T cell suppression by acting positively on effector rather than negatively on regulatory cells. Because non-suppressor effector cells also transcriptionally upregulate CTLA-4 after activation, it is difficult to predict whether antibody treatments that break suppression interrupt the suppressive signal or further activate effector T cells. Still, *in vivo* models suggest involvement of CTLA-4 in $CD4^+CD25^+$T cell function, and the demonstration of constitutive expression of surface CTLA-4 on this population provides a mechanism for short range suppression. These findings make a role for this molecule likely, though it may not be universally involved and will be difficult to decisively demonstrate.

5.2. IL-10

Interleukin-10 (reviewed in 33) is a pleiotropic cytokine with numerous effects on a wide variety of cell lineages. The central role for this molecule appears to be in resolution of inflammatory responses, which is in part accomplished through induction of anti-inflammatory molecules and inhibition of pro-inflammatory cytokine and chemokine expression. The effects of IL-10 on T cell responses are mediated through both the T cell and antigen presenting cell. The cytokine acts directly on T cells to inhibit expression of particular cytokines and chemokine receptors. It also downregulates expression of costimulatory molecules on antigen presenting cells. Because IL-10 can impact both T cells and antigen presenting cells, it possesses vast suppressive potential and could be involved in the function of regulatory T cell populations.

Evidence suggesting the involvement of IL-10 in suppression by $CD25^+$T cells has been acquired exclusively through *in vivo* models. An antibody to IL-10 has been shown to break $CD25^+$T cell-mediated suppression in the inflammatory bowel disease and allograft tolerance models mentioned above (30,34). In addition, Annacker, et. al. showed that anti-IL-10 impaired the capacity of $CD25^+$T cells to prevent proliferation of effector cells in immunodeficient mice (35). Such studies clearly implicate IL-10 in $CD25^+$T cell-mediated suppression but cannot determine whether the regulatory population is the cytokine source. $CD4^+CD25^+$T cells are believed to be the source because these cells express high levels of IL-10, and IL-$10^{-/-}$ $CD25^+$T cells are not suppressive *in vivo* (13,15,29,34).

Nonetheless, additional data from another model suggest that $CD25^+$T cells can function independently of IL-10. Shevach, et. al. reported that IL-$10^{-/-}$ cells remain capable of mediating suppression in an autoimmune gastritis model (31). This finding correlates with *in vitro* results in which IL-$10^{-/-}$ $CD25^+$T cells remained capable of suppression and anti-IL-10 antibody failed to break suppression (13,15,16). It is therefore possible that IL-10 is required in the function of $CD25^+$T cells only under certain conditions, or that heterogeneous populations of $CD25^+$T cells exist which differ in their reliance upon IL-10 for function.

When involved in T cell-mediated suppression, IL-10 could serve a direct or indirect role. First, IL-10 may downregulate APC function, preventing the activation of effector cells. This hypothesis could explain the effects of $CD25^+$T cells on antigen presenting cells (discussed in section 6). Alternatively, IL-10 could serve an indirect role by quieting local inflammation. Our preliminary evidence suggests that $CD4^+CD25^+$T cell-mediated suppression may be abrogated by an inflammatory environment (manuscript in preparation). If an inflammatory milieu inhibits the activity of $CD25^+$T cells, IL-10 could be required for the function of these cells without directly participating in the suppressive event. Such a role could unite findings suggesting cytokine involvement in $CD25^+$T cell function with those demonstrating that $CD25^+$T cells suppress through a cell/cell contact-dependent mechanism. As

suggested by Shevach (31,36), it is possible that IL-10 may help to resolve the inflammatory milieu in order to allow for cell/cell contact-dependent suppression to occur. In this scenario, IL-10 would be required for function in inflammatory disease models but not in non-inflammatory systems like the gastritis model. This function would also explain why IL-10 has been more consistently implicated in studies *in vivo* than *in vitro*, where the inflammatory environment is not present. Finally, this "permissive" role could explain why $CD25^+$T cells from IL-$10^{-/-}$ animals remain capable of suppression *in vitro* and in non-inflammatory *in vivo* models.

5.3. TGF-beta

TGF-beta (reviewed in 37) is a pleiotropic cytokine which is a critical regulator of immune cell differentiation and function. Like CTLA-4, the importance of this molecule in immune cell regulation is evident in its absence, as TGF-$beta^{-/-}$ mice suffer an autoimmune-like inflammatory syndrome. TGF-beta is known to inhibit T cell proliferation, cytokine production, and cytolytic activity, establishing it as a potential mediator of the effects of suppressor cell groups.

A role for TGF-beta in $CD25^+$T cell-mediated suppression is not fully established. Its involvement has been suggested mainly through *in vivo* studies, in which Powrie, et. al. found that an antibody to TGF-beta 1, 2, and 3 broke suppression by CD25+$CD45RB^{lo}$ T cells in the inflammatory bowel disease model (12,38). In support of this data, Nakamura, et. al. implicated TGF-beta in $CD25^+$T cell function *in vitro* and suggested the mechanism for its involvement (29). They found that high concentrations of anti-TGF-beta antibody broke $CD25^+$T cell-mediated suppression *in vitro* in a dose-dependent manner, and that addition of recombinant TGF-beta alone suppressed proliferation of CD25- populations. The group showed further that $CD25^+$T cells constitutively express TGF-beta at the cell surface, and expression of surface TGF-beta is greatly increased with activation. These data were interpreted as the basis for the necessity of cell-cell contact in suppression, as it would be required in order to facilitate the immunoregulatory effects of the surface TGF-beta molecule.

In a separate study, Piccirillo, et. al. recently reported a number of findings suggesting that $CD4^+CD25^+$cells can function independently of TGF-beta *in vitro* (36). This group found that suppression of responding cells by $CD25^+$cells was not abrogated by high concentrations of anti-TGF-beta antibody. Furthermore, $CD4^+$ T cells from $Smad3^{-/-}$ mice deficient in TGF-beta signaling were still capable of responding to (CD25- fraction) or mediating ($CD25^+$fraction) suppression. Finally, $CD25^+$T cells from TGF-$beta^{-/-}$ mice were equivalent to wild type cells in suppressive capacity, suggesting again that these cells can suppress in the absence of TGF-beta. These data exclude a universal requirement for TGF-beta in the function of $CD25^+$T cells.

Given the evidence summarized, the exact role of TGF-beta in suppression remains unclear. Once again, the data appear most compatible with an indirect role. It is possible that TGF-beta helps to resolve the inflammatory response in order to allow for direct cell/cell inhibitory signals to be delivered. In this case, TGF-beta would be necessary in inflammatory *in vivo* models, but not *in vitro* or in non-inflammatory models. Though demonstration of surface TGF-beta suggests a definitive mechanism for involvement of the cytokine in suppression, this finding must be reproduced to better speculate on this mechanism for suppression.

In summary, immunoregulatory molecules are involved in the function of $CD4^+CD25^+$T cells, but direct involvement of any one molecule has yet to be demonstrated definitively. The candidate most likely to be directly involved in suppression is CTLA-4, for which constitutive expression on the surface of regulatory $CD25^+$T cells has been demonstrated repeatedly. The evidence suggests potential involvement of both IL-10 and TGF-beta, but their role may be indirect. By downregulating the inflammatory response, these cytokines may facilitate delivery of the suppressive signal by $CD25^+$T cells.

Establishing the precise role for these three molecules in $CD4^+CD25^+$T cell-mediated suppression may be difficult because of their interplay. Signaling through one of these molecules often induces expression of another. As stated above, CTLA-4 induces expression of TGF-beta (32). In addition, IL-10 stimulates TGF-beta release and vice versa (39,40). This self-amplifying cascade facilitates maximal regulation of the immune response, but hinders the ability to precisely define the effects of an individual cytokine in suppression.

6. TARGET OF SUPPRESSION

In order to mediate suppression, a regulatory T cell must either act directly on responding T cells to modulate their activity or interfere with the function of antigen presenting cells required for T cell activation. Current evidence suggests that $CD25^+$T cells operate at both levels, as data have both implicated antigen presenting cells in suppression and demonstrated suppression in the absence of the presenting cell.

With respect to the role of antigen presenting cells, one could envision three scenarios (Figure 1). First, $CD25^+$T cells may have inhibitory effects on the APC, downregulating costimulatory molecules (Figure 1A). Interaction of naïve effector cells with these deactivated presenting cells promotes T cell anergy, creating a set of effector cells which are hyporesponsive to their cognate antigen (19). Second, the regulatory cells may act strictly through competition, without actively inhibiting antigen presentation or downregulating costimulatory molecules (Figure 1B). In this case, interaction between regulatory T cells and APC would deny the access of naive cells to antigen and costimulatory molecules, preventing T cell activation. Third, the antigen presenting cell may act as a "bridge" to bring regulatory T cells and naïve effector cells

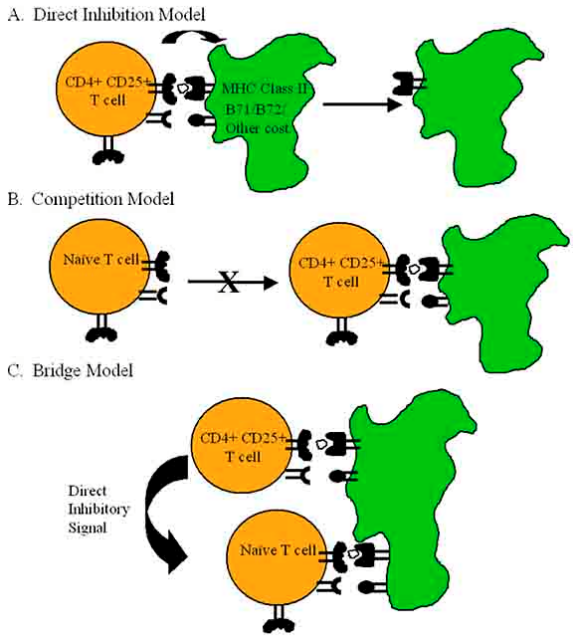

Figure 1. Involvement of the antigen presenting cell in CD4$^+$CD25$^+$T cell-mediated suppression. (A) Direct inhibition model--CD4$^+$CD25$^+$T cells act directly on the APC to inhibit expression of costimulatory molecules and/or antigen presentation. (B) Competition model--Interaction of CD4$^+$CD25$^+$T cells with antigen presenting cells denies access of responding cells to foreign antigen and/or costimulatory molecules. (C) Bridge model--The antigen presenting cell acts as a "bridge" to bring regulatory T cells and naïve effector cells into close proximity, allowing for delivery of a direct signal which inhibits the activity of the latter.

into close proximity, allowing for delivery of a cell-to-cell signal that inhibits the activity of the latter (Figure 1C).

Initial *in vitro* characterization of CD4$^+$CD25$^+$T cells suggested a role for the antigen presenting cell in suppression. Thornton and Shevach reported that suppression by CD25$^+$T cells was observed *in vitro* only when soluble and not plate-bound anti-CD3 was used for stimulation, suggesting the APC as a target (15). Subsequent studies revealed that CD25$^+$T cell-mediated suppression is affected by the number and type of APC, further substantiating participation of APC (13,41). Though these findings suggest that APC are involved in suppression, they do not differentiate between active downregulation, a competitive role, or a passive "bridge" role. Evidence differentiating these alternatives is controversial. One approach to exclude a downregulatory role for CD25$^+$T cells would be to document that these cells have no effects on the antigen presentation capacity or costimulatory molecule expression on APC. To date, evidence indicates that antigen presentation is not altered in the presence of CD25$^+$T cells (13,23), but the effects on costimulatory molecule expression are not fully established. Both Shevach, et. al., and Cederbom, et. al. studied the effects of CD4$^+$CD25$^+$T cells on expression of costimulatory molecules on APC. Thornton and Shevach first reported that CD25$^+$cells did not affect the induction of CD86, CD40, or ICAM-1 on co-cultured antigen presenting cells, arguing that T cell suppression was independent of the presenting cell (23). This finding was corroborated by Ng, et. al., who found that human CD4$^+$CD25$^+$T cells failed to significantly decrease expression of CD80 or CD86 on co-cultured antigen presenting cells (42). In contrast to these findings, Cederbom, et. al. demonstrated decreased expression of CD80 and CD86 on dendritic cells in the presence of the regulatory population (41). This group further illustrated that downregulation occurred at the transcriptional level for CD80, but at an unspecified level for CD86.

Though CD4$^+$CD25$^+$T cells may function in part via the antigen presenting cell, data has also been put forth claiming that suppression can be exerted in the absence of APC. By using tetramers to stimulate T cells rather than antigen presenting cells, Piccirillo, et. al. recently concluded that CD25$^+$T cells inhibit the proliferation of CD8+ populations in the absence of antigen presenting cells (22). This suggests a direct effect of CD25$^+$T cells on effector cells but cannot exclude additional effects on the presenting cell. Perhaps the best model is that the regulatory population both affects effector T cells directly and downregulates interactions with antigen presenting cells. Effects on the antigen presenting cell prevent T cell activation, and delivery of a direct inhibitory signal to responding cells prevents effector T cell function. By this dual capacity, CD25$^+$T cells effect maximal suppression, inhibiting both the induction and effector ends of the T cell response. CD25$^+$T cells would therefore be capable of suppressing in the presence or absence of antigen presenting cells, but would be most effective in their presence. In their absence, anything which brings regulatory and responding cells in close proximity (e.g. tetramers) promotes suppression through the direct inhibitory signal. The antigen presenting cell would be doubly involved in suppression in this scenario. The regulatory cell downregulates costimulation on APC to prevent T cell activation, possibly also inducing anergy in the antigen-specific T cell population. In addition, the presenting cell serves as a bridge used to bring the regulatory cell and responding cell(s) together for delivery of the direct inhibitory signal. The exact molecules used to deliver the respective downregulatory signals are unclear. It is known that IL-10 downregulates costimulation on antigen presenting cells, and that CTLA-4 prevents T cell activation and proliferation. These molecules therefore represent logical candidates, but their involvement in this specific function has yet to be established conclusively.

7. ORIGIN OF CD4$^+$CD25$^+$T CELLS

Although CD4$^+$CD25$^+$regulatory T cells may mediate their effects in the periphery, several lines of investigation indicate they arise within the thymus. Papiernik, et. al. were the first to demonstrate CD4$^+$CD25$^+$T cells in the thymus and documented that they migrate from the thymus to the periphery (43). Subsequently, their thymic origin was more rigorously detailed by Itoh, et. al. (44) They demonstrated that

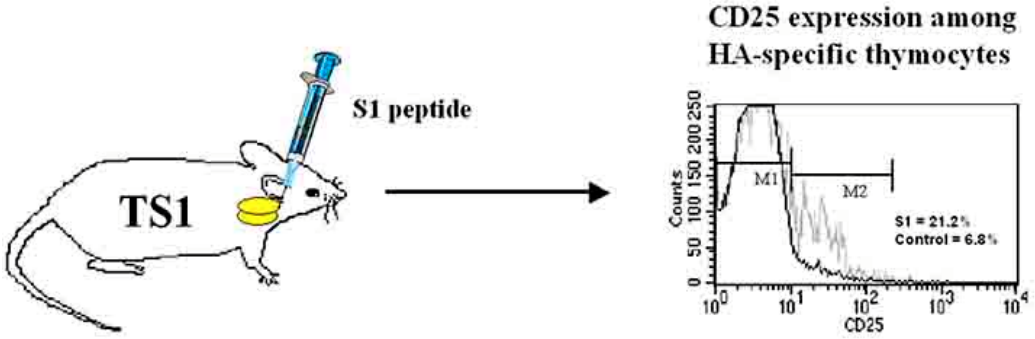

Figure 2. Thymic development of $CD4^+CD25^+$T cells in response to intrathymic peptide inoculation. TS1 mice express a high frequency of T cells specific for the immunodominant (S1) epitope of the influenza virus PR8 hemagglutinin (HA) antigen. We found that intrathymic inoculation of TS1 mice with S1 peptide resulted in a significant increase in CD25 expression among HA-specific (clonotypic antibody positive) thymocytes. While only 6.8% of HA-specific thymocytes were $CD25^+$in naïve mice, 21.2% expressed CD25 in mice tolerized by intrathymic antigen inoculation. Subsequent studies demonstrated that $CD25^+$thymocytes in tolerized mice were suppressive in function and mediated tolerance to HA-expressing allografts.

approximately 5% of CD4 single positive thymocytes express the CD25 molecule (a frequency comparable to that of peripheral $CD4^+$ cells), and the surface phenotype of these cells ($CD5^{hi}CD44^{hi}CD45RB^{lo}CD62L^{hi}$) is similar to that of the peripheral $CD25^+$immunoregulatory population. In addition, transfer of thymocyte suspensions depleted of $CD25^+$thymocytes into athymic nude mice produced autoimmune disease at higher incidence and in a wider spectrum of organs than did transfer of non-depleted suspensions. As further evidence, $CD25^+$T cells developed from CD25- populations injected directly into the thymus *in vivo*, and developed *in vitro* from double negative thymocytes in fetal thymic organ cultures. Finally, $CD4^+CD25^+$thymocytes displayed hyporesponsiveness to TCR stimulation and suppressed the proliferation of other T cells. As was previously noted regarding peripheral regulatory cells (13-15), both suppression and unresponsiveness were broken by IL-2 and anti-CD28 antibodies.

Current data suggests that selection of regulatory $CD25^+$T cells involves both cortical and medullary thymic components. Bensinger, et. al. reported that $CD4^+CD25^+$T cells are selected through interactions with thymic cortical epithelium, as expression of MHC class II on these cells alone was sufficient for development of anergic, suppressive $CD25^+$T cells (45). Their findings also indicated that like effector T cells, regulatory cells are subject to clonal deletion on hematopoietic APC. These results are consistent with those observed by Jordan, et. al., who argued that thymic selection of $CD4^+CD25^+$T cells occurs through high affinity interactions with self-peptide on thymic cortical epithelium (46).

Although the thymic origin of $CD4^+CD25^+$regulatory T cells is convincingly established, approaches to induce thymic generation in adult animals are uncommon. Our laboratory has demonstrated that injection of the relevant antigen into the thymus of TcR transgenic mice increases the percentage of thymic $CD4^+CD25^+$T cells (ref. 47 and Figure 2). To document regulatory function *in vivo*, we demonstrated prolonged survival of allografts expressing the same antigen. These findings indicate that intrathymic presentation of antigen can result in generation of $CD4^+CD25^+$T cells capable of suppressing the immune response to that antigen.

While the thymic origin of CD25+T cells is well documented, whether these cells can be generated peripherally is not certain. Recent data documenting a master regulatory gene programming development of CD4+CD25+T cells provides a potential basis for peripheral development. Several groups have now demonstrated that expression of the Foxp3 gene programs $CD4^+$ cells to acquire the $CD25^+$ phenotype and suppressor function (48-50). $CD25^+$T cells express Foxp3 at a much higher level than $CD25^-$ cells, and transduction of $CD4^+CD25^-$T cells with the Foxp3 gene confers suppressor capacity. This suggests that peripheral generation of regulatory $CD25^+$T cells may be accomplished through induction of Foxp3 expression. What controls induction of Foxp3 is not clear, but the evidence indicates a critical role for this gene in regulatory $CD4^+CD25^+$T cell development and function.

8. DO $CD4^+CD25^+$T CELLS SUPPRESS THE ALLOGRAFT RESPONSE?

The preponderance of evidence demonstrating that $CD4^+CD25^+$T cells regulate the immune response to self and foreign antigen suggests that these cells may be capable of suppressing allograft rejection. $CD25^+$T cells represent an exciting advance in transplant immunology because they carry the potential to induce graft-specific tolerance without suppressing the immune response globally. To this end, studies indicate that $CD4^+CD25^+$T cells are capable of prolonging allograft survival. However, whether $CD25^+$T cells must be previously exposed to alloantigens in order to suppress the allograft response remains unknown. While some studies suggest that "naïve" (referring here to regulatory cells not previously exposed to graft antigen) $CD25^+$T cells can extend allograft survival, others have shown that exposure to graft antigen is required for the regulatory population to function.

Sakaguchi, et. al. first reported a role for CD4$^+$CD25$^+$T cells in prolonging allograft survival (9). This was demonstrated by transfer of either Balb/c CD25- T cells or a mixture of CD25- cells and normal CD4$^+$ cells into T cell-deficient nude mice with allogeneic C57BL/6 skin grafts. Recipients of CD25- cells rejected allografts significantly faster than mice receiving the mixed population, implicating CD25$^+$T cells in regulation of the alloresponse. Moreover, this finding indicates that CD25$^+$T cells isolated from an animal which has not been exposed to graft antigens are nonetheless potent suppressors of the alloantigen response. The observation that "naïve" CD25$^+$T cells can suppress the allograft response was later corroborated by Davies, et. al., who found that CD45RB^{lo} cells from un-manipulated mice were capable of suppressing rejection of a neonatal isl*et al*lograft by a CD45RB^{hi} population (51). As aforementioned, the regulatory subset within this population is believed to be the CD25$^+$fraction (12,13). Finally, Graca, et. al. found that co-transfer of CD25$^+$T cells isolated from a naïve CBA/Ca mouse prevented rejection of B10.BR skin allografts by CBA/Ca splenocytes (52). Collectively, these studies indicate that CD4$^+$CD25$^+$T cells arising in the absence of an allograft can suppress transplant rejection.

In other systems, CD25$^+$T cells have required prior exposure to allograft antigens in order to extend allograft survival. Hara, *et al.* found that co-transfer of "naïve" CD45RB^{lo} cells with the graft-rejecting CD45RB^{hi} population from CBA/Ca mice failed to prevent rejection of C57BL/10 skin grafts, a finding in opposition to that of Davies, et. al. (53) However, this differing result may illustrate variability in the capacity of regulatory populations to prevent rejection of skin versus isl*et al*lografts. In a more comparable report, Gregori, et. al. found that transfer of CD4$^+$25$^+$T cells from Balb/c mice unexposed to B6 antigens failed to prevent rejection of B6 islets by naïve Balb/c mice (54). These findings suggest that "naïve" CD4$^+$CD25$^+$T cells are not effective regulators of all allograft responses. The limit of the suppressive capacity of CD25$^+$T cells remains unknown but is a critical parameter for application in human transplant studies.

These limits appear markedly extended in CD25$^+$cells previously exposed to alloantigen, which have consistently shown suppressive capacity. Hara, et. al. found that CD4$^+$CD25$^+$T cells from operationally tolerant (by use of anti-CD4 antibody and donor-specific antigen) animals effected prolonged survival of cardiac grafts (53). The group subsequently showed that this tolerizing protocol actually induces the formation and/or function of the CD25$^+$regulatory population prior to transplantation so that the allograft is protected from the outset (30). In agreement with these findings, Gregori *et al.* showed that CD4$^+$CD25$^+$T cells from mice rendered tolerant using a short-term treatment of 1alpha,25-Dihydroxyvitamin D_3 and Mycophenolate Mofetil prolonged survival of donor-type islet grafts in naïve syngeneic animals (54).

In view of the data, CD25$^+$T cells from both unmanipulated and tolerized animals appear capable of suppression, but regulatory cells from the latter seem more effective. This enhancement could occur through several mechanisms. The most likely scenario is that alloantigen exposure serves to expand and/or activate suppressor function in the regulatory population. It is clear from previous studies that the method used to induce tolerance is not especially important as long as the recipient is exposed to graft antigen. As detailed above, two different protocols used to induce operational tolerance yielded CD25$^+$T cells which suppressed the immune response to the allograft. In further support of this is our data indicating that CD4$^+$CD25$^+$T cells from mice rendered tolerant through intrathymic inoculation are also capable of promoting allograft tolerance (ref. 47 and Figure 2). In this case, intrathymic injection exposes the recipient mouse to the principle allograft antigen, resulting in induction of CD4$^+$CD25$^+$T cells which suppress allograft rejection. This data is noteworthy because it demonstrates thymic development of CD4$^+$CD25$^+$T cells in response to inoculation with a foreign allograft antigen. While previous studies also demonstrated prolonged allograft survival after tolerizing treatment, the origin and specificity of the CD25$^+$population were unknown. In this set of experiments, both the thymic origin and specificity for the injected antigen are evident.

9. DETERMINING THE MECHANISM BY WHICH CD4$^+$CD25$^+$T CELLS PROLONG ALLOGRAFT SURVIVAL: MOLECULES INVOLVED AND SITE OF SUPPRESSION

As data on the mechanism of CD4$^+$CD25$^+$T cells *in vivo* has largely been limited to autoimmune models, the function of these cells in regulating allograft tolerance has not been well-studied. To this point, the molecules involved in this capacity are similar to those involved in regulating self-tolerance. In the model discussed above, Kingsley, et. al. used antibodies to CTLA-4 and IL-10 to reveal that the capacity of tolerized CD4$^+$CD25$^+$T cells to suppress allograft rejection is dependent on both of these molecules (30). The CD25$^+$population was unable to transfer tolerance in the presence of these antibodies but did transfer tolerance in the presence of anti-IL-4. Similar to the autoimmune models, data acquired in allograft systems has not consistently implicated one molecule in suppression. Data from Graca, et. al. in a transplantation model demonstrated that suppression of allograft rejection by CD4$^+$CD25$^+$T cells occurs even in the presence of antibodies to CTLA-4 or IL-10 (52). Because studies in this area have been limited, the involvement of particular immunoregulatory molecules in suppression is not yet well-established.

Our laboratory is currently assessing the function of CD4$^+$CD25$^+$T cells using a system that allows for direct visualization of the effects of graft-specific CD4$^+$CD25$^+$T cells on the *in vivo* allograft response. To date, we have observed a contracted proliferative response of graft-specific cells *in vivo* in the presence of regulatory CD25$^+$T cells (manuscript in preparation). This finding correlates with the large amount of *in vitro* data showing that CD25$^+$T cells limit proliferation of responding cells. Because proliferation and effector function are linked (55), this

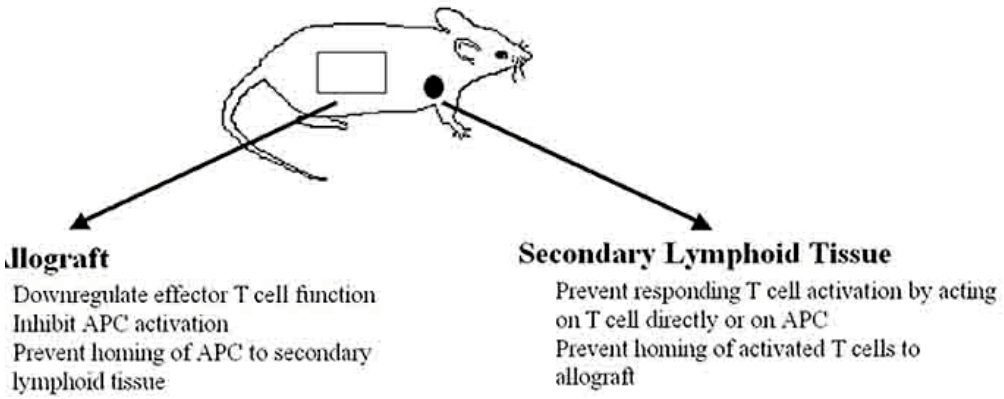

Figure 3. Site of suppression of the allograft response by $CD4^+CD25^+$T cells. Likely roles for $CD4^+CD25^+$T cells within the allograft and the secondary lymphoid tissue in inhibition of the allograft response.

aborted proliferative response may prevent T cells from acquiring effector function to reject the transplant. A further advantage of the system is that it allows visualization of the effects of antibodies on $CD25^+$T cell-mediated suppression. Using this strategy, we hope to clarify the involvement of particular molecules in $CD25^+$T cell-mediated regulation of the allograft response.

The site at which regulatory T cells act to prevent allograft rejection has been in part uncovered in recent studies. Clearly, the most likely sites at which regulatory T cells could inhibit the response of effector populations are within the graft itself (where regulatory cells could prevent the effector function of graft-destructive cells) and the secondary lymphoid tissue (where the regulatory population would prevent the activation of graft-reactive T cells) (Figure 3). Recent evidence indicates that $CD25^+$T cells that prolong survival of skin allografts are at least operative within the graft itself. These results do not exclude parallel function in relevant lymph nodes. To illustrate the presence of regulatory T cells within tolerated skin allografts, Graca, et. al. re-transplanted a tolerated B10.BR skin allograft to a second T cell-deficient CBA mouse (56). After a period of thirty days, the group re-transplanted the CBA mouse with fresh B10.BR skin and adoptively transferred $1x10^7$ CBA splenocytes, a dose which would otherwise reject the transplant. Prolonged survival of both the established and acute allografts was observed, leading to the conclusion that a tolerated skin graft is capable of transferring dominant tolerance. If T cells present in the "empty" CBA recipient (those migrating out of the tolerated allograft) were antibody-depleted, the transferred graft-reactive splenocyte population rejected the fresh allograft. This finding demonstrates that regulatory T cells emigrating from the tolerated graft were likely responsible for preventing allograft rejection. To formally demonstrate emigration of cells from an allograft, the group re-transplanted a tolerated B10.BR skin graft onto a T cell-deficient $CBA/RAG1^{-/-}$ recipient. Thirty days post-transplant, flow cytometry revealed numerous T cells in the periphery of the RAG animal, demonstrating that T cells present in the graft were capable of emigration. Taken together, these results provide strong evidence that regulatory T cells which promote allograft tolerance are at least in part located within the graft. It is important to note, however, that the protective regulatory cells in this population were not conclusively demonstrated to be $CD4^+CD25+$.

Further documentation from this group indicating the presence of regulatory T cells in allografts was provided by Zelenika, et. al., who used reverse transcriptase PCR to compare gene expression in tolerated, rejecting, and syngeneic tissues (57). This group found that gene transcripts characteristic of regulatory T cells were present in tolerated and syngeneic tissues, indirectly confirming that regulatory T cells may be present within tolerated graft tissue. More importantly, we can also infer that T cell suppression is active in maintaining tolerance to both allogeneic and syngeneic tissues, corroborating the hypothesis that mechanisms of self-tolerance and tolerance to alloantigen are similar.

10. CONCLUSIONS AND PERSPECTIVES

For over a decade, a role for regulatory T cell lineages in effecting tolerance to self and foreign antigens has been investigated. The inability to distinguish these regulatory cells from more abundant naïve cells was a hindrance to such studies and discredited the idea that specific T cell populations designated for a regulatory role existed and could be isolated. This concept re-emerged in early work showing that depletion of specific T cell groups could result in autoimmunity and was strengthened greatly with the discovery of the IL-2 receptor alpha chain (CD25) as an improved marker for identification of regulatory cells. From this point, intense investigation attempting to elucidate the origin, mechanism, and properties of regulatory $CD4^+CD25^+$T cells has transpired.

$CD4^+CD25^+$T cells have been established as a naturally-occurring, anergic, suppressive T cell population. While the anergic, suppressive state of these cells can be temporarily overridden by soluble mediators, it is the default state. The mechanism of suppression remains a matter of debate, but immunosuppressive molecules are likely involved. The balance of evidence suggests that

CTLA-4 and IL-10 are involved in the function of these cells, while the involvement of TGF-beta is less certain. It will be difficult to conclusively determine the role of CTLA-4, as activation or inhibition of CTLA-4 signaling affects both regulatory and effector T cells. IL-10 and TGF-beta seem to be excluded by the *in vitro* requirement for cell/cell contact in suppression, but these molecules may act to downregulate the inflammatory response to facilitate the cell/cell signal. This could explain the involvement of these molecules in suppression *in vivo* but not *in vitro*.

To mediate their effects, CD4$^+$CD25$^+$T cells likely act on both effector T cells and antigen presenting cells. To effect the most potent suppression, it is necessary to downregulate both the activation and effector function of naïve T cells. By downregulating costimulation and/or antigen presentation on APC, CD25$^+$T cells can prevent the activation of naïve cells. Furthermore, by delivering an inhibitory cell/cell signal to naïve cells, regulatory cells would be capable of acting in the absence of APC, as has been observed. The antigen presenting cell may also be involved in the cell/cell signal delivered by the regulatory population by bringing the regulatory and effector cells in close proximity to allow the inhibitory signal to be delivered. If CD25$^+$T cells act on both antigen presenting cells and effector cells, they will likely be operative both within the secondary lymphoid tissue (preventing T cell activation) and within the graft itself (preventing effector function). Localization of regulatory cells within the graft has been demonstrated, and our own data documenting inhibited proliferation of graft-specific cells within secondary lymphoid tissues suggest that CD25$^+$T cells are operative outside of the graft as well (manuscript in preparation).

Evidence suggests that both unexposed and antigen-experienced CD25$^+$T cells can suppress the allograft response, although the latter may be more capable. Graft-specific regulatory cells already exposed to antigen may be more effective in prolonging allograft survival if prior exposure expands and/or activates the population. In contrast, regulatory cells unexposed to graft antigen would be capable of suppression only after activation, expansion, and trafficking to the site of the graft.

These hypotheses have in some instances been opposed by studies in alternative systems. This could imply that the CD25$^+$T cell population is heterogeneous in nature, with different groups being distinct in their function. It is also possible that CD25$^+$T cells are capable of functioning in different ways depending on the conditions present. For instance, perhaps IL-10 and TGF-beta are secreted by these cells only in conditions of inflammation, when these molecules are needed to downregulate the inflammatory response. It is clear that many questions regarding the function of CD4$^+$CD25$^+$T cells remain. Moreover, a number of issues which are relevant to the involvement of CD25$^+$T cells in the allograft response remain that have been only marginally addressed. Below, we examine a few of these issues.

10.1. Can graft-specific CD4$^+$CD25$^+$T cells be isolated and/or created?

A major problem with the use of current immunosuppressive therapies is their non-specific impairment of the immune system. Because these agents globally inhibit the immune response, the host's ability to react to microbial or tumor antigens is also depressed. CD25$^+$T cells may also carry the potential to inhibit the immune response non-specifically. If CD25$^+$T cells downregulate the response to allografts, they may also simultaneously inhibit the host response to other non-self antigens. This feature would create the unwanted condition of general immunosuppression, increasing the likelihood of tumor or infection in treated individuals. A solution to this problem lies in the ability to create and isolate CD4$^+$CD25$^+$T cells that inhibit the immune response to the graft specifically. Above, we discussed isolation of graft-specific CD25$^+$T cells from animals undergoing treatments used to induce donor-specific unresponsiveness. However, antigen-specific CD25$^+$T cells have also been generated in systems without the use of antibody or immunosuppressive therapies.

To this point, antigen-specific CD4$^+$CD25$^+$T cells have been generated best by thymic introduction of antigen. This can be accomplished by either transgene expression or peptide inoculation. By mating a hemagglutinin (HA)-specific TcR transgenic mouse (the TS1 mouse) with a mouse expressing HA throughout its periphery (the HA28 mouse), Jordan, et. al. obtained an F1 with numerous self-specific T cells in its periphery (14). Interestingly, the frequency of CD4$^+$CD25$^+$T cells was greatly increased among cells expressing the HA-specific TcR. These cells were important in maintenance of self-tolerance, and have been shown within our laboratory to prolong survival of HA-expressing allografts (47). In a similar system, expression of HA under the immunoglobulin kappa promoter in HA-specific TcR transgenic mice was also shown to upregulate HA-specific CD25$^+$T cells (58). Finally, as detailed above, putative antigen-specific CD25$^+$T cells have been generated in our laboratory using intrathymic antigen injection. We have observed an increased proportion of CD4$^+$CD25$^+$T cells in the thymus of HA-specific TcR transgenic mice injected intrathymically with the immunodominant epitope of HA. These CD25$^+$T cells prolonged survival of HA-expressing allografts, suggesting that they are HA-specific. However, we did not test the ability of these cells to prevent rejection of third party allografts and therefore cannot rule out the possibility that these cells inhibit the immune response non-specifically. Nonetheless, we hypothesize that generation of antigen-specific regulatory cells can be accomplished through intrathymic injection.

10.2. Do CD25$^+$T cells mediate infectious tolerance and/or linked suppression?

Using a short course of anti-CD4 and anti-CD8 antibodies, Waldmann, et. al. have induced donor-specific unresponsiveness to minor and major MHC-mismatched allografts (59-62). This "dominant" tolerance was mediated by CD4$^+$ T cells, as co-transfer of a tolerized lymphocyte population failed to suppress the rejection response of naïve cells when CD4$^+$ cells were removed from the tolerant population (61). In addition to being "dominant," tolerance in this system was also found to be "infectious." Infectious tolerance refers to the capacity of

regulatory T cells to recruit naïve T cells to become regulatory (63). In this system, Chen, et. al. demonstrated infectious tolerance by using Thy-discordant mice to transfer and then remove tolerant cells from otherwise naïve hosts (61). They found that exposure of the naïve host to the tolerant population made naïve cells capable of maintaining long-term tolerance, demonstrating infectious tolerance. Furthermore, regulatory $CD4^+$ T cells arising in this tolerizing protocol were also found to mediate linked suppression (61). Davies, et. al. found that CBA mice made tolerant to a B10.BR heart allograft would also accept a (CBKxB10.BR)F1 skin graft, suggesting that tolerance to B10 antigens had spread to other foreign antigens present on the graft. Mice that had accepted CBKxB10 skin grafts also accepted a subsequent CBK skin allograft, indicating that the regulatory population can link suppression from one antigen set to another co-expressed on the same tissue.

The relation between the regulatory $CD4^+$ population in these studies and regulatory $CD25^+$T cells is currently unknown. However, it will be necessary to determine whether the $CD25^+$population also possesses these properties. Inherent in this question are more important issues regarding the use of $CD25^+$T cells in preventing allograft rejection. For instance, is it possible that $CD25^+$T cells could link suppression in an undesired fashion? If $CD25^+$T cells prevent rejection of an allograft, will they also effect tolerance to microbial or tumor antigens present within the same tissue? The capacity of regulatory cells to link suppression to microbial antigens has yet to be investigated. If $CD25^+$T cells can mediate linked suppression, it is reasonable to expect increased infection rates by pathogens known to localize to the transplanted tissue. The idea of $CD25^+$T cell-mediated infectious tolerance raises questions about the origin of this regulatory population. Is it possible that $CD25^+$T cells can induce naïve cells in the periphery to become regulatory? The aforementioned data indicating that expression of Foxp3 induces naïve cells to become regulatory may be relevant.

10.3. What is the impact of $CD4^+CD25^+$T cells on chronic allograft rejection and the memory response?

To date, studies on $CD4^+CD25^+$T cells in the allograft response have attempted to prolong survival of primary allografts. As discussed, $CD25^+$T cells have shown the potential to prolong primary allograft survival, making them an attractive commodity in transplantation tolerance. However, the principle quandary in modern transplantation is not the ability to extend primary transplant survival. Currently, organ transplantation is limited most by the process of chronic allograft rejection, an insidious course of allograft rejection occurring over an extended period of time.

Chronic allograft rejection (rev. in 65) involves fibrotic replacement of a transplanted organ occurring over months to years. It is characterized by a decrease in the caliber of arterial lumena in the graft, leading to ischemia. The precise immunologic mechanism of chronic rejection is not known, in part because animal models of the process have shown limitations in reproducing the disease. CD8+ T cells are known to be involved, largely in a cytolytic capacity. In addition, $CD4^+$ T cells are believed to have several different functions in the chronic allograft response. These include activation of cytolytic CD8+ T cells, activation of alloantibody-producing B cells, and activation of antigen-independent leukocytes aiding in graft destruction. The integral involvement of CD4 and CD8 T cells suggests that regulatory T cells may have some impact on the chronic rejection response, as they have been shown to inhibit proliferation of both populations. However, in part because animal models are lacking, the ability of regulatory T cells to suppress the chronic rejection response has yet to be investigated. Findings indicating that regulatory T cells can suppress this response would be promising, as current immunosuppressive therapies have been unable to prevent late graft loss.

The inability to prevent chronic allograft rejection often results in cessation of allograft function within the lifetime of the recipient, necessitating re-transplantation. Unfortunately, re-transplantation actualizes another obstacle in transplantation--immunologic memory (rev. in 66). Memory T cells persisting after prior antigenic exposure exhibit accelerated entry into the cell cycle, synthesis of cytokines, differentiation into CTL, and migration to non-lymphoid tissues. This creates an enhanced T cell response which may be less able to be suppressed. In studying the role of $CD4^+CD25^+$T cells in transplantation tolerance, it will be necessary to establish their impact on the memory response. Such studies have been limited thus far in part due to the inability to isolate antigen-specific $CD25^+$T cells. However, such populations have been characterized recently in separate studies, aiding our ability to study suppression of antigen-specific responses (14,58).

10.4. Summary

Since discovery of the CD25 molecule as an improved marker for immunoregulatory T cell populations, intense characterization of the traits and mechanism of $CD4^+CD25^+$T cells has transpired. Because these cells are known to be critical in self-tolerance, the majority of study of $CD4^+CD25^+$T cells continues in autoimmune animal models. These studies have greatly advanced our understanding of the function of this regulatory population, furthering our knowledge of their origin, basic characteristics, and mechanism. It is clear that this population represents a potentially valuable tool in controlling the immune response to particular antigens. As a result, the application of these regulatory cells in transplantation tolerance has begun to be investigated. Because immunosuppression by $CD25^+$T cells may be antigen-specific, this modality could be an upgrade over current immunosuppressive therapies that globally inhibit the immune response.

Despite the clear applicability of these cells in transplantation tolerance, a number of barriers must be surmounted for clinical application. The antigen to which $CD4^+CD25^+$T cells respond is typically difficult to determine, making isolation of graft-specific T cells difficult. While transgenic mouse offspring expressing

large numbers of antigen-specific CD25$^+$T cells exist (14,58), other methods of generating antigen-specific cells or determining the specificity of regulatory cells are lacking. In addition, the anergic phenotype of these cells makes them difficult to expand *in vivo* or *in vitro*. One protocol used to expand regulatory cells while retaining suppressive capacity has been documented (13,23), but the generalizability of this procedure is not fully established and development of other such protocols is lacking. This characteristic likely needs to be overcome in order to acquire large numbers of graft-specific regulatory cells. If large numbers can be acquired, it must also be conclusively proven that their use does not predispose the recipient to malignancy or opportunistic infection. In addition, the pathogenesis of chronic allograft rejection must be further understood in order to determine whether these cells will be effective in its prevention.

To summarize, CD4$^+$CD25$^+$T cells represent a potentially valuable tool in the quest to prolong survival of allografts. However, clinical use requires better understanding of their molecular mechanism, capacities, and limitations, and also necessitates a clearer understanding of all aspects of allograft rejection. We are hopeful that ongoing intense investigation of these cells will continue to clarify their function, and that parallel studies in allograft rejection will further our understanding of the obstacles to be surmounted in their use. In time, application of regulatory CD4$^+$CD25$^+$T cells in extending allograft survival may be a significant improvement over current immunosuppressive therapies.

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