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# Allopeptides and the Alloimmune Response[1]

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## Abstract

The inherent ability of the host immune system to distinguish between self- and non-self forms the basis of allorecognition. T lymphocytes constitute the most important effector arm of allorecognition. Here we describe the fundamentals of direct and indirect pathways by which allopeptides are presented to effector T cells. The nature of allopeptides presented along with tolerogenic strategies like altered peptide ligands and intra- or extra-thymic allopeptide inoculation are discussed. In addition, we speculate on the potential of regulatory T cells to modulate alloimmune responses.

### Keywords

allopeptides; alloimmune response; MHC antigens; rejection; tolerance; regulatory T cells

## Introduction

Allorecognition refers to the phenomenon by which the recipient immune system reacts with donor antigens that are considered to be “non-self”. T cells constitute the principal effector arm of allorecognition. In contrast, there exists a “tolerogenic” arm of regulatory T cells that suppresses the alloimmune response and facilitates tolerance. Nevertheless, the most common natural consequence following transplantation is allograft rejection. This suggests that the alloreactive T cells have a survival advantage following transplantation and are able to predominate. In this review, we discuss the nature of allopeptides recognized by T cells along with the different pathways of allorecognition. In addition, we speculate on the potential role of regulatory T cells in suppressing alloreactive T cells and achieving allograft tolerance.

## Cellular basis of allopeptide recognition

The main targets of the recipient immune response against the allograft are the donor major (MHC) histocompatibility antigens present on the allogeneic tissue. The recognition of mismatched donor histocompatibility antigens is the primary event that ultimately leads to allograft rejection (1–3). Allorecognition occurs through two unique but not mutually exclusive pathways: called direct and indirect pathways of antigen presentation. Direct pathway involves recognition of intact donor MHC molecules on the donor cells, usually the antigen presenting cells (APC). Both $CD8^+$ and $CD4^+$ T cells can directly recognize donor MHC class I and class II, respectively. The indirect pathway, in contrast, involves presentation of processed donor

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antigens by recipient APC to recipient T cells (Figure 1). Again, both $CD4^+$ and $CD8^+$ T cells can mediate indirect allorecognition.

## Direct Allorecognition

Direct pathway involves presentation of intact donor antigens to the recipient T cells. This may seem to contradict the classic self-MHC restriction property of T cells since the peptide being recognized is presented in a non-self MHC. Two models have been proposed to explain this discrepancy (2).

The first, called the “high determinant density” model, proposes that the direct alloreactive T cells recognize amino acid polymorphisms on the MHC molecules of the donor cells and the nature of peptide in the MHC groove is not important. Therefore, all the donor cells of any given MHC act as ligands for the direct alloreactive T cells, thereby creating a very high ligand density. Consequently, the affinity of the alloreactive T cell receptors required to generate an optimal alloimmune response can be significantly lower compared to that required for self-MHC peptide complex (4).

The “multiple binary complex” model proposes that the alloreactive T cells recognize specific peptides in the donor MHC grooves. These peptides are derived from the same normal cellular proteins that are present even in the recipient. However, the differences in the allo-MHC groove causes different set of peptides to be presented from homologous proteins. These peptides can be recognized by the recipient T cells. Therefore, even a single MHC mismatch between the donor and the recipient would be able to stimulate a large number of alloreactive T cells by providinga completely different set of peptides. It is now known that the TCR contact surfaces of many MHC alleles may be similar, thereby providing a degeneracy effect with regards to MHC-restriction and allowing the recipient T cells to cross-react with donor MHC. In another version of this model, any particular cell surface MHC protein is complexed with a naturally arising peptide from the intracellular proteolytic machinery, forming a heterogeneous population of binary complexes. Such a multitude of MHC-peptide complexes could be recognized by many different T cell clones in the recipient (5,6).

It is hypothesized that the allograft brings with it “passenger” APC that are able to stimulate recipient T cells directly. There have been several classical studies to support the concept of passenger APC. Lafferty *et al* demonstrated that cultured thyroid tissue has prolonged survival due to the loss of passenger APC (7). Another important set of experiments revealed that depleted passenger APC survive permanently in allogeneic recipients (8). Importantly, the recipients of the re-transplanted kidneys rapidly rejected the allografts when injected with donor APC (9). These reports also indicate that the allospecific T cells reactive through the direct pathway need to be primed by the donor passenger APC. If this is not achieved while the passenger APC are present, the direct alloreactive T cells cannot mediate rejection. More conclusive evidence of the direct pathway in allograft rejection came from studies from Pietra et al (10). They demonstrated that lymphocyte deficient, SCID or $RAG1^{-/-}$, mice when reconstituted with $CD4^+$ T cells rejected MHC class I but not MHC class II deficient cardiac allografts. Furthermore, $RAG1^{-/-}$ mice that were also MHC class II deficient rejected cardiac allografts when reconstituted with $CD4^+$ T cells. This indicated that $CD4^+$ T cells alone, directly activated by donor MHC class II bearing APC, could mediate rejection. Another interesting observation that emerges from the above studies is that the direct pathway may be of decreasing importance with time after transplantation as the passenger APC are lost.

## Indirect allorecognition

The indirect pathway of allorecognition is more representative of how the immune system typically recognizes an antigen. Here, the T cells recognize the donor antigens that have been

processed and presented in the context of self-MHC on the recipient APC. Using monoclonal antibodies directed against specific MHC-peptide complexes, it was demonstrated that MHC-derived peptides could be presented in the context of other (recipient) MHC molecules. One of the first such monoclonal antibodies developed was the Y-Ae (11,12). This antibody reacts to a peptide derived from the H2-Eα chain presented in the context of H2-$A^b$. This antibody brightly stained dendritic cells (DC) and B cells from murine strains co-expressing H2-$A^b$ and H2-E but not from those expressing either of these alone. When H2-E bearing DCs were injected into the H2-$A^b$ recipients, a significant proportion of recipient DCs in the draining lymph nodes became reactive with the Y-Ae antibody. These studies clearly show that MHC molecules can be processed and presented by self- or by allogeneic MHC.

Seminal studies done by Fangmann *et al* demonstrated that immunization with peptides corresponding to the MHC class I molecules could accelerate rejection of renal allografts in rats (13,14). Furthermore, $CD4^+$ T cells from recipient mice could specifically proliferate in presence of these peptides and recipient APC. Conclusive evidence for the indirect pathway of allorecognition in organ rejection came from the reports of Auchincloss et al (15). They demonstrated that MHC class I deficient mice could reject skin grafts from MHC class II deficient donor mice. The recipient mice in this model lack $CD8^+$ T cells capable of directly recognizing MHC class I of the donors. Furthermore, since the donor lacks MHC class II, direct allorecognition by recipient $CD4^+$ T cells is excluded. Therefore, the rejection of the skin allografts in this model is mediated by indirect allorecognition pathway, namely, recognition of processed donor MHC class I antigens in the context of self-MHC class II molecules present on recipient APC by the recipient $CD4^+$ T cells. Recent evidence also demonstrates that recipient DC can acquire and process intact donor MHC molecules from donor cell debris and stimulate $CD8^+$ T cells by cross-priming (16). Therefore, both $CD4^+$ and $CD8^+$ T cells mediate indirect allorecognition.

While the direct pathway is more important for acute allograft rejection, the indirect pathway is postulated to play a dominant role in chronic allograft rejection (17,18). This hypothesis originates from experiments demonstrating that inhibition of acute rejection by depleting passenger APC significantly delays but not prevent development of chronic rejection. The frequency of direct alloreactive T cells exceeds indirect alloreactive T cells especially in the early post-transplant period. Indeed, draining lymph node analysis demonstrated that more than 90% of allospecific T cells were of direct pathway while only 1–5% represented the indirect pathway of alloreactive T cells (18–20). However, the frequency of direct alloreactive T cells declines with time following transplantation while the continuous influx of the processed donor antigens by the recipient APC through the indirect pathways increases the number of indirect alloreactive T cells. It has also been shown that indirect alloreactive T cells are more resistant to conventional immunosuppression (21). Indeed, we and others, have demonstrated that indirect alloreactive T cells can be readily detected in the peripheral blood of human allograft recipients years after transplantation and are associated with allograft rejection (22–27).

In addition to the above two pathways, transfer of intact MHC molecules between cells has been observed (28–30). DC have been shown to acquire intact MHC class I and II molecules from exosomes secreted by other DC and prime both naïve $CD8^+$ and $CD4^+$ T cells (31–33). Reports from Knight and Lechler's group observed (28–30,34) proposed that this represents a third mode of allorecognition, which Lechler's group has termed "semi-direct" pathway (34). Briefly, through this pathway, DC could simultaneously present intact MHC molecules to directly alloreactive $CD8^+$ T cells as well as internalized and processed donor MHC peptides to indirect alloreactive $CD4^+$ T cells. Further studies are required to establish the clinical significance of this pathway in rejection of organ allografts.

## Role of MHC bound peptides in allorecognition

When considering the phenomenon of allorecognition two main questions emerge. First, are allopeptides truly required to elicit an alloimmune response? And secondly, what is the nature of such allopeptides? Interestingly, there is evidence that allorecognition can occur independent of MHC-bound peptides (35–37). Elliott *et al* denatured purified HLA-A2 protein, separated its heavy (α) and light (β-2 microglobulin) chains and then mixed them in the absence of any peptides. The reconstituted protein, deficient of any peptides, was indistinguishable from native A2 in its reactivity to a monoclonal antibody and ability to activate HLA-A2 specific $CD8^+$ T cells (35). Smith *et al* isolated H2-$K^b$ alloreactive $CD8^+$ T cells clones from skin allograft recipients and demonstrated that some of those clones could react with peptide deficient H2-$K^b$ molecules on T2 and RMA-S cell lines that have defective antigen processing and presentation (37). Furthermore, the reactivity of these $CD8^+$ T cells was not altered by either eluting or adding the alloreactive peptides. However, empty MHC molecules may not have an important role *in vivo* since they are rare and highly unstable under physiological conditions. But recognition of peptide carrying intact MHC molecules on donor APC by recipient T cells, regardless of the nature of peptide, does seem to exist and support the determinant-density hypothesis. In a more recent study, Jankovic *et al* developed an engineered variant of the murine MHC class I molecule H-$2K^b$, called $K^b$W9 (36). The variant was devoid of the central anchor pocket owing to a point mutation on the floor of the peptide binding site. This substitution drastically altered selection of bound peptides and therefore, the peptide repertoires of $K^b$ and $K^b$W9 were nonoverlapping. $K^b$W9 readily served as a restriction element for a peptide-specific syngeneic CTL response suggesting that the mutation did not result in gross distortions of the TCR-interacting surface of class I. When $K^b$W9 was used to stimulate allogeneic T cells, some of the CTL lines induced cross-reacted against the original $K^b$ molecule and demonstrated peptide-independent MHC reactivity.

The peptide-dependent arm of allorecognition plays a role in both the direct and indirect pathways. It also forms the basis for the multiple binary complex model of direct allorecognition. While $CD8^+$ T cells recognize MHC class I bound peptides, $CD4^+$ T cells react with MHC class II peptides. MHC class I molecules preferentially bind peptides from intracellular cytoplasmic and nuclear proteins while class II molecules present peptides from cell surface and extracellular proteins. Importantly both MHC class I and class II antigens can also be presented in the context of self- and non-self MHC molecules. MHC derived peptides are highly represented amongst the naturally processed peptides bound to class I and II molecules, making them accessible to direct alloreative T cells (3). In addition, host APC can process donor MHC molecules and activate alloreactive T cells through the indirect pathway. We and others have previously demonstrated that development of indirect alloreactive T cells specific to donor MHC class I as well as class II antigens correlates with human lung allograft rejection (25–27,38–40). Alloreactive T cells specific to mismatched donor HLA class I and class II were analyzed using limiting dilution or ELISPOT assay. As shown in Figure 2, patients with chronic human lung allograft rejction (bronchiolitis obliterans syndrome, BOS) were found to have a significantly higher frequency of alloreactive T cells (Figure 2). Moreover, the predominant alloreactive T cells were characterized to be of Th1-phenotype (27).

Alloreactive T cell can also recognize peptides from other (non-MHC) polymorphic loci such as HY-encoded proteins, known as minor histocompatibility antigens (MnHC). MnHC antigens are peptides derived from allelically polymorphic host proteins, other than MHC molecules. MnHC play an important role in the development of graft versus host disease after MHC-matched allogeneic bone marrow transplantation (41). MnHC have also been shown to mediate murine skin allograft (42,43) and rat cardiac allograft rejection in MHC-matched recipients (44–46). We have previously cloned mismatched MnHC antigen specific cytotoxic $CD8^+$ T cells from human renal allograft infiltrating cells at the time of acute rejection (47).

We further characterized the role of MnHC in chronic allograft rejection using two well characterized heterotopic murine cardiac (vascularized) and tracheal (non-vascularized) allo-transplantation models. C56BL/10SnJ ($H13^a$) cardiac allografts were transplanted into congenic B10.CE-$H13^b$ $A^w$(30NX)/Sn ($H13^b$) mice (48). The $H13^a$ and $H13^b$ alleles encode the SSVVGVWYL (SVL9) and SSVIGVWYL (SIL9) MnHC antigens bound to the $H2D^b$ molecule, respectively. $H13^a$ cardiac allografts transplanted into $H13^b$ recipients were rejected while isografts survived indefinitely. Pre-transplant sensitization with donor ($H13^a$) antigens accelerated the rejection process. Rejected cardiac allografts revealed histopathological signs of chronic rejection with diffuse mononuclear cell infiltration, concentric intimal hyperplasia, and fibrosis. While both $CD4^+$ and $CD8^+$ T cells were identified in the graft infiltrating cells, $CD8^+$ T cells revealed SVL9 specific cytotoxicity under $H2D^b$ restriction and, furthermore, specifically bound to $H2D^b$/SVL9 tetramers (Table 1). Murine heterotopic tracheal transplantation has been used as a model to investigate the pathogenesis of obliterative airway disease (OAD) following lung transplantation. $H13^a$ tracheal allografts were transplanted into congenic $H13^b$ recipients. The allografts were harvested at different times post transplantation and OAD lesions including epithelial damage, cellular infiltration, and luminal fibrosis were analyzed. In parallel experiments, mice were immunized (subcutaneous injection) or tolerized (intravenous injection) with the SVL9 or SIL9 peptide before transplantation. $H13^a$ tracheal allografts developed OAD in the $H13^b$ recipients within 90 days (Table 2). SVL9 immunization significantly accelerated the kinetics of OAD development. In contrast, tolerization using SVL9 completely abrogated the OAD development. This correlated with significant inhibition of $H13^a$-specific $CD8^+$ T cell cytotoxicity along with suppression of IFN-γ production (49).

Besides the histocompatibility antigens, studies have also demonstrated the role of self-antigens in alloimmunity (50–54). Immunity against the self-antigens can again develop through the direct or indirect pathway. Each MHC allele has a very extensive array of peptides that it can present. These peptides are derived from a variety of intra- and extra-cellular protein sources. The thymic selection eliminates a significant proportion of T cells that react to self-peptides. However, different MHC alleles would present different peptide sequences from homologous proteins. Hence, the host thymus would not eliminate T cells reactive to peptides from self-proteins that are presented in the context of donor MHC. Therefore, donor passenger APC may have the potential to induce an immune response against conserved self-proteins through the direct pathway. In addition, physiological stresses (like hypoxia and ischemia-reperfusion) can significantly alter the nature of peptides presented on the MHC surface and promote the expression of rare peptides against which central tolerance may not have been achieved (55,56). This also increases the probability of developing immunity against self-proteins. Further studies demonstrating direct alloimmune responses against peptides derived from conserved non-polymorphic proteins are required. Alternatively, the intense inflammation associated with transplantation may activate indirect auto-reactive T cells (discussed further in subsequent text). Autoreactive T cell responses to cardiac myosin (57), vimentin (58), collagen V (59–61), heat shock protein (62) have all been shown to contribute to allograft rejection. We analyzed the collagen type V (col-V) specific response longitudinally in human lung transplant patients. Col-V reactive $CD4^+$ T cells could be detected in the peripheral blood of lung transplant recipients. Importantly, the $CD4^+$ T recognized col-V through the indirect pathway. There was a predominance of IL-10 producing T cells ($T_{IL\text{-}10}$) reactive to col-V with significantly lower levels of IFN-γ and IL-2 producing T cells (Th1-cells). The col-V specific $T_{IL\text{-}10}$ cells suppressed the proliferation and expansion of col-V specific Th1-cells by IL-10 dependent but contact-independent pathways. Furthermore, during chronic lung allograft rejection there was a significant decline of $T_{IL\text{-}10}$ cells with concomitant expansion of col-V-specific IFN-γ producing Th1-cells. We have also obtained evidence for the role of *de novo* antibodies produced against non-MHC, tissue restricted antigens in the pathogenesis of chronic allograft rejection (38,63–66). For example, sequence analysis of a target antigen present on airway epithelial cells recognized by sera from patients with chronic

lung allograft rejection identified Kα1-tubulin (manuscript under preparation) as an immunogenic protein.

## Recognition of antigens by alloreactive T cells

The interactions between the T cell receptor and donor MHC during allorecognition are similar to the conventional recognition by self-MHC (67–75). The initial crystallographic studies were performed using the BM3.3 TCR and peptide pBMI bound to allogeneic class I H2-$K^b$ (68, 69,75). It was shown that the TCR interacted with MHC-peptide complexes in a conserved diagonal orientation across the long axis of the peptide-binding site. The interactions between the TCR and the MHC-peptide complex almost exclusively involved the β-chain CDR3. The complimentarity-determining regions CDR1 and CDR2 predominantly interacted with the α-helical portions of the MHC molecule and the contact with CDR3 segments lead to the exposure of the peptide. There seems to be degeneracy between the TCR and MHC-peptide complex interactions. This may be due to structural similarities between different MHC-peptide complexes or, alternatively, the TCR may exhibit conformational flexibility. Crystallographic studies prove that alloreactive T cells can demonstrate flexibility. For example, the 2C TCR can engage the same peptide presented in the context of self-H2$K^b$ or H2$K^{bm3}$ by rearranging the peptide contacting CDR3 portion of TCR. Therefore, CDR3 seems to play a crucial in the reorganization of the TCR. Interestingly, it was also demonstrated that water molecules fill the empty gaps between the TCR, MHC and the peptide providing a form of “padding”. The shape of this water padding also promoted the interactions of the TCR with different MHC-peptide complexes (68,69,75). Allorecognition gets even more complex as conformational determinants are applied to MHC-peptide complexes (76).

## Initiation of alloimmune response

The alloimmune response is postulated to be initiated in the lymphoid tissue of the recipient (77). Recipients that lacked secondary lymphoid organs were unable to reject cardiac allografts (78). However, others have challenged this view and one recent study by Zhou *et al* suggests that lymphoid organs may not be absolutely required for the development of alloimmunity (79). Another interesting study from Kreisel *et al* demonstrates that non-hemopoietic cells, vascular endothelial cells in their case, present on the allograft are sufficient to generate direct alloimmune responses and mediate allograft rejection (80).

Allorecognition, however, in the absence of an inflammatory milieu and co-stimulation may not be able to generate an alloimmune response. Following organ transplantation, activation of the recipient immune response is initiated by the surgical stress and ischemia-reperfusion injury. This is accompanied by the production of several chemokines and cytokines that leads to the recruitment of recipient immune cells into the allograft (81,82). Several inflammatory mediators including MCP-1, IP-10, and Th1-cytokines are induced following transplantation (81,82). These can upregulate costimulatory molecules such as CD80 and CD86, and cell adhesion molecules such as CD54 and CD58 on both donor and recipient cells including APC, epithelial, and endothelial cells (83–85). We have recently demonstrated that the early cytokine release plays a crucial role in the development of alloimmunity which may lead to allograft rejection (27). Interestingly, reports by Sayegh’s group have challenged the traditional concept of antigen presentation to T cells wherein both the MHC-peptide complex (signal 1) and co-stimulation (signal 2) are present on the same APC (86). They used B7-1/B7-2 knockout mice as donors for cardiac allografts for MHC class II knockout recipients. In this model system, the donor APC would present the MHC-peptide complex directly to the recipient T cells without any co-stimulation. In contrast, the recipient APC cannot indirectly present the donor peptides but can provide the costimulation. The cardiac allografts in these recipients were

rejected as rapidly as the wild-type recipients demonstrating that the antigen and co-stimulation necessarily need not be on the same APC.

## Tolerization strategies using allo-peptides

Allopeptides have been used to develop strategies to induce allograft tolerance. Some of these widely investigated include intra- or extra- thymic inoculation of the allopeptides and altered peptide ligands.

### Intrathymic inoculation of allopeptides

The thymus plays a central role in the development of self-tolerance. T cells with high reactivity to antigens presented in the thymus are eliminated. On this basis it was investigated whether injection of donor antigens in the thymus would eliminate the donor alloreactive T cells. Several investigators have shown that intrathymic administration of donor antigens in various forms, including donor spleen cells, soluble MHC molecules, or allo-MHC peptides can induce prolonged tolerance to allografts. Sayegh's group demonstrated that pre-transplant intrathymic administration of polymorphic peptides derived from the (Wistar- Furth) [WF]) RT1.Bu and RT1.Du class II MHC molecules prevented acute rejection and induced long-term graft survival in the WF-to–Lewis RT11 (LEW) kidney transplant model (87). They further demonstrated that the onset of acute rejection of cardiac allograft can be prolonged by intrathymic injection of unmodified donor splenocytes (88). Data from Hardy's laboratory has also provided compelling evidence about the efficacy of intrathymic inoculation strategy (89–94). They demonstrated that intrathymic inoculation of donor soluble Ag (obtained from resting donor T cells using alkali-extraction) seven days prior to transplantation combined with anti-lymphocytic serum lead to indefinite and donor-specific cardiac allograft tolerance in Lewis rat recipients. The peptide inoculation was also efficacious when combined with total body radiation. The tolerized recipients specifically and permanently accepted donor-type, second-set cardiac allografts. Interestingly, thymectomy performed 7 days after peptide inoculation led to graft rejection suggesting that the early phase of induction of donor-specific tolerance is dependent on the presence of donor alloantigens in the host thymus (89–94). Flye and colleagues also demonstrated that intrathymic injection of donor antigens induces tolerance to both allo- and xeno-transplantation (95–101). Furthermore, adoptive transfer of splenocytes isolated from the tolerized recipients confers tolerance (102). Other investigators have supported these findings (103). Shirwan's group reported that tolerization using intrathymic inoculation of donor antigens was mediated by an increased production of $CD4^+CD25^+$ regulatory T cells (104). The recipients that had received intrathymic injection of donor antigens were found to develop increase in $CD4^+CD25^+$ regulatory T cells ($T_{regs}$) within the lymphoid organs. These tolerized recipients also had significantly higher levels of IL-10. Further, $T_{regs}$ from the tolerized recipients were found to suppress donor–specific proliferative responses. Importantly, depletion of $T_{regs}$ from the tolerized recipients abrogated donor specific tolerance to cardiac allografts after intrathymic modulation. Other mechanisms that have been postulated to play a role in donor specific tolerance after intrathymic immunomodulation include clonal anergy, T cells deletion, and active suppression (105,106).

### Extra-thymic inoculation of allopeptides

HG Wells in 1911, made a remarkable observation that anaphylactic reactions to ovalbumin in guinea pigs could be inhibited by prior oral administration of OVA (107,108). Subsequent systemic immunizations failed to elicit a response to OVA. Furthermore, the unresponsiveness was OVA specific. Sayegh *et al* tested whether oral tolerance could be achieved in the context of allo-transplantation (109,110). They demonstrated that oral administration of MHC antigens in the form of donor splenocytes significantly inhibited the MLR reaction of unsensitized allo-recipients. Furthermore, this strategy prevented the accelerated rejection observed in cardiac

allograft recipients pre-sensitized with donor skin allografts (109,110). Kahan and Streptowski used an interesting strategy of inducing donor-specific tolerance by allochimeric MHC class I proteins bearing donor-type amino acid epitope substitutions for the host-type sequences. For example, they produced an allochimeric protein (α1H u70–77-RT1.Aa) by superimposing the nucleotides encoding last four (His70, Val73, Asn74, and Asn77) of nine (Arg62, Glu63, Gln65, Gly66, Gly69, His70, Val73, Asn74, and Asn77) polymorphic amino acids in the α1-helical region of donor-type RT1.Au onto the host RT1.Aa backbone using gene splicing and overlap extension. Intraportal injection of this allochimeric protein significantly prolonged the survival of Wistar-Furth (WF, RT1u) cardiac allografts in ACI (RT1a) rats (111,112). They have further used allochimeric proteins via the oral route to induce allo-specific tolerance (113). More recently, Wilkes' group reported that tolerance against fully-mismatched lung allografts could be achieved using a self-protein, collagen type V (col-V) (114). Administration of donor antigens has also been found to be effective using intragastric (115), intra-jejunal (116), transrectal, and intratracheal routes (107). The tolerance induced using such transmucosal routes has been shown to be T cell dependant as T cells, but not serum, from tolerized recipients could transfer tolerance. The regulatory T cells induced have been classified into TH3 and Tr1 that secrete TGF-β and IL-10, respectively. However, the success from using these strategies in humans has been limited due to the genetic diversity, complex tolerogenic mechanism, and differences in the pathogenesis of allograft rejection. Nevertheless, this phenomenon appears real and may have some clinical utility in the future (107).

### Altered peptide ligands

In general, a peptide has one MHC binding site and another that comes in contact with the TCR. In 1991, Paul Allen's laboratory reported a seminal observation that a cognate murine hemoglobin peptide with a TCR site-specific single amino acid alteration caused a reversible suppression of T cells (117). Subsequent studies from the same group provided compelling evidence that such altered peptide analogues (or ligands) could result in partial or complete T cells anergy (118,119). Altered peptide ligands contain alterations in the sites of the wild-type peptide that comes in contact with the TCR. Therefore, there is no difference in the MHC binding capacity of the peptide but the alterations significantly change the outcome of TCR recognition. APL can be classified as antagonistic or partial agonistic depending upon the response they elicit. Antagonistic APL fail to activate the T cells and rather suppress the T cells response when presented with the wild-type peptide. However, the T cell response is fully reversible after re-stimulation with the wild-type agonistic peptide in the absence of antagonistic APL. In contrast, partial agonistic APL partially activate T cells, resulting in induction of anergy.

Unlike the antagonistic peptides, T cells that have been stimulated with partial agonistic peptides do not proliferate on re-stimulation with the wild-type agonistic peptide. Suciu-Foca's group substituted multiple amino acids in the TCR binding site of wild type peptides to test the ability to generate APL (120). They examined whether analogues of the dominant determinant of HLA-DRpl*0101 molecule (peptide DR1/22–35), recognized in the context of HLA-DRj31*1101 protein, could modulate the T cell response against the wild-type peptide Ag. Using this strategy they were able to generate both antagonistic and partial agonistic APL that suppressed the T cell response to the wild-type agonistic peptide. The efficacy of APL in suppressing T cells responses has been well documented *in vitro* and in some autoimmune models in vivo (121). However, their efficacy in inducing transplantation tolerance *in vivo* needs to be further examined.

## A unified model of $T_{reg}$ mediated modulation of allorecognition

$CD4^+CD25^+$ regulatory T cells have been identified as a dominant subset of tolerogenic T cells (122). $T_{regs}$ are selected in the thymus in the context of self-peptides and predominantly

recognize cognate antigens through the indirect pathway. Since natural $T_{regs}$ are selected in the thymus in the context of "self-proteins", the natural presence of allo-specific $T_{regs}$ in naïve hosts is difficult to explain. Nevertheless, using animal models, allospecific $T_{regs}$ have been demonstrated in naïve recipients, although at low frequency. However, considering the diversity of TCR expressed by $T_{regs}$ cross-reactivity of $T_{regs}$ with alloantigens cannot be excluded. Other mechanisms that may be involved in the generation of allo-specific $T_{regs}$ following transplantation may include epitope-spreading from auto- to allo-antigens, TCR-affinity maturation or expansion of cross-reactive $T_{regs}$ (122). In addition, as shown in Figure 3, tolerogenic properties can be imparted by $T_{regs}$ into conventional T-cells (59,60,123,124). $T_{regs}$ impart tolerance by working through the indirect pathway and have been shown to contribute to indirect allospecific hyporesponsiveness, and more recently by us towards direct allospecific hyporesponsiveness using anti-donor MLR (59,60,122). In addition, autospecific Tregs were found to suppress anti-donor as well as mitogen induced proliferation of bystander host T cells thereby providing a powerful tolerogenic mechanism (59).

It has always been intriguing why, despite the presence of such a dominant subset of regulatory T cells, the most common natural consequence following transplantation is rejection. Multiple hypotheses can be postulated to explain the natural rejection of allografts in the absence of immunosuppression. Alloreactive T-cells may "overpower" $T_{regs}$ in the absence of immunosuppression probably due to rapid proliferation (125), as alloreactive $T_{regs}$ are present in low frequencies in the recipient compared to direct alloreactive T cells (126). Further, graft infiltrating alloreactive T-cells may induce apoptosis via CD95 in freshly recruited $T_{regs}$ (127). This would mediate acute allograft rejection. However contemporary immunosuppression is highly effective on the directly alloreactive T cells and therefore has significantly reduced the incidence of acute rejection. It is interesting that immunosuppression has had a much less impact on chronic allograft rejection. It is noteworthy here that indirect alloreactive T cells are more resistant to immunosuppression. In fact, as previously discussed, we have demonstrated that indirect alloreactive T cells can be detected in human lung allograft recipients years after transplantation and correlate with the development of chronic lung allograft rejection (Figure 2). At the same time there is a constant influx of new $T_{regs}$ in the recipients. However, $T_{regs}$ are more sensitive to contemporary immunosuppression, specially the calcineurin inhibitors like cyclosporine and tacrolimus, since the function and survival of $T_{regs}$ is dependent on interleukin-2 (128). Calcineurin inhibitors not only inhibit the development of long-term allograft tolerance (129) but can actually induce autoimmunity (130). Demirkiran *et al* (131) and Ciancio *et al* (132) demonstrated a decline in Tregs with calcineurin inhibitors in human liver and renal allograft recipients, respectively. Furthermore, we postulate that there may be additional mechanisms that favor the survival of effector T cells over $T_{regs}$.

Using a unique orthotopic tracheal transplant model for investigating the pathogenesis of BOS, we found that immediately following transplantation there was a significant loss of $T_{regs}$ in the draining lymph nodes due to apoptosis, most likely induced by proliferating alloreactive T cells. However, as the recipient epithelium repopulated the tracheal allograft, there was a loss of allogeneic stimuli and restoration in the frequency of $T_{regs}$. Interestingly, respiratory viral infections were found to upregulate death receptors on airway epithelial cells *in vitro* that induced rapid apoptosis of $T_{regs}$ (Bharat A et al, manuscript under preparation). It is noteworthy that respiratory viral infections predispose to chronic human lung allogaft rejection (133). Therefore, there are events that can lead to $T_{reg}$ cell death and favor proliferation of alloreactive T cells, thereby predisposing to chronic allograft rejection. Indeed, we recently found that patients with chronic human lung allograft rejection developed a significant loss of $T_{regs}$ that was associated with concomitant expansion of both indirect alloreactive, and autoreactive, Th1-cells (59). Chronic allograft rejection may therefore be the "function of predominance" between (indirect) alloreactive effector T cells and $T_{regs}$ (Figure 4).

## Pathogenesis of chronic human lung allograft rejection (BOS)

We have summarized these results into a unifying hypothesis towards the immunopathogenesis of chronic rejection, ie, BOS following human lung transplantation. Following transplantation, there is an inflammatory milieu within the allograft that promotes allo-antigen presentation through both direct and indirect pathways. In addition, inflammation also leads to the release of sequestered, but immunogenic, self-antigens. Furthermore, there is epitope spreading from auto- to allo- antigens combined with activation of autoreactive "low-affinity" T cells that escaped the thymic deletion. Activation of both allo- and auto- reactive T cells promotes allograft rejection. However, inflammation also leads to the recruitment of $T_{regs}$ that are suppressive in nature (Figure 3). Autospecific $T_{regs}$ can inhibit both autoreactive as well as alloreactive effector T cells. In a naïve host, the combined frequency of allo- and auto-reactive T cells far exceeds $T_{regs}$ and therefore, the most common natural consequence following transplantation is rejection. Immunosuppression depletes both T cells and $T_{regs}$ maintaining the allograft on neutral grounds between rejection and tolerance. Following transplantation there is influx of new indirect alloreactive T cells as the recipient APC process and present the allograft antigens. Simulataneously, new $T_{regs}$ originate from the central (thymic) and peripheral mechanisms. Modulating the factors that favor $T_{reg}$ survival would promote tolerance (Figure 4). However, if the effector T cells predominate they activate other arms of the immune system and initiate a cascade of cytokines and growth factors that ultimately lead to the development of chronic (lung) allograft rejection (Figure 5).

## Summary

Allorecognition leads to the generation of an extremely powerful anti-donor immune response that ultimately leads to rejection of the transplanted organ. The peptides recognized by the alloreactive T cells include the polymorphic major and minor histocompatibility antigens as well as conserved self-antigens. Direct and indirect pathways form the fundamental basis of allorecognition but are not mutually exclusive. Ongoing research to further characterize the complexities within the pathways of allorecognition and the interactions between autoreactive T cells, alloreactive T cells and $T_{regs}$ is imperative to promote long term success following transplantation.

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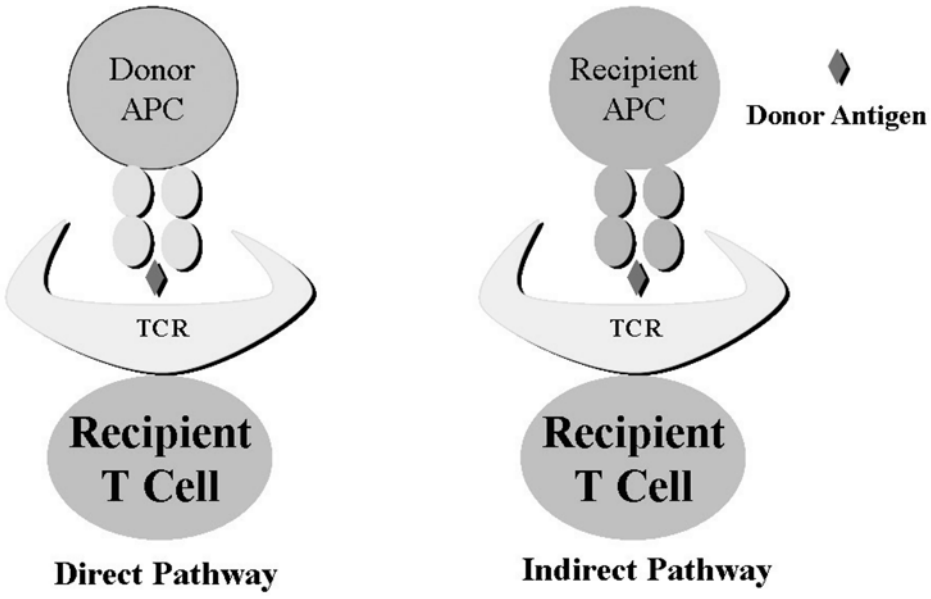


**Figure 1.**
Direct and Indirect Allorecognition

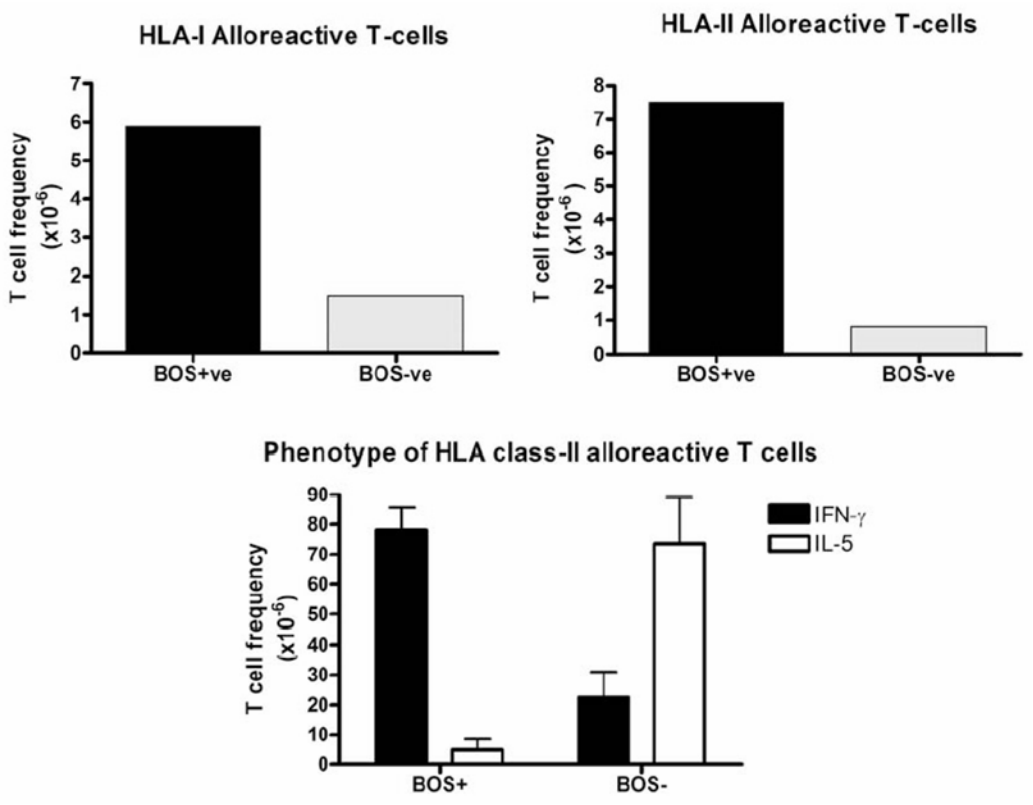


**Figure 2.**
Chronic lung allograft rejection is association with an expansion of Th1-predominant mismatched donor HLA class I and II allospecific T cells

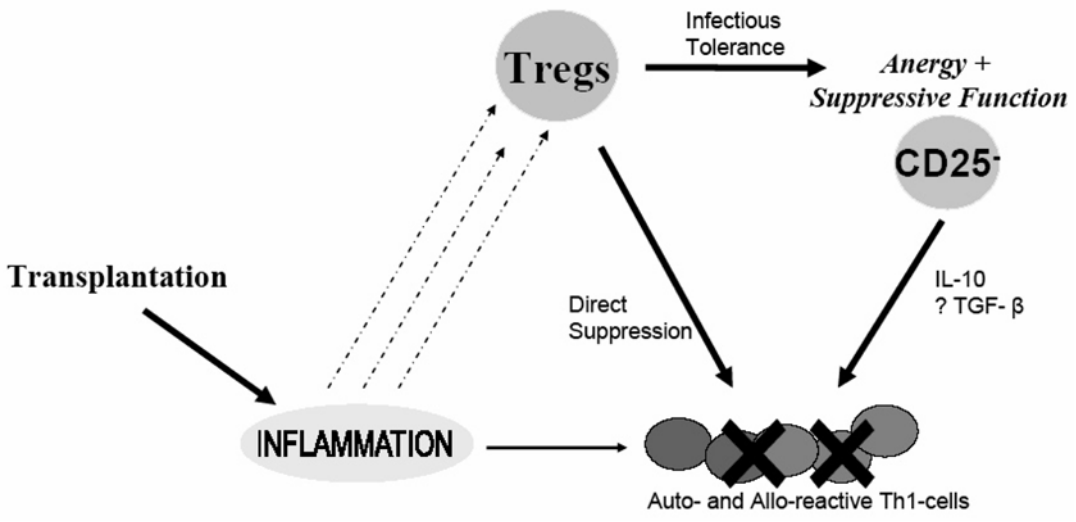


**Figure 3.**
Regulation of alloimmunity by $T_{regs}$ following transplantation

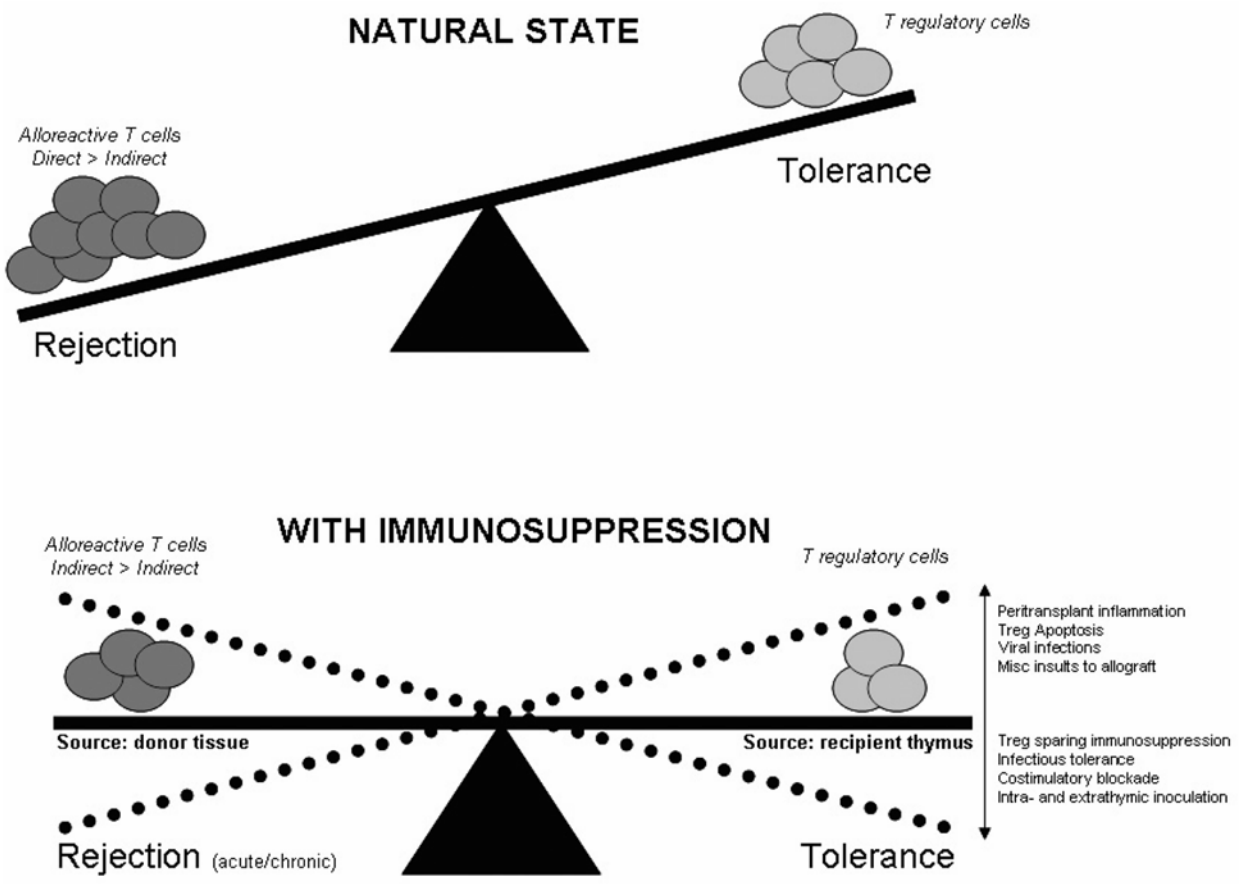


**Figure 4. Balance between Alloreactive T cells and Tregs in transplant outcome**

In the natural state, without any immunosuppression, there is a significantly greater frequency of directly alloreactive T cells that overcome the tolerogenic potential of Tregs and lead to allograft rejection. Administration of immunosuppression eliminates a significantly proportion of directly alloreactive T cells as well as Tregs. Nevertheless, there is influx of donor alloantigens through the indirect pathway that maintains alloreactive T cells. Since indirectly alloreactive T cells are more resistant to immunosuppression, the long-term sequelae of this influx is chronic rejection. Some important factors that can favor Tregs or alloreactive T cells following transplantation are represented.

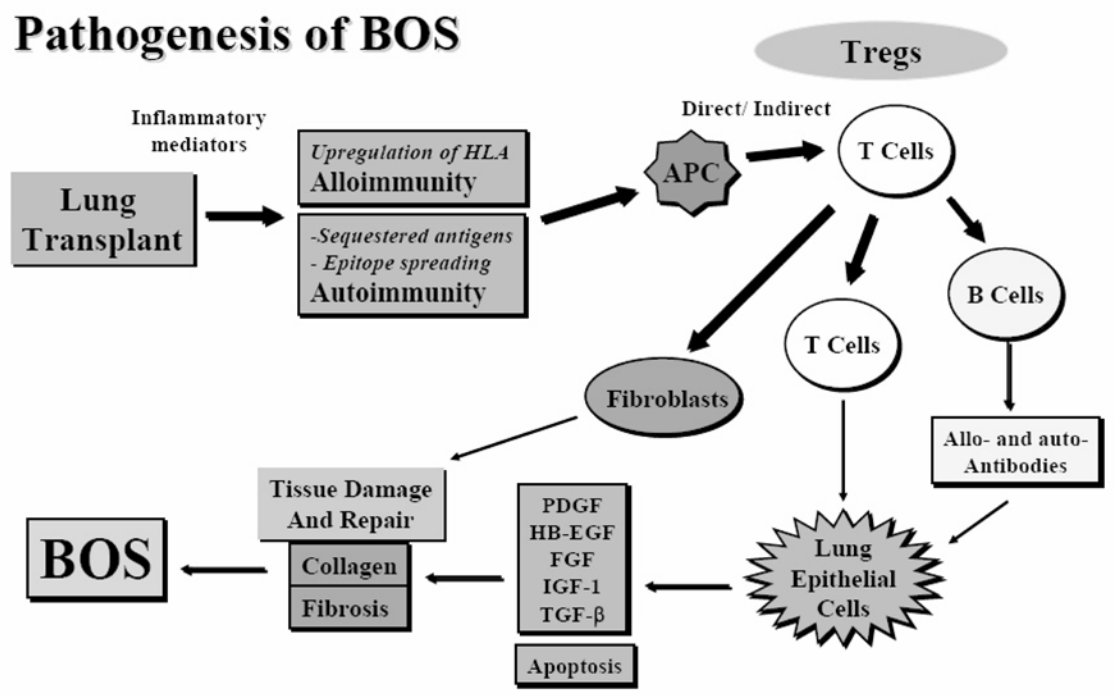


**Figure 5.**
Pathogenesis of BOS

**Table 1**

Development of H13[a] cardiac allograft rejection in H13[b] recipients

| Group | Donor | Recipient | H13[a] immunization | Mean graft survival (days ± SD) |
|---|---|---|---|---|
| 1 | H13[b] | H13[b] | − | >100 |
| 2 | H13[a] | H13[b] | − | 37.0 ± 14.5 |
| 3 | H13[a] | H13[b] | + | 27.6 ± 15.9 |

**Table 2**

Development of H13[a] tracheal allograft rejection in H13[b] recipients

| Donor | Recipient | Time post-Tx (days) | Treatment | OAD Lesions (% of Allografts) | | |
|---|---|---|---|---|---|---|
| | | | | Epithelial Damage | Cellular Infiltration | Lumen Occlusion |
| H13[a] | H13[b] | 30 | - | 25 | 25 | 0 |
| H13[a] | H13[b] | 60 | - | 100 | 100 | 0 |
| H13[a] | H13[b] | 90 | - | 100 | 100 | 100 |
| H13[b] | H13[b] | 30 | - | 0 | 0 | 0 |
| H13[b] | H13[b] | 60 | - | 0 | 0 | 0 |
| H13[b] | H13[b] | 90 | - | 0 | 0 | 0 |
| ***Pre-transplant sensitization (subcutaneous injection of peptide)*** | | | | | | |
| H13[a] | H13[b] | 30 | SVL9 | 100 | 100 | 50 |
| H13[a] | H13[b] | 60 | SVL9 | 100 | 100 | 75 |
| H13[a] | H13[b] | 90 | SVL9 | 100 | 100 | 100 |
| H13[a] | H13[b] | 30 | SIL9 | 25 | 25 | 0 |
| H13[a] | H13[b] | 60 | SIL9 | 100 | 100 | 0 |
| H13[a] | H13[b] | 90 | SIL9 | 100 | 100 | 100 |
| ***Pre-transplant tolerization (intravenous injection of peptide)*** | | | | | | |
| H13[a] | H13[b] | 90 | SVL9 | 0 | 0 | 0 |
| H13[a] | H13[b] | 90 | SIL9 | 100 | 100 | 100 |
| BALB/c | H13[b] | 30 | SVL9 | 100 | 100 | 100 |