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Oral administration of alloantigen prolongs kidney allograft survival by generating intragraft regulatory cells

by

Juan Zhou

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Oral administration of alloantigen prolongs kidney allograft survival by generating intragraft regulatory cells" by Juan Zhou in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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External Examiner:

Research Supervisor:

Examining Committee:

Dalhousie University
Date: May 15, 2001
Author: Juan Zhou
Title: Oral administration of alloantigen prolongs kidney allograft survival by generating intragraft regulatory cells
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dedication

To my mother and father

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Abstract

A major goal of transplantation research is to develop a specific immune unresponsiveness to alloantigen. Oral administration of antigen prior to systemic challenge has been demonstrated to suppress systemic immune responses, and this antigen specific suppression is termed oral tolerance. This thesis assesses whether oral tolerance can be used to prolong kidney allograft survival and investigates the mechanisms by which this survival is prolonged.

It was found that oral administration of allogeneic splenocytes prior to kidney allograft transplantation dramatically prolonged allograft survival in rats in an allospecific manner. Preservation of structural elements could be observed in kidneys transplanted into fed animals long after control transplants were destroyed. In mixed lymphocytes reaction experiments, splenocytes from fed and kidney transplanted animals exhibited increased, rather than decreased cell proliferation comparing to unfed control, confirming that prolongation of the kidney allografts was not due to a masking of allorecognition but to immunomodulation of the immune response.

To assess the immune response in the kidney allograft, graft infiltrating cells (GIC) were isolated day-5 post transplant and extensively studied. It was found that decreased numbers of GIC were present in the allografts taken from fed animals compared to unfed controls. The decreased number of GIC is mirrored by a decreased number of T cells, especially CD4+ T cells, in the GIC population. The number of CD8+ GIC T cells was similar in both fed animals and controls. To investigate whether CD8+ T cells were allo-active CTL, expression of killer mediators and CTL activity of CD8+ GIC were examined. These CD8+ GIC from fed animals transcribed substantial levels of perforin, granzyme and FasL mRNA and exhibited higher allo-CTL activity than CD8+ GIC from control unfed recipients, suggesting the presence of mature allo-CTL in the graft in fed animals. CD8+ GIC from fed animals also exhibited high levels of IL-4 mRNA, suggesting a Tc2 type regulatory cells. These data are consistent with a hypothesis that CD8+ GIC are regulatory cells that mediate oral transplantation tolerance. To confirm this, adoptive transfer experiments were performed. Prolongation of graft survival could be transferred from rats orally exposed to alloantigen, to naive animals by transfer of CD8+ GIC. These data confirm that intragraft CD8+ T cells are indeed regulatory cells that mediate oral transplantation tolerance. In addition to GIC, it was also demonstrated that regulatory cells are present in spleen and MLN by adoptive transfer of cells from spleen and MLN.

These studies suggest that oral exposure to alloantigen induce the generation of intragraft CD8+ regulatory cells which prolong allograft survival by shifting immune responses toward to type 2 responses and/or by deleting alloreactive T lymphocytes through Fas/FasL interaction.

List of abbreviations

ACK	ammonium chloride potassium
ADCC	antibody-dependent cell-mediated cytotoxicity
AICD	activation induced cell death
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
BN	Brown Norway;
CD	cluster determinant
cDNA	complementary deoxyribonucleic acid
Con A	Concanavalin A
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveitis
EDTA	ethylenediamine tetra-acetic acid
FACS	fluorescence-activated cell sorter
Fas	Fas receptor
FasL	Fas ligand
FBS	fetal bovine serum
GALT	gut associated lymphoid tissue
GI	gastrointestinal
GIC	graft infiltrating cells
GIL	graft infiltrating leukocytes
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2ethanesulfonic acid
HIV	Human Immunodeficiency Virus
ICAM-1	intercellular adhesion molecule
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocytes
IFN-	interferon-gamma
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
KO	knock out
LFA-1	lymphocyte function in adhesion-1
LP	lamina propria
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBP	myelin basic protein
MHC	major histocompatibility complex
MLN	mesenteric lymph node

MLR	mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
Nb	*Nippostrongylus brasiliensis*
OVA	ovalbumin
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PP	Peyer's patches
pv	portal venous
RBC	red blood cells
rIL	recombinant interleukin
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SEA	staphylococcal enterotoxin A
Tc	T cytotoxic cell
TCR	T cell receptor
Th	T helper cell
TNF	tumor necrosis factor
Tx	transplantation
Ts	T suppressor cells

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1.0 INTRODUCTION

Organ transplantation is the most effective therapy for organ failure and has been widely applied in clinical medicine. However, transplants are recognized as foreign by the recipient immune system and are rejected a short time after transplantation. To prevent graft rejection, a variety of immunosuppressive drugs, such as cyclosporin, rapamycin and FK-506, have been developed and are in wide use in clinical transplantation. Use of such drugs significantly increases 1-year graft survival rates. However, these immunosuppressive drugs non-specifically inhibit cell-mediated immunity, resulting in very significant side effects, such as increased opportunistic infections and malignancy (Barry 1992, Shapiro et al 1990, Kusne et al 1991). In addition, the drugs exhibit significant organ cytotoxicity, most commonly nephrotoxicity (Platz et al 1994, Barry 1992). Further, the available drugs do not prevent late rejection events such that 10 year graft survival rates have not improved. Novel adjuvant therapies are, therefore, required to induce immune specific unresponsiveness to tissue antigens so that the transplanted organ is protected from rejection but the recipient is not overly immune compromized.

This specific unresponsiveness is commonly seen as the "Holy Grail" of transplantation immunology. In this thesis a strategy is explored to obtain this transplant specific tolerance. The strategy is tested in the first chapter and a mechanism is investigated in the second chapter.

Immune responses are designed to recognize and eliminate foreign but not self-antigens. However, the immune system exhibits tight regulation of the response to soluble protein antigens that gain access through the intestinal tract. The manner in which these food antigens are processed in the gastrointestinal tract appears distinct from the process

elsewhere in the body. Such antigens generally do not elicit systemic protective immune responses. It appears that a local humoral response precedes a systemic "unresponsiveness". This unresponsiveness is antigen specific and has been termed oral tolerance. Oral tolerance is an important mechanism of immune regulation that prevents inappropriate immune responses targeted toward non-harmful extrinsic antigens that are ingested through the intestinal system (reviewed Mowat 1987). Because of the specific nature of oral tolerance, researchers have investigated the effects of oral tolerance on ablating immune responses to autoantigens that induce autoimmune disease. For example, experimental autoimmune encephalomyelitis (EAE) is an animal model of the human autoimmune disease multiple sclerosis (MS). MS is a disease in which the myelin sheath of nerves is attacked by immune effector cells. EAE can be induced by injection of myelin basic protein (MBP), which activates a cell-mediated immune response against the myelin sheath. Oral administration of MBP prior to injection of MBP prevents this induction of EAE (Weiner et al 1994). The protection provided against autoimmunity by oral administration of the autoantigen (Miller et al 1991, Yoshino 1998) or autoantigen peptides (Gregerson et al 1993, Miller et al 1993) prior to systemic immunization has been reported in other experimental models, such as experimental autoimmune uveitis (Singh et al 1992, Wildner et al 1996), collagen-induced arthritis (Yoshino 1998, Thorbecke et al 1998) and pristane-induced arthritis (Thompson et al 1993). Because of the positive results obtained with oral tolerance in ameliorating autoimmune disease in pre-clinical animal models, clinical trials to assess the feasibility of oral tolerance in the reduction of human autoimmune disease have been initiated (Fukaura et al 1996, Weiner 1997, Nussenblatt et al 1997).

Since oral administration of antigen can induce specific immune suppression and the

clinical goal of transplantation tolerance research is to induce such specific immune unresponsiveness (towards allogeneic antigens), it is plausible to postulate that oral tolerance could be used as an adjuvant therapy to induce transplantation tolerance. Although the effect of oral tolerance has been extensively studied in the suppression of autoimmune disease, the effect of oral tolerance on primary organ allograft rejection has not been established. Further, the mechanisms responsible for oral tolerance are unclear. Some workers (Sayegh et al 1992a, b) have reported that feeding allopeptides or allogeneic cells resulted in a decreased recognition of alloantigen as measured by decreased mixed lymphocyte reaction (MLR) in vitro. In addition, decreased delayed type hypersensitivity (DTH) responses to alloantigen in vivo have been demonstrated (Sayegh et al 1992a, b). Weiner and colleagues reported that oral administration of allogeneic splenocytes prevented sensitisation by skin grafts and transformed accelerated (second set) rejection of cardiac allografts to an acute (primary) response, typical of un-sensitized recipients (Hancock et al 1993, Sayegh et al 1992b). These findings suggest that oral tolerance may modulate allograft rejection, but do not determine whether oral tolerance could be used to prevent primary organ graft rejection. Nor do they provide convincing evidence as to the mechanisms by which oral tolerance may affect the perception of, and response to, alloantigens.

In this thesis, the effect of oral tolerance on kidney transplant survival is investigated. The kidney was chosen as a model organ for a number of reasons. First, kidney transplantation is the best long-term option to remedy kidney failure and, as such, this model is directly relevant to clinical medicine. Second, kidney transplants can be performed in such a manner as to be normally perfused with blood and be able to

function in a normal physiological manner (as opposed to animal models of skin or heart transplantation). Third, the transplanted kidneys can be removed from the animals and graft-infiltrating cells can be collected from it. It is the local response in the allograft which is the focus of this research and thus the availability of graft infiltrating cells is essential. In this thesis research, the influence of oral tolerance on kidney graft survival is examined as well as the mechanisms by which this survival is influenced.

2.0 BACKGROUND

2.1 Mechanisms of Allograft Rejection

Grafts transplanted into genetically different individuals are recognized as foreign, immune responses against the grafts are induced and organ failure results. Depending on the genetic difference between the donor and recipient, grafts can be classified as syngeneic, allogeneic or xenogeneic. Organs transplanted between genetically identical or disparate individuals of the same species are termed syngrafts or allografts, respectively. Syngeneic grafts are normally only used in pre-clinical animal models but grafts between human identical twins, which have extremely high long term success rates, are also classed as syngeneic grafts. Organs transplanted between different species are termed xenografts (Auchincloss et al 1999).

Although humoral responses have been reported to be associated with allograft rejection (Hancock et al 1998, Leprince et al 1999), cell mediated immune responses, including DTH and CTL responses, are currently thought to be the predominant mechanisms involved in acute allograft rejection (Hall 1991). This rejection is initiated by T cell recognition of alloantigen, expansion of the alloreactive T cell population and infiltration into the grafts of effector T cells and macrophages.

The recognition of alloantigen occurs by two distinct pathways: an indirect and a direct pathway. Generally, recognition of foreign antigen by a CD4+ T cell requires that peptides derived from the antigen be displayed in the cleft of a self class-II major histocompatibility complex (MHC) molecule expressed on an antigen presenting cells (APC). This recognition has long been described as self-MHC restricted. In this classical form of T cell activation, T cells are able to recognize foreign peptides, derived

from the processing of infective particles or allogeneic molecules, expressed within the groove of self-MHC class II (Gould and Auchincloss 1999). This manner of allorecognition and activation is termed indirect recognition. However, in transplantation, CD4+ and CD8+ T cells can both recognize alloantigen directly (foreign MHC expressed by donor cells) without the requirement of processing and presentation by self-APC (Auchincloss and Sultan 1996). This manner of allorecognition and activation is termed direct allorecognition.

Both direct and indirect recognition have been suggested to play major roles in allograft rejection. For example, depleting donor APC from kidney allografts protects the allografts from rejection, whereas injection of donor APC back into the grafts restores acute rejection (Lechler and Batchelor 1982). These data would suggest that acute allograft rejection is dependent on donor APC, which may be recognized by recipient T cells in a direct pathway. In contrast, in another experimental model, APC depleted donor cardiac allografts were rejected in 2-3 weeks (Yin and Fathman 1995), suggesting that donor APC are not necessarily required for allograft rejection, and indirect recognition of alloantigen in the absence of donor APC is sufficient to initiate acute rejection. However, when compared to control groups, where allografts were rejected in about 7-9 days, the APC depleted grafts exhibited a delayed rejection. These data suggest that the rejection induced by indirect recognition may be weaker and/or later than that induced by direct recognition of alloantigen. Therefore, both direct and indirect recognition are likely involved in graft rejection but at different times and perhaps with different efficiencies. Direct recognition may be more effective than indirect recognition in initiating early immune responses against alloantigens. Indirect recognition may play

a greater role in later graft rejection events, after most donor APC are eliminated. Several experiments (Benichou et al 1996, Vella et al 1997, 1999) support this hypothesis. The delayed rejection of allografts in animals where direct recognition has been substantially reduced may be due to the time required for antigen uptake, processing and presentation by self-APC. Moreover, the frequency of T cells directly recognizing allogeneic MHC has been suggested to be 100-fold higher than that of T cells recognizing specific antigen peptides in the context of self-MHC (Liu et al 1993).

In the direct pathway, after revascularization of the allograft, donor APC, mainly dendritic cells, migrate from the graft to the spleen (Austyn and Larsen 1990), where host T cells are activated by recognition of class I and class II MHC molecules expressed on the donor APC. Following T cell activation, a variety of effector mechanisms participates in allograft rejection (Hall 1991). Although both CD4+ and CD8+ T cells are activated during allograft rejection (Austyn and Larsen 1990, Hall 1991), the relative importance of the two subsets of T cells in the effector arm of allograft rejection remains controversial. Many experiments have shown that prolonged allograft survival occurs in CD4-depleted (Newell et al 1997, Morton et al 1993, Bushell et al 1995) or CD4-knockout animals (Krieger et al 1996, Hall 1991). For example, CD4+ T cells were found to be the predominant T cells infiltrating grafts early after cardiac allograft transplantation (Bishop et al 1992). Depletion of CD4+ T cells from recipient mice prior to transplantation resulted in prolongation of allograft survival. In addition, skin and heart allografts have been demonstrated to be permanently accepted in CD4 knockout mice, even though CD8+ T cells were present, suggesting that CD4+, but not CD8+, T cells are required for allograft rejection (Krieger et al 1996). Furthermore, reconstitution of CD4-KO mice with naïve CD4+ T cells prior to

engraftment restored allograft rejection, confirming that CD4+ T cells are essential for allograft rejection.

Since CD4+ T cells are critical for CD8+ T effector cell generation, the influence of CD4+ T cells on graft rejection may be to provide help for generating CD8+ CTL. CTL generation requires cytokines produced by CD4+ T cells. It has been shown, for example, that depletion of CD4+ T cells from allograft recipients eliminated CD8+ T cell infiltration of the graft (Bishop et al 1992). In addition, injection of rIL-2 into recipients depleted of CD4+ T cells restored CD8+ T cell infiltration (Bishop et al 1993). However, this treatment did not restore graft rejection (Bishop et al 1993). These data, and the evidence that depletion of CD8+ T cells (or use of CD8 KO animals) did not protect allografts from acute rejection (Bishop et al 1993, Gracie et al 1990, Bradley et al 1992, Mannon et al 1995) suggest that mechanisms other than CD8+ CTL are involved in acute rejection of solid organs. An alternate hypothesis is that CD8+ T cells and CD4+ T cells co-operate in graft rejection and that the dependence of CD8+ CTL generation and function on CD4+ T cell activity leads to a dependence on CD4+ T cell presence for graft rejection to occur. Clearly, graft rejection can occur in the absence of CD8+ T cells but this does not rule out a role for CD8+ CTL under normal condition nor does it rule out a role for CTL even in the absence of CD8+ T cells. Alloreactive CD4+ CTL (Williams and Engelhardt 1997, Yi et al 1999) and CD4-CD8- CTL (Dalloul et al 1996, Schilham et al 1993) have been shown to be generated in the absence of CD8+ T cells and these CTL may mediate allograft rejection.

Support for a role of CD8+ T cell involvement in graft rejection in fully immuno-competent animals comes from the adoptive transfer experiments of Prowse and co-

workers (1983) where CD8+ T cells derived from animals previously sensitized with alloantigen, induced allograft rejection when transferred into naïve animals. In addition, CD8+ T cells have been implicated in the rejection of skin allografts in the absence of CD4+ T cells (Rosenberg et al 1986). Further, in contrast to other work, He and colleagues (1999) have recently demonstrated that depletion of CD8+ T cells by mAb prolonged intestinal allograft survival in rats.

2.2 Immunomodulation

2.2.1 Type 1 and type 2 responses

a) Concept of Th1/Th2 paradigm

Using a panel of antigen specific murine CD4+ T cell clones, Mosmann and co-workers (1986) observed that CD4+ T cells could be subdivided into two distinctive cell types according to their patterns of cytokine production. The clones secreting IL-2 and IFN-γ were termed Th1 cells, while the clones secreting IL-4 were termed Th2 cells. This distinctive pattern of cytokine production suggested to Mosmann and colleagues that two functionally different subsets of T cells may exist in vivo and that these may exhibit different effector functions. Subsequent work (Romagnani 1997, Constant and Bottomly 1997) showed that Th1 cells also produce TNF, and that Th2 cells also produce IL-5, IL-10, and IL-13.

Different functional roles for Th1 and Th2 cells were first illustrated by examining the help provided for B cell activity. In the presence of a supernatant obtained from Th2 cell cultures, LPS-stimulated murine B cells showed enhanced Ig-E and IgG1 production. However, in the presence of a supernatant obtained from Th1 cell cultures,

Ig-E and IgG1 production was undetectable, but instead, a significant amount of IgG2a was found (Mosmann et al 1986). These data suggest that both Th1 and Th2 have effects on the humoral response, but induce different isotypes. By reconstitution of nude mice or lethally irradiated mice with antigen-specific Th1 or Th2 clones, Rizzo and co-workers (1995) confirmed the effect of Th1 and Th2 cells on isotype differentiation in vivo.

Different functional roles for Th1 and Th2 cells were also demonstrated in cell-mediated responses. By injection of relevant antigen into footpads, Cher and Mosmann (1987) found that Th1 clones, but not Th2 clones, induced DTH. Further evidence to support the concept of Th1 mediating DTH responses came from extensive studies in a *Leishmania major* infection model in mice. It had long been known that *L. major* was efficiently cleared in resistant strains, such as C57BL/6 or CBA, but that this protozoan parasite induced a fatal infection in susceptible strains, such as BALB/c. The inability to efficiently clear the infection in susceptible strains was shown to be due to a decreased cell-mediated immune response, as measured by DTH responses (Howard et al 1980). To confirm the relationship of Th1 cells with DTH-like responses, Heinzel et al (1989) examined IL-4 and IFN-γ mRNA levels in the lymph nodes and spleens from BALB/c and C57BL/6 mice during the *L. major* infection. The presence of IL-4, and absence of IFN-γ, were observed in susceptible, but not in resistant, strains. Furthermore, treatment of susceptible BALB/c mice with anti-IL-4 antibody or injection with IFN-γ resulted in resistance to infection (Sadick et al 1990). Transfer of *Leishmania* reactive Th1 clones, but not Th2 clones, was able to induce resistance in infected T cell deficient mice (Scott et al 1987). In addition to suppressing DTH-like responses, IL-4 has also been demonstrated to suppress antiviral CTL responses, resulting in delayed viral clearance

(Sharma et al 1996). These data clearly demonstrate that Th1 cells mediate significant cell mediated responses whereas Th2 cells suppress cell mediated responses.

Subsequent experimentation demonstrated that Th1 cells and Th2 cells exhibit cross-regulation. Th2 cytokines, for example, ablate the activation of macrophages mediated by the Th1 cytokine IFN-γ (Morel and Oriss 1998). In addition, Th2 cytokines are able to inhibit Th1 activities by down regulating Th1 cell differentiation (Mosmann and Moore 1991).

b) Th1/Th2 paradigm in allograft rejection and transplantation tolerance

Since Th1 cells are associated with enhancing cell-mediated immune responses, and Th2 cells are associated with decreasing cell-mediated responses by down regulating Th1 cells, it has been speculated that the balance between these cells may play an important role in the development of immunity and immune tolerance. In transplantation, acute allograft rejection is thought to be predominantly mediated by cell mediated responses, including DTH and CTL activities (Hall 1991). Therefore acute rejection is thought to be primarily a Th1 (or Type 1) immune response.

A large amount of evidence supports the position that Th1 cytokines are associated with graft rejection (Gorczynski et al 1995, Dallman 1993) and that Th2 cytokines are associated with graft prolongation (Gorczynski et al 1994, 1995, Ledingham et al 1996, Liwski et al 2000). A shift from Th1 towards Th2 has been demonstrated in a number of experimental models in which transplantation tolerance was induced (Gorczynski et al 1995, Siegling et al 1994). For example, treatment with anti-LFA-1 and anti-ICAM-1 mAb prolongs cardiac allograft survival (Xu et al 1997). This

prolongation corresponds with a significant increase in IL-4 and IL-10 secreting cells, but a decrease in IFN-γ and IL-2 secreting cells, in the grafts. This data is correlative but when anti-IL-4 or anti-IL-10 mAb were injected into these animals prolonged graft survival was abrogated (Xu et al 1997). This confirms a role for Th2 cytokines in this transplantation tolerance. Similar observations of a shift in the Th1/Th2 balance in the induction and maintenance of transplantation tolerance were obtained from studies using anti-CD4 mAb (Mottram et al 1995, Siegling et al 1994), anti-CD2 and anti-CD3 mAb (Punch et al 1998), CTLA-4 Ig (Sayegh et al 1995), and portal vein inoculation of allogeneic cells (Gorczyski et al 1994, 1998a,b). In all of these studies tolerance induction was associated with a shift toward Th2 (or Type 2) cytokines.

Maeda and co-workers (1994a, b) demonstrated that injection of a foreign class II MHC-specific Th2 cell line that secreted IL-4 and IL-10 into naïve mice markedly delayed rejection of skin allograft bearing the specific foreign class II MHC. This delayed allograft rejection was associated with inhibition of induction of allo-specific CTL activity, suggesting an important role of Th2 cells in preventing allograft rejection through suppressing cell mediated immune responses.

There is, however, evidence that transplantation tolerance is not always associated with immunomodulation towards type 2 responses. For example, a high frequency of IL-10 secreting CD4+ cells was found in rejecting, but not in nonrejecting, human kidney allografts at biopsy (Merville et al 1995). In mice, administration of IL-10 exacerbated, rather than prevented, cardiac allograft rejection (Qian et al 1996). However, IL-10 can be secreted by both Th1 and Th2 cells, as well as by macrophages. Thus an increase in IL-10 associated with graft rejection does not support or refute a Th1/Th2 bias. In contrast, the

fact that induction of Th2 responses by IL-12 antagonists exacerbated cardiac allograft rejection (Piccott et al 1996) suggests that type-2 responses could be involved in graft rejection rather than tolerance. Less direct evidence, showing that high levels of IL-5 were found associated with allograft rejection, supports this postulation (Martinez et al 1993a,b). These data provide evidence that, in some cases at least, Th2 cytokines may not induce or maintain transplantation tolerance. Allograft rejection is a complex process, which involves T cells and other immune elements. Therefore, pathways downstream to T cell maturation could be affected by cytokine activities. For example, IL-10 is known to inhibit IFN-γ secretion by T cells and macrophages, and to inhibit MHC class II expression on antigen presenting cells, resulting in down regulation of cell-mediated responses (Moore et al 1993, Groux et al 1996). However, IL-10 is also a growth factor for B cells, resulting in increased antibody production. Although cell mediated immune responses are the predominant mechanism of acute allograft rejection, humoral immune responses probably play some role in graft rejection. Therefore, high levels of IL-10 may increase antibody production against alloantigens, resulting in allograft rejection, rather than acceptance. In fact, anti-HLA antibodies were found in patients who had rejecting allografts expressing significant levels of IL-10 (Merville et al 1995). IL-5, another type-2 cytokine, has been found to recruit and activate eosinophils (Sanderson et al 1988), which may contribute to graft rejection by release of their toxic granule constituents (Martinez et al 1993 a, b, Kita et al 1992). Therefore, elevation of IL-5 in grafts may result in eosinophil mediated rejection (Martinez et al 1993b, Le Moine et al 1999). Taken together, these data suggest that association of the Th1/Th2 balance, rather than observation of the presence of type 2 cytokines alone, may be important in developing an understanding of the role of type 1 and type 2 immunity in the

generation of transplantation tolerance.

Although it is generally believed that type 1 cytokines mediate acute allograft rejection, recent data indicate that rejection can be induced in the absence of IFN-γ (Saleem et al 1996, Nagano et al 1997, Steiger et al 1998) or IL-2 (Dai et al 1998), suggesting that the cytokines generally accepted as the most important type 1 cytokines are not essential for allograft rejection. Type 1 cytokines have even been suggested to be involved in the induction of transplantation tolerance. There is evidence that transplantation tolerance cannot be established in the absence of IL-2 or IFN-γ (Li et al 1998, Raisanen-Sokolowski et al 1997, Konieczny et al 1998). For example, treatment of recipients with a tolerogeneic protocol utilizing CTLA-4Ig and anti-CD40 mAb prolongs skin and cardiac allograft survival in wild type mice but not in IFN-γ KO mice (Konieczny et al 1998). In addition, injection of anti IFN-γ mAb into wild type allograft recipients during such tolerance induction abrogated transplantation tolerance, indicating that IFN-γ is required for tolerance induction using this protocol (Konieczny et al 1998). The nature of the involvement of IFN-γ in this tolerance induction is unclear, but it has been suggested that IFN-γ may act to limit alloreactive T cell expansion, resulting in allograft acceptance (Konieczny et al 1998).

c) The role of IL-4 in transplantation tolerance

IL-4 is believed to be the central factor in the development of type 2 immunity and to play a critical role in transplantation tolerance (Mosmann and Sad 1996). For example, enhanced IL-4 mRNA, or protein, has often been found in the spleen or grafts of tolerized animals treated with anti-CD4 mAb (Takeuchi et al 1992, Mottram et al 1995), portal vein inoculation of alloantigen (Gorczynski 1992, 1995), or parasite infection (Ledingham et al

1996, Liwski et al 2000). In addition, anti-IL-4 mAb applied during tolerance induction abrogates transplantation tolerance induced by anti-CD2 and anti-CD4 mAb treatment (Punch et al 1998). Furthermore, long-term cardiac allograft survival induced by treatment with anti-CD4 mAb (Sirak et al 1998) or anti-CD2 plus anti-CD3 treatment (Punch et al 1998) cannot be established in IL-4 knockout mice.

IL-4 is also suggested to play a role in generation of regulatory cells. For example, anti-CD4 mAb treatment prolongs cardiac (Kupiec-Weglinski et al 1993, Lehmann et al 1997) and renal (Siegling et al 1994, Lehmann et al 1997) allograft survival in rats. This prolongation is mediated by regulatory T cells which are able to adoptively transfer transplantation tolerance to naïve engrafted recipients (Onodera et al 1996, 1997, Bushell et al 1999). Neutralizing antibody against IL-4 used at the time of tolerance induction in the primary recipient abrogated the ability to transfer tolerance indicating that IL-4 is critical for generating regulatory cells at least in this form of transplantation tolerance (Bushell et al 1999). However, anti-IL-4 mAb given to the secondary recipient, at the time of the cell transfer, did not abrogate the tolerance (Chen et al 1996). This suggests that IL-4 is not required once the regulatory cells have been generated. Therefore, these data suggest that IL-4 may play an important role in tolerance induction by driving the differentiation and expansion of Th2 like cells. However, once the tolerance has been established, IL-4 is no longer required.

In contrast to the work discussed above, there is also evidence that IL-4 is not always necessary for the generation of transplantation tolerance. For example, acceptance of cardiac allografts can be generated in IL-4 deficient mice by treatment with CTLA4Ig (Lakkis et al 1997). IL-4 expression by cardiac allografts (from IL-4 transgenic donors)

does not protect the grafts against rejection (Mueller et al 1997, Ritter et al 1999). However, the role of IL-4 is still unclear since there is evidence that expression of IL-4 by cardiac allografts prolongs graft survival (Takeuchi et al 1997). These studies reflect a differential requirement for IL-4 in tolerance induction in different experimental regimes that may be influenced by a variety of factors including the treatment regimes and genetic background of the animals used. In addition, in some situations other cytokines, such as IL-13, may substitute for the absence of IL-4.

IL-4 has been suggested to suppress DTH responses (Sirak et al 1998), inhibit CTL activity (Meada et al 1994) and down-regulate Th1 cytokine production (Punch et al 1998, Davies et al 1996, Takeuchi et al 1997). All of these would have a negative effect on transplant rejection. However, IL-4 has also been demonstrated to promote CD8+ CTL generation in response to alloantigen (Spits et al 1988, Bertagnolli 1991, Widmer and Grabstein 1987, Villacres and Bergmann 1999). Thus the role of IL-4 in the establishment of transplantation tolerance is likely to be complex and multifactoral and depend on the manner of tolerance induction.

d) Tc1 and Tc2

Recently, it was found that CD8+ T cells, often referred to as T cytotoxic (Tc) cells, selectively secrete distinct cytokines. This has been observed in both humans (Halverson et al 1997) and rodents (Li et al 1997). For example, progression toward frank AIDS in HIV infected individuals is often associated with a shift toward Th2 type cytokine secretion in the remaining T cells. These Th2 type cytokines, including IL-4, IL-5 and IL-6, are derived from CD8+ T cells (Paganelli et al 1995). In addition, IL-5

secreting CD8+ T cells are found to be associated with the airway eosinophilia that is believed to be the central event in pathogenesis of asthma (Colye et al 1995a, b, 1996, Schwarze et al 1999). In in vitro experiments, naïve CD8+ T cells can be induced to differentiate into Th1 or Th2-like phenotypes in the presence of IL-2 (or IFN-γ) or IL-4, respectively (Colye et al 1995b). These cytokine secreting CD8+ Tc cells have been classified as Tc1 and Tc2 depending on the pattern of cytokine secretion. Tc1 cells secrete IL-2 and IFN-γ; whereas Tc2 cells produce IL-4, IL-5 and IL-10 (Halverson et al 1997, Li et al 1997).

It is somewhat controversial whether the cytokine secreting CD8+ T cells maintain their cytotoxic activity and are able to effectively kill their targets. Some workers have demonstrated that they remain cytotoxic (Li et al 1997, Noble et al 1998), but others (Erard et al 1993) have found that they lose their cytotoxicity. The progression of AIDS has been linked to the loss of such CD8+ T cell cytotoxic activities (Switzerland 1994). Most evidence, however, suggests that Tc1 cells appear to be much more efficient CTL than Tc2 cells (Matesic et al 1998, Cerwenka et al 1999, Dobrzanski et al 1999). However, there is some evidence that, in some models, both Tc1 and Tc2 retain equal CTL activity (Fowler et al 1998).

Tc2 cells help B cells to proliferate and produce antibody (Cronin et al 1995, Paganelli et al 1995). Tc2 cells have also been suggested to down regulate cytolytic responses by increasing type 2 responses and concomitantly decreasing CTL activity (Erard et al 1993, Kanagawa et al 1993, Actor et al 1993). This has been shown to result in delayed virus clearance and more rapid disease progression. Recently, Fowler and colleagues (1998) demonstrated that Tc2, but not Tc1, cells prevent bone marrow

allograft rejection, even though both Tc1 and Tc2 exhibited similar CTL activity to alloantigen in vitro. These data suggest that Tc2 cells have a modulatory effect on immune responses and may play an important, previously unrecognized role in transplantation tolerance.

2.2.2 Fas/FasL interaction

a) Concept of Fas/FasL interaction

As part of the normal immune response immune regulation must occur. After initial activation and amplification, regulatory mechanisms must be in place to dampen down the response after antigenic challenge has been eliminated. Defects in this down regulation result in disorders of the immune system such as lymphoproliferative and/or autoimmune diseases. It is generally believed that the engagement of FasL on killer cells with cell bound Fas on the target cells plays a critical role in eliminating lymphocytes and maintaining normal homeostasis in the immune system.

Fas was originally described as a transmembrane protein that, when engaged by its ligand, can induce apoptotic cell death of transformed cells (Trauth et al 1989). Fas is constitutively expressed on resting T cells and is upregulated following cell activation (Krammer 1999). FasL is a transmembrane protein that can induce cell apoptosis in other cells by binding to membrane bound Fas on the target cells (Nagata and Suda 1995). Under normal conditions, resting T cells do not express FasL constitutively. However, it is rapidly upregulated following T cell activation (reviewed by Lynch et al. 1995).

A number of elegant experiments have demonstrated that Fas/FasL interactions are critical to the normal regulation of the T cell response. For example, although activation of primary T cells in vitro (by engagement of TCR/CD3) results in cell

proliferation and cytokine production, similar treatment of previously activated T cells leads to apoptotic cell death, termed activation induced cell death (AICD) (Kabelitz, et al. 1993). In contrast, previously activated T cells from Fas deficient mice (*lpr*) or FasL deficient mice (*gld*) do not undergo apoptosis after restimulation, confirming that Fas/FasL interaction is important in AICD (Bossu et al 1993, Russell et al 1995). Further experimentation showed that AICD could be blocked by antagonists of Fas, such as soluble Fas and a Fas-Fc fusion protein (Alderson et al 1995, Dhein et al 1995).

Since both *lpr* and *gld* mice exhibit lymphoproliferation and autoimmune disease (Cohen and Eisenberg 1991, Suda and Nagata 1997), and express defects in AICD, a link has been proposed between this phenomenon which includes a role of Fas/FasL interaction in the maintenance of normal immune homeostasis. However, the nature of this role is still controversial. In in vitro experimentation, Piazza and coworkers (1997) demonstrated that activated human CD4+ and CD8+ T cells expressed similar levels of Fas and FasL and exhibited similar sensitivity to anti-Fas mAb mediated apoptosis. However, after restimulation, CD4+ T cells killed CD8+ T cells and Fas-sensitive human Jurkat cells, but not CD4+ cells. These data indicated that activated CD4+ T cells were able to induce AICD of CD8+, but not CD4+ T cells. The fact that this killing was inhibited by cyclosporine treatment, which inhibits FasL expression on CD4 cells, suggested to Piazza and coworkers that this cell death was mediated through the Fas/FasL interaction. Since cyclosporine is not a specific inhibitor of Fas/FasL interaction, these experiments cannot rule out the effect of other apoptotic factors, such as TNF, on AICD induction (Zheng et al 1995). In addition, since cyclosporine inhibits T cell activation, it

may directly inhibit AICD by inhibiting T cell activation without involvement of Fas/FasL interaction.

There is also evidence that activated CD8+ T cells are able to induce AICD of CD4+ T cells. Indeed, it would be surprising if they could not since FasL was originally identified on a CD8+ T cell CTL hybridoma that kills target cells in a Ca++-independent, Fas-dependent fashion (Rouvier et al. 1993). Further, CD8+ CTL are well known to kill target cells by Fas/FasL pathways (Henkart 1999). This regulation of the CD4+ T cell population has been amply demonstrated in CD8+ T cell deficient (β2m-/-) and MRL/lpr mice. Noble and coworkers (1998) demonstrated that the superantigen SEA (staphylococcal enterotoxin A), specific to Vβ3 bearing CD4+ T cells, causes the loss of these cells in wild type mice but not in β2m-/- or lpr mice. This demonstrates that the elimination of activated CD4+ T cells requires the presence of CD8+ T cells and Fas. Further evidence of this interaction comes from studies with Vβ3 transgenic mice, which, for obvious reasons, are hyper-responsive to superantigen SEA. In these mice, cell proliferation of CD4+ T cells was significantly suppressed by activated CD8+ T cells after restimulation. This suppression was blocked by Fas Ig fusion protein, suggesting Fas dependent suppression (Noble et al 1998). Interestingly, the injection of SEA into these transgenic mice induced a greater increase of cell surface expression of FasL on CD8+ T cells than on CD4+ T cells, and an enhanced cytolytic activity of CD8+ T cells. The preferential expression of FasL on activated CD8+ T cells is consistent with CD8+ T cells limiting immune responses by induction of CD4+ T cell apoptosis through Fas/FasL interaction.

One could argue that since both CD4+ T cells and CD8+ T cells express equal levels of Fas, they should be equally sensitive to Fas/FasL mediated elimination. However, it has been demonstrated that anti-Fas mAb suppresses CD4+ T cell proliferation, but enhances CD8+ T cell proliferation (Noble et al 1998). These data suggest that the responses to Fas activation by CD4+ and CD8+ T cells are different. CD4+ T cells may be much more susceptible to Fas induced AICD. Inherent resistance of CD8+ T cells to Fas-mediated cell death may provide a regulatory mechanism that prevents the suicide of individual CD8+ T cells and allows the development of effective Fas/FasL interaction that suppresses CD4+ T cell expansion by CD8+ T cell regulation. The elimination of CD8+ T cells in late stage immune responses may be TNF mediated, rather than Fas mediated, since CD8+ T cells are very susceptible to AICD through TNF-dependent pathways (Noble et al. 1998)

There are interesting differences between the results of Piazza (1997) and Noble (1998) described above. It is worth remembering that Piazza and colleagues activated their T cells with anti-CD3 mAb or PMA plus ionomycin in vitro. This stimulates T cells without TCR recognition of antigen. Noble and colleagues, in contrast, activated their T cells with superantigen in vivo. This engages the TCR directly through engagement of Vβ3. The requirement of TCR engagement for activation of the FasL lytic system has been previously demonstrated (Lowin et al. 1995). Interestingly, both experiments (Piazza et al 1997, Noble et al 1998) demonstrated specificity in the induced killing, indicating that a mechanism of cell recognition must be involved in Fas/FasL induced apoptosis.

Antigen presenting cells, such as dendritic cells (DC) (Suss and Shortman 1996) and macrophages (Zhang et al. 1999, 2000), have also been suggested to be involved in the elimination of activated T cells by Fas/FasL interactions. It was found that two subpopulations of DC cells, one bearing CD8 and the other lacking CD8, are present in murine lymphoid organs (Suss and Shortman 1996). CD8- DC can efficiently stimulate alloreactive CD4+ T cells to proliferate, whereas CD8+ DC are less able to induce such CD4+ T cell proliferation. This appears to be due to the induction of apoptosis of CD4+ T cells in the presence of CD8+ DC (Suss and Shortman 1996). The apoptosis of CD4+ T cells, induced by CD8+ DC, is dependent on Fas/FasL interaction since the effect was not observed when CD4+ T cells were isolated from *lpr* mice (Fas-), or when DC were isolated from *gld* mice (FasL-). In support of this observation, high levels of FasL were found on CD8+DC, but not on CD8- DC (Suss and Shortman 1996). These data suggest that CD8+DC have the ability to eliminate activated CD4+ T cells, and may efficiently terminate ongoing immune responses.

Since CD4+ helper T cells play a critical role in immune responses by providing help to other cells, such as CD8+ T cells and B cells, by cytokine secretion and cell-cell interaction, the elimination of activated CD4+ T cells is an efficient pathway to terminate the immune response after resolution of antigen challenge.

Although there is a large amount of evidence to suggest that signal transduction through Fas induces cell apoptosis, it has also been reported that some cells are resistant to Fas mediated apoptosis (Zhang et al 1996, Jenkins et al 1999). The mechanisms involved in resistance to Fas induced apoptosis on lymphocytes are unclear. Down regulation of expression of Fas is as likely a protective event as is upregulation of

proteins such as Bcl-2 or death-effector-domain-containing proteins, which block the transmission of death signals (Tschopp et al 1998, Algeciras-Schimnich et al 1999). Bcl-2 is well known to be able to prevent cells from apoptosis and prolong cell survival time (Perlman et al 2000). However, its role in rescuing T cells from Fas mediated apoptosis is still controversial (Parijs et al 1998, Chiu et al 1995, Schroter et al 1995).

Taken together, these data suggest that the expression of Fas and FasL are important in maintaining normal homeostasis of the immune system. It follows that modification of this interaction could lead to a major shift in the regulatory pathways that define these responses. It has been speculated that just such a modification is the core element in the maintenance of immunoprivileged sites.

b) Effects of Fas/FasL interaction on immune privilege

It is well known that certain areas of the body are more permissive to allografts than others. These sites display the unusual characteristic that only limited immune reactions are initiated in response to alloantigen challenge. Sites, such as the ovary, testis, placenta and eye, appear to be exempt from immune responses and are thus referred to as immunoprivileged sites. The most extensively studied of these sites are the eye and the testis (reviewed by Griffith and Ferguson 1997).

The best demonstration of immune privilege in the eye is the remarkable success of corneal allografts in the absence of tissue matching and immunosuppressive therapy (Griffith and Ferguson 1997). The mechanisms of immune privilege in the eye are very complex, including the lack of lymphatic drainage and the presence of the blood-ocular barrier (Streilein et al 1995), local production of immunosuppressive cytokines, such as

TGF-β and IL-10 (D'Orazio and Niederkorn 1998), and limited MHC expression (Niederkorn 1999). In addition to these factors, however, FasL expression on the cells of eye tissue has been shown to be an important mechanism in protecting corneal allografts from rejection. Stuart and coworkers (1997) demonstrated that human corneas both express functional FasL and are capable of killing Fas bearing lymphoid cells. Rejection of corneal allografts from *gld* (FasL-) donor mice was much more vigorous than was rejection of corneal allografts from wild-type (FasL+) donors. Grafts from wild-type donors contained apoptotic mononuclear cells indicating the induction of apoptosis of infiltrating cells by the grafts, whereas rejecting FasL- grafts contained numerous inflammatory cells without sign of apoptosis (Stuart et al 1997, Yamagami et al 1997). These data suggest that FasL expression on the corneal graft cells plays a critical role in protection of such grafts from rejection by inducing apoptosis of infiltrating cells through the interaction with surface Fas on recipient infiltrating leukocytes, presumably alloreactive T cells.

The effect of FasL expression on graft protection was also observed on testis transplantation using *gld* or wild-type mice as organ donors (Bellgrau et al 1995). Similar to corneal allografts, the indefinite survival of testis allografts from wild type mice was due to apoptosis of infiltrating cells induced by the interaction of FasL on testis cells with Fas expressed on alloreactive T cells (Bellgrau et al 1995). These data suggest that FasL expression on donor tissue prevents allograft rejection by eliminating alloreactive T cells through Fas/FasL interaction, therefore down regulating the immune response to the allograft.

c) The effects of Fas/FasL interaction on allograft rejection

CTL mediated graft damage has been shown to be associated with granzyme and perforin release from CTL (Henkart 1999). Recently, the interaction of FasL on alloreactive cells with Fas on donor cells has also been reported to contribute to allograft rejection (Josien et al. 1998, Matsuno et al. 1998, Wang et al. 1997, Sharma et al. 1996). However, the role of Fas/FasL interactions in graft rejection remains controversial.

It has been reported that Fas protein (and mRNA for Fas) is expressed in cardiac and kidney allografts regardless of whether they undergo acute rejection or not. However, the expression of FasL was greatly upregulated during rejection (at 5-9 days posttransplantation), but was less so in non-rejected grafts (Josien et al. 1998, Matsuno et al. 1998, Wang et al. 1997, Sharma et al. 1996). The increased FasL expression was seen on graft infiltrating cells (GIC), and was closely correlated with the level of apoptosis detected by the TUNEL technique (Josien et al 1998), and the histologic score of acute rejection (Sharma et al. 1996). From this, it was suggested that FasL mediated apoptosis in the graft is involved in acute allograft rejection (Josien et al. 1998, Matsuno et al. 1998, Wang et al. 1997, Sharma et al. 1996).

Other studies have demonstrated that although Fas/FasL interaction may play a role in graft rejection, this role is not essential for rejection. Selvaggi and coworkers (1996) demonstrated that skin allografts transplanted into *gld* (FasL-) recipients were rejected in a similar manner to grafts transplanted into wild-type mice. Further, the survival of grafts from *lpr* (Fas-) donors was not prolonged in wild-type or *gld* mice, indicating that the Fas/FasL pathway is not required for skin graft rejection. Similar observations were obtained with cardiac allografts (Larsen et al 1995). However, these

data must be interpreted with caution because experiments performed on genetically modified animals may provide misleading results. For example, the genetic depletion of FasL expression in *gld* mice may induce a compensatory mechanism to substitute for the lack of the FasL induced immune response. This would result in an unchanged immune outcome but not necessary reflect the normal role of FasL in wild-type mice.

2.3 Oral Tolerance

The immune system is designed to recognize and eliminate "foreign" antigen challenge. The gastrointestinal (GI) tract is a major site of antigenic challenge to the body, where the immune system is continually exposed to a variety of foreign antigens, including antigens from dietary elements, commensal bacteria and a variety of pathogens. The GI tract employs a complex mechanism to counter the threat of pathogens while at the same time limiting the response to "non hazardous" dietary elements (Mowat 1987). Breakdown of this complex regulation results in food hypersensitivities such as coeliac disease (review Mowat 1987). To maintain this regulation, soluble food antigens absorbed from GI tract must be processed in such a manner as to generate suppressed immune responses to subsequent exposure. This suppressed (or depressed) immune response following soluble antigen exposure via the GI tract is termed oral tolerance. A well-described example is that of feeding the soluble protein ovalbumin (OVA). This results in almost complete suppression of anti-OVA responses, including cell proliferation, antibody production and DTH responses, upon subsequent exposure to this antigen (see, for example, Garside et al 1995).

2.3.1 Mechanisms of oral tolerance

Although oral tolerance has been observed for many years and has been studied extensively, the exact mechanisms for the induction and maintenance of this complex phenomenon are still controversial, and incompletely elucidated. A number of different mechanisms have been implicated. These can be divided into two principal groups: active suppression (or active modulation) and direct inactivation of responding lymphocytes (anergy/deletion) (Garside and Mowat 1997). The first mechanism refers to active suppression of responding lymphocytes by antigen-reactive regulatory cells. The secondary mechanism refers to the deletion, or rendering functionally inactive (anergy) of the responding lymphocytes as a direct consequence of antigen perception. Uncovering the complex mechanisms of oral tolerance is complicated by a myriad of factors such as the dosage and timing of antigen administration (Friedman and Weiner 1994). For example, feeding high doses of antigen has been suggested to provide a strong TCR signal to T cells and induce anergy or apoptosis of antigen specific cells (Whitacre et al 1991, Chen et al 1995, Gregerson et al 1993). In contrast, feeding multiple low doses of antigen has been suggested to generate active suppression by inducing regulatory T cells, including both CD8 and CD4+ T cells (Ke and Kapp 1996, Miller et al 1992, Lider et al 1989, Chen et al 1994, 1995).

The basis of active suppression depends on inhibitory cytokines produced by regulatory T cells following antigen specific activation (Faria and Weiner 1999). These are suggested to be critical in mediating immune suppression induced by multiple low dose feeding (Faria and Weiner 1999, Strober et al 1998). A variety of these regulatory cells have been suggested, including TGF-β secreting CD8+ T cells (Miller et al 1991, 1992,

Blank et al 1998); IL-4 and IL-10 producing Th2 cells (Chen et al 1994); and IL-4 and TGF-β secreting Th3 cells (Chen et al 1994).

Although both B cells and T cells can be tolerized following oral administration of antigens, it is generally believed that the role of the T cell in oral tolerance is paramount (Garside and Mowat 1997) and this would be especially so in oral transplantation tolerance.

a) Active suppression

Many studies demonstrate that oral administration of antigen generates regulatory T cells (Santos et al 1994, Lider et al 1989). These regulatory T cells from tolerized animals are able to actively suppress antigen specific antibody production and antigen primed T cell proliferation in a co-culture system (Miller et al 1991, 1992), and also, and very importantly, transfer tolerance to naïve recipients by cell transfer (Santos et al 1994). Both CD8+ (Blank et al 1998, Ke and Kapp 1996, Lider et al 1989) and CD4+ (Chen et al 1994, Barone et al 1995) T cells have been implicated in this regulatory response.

b) CD8+ T cells

Early studies implicated CD8+ regulatory cells in the tolerance that results from oral administration of antigen. Oral administration of myelin basic protein (MBP) prior to systemic immunization with MBP protects rats from the development of experimental autoimmune encephalomyelitis (EAE) (Lider et al 1989, Miller et al 1993, Whitacre et al 1991). The protection can be adoptively transferred from tolerized rats to naïve recipients

with mesenteric lymph node (MLN) or spleen cells from tolerized animals but this transfer is ineffective if the cell population is depleted of CD8+ T cells (but not if it is depleted of CD4+ T cells) (Lider et al 1989, Miller et al 1991, 1992). In vitro, CD8+ T cells from tolerized animals were able to suppress anti-MBP antibody production and lymphocyte proliferation of MBP specific T cells (Lider et al 1989), suggesting that CD8+ T cells exhibit active suppression. In addition, suppression of the DTH response to MBP following oral administration of this antigen, could be transferred by adoptive transfer of CD8+ T cells from tolerized animals (Miller et al 1992). Similar observations that CD8+ regulatory T cells prevent the induction of autoimmune diseases, (including collagen induced arthritis, Thorbecke et al 1998; and β2-glycoprotein induced experimental antiphospholipid syndrome, Blank et al 1998), following oral administration of autoantigens were also reported. In an antiphospholipid syndrome model, CD8+ T cells from tolerized animals not only suppressed primed lymphocyte proliferation, but also suppressed antigen primed CTL activity when co-cultured with antigen primed lymphocytes (Blank et al 1998). Taken together, these data suggest that oral administration of autoantigens generates CD8+ regulatory T cells which are able to suppress both humoral and cell mediated responses in vitro and inhibit the development of autoimmune diseases in vivo.

Miller and colleagues (1991), working in the EAE model (with MBP) and using a transwell system, demonstrated that the in vitro suppression seen with isolated CD8+ regulatory T cells derived from the spleens of tolerized animals was mediated by a soluble factor rather than cell-cell contact. The evidence provided by Miller and colleagues (1992) implicated TGF-β since splenocytes isolated from MBP fed animals produced large amounts of TGF-β in vitro in the presence of MBP, and anti-TGF-β mAb applied in culture

abrogated the suppression of antigen-primed lymphocyte proliferation and antibody production. In addition, TGF-β injection into animals during the induction of tolerance enhanced the tolerance (Thorbecke et al 1999). In contrast, anti-TGF-β mAb injection abrogated the oral tolerance induced protection from EAE (Miller et al 1992). A similar result was found in the antiphospholipid syndrome model (Blank et al 1998). The oral tolerance in the experiments described above was induced by low dose feeding and mediated by CD8+ regulatory T cells. Such data suggest that active suppression induced by low dose feeding is TGF-β mediated. Recently, however, induction of oral tolerance by low dose feeding was demonstrated in TGF-β1 null mice (Barone et al 1998), suggesting that mechanisms other than TGF-β may be involved in low dose induced active suppression.

Since CD8+ T cells have been generally described as cytotoxic lymphocytes, the cytotoxic potential of CD8+ regulatory T cells generated by oral tolerance is of considerable interest. In the experimental model of feeding β2-glycoprotein to prevent induction of antiphospholipid syndrome described above (Blank et al 1998), the CD8+ regulatory T cells demonstrated were antigen specific and MHC class I restricted, but they did not exhibit CTL activity against β2GPI bearing target in a conventional CTL assay. This indicates that oral administration of β2-glycoprotein did not activate CTL precursors (Blank et al 1998).

In an OVA model of oral tolerance, it has been observed that MHC class I restricted CTL activity was significantly suppressed in orally tolerized mice (Garside et al 1995). It is suggested that CD8+ suppressor cells generated by feeding low dose OVA are distinguishable from conventional CTL by their expression of a detectable (by mAb) carbohydrate epitope (Ke and Kapp_1996). Such CD8+ suppressor cells do not exhibit CTL activity, but suppress OVA primed CTL (Ke and Kapp 1996). Of significant interest,

several experiments suggest that the CD8+ regulatory T cells mediating oral tolerance are γδ T cells. Both transfer and depletion experiments support this hypothesis (Ke et al 1997, Wildner et al 1996).

As described above, CD8+ regulatory cells generated by oral administration of antigens are able to suppress immune responses in an antigen specific manner, and this suppression is mainly cytokine mediated. In contrast to this antigen specific effect, CD8+ T cells have also been reported to induce bystander suppression. In this case, CD8+ T cells activated by an initial tolerizing antigen suppress immune responses to another antigen in an antigen nonspecific manner. This bystander suppression appears to be triggered by specific antigen but the effect is mediated by cytokines (TGF-β; Miller et al 1991, 1993, Weiner et al 1994) which act in a nonspecific manner. The evidence for this bystander suppression comes primarily from EAE models. In these experiments, CD8+ T cells isolated from MBP-fed animals not only specifically suppressed MBP-specific T cell proliferation, but also nonspecifically suppressed OVA-specific T cell proliferation if both MBP and OVA were present in the culture (Miller et al 1991). This in vitro bystander suppression was abrogated with anti-TGF-β mAb (Miller et al 1992). The same group also demonstrated bystander suppression in vivo by suppressing DTH responses and inhibiting EAE development, and this suppression could be transferred with CD8+ T cells from tolerized animals to naïve animals (Miller et al 1991, 1993).

Although many studies indicate that CD8+ T cells mediate oral tolerance, others demonstrate that oral tolerance can be generated in the absence of CD8+ T cells either by mAb depletion (Garside et al 1995, Barone et al 1995) or by genetic manipulation (Tada et al 1996, Garside and Mowat 1997), suggesting that CD8+ T cells are not required for oral

tolerance induction. Therefore, other mechanisms rather than CD8+ T cells may be involved in oral tolerance.

c) CD4+ T cells

Generation of regulatory CD4+ T cells by oral administration of antigens has also been reported in a number of experimental models of oral tolerance (see Desvignes et al 2000). One of the most studied is the EAE model. Suppression of EAE induction can be generated in CD8 depleted mice following oral administration of MBP (Chen et al 1994). Splenocytes isolated from these CD8-depleted, orally tolerized animals were able to suppress MBP primed lymphocyte proliferation in vitro and transfer protection from EAE to naive animals in vivo, suggesting the presence of active suppression. In addition, MBP-specific CD4+ T cells were isolated from the MLN of MBP fed mice. These cells secrete high levels of TGFβ, IL-4 and IL-10, but low levels of IFN-γ in comparison to control animals fed hen egg lysozyme (Chen et al 1994). These CD4+ T cells that secreted TGF-β and IL-4 (or IL-10) were termed Th3 cells. These Th3 cells have also been reported to suppress EAE induction after transfer to naïve animals (Chen et al 1994), although there is some controversy over which cells were able to exhibit this transfer of tolerance.

In this EAE model, injection of IL-4 at the time of feeding increased TGF-β secreting T cells and enhanced tolerance induction, whereas injection of anti-TGF-β mAb abrogated tolerance (Inobe et al 1998). In other experiments, injection of anti-IL-4 mAb at the time of antigen feeding abrogated the oral tolerance (Yoshino 1998). These data suggest that CD4+ T cells mediate active suppression through cytokine secretion, most notable type 2 cytokines (Weiner 1998, Strober et al 1998). However, it has also been reported that oral

administration of antigens suppresses antigen specific Th2 cells (putatively the source of the type 2 cytokines) but not Th1 cells (Wu et al 1998). In addition, oral tolerance can be induced in the absence of Th2 cells (Shi et al 1999) and in IL-4 deficient animals (Kweon et al 1998). Moreover, in some cases, anti-IL-4 mAb (ref) and anti-IL-10 mAb (Aroeira et al 1995) treatment during tolerance induction do not block oral tolerance. Further evidence of the complexity of this response comes from experimentation which showed that although decreased type 1 cytokines (including IFN-γ) were observed in many orally tolerized animals, IFN-γ appears to be critical for oral tolerance induction since oral tolerance cannot be expressed in IFN-γ deficient animals (Kweon et al 1998). Indeed, it has recently been suggested, based on work with IL-4 and IL-10 deficient mice, that both type 2 and type 1 pathways must be intact for effective induction of oral tolerance (Rizzo et al 1999).

2.3.2 Mechanisms of oral tolerance induction

The initial events which lead to the generation of oral tolerance are unclear. It has been proposed that some intrinsic property of the intestinal immune system must contribute to oral tolerance induction. The generation of oral tolerance has been suggested to be determined by some unique manner of antigen presentation in the GI tract, potentially resulting from activation of T cells in the presence of modified costimulatory signals, or in the absence of costimulation (Garside and Mowat 1997). Oral tolerance is a complex phenomena and it is probable that multiple sites are involved in it's development. Many factors, including the nature and dose of antigen, the age and genetic background of the animals as well as other factors influence the development of oral tolerance (Garside and Mowat 1997). Furthermore, there is evidence that the portal circulation of antigen to the

liver is critical to oral tolerance induction, indicating an additional level of regulatory control (Garside and Mowat 1997).

a) Basic structure of the intestinal immune system

The intestinal tract is covered by a single, continuous layer of intestinal epithelial cells (IEC), which separates the tissue from the lumen. Together with the IEC, organized secondary lymphoid tissues under the epithelium facilitate antigen uptake, processing and presentation, which leads to the induction of an intestinal immune response. The most prominent organized component of the intestinal immune system is the gut associated lymphoid tissue (GALT), which contains the Peyer's patches (PP) and other solid follicles. PP contain specialized M cells and closely associated lymphoreticular cells, with lymphoid follicles. Between the IEC, there are scattered lymphocytes, termed intraepithelial lymphocytes (IEL). Furthermore, the intestinal lymphatic drainage connects to lymph nodes located in the mesentery, termed mesenteric lymph nodes (MLN) (McGhee and Kiyono et al 1999).

b) Effects of IEC and IEL on oral tolerance induction

Antigens present in the intestine have three routes to access the GALT, 1) by a transcellular route though IEC; 2) through M cells to enter PP; 3) by a paracellular route by entry between epithelial cells. Under normal conditions, antigens are unlikely to penetrate through the paracellular route, due to tight junctions joining the apices of the IEC (Mayer 1999).

It is well known that conventional antigen presentation by "professional" antigen presenting cells (macrophages and dendritic cells) requires two signals. One signal is transmitted through the TCR/CD3 complex on T cells (by recognition of peptide-MHC complex on APC). The second signal is a non-specific co-stimulatory signal delivered by the interaction of CD28 on T cells with B7 molecules on the APC. In the absence of costimulation, T cells stimulated by the TCR/CD3 complex will be only partially activated, resulting in refractiveness to further stimulation through the TCR/CD3, a phenomenon known as anergy (Weiss 1999).

IEC are epithelial cells and are not considered professional or conventional APC. However, IEC are believed to be able to present antigens to T cells and induce T cell activation (Mayer and Shlien 1987). However, IEC-induced T cell proliferation is much less robust than proliferation induced by splenic APC (Bland and Warren 1986a). This lower efficiency of stimulation by IEC is thought to be due to lower expression of MHC-II on IEC (Vidal et al 1993, Hershberg et al 1997, 1998). In addition, IEC do not appear to express the costimulatory molecule B7 (Sanderson et al 1993, Hershberg et al 1997, Framson et al 1999). The lack of costimulatory molecules on IEC may indicate that these cells could be involved in the mediation of clonal anergy and thus be involved in oral tolerance. In addition to this, IEC have been reported to secrete IL-10 and TGF-β (Strober et al 1998). This suggests that IEC may also play a role in the generation of Th2 (IL-4, IL-10), Th3 (IL-4 and TGF-β) or Tr (IL-10, TGF-β) regulatory T cells.

Recently, it has been found that feeding of the hapten dinitrochlorobenzene prevented later contact sensitivity to this hapten (Galliaerde et al 1995). The hapten was rapidly captured by both IEC and dendritic cells in the PP and lamina propria (LP) of the

gut. When cultured with hapten-specific T cells, PP APC isolated from fed animals promoted T cell proliferation. IEC from the same animals did not. In fact, these IEC from hapten fed animals inhibited hapten specific T cell proliferation induced by hapten-pulsed splenic APC. This inhibitory effect was mediated by IL-10 and TGF-β secreted by IEC and resulted in T cell anergy, since the T cell proliferation could be reversed by addition of IL-2 (Kaiserlian 1999), which is characteristic of anergic T cells. These data confirm that IEC are able to induce T cell anergy.

Of significant interest, IEC can also take up, process and present exogenous soluble antigen (OVA) to CD8+ T cells and induce CD8+ T cell activation (Bland and Warren 1986b). These activated CD8+ T cells display a suppressor function and lack cytolytic activity. Both IEC and splenic APC stimulate OVA-primed spleen T cells, however, only IEC stimulated T cells suppress APC induced T cell proliferation, and this suppression is antigen specific (Bland and Warren 1986b). Interestingly, using mAb-blocking experiments, it was found that activation of these CD8+ suppressor cells required signaling through CD8 and TCR, but not MHC molecules (Li et al 1995, Campbell et al 1999). These data suggest that IEC induced CD8+ T cell activation may use an alternative mechanism for T cell activation. In fact, two ligands, CD1, a class I-MHC like molecule, (Bleicher et al 1990, Panja et al 1993) and gp180 (Li et al 1995, Mayer 1998) were found to be expressed on IEC and these two molecules are involved in the stimulation pathway of CD8+ T cells (Panja et al 1993, Li et al 1995).

It is very possible, if not probable, that the target for IEC presentation is the IEL. These IEL are mostly T cells and the IEL compartment includes both CD4+ and CD8+ T cells. Moreover, a large number of γδ T cells are also present in IEL (about 50% of IEL

T cells are γδ T cells in mice). A large portion of the γδ T cell population is CD8+ (McGhee and Kiyono 1998). Perhaps the most intriguing property of γδ T cells is their ability to recognize antigen in an MHC unrestricted manner and to recognize CD1 molecules (Groh et al 1998, Boismenu and Havran 1998, Chien et al 1996). Therefore, it is conceivable that this alternative presentation by IEC may lead to the generation of CD8+ γδ T regulatory cells.

From the above it is evident that the engagement of antigen by IEC and the presentation to IEL could influence the development of oral tolerance in a number of ways. First, the lack of costimulation by IEC may contribute to the generation of T cell anergy. Second, low levels of class II MHC expression on IEC may inhibit CD4+ T cell activation. Third, IEC secretion of IL-10 and TGF-β (Strober et al 1998) may induce the generation of Th2 (IL-4, IL-10), Th3 (IL-4 and TGF-β) or Tr (IL-10, TGF-β) regulatory T cells. Fourth, IEC may be able to interact with CD8+ T cells to generate CD8+Ts in an MHC unrestricted manner, potentially by the involvement of CD1 and gp180 on IEC. CD8+ γδ IEL may be the major source of this Ts generated by IEC stimulation. The fate of these IEL which have encountered antigen presented by IEC is unclear. They may migrate to local lymphoid tissue or to draining lymph nodes such as the MLN and from there to the spleen and systemic circulation.

c) Effects of PP, MLN and spleen on oral tolerance induction

PP are one of the major lymphoid structures of GALT and have been suggested by some to play a critical role in the induction of oral tolerance (Mattingly 1984, Richman et al 1981, Gonnella et al 1998). M cells overlie the dome of PP and are able to

take up and transport lumenal antigens (Neutra 1998, 1999) into the underlying lymphoid tissues. It has been suggested that M cells do not process and present antigen to lymphocytes (Wolf et al 1984) but transport antigens to underlying APC for antigen presentation to lymphocytes in PP (McGhee and Kiyono 1998). B cells, macrophages and dendritic cells in the PP are suggested to play a role in antigen processing and presentation (Ruedl and Hubele 1997, Kelsall and Strober 1996, 1997, Van Wilsen et al 1994).

It has been demonstrated that after feeding antigen, antigen specific "suppressor" T cells can be isolated from PP. Later such cells can be found in the MLN and spleen (Mattingly and Waksman 1978, Mattingly 1984, Richman et al 1981). These regulatory T cells suppress antibody (IgG) production (Richman et al 1981) and inhibit antigen-primed lymphocyte proliferation (Mattingly and Waksman 1978) in an antigen specific manner. In addition, these cells are capable of transferring suppression, including suppression of antibody production, cell proliferation and DTH responses (Mattingly and Waksman 1978, Richman 1981). Recently, regulatory CD4+ T cells that secrete IL-4, IL-10 and TGF-β were obtained from PP in OVA TCR-transgenic mice after a single low dose feeding, but not after high dose feeding of OVA (Gonnella et al 1998). In another oral tolerance model, feeding of interphotoreceptor retinoid-binding protein prevented the induction of experimental autoimmune uveitis (EAU) (Rizzo et al 1994). This protection was correlated with the production of TGF-β, IL-4 and IL-10 by cells from the PP, and these cytokines were found to be mainly secreted by CD4+ T cells of the PP (Rizzo et al 1994, 1999). Interestingly, injection of human recombinant IL-2 enhances protection from EAU and increases the number of IL-4, IL-10 and TGF-β secreting cells in the PP

(Rizzo et al 1999). It has been suggested that this enhancement by IL-2 treatment is due, at least in part, to the expansion of a population of regulatory cells in the PP which results in increased production of anti-inflammatory cytokines. In the EAE oral tolerance model, TGF-β secreting regulatory cells appear in the PP. These cells suppress lymphocyte proliferation in vitro and prevent the induction of EAE when transferred into naïve animals (Santos et al 1994).

Taken together, these data suggest that, after low dose feeding, the suppressor cells are generated in PP and that these suppressor cells are predominately Th2 type cells (Rizzo et al 1994, 1998) and/or TGF-β secreting cells (Santos et al 1994, Gonnella et al 1998). Although IFN-γ secreting cells were also found in PP in some situations, their induction appears to be related to high dose feeding of antigens (Hoyne et al 1993, Marth et al 1996).

The data described above suggest that suppressive regulatory cells can be generated in PP following the feeding of antigens and these cells are predominantly Th2 cells and/or TGF-β secreting cells. However, it was recently demonstrated that oral tolerance could be generated in mice that are deficient in PP, but not in mice that are deficient in both PP and MLN, suggesting that the PP is not necessary for the induction of oral tolerance (Spahn et al 2001, Yamamoto et al 2000). In fact, evidence from several studies has suggested that the MLN is critical for induction of oral tolerance. Suppressive T cells could be isolated from MLN after oral administration of antigens and these cells are able to transfer tolerance to naïve animals (Mattingly 1984, Richman et al 1981, Franco et al 1998). It was found that type 2 regulatory cells were present in the MLN after low dose, but not high dose, feeding of OVA-DNP and that these cells

respond to OVA stimulation as measured by cell proliferation (Franco et al 1998). In addition, antigen-specific T cells that produce IFN-γ were found in the MLN after high dose feeding of OVA (Hoyne and Thomas 1995). Furthermore, suppressor cells generated in PP are suggested to migrate to MLN. This comes from evidence that the suppressor cells appeared early in PP but later in MLN, and at the later time point, suppressor cells disappeared from the PP (Mattingly 1984, Richman et al 1981, Mattingly and Waksman 1978). However, these data cannot rule out the possibility that the suppressor cells from MLN are generated from sites other than PP.

The spleen has also been suggested to be a site of oral tolerance induction. Much evidence has shown that splenocytes from orally tolerized animals are able to suppress immune responses, including cell mediated and humoral responses, in vitro. These splenocytes are able to transfer tolerance to naïve animals (Singh et al 1992). Although there is evidence to suggest that the regulatory cells present in spleen have migrated there from the PP or the MLN (Mattingly and Waksmen 1978, Mattingly 1984, Richman et al 1978), there is also evidence to suggest that the regulatory cells present in spleen, MLN or PP are not the same population (Richman et al 1981, Franco et al 1998). Cells isolated from the spleen exhibit different functions than cells from the MLN after the same feeding regime of OVA-NDP (Richman et al 1981, Franco et al 1998). For example, regulatory cells from the PP and MLN of OVA fed animals induce decreased cell proliferation but enhanced IgA production when these cells are transferred into naïve animals. Regulatory cells from the spleen of fed animals, however, suppress cell proliferation but do not enhance IgA production upon transfer (Richman et al 1981). These data suggest that the regulatory cells in the PP, MLN and spleen may not be the

same population (Richman et al 1981). Further to this, Franco and colleagues have shown that cells from the MLN of OVA-DNP fed animals suppress DTH responses in vivo (and cell proliferation in vitro) after transfer into naïve animals, whereas cells from the spleen of fed animals do not transfer tolerance, confirming that two functionally different cell populations were present in the MLN and spleen (Franco et al 1998). This led Franco and colleagues to suggest that more than one mechanism may be involved in oral tolerance induction and that oral tolerance generated in different sites may reflect different mechanism. They suggested that regulatory cells develop in the MLN that mediate active suppression, whereas anergy or clonal deletion might occur in the spleen.

A role for the spleen in oral tolerance induction has been amply demonstrated by using splenectomized mice and the EAU model (Ma et al 1997, Suh et al 1993). Oral administration of autoantigen prevents the induction of EAU only in eusplenic mice, not in asplenic mice. These data confirm the spleen as an important site for oral tolerance induction.

Taken together, the data described above suggest that multiple sites are involved in induction of oral tolerance. However, it is still unclear how oral tolerance is generated at these sites. Several factors may contribute to oral tolerance induction, but APC, especially DC, in the GALT have been suggested to play a critical role in tolerance induction (Garside and Mowat 1997, Van Wilsen et al 1994, Ruedl and Hubele 1997). Oral tolerance can be enhanced after expanding the DC population in mice by treatment with Flt3L (Willamson et al 1999, Viney et al 1998), suggesting that DC play a role in oral tolerance induction. DC isolated from GALT, including from PP, acquire and present antigens to T cells and induce T cell activation (Liu et al 1991, Van Wilsen et al

1994, Ruedl and Hubele 1997, Kelsall and Strober 1996). Both mature and immature DC have been described at different sites in the PP. Immature DC excel at antigen uptake and processing whereas mature DC are potent T cell stimulators (Ruedl and Hubele 1997). Indeed, when compared to splenic DC, DC isolated from PP express 5-10 fold higher levels of MHC class II associated antigens (Kelasll and Strober 1996). These data suggest that the DC found in the PP are potent APC and should be easily able to induce T cell activation. Interestingly, it was found that splenic DC induced mainly Th1 cells whereas PP DC stimulated predominantly Th2 cells (Everson et al 1996). These data indicate that PP DC may have the ability to shift T cell differentiation toward a Th2 response, a phenomenon associated with the induction of oral tolerance. Recently, it was demonstrated that the continuous feeding of microdoses of OVA induces tolerance (and enhanced type 2 response) by down-regulation of B7 molecules on APC and down-regulation of antigen specific TCR (Wu et al 1998).

d) Effects of the liver on oral tolerance induction

There is considerable evidence that the liver plays a significant role in the induction of oral tolerance. Oral tolerance to dinitrophenol (DNP), for example, is completely abrogated by portal venous shunt (to re-route the portal circulation to exclude passage through the liver) prior to feeding (Cantor and Dumont 1967). It was also found that injection of antigen directly into the portal vein generates a marked reduction in antigen specific immune responsiveness as demonstrated by decreased antibody production, reduced DTH responses (Fujiwara et al 1986, Sato et al 1988) and prolonged allograft survival (Gorczynski 1992). The characteristics of the immune suppression induced by injection of

antigen into the portal vein (portal tolerance) or by delivery via the oral route (oral tolerance) are similar (Gorczynski et al 1998). Since intestinal blood drains first into the liver via the portal vein, antigens absorbed from the intestine are transported into the liver by the portal route. This has led to the suggestion that portal tolerance is a version of oral tolerance and that resident liver macrophages (Kupffer cells) and liver DC are critical in both portal and oral tolerance induction.

Kupffer cells are potent phagocytic cells and are potential APC. They express high levels of MHC-II and B7 molecules (Lohse et al 1996). They have been shown to stimulate T lymphocytes and produce a variety of important cytokines (Gregory and Wing 1998).

It was found that blocking Kupffer cell function by intravenous injection of gadolinium chloride blocked portal tolerance, including alloantigen specific portal tolerance, (Callery et al 1989, Yu et al 1997). Prolonged cardiac allograft survival induced by portal tolerance, for example, was abolished by injection of gadolinium chloride (Squiers et al 1990). These data confirm that Kupffer cells may play an important role in portal tolerance induction and potentially in oral tolerance.

Gadolinium chloride was demonstrated to inhibit antigen-presentation by Kupffer cells by blocking phagocytosis without influencing the surface expression of MHC class II (Roland et al 1993). This suggests two possible interpretations of the tolerance induction by Kupffer cells, one passive, and one active. First, blocking antigen uptake by Kupffer cells in the liver may prevent tolerance simply by allowing antigen to enter the systemic circulation and peripheral lymph tissue, resulting in priming of the immune response. Second, blocking antigen uptake may prevent tolerance by blocking the

processing and presentation of antigen by Kupffer cells which is required for tolerance induction (Roland et al 1993).

Given that both portal and oral tolerance lead to long term immune modulation, even if antigen is re-introduced by a different route, it is reasonable to assume that the latter interpretation is more likely. In addition, as outlined in this thesis, oral transplantation tolerance can be transferred to naïve animals, confirming that the former hypothesis is unlikely. Recently, Gorczynski and colleagues demonstrated that DC from the liver play a critical role in portal tolerance. They associated this role with the expression of surface OX-2 on the APC in the liver. They found that portal transplantation tolerance was associated with increased Th2 cytokine secretion (Gorczynski et al 1998a,b). Both Th2 induction and resulting graft prolongation were abrogated by injecting the recipient with anti-OX-2 mAb (Gorczynski et al 1998b).

2.3.3 Effects of oral tolerance on inhibiting allograft rejection

Early studies showed that neonatal exposure of mice to breast milk from allogeneic foster mothers slightly prolonged skin allograft survival in the fed recipients (Beer and Billingham 1975). In addition, breast-fed humans who received living-related maternal kidney transplants showed fewer rejection episodes and better one-year allograft survival (Campbell et al 1984). This prolonged graft survival may be related to the abundant mononuclear cells contained in breast milk.

Later, Weiner and colleagues (Sayegh et al 1992a, b) studied the effects of oral administration of allogeneic splenocytes on the systemic immune response to alloantigens. They demonstrated that feeding allogeneic splenocytes suppressed alloimmune responses in

vitro (lymphocyte proliferation) and in vivo (DTH responses). The suppression was antigen specific in vivo but non-specific in vitro, which might suggest the involvement of a soluble suppressor factor (Sayegh et al 1992b). In addition, feeding allogeneic cells was shown to prevent accelerated rejection of cardiac allografts that had been induced by presensitization, essentially transforming accelerated rejection to acute rejection in an antigen specific manner (Sayegh et al 1992b, Hancock et al 1993). This group, however, was not successful in prolonging primary allograft survival as demonstrated in this thesis (Weiner, personal communication). Weiner and colleagues suggested that the observed effects were mediated by up-regulation of Th2 cells and down-regulation of the Th1 response. This was because the cardiac allografts harvested from fed animals exhibited reduced IL-2 and IFN-γ expression but showed markedly increased IL-4 expression (Hancock et al 1993).

Recently, the effect of oral tolerance on prolongation of first set allograft survival was demonstrated in an immunoprivileged corneal transplantation model (He et al 1996, Ding et al 1997). Corneal allografts have the highest success rate in transplantation, but approximately 10% of human corneal allografts are still rejected due to immunologic responses (He et al 1996). By feeding allogeneic cells (cultured murine corneal cells and/or freshly isolated keratinocytes) to mice, corneal allograft survival was prolonged (He et al 1996, Ding et al 1997). The prolongation was correlated with decreased DTH and CTL activity, suggesting that the prolongation is due to decreased alloimmune responses following oral administration of alloantigens. In addition, the prolongation of allografts could be transferred to naïve animals by transferring lymphocytes from the MLN or spleens of fed animals, suggesting that the suppression was mediated by regulatory cells (He 1996).

Our laboratory was the first to demonstrate that oral tolerance can prolong primary

allograft survival (Carr et al 1996, 1998). Since that discovery, there has been an increase in experimentation exploring the effects of oral tolerance on transplantation, including cell (Senesi et al 1998, Ilan et al 2000) and solid organ (Ishido et al 1999, Zavazava et al 2000, Niimi et al 2000) transplantation. In solid organ transplantation, oral administration of allogeneic splenocytes (Ishido et al 1999, Niimi et al 2000) or synthetic MHC peptides (Zavazava et al 2000) prolonged cardiac allograft survival. Taken together the available data suggest that oral transplantation tolerance is associated with decreased alloresponsive proliferation in vitro and decreased DTH responses in vivo (Ishido et al 1999, Zavazava et al 2000, Saygh et al 1992, He et al 1996). In addition, in these experiments, a bias toward type 2 cytokines is shown by cells isolated from spleens or MLN from orally tolerized animals. However, the mechanisms of induction of oral tolerance at the level of the GI tract and the expression of oral tolerance at the level of the transplanted organ are unknown. In this thesis, experimentation is described which deals with the expression of the oral tolerance at the level of the transplanted organ.

2.4 Objectives

This study was designed i) to confirm the hypothesis that oral administration of alloantigen would prolong primary kidney allograft survival in rats, and ii) to elucidate the mechanisms by which this prolongation is mediated. The mechanisms involved in the induction of oral tolerance of any kind (not only transplantation tolerance) at the level of the GI tract are complex, controversial and mostly unclear. For that reason this study was designed to address the mechanisms behind oral transplantation tolerance at the level of the graft, looking for the expression of the pathways initiated upstream. In this thesis

experimentation, kidney transplants are used since graft-infiltrating cells can be readily obtained, quantified and characterized. The central concept driving this experimentation is the position that until the manner of expression of oral transplantation tolerance can be defined there can be no productive investigation of the induction of that expression. This thesis experimentation deals with the expression of oral transplantation tolerance in the kidney.

3.0 PROLONGATION OF SURVIVAL OF PRIMARY RENAL ALLOGRAFTS BY FEEDING OF DONOR SPLEEN CELLS. [1]

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The Full title: Prolongation of survival of primary renal allografts by feeding of donor spleen cells.[1]

Ronald I. Carr[2,3], Juan Zhou[2], Joy A. Kearsey[4], Andrew W. Stadnyk[2,4], and Timothy D.G. Lee[2,5].

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[2] Department of Microbiology and Immunology, Dalhousie University.

[3] Department of Medicine, Dalhousie University

[4] Department of Pediatrics, Dalhousie University

[5] Department of Surgery, Dalhousie University

Abbreviations used in this paper: BN, Brown Norway; FBS, fetal bovine serum; GIL, graft infiltrating leukocytes; IL, interleukin; PBS, phosphate buffered saline; PV, portal vein; RT-PCR, reverse transcription polymerase chain reaction; Tx, transplantation.

Abstract

We have found that feeding Brown Norway rat (BN) spleen cells to Lewis rats prior to transplanting BN kidneys, prolonged allograft survival (mean of 8.8 days in unfed rats; 21 days in the BN cell-fed rats; longest survival 11 days without allo-feeding, vs 37 days with feeding.) We have also found that feeding BN cells both pre- and post-transplant further extends survival (mean of 38 days; longest survival 105 days). We also examined the cells infiltrating the grafts during the early stages of the allograft response (day 5). Using flow cytometry, we found a significant decrease in the number of leukocytes infiltrating the transplanted kidneys of fed animals. This decrease was mainly due to a drop in the number of infiltrating T cells. We also found that cytokine mRNA production by the graft-infiltrating lymphocytes (GIL), assessed by RT-PCR, showed a significant increase in IL-4 and TGF-β mRNA in the GIL of fed animals compared to the controls.

Introduction

In the last decade, the development of new immunosuppressive drugs producing blanket immunosuppression has resulted in a significant prolongation in the length of survival of kidney transplants. However, the profound immunosuppression produced by these drugs results in major undesirable side effects, such as an increase in susceptibility to infections and tumours, and in some cases nephrotoxicity. In addition, they have proven of limited effectiveness in the prevention of late graft (chronic) rejection. If methods of allograft-specific immunosuppression could be developed, such profound blanket immunosuppressive treatments, with their inherent increased risk of infection and tumours could be avoided, and chronic rejection might be ameliorated.

Oral tolerance is a state of antigen-specific decreased responsiveness in cell mediated and/or humoral immunity, induced by feeding antigen (reviewed in 1 and 2). Since oral tolerance is antigen specific, this approach to immunosuppression, if effective in suppressing graft rejection, would avoid the problems associated with non-specific immunosuppression. We have begun to examine the induction of "oral tolerance" for kidney transplantation, to assess the potential value of this approach for modulating kidney allograft rejection. Our approach has been by feeding donor spleen cells, to trigger oral tolerance by gastric exposure to the donor alloantigens.

We have also tested tolerance induced by portal vein injection of the alloantigens, to compare the effectiveness of feeding with that route, since there have been several reports of prolongation of allograft survival induced by that approach (3-6).

Oral tolerance has been demonstrated in a variety of experimental animals including mice, rats, rabbits, and guinea pigs (reviewed in 1 and 2). There is also substantial suggestive evidence (7-9) and direct evidence (10-11) of its occurrence in

man. We report here that oral tolerance induction significantly retards the **primary rejection of kidney allografts** in rats.

Materials and Methods

Animals

Male Brown Norway (BN, $RT1^n$) and Lewis (LEW, $RT1^l$) rats purchased from Harlan Sprague Dawley (Indianapolis, IN) were used as donors and recipients, respectively. Animals were maintained in the Dalhousie University Faculty of Medicine animal care facility and provided with water and rodent chow *ad libitum*. All animals were cared for in compliance with the guidelines established by the Canadian Council on Animal care.

Preparation of cells for feeding and pv injection

Brown Norway (BN) spleen cells were isolated from BN rats (Harlan) by standard protocols. Briefly, animals were anaesthetized with halothane and sacrificed by cervical dislocation. Animals were completely submersed in 70% ethanol for 30 seconds before being placed on sterile toweling in a laminar flow hood. The spleen was removed after a sagital incision, and placed in sterile RPMI-1640 and cut into several pieces. To produce single cell suspensions, the pieces were crushed with the ribbed sterile back ends of plastic syringe plungers and clumps were broken up by aspirating and ejecting 5 times with sterile Pasteur pipettes. After washing, the cells were purged of red blood cells by lysis for 5 minutes with ACK erythrocyte lysing buffer (0.15M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) at room temperature (12). The cells were washed again, resuspended in sterile phosphate buffered saline (PBS: 0.15 M NaCl, 0.05 M Na_2PO_4 pH 7.4) and counted using trypan blue. Viability was always greater than 95%. The concentration was adjusted to 3.3×10^8 live cells per ml of PBS for feeding. For pv injection, after ACK lysing and washing as above, the cells were resuspended in PBS, and rapidly passed through a loosely packed column of sterile glass wool to remove any

residual cell clumps and debris. After counting as above, the concentration was adjusted with sterile saline, to 2 x 10^8 live cells per ml.

Gastric intubation

Unanaesthetized rats were immobilized and fed using a plastic gag between the upper and lower front teeth. A 3 1/2 Fr premature human infant feeding tube (Sherwood Industries, St. Louis, MO) was passed into the stomach through a small hole in the centre of the gag. Three hundred microlitres of spleen cell suspension in PBS (1 x 10^8 cells) was given to each rat at each feeding.

Portal venous administration of cells

Brown Norway spleen cells were prepared as above. Lewis rats were anaesthetized with sodium pentobarbital and a mid-abdominal incision made. The portal vein was exposed and 0.5 ml of sterile saline containing 1 x 10^8 cells was injected using a 27 gauge needle, through an area of mesenteric fatty tissue to contain bleeding. Successful transfer of the cells was assured by observing a transient blanching of the liver. Post-injection pressure was applied with a sterile Q-tip. No significant bleeding occurred. Closure was carried out using standard procedures.

Kidney transplantation

Kidney transplantation was performed as described previously (13). Briefly, BN rats were anaesthetized with pentobarbital and a midline abdominal incision was made. The left kidney was exposed, freed of connections, perfused with heparinized cold saline and removed with a cuff of the aorta and the renal vein, as well as a long piece of ureter. This donor kidney was kept in ice cold saline while the recipient was prepared. Left

nephrectomy was performed on the recipient. The donor kidney was then attached in an orthotopic position via end to side anastomosis of the cuff of renal artery with the abdominal aorta and the renal vein to the recipient vena cava. The ureter was attached by end-to-end anastomosis. After 3 days the animals were anaesthetized and a right nephrectomy performed to remove the remaining native kidney. This three day delay in the removal of the other native kidney allowed the functional recovery of the transplanted kidney. Simultaneous contra-lateral nephrectomy at the time of transplant would render the animal uremic for at least 24 hours while the transplanted kidney recovers full function.

Experimental protocols

Protocol 1 (prior feeding): Lewis rats were fed $1x10^8$ Brown Norway (BN) splenocytes every day for 5 days. Ten days later, the rats were given a BN left kidney transplant as described above.

Protocol 2 (prior and post-feeding): Lewis rats were fed $1x10^8$ Brown Norway (BN) splenocytes every day for 5 days. Nine days later they were fed $1x10^8$ BN splenocytes and the following day they received the BN transplant. Thereafter, once every week they were fed $1x10^8$ BN splenocytes until death or sacrifice.

Protocol 3 (portal vein injection): Lewis rats were given a single pv inoculation of $1x10^8$ BN splenocytes and ten days later, the rats were transplanted with a BN kidney.

Protocol 4 (portal vein injection and post transplant feeding): Lewis rats were given a single pv inoculation of $1x10^8$ BN splenocytes and ten days later, the rats were transplanted with a BN kidney. Thereafter, they were fed $1x10^8$ BN splenocytes once every week until death or sacrifice.

Protocol 5 (prior feeding for assessment of graft infiltrating leukocytes (GIL)): Lewis rats were fed $1x10^8$ Brown Norway (BN) splenocytes every day for 5 days. Nine days later they were fed $1x10^8$ BN splenocytes and the following day they received the BN transplant. Five days later the rats were sacrificed and the kidneys removed for assessment of the GIL.

In one experiment a group of 5 Lewis rats received syngeneic (Lewis) spleen cells, and another group of Lewis rats, received PVG spleen cells as in protocol 1 above.

Histology

Transplanted kidneys were flushed with cold saline and cut into 2-3mm thick slices which were fixed in 10% neutral buffered formalin. Tissue was embedded in wax and sections were taken at 5μm. The sections were stained with hematoxylin and eosin using standard procedures.

Cytokine RT-PCR of GIL

GIL were isolated from the kidneys as previously described (13). Briefly, kidneys were removed from experimental animals 5 days post transplant. Kidneys were first flushed *in situ*, with ice cold heparinized saline until completely blanched. This ensured that circulating blood did not contaminate the cell suspension. The volume and weight of the kidneys was determined. The tissue was minced and then digested with collagenase, followed by a two step percoll centrifugation to remove tubular epithelial cells (13). Total cellular RNA was obtained using Trizol (BRL, Canadian Life Technologies, Burlington, Ont.). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (BRL) and random hexamer primers (Pharmacia Biotech Inc. Baie D'Urfe, Que.). PCR amplification (40 cycles) of the first strand cDNA product of

the reverse transcription was performed as previously described (14) using commercially synthesized primers (BioCan Scientific, Mississauga, Ont.) and Taq polymerase (BRL). The primers were constructed to span introns to exclude PCR amplification products from genomic DNA, and yield approximately similar sized amplicons (between 311-380 bp).

Statistical analysis

Survival curves were plotted using the Kaplan-Maier product limit estimate (15), and differences analyzed by the log-rank test for comparing survival curves (15). Differences in cellular infiltrates were analyzed using the Mann Whitney rank sum test (16).

Results

Survival of control rats and rats fed donor spleen cells either only PRE-Tx or both PRE- and POST-Tx

The survival curves for rats fed only pre-Tx (protocol 1) and both pre- and post-Tx (protocol 2) are shown in Figure 1 compared to the survival of animals which were untreated. As can be seen there is a marked prolongation of survival in the fed animals with several of them surviving beyond 50 days without any supplemental immunosuppressive therapy. The mean survival in the control group was 8.8±1.9 days, with the longest survival 11 days. In the group receiving pre-feeding only, all animals lived at least 15 days, with a mean survival of 21±8. The longest survival was 37 days. In the group receiving pre- and post- feeding the survival was very variable, but all animals lived at least 15 days with the longest survival 105 days. The mean for the group was 38.6±31.1 days.

Rats fed syngeneic or PVG spleen cells prior to transplant, rejected the BN spleens at the same rate as rats which were not fed allogeneic spleen cells (data not shown).

Survival of control rats, and rats pv injected with donor spleen cells pre-Tx (protocol 3) and of rats pv injected with donor spleen cells pre-Tx and fed donor spleen cells post-Tx (protocol 4)

The survival curves for rats pv injected pre-Tx only (protocol 3), and for rats pv injected pre-Tx and fed post-Tx (protocol 4) are shown in Figure 2 compared to the survival of animals which were untreated. There is a marked prolongation of survival in the pv injected animals with or without post-Tx feeding, although the latter showed slightly longer survival.

Histology of the kidneys

In the unfed animals, 5 days after transplantation, there is a marked mononuclear cell infiltration, although tubular and glomerular structures are still preserved (Figure 3A). However by day 10, there is obvious tubular destruction, as well as glomerular sclerosis (Figure 3B). There also appears to be a substantial mononuclear cell infiltrate on day 5 in the fed animals (Figure 4A), so that the histological picture on day 5 appears similar to that in the unfed animals. However, in the fed animals, there was still a marked preservation of tubular and glomerular structure on day 23 (Figure 4B), and even in an animal sacrificed on day 105 (Figure 4C), tubular and glomerular structure, although now abnormal, was conserved.

Assessment of the graft infiltrating cells

As can be seen in Figure 5A, there was a significant decrease in the number of recoverable graft infiltrating cells per kidney from the fed compared to the unfed rats ($p < 0.05$). The data regarding T cell infiltration of the graft (Fig. 5A) mirrors the drop in total leukocytes. Analysis of T cells as a percentage of GIL (Fig. 5B) confirms the association between feeding and the generalized reduction of leukocyte infiltration in the graft since T cells represent a nearly similar percentage of the GIL in both groups. Since our previous experimentation (13) has shown that GIL are almost exclusively T cells and macrophages, this data is consistent with a similar reduction in macrophage numbers in the graft.

Since kidneys showed a variable amount of edema during rejection, the expression of GIL per unit volume, or per gram wet weight, is an unreliable measure of recording infiltrating cells. The GIL per kidney gives values relevant to the influx of cells into the kidney.

RT-PCR

To begin to determine the cytokine secretion profile of the GIL, RT-PCR was performed using primers specific for IL-2, IL-4, IL-6, IFN-γ and TGF-β1. In the untreated allograft control animals IL-2, IL-6 and IFN-γ mRNAs were detectable but there was little or no IL-4 or TGF-β1 mRNA in the GIL. However, in all the Lewis rats fed with BN spleen cells prior to transplantation, in addition to the IL-2 and IFN- mRNA, there was also substantial levels of IL-4 and TGF-β (Figure 6).

Figure 1.The survival for rats fed both pre (protocol 1) and pre- and post-Tx (protocol 2) is shown compared to the survival of animals which were untreated. As can be seen there is a marked prolongation of survival in the fed animals with 25% of the pre- and post-Tx fed animals surviving beyond 50 days, without any other immunosuppressive therapy. There is a significant increase in kidney graft survival in animals pre-fed only ($p < 0.005$) and in animals both pre- and post-Tx fed ($p < 0.001$) compared to unfed animals. There is a trend towards longer survival in the pre- and post-Tx fed animals compared to the pre-fed only animals ($p = 0.077$). At 15 days 44% of the pre-fed only animals were still alive, compared to 75% of the animals pre- and post-fed.

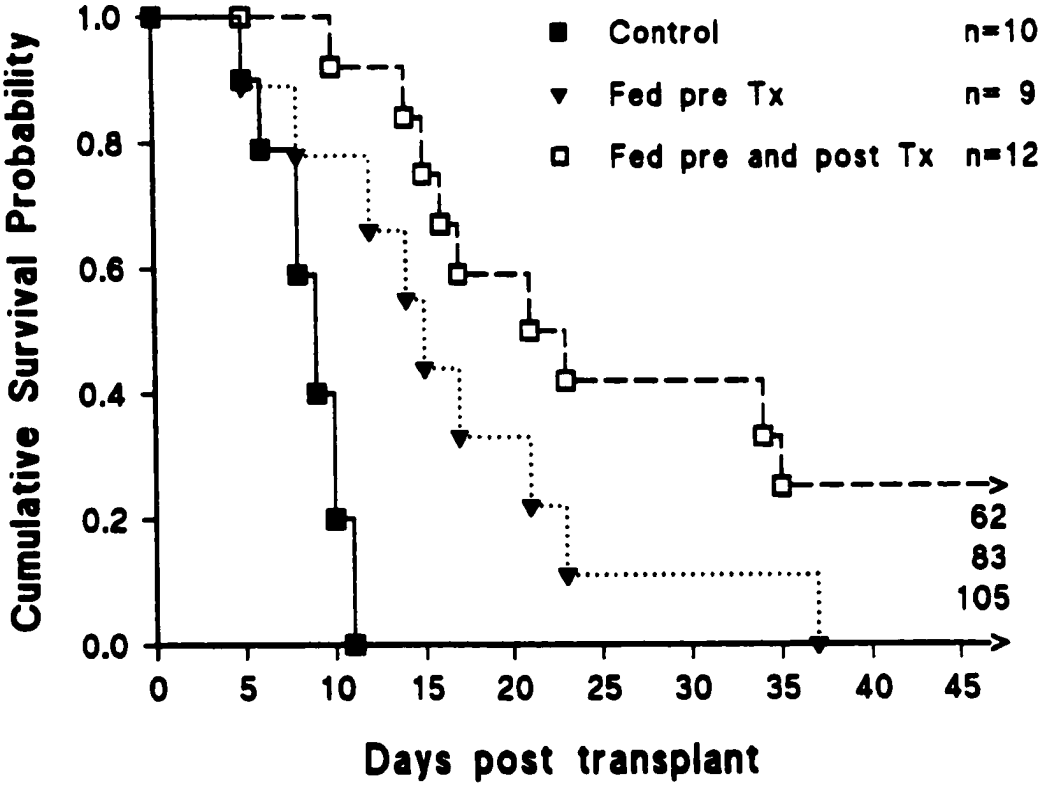
Cumulative Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
45
Days post transplant
Control n=10
Fed pre Tx n= 9
Fed pre and post Tx n=12
62
83
105

Figure 1

Figure 2. The survival curves for rats pv injected pre-Tx (protocol 3) and for rats pv injected pre-Tx and fed post-Tx (protocol 4) are shown compared to the survival of animals which were untreated. As can be seen, there was a marked prolongation of survival in the pv injected animals with or without post-Tx feeding ($p < 0.001$ for both groups) compared to untreated rats. There is no significant difference between the survival of PV-only injected animals and the animals which were pv injected and post-Tx fed.

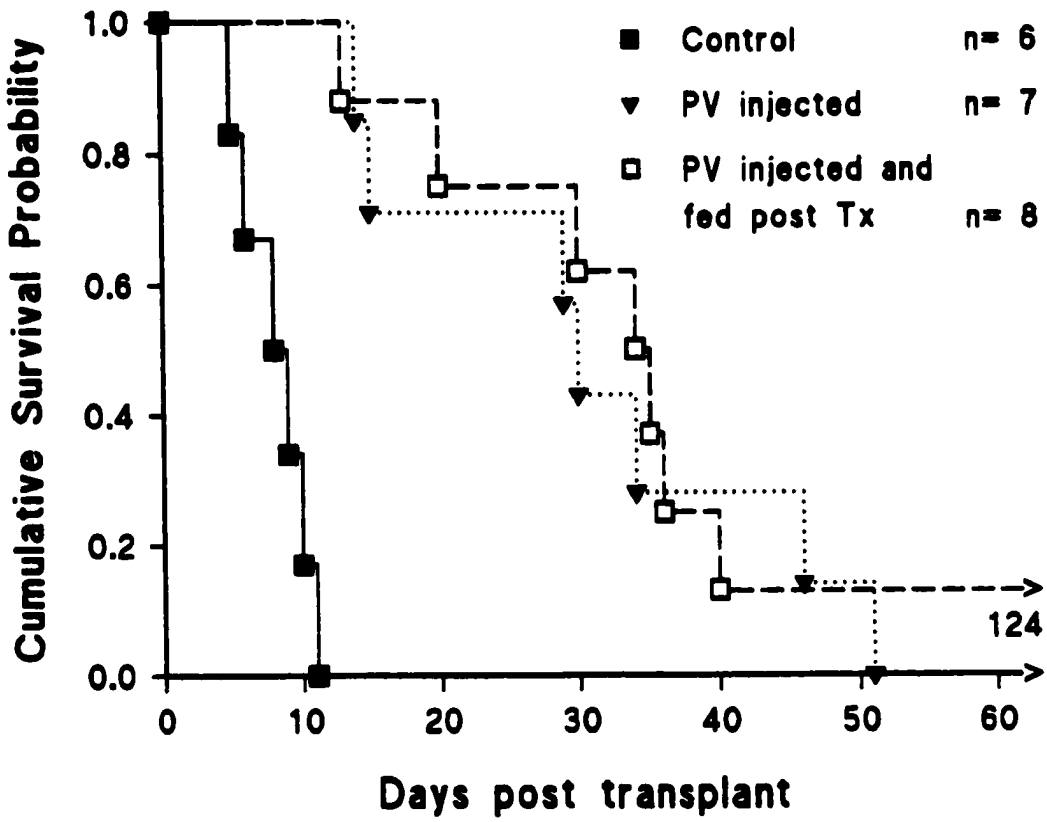
Cumulative Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Days post transplant
Control n= 6
PV injected n= 7
PV injected and fed post Tx n= 8
124

Figure 2

Figure 3. A) Hematoxylin and eosin (H&E) stained section of transplanted kidney, 5 days after transplantation into an unfed control. B) H&E section 10 days after transplant into an unfed control.

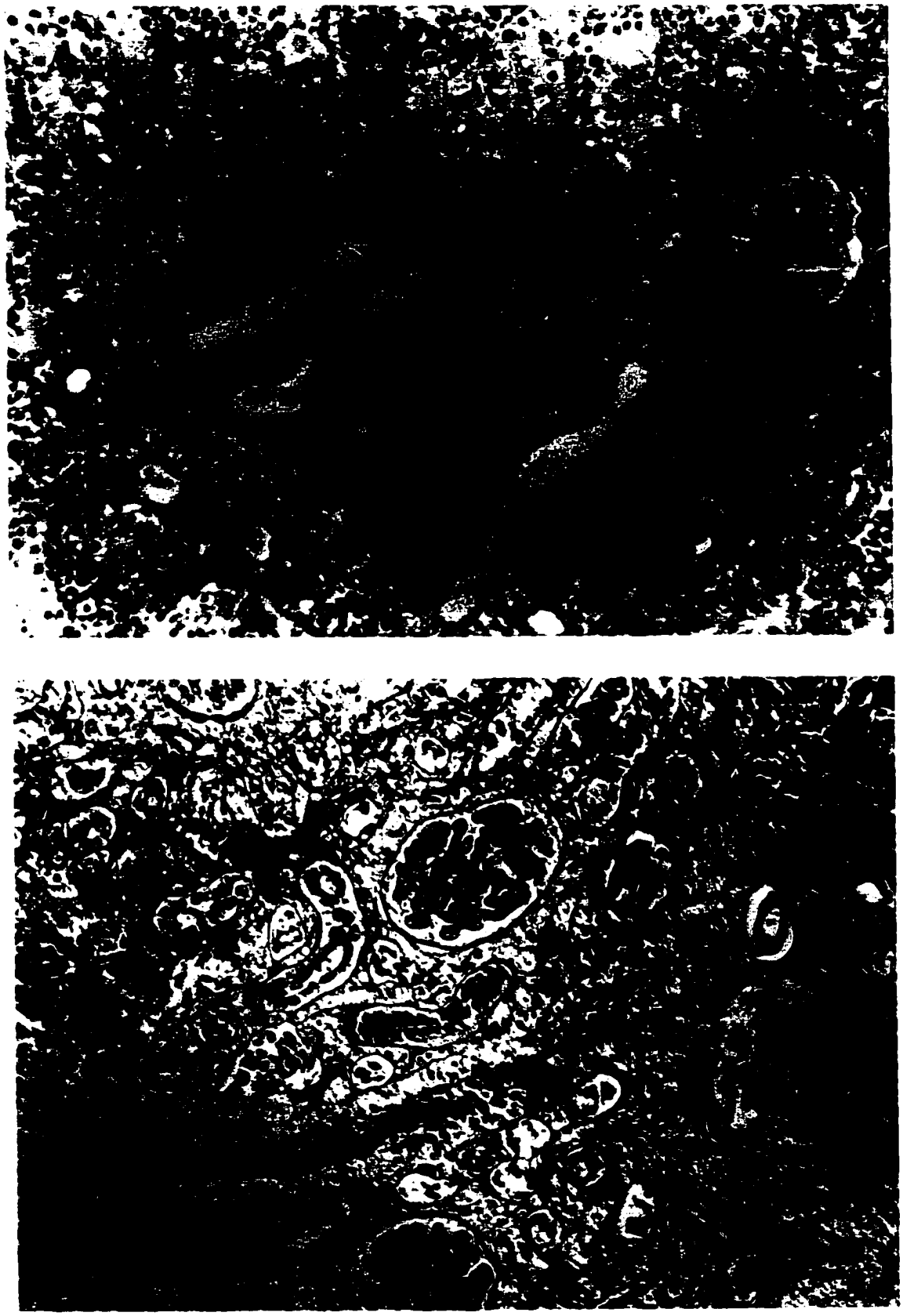

Figure 3

Figure 4. A) H&E section of transplanted kidney, 5 days after transplanting into a recipient fed donor spleen cells. B) H&E section of transplanted kidney, 23 days after transplantation into a fed animal. C) H&E section of transplanted kidney, 105 days after transplantation into a fed animal.

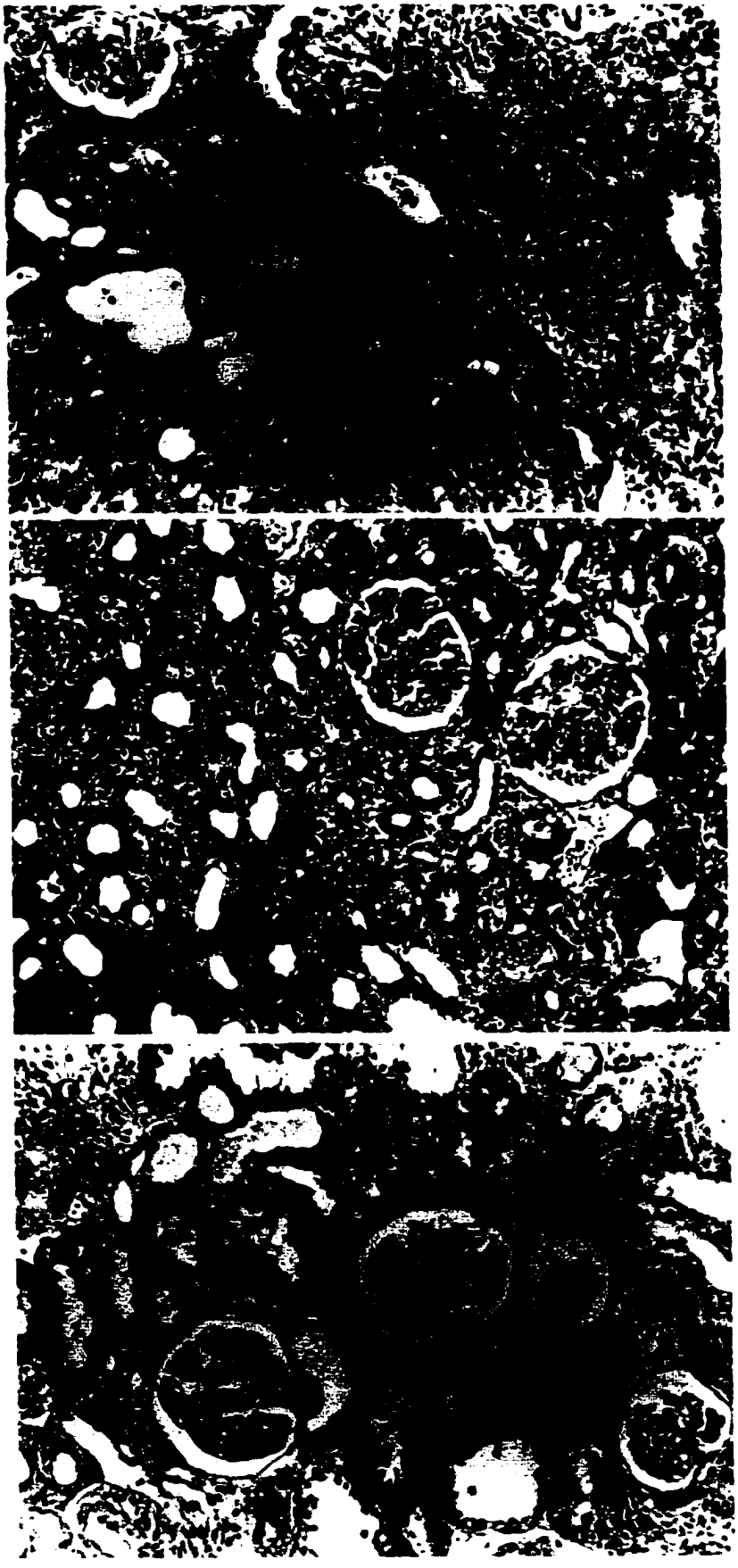

Figure 4

Figure 5. A) Examination of the total number of graft infiltrating cells and of T cells recoverable from the transplanted kidneys of orally treated and control transplant recipients. B) T cells as a % of total GIL. * indicates a significant difference ($p < 0.05$) between the control and the pre-fed animals for the parameter being measured.

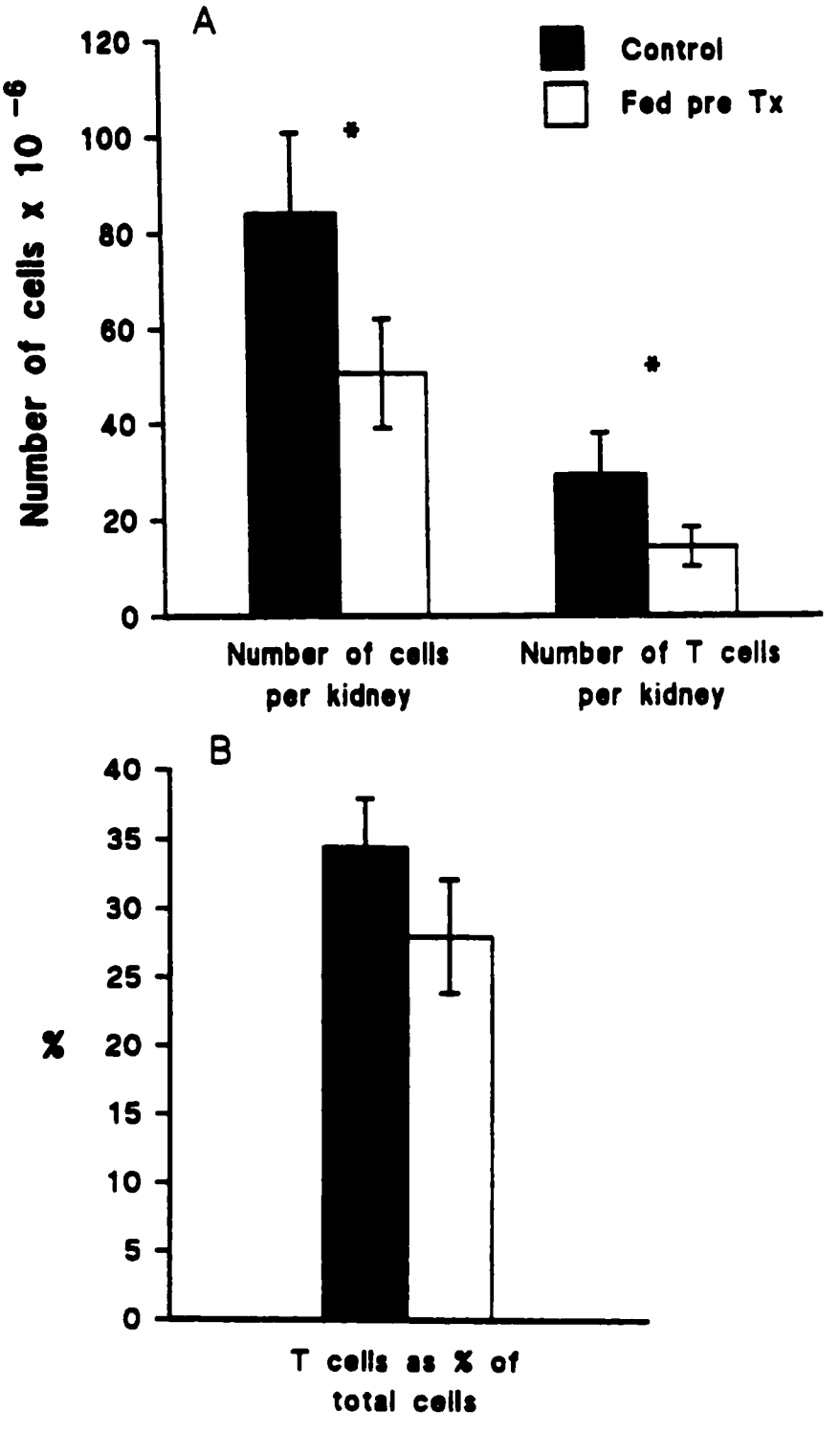
A
Control
Fed pre Tx
Number of cells x 10 -6
120
100
80
60
40
20
0
*
*
Number of cells per kidney
Number of T cells per kidney
B
%
40
35
30
25
20
15
10
5
0
T cells as % of total cells

Figure 5

Figure 6. RT-PCR of graft infiltrating leukocytes from kidney allografts in control (A) unfed and (B) orally treated recipients at day 5 post transplant. Lanes containing no DNA (-) and β-actin cDNA (+) were used as negative and positive controls for the PCR reaction respectively. RT-PCR of β-actin mRNA was used to control for relative mRNA abundance in the initial cell RNA preparations.

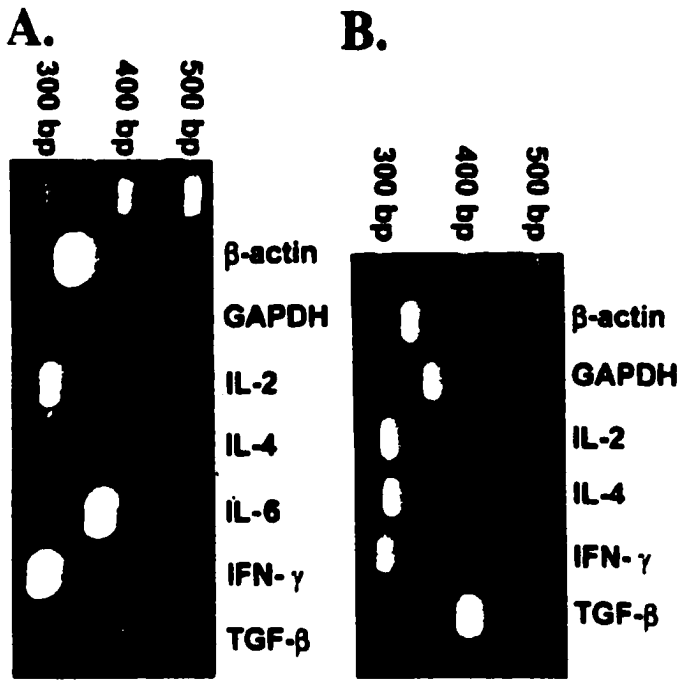
A.
300 bp
400 bp
500 bp
β-actin
GAPDH
IL-2
IL-4
IL-6
IFN-γ
TGF-β
B.
300 bp
400 bp
500 bp
β-actin
GAPDH
IL-2
IL-4
IFN-γ
TGF-β

Figure 6

Discussion

In 1978, Kagnoff (17) found priming of precursor cytotoxic T cells by antigen feeding, and failed to find suppression of allograft cytotoxicity by attempts to induce oral tolerance (18). However a 1984 study by Hashimoto (19), although not of oral induction of tolerance to histocompatibility antigens *per se*, was clearly suggestive that such might be a possibility. In that study, syngeneic tumours were transplanted into animals fed high doses of killed tumour cells. Such animals showed significant suppression of the normal rejection response expected to occur against the tumour specific transplantation antigens (19). In preliminary studies, we found suppression of alloreactivity in mice after feeding allogeneic spleen cells (20). Thus we decided to pursue the possibility that by feeding donor spleen cells to recipient rats, we might be able to prolong kidney graft survival. The results reported here do indeed support the concept that oral administration of allogeneic splenocytes can markedly prolong kidney allograft survival without additional immunosuppressive therapy. Conversely, feeding syngeneic (Lewis) or MHC unrelated (PVG) spleen cells, had no effect on the rate of BN kidney allograft rejection (data not shown).

Recently He and colleagues reported that oral tolerance, induced by feeding donor keratinocytes, epithelial cells, or endothelial cells, significantly prolonged corneal allograft survival in mice (21), but the results presented in this paper are the first demonstrating a marked prolongation of solid organ primary allograft survival induced by oral tolerance.

Others have demonstrated solid organ allograft prolongation in mice with portal venous inoculation of allogeneic cells (22,23). We have confirmed that finding using the portal vein rather than the oral route to deliver allogeneic splenocytes. There has long been a suggestion that oral tolerance and portal tolerance are linked. Early studies

indicated that bypassing the portal system by porta-caval shunting of the intestinal venous drainage reduced or abrogated orally induced tolerance (24,25). We found that oral tolerance appeared to be more effective than portal tolerance, but given the recent findings by Gorczynski et al. (26,27) it appears likely that both routes of tolerance induction are associated with the transcription of Th2 cytokines.

A number of mechanisms have been suggested to be involved in the development of oral tolerance. These include the generation of T suppressor cells (Ts) (28-30), development of helper T cell anergy (31,32), immune complex formation (33), production of suppressive serum factors (34,35), antigen alteration by intestinal processing (36-38), and production of anti-idiotype antibody (39,40).

In light of the description of Th1 and Th2 subsets of T cells (41) and the subsequent demonstration of their cross regulation (42), as well as substantial evidence that cell mediated immunity (involving type-1 pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α), is involved in kidney allograft rejection (reviewed in 43, 44), it is possible to speculate that the oral tolerance observed here, results from a shift in the bias in GIL from Th1 type cells in the acutely rejecting allograft in the unfed animals, to Th2 type cells in the allografts of orally tolerized animals. The increase in IL-4 mRNA in the graft infiltrating lymphocytes (typical of Th2 type cells) reported here, is supportive of this hypothesis. The presence of IFN-γ and IL-2 in GIL preparations is not inconsistent with this hypothesis since the paradigm postulated by Lowry (45) and others suggests that the "shift" is certainly associated with transcription of IL-4, but other cytokines are also transcribed. This is consistent with the evidence that shows Th2 cell generation *in vitro* in the presence of IFN-γ and IL-4, but Th1 generation in the presence of IFN-γ alone (46). Thus the presence or absence of IL-4 is more important in the Th2 shift, than is a reduction of IFN-γ levels. Our findings are consistent with the work of others (47)

who have demonstrated an increase in the immunohistochemical staining for IL-4 protein in the allograft tissue, in a heterotopic cardiac graft model in which accelerated second set rejection was somewhat retarded when recipients were fed purified or synthesized donor MHC alloantigens.

In the case of orally induced suppression of autoimmune encephalomyelitis, Chen et al. (48) found evidence that another T cell subset secreting high quantities of TGF-β (which they referred to as a Th3 type cell), might play a critical suppressive role. Our finding of increased TGF-β mRNA (typical of the putative Th3 type cells) in the GIL suggests that the observed suppression may be multifactorial. Of considerable interest, Sayegh and colleagues (49) have reported that rats fed allogeneic cells, showed an alloantigen specific reduction in delayed type hypersensitivity reaction *in vivo*, and in mixed lymphocyte reactions *in vitro*. Using an accelerated allograft rejection model, they also showed suppression of accelerated rejection of heterotopic cardiac grafts by feeding cells from Brown Norway (BN) rats to Lewis Rats (50). Examination of the grafts in treated animals showed decreased infiltration with macrophages, T cells in general, and IL-2 receptor positive T cells. There was also decreased mononuclear cells staining for IL-1, IL-2, IL-6, IL-8, and TNF-α. In contrast IL-4 was markedly increased (47). We likewise found a generalized drop in cellular influx into our primary kidney allografts. Our previous work (13) has shown that kidney GIL are predominantly macrophages and T cells and our data here point to a reduction in both of these cell types in the GIL of the fed animals, consistent with a T cell mediated TGF-β effect.

Weiner's group suggested that oral alloantigen appears to inhibit the function of $CD4^+$ Th1 like cells (IL-2 and IFN-γ producing) but does not inhibit the function of Th2 cells, which may be secreting protective cytokines (such as IL-10 and/or IL-4) (39). They have not however demonstrated increased production of TGF-β in their cardiac

transplant model, which may be related to their failure thus far to show suppression of a first set rejection. In addition, they found decreased amounts of IL-2 and IFN-γ, whereas we did not find a significant decrease in transcript levels of these two cytokines in the graft infiltrating lymphocytes in our model (by RT-PCR).

Friedman and Weiner have recently reported that, high dose feeding induced only anergy, but low dose feeding induced active suppression (51). Their work also suggested that TGF-β is the non-specific suppressor effector factor secreted by Ts. Although these authors originally suggested that a high dose single feed results in both Ts induction and Th anergy, their recent results suggest that T cell anergy, without induction of Ts is induced by high dose feeding (51).

Our data is consistent both with a shift in bias from Th1 to Th2 type GIL and also the induction of TGF-β secreting Ts, supporting the possibility of a multifactorial process in the development of orally induced transplantation tolerance. Two recent reviews have also pointed out the probability that both transplantation rejection and the induction of transplantation tolerance are indeed multifactorial (52,53). Further experimentation will be needed to elucidate the mechanism(s) of oral tolerance responsible for the marked prolongation in graft survival we have found.

References

1. Weiner, HL, Friedman, A, Miller, A, et al. Oral tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu Rev Immunol 1994; 12:809.
2. Weiner, HL, Mayer, LF (editors). Oral Tolerance: Mechanisms and applications. Ann NY Acad Sci 1996; 778:1.
3. Kenick, S, Lowry, RP, Forbes, RDC, Lisbona, R. Prolonged cardiac allograft survival following portal venous inoculation of allogeneic cells: What is "Hepatic Tolerance"? Transplant Proc 1987; XIX:478.
4. Kamei, T, Callery, MP, Flye, MW. Pretransplant portal venous administration of donor antigen and portal venous allograft drainage synergistically prolong rat cardiac allograft survival. Surgery 1990; 108:415.
5. Oka, T, Yoshimura, N, Matsui, S, Hamashima, T, Lee, CJ, Ohsaka, Y. The effects of peri-operative portal venous inoculation with donor lymphocytes on renal allograft survival in the rat. I. Specific prolongation of donor grafts and suppressor factor in serum. Transplantation 1990; 49:167.
6. Hamashima T, Yoshimura N, Matsui S, Lee CJ, Ohsaka Y, Oka T. The effects of peri-operative portal venous inoculation with donor lymphocytes on renal allograft survival in the rat II. Phenotypic and functional analyses of graft-infiltrating cells. Transplantation 1990; 49:171.
7. Campbell, DA, Lorber, MI, Sweeton, JC, Turcotte, JG, Niederhuber, JE, Beer, AE. Breast feeding and maternal-donor renal allografts: Possibly the original donor-specific transfusion. Transplantation 1984; 37:340.

8. Zhang, L, Van Bree, S, Van Rood, JJ, Claas, FHJ. Influence of breast feeding on the cytotoxic T cell allorepertoire in man. Transplantation 1991; 52:914.

9. Bierme, SJ, Blanc, M, Abbal, M, Fournie, A. Oral Rh treatment for severely immunized mothers. Lancet 1979; I:604.

10. Korenblatt, EE, Rothberg, RM, Minden, P, Farr, RS. Immune responses of human adults after oral and parenteral exposure to bovine serum albumin. J Allergy 1968; 41:226.

11. Husby, S, Mestecky, J, Moldoveanu, Z, Holland, S, Elson, CO. Oral tolerance in humans: T cell but not B cell tolerance after antigen feeding. J Immunol 1994; 152:4663.

12. Kruisbeck, AM. Isolation and fractionation of mononuclear cell populations. In: Coligan, JE, Kruisbeck, AM, Margulies, DH, Shevach, EM, Strober, W, eds. Current Protocols in Immunology. New York: John Wiley and Sons 1991:3.1.1.

13. Ledingham, DL, McAlister, VC, Ehigator, HN, Giacomantonio, C, Theal, M, Lee, TDG. Prolongation of rat kidney allograft survival by nematodes. Transplantation 1996; 61:184.

14. Kearsey, JA, Stadnyk, AW. Isolation and characterization of highly purified rat intestinal intraepithelial lymphocytes. J Immunol Methods 1996; 194:35.

15. Glantz, SA. How to analyze survival data. In: Primer of Biostatistics 4th ed. New York: McGraw Hill 1997:371.

16. Glantz, SA. Alternatives to analysis of variance and the *t* test based on ranks. In: Primer of Biostatistics 4th ed. New York: McGraw Hill 1997:323.

17. Kagnoff, M.F. Effects of antigen feeding on intestinal and systemic responses. I: priming of precursor cytotoxic T lymphocytes by antigen feeding. J Immunol 1978; 120:395.

18. Kagnoff, MF. Oral tolerance. Ann NY Acad Sci 1982; 392:248.

19. Hashimoto, T, Okuno, K, Tsuchida, T, Fujiwara, H, Hamaoka, T. Tolerance induction in tumor-specific effector T cells by presensitization with tumor antigens via the intragastric route. Gann 1984; 75:610.

20. Carr, RI, Ledingham, D, Maloney, C, Akhi, K, McAlister, V, Bitter-Suermann, H, Lee, TDG. Induction of transplantation tolerance by prior feeding or portal vein injection of recipient with donor cells. Henry G. Kunkel Symposium, Rockefeller University, NY 1994.

21. He, YG, Mellon, J, Niederkorn, JY. The effect of oral immunization on corneal allograft survival. Transplantation 1996; 61:920.

22. Kamei, T, Callery, MP, Flye, MW. Pretransplant portal venous administration of donor antigen and portal venous allograft drainage synergistically prolong rat. Surgery 1990; 108:415.

23. Gorczynski, RM, Chan, Z, Chung, S, Cohen, Z, Levy, G, Sullivan, B, Fu, X-M. Prolongation of rat small bowel or renal allograft survival by pretransplant transfusion and/or by varying the route of allograft venous drainage. Transplantation 1994; 58:816.

24. Callery, MP, Kamei, T, Flye, MW. The effect of portacaval shunt on delayed-hypersensitivity responses following antigen feeding. J. Surg. Res. 1989; 46:391.

25. Yang, R., Liu, Q, Grosfeld, JL, Pescovitz, MD. Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction. J. Pediatr. Surg. 1994; 29:1145.

26. Chung, SW, Gorczynski, RM, Dziadkowiec, M, Levy, GA. Induction of T-cell hypo-responsiveness by intrahepatic modulation of donor antigen-presenting cells. Immunology 1994; 85:582.

27. Gorczynski, RM, Chen, Z, Hoang, Y, Rossi-Bergman, B. A subset of γδ T-cell receptor-positive cells produce T-helper type-2 cytokines and regulate mouse skin graft rejection following portal venous pretransplant preimmunization. Immunology 1996; 87:381.

28. Richman, LK, Chiller, JM, Brown, WR, Hanson, DG, Vaz, NM. Enterically induced immunologic tolerance. I: Induction of suppressor T lymphocytes by intragastric administration of soluble proteins. J Immunol 1978; 121:2429.

29. Mattingly, JA, Waksman, BH. Immunologic suppression after oral administration of antigen I: Specific suppressor cells formed in rats Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. J Immunol 1978; 121:1878.

30. Mattingly, JA. Immunologic suppression after oral administration of antigen III: Activation of suppressor-inducer cells in the Peyer's patches. Cell Immunol 1984; 86:46.

31. Whitacre, CC, Gienapp, IE, Orosz, CG, Bitar, DM. Oral tolerance in experimental autoimmune encephalomyelitis III: Evidence for clonal anergy. J Immunol 1991; 147:2155.

32. Melamed, D, Friedman, A. Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. Eur J Immunol 1993; 23:935.

33. Andre, C, Heremans, JF, Vaerman, JP, Cambiaso, CL. A mechanism for the induction of immunologic tolerance by feeding antigen-antibody complexes. J Exp Med 1975; 142:1509.

34. Kagnoff, MF. Effects of antigen-feeding on intestinal and systemic immune responses III: Antigen-specific serum-mediated suppression of humoral antibody responses after antigen feeding. Cell Immunol 1978; 40:186.

35. Chalon, MP, Milne, RW, Vaerman, JP. *In vitro* immunosuppressive effect of serum from orally immunized mice. Eur J Immunol 1979; 9:747.

36. Strobel, S, Mowat, AM, Drummond, HE, Pickering, MG, Ferguson, A. Immunological responses to fed protein antigens in mice II: Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen. Immunology 1983; 49:451.

37. Mowat, AM, Lamont, AG, Strobel, S, MacKenzie, S. The role of antigen processing and suppressor T cells in immune responses to dietary proteins in mice. Adv Exp Med Biol 1987; 216A:709.

38. Bland, PW, Kambarage, DM. Antigen handling by the epithelium and lamina propria macrophages. Gastroenterol Clin North Am 1991; 20:577.

39. Bhogal, BS, Karkhanis, YD, Bell, MK, et al. Production of auto-anti-idiotypic antibody during the normal immune response. XII. Enhanced auto-anti-idiotypic-antibody production as a mechanism for apparent B cell tolerance in rabbits after feeding antigens. Cell Immunol 1986; 101:93

40. Chalon, MP, Milne, RW, Vaerman, J-P. In vitro immunosuppressive effect of serum from orally immunized mice. Eur J Immunol 1979; 9:747.

41. Mosmann, TR, Coffman, RL. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7:145.

42. Coffman, RL. Regulation and functional significance of T cell subsets. Chem Immunol 1992; 54:1.

43. Sykes, M. Immunobiology of transplantation. FASEB J 1996; 10:721.

44. Chen, N, Gao, Q, Field, EH. Prevention of Th1 responses is Critical for Tolerance. Transplantation 1997; 61:1076.

45. Lowry, RP, Takeuchi, T. The Th1, Th2 paradigm and transplantation tolerance. (Medical intelligence Unit) Austin: R.G. Landes Co. 1994:1

46. Abehsira-Amar, O, Gibert, M, Joliy, M, Thèze, J, Jankovic, DL. IL-4 plays a dominant role in the differential development of Tho into Th1 and Th2 cells. J. Immunol. 1992; 148:3820.

47. Hancock, WW, Sayegh, MH, Kwok, CA, Weiner, HL, Carpenter, CB. Oral, but not intravenous, alloantigen prevents accelerated allograft rejection by selective intragraft TH2 cell activation. Transplantation 1993; 55:1112.

48. Chen, Y, Kuchroo, VK, Inobe, J, Hafler, DA, Weiner, HL. Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. Science 1994; 265:1237.

49. Sayegh, MH, Zhang, ZJ, Hancock, WW, Kwok, CA, Carpenter, CB, Weiner, HL. Down-regulation of the immune response to histocompatibility antigens and prevention of sensitization by skin allografts by orally administered alloantigen. Transplantation 1992; 53:163.

50. Sayegh, MH, Khoury, SJ, Hancock, WW, Weiner, HL, Carpenter, CB. Induction of immunity and tolerance with polymorphic class II major histocompatibility complex allopeptides in the rat. Proc Natl Acad Sci USA 1992; 89:7762.

51. Friedman, A, Weiner, HL. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc Natl Acad Sci USA 1994; 91:6688.

52. Nickerson, P, Steiger, J, Zheng, XX, Steele, AW, Steurer, W, Roy-Chaudhury, P, Strom, TB. Manipulation of cytokine networks in transplantation False hope or realistic opportunity for tolerance? Transplantation 1997; 63:489.

53. Piccotti, JR, Chan, SY, Van Buskirk, AM, Eichwald, EJ, Bishop, DK. Are Th2 helper T lymphocytes beneficial, deleterious, or irrelevant in promoting allograft survival? Transplantation 1997; 63:619.

4.0 ORAL EXPOSURE TO ALLOANTIGEN GENERATES INTRAGRAFT $CD8^+$ REGULATORY CELLS

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The Full Title: Oral exposure to alloantigen generates intragraft CD8+ regulatory cells[1]

Juan Zhou*†, Ronald I. Carr*[2]‡, Robert S. Liwski*†, Andrew W. Stadnyk*§ and Timothy D. G. Lee*†.

Departments of *Microbiology and Immunology, †Surgery, ‡Medicine, and §Pediatrics, Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4H7.

Key words: transplantation, Th1/Th2, T lymphocytes, mRNA.

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2. Address correspondence and reprint requests to Dr. Ronald I. Carr, Department of Microbiology and Immunology, Transplantation and Immunology Research Laboratory, Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7. Phone: 902-494-7017, Fax: 902-494-5125, e-mail: ronald.carr@dal.ca.

Abbreviations used in this paper: GIC, graft infiltrating cells; MLN, mesenteric lymph nodes; BN, Brown Norway.

Abstract

We have previously reported that oral administration of allogeneic rat spleen cells prior to kidney allotransplantation significantly prolongs graft survival. This prolongation was alloantigen specific, and was associated with a decrease in graft infiltrating cells (GIC), and an increase in transcription of IL-4 mRNA in the GIC. In this study, increased splenic mixed lymphocyte responses from animals orally exposed to alloantigen prior to kidney transplantation suggested that the kidney allograft prolongation was not due to a masking of allorecognition but to an immunomodulation of the immune response. We have assessed GIC T cell subsets at day 5 post transplant and found decreased numbers of CD4+ T cells in fed animals compared to controls, but there was no change in CD8+ T cell numbers. The CD8+ GIC from fed animals transcribed substantial levels of perforin, granzyme and FasL mRNA indicating the presence of active cytotoxic T lymphocytes (CTL). Direct CTL assays showed that the GIC from fed recipients exhibited higher allo-CTL activity than GIC from control unfed recipients. In addition, the CD8+ GIC exhibited high levels of IL-4 mRNA, suggesting a Tc2 type regulatory cells. Prolonged graft survival in the face of active CTL and Tc2 cells suggests the presence of a CD8+ regulatory cell population in the allograft. To confirm this, cell transfer experiments were performed. Prolongation of graft survival was transferred from rats orally exposed to alloantigen, to naive animals by transfer of CD8+ GIC. This is the first report that oral exposure to alloantigen prolongs kidney allograft survival by the generation of intragraft CD8+ regulatory cells.

Introduction

Oral administration of antigen to ameliorate autoimmune disease has been demonstrated in a number of experimental animal models [1,2,3]. Recently, trials have been initiated to assess the feasibility of such treatments in the reduction of human disease [4]. However, prolongation of allograft survival by oral administration of alloantigen has been less well established. Weiner and colleagues reported that oral administration of allogeneic splenocytes ameliorated allo-sensitisation by skin grafting but only observed effects on second set (not primary) rejection of solid organ allografts with their feeding protocol [5,6]. We subsequently demonstrated, using a different protocol, that feeding allosplenocytes significantly prolonged kidney allograft survival in rats [7,8]. This prolongation was alloantigen specific [8]. Recently, this oral tolerance to primary allografts has been confirmed, by Ishido and colleges, in a rat cardiac model [9].

Oral tolerance has been suggested to be mediated by clonal deletion/anergy [1,4] or active suppression [4,10,11]. In active suppression, regulatory cells are thought to play a critical role by modulating the immune response through secretion of Th2 cytokines and/or TGF-β [4,12,13]. Such regulatory cells have been shown to be capable of transferring tolerance to naive recipients. In an experimental allergic encephalomyelitis (EAE) model, the cells that transferred this tolerance following oral administration of antigen were $CD8^+$ T cells [14]. An important role of $CD8^+$ regulatory T cells in the generation and maintenance of oral tolerance has also been suggested by experimentation in other models [15,16]. Recently, however, $CD4^+$ T regulatory cells have also been suggested to play an important role in the development of oral tolerance [17].

Although oral tolerance has been extensively studied in autoimmune disease models, it is only recently that oral tolerance has been demonstrated to prolong first set transplant survival [7,8,9]. As such, the mechanisms responsible for this graft prolongation by oral administration of alloantigen are still unclear. It has been reported that oral administration of alloantigen induces an antigen specific reduction in delayed type hypersensitivity responses [5,9,18], a decreased mixed lymphocyte response (MLR) [5,9] and a reduction in allospecific cytotoxic T lymphocyte (CTL) activity [18]. Feeding alloantigen has also been suggested by some to induce a Th subset "shift" from the type 1 (Th1) responses to type 2 (Th2) responses [5,6,9] and that this is associated with prolonged allograft survival [6,9,12]. In this study, we demonstrate the presence of active $CD8^+$ regulatory cells infiltrating grafts in orally tolerized animals. Adoptive transfer of these $CD8^+$ cells to naive rats transfers the graft tolerance seen in the original fed animals. The presence of high levels of mRNA for IL-4 in this $CD8^+$ population suggests that Tc2 graft infiltrating cells result from oral transplantation tolerance and mediate the graft prolongation.

This is the first report to demonstrate that oral exposure to alloantigen generates intragraft regulatory cells which are capable of transferring prolongation of allograft survival to naive animals. The intragraft regulatory cells are $CD8^+$ T cells, which may regulate rejection responses by Tc2 type activities or by Fas/FasL interaction with alloreactive T cells.

Materials and Methods

Animal model

Male Brown Norway (BN, $RT1^n$) and Lewis rats (LEW, $RT1^l$) purchased from Harlan Sprague Dawley (Indianapolis, IN) were used as donors and recipients, respectively. This strain combination is fully disparate at both major and minor histocompatibility complex loci. Animals were maintained in the Dalhousie University Faculty of Medicine animal care facility and provided with water and rodent chow *ad libitum*. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council on Animal Care.

Single cell suspensions

Lymphocytes were isolated from the spleen or mesenteric lymph nodes (MLN) and single cell suspensions prepared following standard protocols (as we have described previously, [8]). Cells were prepared, washed and used for in vitro experiments in RPMI 1640 medium (Gibco BRL, Life Technologies, Burlington, ON, Canada) supplemented with 20 mM HEPES (USB, Cleveland, OH), 100 U/ml Penicillin, 100μg/ml streptomycin and 10% fetal bovine serum (Gibco BRL). Spleen cells were purged of red blood cells (RBC) by lysis for 5 min with ACK erythrocyte lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). After lysis of RBC, spleen cells were washed in RPMI.

Oral tolerance induction

Lewis rats were fed, without anaesthetic, by intragastric intubation using a 3 1/2 Fr premature human infant feeding tube (Sherwood Industries, St. Louis, MO). All rats were

fed $1x10^8$ BN splenocytes suspended in 300 µl phosphate buffered saline (PBS; 0.15 M NaCl, 0.05 M Na_2PO4, pH 7.4) on days -14, -13, -12, -11, -10, and -1, with the day of kidney transplantation being day 0.

Kidney transplantation

Kidney transplantation was performed as described previously [8]. Briefly, BN rats were anaesthetized with sodium pentobarbital. After ligation of the abdominal aorta and vena cava proximal and distal to the renal artery and vein, the left kidney was perfused in situ with cold (4°C) heparinized 0.9% saline and removed with a cuff of the aorta and the renal vein, as well as a long piece of the ureter. The donor kidney was kept in cold saline during the preparation of the Lewis recipient. Following left nephrectomy of the recipient, the donor kidney was transplanted in an orthotopic position by end to side anastomosis of the cuff of renal artery to the abdominal aorta, and the renal vein was connected to the inferior vena cava. The ureter was attached by end to end anastomosis. Three days after the transplant the right native kidney was removed, leaving rat survival dependent on the transplanted kidney.

Mixed lymphocyte response (MLR) reaction

Lewis rat spleen, and MLN, responder cells were recovered from kidney transplant recipients at day 5 post-transplant. BN spleen cells were used as stimulators. Responder cells ($1x10^5$ cells/well) were cultured in NUNC 96-well round-bottomed plates with or without equal numbers of mitomycin-C (25µg/ml) treated stimulator cells in RPMI media. The plates were incubated at 37°C for 72 h, then pulsed with ^{3}H-thymidine (1µCi/well) for

18 h and harvested with an automated cell harvester (Skatron). Proliferation was assayed by ^{3}H-thymidine incorporation.

Isolation of graft infiltrating cells (GIC)

GIC were isolated as we have described previously [8]. Briefly, at 5 days post-transplantation, rats were sacrificed and kidney allografts were flushed in situ with heparinized (100 U/ml) 0.9% saline and removed from the rat. The kidney was minced and then digested in 25 ml collagenase solution (50 μg/ml collagenase (Gibco BRL), 20% FBS in RPMI). To clear the debris including dead tubular epithelial cells, the cell suspensions were rapidly passed down a loosely packed glass wool column (300 mg sterile glass wool in a 10 ml syringe), then mixed with isotonic Percoll (Pharmacia, Piscatawa, NJ) to a concentration of 30% Percoll and centrifuged at 460xg for 20 min at 10°C. The pellet was resuspended in RPMI, then loaded on top of a cushion of 70% Percoll (in RPMI) and centrifuged as above. The cells overlying the cushion were removed for GIC examination.

Flow cytometry

The leukocytes isolated from the kidney graft were incubated with the following primary antibodies: R73 (anti-αβ T cell receptor; PharMingen; San Diego, CA), OX-8 (anti-CD8; Serotec; Oxford, England), W3/25 (anti-CD4; Serotec) at a concentration of 10 μg/ml in PBS solution containing 0.5% w/v bovine serum albumin (Boehringer Mannheim, Indianapolis, IN). The secondary antibody was a polyclonal FITC-labelled anti-mouse IgG (Organon Teknika, Durham, NC). Following incubation with the FITC-labelled antibody at a 1:500 dilution, the cells were fixed with 1 % paraformaldhyde in PBS and examined on a

BD-FACScan.

Purification of CD8+ or CD4+ T cells from GIC

Graft infiltrating T cells were enriched by incubating GIC in warm nylon wool (Polysciences, Warrington, PA) columns (1 g nylon wool in a 10 ml syringe) with RPMI at 37 °C for 1 h to remove the majority of the B cells, macrophages and other accessory cells. The enriched population was usually 85% to 90 % T cells as analysed by flow cytometry. Enriched T cells were incubated with W3/25 or OX-8 (anti-CD4 or anti-CD8 antibody, supplied by Biotex, Edmonton, Al, Canada) for 30 min, before passage through immuno-enrichment columns (Biotex), which enrich for CD8+ or CD4+ cells by negative selection. Purity of CD8+ or CD4+ cells obtained by this method was normally greater than 97% (assessed by flow cytometry).

Direct cytotoxic T lymphocyte assay

Five days after transplantation, allograft kidneys were removed. Single cell suspensions of CD8+ cells from the kidney GIC were prepared as effector cells. ^{51}Cr (100 μCi for 1 h at 37°C) labelled BN spleen Con A (2 μg/ml for 72 h) blasts were used as target cells. The effector cells and target cells were plated in V-bottomed 96-well microtiter plates ($1x10^4$ target cells/well) at effector: target ratios ranging from 100:1 to 12.5:1 in RPMI media. After incubation at 37°C for 12 h, the plates were centrifuged and lysis was measured by ^{51}Cr release.

RT-PCR

Relative (rather than quantitative) RT-PCR was performed as we have described previously [8]. Briefly, total RNA was obtained from the CD8+ GIC (1-2 x10^6 cells), using Trizol (Gibco BRL) as suggested by the manufacturer. RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and random hexamer primers (Gibco BRL) according to the manufacturer's instructions. PCR amplification of the product of the reverse transcription was achieved with a mixture containing 1 x PCR buffer, 0.2 mM dNTP, 0.4 μm each of sense and antisense primers and 2.5 U of Taq polymerase (BRL) for 40 cycles. Ten μl aliquots of PCR products were analysed in ethidium bromide stained agarose gels. Primers were developed from the published sequences for the respective rat cytokines as we have previously described [8]. Experimental amplicons were compared to the housekeeping β-actin amplicon and relative amplification within samples was assessed.

Cell transfer

To generate GIC for cell transfer, Lewis rats were fed with 1x10^8 BN splenocytes on days -14, -13, -12, -11, -10, and –1 and given kidney transplants on day 0 as described above. For control GIC, unfed Lewis rats were used as recipients. At day 5 post transplantation, whole GIC, CD8+, or CD4+ cell populations were isolated from kidneys of fed and unfed animals. GIC were transferred intravenously into naive rats that had received a kidney transplant one day before the GIC injection. The GIC population isolated from one kidney was transferred into a single paired naive Lewis rat. Three days after kidney

transplantation, the recipient native right kidney was removed, leaving rat survival dependent only on the transplanted BN kidney.

Results

Effects of oral exposure to alloantigen on allorecognition

Five days after kidney transplantation, the spleen and MLN were removed from the rats orally exposed or not exposed to alloantigen. Cell proliferation was assessed by MLR using mitomycin C treated donor spleen cells as stimulators. In this experiment sensitization to alloantigen occurs in vivo in response to a kidney transplant. The MLR was used to assess whether prior oral exposure to alloantigen would decrease this sensitization induced by the graft. Fig.1 shows the results of the MLR using spleen cells (Fig. 1A) or MLN cells (Fig. 1B) derived from fed (orally exposed to alloantigen prior to kidney transplantation) or unfed rats. As shown in Fig. 1, prior oral exposure to alloantigen led to increased allosensitization, as seen by an increased response to allochallenge. Data is shown for a representative experiment and also shown is the mean percentage increase for 5 experiments. Previous alloexposure by the oral route does not lead to reduced allorecognition and activation, which would be indicated by a proliferative response lower than that seen in the control MLR where sensitization is generated by the kidney transplant. In fact in all experiments there was a significant increase over control MLR with effector cells from the animals previously exposed to alloantigen by the oral route (271.3 ± 77.4 % for spleen, 302.7 ± 56.2 % for MLN, taking the control mean as 100%). In contrast, effector cells from animals orally exposed to syngeneic spleen cells, or to third party (PVG) spleen cells did not show an increased MLR after kidney transplantation in comparison to control animals without prior alloexposure before kidney transplantation (syngeneic = 137.2 ± 41.4 % of control; third party exposure = 113.3 ± 28.6 % of control). These results show that the feeding of

allogeneic cells to induce antigen specific immune regulation does not prolong allograft survival by decreasing allorecognition. On the contrary, the treatment regimen appears to increase alloreactivity.

Phenotype of graft infiltrating cells

The data above show that increased survival of kidney allografts by prior alloexposure through the oral route was not due to decreased recognition of the allochallenge. Therefore we hypothesized that the increased survival would be due to a modulation in the generation, or effector activity, of CD8+ CTL in the graft. To examine T cell responses in the graft, we isolated GIC at day 5 post transplant. Using flow cytometry, we found that the GIC contained predominately T cells (approximately 65%; labelled with anti-TCR mAb, data not shown) and some macrophages (about 35%, labelled with anti-Mac-1 mAb, data not shown). We have previously observed that at day 5 post transplant, the total number of graft infiltrating T cells was decreased by 30% in comparison to controls [8]. The data in Fig. 2 shows that this decreased number of T cells is due to a decrease in the number of CD4+ cells. In this figure, data from a representative experiment (Fig. 2A) and the mean of 5 separate experiments (Fig. 2B) are shown. In fed animals CD4+ GIC are present at only 60% of control levels whereas there is no significant difference in the CD8+ GIC levels from fed and control animals. Thus, there appears to be no defect in the ability of CD8+ T cells to transit to the kidney allograft. This suggests either that this CD8+ T cell population does not contain CTL or that the CTL activity is somehow modulated.

CTL activity of GIC

To examine whether the CD8+ T cell population in the GIC of the animals orally exposed to alloantigen contained alloresponsive CTL, we assessed the expression of cytotoxic mediators in the CD8+ T cell population of the GIC and we also conducted a direct CTL assay. The mediators we assessed (by RT-PCR) were those well known to be associated with CTL activity, granzyme B and perforin. In addition, we assessed the presence of mRNA for FasL which mediates apoptosis in target cells by ligation of Fas. The results, shown in Fig. 3, clearly demonstrate the presence of abundant mRNA for the CTL mediators granzyme B and perforin in the CD8+ GIC T cell population from both control and fed animals. There is no obvious diminution in the expression of the message for these important cytotoxic mediators suggesting that CTL are present in the CD8+ T cell compartment of the GIC. Furthermore, FasL expression appears to be increased, rather than decreased, in the CD8+ T cell compartment of the GIC from the transplanted kidneys of animals previously exposed to alloantigen. These data indicate that CTL are present in the CD8+ T cell compartment of these GIC.

To directly assess CTL presence in the CD8+ T cell GIC population we performed a direct CTL assay [19] using BN allogeneic cells as targets. In this assay, CD8+ T cells from GIC were immediately incubated with allogeneic target cells, without an in vitro expansion of CTL precursors, to assess for the presence of mature fully active CTL in the fresh GIC population. The results, shown in Fig. 4, clearly demonstrate that the CD8+ T cell population in the GIC of both fed and unfed animals contains mature alloresponsive CTL. Indeed the CTL activity in the animals which had been orally exposed to alloantigen prior to

kidney transplant is consistently higher than the CTL activity in the animals without prior exposure to alloantigen by the oral route ($p<0.04$).

These data confirm that the prolongation of allograft survival mediated by allogeneic exposure by the oral route is not due to a deficiency in the generation of competent alloresponsive CTL or their presence to the graft. This indicates that this population must be exposed to intragraft modulatory effects.

Cytokine transcription of CD8+ GIC

It has been suggested that oral tolerance can generate CD8+ regulatory cells [14,20] and/or switch immune responses toward type 2 responses [7,12,17]. Since, in our experiments, the number of CD8+ GIC T cells remains the same in fed and unfed animals, but grafts survived much longer in the fed animals, we postulated that the phenotype of the CD8+ GIC T cells may have shifted to a type 2 CD8+ T cell phenotype (Tc2). Therefore, we investigated cytokine mRNA from these cells. As shown in Fig. 5, IFN-γ and TGF-β mRNA level were similar in CD8+ GIC T cells from both fed and unfed animals. However, IL-4 mRNA was only detectable in the CD8+ GIC T cells from fed animals, but not in the CD8+ GIC T cells from unfed controls. This data suggests that, in fed animals, at least some of the CD8+ graft infiltrating cells are Tc2 cells and potentially regulatory cells.

Transfer of CD8+ intragraft regulatory cells

The data above suggest that the CD8+ GIC may be involved in intragraft modulation as a regulatory cell in animals which have been exposed to alloantigen by the

oral route. To investigate the existence of regulatory T cells in the kidney, we transferred allograft GIC from fed or unfed animals into animals which had received a kidney allograft one day before. GIC for transfer were taken at day 5 post transplant, because our previous experimentation has shown significant damage to kidney grafts at this time point, in control animals, but preservation of kidney architecture in animals orally exposed to alloantigen (thus indicating the presence of active regulatory events at this time point).

The results, shown in Fig. 6, confirm the presence of a regulatory cell in the kidney allograft after oral exposure to alloantigen. Survival of the kidney grafts in the animals receiving GIC from allogeneic kidney grafts of animals orally exposed to alloantigen was significantly increased when compared to survival of grafts in animals receiving GIC from control grafts. The mean survival in the control group was 9 days with the longest survival being 12 days. The mean survival of the kidneys in animals receiving GIC transferred from orally treated animals was 22 days with the longest survival being 46 days ($p<0.03$). This data suggests that regulatory cells are present in the GIC of the allograft kidney of orally treated animals.

To confirm that the regulatory GIC are CD8+ T cells, we transferred purified CD8+ or CD4+ GIC populations from allogeneic kidney grafts of animals orally exposed to alloantigen. As shown in Fig. 7, transfer of CD8+ allograft graft infiltrating cells from orally pre-treated rats into naive rats significantly prolonged kidney allograft survival in those rats (mean survival = 89 d; longest survival = 215 d; $p<0.01$). CD4+ graft infiltrating cells, in contrast, did not confer increased survival (mean survival 10 d, longest survival 13 d; $p>0.05$). These data indicate that a population of CD8+, but not CD4+, intragraft

regulatory T cells are present in the kidney allografts of animals orally exposed to alloantigen. Note that purified CD8+ regulatory cells showed much more modulatory activity than whole GIC (compare Fig. 6 and 7).

Figure 1. Cell proliferation of lymphocytes harvested from Lewis rat spleen (A) and MLN (B) in response to mitomycin-C treated allogeneic BN splenocytes. Spleen and MLN were harvested 5 days post transplantation. The proliferation of splenocytes and MLN cells from fed animals significantly increased compared to unfed controls. In A (a) and B (a) the data are presented as mean (+/- SD) of triplicate wells from a representative experiment. A(b) and B(b) show the mean (+/- SE) of all 5 experiments, depicted as a percentage of proliferation in comparison to cells isolated from the unfed control. ($p<0.02$, One-way ANOVA).

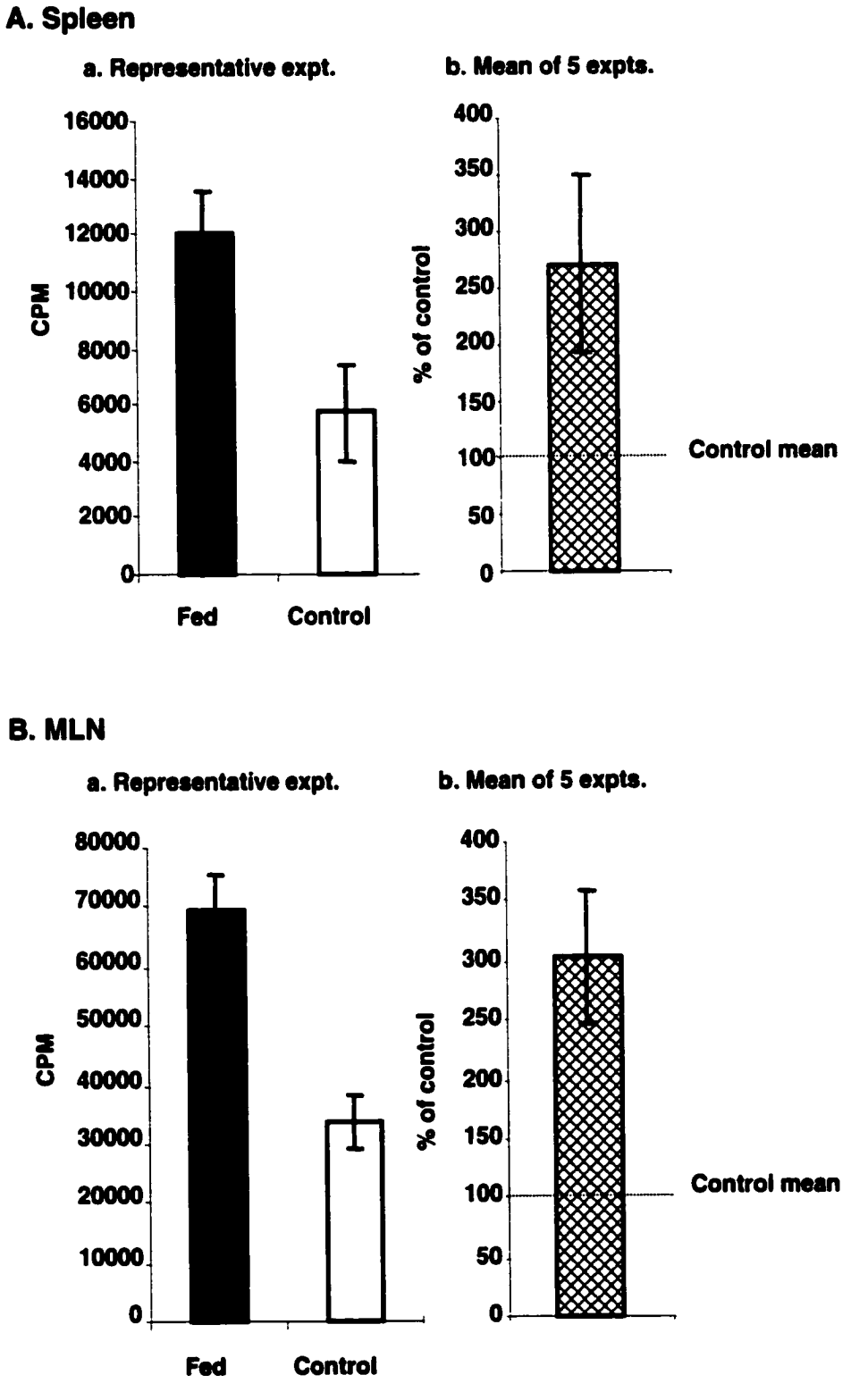
A. Spleen
a. Representative expt.
b. Mean of 5 expts.
CPM
16000
14000
12000
10000
8000
6000
4000
2000
0
Fed
Control
% of control
400
350
300
250
200
150
100
50
0
Control mean
B. MLN
a. Representative expt.
b. Mean of 5 expts.
CPM
80000
70000
60000
50000
40000
30000
20000
10000
0
Fed
Control
% of control
400
350
300
250
200
150
100
50
0
Control mean

Figure 1

Figure 2. Phenotype of graft infiltrating cells from day 5 post-transplanted kidneys. GIC were isolated and labeled with w3/25 (anti-CD4) or OX-8 (anti-CD8) mAb and analyzed by flow cytometry. A: A representative experiment of 5 separate experiments shows the total number of infiltrating T cells obtained from kidney transplanted into fed and control animals. B: Mean (+/- SE), from 5 separate experiments, of the percentage of cells of the particular phenotype in comparison to GIC isolated from unfed controls (CD4 vs control, $p<0.01$; CD8 vs control, $P>0.05$, One-way ANOVA).

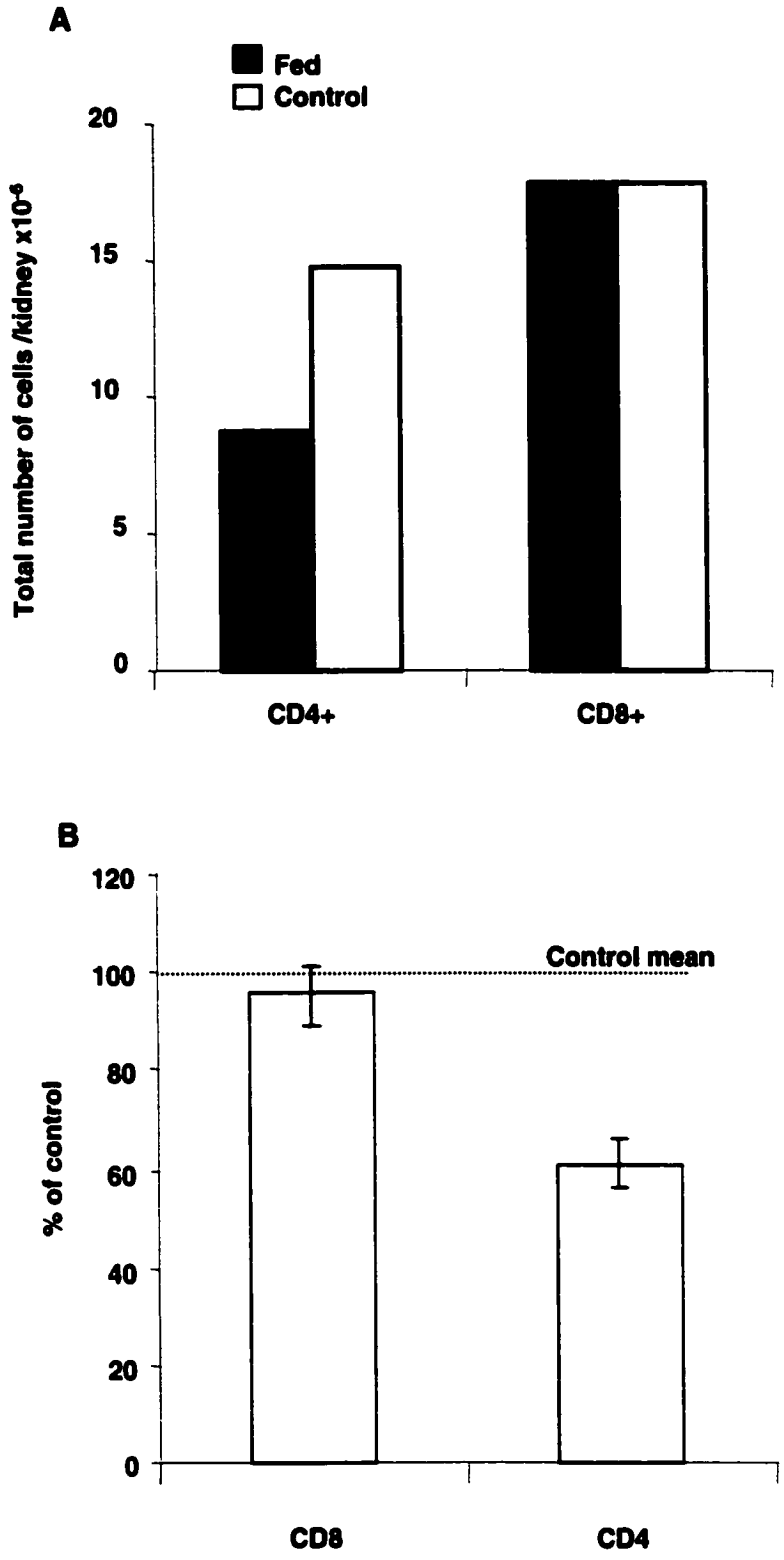
A
Fed
Control
Total number of cells /kidney x10⁻⁶
20
15
10
5
0
CD4+
CD8+
B
120
100
80
60
40
20
0
% of control
Control mean
CD8
CD4

Figure 2

Figure 3. Expression of perforin, granzyme and FasL mRNA (by RT-PCR) in CD8+ graft infiltrating T cells. T cells were harvested from the transplanted kidneys at day 5 post transplant from control (left) or fed (right) animals. β-actin amplification was used as a control.

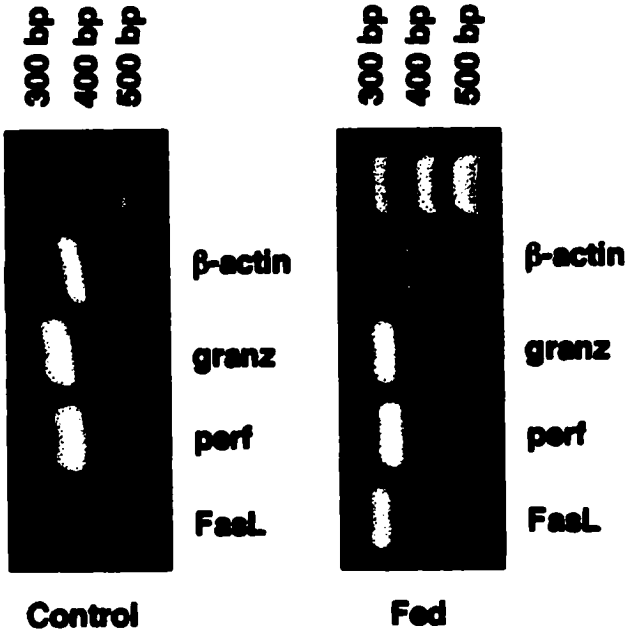
300 bp
400 bp
500 bp
β-actin
granz
perf
FasL
Control
300 bp
400 bp
500 bp
β-actin
granz
perf
FasL
Fed

Figure 3

Figure 4. Direct CTL activity of CD8+ GIC isolated from day 5 post-transplanted kidney allografts from fed and control animals. ^{51}Cr labeled allogeneic spleen Con A blasts were used as targets. These data are representative of 3 separate experiments. ($P<0.04$, Mann-Whitney)

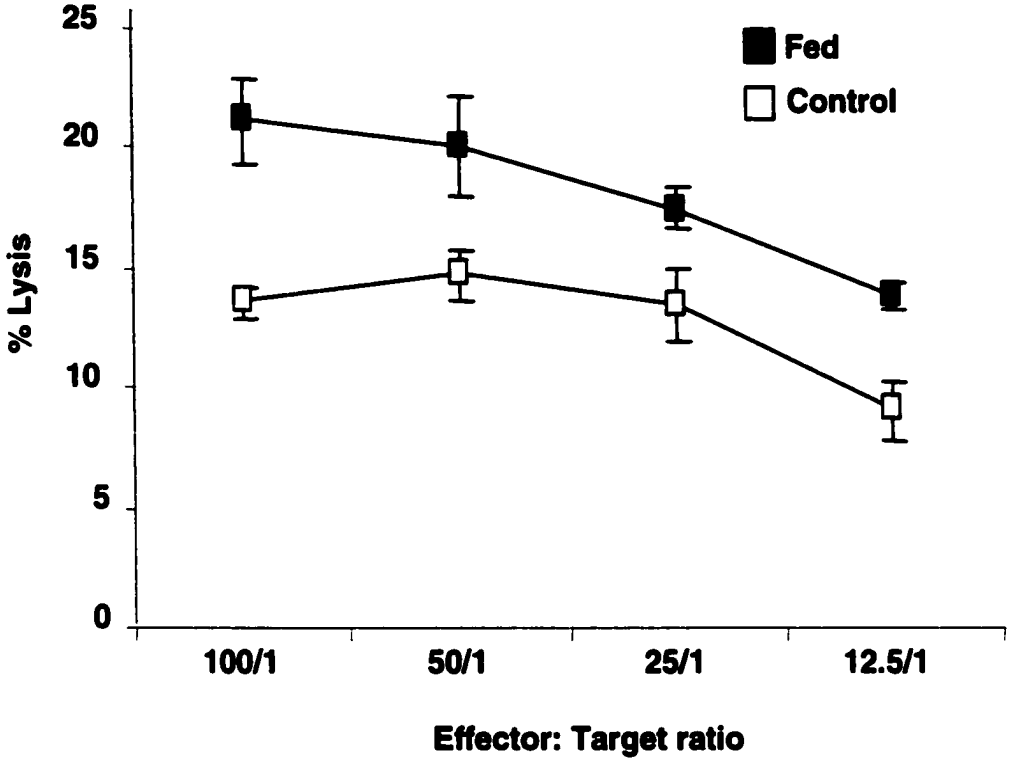
25
20
15
10
5
0
% Lysis
Fed
Control
100/1
50/1
25/1
12.5/1
Effector: Target ratio

Figure 4

Figure 5. Expression of IL-4, IFN-γ and TGF-β mRNA (by RT-PCR) in CD8+ graft infiltrating T cells. T cells were harvested from kidney at day 5 post transplant from control (left) and fed (right) animals. β-actin amplification was used as a control.

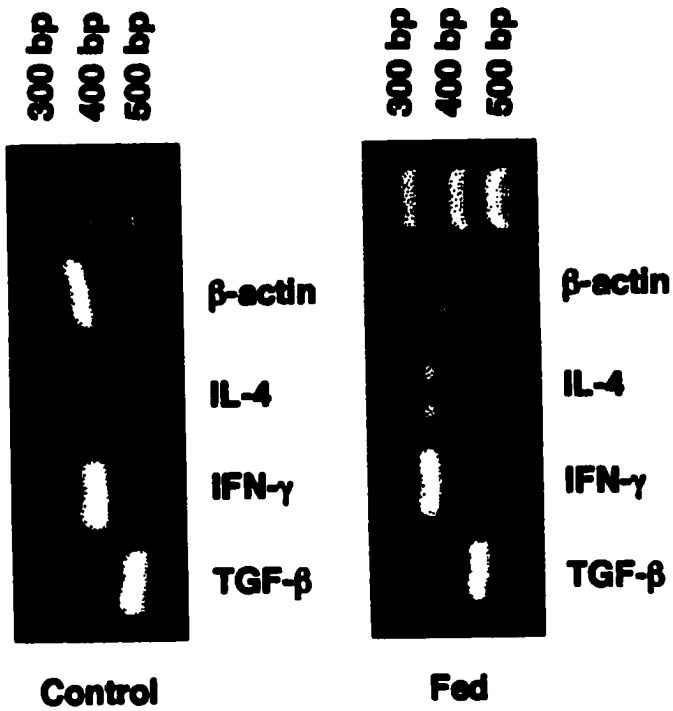
300 bp
400 bp
500 bp
β-actin
IL-4
IFN-γ
TGF-β
Control
300 bp
400 bp
500 bp
β-actin
IL-4
IFN-γ
TGF-β
Fed

Figure 5

Figure 6. Kidney allograft survival is enhanced by transferring total GIC derived from allograft kidneys in rats which had been orally exposed (▲, n=10) to alloantigen, but not by transferring total GIC from unfed control rats (■, n=5). Total GIC isolated from a single kidney allograft were transferred into a single recipient naive rat which had received a transplant one day earlier. ($p<0.03$, Mann-Whitney).

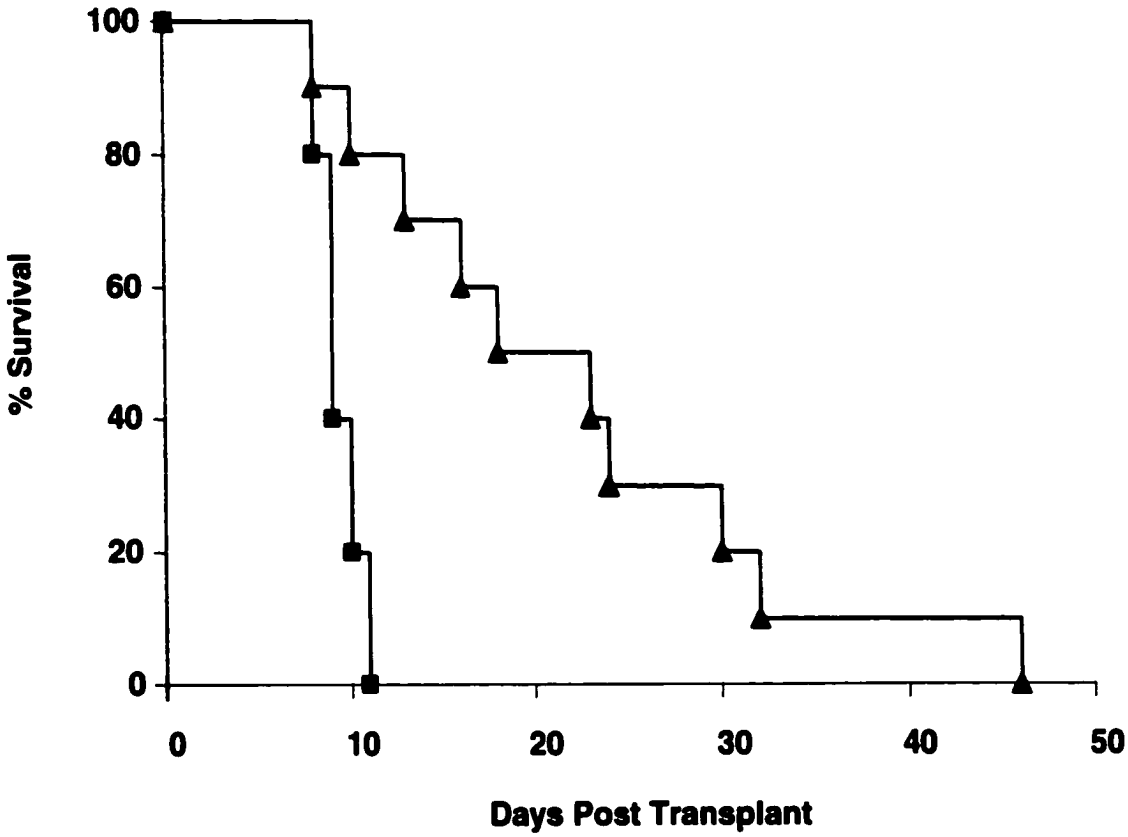

Figure 6

Figure 7. Kidney allograft survival is enhanced by transferring CD8+ GIC (♦, n=8), but not CD4+ GIC (0, n=5) or control GIC (■, n=5). Total GIC isolated from one kidney allograft were transferred into a recipient naive rat which had received a transplant one day earlier. (CD8 vs control, $P<0.001$; CD4 vs control, $p>0.05$, Kruskal-Wallis).

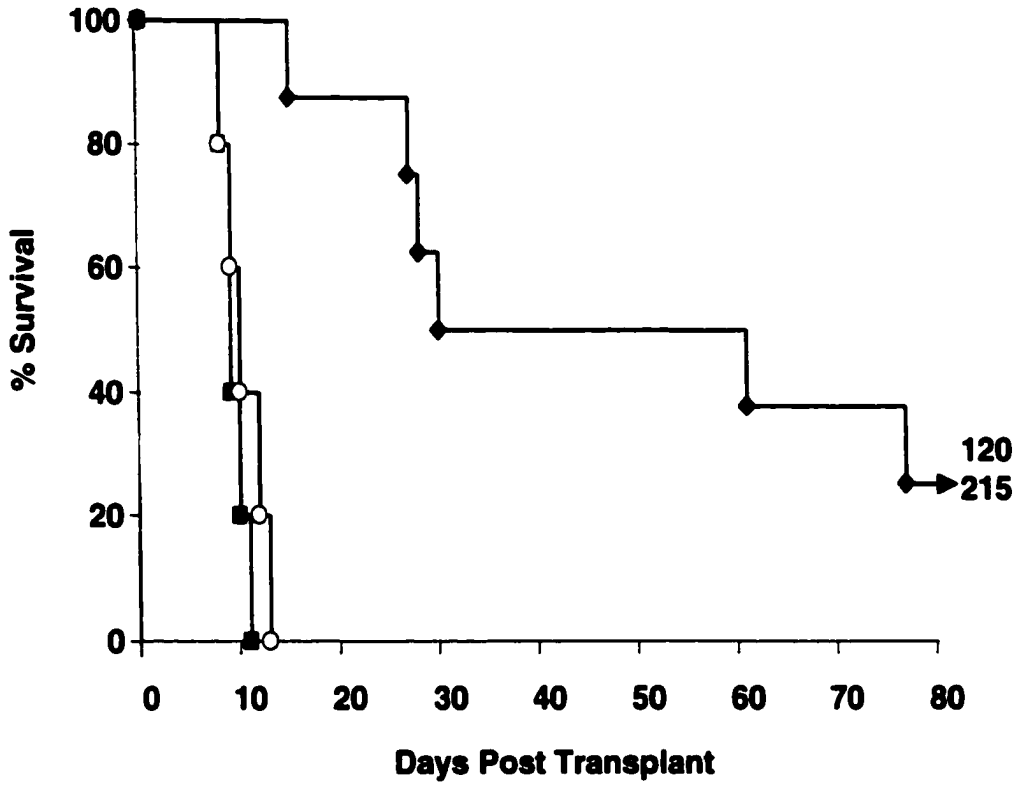
100
80
60
40
20
0
% Survival
0
10
20
30
40
50
60
70
80
Days Post Transplant
120
215

Figure 7

Discussion

Oral tolerance has been extensively studied in a variety of experimental models [4,10,11]. Depending on the dose and timing of the antigenic challenge, oral tolerance has been reported to result in clonal anergy or active suppression [4,10,11]. Recently, positive results ameliorating experimental immune encephalomyelitis (EAE) by inducing oral tolerance to myelin basic protein (MBP) have led to clinical trials of oral tolerance as a potential therapy for multiple sclerosis [4,13]. There is considerable evidence [15,16] that $CD8^+$ regulatory T cells play an important role in the generation and maintenance of oral tolerance. In the EAE model, oral tolerance-induced protection from disease could be adoptively transferred to naive hosts with $CD8^+$ T cells [14]. This is not to say that oral tolerance is absolutely dependent on the ability to generate $CD8^+$ regulatory cells. Weiner and colleagues [20], for example, have provided evidence that both $CD4^+$ and $CD8^+$ T cells play a role in the induction of oral tolerance in EAE and have also postulated the existence of a Th3, TGF-β secreting, T cell as a mediator in this complex interaction [17]. Others [11,21,22] have demonstrated oral tolerance can be induced in CD8-knockout mice and in mice depleted of $CD8^+$ T cells by anti-CD8 antibody treatment. Thus it appears that the generation of $CD8^+$ regulatory cells may be sufficient for, but not essential to, the development of oral tolerance.

In the experimentation described here, we have examined whether the generation of such regulatory cells plays a role in the significant prolongation of primary kidney allograft survival that we have observed following oral exposure to alloantigen [7,8]. We first assessed allorecognition by MLR in fed versus control animals. The MLR showed an increase in reactivity in the cells from the animals which had been previously exposed

to alloantigen by the oral route before transplantation. From this we conclude that there is no defect in allorecognition produced by induction of immune modulation by pre-exposure to alloantigen by the oral route. These data confirm that prolongation of kidney allograft survival is not due to masking of allorecognition, but to an immunomodulatory effect on the immune response, presumably the development of an immunomodulatory cell.

Past research in oral tolerance has primarily concentrated on events in the draining node (MLN) or spleen. The response to allogeneic transplants, however, is defined at the level of the organ transplant. We were the first to show that oral tolerance could prolong primary solid organ transplants [7,8] and we chose to use the kidney as our model so that cells infiltrating the graft would be readily available. On the premise that rejection is defined at the level of the organ we postulated that critical immunomodulatory events would also be defined at the level of the transplanted organ, and examining the graft infiltrating lymphocytes would be of prime importance for developing an understanding of oral transplantation tolerance. We isolated GIC at day 5 post transplant. This is the latest time point at which we can obtain GIC from control animals. We have reported previously that the total number of allograft infiltrating T cells in orally treated animals was decreased at this time by approximately 30% in comparison to normal untreated control allografts [8]. In the current study, we have found that this decreased number of T cells is due to a decrease in the number of $CD4^+$ cells. In contrast we found that the $CD8^+$ cells, a subset of which are presumably allospecific CTL, remain intact. $CD8^+$ T cells are the major source of CTL and have been suggested to destroy graft tissue predominantly through direct cytotoxic effects

against foreign class I MHC expressing cells [23]. Many treatments that prolong allograft survival, including anti-CD4 mAb [24], portal venous inoculation with alloantigen [25] and nematode infection [26] are associated with decreased numbers of $CD8^+$ T cells or decreased CTL activity. The undiminished numbers of intragraft $CD8^+$ T cells in our study suggests that the animals that have been orally pre-exposed to alloantigen do not have a defect in the generation or transit of active CTL to the allograft. However, it was possible that the $CD8^+$ T cells in the graft did not contain active CTL and that the modulation lay in a defect in $CD8^+$ CTL differentiation.

To address this we examined whether that the known characteristics of CTL, that is the expression of perforin and granzyme as well as FasL [27], were present in a highly purified $CD8^+$ T cell population from the GIC derived from allografts in orally treated animals. We found no substantial difference in the level of mRNA for the cytotoxic mediators perforin and granzyme in the GIC from orally treated animals versus controls. Further, FasL expression appeared to be increased, rather than decreased, in these highly enriched $CD8^+$ GIC T cell populations. This supports the hypothesis that similar numbers of potentially active mature CTL are present in the grafts of orally treated animals, even in the face of prolonged graft survival. This hypothesis was confirmed by our experimentation showing increased, rather than decreased, cytotoxicity to allogeneic targets in a direct CTL assay, thus amply demonstrating the presence of mature CTL in the GIC population. This increase in cytotoxicity could be due to the increased levels of FasL on these cells.

Although many studies have demonstrated that $CD8^+$ T cells are not absolutely essential for allograft rejection, including heart, kidney and skin allografts [28,29], CTL

have been suggested to play an important role in normal MHC-mismatched allograft rejection [30]. The potential mechanism of CTL involvement in acute renal allograft rejection has been suggested to be mediated by cytotoxic granule-based killing but not FasL induced killing. This is because granule proteins are expressed on biopsies of kidney undergoing acute rejection whereas FasL is expressed mostly in the absence of acute rejection episodes [31,32]. In addition, there is a paucity of FasL induced apoptosis seen in renal allograft rejection, which may be due to low levels of Fas expression on graft cells [31] or resistance of kidney tubule cells to FasL mediated events [33]. In our experiments, the presence of these mature, alloreactive, CTL in the orally treated animals exhibiting prolonged graft survival was perplexing. Interestingly, similar observations of increased CTL activity exhibited by GIC from kidney allografts have also been reported in studies of blood transfusion transplantation tolerance, although further characterization of the cells was not performed [34]. One potential explanation could be that the mature, alloreactive CTL fall under the control of regulatory elements in situ, and that they are somehow freed from those regulatory elements in the artificial conditions of the in vitro assay. Another possibility is that the increased CTL activity observed in our in vitro experiments is mediated by increased FasL expression, which may be less significant in vivo, as describe above. However, increased FasL expression by $CD8^+$ intragraft regulatory cells may in fact represent another level of regulation. Some immune privileged sites, such as the testes and the anterior chamber of eye, are thought to express their privileged phenotype because of the high levels of FasL expression in these sites [35-38]. Indeed, Swenson and co-workers [39] have provided evidence that kidney allografts transfected with FasL cDNA were protected from rejection, presumably

through interference with alloreactive T cells by a Fas/FasL interaction. In addition, high levels of FasL expression in renal biopsies from kidneys that are not undergoing acute rejection support the idea that some degree of FasL dependent immune privilege occurs in renal graft protection [32]. Therefore, it is possible that the observed $CD8^+$ T cells may be involved in graft protection, rather than destruction, due to their level of FasL expression which may interact with alloreactive Fas bearing T cells.

This study focused on $CD8^+$ GIC T cells isolated from allografts at day 5 post transplant, a time point where robust graft destruction occurs in control animals and the latest time point that we could obtain GIC from control transplants. We currently have no information on whether these $CD8^+$ T cells persist throughout the entire period of graft prolongation.

It has been suggested that type 1 immunity, mediated mainly by $CD4^+$ Th1 cells, is the major effector mechanism of allograft rejection, whereas type 2 immunity favors allograft survival [40,41]. A shift from type 1 T cell to type 2 T cell responsiveness has been reported to prolong allograft survival in several experimental models [6,12,26] and this shift has been associated with an increased presence of IL-4 [6,9,42]. Since IL-4 is the only type 2 cytokine which can be reliably correlated with type 2 T cell activity, the presence of increased levels of IL-4 mRNA in the $CD8^+$ GIC from allografts of fed animals indicates an increased intragraft type 2 response. IL-4 mRNA has often been found in whole graft extracts or whole GIC population of control rejecting grafts and in biopsies of human grafts, but has not been previously demonstrated in a graft infiltrating $CD8^+$ T cell population. This finding, in the context of the tolerizing protocol, suggests that the regulatory cell is a $CD8^+$ Tc2 cell [43,44].

The presence of regulatory cells can be demonstrated by in vivo cell transfer [45,46]. Others have shown that oral tolerance can induce regulatory cells in the spleen and draining lymph nodes, using autoimmune disease models [14,17]. We also found that regulatory cells are present in the spleen (the reactive node for kidney transplants) and MLN (the draining node of the gastrointestinal tract) following oral exposure to alloantigens. We were able to transfer graft protection using cells from these sites from fed animals but not from unfed control animals (Mean survival of rats that received splenocytes or MLN cells from fed animals was 38 and 24 d respectively, whereas the mean of survival of rats that received splenocytes or MLN cells from unfed control rats was 9 d). The presence of regulatory cells in the reactive node may indicate an effect of oral tolerance and the inductive phase of the responses. Importantly, however, we demonstrated that regulatory cells were also present in the kidney allograft of fed animals. Transfer of kidney graft infiltrating cells from orally tolerized animals into naive animals prolonged graft survival in these animals. This confirms that oral transplantation tolerance mediates a change at the effector site (i.e. within the transplant). We chose to harvest cells from the kidney allografts of orally pre-exposed animals at day 5 post transplant because (i) our preliminary histologic evidence has shown that immune modulation is ongoing at this point [8]; (ii) this is the point at which we know there are mature CTL in the GIC population of the graft; (iii) this is the latest time point that we could reliably obtain GIC from control transplants. We compared the effect of transfer of these cells harvested from orally treated animals with cells obtained from control, non pre-treated, animals on allograft survival in naïve recipients. Our results confirmed that

oral exposure to alloantigen generates intragraft regulatory cells which are present in the kidney allograft.

Once we had demonstrated that regulatory T cells exist in the kidney allograft of animals orally exposed to alloantigen it was important to differentiate between $CD4^+$ and $CD8^+$ effects. Since others have previously shown the existence of a $CD8^+$ regulatory cell in the spleens and lymph nodes of orally tolerized animals in other models [14], we suspected that regulation would occur in this compartment. The data obtained from our subsequent transfer experiments confirmed that $CD8^+$, but not $CD4^+$, T cells in the GIC transfer tolerance. Thus, animals which have been orally exposed to alloantigen develop $CD8^+$ regulatory cells which are present in the kidney at day 5 post transplant. This cell population will transfer graft prolongation to a naive animal receiving a kidney transplant. Interestingly, the transferred $CD8^+$ GIC were much better at transferring graft prolongation than transferred whole GIC, even though the same number of $CD8^+$ T cells were transferred in each instance (compare Fig. 6 and Fig. 7). This suggests that the whole GIC population contains both effector and regulatory cells.

These data do not rule out the possibility that of $CD4^+$ T cells may contribute to oral transplantation tolerance in ways other than active regulation. However, these data confirm that generation of CD8+ regulatory cells in response to oral pre-exposure to alloantigen is sufficient to induce graft prolongation. These intragraft regulatory cells are capable of transferring prolongation of graft survival to naive animals and may mediate their effects by IL-4 secretion and/or Fas/FasL interaction with alloreactive T cells.

References

1. Whitacre, C. C., I. E. Gienapp, C. G. Orosz and D. M. Bitar. 1991. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. *J. Immunol. 147:2155.*

2. Singh, V. K., H. K. Kalra, K. Yamaki and T. Shinohara. 1992. Suppression of experimental autoimmune uveitis in rats by the oral administration of the uveitopathogenic S-antigen fragment or a cross-reactive homologous peptide. *Cellular Immunol. 139:81.*

3. Thompson S. J., H. S. G. Thompson, N. Harper, M. J. Day, A. J. Coad, C. J. Elson and N. A. Staines. 1993. Prevention of pristane-induced arthritis by the oral administration of type II collagen. *Immunology 79:152.*

4. Weiner, H. L., A. Friedman, A. Miller, S. J. Khoury, A. Al-Sabbagh, L. Santos, M. Sayegh, R. B. Nussenblatt, D. E. Trentham, D. A. Hafler. 1994. Oral tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu. Rev. Immunol. 12:809.*

5. Sayegh, M. H., Z. J. Zhang, W. W. Hancock, C. A. Kwok, C. B. Carpenter and H. L. Weiner. 1992. Down-regulation of the immune response to histocompatibility antigens and prevention of sensitization by skin allografts by orally administered alloantigen. *Transplantation 53:163.*

6. Hancock W. W., M. H. Sayegh, C. A. Kwok, H. L. Weiner and C. B. Carpenter. 1993. Oral, but not intravenous, alloantigen prevents accelerated allograft rejection by selective intragraft Th2 cell activation. *Transplantation 55:1112.*

7. Carr, R. I., J. Zhou, D. Ledingham, C. Maloney, V. McAlister, M. Samson, H. Bitter-Suermann and T.D.G. Lee. 1996. Induction of transplantation tolerance by feeding or portal vein (PV) injection pretreatment of recipient with donor cells. *Annals of the New York Academy of Sciences. 778: 368-370.*

8. Carr, R. I., J. Zhou, J. A. Kearsey, A. W. Stadnyk and T. D. G. Lee. 1998. Prolongation of survival of primary renal allografts by feeding or portal vein injection of donor spleen cells. *Transplantation 66:976.*

9. Ishido, N., J. Matsuoka, T. Matsuno, K. Nakagawa and N. Tanaka. 1999. Induction of donor-specific hyporesponsiveness and prolongation of cardiac allograft survival by jejunal administration of donor splenocytes. *Transplantation 68:1377.*

10. Strober, W., B. Kelsall and T. Marth. 1998. Oral tolerance. *J. Clin. Immunol. 18:1.*

11. Garside, P and A. M. Mowat. 1997. Mechanisms of oral tolerance. *Critical Reviews Immunology. 17:119.*

12. Gorczynski, R. M., Z. Chen, H. Zeng, X. M. Fu. 1998. A role for persisting antigen, antigen presentation, and ICAM-1 in increased renal graft survival after oral or portal vein donor-specific immunization. *Transplantation 66:339.*

13. Weiner, H. L. 1997. Oral tolerance: Immune mechanisms and treatment of autoimmune disease. *Immunology today 18:335.*

14. Lider, O., L. M. B. Santos, C. S. Y. Lee, P. J. Higgins and H. L. Weiner. 1989. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. II. Suppression of disease and in vitro immune responses is mediated by antigen-specific CD8+ T lymphocytes. *J. Immunol. 142:748.*

15. Ke, Y., K. Pearce, J. P. Lake, H. K. Ziegler and J. A. Kapp. 1997. γδ T lymphocytes regulate the induction and maintenance of oral tolerance. *J. Immunol. 158:3610.*

16. Ke, Y. and J. A. Kapp. 1996. Oral antigen inhibits priming of CD8+ CTL, CD4+ T cells, and antibody responses while activating CD8+ suppressor T cells. *J. Immunol. 156:916.*

17. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. *Science 265:1237.*

18. He, Y. G., J. Mellon, J. Y. Niederkorn. 1996. The effect of oral immunization on corneal allograft survival. *Transplantation 61:920.*

19. Liwski, R., J. Zhou, V. McAlister and T. D. G. Lee. 2000. Prolongation of allograft survival by *Nippostrongylus brasiliensis* is associated with decreased allospecific cytotoxic T lymphocyte activity and development of T cytotoxic cell type 2 cells. *Transplantation 69:1912*

20. Chen, Y., J. I. Inobe, H. L. Weiner. 1995. Induction of oral tolerance to myelin basic protein in CD8-depleted mice: Both $CD4^+$ and $CD8^+$ cells mediate active suppression. *J. Immunol. 155:910.*

21. Garside, P., M. Steel, F. Y. Liew and A. M. Mowat 1995. CD4+ but not CD8+ T cells are required for the induction of oral tolerance. *Internation. Immunol. 7:501.*

22. Barone, K. S., S. L. Jain and J. G. Michael. 1995. Effect of in vivo depletion of CD4+ and CD8+ cells on the induction and maintenance of oral tolerance. *Cellul. Immunol. 163:19.*

23. Hall, B. M. 1991. Cells mediating allograft rejection. *Transplantation. 51:1141.*

24. Alters, S. E., J. A. Shizuru, J. Ackerman, D. Grossman, K. B. Seydel and C. G. Fathman. 1991. Anti-CD4 mediates clonal anergy during transplantation tolerance induction. *J. Exp. Med. 173:491.*

25. Gorczynski, R. M., Z. Chan, S. Chung, Z. Cohen, G. Levy, B. Sullivan and X. M. Fu. 1994. Prolongation of rat small bowel or renal allograft survival by pretransplant transfusion and/or by varying the route of allograft venous drainage. *Transplantation 58: 816.*

26. Ledingham, D. L., V. C. McAlister, H. N. Ehigiator, C. Giacomantonio, M. Theal, and T. D. G. Lee. 1996. Prolongation of Rat Kidney Allograft Survival by Nematodes. *Transplantation 61:184.*

27. Henkart, P. A. 1998. Cytotoxic T lymphocytes. In *Fundamental Immunology.* W. E. Paul, ed. Lippincott-Raven, Philadelphia, NY. p 1021.

28. Krieger, N. R., D. Yin and C. G. Fathman. 1996. CD4+ but not CD8+ cells are essential for allorejection. *J.Exp. Med. 184:2013.*

29. Gracie, J. A., E. M. Bolton, C. Porteous and J. A. Bradley. 1990. T cell requirements for the rejection of renal allografts bearing an isolated class I MHC disparity. *J. EXP. Med. 172:1547.*

30. Mannon, R. B., B. L. Kotzin, E. Roper, C. Nataraj, R. J. Kurlander and T. M. Coffman. 1996. The intragraft CD8+ T cell response in renal allograft rejection in the mouse. *Transplantation 62:96*

31. Olive, C., C. Cheung and M. C. Falk. 1999. Apoptosis and expression of cytotoxic T lymphocyte effector molecules in renal allografts. *Transplant Immunology 7:27.*

32. Porter, C. J., J. E. Ronan and M. J. D. Cassidy. 2000. Fas-fas-ligand antigen expression and its relationship to increased apoptosis in acute renal transplant rejection. *Transplantation 69:1091.*

33. Wever, P. C., J. G. Boonstra, J. C. Laterveer, C. E. Hack, F. J. Woude, M. R. Daha and I. J. M. Berge. 1998. Mechanisms of lymphocyte-mediated cytotoxicity in acute renal allograft rejection. *Transplantation 66:259.*

34. Dallman, M. J., K. J. Wood and P. J. Morris. 1987. Specific Cytotoxic T cells are found in the nonrejected kidney of blood-transfused rats. *J. Exp. Med. 165:566.*

35. Streilein, J. W. 1995. Unravelling immune privilege. *Science 270:1158.*

36. Bellgrau, D., D. Gold, H. Selawry, J. Moore, A. Franzusoff and R. C. Duke 1995. A role for CD95 ligand in preventing graft rejection. *Nature 377:630.*

37. Griffith, T. S. and T. A. Ferguson. 1997. The role of FasL-induced apoptosis in immune privilege. *Immunol. Today. 18:240.*

38. Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green and T. A. Ferguson. 1995. FasL-induced apoptosis as a mechanism of immune privilege. *Science 270:1189.*

39. Swenson, K. M., B. Ke, T. Wang, J. S. Markowitz, M. A. Maggard, G. S. Spear, D. K. Imagawa, J. A. Goss, R. W. Busuttil and P. Seu. 1998. Fas ligand gene transfer to renal allografts in rats. *Transplantation 65:155.*

40. Chen, N., Q. Gao and E. H. Field. 1996. Prevention of Th1 responses is critical for tolerance. *Transplantation 61:1076.*

41. Cobbold, S. P., E. Adams, S. E. Marshall, J. D. Davies, H. Waldmann. 1996. Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8. *Immunol. Rev. 149:5.*

42. Zavazava, N., F. Fandrich, X. Zhu, A. Freese, D. Behrens and K.A. Too-Ott. 2000. Oral feeding of an immunodominant MHC donor-derived synthetic class I peptide prolongs graft survival of heterotopic cardiac allografts in a high-responder rat strain combination. *Journal of Leukocyte Biology 67:793*

43. Halverson, D. C., G. N. Schwartz, C. Carter, R. E. Gress and D. H. Fowler. 1997. In vitro generation of allospecific human CD8+ T cells of Tc1 and Tc2 phenotype. *Blood 90:2089.*

44. Li, L., S. Sad, D. Kagi and T. R. Mosmann. 1997. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J. Immunol. 158:4152.*

45. Gorczynski, R. M. 1994. Adoptive transfer of unresponsiveness to allogeneic skin grafts with hepatic γδ+ T cells. *Immunology 81:27.*

46. Chen, Z. K., S. P. Cobbold, H. Waldmann and S. Metcalfe. 1996. Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation 62:1200.*

5.0 DISCUSSION

5.1 Effect of Oral Administration of Alloantigen on Prolongation of Kidney Survival

Based on the evidence available at the onset this research program it appeared likely that oral administration of alloantigen should ablate, to some extent, primary solid organ allograft rejection. Others had demonstrated that oral administration of allogeneic splenocytes prevented allo-sensitization by skin allografts and reduced second set rejection responses to cardiac allografts to a level which is more characteristic of acute (primary) responses (Sayegh et al 1992, Hancock et al 1993). In these latter experiments, the effect of oral tolerance on a cardiac allograft rejection was demonstrated on second set, but not primary, responses. The only data available for amelioration of primary responses came from experimentation with transplantation of "immunoprivileged" tissue. He and colleagues showed that feeding donor fresh or cultured keratinocytes or corneal cells prolonged corneal allograft survival in mice (He et al 1996).

In the research presented here, the primary immune response to a solid organ allograft with significant clinical importance (the kidney) is assessed. BN and Lewis rats were selected as donors and recipients respectively since they are fully MHC disparate. The data presented here demonstrates that feeding recipients $1x10^8$ live donor splenocytes five consecutive times starting 2 wk prior to kidney transplantation leads to kidney allograft survival that was significantly prolonged in comparison to PBS fed or non fed controls. This prolongation is antigen specific since the feeding of third party PVG splenocytes did not prolong BN kidney allograft survival. These data confirm that

oral administration of alloantigen is able to markedly prolong primary kidney allograft survival in an antigen specific manner.

Recently, using the same rat strain combination, Ishido and co-workers (1999) confirmed our central hypothesis and provided supportive data by demonstrating that oral administration of donor splenocytes prolongs primary cardiac allograft survival. Although their data support the hypothesis that feeding allosplenocytes can prolong the survival of primary solid organ allografts, the survival of their cardiac allografts was only prolonged by a few days. There are two potential reasons for this reduced efficiency of oral tolerance in comparison to our results. The first is that their feeding regime was less effective than the one used in our experimentation. The second is that they used CsA to help induce tolerance and may, indeed, have done the opposite. Since the mechanisms underlying oral tolerance are poorly understood, and data presented in this thesis suggest it may involve the generation of active regulatory cells, then CsA may decrease rather than increase this tolerogenic effect.

With respect to the feeding regime, tolerance induced by oral administration of antigens (Hoyne et al 1995) is known to fade over time. This is also true for tolerance induced by donor blood transfusion (Fabre and Morris 1972). Multiple pre-exposure to alloantigen is more effective than a single pre-exposure in generating transplantation tolerance (Fabre and Morris 1972, Hoyne et al 1995). This suggests that continuous exposure to antigen may enhance tolerance. Therefore, our final feeding regime was designed to boost the tolerance by repeated exposure to alloantigen by the oral route. This was accomplished not only by multiple pre-feeding but also by "post-feeding" the rats after transplantation until the termination of the experiment. Such post feeding

markedly enhanced the prolongation of kidney allografts compared to the prolongation induced by pre-feeding alone. These data demonstrate, for the first time, that continuous exposure to alloantigen through the gut can enhance the effect of oral transplantation tolerance.

Both antigen dose and time of administration influence the induction of oral tolerance (Strobel and Mowat 1998). In our experiments, we did not investigate the influence of antigen dose and time of administration on prolongation of allograft survival. This study focused on the feasibility of establishing oral transplantation tolerance to kidney allografts and, once established, the mechanism of expression of that tolerance.

An important role of the liver in oral tolerance has been suggested by evidence that bypassing the portal system by portacaval shunting reduced or abrogated orally induced tolerance (Cantor and Dumont 1967, Callery et al 1989). One way in which this role has been investigated in the transplantation arena is by portal venous inoculation of donor cells or antigens to establish "portal tolerance". For example, portal vein inoculation of alloantigens significantly suppresses both humoral and cell mediated responses to alloantigen (Fujiwara et al 1986, Sato et al 1988, Gorczynski 1992, 1995). Portal tolerance has been reported to prolong survival of allografts, including skin, heart and kidney (Kamei 1990, Gorczynski 1992, 1998).

However, there is controversy about the importance of the liver in the induction of oral tolerance. Enzymic digestion of proteins in the GI tract has been suggested to be critical for the induction of oral tolerance (Michael 1989, Hanson et al 1993, Jain et al 1996, Barone et al 2000). As an example, oral administration of bovine serum albumin (BSA) suppresses anti-BSA antibody production in mice. However, when BSA is

delivered directly into the ileum (bypassing the stomach, duodenum and jejunum), tolerance induction does not occur (Michael 1989, Stransky et al 1998). In marked contrast, delivery of enzymatically digested BSA into the ileum did induce tolerance. Others have shown that oral tolerance (indicated by suppressed humoral responses and spleen cell proliferation) can be induced by feeding OVA, but not by feeding encapsulated OVA. Encapsulation protects OVA from digestion in the upper GI tract (Jain et al 1996, Barone et al 2000, Vogel et al 1998). These data suggest that digestion in the GI tract is critical for the induction of oral tolerance and further suggest that oral tolerance and portal tolerance may differ in significant ways. On the other hand, undigested antigen delivered rectally (Witkin et al 1984, Richards et al 1984) or jejunally (Ishido et al 1999) induces tolerance. The role of these various organs in the induction of oral tolerance clearly remains to be completely elucidated.

Unlike the observation that induction of oral tolerance requires enzymatic digestion of antigen in GI tract in the OVA model described above, we demonstrate that direct delivery of alloantigen through portal vein (to avoid digestion of alloantigens in the GI tract) can induce transplantation tolerance. In congruence with our results, Ishido and co-workers (1999) demonstrated that both jejunal and oral administration of allosplenocytes induces transplantation tolerance. Such differences in observed induction of oral tolerance between feeding OVA or alloantigens may reflect intrinsic differences in the antigens responsible for tolerance induction. It is also possible that the mechanism of tolerance induced by oral or portal administration of allogeneic cells may be completely different. Allogeneic cells administrated by the oral route may be destroyed and degraded, and alloantigens are likely presented as peptides by recipient antigen

presenting cells to the gut-associated lymphoid tissue. This implicates indirect recognition of alloantigen in the generation of tolerance. The evidence that oral tolerance to alloantigen can be equally generated by feeding live or dead allogeneic cells or allogeneic peptides (Sayegh et al 1992 a, b) supports the position that intact allogeneic cells are not required for tolerance induction through the oral route. In contrast, allogeneic cells administrated through the portal route may present alloantigens to the recipient immune system directly and generate tolerance by direct recognition of alloantigen without the requirement of recipient antigen presenting cells.

5.2 Active Suppression Mediates Oral Transplantation Tolerance

Oral tolerance has been suggested to result from the generation of regulatory T cells that mediate active suppression. Active suppression can be demonstrated by decreased cell proliferation of splenocytes upon re-challenge in vitro (Miller et al 1991, 1992) as well as by the ability to transfer this effect to naïve animals by cell transfer (Santos et al 1994, Franco et al 1998). Such regulatory cells have been found in Peyer's patches, MLN and the spleen (Mattingly 1984, Gonnella et al 1998). Active suppression has been associated with type 2 immune responses, presumably mediated by regulatory cells that secrete IL-4, IL-10 and TGF-β (Rizzo et al 1994, Gonnella et al 1998).

In the experimentation presented here, tolerance could be transferred to naïve animals with cells from MLN, spleen or GIC. This suggests that regulatory cells are present in these sites. Since regulatory cells have been previously reported to be generated in PP, and then to migrate to the MLN and the spleen (Richman et al 1978, Mattingly 1984), it is possible that the regulatory cells seen in the spleen and MLN in this

experimentation follow the same pattern and are essentially the same phenotype of cells but in transit to the allograft. This is of some importance since the GIC in the kidney are likely alloreactive cells derived from allosensitization in the spleen, but given the transit noted above, GIC may also contain regulatory cells originating in the gut associated lymph tissue, including the MLN. It is thus possible that the regulatory cells in the GIC in these experiments come from the spleen and/or the MLN.

It has been reported that regulatory cells generated by oral tolerance mediate active suppression mainly by their cytokine secretion. IL-4 and TGF-β are suggested to be the most important cytokines that mediate this active suppression. For example, feeding MBP generates MPB-specific regulatory cells that secrete high levels of IL-4 and TGF-β in the fed animals (Miller 1992, Inobe et al 1998). These regulatory cells can transfer oral tolerance to naïve animals. Injection of anti-IL-4 mAb (Yoshino 1998) or anti-TGF-β mAb (Inobe et al 1998) at the time of antigen feeding abrogated the induction of oral tolerance. It is unclear whether IL-4 and TGF-β are required for, or result from, oral tolerance. However, enhanced IL-4 and TGF-β expression is often associated with established oral tolerance.

Likewise, in the present study, the regulatory cells in the GIC isolated from orally tolerized animals express significantly increased levels of IL-4 and TGF-β. These data are consistent with an important role for IL-4 and/or TGF-β in the establishment and maintenance of oral transplantation tolerance.

In transplantation tolerance, various immunosuppressive regimens applied for the induction of transplantation tolerance have been demonstrated to generate a type 2 immune response, indicated by increased type 2 (especially IL-4) but decreased type 1

cytokine secretion (Hall 2000). These transplantation tolerance models often exhibit a decreased allo-specific cell proliferation of splenocytes in vitro following alloantigen challenge (Gorczynski 1992, Chen et al 1996, Lehmann et al 1997). However, increased cell proliferation has also been reported in several transplantation tolerance models (Yoshimura et al 1990, Tomura et al 1997, Liwski et al 1999). This may be due to enhanced type 2 cytokine expression. Recent evidence suggests that type 2 cytokine expression can enhance T cell proliferation and induce resistance to AICD (Zhang et al 1997, Varadhachary et al 1997, Liwski et al 1999). In addition, IL-4 has been suggested to be a primary T cell growth factor (Weiner et al 1997). As an example, infection of mice with the nematode parasite *Nippostrongylus brasiliensis* (*Nb*) prolongs cardiac allograft survival. This prolongation is associated with increased antigen-specific cell proliferation and enhanced type 2 immune responses (Liwski et al 2000). The increased cell proliferation of splenocytes in *Nb* infected mice was shown to be due to IL-4 since anti-IL-4 treatment in these experiments blocked the T cell proliferation, and rIL-4 enhanced the proliferation (Liwski et al 1999, 2000). In addition to IL-4, IL-6 has also been shown to enhance cell proliferation when added to cultures of splenocytes from alloantigen exposed animals, and injection of IL-6 prolongs skin allograft survival. This later prolongation was associated with increased, rather than decreased, splenocyte proliferation (Tomura et al 1997).

In the experimental data presented here, spleen and MLN cells from orally tolerized animals exhibited increased cell proliferation when compared to the cells from non-fed or third party fed control animals. Although the cytokine secretion pattern from spleen and MLN was not examined, it was clear that GIC from orally tolerized animals

expressed enhanced IL-4 mRNA in comparison to unfed controls. Since the regulatory cells present in the spleen, the MLN and the GIC are likely the same phenotype (as described above), it is possible that regulatory cells in the spleen and MLN from tolerized animals also express enhanced IL-4. Therefore, the increased cell proliferation of lymphocytes from spleen and MLN in orally tolerized animals may be due to the increased IL-4 expression on these regulatory cells.

Oral tolerance has also been suggested to be mediated by clonal anergy/deletion. However, in this experimentation, both the increased MLR and the ability to transfer tolerance with T cells strongly suggest a regulatory mechanism, rather than anergy/deletion as the primary contribution to the oral transplantation tolerance described here.

Since oral tolerance is a complex phenomenon and several sites can be involved in tolerance induction, one cannot rule out the possibility that the regulatory cells found in the MLN, the spleen and the GIC are not phenotypically similar. There is evidence to support this position since, in some cases, they exhibit different effects when transferred to naïve animals (Richman et al 1981, Franco et al 1998). It has been suggested that active suppression occurs in PP and MLN whereas anergy/depletion occurs in spleen (Franco et al 1998). This position is clearly in opposition to what we have found in the oral transplantation tolerance model. In the data presented here, both spleen and MLN cells transferred tolerance although it is unknown if the transfer is by the same mechanism. To confirm the relationship between these cells, the phenotype of the regulatory cells in MLN and in the spleen must be characterized. Of significant interest would be a comparison of the cytokine secretion pattern and/or mRNA expression from

lymphocytes from the spleen and the MLN of orally tolerized animals. Further, since the MLR for both spleen and MLN is increased, it would be of interest to examine the effect of anti-IL-4 mAb treatment of the MLR culture on cell proliferation. However, such experiments were beyond the scope of the present research project, since the focus of this research was the expression of tolerance at the level of the transplanted organ (in the GIC).

It is worth mentioning that there is a dissociation between the MLR experiments and the cell transfer experiments in terms of the presence of regulatory cells from spleen, MLN and GIC. Note that if regulatory cells are present in the spleen (as indicated by the ability to transfer tolerance with these cells) it might be expected that MLR in this population would be decreased in vitro as demonstrated in other oral tolerance models (Sayegh et al 1992). A possible explanation is that in the cell transfer experiments, spleen or MLN cells were transferred from animals that were orally tolerized without receiving kidney transplants. The spleen and the MLN from these animals clearly contain regulatory cells. However, after kidney transplantation, the regulatory cells in the spleen and MLN of tolerized animals may migrate to the allograft and play a role in preventing graft rejection by modulation of the immune response in the local site. Therefore, it is possible that at day 5 post transplantation, GIC from tolerized animals contain regulatory cells that have migrated from the spleen and the MLN. However, the spleen and the MLN may not retain regulatory cells. If this is the case, MLR performed at day 5 transplantation from orally tolerized and kidney transplanted animals may reflect proliferation in the absence of regulatory cells. Therefore, the increased cell proliferation of lymphocytes from spleen and MLN in these animals may be due simply to a normal

primed response due to pre-exposure to alloantigen. Interestingly, several observations of increased cell proliferation of splenocytes in transplantation tolerance models were obtained post transplantation (Tomura et al 1997, Yoshimura et al 1990, Liwski et al 1999).

5.3 Graft Infiltrating Cells

Graft rejection is predominantly induced by cell mediated immune responses. After revascularization of the allograft, allosensitization probably occurs in the spleen to generate alloreactive cells. These alloreactive cells infiltrate the graft and play a effective role in destroying the grafts. It has been demonstrated that dramatic cellular infiltration is associated with acute rejection (Hall 1991), and the effector responses which destroy allogeneic transplants occur locally (Hall 1991). Therefore, to evaluate the mechanism of prolongation of kidney allograft survival induced by oral exposure to alloantigens, graft-infiltrating cells must be analyzed. Although most research in oral tolerance has primarily concentrated on events in the draining nodes or spleen, we postulated that critical immunomodulatory events would also be defined at the level of the transplanted organ, and that examining the graft infiltrating lymphocytes would be of prime importance for developing an understanding of oral transplantation tolerance. Using collagenase to digest the kidney graft, we were able to isolate GIC at day 5 post transplant from orally tolerized and non-tolerized control animals. This is the latest time point at which we can obtain GIC from control transplants because of the extent of transplant fibrosis present at later time points. It has been reported that the enzyme treatment does not affect the surface glycoprotein pattern of the treated cells and does not

affect the surface receptor structure of the infiltrating leukocytes necessary for their classification and functional analysis (von Willebrand and Hayry 1978). The transfer experiments confirmed that the digestion process did not significantly alter the function of the GIC regulatory cells, since we were able to transfer tolerance with GIC obtained by the enzyme digestion.

In the experimentation described in this thesis, T cells were the most prominent cell population in the immune cell infiltrate of the kidney allografts. These results are consistent with the findings obtained in other kidney transplantation models in rats (von Willebrand and Hayre 1978). However, the data in this thesis demonstrates that there is a significant decrease in the number of T cells infiltrating kidney allografts in the orally tolerized animals as compared to control groups. There are a number of potential reasons for this decrease in graft infiltrating T cells in the orally tolerized animals. There could be a decrease in the number of alloreactive lymphocytes generated in response to the transplant challenge in the animals which had previous oral exposure to alloantigens. Such deletion of antigen specific lymphocytes has been suggested to be one mechanism of oral tolerance (Strober et al 1998, Faria and Weiner 1999). However, this seems an unlikely explanation for the data presented here because we found increased, rather than decreased, spleen and MLN cell proliferation in response to alloantigen challenge in the fed animals. Another possibility is that lymphocytes are generated normally but their infiltration into the grafts is blocked. This could occur if adhesion molecules on the lymphocytes or on graft cells were modulated since adhesion molecules are suggested to be critical to recruit lymphocytes into the graft (Grau et al 2000, Robertson et al 2000). There is no evidence to support or refute this conjecture at this time. A third possibility is

that alloreactive lymphocyte infiltration is not reduced but that lymphocytes are eliminated in the allograft. Although we do not have direct evidence to prove this, histological examination of the allografts at day 5 post transplants suggests that more GIC are present than are obtained as viable cells after enzymatic digestion. It is therefore possible that a significant amount of cell death may be occurring in the graft. To confirm such elimination of lymphocytes in the local grafts, double staining by TUNEL and anti-CD3 would need to be performed to examine whether there is an increase in apoptosis of lymphocytes in the grafts from orally tolerized animals.

By histological analysis, there is a substantial mononuclear cell infiltrate on day 5 post transplant in the kidney grafts of both fed and unfed animals, but there is decreased cell infiltration at later time points in the tolerized animals (day 23 and 105). This decreased cellular infiltration correlated with marked preservation of tubular and glomerular structure. Our results are consistent with observations obtained from experiments with blood transfusion induced transplantation tolerance by Armstrong and colleagues (1987). In their experiments, preoperative injection of donor strain blood significantly prolonged kidney allograft survival in rats. Histological examination of the grafts at various time points after kidney transplantation showed dynamic changes in cellular infiltration in tolerized animals in comparison to control animals. Grafts from tolerized animals exhibited a greater cellular infiltration at day 3 post transplant. However, similar levels of infiltration were seen at day 5 post transplant and fewer infiltrating cells were seen at later time points (Armstrong et al 1987). An early increase in cellular infiltration has been suggested to be required for the induction of

transplantation tolerance (Armstrong et al 1987). It is possible that this early infiltration represents the activity of a suppressive phenotype of infiltrating cell.

5.4 CD8+ GIC

Both CD4+ and CD8+ T cells are critical for graft rejection. CD4+ T cells provide help for generating effector cells, including activated macrophages (in DTH-like responses) and CTL. CD8 + T cells are thought to be the major source of CTL in transplantation. To investigate the expression of oral transplantation tolerance induced in our experiments, we characterized the phenotype of graft infiltrating cells from day 5 transplants. We found a significantly decreased number of CD4+ T cells in the kidneys of orally tolerized animals in comparison to controls. The numbers of CD8+ T cells in both groups were similar. This was surprising since CD8+ T cells have been suggested to play a major role in graft rejection through direct cytotoxic effects against foreign class I MHC expressing donor kidney tubule cells (23). Many treatments that prolong allograft survival, including cyclosporine (Bradley et al 1985, Mason and Morris 1984), anti-CD4 mAb (Motoyama et al 2000), portal venous inoculation with alloantigen (Gorczynski et al 1992, 1995) and nematode infection (Ledingham et al 1996, Liwski et al 1999) are associated with decreased numbers of CD8+ T cells or decreased CTL activity. These data suggest that other methods of establishing transplantation tolerance may depend on the inhibition of CD8+ T cell infiltration and/or suppression of CTL activity.

However, in tolerance models using donor blood transfusion, kidney allografts showed high levels of cellular infiltration early after transplantation with the majority of the cells being CD8+T cells, not CD4+ T cells (Armstrong et al 1987, Wood et al 1987,

1988). This observation is similar to our finding. In our experiments, undiminished numbers of intragraft CD8+ T cells in the kidneys of tolerized animals suggest that such animals do not have a defect in the generation or transit of alloreactive CD8+ T cells to the allograft. However, it is possible that these CD8+ T cells did not contain active CTL and that the modulation observed lay in a defect in CD8+ T cell differentiation to active CTL.

To address this we examined the expression of perforin, granzyme and FasL, characteristic of mature CTL activity (Henkart 1999), in a highly purified CD8+ GIC population. We found no substantial difference in the level of mRNA for the cytotoxic mediators perforin and granzyme in the GIC from orally treated animals versus controls. Further, FasL expression appeared to be increased, rather than decreased, in these highly enriched CD8+ GIC T cell populations from animals orally exposed to alloantigen before transplantation. These findings support the hypothesis that similar numbers of potentially active mature CTL are present in the grafts of orally treated animals, even in the face of prolonged graft survival. This hypothesis was confirmed by our experimentation showing marked cytotoxicity to allogeneic targets in a direct CTL assay, thus amply demonstrating the presence of mature CTL in the GIC population.

Clearly, once removed from the recipient, the CD8+ GIC are relieved of any suppressive effects with regard to their cytotoxicity. Indeed, when purified from the kidneys, CD8+ GIC from orally tolerized animals are more active killers than GIC from normal rejecting allografts. The fact that there is no drop in these competent CD8+ CTL numbers in the kidneys of orally tolerized animals, but yet there is clear protection from immune mediated destruction in these organs is difficult to explain. One possibility is

that the reduced numbers of CD4+ T cells in these grafts may limit CD4+ T cell help for CTL activity but this is doubtful given the ability of these enriched CD8+ CTL to kill, in vitro, in the absence of CD4+ help. It is more probable that this population of graft infiltrating CD8+ T cells is heterogeneous and contains both active allo CTL and regulatory T cells. Freed from the environment of the graft, the CTL are effective allo killers. In the graft environment the allo killing activity is somehow blocked or regulated. Another possibility is that CTL activity against the Con A stimulated splenocyte blasts examined in vitro does not accurately predict killing of allogeneic grafts. It has been suggested that there is a dissociation between tissue destruction induced by CTL in vivo and cytotoxicity measured in vitro (Steinmuller et al 1990, Hadley et al 1996). For example, graft-infiltrating cells isolated from skin allografts exhibit high cytotoxic activity against allo-Con A blasts, but do not kill skin epithelium cells effectively (Yamamoto et al 1998). Furthermore, graft infiltrating cells isolated from rejecting human kidney allografts exhibit high levels of cytotoxicity to donor-derived Con A blast cells, but exhibit weak cytotoxicity to kidney parenchymal cells (von Willebrand and Hayry 1978). Thus there is a possibility that the CTL activity we have seen in vitro does not accurately reflect the ability of these cells to kill in vivo. This could explain the discrepancy between the in vitro killing seen in our experiments and the lack of tissue destruction in the kidney allografts in orally tolerized animals.

There are other potential explanations of why high in vitro CTL activity does not correlate with graft destruction. Perhaps CD8+ CTL are not critical for allograft rejection. There is evidence to support this possibility. For example, depletion of CD8+ T cells does not protect allografts from acute rejection in several transplantation models,

including skin, heart and kidney (Gracie et al 1990, Bishop, et al 1993, Mannon et al 1995, Douillard et al 1999). In addition, CD4 deficient mice did not reject skin and heart allografts even though CD8+ T cells are present (Krieger et al 1996). However, there is evidence that CD8+ T cells do play a role in allograft rejection. First, adoptive transfer of allo-sensitized CD8+ T cells into nude animals induces allograft rejection (Prowse et al 1983). Second, CD8+ T cells have been implicated in the rejection of skin allografts in the absence of CD4+ T cells (Rosenberg et al 1986). Third, although depletion of CD8+ T cells by mAb does not prevent graft rejection in certain models, it does prolong allograft survival (Douillard et al 1999, He et al 1999). In fact, the data available on the role of CD8+ T cells in graft rejection is mixed and often contradictory. This may be because CD8+ T cells from some animal strains are unable to be activated without CD4 help, whereas CD8+ T cells from other stains can be activated and proliferate independent of CD4+ T cell help. These latter CD8+ T cells are capable of generating CTL by their own cytokine production (Hall 1991). Therefore, CD8+CTL generated in a CD4 independent manner may be responsible for skin allograft rejection in the absence of CD4+ T cells (Andrus et al 1984, Rosenberg et al 1986).

It is also possible that the CD8+ CTL generated by oral administration of allo-antigen in our experimental model may have low avidity to alloantigen, resulting in limited damage to the allograft. It has been demonstrated previously that the avidity of CTL to allo-antigen is important for allograft rejection (Van Emmerik et al 1997). In their cardiac allograft model, high avidity CD8+ or CD4+ CTL were implicated in transplant rejection, whereas low-avidity CTL were not (Van Emmerik et al 1996, 1997).

In humans, CD8+ T cells can be isolated from human renal allografts (von Willebrand and Hayry 1978, Tilney et al 1979). Such infiltrating cells exhibit greater cytotoxicity to donor leukocytes than circulating cells, suggesting an enrichment of active CTL in the grafts. However, since all patients who receive transplants are treated with immunosuppressive drugs, the presence of CTL in the grafts does not eliminate a potential role for them in transplantation tolerance. In fact, increased cytotoxic activity has been reported in GIC from blood transfusion transplantation tolerance experiments (Armstrong et al 1987, Dallman et al 1987, 1988, Quigley et al 1987, 1988). These data are consistent with our finding that GIC from kidneys of tolerized animals exhibit high in vitro CTL activity.

To investigate the relationship between CTL activity in vitro and transplantation tolerance in vivo, Wood and co-workers (1989) transferred GIC from kidney transplants from blood transfusion treated and untreated control rats into blood transfusion tolerized animals which had received kidney transplants. They found that GIC from the kidneys of control rats broke the transplantation tolerance in the recipients. In contrast, GIC from kidney transplants of tolerized animals did not break tolerance (Wood et al 1989). Several possibilities were suggested based on these observations. First, the target antigen expressed on in vitro targets might not be expressed on the cells in the nonrejected kidney grafts. Second, the activity of cytotoxic cells might be blocked, in vivo, in tolerized animals. The blocking could be mediated by the presence of donor specific antibodies or by suppressor cells, although it is unclear if suppressor cells can block fully activated cells. Third, it is possible that the cytotoxicity observed in vitro is irrelevant to graft rejection. Therefore, these authors suggested that cytotoxic cells examined in vitro alone

are not sufficient to predict kidney allograft rejection. However, in their experiments, they did not comment on whether these cytotoxic GIC are able to induce suppression. In addition, they did not further investigate which cell population in the GIC is responsible for maintaining the transplantation tolerance.

A role for CD8+ T cells in tolerance has been suggested from work in many experimental models. For example, oral administration of MBP prior to systemic immunization with MBP protected rats from developing EAE. The protection could be adoptively transferred from tolerized rats to naïve recipients with MLN or spleen CD8+ T cells, but not with CD4+ T cells (Lider et al 1989, Miller et al 1991, 1992). Likewise oral tolerance induced by feeding mice OVA could be adoptively transferred to naïve animals by CD8+ T cells, but not CD4+ T cells (Ke et al 1996, 1997). In a transplantation model, transfusion tolerance was abrogated by depleting CD8+ T cells with anti-CD8 mAb prior to blood transfusion, suggesting that CD8+ T cells are required for the induction of transfusion tolerance (Douillard et al 1996, 1999). When the anti-CD8 mAb was given after blood transfusion but before the engraftment, transfusion tolerance was not abrogated. Clearly once transfusion tolerance has been established, the alloactivated CD8+ T cells are resistant to depletion by mAb (Douillard et al 1999). Alloactivated CD8+ T cells resistant to anti-CD8 mAb depletion have been confirmed by FACS analysis (Douillard et al 1999).

We have demonstrated that CD8+ GIC from orally tolerized animals are able to transfer transplantation tolerance to naïve animals, suggesting that these CD8+ GIC are regulatory cells. These regulatory cells exhibited increased CTL activity to allolymphocytes and increased FasL expression. Since we used a direct CTL assay to

assess cytotoxicity of CD8+ GIC in our experiments, we avoided potentially misleading results which could be expressed in a conventional CTL assay. Conventional assessment of CTL activity is carried out after in vitro expansion of CTL precursors. However, the characteristics of allo-reactive cells in vivo may be functionally changed by expansion in vitro. Precursors ex vivo may differentiate into different cells in vitro than they would in the in vivo milieu. For example, CD8+ T cells derived from kidney allografts spontaneously surviving for more than 3 weeks express low levels of TCR. However, after 4 days stimulation in culture with alloantigen, TCR expression increases dramatically and allospecific CTL activity is generated (Mannon et al 1998). These data suggest that an in vitro expanded population may not reflect the situation in vivo, especially with respect to cytotoxic activity. Therefore, in our experiments, CTL activity was evaluated directly from freshly isolated GIC. The in vitro cytotoxic data here, however, does have a potential drawback. For assessment of cytotoxicity the target cells used are mitogen stimulated lymphocyte blasts, a standard technique for such assays in that it allows the more accurate JAM assay to be used as a measure of cytotoxicity. It is possible, however, that although these CD8+ CTL are effective killers against lymphocytes in vitro they do not kill kidney tubular epithelial cells in vivo. It is also possible that these cells kill lymphocytes in vivo. If this is the case, CD8+ CTL may kill alloreactive Fas bearing T cells, potentially CD4+ T cells, in the grafts, resulting in protection of the graft.

5.5 Effects of Fas/FasL Interaction on Transplantation Tolerance

We hypothesized above that regulatory CD8+ T cells may kill alloreactive CD4+

lymphocytes in the kidney grafts by Fas/FasL interaction. This hypothesis is based on our observation that CD8+ regulatory GIC from orally tolerized animals express higher levels of FasL mRNA and exhibit increased CTL activity compared to cells from unfed controls. These CD8+ T cells, however, did not induce graft rejection but protected grafts when transferred to naïve animals. Therefore, high levels of FasL expression and CTL activity may contribute to the oral transplantation tolerance.

This hypothesis is supported by a large body of evidence for a critical role of Fas/FasL interaction in maintaining immune homeostasis, immune privilege and transplantation tolerance. For example, expression of Fas and FasL on lymphocytes is necessary for maintaining normal immune homeostasis since mice deficient in Fas (*lpr*) or FasL (*gld*) exhibit lymphoproliferation and autoimmune disease (Suda and Nagata 1997). This is due to the lack of apoptosis of lymphocytes. Further, it has been reported that superantigen activated CD8+ T cells can kill antigen activated CD4+ T cells in a Fas/FasL dependent manner (Noble et al 1998).

The testis and eye are believed to be immunoprivileged sites where limited immune reactions are initiated in response to antigen challenge. The limited immune reaction is due to the constitutive expression of FasL on sertoli cells of the testes and parenchymal cells of the eye. Lymphocytes infiltrating the eye in response to viral challenge are eliminated by apoptosis, whereas viral challenge in FasL deficient mice (*gld*) results in a severe ocular inflammatory response (Griffith et al 1995). FasL+ sertoli cells from the testes transplanted under the kidney capsule of allogeneic recipients are resistant to graft rejection, whereas cells from the testes of FasL deficient mice (gld) undergo acute rejection (Bellgrau et al 1995). Similar observations of FasL expression on allografts have been reported in the

corneal transplantation model (Stuart et al 1997, Yamagami et al 1997). In this model, grafts from wild type donor mice contained apoptotic mononuclear cells, indicating the induction of apoptosis of infiltrating cells by the grafts, whereas rejecting grafts from FasL- donors contained numerous inflammatory cells without sign of apoptosis (Stuart et al 1997, Yamagami et al 1997). These data suggest that FasL expression on the grafts protects the grafts from rejection by inducing apoptosis of infiltrating cells through the interaction with surface Fas molecules on infiltrating alloreactive T cells.

The effect of Fas/FasL interaction on graft protection can be further demonstrated by the co-transplantation of allografts along with FasL+ cells. Islet allografts co-transplanted with genetically engineered syngeneic myoblasts expressing FasL under the same kidney capsule do not exhibit rejection. However, when islets and FasL+ myoblasts are transplanted under the capsules of contralateral kidneys, the islet grafts are rejected (Lau et al. 1996). These results suggest an important role of local Fas/FasL interaction in graft protection. Presumably the provision of the FasL signal by the transfected syngeneic myoblasts within the local environment of an islet allograft depletes infiltrating allo-activated T cells that express Fas and therefore protects the islet allograft from destruction by the alloreactive lymphocytes. However, others have suggested that the Fas/FasL effect need not be local. FasL bearing allogeneic testes cells transplanted under one kidney capsule protect islet grafts transplanted under the capsule of the contralateral kidney. This protection is dose dependent (Takeda et al 1999). The reasons for this systemic effect are unclear and explanations have been unconvincing. For example, it has been suggested that because in this case the FasL+ testes cells were allogeneic, not syngeneic, these cells attract alloreactive T cells to the site and eliminate these T cells by Fas/FasL

interaction. This results in a generalized decrease in alloreactive T cells in the immune system and protects the contralateral islet grafts from rejection. Given that these transplants are into immune privileged sites (kidney capsule) this may be possible but it is unlikely.

Solid organs genetically engineered to express high levels of FasL can also be protected from rejection. Swenson and coworkers (1998) demonstrated that kidney allografts transfected with FasL were resistant to acute rejection. The amount of cellular infiltration was similar in both FasL transfected and control grafts, but the FasL transfected grafts exhibited well preserved structure and prolonged graft survival. These data suggest that FasL expression in the solid organ can regulate alloimmunity, presumably by eliminating alloreactive lymphocytes in the local environments.

However, in a cardiac allograft model, FasL transfected grafts, either syngeneic or allogeneic, are not protected from the rejection. Instead, FasL transfection accelerates graft rejection (Takeuchi et al. 1999). This accelerated rejection is associated with massive neutrophil infiltration. Similar observations of accelerated rejection have also been reported in experiments involving transplantation of syngeneic tumor cells or allogeneic pancreatic islet cells engineered to express FasL (Seino et al. 1997, Kang et al. 1997, 2000). In these experiments, the grafts are rejected by neutrophils. It is speculated that FasL itself may function as a chemokine for neutrophils or that it, directly or indirectly, activates neutrophils (Turvey et al 2000). It is unclear why FasL expression in some experiments induces apoptosis of alloreactive T cells without activating neutrophils, but in other experiments, the FasL expression attracts and activates neutrophils, resulting in accelerated graft rejection. It may be dependent on numerous

factors, such as the level of FasL expressed on the cells or grafts, method of gene transfection used and immune status of the host (Li et al 1998, Turvey et al 2000). For example, liver allografts transfected with 180 μg of FasL-expressing plasmid vector (pFasL) are protected from immune rejection, whereas similar allografts transfected with 240 or 360 μg of pFasL exhibit hepatocyte apoptosis and graft rejection (Li et al 1998). In addition, tumor cells transfected with FasL are rejected when transplanted subcutaneously or intraperitoneally, but are protected when transplanted under the kidney capsule (Seino et al 1997).

Recent evidence revealed that Fas/FasL interaction is not absolutely necessary for the induction of transplantation tolerance because transplantation tolerance can be induced in Fas deficient animals (Xian Chang Li et al 1999, Wagener et al 2000).

There is no direct evidence, as yet, to support a hypothesis that regulatory CD8+ T cells play a central role in transplantation tolerance by killing allo-reactive syngeneic CD4+ lymphocytes by Fas/FasL interaction. However, Fas/FasL interaction is critical in eliminating syngeneic clonal expansion of CD4+T cells by CD8+ T cells after viral or superantigen infection (Noble et al 1998), and is important for maintaining normal homeostasis (Sakata et al 1998). This suggests that T cells are indeed able to induce apoptosis of syngeneic T cells. In in vitro experimentation, T cells activated by alloantigen express upregulated Fas and FasL on the cell surface, and are susceptible to apoptosis induced by anti-Fas mAb or FasL expressing tumor cells (O'Flaherty et al 1998). In the transplantation tolerance induced by donor specific blood transfusion, there are more apoptotic cells present in accepted, compared to rejected, cardiac allografts, especially in the periarterial areas (Bergese et al 1997), suggesting apoptosis of

alloreactive lymphocytes, presumably by syngeneic lymphocytes. In the transfusion tolerance model, FasL is expressed on GIC from non-rejecting cardiac allografts (Josien et al 1998). Furthermore, the protection of skin allografts, induced by donor bone marrow (DBM) transfusion, required FasL expression on the DBM (since DBM from *gld* mice did not induce the protection; George et al. 1998). This suggests that the expression of FasL on DBM plays a role in tolerance induction, presumably by inducing apoptosis of alloreactive T cells resulting in prolongation of allograft survival.

It is possible that FasL expression on the CD8+ T cells is able to induce apoptosis of syngeneic alloreactive CD4+ T cells in the oral transplantation tolerance described in this thesis. The evidence of decreased numbers of CD4+ GIC, but not CD8+ GIC from fed animals compared to control animals, suggests that CD4+, not CD8+ T cells, could be the major target for FasL induced apoptosis. To confirm this hypothesis, CTL activity of CD8+ GIC against alloreactive syngeneic CD4+ T cells must be examined. In addition, to assess the role of Fas/FasL interaction in the generation and maintenance of transplantation tolerance, soluble Fas protein could be used, especially in the cell transfer experiments.

CD8+ dendritic cells (DC) are also reported to express FasL and to be able to induce apoptosis of CD4+ T cells (Suss and Shortman 1996). In our experimentation, we use nylon wool columns to enrich for T cells and CD8 immuno-columns to select for CD8+ GIC. These columns involve negative selection and are designed to eliminate adherent cells, including macrophages and DC, but it is possible that this isolation process does not completely deplete CD8+ DC from total GIC. Therefore, we cannot rule out a role of CD8+ DC in our oral transplantation tolerance, especially in the transfer of tolerance to naïve

animals. To evaluate a role for DC in oral transplantation tolerance, transfer of T cell depleted GIC from tolerized animals into naïve animals could be performed immediately following kidney transplantation. Whether this is practically possible remains to be seen.

If killing of Fas bearing CD4+ T cells by FasL bearing CD8+ T cells is occurring in the grafts, it is not clear why these CD8+ cells do not also induce apoptosis of kidney allograft cells and thus accelerate rejection. Fas molecules are expressed on human kidney epithelium (Ortiz-Arduan et al 1996, Kato et al 1997), suggesting that kidney cells can be targets of the Fas/FasL pathway resulting in graft damage. Indeed, upregulation of FasL expression has been detected in biopsies of kidneys undergoing acute and chronic rejection (Sharma et al 1996, Wang et al 1997, Josien et al 1998, Matsuno et al 1998). Increased FasL expression can be found on graft infiltrating cells and is closely correlated with the level of apoptosis, detected by TUNEL technique (Josien et al 1998), and the histologic score of acute rejection (Sharma et al 1996). These correlative data suggest that FasL mediated apoptosis plays a role in acute rejection. However, correlative data such as this has significant limitations. These data do not identify the type of the apoptotic cells in the graft (infiltrating leukocytes vs. graft cells). It is possible that increased FasL expression may induce apoptosis of graft infiltrating leukocytes, rather than graft tissue cells. In cardiac (Bergese et al 1997) and renal (Matsuno et al 1998) transplantation models in rats, upregulated FasL expression appears at a time when allografts were almost completely destroyed. Therefore, increased FasL expression on lymphocytes at this time point might be better explained as a mechanism for eliminating alloreactive lymphocytes generated by allograft transplantation.

Cytotoxic discrimination may, in fact, be due to the level, rather than the presence, of Fas, or perhaps to susceptibility to FasL induced killing. In animal models, the level of Fas expressed on normal kidney cells is upregulated in the presence of inflammatory cytokines, such as IFN-γ and TNF-α (Kato et al 1997, Ortiz-Arduan et al 1996). Furthermore, although Fas is expressed on kidney tubular epithelial cells, epithelial cells are relatively resistant to anti-Fas mAb induced apoptosis (Wever et al 1998).

The importance of Fas/FasL interaction in transplant rejection is still unclear. Granzyme and perforin have been demonstrated to be the predominant pathway mediating kidney allograft rejection (Wever et al 1998). Indeed, skin allografts transplanted into *gld* (FasL-) recipients are rejected in a manner similar to grafts transplanted into wild type mice and the survival of grafts from *lpr* (Fas-) donors is not prolonged (Selvaggi et al 1996). Similar observations were reported in a cardiac transplantation model (Larsen et al 1995). It should be noted here that the knockout experimentation must be interpreted with caution because experiments performed on genetically modified animals may provide misleading results. These data do confirm, however, that the roles of Fas/FasL interaction in graft rejection and tolerance induction are still unclear. The data are also consistent with the hypothesis that the increased FasL seen in the GIC in this experimentation could play a significant role in oral tolerance induction and do not represent an epiphenomenon.

5.6 Role of Type 2 Cytokines in Oral Transplantation Tolerance

By transfer experiments, we have concluded that prolongation of kidney allograft survival is due to CD8+ regulatory cells that express high levels of FasL and IL-4 mRNA after oral administration of alloantigens. It is unclear whether these regulatory CD8+ T cells express both enhanced FasL and IL-4 mRNA, or whether the GIC contain two different functional populations of CD8+ T cells that express FasL and IL-4 mRNA, respectively. To address this, double staining should be used to measure the co-expression of surface FasL and intracellular IL-4 on CD8+ GIC. Based on the results we obtained here, we hypothesize that oral administration of alloantigens generates intragraft regulatory cells that eliminate alloreactive cells through their Fas/FasL interaction, and/or interfere with type 1 allo-responses that are important in graft rejection.

Type 2 cytokines have been suggested to play a critical role in oral tolerance. For example, spleen cells from mice fed with MBP secrete large amounts of IL-4 and IL-10 as well as TGF-β (Rizzo et al 1994, Santos et al 1994, Miller et al 1992). These cells are able to transfer oral tolerance to naïve animals. Anti-IL-4 mAb treatment abrogates the transfer of tolerance, suggesting that IL-4 is critical in tolerance transfer (Yoshino 1998, Faria and Weiner 1999). In addition, IL-4 secreting cells were found in PP and MLN soon (2-6 h) after feeding OVA (Gonnella et al 1998) and administration of IL-4 enhanced oral tolerance (Inobe et al 1998), further supporting a role for IL-4 in oral tolerance. In our experiments, graft infiltrating cells isolated from the transplanted kidneys of fed animals express high levels of IL-4 mRNA which is consistent with these findings. Although other type 2 cytokines, such as IL-5 and IL-10, were also reported to be associated with oral tolerance (Garside et al 1999), we did not evaluate their

expression for several reasons. First, IL-10 is known to be secreted by Th2 cells in mice but can be secreted by both Th1 and Th2 cells in humans and by macrophages in a variety of species. The source of IL-10 in rats is not clear. Second, although IL-10 and IL-5 are found to be associated with oral tolerance in some experiments, they do not show consistent upregulation following oral administration of antigens. For example, only IL-4, not IL-10 or IL-5, are increased in the spleen in orally tolerized animals in some models (Zavazava et al 2000). Third, knowledge of the effects of IL-10 and IL-5 on transplantation tolerance is incomplete and the area remains controversial. IL-4 is the most well-established type 2 cytokine that is consistently upregulated in many forms of oral tolerance and transplantation tolerance. Therefore, evaluation of the levels of IL-4 on the GIC was seen as important for elucidating the mechanisms of oral transplantation tolerance.

IL-4 plays a critical role in transplantation tolerance. Its role has been demonstrated in many experimental models including transfusion tolerance (Takeuchi et al 1992), treatment with anti-CD4 mAb (Mottram et al 1995, Takeuchi et al 1992), portal vein inoculation of alloantigens (Gorczynski 1992, 1995) and nematode infection (Ledingham et al 1996, Liwski et al 2000). In these models, IL-4 is thought to shunt the developing allo-immune response toward type 2 and away from type 1 responses, indicated by a decreased IFN-γ expression. For example, consistent expression of IL-4 and IL-10 but diminished IL-2 and IFN-γ are found in long-term cardiac and renal allografts in mice treated with anti-CD4 mAb (Takeuchi et al 1992, Siegling et al 1994). This treatment generates CD4+ regulatory cells that are able to adoptively transfer the tolerant state to naïve engrafted recipients (Onodera et al 1997). In addition, evidence

that transplantation tolerance cannot be established in IL-4 knockout mice after anti-CD2 and anti-CD3 mAb treatment (Punch et al 1998) further confirms the important role of IL-4 in transplantation tolerance.

In our experiments, CD8+ GIC from tolerized animals exhibited high levels of IL-4 and these cells could transfer oral transplantation tolerance to naïve animals. Our data suggest that oral administration of alloantigen generates Tc2 type regulatory cells that drive immune responses toward type 2 response. However, in our experimentation, the levels of IFN-γ are not significantly inhibited in the tolerized animals, although there is an increased type 2 response in these animals. The lack of a decrease in expression of IFN-γ in the face of increased IL-4 may be interpreted in two ways. First, although oral tolerance is often suggested to generate type 2 responses and IL-4 is a critical differential factor in oral tolerance induction, IFN-γ is also suggested to be a critical cytokine in the induction of oral tolerance, since oral tolerance cannot be induced in IFN-γ deficient animals (Kweon et al 1998). Second, in transplantation tolerance, suppression of IFN-γ is often associated with graft acceptance but grafts in IFN-γ deficient mice did not avoid rejection. In fact, IFN-γ is required for long-term graft survival after blocking the CD28 and CD40 co-stimulatory pathway (Konieczny et al 1998). These data suggest that the presence of IFN-γ is not necessary for allograft rejection but is required for tolerance induction.

Increased IL-4 concomitant with high levels of IFN-γ has been observed in other models of transplantation tolerance (Hall 2000). In fact, there is evidence indicating that oral administration of antigen does not reduce the levels of IFN-γ, but shifts the ratio of type1/type2 cytokines (Faria and Weiner 1999). For example, feeding allogeneic

peptides is associated with increased IL-4 expression with no significant decrease of IFN-γ resulting in a shift in the ratio of IL-4 and IFN-γ (Zavazava et al 2000).

The results of this cytokine shift are likely to be complex. For example, the effect of IL-4 on the generation of CTL is controversial. Experiments have shown that increased IL-4 expression is correlated with prolonged cardiac allograft survival induced by anti-CD4 mAb treatment (Lehmann et al 1997) or *Nb* infection (Liwski et al 1999). Spleen cells from these animals exhibited decreased CTL activity, suggesting that IL-4 may downregulate allospecific CTL activity (Liwski 1999). However, these data are only correlative. In contrast, IL-4 has been suggested to be a potent helper factor for generating CTL in vitro (Widmer and Grabstein 1987, Trenn et al 1988, Spits et al 1988, Bertagnolli et al 1991). Widmer and Grabstein (1987) demonstrated that allospecific CTL can be generated in culture by stimulation of allogeneic, irradiated splenocytes in the presence of IL-4. The effect of IL-4 on the generation of CTL is much greater than that of IL-2. In addition, IL-4 can enhance the generation of CD8+ CTL in the absence of CD4+ T cells (Widmer and Grabstein 1987). In our experiments, CD8+ GIC isolated from kidneys of tolerized animals expressed higher levels of IL-4 and enhanced CTL activity when compared to CD8+ GIC from control animals. It could be argued that the enhanced CTL activity is due to the increased IL-4 expression. Therefore, in our transplantation tolerance studies, IL-4 may enhance, rather than suppress, CTL activity, at least as measured in vitro.

DTH-like responses have also been suggested to play a critical role in graft rejection (Mason et al 1984, Hall 1991). Moreover, oral administration of allogeneic peptides or allosplenocytes suppress DTH-like responses (Sayegh et al 1992, Zavazava et

al 2000, Miller et al 1993). Since DTH responses are known to be depressed by type 2 cytokines, it is possible that type 2 responses induced by our feeding regimen result in prolongation of kidney allograft survival by suppression of DTH responses.

In contrast to the role of IL-4 in transplantation tolerance, IL-4 has also been suggested to play an active role in graft rejection. Increased IL-4 and IFN-γ can be found in rejecting allografts including liver, lung and heart grafts in mice and rats (Papp et al 1992, Dallman et al 1991, 1995). In addition, IL-4 mRNA is also found in some rejected or rejecting human renal (Krams et al 1992) and lung (Whitehead et al 1993) allografts. In our experiments, detectable IL-4 mRNA can be found in whole GIC, but not CD8+ GIC, from rejecting grafts of control animals. In contrast, IL-4 mRNA was detectable in the CD8+ GIC population in tolerized grafts. This points to the importance of interpreting with care cytokine expression data from human biopsies or whole organ extracts from rodents without knowledge of the cytokine source. In human biopsies, this interpretation is confounded by the fact that the patients are receiving immunosuppressive drugs that can induce limited immune tolerance. Thus, although graft rejection is ongoing, limited expression of a type 2 response may still be occurring in the grafts.

5.7 Effect of TGF-β on Oral Tolerance and Transplantation Tolerance

TGF-β has been linked to the expression of oral tolerance in some models, especially the EAE model. Feeding mice with low dose MBP significantly increases TGF-β expression in PP, MLN and spleen and suppresses the induction of EAE (Miller et al 1991, 1992, Santos et al 1994). Administration of anti-TGF-β during tolerance

induction blocks the suppressive effect in fed animals, resulting in induction of EAE after MBP challenge in vivo (Miller et al 1992). TGF-β secreting cells can be isolated in PP and MLN after oral administration of antigens and are able to transfer oral tolerance to naïve animals (Chen et al 1994). Therefore, TGF-β has been suggested to be a critical differential factor for generating oral tolerance (Gonnella et al 1998, Faria and Weiner 1999). Increased TGF-β expression has also been shown in other oral tolerance models (Caspi et al 1996). Consistent with these data, GIC from orally tolerized rats in our experiments exhibited increased TGF-β, which may play a role in suppression of immune responses as suggested by others (Faria and Weiner 1999).

There is evidence that TGF-β may also play a role in transplantation tolerance. First, TGF-β expression is increased in cardiac allografts that do not undergo acute rejection in CsA treated rats (Waltenberger et al 1993). Second, patients who received CsA treatment before their living donor renal transplantation show increased levels of TGF-β mRNA and protein in their peripheral blood mononuclear cells that corresponds with their stable renal function (Shin et al 1998). Recently, CsA has been suggested to be able to stimulate TGF-β expression, but inhibit IL-2, IL-10 and TNF-α expression in human T lymphocytes (Shin et al 1998). Third, treatment with recombinant TGF-β (Wallick et al 1990, Raju et al 1994) or TGF-β encoding vectors (Qin et al 1995, Douillard et al 1999) results in reduced graft rejection and prolonged survival of allografts in different transplantation models. For example, heart allografts injected with TGF-β encoding adenovirus survive much longer than untreated grafts (Qin et al 1995), suggesting that the overexpression of TGF-β is important for prolongation of cardiac allograft survival. Fourth, increased rejection of cardiac transplants is observed in TGF-β

deficient mice and corresponds with increased Th1 but decreased Th2 cytokine secretion (Koglin et al 1998).

Recently, cardiac allografts from donor blood transfusion treated animals have been shown to exhibit increased expression of TGF-β mRNA (Josien et al 1998). The increased TGF-β in the grafts is due to graft infiltrating cells producing large amounts of TGF-β. Neutralization of TGF-β partially abrogated transfusion tolerance. However, anti-TGF-β did not fully abrogate transfusion tolerance. Moreover, TGF-β was also present in grafts in untreated animals. Thus mechanisms other than, or in addition to, TGF-β must be involved in transfusion tolerance induction.

A role for TGF-β in oral transplantation tolerance has not been reported. Oral tolerance to cardiac allografts correlates with enhanced IL-4, but not TGF-β (Zavazava et al 2000). In our experimentation, we demonstrate that total GIC isolated from allografts of fed animals exhibit significantly enhanced levels of TGF-β mRNA, but CD8+ GIC, which transfer tolerance, did not exhibit significant increased levels of TGF-β mRNA. Thus the TGF-β in GIC of fed animals may be from CD4+ T cells and not related to graft prolongation.

Taken together these data indicate that unlike EAE, TGF-β is not a critical player in oral transplantation tolerance. However, it is possible that in our tolerance regime, although the regulatory cells are predominantly CD8+ T cells, there may be a small population of CD4+ T cells that secrete TGF-β that also exhibit regulatory function. Since the number of CD4+ T cells that could be recovered from the grafts in orally tolerized animals was lower than the CD8+ cells, it is possible that the number transferred might not be sufficient to transfer tolerance. However, we demonstrated that

transfer CD8+ GIC is significantly better for tolerance induction than transfer of whole GIC, suggesting that CD4+ T cells do not contribute in a positive manner to tolerance induction. This is in contrast to other models where CD4+ regulatory cells are thought to play a major role in tolerance induction (Barone et al 1995).

6.0 CONCLUSIONS

The evidence presented in this thesis demonstrated that oral administration of allosplenocytes can significant prolong allograft kidney survival in rats in an antigen specific manner. This prolongation is due to generation of CD8+ regulatory T cells that are not only present in spleen and MLN, but also present in allograft kidneys after oral administration of alloantigen. The presence of the regulatory cells can be demonstrated by cell transfer experiments. The regulatory T cells isolated from allograft kidney express high levels of IL-4 mRNA that may prevent allograft rejection by shifting the immune response toward to a type 2 response and away from a type 1 response. In addition, IL-4 may directly suppress DTH like responses that have been suggested to play a critical role in allograft rejection. The CD8+ regulatory graft infiltrating cells also exhibit increased FasL mRNA expression that may contribute to the deletion of alloreactive T lymphocytes in the local allografts by interaction with Fas molecules on T lymphocytes. These data suggest that oral exposure to alloantigen induce the generation of CD8+ regulatory cells which prolong allograft survival by shifting immune responses toward to type 2 responses or by deleting alloreactive T lymphocytes through Fas/FasL interaction.

7.0 REFERANCES

Actor,J.K., Shirai,M., Kullberg,M.C., Buller,R.M., Sher,A., and Berzofsky,J.A. (1993). Helminth infection results in decreased virus-specific CD8+ cytotoxic T-cell and Th1 cytokine responses as well as delayed virus clearance. Proc. Natl. Acad. Sci. U. S. A *90*, 948-952.

Alderson,M.R., Tough,T.W., Davis-Smith,T., Braddy,S., Falk,B., Schooley,K.A., Goodwin,R.G., Smith,C.A., Ramsdell,F., and Lynch,D.H. (1995). Fas ligand mediates activation-induced cell death in human T lymphocytes. J. Exp. Med. *181*, 71-77.

Algeciras,S.A., Griffith,T.S., Lynch,D.H., and Paya,C.V. (1999). Cell cycle-dependent regulation of FLIP levels and susceptibility to Fas-mediated apoptosis. J. Immunol. *162*, 5205-5211.

Andrus,L., Granelli,P.A., and Reich,E. (1984). Cytotoxic T cells both produce and respond to interleukin 2. J. Exp. Med. *159* , 647-652.

Armstrong,H.E., Bolton,E.M., McMillan,I., Spencer,S.C., and Bradley,J.A. (1987). Prolonged survival of actively enhanced rat renal allografts despite accelerated cellular infiltration and rapid induction of both class I and class II MHC antigens. J. Exp. Med. *165*, 891-907.

Aroeira,L.S., Cardillo,F., De Albuquerque,D.A., Vaz,N.M., and Mengel,J. (1995). Anti-IL-10 treatment does not block either the induction or the maintenance of orally induced tolerance to OVA. Scand. J. Immunol. *41*, 319-323.

Auchincloss,H. and Sultan,H. (1996). Antigen processing and presentation in transplantation. Curr. Opin. Immunol. *8*, 681-687.

Austyn,J.M. and Larsen,C.P. (1990). Migration patterns of dendritic leukocytes. Implications for transplantation. Transplantation *49*, 1-7.

Barone,K.S., Jain,S.L., and Michael,J.G. (1995). Effect of in vivo depletion of CD4+ and CD8+ cells on the induction and maintenance of oral tolerance. Cell Immunol. *163*, 19-29.

Barone,K.S., Reilly,M.R., Flanagan,M.P., and Michael,J.G. (2000). Abrogation of oral tolerance by feeding encapsulated antigen. Cell Immunol. *199*, 65-72.

Barone,K.S., Tolarova,D.D., Ormsby,I., Doetschman,T., and Michael,J.G. (1998). Induction of oral tolerance in TGF-beta 1 null mice. J. Immunol. *161*, 154-160.

Barry,J.M. (1992). Immunosuppressive drugs in renal transplantation. A review of the regimens. Drugs *44*, 554-566.

Beer,A.E. and Billingham,R.E. (1975). Immunologic benefits and hazards of milk in maternal-perinatal relationship. Ann. Intern. Med. *83*, 865-871.

Bellgrau,D., Gold,D., Selawry,H., Moore,J., Franzusoff,A., and Duke,R.C. (1995). A role for CD95 ligand in preventing graft rejection. Nature *19,377*, 630-632.

Bergese,S.D., Klenotic,S.M., Wakely,M.E., Sedmak,D.D., and Orosz,C.G. (1997). Apoptosis in murine cardiac grafts. Transplantation *63*, 320-325.

Bertagnolli,M.M., Takai,Y., and Herrmann,S.H. (1991). IL-4-supported induction of cytolytic T lymphocytes requires IL-2 and IL-6. Cell Immunol. *133*, 327-341.

Bishop,D.K., Chan,S., Li,W., Ensley,R.D., Xu,S., and Eichwald,E.J. (1993). CD4-positive helper T lymphocytes mediate mouse cardiac allograft rejection independent of donor alloantigen specific cytotoxic T lymphocytes. Transplantation *56*, 892-897.

Bishop,D.K., Shelby,J., and Eichwald,E.J. (1992). Mobilization of T lymphocytes following cardiac transplantation. Evidence that CD4-positive cells are required for cytotoxic T lymphocyte activation, inflammatory endothelial development, graft infiltration, and acute allograft rejection. Transplantation *53*, 849-857.

Bland,P.W. and Warren,L.G. (1986a). Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. Immunology *58*, 9-14.

Bland,P.W. and Warren,L.G. (1986b). Antigen presentation by epithelial cells of the rat small intestine. I. Kinetics, antigen specificity and blocking by anti-Ia antisera. Immunology *58*, 1-7.

Blank,M., George,J., Barak,V., Tincani,A., Koike,T., and Shoenfeld,Y. (1998). Oral tolerance to low dose beta 2-glycoprotein I: immunomodulation of experimental antiphospholipid syndrome. J. Immunol. *161*, 5303-5312.

Bleicher,P.A., Balk,S.P., Hagen,S.J., Blumberg,R.S., Flotte,T.J., and Terhorst,C. (1990). Expression of murine CD1 on gastrointestinal epithelium. Science *250*, 679-682.

Boismenu,R. and Havran,W.L. (1998). Gammadelta T cells in host defense and epithelial cell biology. Clin. Immunol. Immunopathol. *86*, 121-133.

Bossu,P., Singer,G.G., Andres,P., Ettinger,R., Marshak,R.A., and Abbas,A.K. (1993). Mature CD4+ T lymphocytes from MRL/lpr mice are resistant to receptor-mediated tolerance and apoptosis. J. Immunol. *151*, 7233-7239.

Bradley,J.A., Mason,D.W., and Morris,P.J. (1985). Evidence that rat renal allografts are rejected by cytotoxic T cells and not by nonspecific effectors. Transplantation *39*, 169-175.

Bradley,J.A., Mowat,A.M., and Bolton,E.M. (1992a). Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. Immunol. Today *13*, 434-438.

Bradley,J.A., Sarawar,S.R., Porteous,C., Wood,P.J., Card,S., Ager,A., Bolton,E.M., and Bell,E.B. (1992b). Allograft rejection in CD4+ T cell-reconstituted athymic nude rats--the nonessential role of host-derived CD8+ cells. Transplantation *53*, 477-482.

Braun,M.Y., Lowin,B., French,L., Acha-Orbea,H., and Tschopp,J. (1996). Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. J. Exp. Med. *183*, 657-661.

Bushell,A., Morris,P.J., and Wood,K.J. (1995a). Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4+ T cell regulation during the induction phase of the response. Eur. J. Immunol. *25*, 2643-2649.

Bushell,A., Niimi,M., Morris,P.J., and Wood,K.J. (1999). Evidence for immune regulation in the induction of transplantation tolerance: a conditional but limited role for IL-4. J. Immunol. *162*, 1359-1366.

Bushell,A., Pearson,T.C., Morris,P.J., and Wood,K.J. (1995b). Donor-recipient microchimerism is not required for tolerance induction following recipient pretreatment with donor-specific transfusion and anti-CD4 antibody. Evidence of a clear role for short-term antigen persistence [see comments]. Transplantation *59*, 1367-1371.

Callery,M.P., Kamei,T., and Flye,M.W. (1989). Kupffer cell blockade inhibits induction of tolerance by the portal venous route. Transplantation *47*, 1092-1094.

Campbell,D.A., Lorber,M.I., Sweeton,J.C., Turcotte,J.G., Niederhuber,J.E., and Beer,A.E. (1984). Breast feeding and maternal-donor renal allografts. Possibly the original donor-specific transfusion. Transplantation *37*, 340-344.

Campbell,N., Yio,X.Y., So,L.P., Li,Y., and Mayer,L. (1999). The intestinal epithelial cell: processing and presentation of antigen to the mucosal immune system. Immunol. Rev. *172*, 315-324.

Cantor,H.M. and Dumont,A.E. (1967). Hepatic suppression of sensitization to antigen absorbed into the portal system. Nature *215*, 744-745.

Caspi,R.R., Stiff,L.R., Morawetz,R., Miller-Rivero,N.E., Chan,C.C., Wiggert,B., Nussenblatt,R.B., Morse,H.C., and Rizzo,L.V. (1996). Cytokine-dependent modulation of oral tolerance in a murine model of autoimmune uveitis. Ann. N. Y. Acad. Sci. *778:* 315-324.

Cerwenka,A., Morgan,T.M., Harmsen,A.G., and Dutton,R.W. (1999). Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. J. Exp. Med. *189*, 423-434.

Chan,S.Y., DeBruyne,L.A., Goodman,R.E., Eichwald,E.J., and Bishop,D.K. (1995). In vivo depletion of CD8+ T cells results in Th2 cytokine production and alternate mechanisms of allograft rejection. Transplantation *59*, 1155-1161.

Chen,N., Gao,Q., and Field,E.H. (1996a). Prevention of Th1 response is critical for tolerance. Transplantation *61*, 1076-1083.

Chen,W., Murphy,B., Waaga,A.M., Willett,T.A., Russell,M.E., Khoury,S.J., and Sayegh,M.H. (1996b). Mechanisms of indirect allorecognition in graft rejection: class II MHC allopeptide-specific T cell clones transfer delayed-type hypersensitivity responses in vivo. Transplantation *62*, 705-710.

Chen,Y., Inobe,J., and Weiner,H.L. (1995a). Induction of oral tolerance to myelin basic protein in CD8-depleted mice: both CD4+ and CD8+ cells mediate active suppression. J. Immunol. *155*, 910-916.

Chen,Y., Inobe,J., and Weiner,H.L. (1997). Inductive events in oral tolerance in the TCR transgenic adoptive transfer model. Cell Immunol. *178*, 62-68.

Chen,Y., Inobe,J., Kuchroo,V.K., Baron,J.L., Janeway,C.A., and Weiner,H.L. (1996a). Oral tolerance in myelin basic protein T-cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells. Proc. Natl. Acad. Sci. U. S. A *93*, 388-391.

Chen,Y., Inobe,J., Marks,R., Gonnella,P., Kuchroo,V.K., and Weiner,H.L. (1995b). Peripheral deletion of antigen-reactive T cells in oral tolerance [published erratum appears in Nature 1995 Sep 21;377(6546):257]. Nature *376*, 177-180.

Chen,Y., Kuchroo,V.K., Inobe,J., Hafler,D.A., and Weiner,H.L. (1994). Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science *265*, 1237-1240.

Chen,Y.H. and Weiner,H.L. (1996). Dose-dependent activation and deletion of antigen-specific T cells following oral tolerance. Ann. N. Y. Acad. Sci. *778:111-21*, 111-121.

Chen,Z.K., Cobbold,S.P., Waldmann,H., and Metcalfe,S. (1996b). Amplification of natural regulatory immune mechanisms for transplantation tolerance. Transplantation *62*, 1200-1206.

Cher,D.J. and Mosmann,T.R. (1987). Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. J. Immunol. *138*, 3688-3694.

Chien,Y.H., Jores,R., and Crowley,M.P. (1996). Recognition by gamma/delta T cells. Annu. Rev. Immunol. *14*, 511-532.

Chiu,V.K., Walsh,C.M., Liu,C.C., Reed,J.C., and Clark,W.R. (1995). Bcl-2 blocks degranulation but not fas-based cell-mediated cytotoxicity. J. Immunol. *154*, 2023-2032.

Cohen,P.L. and Eisenberg,R.A. (1991). Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu. Rev. Immunol. *9*, 243-269.

Constant,S.L. and Bottomly,K. (1997). Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu. Rev. Immunol. *15*, 297-322.

Coyle,A.J., Bertrand,C., Tsuyuki,S., Pircher,H., Walti,S., Le Gros,G., and Erard,F. (1996). IL-4 differentiates naive CD8+ T cells to a "Th2-like" phenotype: a link between viral infections and bronchial asthma. Ann. N. Y. Acad. Sci. *796*, 97-103.

Coyle,A.J., Erard,F., Bertrand,C., Walti,S., Pircher,H., and Le Gros,G. (1995). Virus-specific CD8+ cells can switch to interleukin 5 production and induce airway eosinophilia. J. Exp. Med. *181*, 1229-1233.

Cronin,D.C., Stack,R., and Fitch,F.W. (1995). IL-4-producing CD8+ T cell clones can provide B cell help. J. Immunol. *154*, 3118-3127.

Dai,Z., Konieczny,B.T., Baddoura,F.K., and Lakkis,F.G. (1998). Impaired alloantigen-mediated T cell apoptosis and failure to induce long-term allograft survival in IL-2-deficient mice. J. Immunol. *161*, 1659-1663.

Dallman,M.J. (1993). Cytokines as mediators of organ graft rejection and tolerance. Curr. Opin. Immunol. *5*, 788-793.

Dallman,M.J. (1995). Cytokines and transplantation: Th1/Th2 regulation of the immune response to solid organ transplants in the adult. Curr. Opin. Immunol. *7*, 632-638.

Dallman,M.J., Larsen,C.P., and Morris,P.J. (1991). Cytokine gene transcription in vascularised organ grafts: analysis using semiquantitative polymerase chain reaction. J. Exp. Med. *174*, 493-496.

Dallman,M.J., Wood,K.J., and Morris,P.J. (1987). Specific cytotoxic T cells are found in the nonrejected kidneys of blood-transfused rats. J. Exp. Med. *165*, 566-571.

Dallman,M.J., Wood,K.J., and Morris,P.J. (1988). Cytotoxicity and IL 2 reactivity of cells from rejected or nonrejected allografts. Transplant. Proc. *20*, 226-228.

Dalloul,A.H., Chmouzis,E., Ngo,K., and Fung-Leung,W.P. (1996a). Adoptively transferred CD4+ lymphocytes from CD8 -/- mice are sufficient to mediate the rejection of MHC class II or class I disparate skin grafts. J. Immunol. *156*, 4114-4119.

Dalloul,A.H., Ngo,K., and Fung-Leung,W.P. (1996b). CD4-negative cytotoxic T cells with a T cell receptor alpha/beta intermediate expression in CD8-deficient mice. Eur. J. Immunol. *26*, 213-218.

Davies,J.D., Leong,L.Y., Mellor,A., Cobbold,S.P., and Waldmann,H. (1996b). T cell suppression in transplantation tolerance through linked recognition. J. Immunol. *156*, 3602-3607.

Davies,J.D., Martin,G., Phillips,J., Marshall,S.E., Cobbold,S.P., and Waldmann,H. (1996a). T cell regulation in adult transplantation tolerance. J. Immunol. *157*, 529-533.

Desvignes,C., Etchart,N., Kehren,J., Akiba,I., Nicolas,J.F., and Kaiserlian,D. (2000). Oral administration of hapten inhibits in vivo induction of specific cytotoxic CD8+ T cells mediating tissue inflammation: a role for regulatory CD4+ T cells. J. Immunol. *164*, 2515-2522.

Dhein,J., Walczak,H., Baumler,C., Debatin,K.M., and Krammer,P.H. (1995). Autocrine T-cell suicide mediated by APO-1/(Fas/CD95) [see comments]. Nature *373*, 438-441.

D'Orazio,T.J. and Niederkorn,J.Y. (1998). A novel role for TGF-beta and IL-10 in the induction of immune privilege. J. Immunol. *160*, 2089-2098.

Douillard,P., Pannetier,C., Josien,R., Menoret,S., Kourilsky,P., Soulillou,J.P., and Cuturi,M.C. (1996). Donor-specific blood transfusion-induced tolerance in adult rats with a dominant TCR-Vbeta rearrangement in heart allografts. J. Immunol. *157*, 1250-1260.

Douillard,P., Vignes,C., Josien,R., Chiffoleau,E., Heslan,J.M., Proust,V., Soulillou,J.P., and Cuturi,M.C. (1999). Reassessment of the role of CD8+ T cells in the induction of allograft tolerance by donor-specific blood transfusion. Eur. J. Immunol. *29*, 1919-1924.

Erard,F., Wild,M.T., Garcia-Sanz,J.A., and Le Gros,G. (1993). Switch of CD8 T cells to noncytolytic CD8-CD4- cells that make TH2 cytokines and help B cells. Science *260*, 1802-1805.

Everson,M.P., McDuffie,D.S., Lemak,D.G., Koopman,W.J., McGhee,J.R., and Beagley,K.W. (1996). Dendritic cells from different tissues induce production of different T cell cytokine profiles. J. Leukoc. Biol. *59*, 494-498.

Fabre,J.W. and Morris,P.J. (1972). The effect of donor strain blood pretreatment on renal allograft rejection in rats. Transplantation *14*, 608-617.

Faria,A.M. and Weiner,H.L. (1999). Oral tolerance: mechanisms and therapeutic applications. Adv. Immunol. *73:153-264*, 153-264.

Field,E.H., Gao,Q., Chen,N.X., and Rouse,T.M. (1997). Balancing the immune system for tolerance: a case for regulatory CD4 cells. Transplantation *64*, 1-7.

Fowler,D.H., Breglio,J., Nagel,G., Eckhaus,M.A., and Gress,R.E. (1996). Allospecific CD8+ Tc1 and Tc2 populations in graft-versus-leukemia effect and graft-versus-host disease. J. Immunol. *157*, 4811-4821.

Fowler,D.H., Whitfield,B., Livingston,M., Chrobak,P., and Gress,R.E. (1998). Non-host-reactive donor CD8+ T cells of Tc2 phenotype potently inhibit marrow graft rejection. Blood *91*, 4045-4050.

Framson,P.E., Cho,D.H., Lee,L.Y., and Hershberg,R.M. (1999). Polarized expression and function of the costimulatory molecule CD58 on human intestinal epithelial cells. Gastroenterology *116*, 1054-1062.

Franco,L., Benedetti,R., Ferek,G.A., Massouh,E., and Flo,J. (1998). Priming or tolerization of the B- and Th2-dependent immune response by the oral administration of OVA-DNP is determined by the antigen dosage. Cell Immunol. *190*, 1-11.

Friedman,A. and Weiner,H.L. (1994). Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. Proc. Natl. Acad. Sci. U. S. A *91*, 6688-6692.

Fu,F., Thai,N.L., Li,Y., Lu,L., Thomson,A.W., Fung,J.J., and Qian,S. (1996). Second-set rejection of mouse liver allografts is dependent on radiation-sensitive nonparenchymal cells of graft bone marrow origin. Transplantation *61*, 1228-1233.

Fujiwara,H., Qian,J.H., Satoh,S., Kokudo,S., Ikegami,R., and Hamaoka,T. (1986). Studies on the induction of tolerance to alloantigens. II. The generation of serum factor(s) able to transfer alloantigen-specific tolerance for delayed-type hypersensitivity by portal venous inoculation with allogeneic cells. J. Immunol. *136*, 2763-2768.

Fukaura,H., Kent,S.C., Pietrusewicz,M.J., Khoury,S.J., Weiner,H.L., and Hafler,D.A. (1996). Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. J. Clin. Invest *98*, 70-77.

Galliaerde,V., Desvignes,C., Peyron,E., and Kaiserlian,D. (1995). Oral tolerance to haptens: intestinal epithelial cells from 2,4-dinitrochlorobenzene-fed mice inhibit hapten-specific T cell activation in vitro. Eur. J. Immunol. *25*, 1385-1390.

Gao,Q., Chen,N., Rouse,T.M., and Field,E.H. (1996). The role of interleukin-4 in the induction phase of allogeneic neonatal tolerance. Transplantation *62*, 1847-1854.

Garside,P. and Mowat,A.M. (1997). Mechanisms of oral tolerance. Crit Rev. Immunol. *17*, 119-137.

Garside,P., Mowat,A.M., and Khoruts,A. (1999). Oral tolerance in disease. Gut *44*, 137-142.

Garside,P., Steel,M., Liew,F.Y., and Mowat,A.M. (1995b). CD4+ but not CD8+ T cells are required for the induction of oral tolerance. Int. Immunol. *7*, 501-504.

Garside,P., Steel,M., Worthey,E.A., Satoskar,A., Alexander,J., Bluethmann,H., Liew,F.Y., and Mowat,A.M. (1995a). T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. J. Immunol. *154*, 5649-5655.

George,J.F., Sweeney,S.D., Kirklin,J.K., Simpson,E.M., Goldstein,D.R., and Thomas,J.M. (1998). An essential role for Fas ligand in transplantation tolerance induced by donor bone marrow. Nat. Med. *4*, 333-335.

Gonnella,P.A., Chen,Y., Inobe,J., Komagata,Y., Quartulli,M., and Weiner,H.L. (1998). In situ immune response in gut-associated lymphoid tissue (GALT) following oral antigen in TCR-transgenic mice. J. Immunol. *160*, 4708-4718.

Gorczynski,R.M. (1992). Immunosuppression induced by hepatic portal venous immunization spares reactivity in IL-4 producing T lymphocytes. Immunol. Lett. *33*, 67-77.

Gorczynski,R.M. (1995). Regulation of IFN-gamma and IL-10 synthesis in vivo, as well as continuous antigen exposure, is associated with tolerance to murine skin allografts. Cell Immunol. *160*, 224-231.

Gorczynski,R.M. and Wojcik,D. (1994). A role for nonspecific (cyclosporin A) or specific (monoclonal antibodies to ICAM-1, LFA-1, and IL-10) immunomodulation in the prolongation of skin allografts after antigen-specific pretransplant immunization or transfusion. J. Immunol. *152*, 2011-2019.

Gorczynski,R.M., Chen,Z., Fu,X.M., and Zeng,H. (1998a). Increased expression of the novel molecule OX-2 is involved in prolongation of murine renal allograft survival. Transplantation *65*, 1106-1114.

Gorczynski,R.M., Chen,Z., Zeng,H., and Fu,X.M. (1998b). A role for persisting antigen, antigen presentation, and ICAM-1 in increased renal graft survival after oral or portal vein donor-specific immunization. Transplantation *66*, 339-349.

Gorczynski,R.M., Hozumi,N., Wolf,S., and Chen,Z. (1995). Interleukin 12 in combination with anti-interleukin 10 reverses graft prolongation after portal venous immunization. Transplantation *60*, 1337-1341.

Gracie,J.A., Bolton,E.M., Porteous,C., and Bradley,J.A. (1990). T cell requirements for the rejection of renal allografts bearing an isolated class I MHC disparity. J. Exp. Med. *172*, 1547-1557.

Grau,V., Gemsa,D., Steiniger,B., and Garn,H. (2000). Chemokine expression during acute rejection of rat kidneys. Scand. J. Immunol. *51*, 435-440.

Gregerson,D.S., Obritsch,W.F., and Donoso,L.A. (1993). Oral tolerance in experimental autoimmune uveoretinitis. Distinct mechanisms of resistance are induced by low dose vs high dose feeding protocols. J. Immunol. *151*, 5751-5761.

Gregory,S.H. and Wing,E.J. (1998). Neutrophil-Kupffer-cell interaction in host defenses to systemic infections. Immunol. Today *19*, 507-510.

Griffith,T.S. and Ferguson,T.A. (1997). The role of FasL-induced apoptosis in immune privilege. Immunol. Today *18*, 240-244.

Griffith,T.S., Brunner,T., Fletcher,S.M., Green,D.R., and Ferguson,T.A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*, 1189-1192.

Griffith,T.S., Yu,X., Herndon,J.M., Green,D.R., and Ferguson,T.A. (1996). CD95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance. Immunity. *5*, 7-16.

Groux,H., Bigler,M., de Vries,J.E., and Roncarolo,M.G. (1996). Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J. Exp. Med. *184*, 19-29.

Hadley,G.A., Rostapshova,E.A., and Bartlett,S.T. (1996). Dominance of tissue-restricted cytotoxic T lymphocytes in the response to allogeneic renal epithelial cell lines. Transplantation *62*, 75-83.

Hall,B.M. (1991). Cells mediating allograft rejection [published erratum appears in Transplantation 1991 Nov;52(5):936]. Transplantation *51*, 1141-1151.

Halverson,D.C., Schwartz,G.N., Carter,C., Gress,R.E., and Fowler,D.H. (1997). In vitro generation of allospecific human CD8+ T cells of Tc1 and Tc2 phenotype. Blood *90*, 2089-2096.

Hancock,W.W., Buelow,R., Sayegh,M.H., and Turka,L.A. (1998). Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. Nat. Med. *4*, 1392-1396.

Hancock,W.W., Sayegh,M.H., Kwok,C.A., Weiner,H.L., and Carpenter,C.B. (1993). Oral, but not intravenous, alloantigen prevents accelerated allograft rejection by selective intragraft Th2 cell activation. Transplantation *55*, 1112-1118.

Hanson,D.G., Roy,M.J., Green,G.M., and Miller,S.D. (1993). Inhibition of orally-induced immune tolerance in mice by prefeeding an endopeptidase inhibitor. Reg Immunol. *5*, 76-84.

He,G., Hart,J., Kim,O.S., Szot,G.L., Siegel,C.T., Thistlethwaite,J.R., and Newell,K.A. (1999). The role of CD8 and CD4 T cells in intestinal allograft rejection: a comparison of monoclonal antibody-treated and knockout mice. Transplantation *67*, 131-137.

He,Y.G., Mellon,J., and Niederkorn,J.Y. (1996). The effect of oral immunization on corneal allograft survival. Transplantation *61*, 920-926.

Heinzel,F.P., Sadick,M.D., Holaday,B.J., Coffman,R.L., and Locksley,R.M. (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J. Exp. Med. *169*, 59-72.

Hershberg,R.M., Cho,D.H., Youakim,A., Bradley,M.B., Lee,J.S., Framson,P.E., and Nepom,G.T. (1998). Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. J. Clin. Invest *102*, 792-803.

Hershberg,R.M., Framson,P.E., Cho,D.H., Lee,L.Y., Kovats,S., Beitz,J., Blum,J.S., and Nepom,G.T. (1997). Intestinal epithelial cells use two distinct pathways for HLA class II antigen processing. J. Clin. Invest *100*, 204-215.

Hoyne,G.F. and Thomas,W.R. (1995). T-cell responses to orally administered antigens. Study of the kinetics of lymphokine production after single and multiple feeding. Immunology *84*, 304-309.

Hoyne,G.F., Callow,M.G., Kuhlman,J., and Thomas,W.R. (1993). T-cell lymphokine response to orally administered proteins during priming and unresponsiveness. Immunology *78*, 534-540.

Ilan,Y., Gotsman,I., Pines,M., Beinart,R., Zeira,M., Ohana,M., Rabbani,E., Engelhardt,D., and Nagler,A. (2000). Induction of oral tolerance in splenocyte recipients toward pretransplant antigens ameliorates chronic graft versus host disease in a murine model. Blood *95*, 3613-3619.

Inobe,J., Slavin,A.J., Komagata,Y., Chen,Y., Liu,L., and Weiner,H.L. (1998). IL-4 is a differentiation factor for transforming growth factor-beta secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. Eur. J. Immunol. *28*, 2780-2790.

Ishido,N., Matsuoka,J., Matsuno,T., Nakagawa,K., and Tanaka,N. (1999). Induction of donor-specific hyporesponsiveness and prolongation of cardiac allograft survival by jejunal administration of donor splenocytes. Transplantation *68*, 1377-1382.

Jain,S.L., Barone,K.S., Flanagan,M.P., and Michael,J.G. (1996). Activation patterns of murine B cells after oral administration of an encapsulated soluble antigen [corrected and republished article originally printed in Vaccine 1996 Jan;14(1):42-8]. Vaccine *14*, 1291-1297.

Jenkins,M., Keir,M., and McCune,J.M. (1999). Fas is expressed early in human thymocyte development but does not transmit an apoptotic signal. J. Immunol. *163*, 1195-1204.

Josien,R., Cuturi,M.C., Douillard,P., Heslan,M., Heslan,J.M., and Soulillou,J.P. (1999). Recombinant IFN-gamma abrogates allograft tolerance induced by donor-specific blood transfusion by restoring alloantibody production. Eur. J. Immunol. *29*, 317-326.

Josien,R., Douillard,P., Guillot,C., Muschen,M., Anegon,I., Chetritt,J., Menoret,S., Vignes,C., Soulillou,J.P., and Cuturi,M.C. (1998b). A critical role for transforming growth factor-beta in donor transfusion-induced allograft tolerance. J. Clin. Invest *102*, 1920-1926.

Josien,R., Heslan,M., Brouard,S., Soulillou,J.P., and Cuturi,M.C. (1998a). Critical requirement for graft passenger leukocytes in allograft tolerance induced by donor blood transfusion. Blood *92*, 4539-4544.

Kabelitz,D., Pohl,T., and Pechhold,K. (1993). Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. Immunol. Today *14*, 338-339.

Kaiserlian,D. (1999). Epithelial cells in antigen. Sampling and presentation in mucosal tissues. Curr. Top. Microbiol. Immunol. *236*, 55-78.

Kamei,T., Callery,M.P., and Flye,M.W. (1990). Pretransplant portal venous administration of donor antigen and portal venous allograft drainage synergistically prolong rat cardiac allograft survival. Surgery *108*, 415-421.

Kanagawa,O., Vaupel,B.A., Gayama,S., Koehler,G., and Kopf,M. (1993). Resistance of mice deficient in IL-4 to retrovirus-induced immunodeficiency syndrome (MAIDS). Science *262*, 240-242.

Kang,S.M., Braat,D., Schneider,D.B., O'Rourke,R.W., Lin,Z., Ascher,N.L., Dichek,D.A., Baekkeskov,S., and Stock,P.G. (2000). A non-cleavable mutant of Fas ligand does not prevent neutrophilic destruction of islet transplants. Transplantation *69*, 1813-1817.

Kang,S.M., Hoffmann,A., Le,D., Springer,M.L., Stock,P.G., and Blau,H.M. (1997a). Immune response and myoblasts that express Fas ligand [letter; comment]. Science *278*, 1322-1324.

Kang,S.M., Schneider,D.B., Lin,Z., Hanahan,D., Dichek,D.A., Stock,P.G., and Baekkeskov,S. (1997b). Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction [see comments]. Nat. Med. *3*, 738-743.

Kato,S., Akasaka,Y., and Kawamura,S. (1997). Fas antigen expression and its relationship with apoptosis in transplanted kidney. Pathol. Int. *47*, 230-237.

Ke,Y. and Kapp,J.A. (1996). Oral antigen inhibits priming of CD8+ CTL, CD4+ T cells, and antibody responses while activating CD8+ suppressor T cells. J. Immunol. *156*, 916-921.

Ke,Y., Pearce,K., Lake,J.P., Ziegler,H.K., and Kapp,J.A. (1997). Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance. J. Immunol. *158*, 3610-3618.

Kelsall,B.L. and Strober,W. (1996). Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. J. Exp. Med. *183*, 237-247.

Kelsall,B.L. and Strober,W. (1997). Peyer's patch dendritic cells and the induction of mucosal immune responses. Res. Immunol. *148*, 490-498.

Kita,H., Weiler,D.A., Abu,G.R., Sanderson,C.J., and Gleich,G.J. (1992). Release of granule proteins from eosinophils cultured with IL-5. J. Immunol. *149*, 629-635.

Koglin,J., Glysing,J.T., Raisanen,S.A., and Russell,M.E. (1998). Immune sources of transforming growth factor-beta1 reduce transplant arteriosclerosis: insight derived from a knockout mouse model. Circ. Res. *83*, 652-660.

Konieczny,B.T., Dai,Z., Elwood,E.T., Saleem,S., Linsley,P.S., Baddoura,F.K., Larsen,C.P., Pearson,T.C., and Lakkis,F.G. (1998). IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. J. Immunol. *160*, 2059-2064.

Krammer,P.H. (1999). CD95(APO-1/Fas)-mediated apoptosis: live and let die. Adv. Immunol. *71*, 163-210.

Krams,S.M., Falco,D.A., Villanueva,J.C., Rabkin,J., Tomlanovich,S.J., Vincenti,F., Amend,W.J., Melzer,J., Garovoy,M.R., Roberts,J.P., and et,a. (1992). Cytokine and T cell receptor gene expression at the site of allograft rejection. Transplantation *53*, 151-156.

Krams,S.M., Hayashi,M., Fox,C.K., Villanueva,J.C., Whitmer,K.J., Burns,W., Esquivel,C.O., and Martinez,O.M. (1998). CD8+ cells are not necessary for allograft rejection or the induction of apoptosis in an experimental model of small intestinal transplantation. J. Immunol. *160*, 3673-3680.

Krieger,N.R., Yin,D.P., and Fathman,C.G. (1996). CD4+ but not CD8+ cells are essential for allorejection. J. Exp. Med. *184*, 2013-2018.

Kupiec-Weglinski,J.W., Wasowska,B., Papp,I., Schmidbauer,G., Sayegh,M.H., Baldwin,W.M., Wieder,K.J., and Hancock,W.W. (1993). CD4 mAb therapy modulates alloantibody production and intracardiac graft deposition in association with selective inhibition of Th1 lymphokines. J. Immunol. *151*, 5053-5061.

Kusne,S., Martin,M., Shapiro,R., Jordan,M., Fung,J., Alessiani,M., Jain,A., Todo,S., and Starzl,T. (1991). Early infections in kidney transplant recipients under FK 506. Transplant. Proc. *23*, 956-957.

Kweon,M.N., Fujihashi,K., VanCott,J.L., Higuchi,K., Yamamoto,M., McGhee,J.R., and Kiyono,H. (1998). Lack of orally induced systemic unresponsiveness in IFN-gamma knockout mice. J. Immunol. *160*, 1687-1693.

Lakkis,F.G., Konieczny,B.T., Saleem,S., Baddoura,F.K., Linsley,P.S., Alexander,D.Z., Lowry,R.P., Pearson,T.C., and Larsen,C.P. (1997). Blocking the CD28-B7 T cell costimulation pathway induces long term cardiac allograft acceptance in the absence of IL-4. J. Immunol. *158*, 2443-2448.

Larsen,C.P., Alexander,D.Z., Hendrix,R., Ritchie,S.C., and Pearson,T.C. (1995). Fas-mediated cytotoxicity. An immunoeffector or immunoregulatory pathway in T cell-mediated immune responses? Transplantation *60*, 221-224.

Larsen,C.P., Morris,P.J., and Austyn,J.M. (1990). Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. J. Exp. Med. *171*, 307-314.

Lau,H.T., Yu,M., Fontana,A., and Stoeckert,C.J. (1996). Prevention of islet allograft rejection with engineered myoblasts expressing FasL in mice. Science *273*, 109-112.

Le Gros,G. and Erard,F. (1994). Non-cytotoxic, IL-4, IL-5, IL-10 producing CD8+ T cells: their activation and effector functions. Curr. Opin. Immunol. *6*, 453-457.

Le Moine,A., Flamand,V., Demoor,F.X., Noel,J.C., Surquin,M., Kiss,R., Nahori,M.A., Pretolani,M., Goldman,M., and Abramowicz,D. (1999a). Critical roles for IL-4, IL-5, and eosinophils in chronic skin allograft rejection. J. Clin. Invest *103*, 1659-1667.

Le Moine,A., Surquin,M., Demoor,F.X., Noel,J.C., Nahori,M.A., Pretolani,M., Flamand,V., Braun,M.Y., Goldman,M., and Abramowicz,D. (1999b). IL-5 mediates eosinophilic rejection of MHC class II-disparate skin allografts in mice. J. Immunol. *163*, 3778-3784.

Lechler,R.I. and Batchelor,J.R. (1982). Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. J. Exp. Med. *155*, 31-41.

Ledingham,D.L., McAlister,V.C., Ehigiator,H.N., Giacomantonio,C., Theal,M., and Lee,T.D. (1996). Prolongation of rat kidney allograft survival by nematodes. Transplantation *61*, 184-188.

Lehmann,M., Graser,E., Risch,K., Hancock,W.W., Muller,A., Kuttler,B., Hahn,H.J., Kupiec-Weglinski,J.W., Brock,J., and Volk,H.D. (1997). Anti-CD4 monoclonal antibody-induced allograft tolerance in rats despite persistence of donor-reactive T cells. Transplantation *64*, 1181-1187.

Lehmann,M., Graser,E., Risch,K., Hancock,W.W., Muller,A., Kuttler,B., Hahn,H.J., Kupiec-Weglinski,J.W., Brock,J., and Volk,H.D. (1997). Anti-CD4 monoclonal antibody-induced allograft tolerance in rats despite persistence of donor-reactive T cells. Transplantation *64*, 1181-1187.

Leprince,P., Fretz,C., Dorent,R., Boudifa,A., Jourdan,J., Youssoub,J.J., Bors,V., Nataf,P., Pavie,A., and Gandjbakhch,I. (1999). Posttransplantation cytotoxic immunoglobulin G is associated with a high rate of acute allograft dysfunctions in heart transplant recipients. Am. Heart J. *138*, 586-592.

Li,J.H., Rosen,D., Ronen,D., Behrens,C.K., Krammer,P.H., Clark,W.R., and Berke,G. (1998b). The regulation of CD95 ligand expression and function in CTL. J. Immunol. *161*, 3943-3949.

Li,L., Sad,S., Kagi,D., and Mosmann,T.R. (1997). CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. J. Immunol. *158*, 4152-4161.

Li,W.M., Laur,O., Hekele,A., Coy,J., Walczak,H., and Krammer,P.H. (1998c). A regulatory element in the CD95 (APO-1/Fas) ligand promoter is essential for responsiveness to TCR-mediated activation. Eur. J. Immunol. *28*, 2373-2383.

Li,X.C., Li,Y., Dodge,I., Wells,A.D., Zheng,X.X., Turka,L.A., and Strom,T.B. (1999). Induction of allograft tolerance in the absence of Fas-mediated apoptosis. J. Immunol. *163*, 2500-2507.

Li,X.C., Roy,C.P., Hancock,W.W., Manfro,R., Zand,M.S., Li,Y., Zheng,X.X., Nickerson,P.W., Steiger,J., Malek,T.R., and Strom,T.B. (1998a). IL-2 and IL-4 double knockout mice reject islet allografts: a role for novel T cell growth factors in allograft rejection. J. Immunol. *161*, 890-896.

Li,X.K., Okuyama,T., Tamura,A., Enosawa,S., Kaneda,Y., Takahara,S., Funashima,N., Yamada,M., Amemiya,H., and Suzuki,S. (1998). Prolonged survival of rat liver allografts transfected with Fas ligand-expressing plasmid. Transplantation *66*, 1416-1423.

Lider,O., Santos,L.M., Lee,C.S., Higgins,P.J., and Weiner,H.L. (1989). Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. II. Suppression of disease and in vitro immune responses is mediated by antigen-specific CD8+ T lymphocytes. J. Immunol. *142*, 748-752.

Liu,L.M. and MacPherson,G.G. (1991). Lymph-borne (veiled) dendritic cells can acquire and present intestinally administered antigens. Immunology *73*, 281-286.

Liu,Z., Sun,Y.K., Xi,Y.P., Maffei,A., Reed,E., Harris,P., and Suciu,F.N. (1993). Contribution of direct and indirect recognition pathways to T cell alloreactivity. J. Exp. Med. *177*, 1643-1650.

Liwski,R., Zhou,J., McAlister,V., and Lee,T.D. (2000). Prolongation of allograft survival by Nippostrongylus brasiliensis is associated with decreased allospecific cytotoxic T lymphocyte activity and development of T cytotoxic cell type 2 cells. Transplantation *69*, 1912-1922.

Lohse,A.W., Knolle,P.A., Bilo,K., Uhrig,A., Waldmann,C., Ibe,M., Schmitt,E., Gerken,G., and Meyer Zum Buschenfelde,K.H. (1996). Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. Gastroenterology *110*, 1175-1181.

Lowin,B., Hahne,M., Mattmann,C., and Tschopp,J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature *370*, 650-652.

Lynch,D.H., Ramsdell,F., and Alderson,M.R. (1995). Fas and FasL in the homeostatic regulation of immune responses. Immunol. Today *16*, 569-574.

Ma,D., Mellon,J., and Niederkorn,J.Y. (1997). Oral immunisation as a strategy for enhancing corneal allograft survival [see comments]. Br. J. Ophthalmol. *81*, 778-784.

Maeda,H., Ogoshi,S., and Fujimoto,S. (1994b). Involvement of Th2-like T cells in regulating allograft reactivity against MHC class II-disparate antigen. Transplant. Proc. *26*, 847-848.

Maeda,H., Takata,M., Takahashi,S., Ogoshi,S., and Fujimoto,S. (1994a). Adoptive transfer of a Th2-like cell line prolongs MHC class II antigen disparate skin allograft survival in the mouse. Int. Immunol. *6*, 855-862.

Mannon,R.B., Kotzin,B.L., Nataraj,C., Ferri,K., Roper,E., Kurlander,R.J., and Coffman,T.M. (1998). Downregulation of T cell receptor expression by CD8(+) lymphocytes in kidney allografts. J. Clin. Invest *101*, 2517-2527.

Mannon,R.B., Kotzin,B.L., Roper,E., Nataraj,C., Kurlander,R.J., and Coffman,T.M. (1996). The intragraft CD8+ T cell response in renal allograft rejection in the mouse. Transplantation *62*, 96-104.

Mannon,R.B., Nataraj,C., Kotzin,B.L., Griffiths,R., Geier,S., Ibrahim,S., Sanfilippo,F.P., Platt,J.L., Kurlander,R., and Coffman,T.M. (1995). Rejection of kidney allografts by MHC class I-deficient mice. Transplantation *59*, 746-755.

Marth,T., Strober,W., and Kelsall,B.L. (1996). High dose oral tolerance in ovalbumin TCR-transgenic mice: systemic neutralization of IL-12 augments TGF-beta secretion and T cell apoptosis. J. Immunol. *157* , 2348-2357.

Martinez,O.M., Ascher,N.L., Ferrell,L., Villanueva,J., Lake,J., Roberts,J.P., and Krams,S.M. (1993). Evidence for a nonclassical pathway of graft rejection involving interleukin 5 and eosinophils. Transplantation *55*, 909-918.

Martinez,O.M., Krams,S.M., Sterneck,M., Villanueva,J.C., Falco,D.A., Ferrell,L.D., Lake,J., Roberts,J.P., and Ascher,N.L. (1992). Intragraft cytokine profile during human liver allograft rejection. Transplantation *53*, 449-456.

Martinez,O.M., Villanueva,J.C., Lake,J., Roberts,J.P., Ascher,N.L., and Krams,S.M. (1993). IL-2 and IL-5 gene expression in response to alloantigen in liver allograft recipients and in vitro. Transplantation *55*, 1159-1166.

Mason,D.W., Dallman,M.J., Arthur,R.P., and Morris,P.J. (1984). Mechanisms of allograft rejection: the roles of cytotoxic T-cells and delayed-type hypersensitivity. Immunol. Rev. *77*, 167-184.

Matesic,D., Valujskikh,A., Pearlman,E., Higgins,A.W., Gilliam,A.C., and Heeger,P.S. (1998). Type 2 immune deviation has differential effects on alloreactive CD4+ and CD8+ T cells. J. Immunol. *161*, 5236-5244.

Matsuno,T., Sasaki,H., Nakagawa,K., Okada,Y., Endo,A., Fujiwara,T., Oishi,M., Saito,S., Yagi,T., Haisa,M., and Tanaka,N. (1998). Expression of Fas/Fas ligand and apoptosis induction during renal allograft rejection. Transplant. Proc. *30*, 2947-2949.

Mattingly,J.A. (1984). Immunologic suppression after oral administration of antigen. III. Activation of suppressor-inducer cells in the Peyer's patches. Cell Immunol. *86*, 46-52.

Mattingly,J.A. and Waksman,B.H. (1978). Immunologic suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. J. Immunol. *121*, 1878-1883.

Mayer,L. (1999).tight junction between IEC.

Mayer,L. (2000). Oral tolerance: new approaches, new problems. Clin. Immunol. *94*, 1-8.

Mayer,L. and Shlien,R. (1987). Evidence for function of Ia molecules on gut epithelial cells in man. J. Exp. Med. *166*, 1471-1483.

Merville,P., Lambert,C., Durand,I., Pouteil,N.C., Touraine,J.L., Berthoux,F., and Banchereau,J. (1995). High frequency of IL-10-secreting CD4+ graft-infiltrating T lymphocytes in promptly rejected kidney allografts. Transplantation *59*, 1113-1119.

Michael,J.G. (1989). The role of digestive enzymes in orally induced immune tolerance. Immunol. Invest *18*, 1049-1054.

Miller,A., al Sabbagh,A., Santos,L.M., Das,M.P., and Weiner,H.L. (1993). Epitopes of myelin basic protein that trigger TGF-beta release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression. J. Immunol. *151*, 7307-7315.

Miller,A., Lider,O., al Sabbagh,A., and Weiner,H.L. (1992a). Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. V. Hierarchy of suppression by myelin basic protein from different species. J. Neuroimmunol. *39*, 243-250.

Miller,A., Lider,O., and Weiner,H.L. (1991). Antigen-driven bystander suppression after oral administration of antigens. J. Exp. Med. *174*, 791-798.

Miller,A., Lider,O., Roberts,A.B., Sporn,M.B., and Weiner,H.L. (1992b). Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. Proc. Natl. Acad. Sci. U. S. A *89*, 421-425.

Moore,K.W., O'Garra,A., de Waal,M.R., Vieira,P., and Mosmann,T.R. (1993). Interleukin-10. Annu. Rev. Immunol. *11*, 165-190.

Morel,P.A. and Oriss,T.B. (1998). Crossregulation between Th1 and Th2 cells. Crit Rev. Immunol. *18*, 275-303.

Morton,A.L., Bell,E.B., Bolton,E.M., Marshall,H.E., Roadknight,C., McDonagh,M., and Bradley,J.A. (1993). CD4+ T cell-mediated rejection of major histocompatibility complex class I-disparate grafts: a role for alloantibody. Eur. J. Immunol. *23*, 2078-2084.

Mosmann,T.R. (1991). Cytokine secretion phenotypes of TH cells: how many subsets, how much regulation? Res. Immunol. *142*, 9-13.

Mosmann,T.R. and Moore,K.W. (1991). The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol. Today *12*, A49-A53.

Mosmann,T.R. and Sad,S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today *17*, 138-146.

Mosmann,T.R., Cherwinski,H., Bond,M.W., Giedlin,M.A., and Coffman,R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. *136*, 2348-2357.

Motoyama,K., Arima,T., Yu,S., Lehmann,M., and Flye,M.W. (2000). The kinetics of tolerance induction by nondepleting anti-CD4 monoclonal antibody (RIB 5/2) plus intravenous donor alloantigen administration. Transplantation *69*, 285-293.

Mottram,P.L., Han,W.R., Purcell,L.J., McKenzie,I.F., and Hancock,W.W. (1995). Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and interferon-gamma in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy. Transplantation *59*, 559-565.

Mueller,R., Davies,J.D., Krahl,T., and Sarvetnick,N. (1997). IL-4 expression by grafts from transgenic mice fails to prevent allograft rejection. J. Immunol. *159*, 1599-1603.

Nagano,H., Mitchell,R.N., Taylor,M.K., Hasegawa,S., Tilney,N.L., and Libby,P. (1997). Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. J. Clin. Invest *100*, 550-557.

Neutra,M.R. (1998). Current concepts in mucosal immunity. V Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. Am. J. Physiol *274*, G785-G791.

Neutra,M.R. (1999). M cells in antigen sampling in mucosal tissues. Curr. Top. Microbiol. Immunol. *236*, 17-32.

Newell,K.A., He,G., Hart,J., and Thistlethwaite-JR,J. (1997). Treatment with either anti-CD4 or anti-CD8 monoclonal antibodies blocks alphabeta T cell-mediated rejection of intestinal allografts in mice. Transplantation *64*, 959-965.

Niederkorn,J.Y. (1999). The immune privilege of corneal allografts. Transplantation *67*, 1503-1508.

Niimi,M., Witzke,O., Bushell,A., Hara,M., Morris,P.J., and Wood,K.J. (2000). Nondepleting anti-CD4 monoclonal antibody enhances the ability of oral alloantigen delivery to induce indefinite survival of cardiac allografts: oral tolerance to alloantigen. Transplantation *70*, 1524-1528.

Noble,A., Pestano,G.A., and Cantor,H. (1998). Suppression of immune responses by CD8 cells. I. Superantigen-activated CD8 cells induce unidirectional Fas-mediated apoptosis of antigen-activated CD4 cells. J. Immunol. *160*, 559-565.

Nussenblatt,R.B., Caspi,R.R., Mahdi,R., Chan,C.C., Roberge,F., Lider,O., and Weiner,H.L. (1990). Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen. J. Immunol. *144*, 1689-1695.

Nussenblatt,R.B., Gery,I., Weiner,H.L., Ferris,F.L., Shiloach,J., Remaley,N., Perry,C., Caspi,R.R., Hafler,D.A., Foster,C.S., and Whitcup,S.M. (1997). Treatment of uveitis by oral administration of retinal antigens: results of a phase I/II randomized masked trial [see comments]. Am. J. Ophthalmol. *123*, 583-592.

O'Flaherty,E., Ali,S., Pettit,S.J., and Kirby,J.A. (1998). Examination of the sensitivity of T cells to Fas ligation: induction of allospecific apoptosis. Transplantation *66*, 1067-1073.

Onodera,K., Hancock,W.W., Graser,E., Lehmann,M., Sayegh,M.H., Strom,T.B., Volk,H.D., and Kupiec-Weglinski,J.W. (1997). Type 2 helper T cell-type cytokines and the development of "infectious" tolerance in rat cardiac allograft recipients. J. Immunol. *158*, 1572-1581.

Onodera,K., Lehmann,M., Akalin,E., Volk,H.D., Sayegh,M.H., and Kupiec-Weglinski,J.W. (1996). Induction of "infectious" tolerance to MHC-incompatible cardiac allografts in CD4 monoclonal antibody-treated sensitized rat recipients. J. Immunol. *157*, 1944-1950.

Ortiz-Arduan,A., Danoff,T.M., Kalluri,R., Gonzalez-Cuadrado,S., Karp,S.L., Elkon,K., Egido,J., and Neilson,E.G. (1996). Regulation of Fas and Fas ligand expression in cultured murine renal cells and in the kidney during endotoxemia. Am. J. Physiol *271*, F1193-F1201.

Paganelli,R., Scala,E., Aiuti,F., and Fiorilli,M. (1998). B-cell help by Tc2 cells. Immunol. Today *19*, 142.

Paganelli,R., Scala,E., Ansotegui,I.J., Ausiello,C.M., Halapi,E., Fanales-Belasio,E., D'Offizi,G., Mezzaroma,I., Pandolfi,F., Fiorilli,M., and . (1995). CD8+ T lymphocytes provide helper activity for IgE synthesis in human immunodeficiency virus-infected patients with hyper-IgE. J. Exp. Med. *181*, 423-428.

Panja,A., Blumberg,R.S., Balk,S.P., and Mayer,L. (1993). CD1d is involved in T cell-intestinal epithelial cell interactions. J. Exp. Med. *178*, 1115-1119.

Papp,I., Wieder,K.J., Sablinski,T., O'Connell,P.J., Milford,E.L., Strom,T.B., and Kupiec-Weglinski,J.W. (1992). Evidence for functional heterogeneity of rat CD4+ T cells in vivo. Differential expression of IL-2 and IL-4 mRNA in recipients of cardiac allografts. J. Immunol. *148*, 1308-1314.

Perlman,H., Georganas,C., Pagliari,L.J., Koch,A.E., Haines,K., III, and Pope,R.M. (2000). Bcl-2 expression in synovial fibroblasts is essential for maintaining mitochondrial homeostasis and cell viability. J. Immunol. *164*, 5227-5235.

Piazza,C., Gilardini Montani,M.S., Moretti,S., Cundari,E., and Piccolella,E. (1997). Cutting edge: CD4+ T cells kill CD8+ T cells via Fas/Fas ligand-mediated apoptosis. J. Immunol. *158*, 1503-1506.

Piccotti,J.R., Chan,S.Y., Goodman,R.E., Magram,J., Eichwald,E.J., and Bishop,D.K. (1996). IL-12 antagonism induces T helper 2 responses, yet exacerbates cardiac allograft rejection. Evidence against a dominant protective role for T helper 2 cytokines in alloimmunity. J. Immunol. *157*, 1951-1957.

Platz,K.P., Mueller,A.R., Bechstein,W.O., Blumhardt,G., and Neuhaus,P. (1994a). Causes of death following liver transplantation in FK 506- and cyclosporine-treated patients. Transplant. Proc. *26*, 3133-3134.

Platz,K.P., Mueller,A.R., Blumhardt,G., Bachmann,S., Bechstein,W.O., Kahl,A., and Neuhaus,P. (1994b). Nephrotoxicity following orthotopic liver transplantation. A comparison between cyclosporine and FK506. Transplantation *58*, 170-178.

Prowse,S.J., Warren,H.S., Agostino,M., and Lafferty,K.J. (1983). Transfer of sensitised Lyt 2+ cells triggers acute rejection of pancreatic islet allografts. Aust. J. Exp. Biol. Med. Sci. *61*, 181-185.

Punch,J.D., Tono,T., Qin,L., Bishop,D.K., and Bromberg,J.S. (1998). Tolerance induction by anti-CD2 plus anti-CD3 monoclonal antibodies: evidence for an IL-4 requirement. J. Immunol. *161*, 1156-1162.

Qian,S., Li,W., Li,Y., Fu,F., Lu,L., Fung,J.J., and Thomson,A.W. (1996). Systemic administration of cellular interleukin-10 can exacerbate cardiac allograft rejection in mice. Transplantation *62*, 1709-1714.

Qin,L., Chavin,K.D., Ding,Y., Favaro,J.P., Woodward,J.E., Lin,J., Tahara,H., Robbins,P., Shaked,A., Ho,D.Y., and et,a. (1995). Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model. Immunosuppression with TGF-beta 1 or vIL-10. Transplantation *59* , 809-816.

Qin,L., Chavin,K.D., Ding,Y., Tahara,H., Favaro,J.P., Woodward,J.E., Suzuki,T., Robbins,P.D., Lotze,M.T., and Bromberg,J.S. (1996b). Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. J. Immunol. *156*, 2316-2323.

Qin,L., Ding,Y., and Bromberg,J.S. (1996a). Gene transfer of transforming growth factor-beta 1 prolongs murine cardiac allograft survival by inhibiting cell-mediated immunity. Hum. Gene Ther. *7*, 1981-1988.

Quigley,R.L., Wood,K.J., and Morris,P.J. (1987). Prolongation of renal allograft survival by adoptive transfer of TDL from blood transfused hosts. Transplant. Proc. *19*, 4276-4278.

Quigley,R.L., Wood,K.J., and Morris,P.J. (1988). Investigation of the mechanism of active enhancement of renal allograft survival by blood transfusion. Immunology *63*, 373-381.

Raisanen-Sokolowski,A., Mottram,P.L., Glysing-Jensen,T., Satoskar,A., and Russell,M.E. (1997). Heart transplants in interferon-gamma, interleukin 4, and interleukin 10 knockout mice. Recipient environment alters graft rejection. J. Clin. Invest *100*, 2449-2456.

Raju,G.P., Belland,S.E., and Eisen,H.J. (1994). Prolongation of cardiac allograft survival with transforming growth factor-beta 1 in rats. Transplantation *58*, 392-396.

Richards,J.M., Bedford,J.M., and Witkin,S.S. (1984). Rectal insemination modifies immune responses in rabbits. Science *224*, 390-392.

Richman,L.K., Chiller,J.M., Brown,W.R., Hanson,D.G., and Vaz,N.M. (1978). Enterically induced immunologic tolerance. I. Induction of suppressor T lymphoyctes by intragastric administration of soluble proteins. J. Immunol. *121*, 2429-2434.

Richman,L.K., Graeff,A.S., Yarchoan,R., and Strober,W. (1981). Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. J. Immunol. *126*, 2079-2083.

Ritter,T., Risch,K., Schroder,G., Kolls,J., Siegling,A., Graser,E., Reinke,P., Brock,J., Lehmann,M., and Volk,H.D. (1999a). Intragraft overexpression of interleukin-4 is neither sufficient nor essential for tolerance induction to cardiac allografts in a high-responder strain combination. Transplantation *68*, 1427-1431.

Ritter,T., Seifert,M., Risch,K., Fretz,A., Tanzmann,H., Brock,J., Volk,H.D., and Lehmann,M. (1999b). Depletion of IL-4 does not prevent tolerance induction in an allogeneic rat kidney transplantation model. Transplant. Proc. *31*, 887-888.

Rizzo,L.V., DeKruyff,R.H., Umetsu,D.T., and Caspi,R.R. (1995). Regulation of the interaction between Th1 and Th2 T cell clones to provide help for antibody production in vivo. Eur. J. Immunol. *25*, 708-716.

Rizzo,L.V., Miller-Rivero,N.E., Chan,C.C., Wiggert,B., Nussenblatt,R.B., and Caspi,R.R. (1994). Interleukin-2 treatment potentiates induction of oral tolerance in a murine model of autoimmunity. J. Clin. Invest *94*, 1668-1672.

Rizzo,L.V., Morawetz,R.A., Miller-Rivero,N.E., Choi,R., Wiggert,B., Chan,C.C., Morse,H.C., Nussenblatt,R.B., and Caspi,R.R. (1999). IL-4 and IL-10 are both required for the induction of oral tolerance. J. Immunol. *162*, 2613-2622.

Robertson,H., Morley,A.R., Talbot,D., Callanan,K., and Kirby,J.A. (2000). Renal allograft rejection: beta-chemokine involvement in the development of tubulitis. Transplantation *69*, 684-687.

Roland,C.R., Mangino,M.J., Duffy,B.F., and Flye,M.W. (1993). Lymphocyte suppression by Kupffer cells prevents portal venous tolerance induction: a study of macrophage function after intravenous gadolinium. Transplantation *55*, 1151-1158.

Romagnani,S. (1997). The Th1/Th2 paradigm. Immunol. Today *18*, 263-266.

Rosenberg,A.S., Mizuochi,T., and Singer,A. (1986). Analysis of T-cell subsets in rejection of Kb mutant skin allografts differing at class I MHC. Nature *322*, 829-831.

Rouvier,E., Luciani,M.F., and Golstein,P. (1993). Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. J. Exp. Med. *177*, 195-200.

Ruedl,C. and Hubele,S. (1997). Maturation of Peyer's patch dendritic cells in vitro upon stimulation via cytokines or CD40 triggering. Eur. J. Immunol. *27*, 1325-1330.

Russell,J.H. (1995). Activation-induced death of mature T cells in the regulation of immune responses. Curr. Opin. Immunol. *7*, 382-388.

Sakata,K., Sakata,A., Kong,L., Dang,H., and Talal,N. (1998). Role of Fas/FasL interaction in physiology and pathology: the good and the bad. Clin. Immunol. Immunopathol. *87*, 1-7.

Saleem,S., Konieczny,B.T., Lowry,R.P., Baddoura,F.K., and Lakkis,F.G. (1996). Acute rejection of vascularized heart allografts in the absence of IFNgamma. Transplantation *62*, 1908-1911.

Sanderson,C.J., Campbell,H.D., and Young,I.G. (1988). Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. Immunol. Rev. *102*, 29-50.

Sanderson,I.R., Ouellette,A.J., Carter,E.A., Walker,W.A., and Harmatz,P.R. (1993). Differential regulation of B7 mRNA in enterocytes and lymphoid cells. Immunology *79*, 434-438.

Santos,L.M., al Sabbagh,A., Londono,A., and Weiner,H.L. (1994). Oral tolerance to myelin basic protein induces regulatory TGF-beta-secreting T cells in Peyer's patches of SJL mice. Cell Immunol. *157*, 439-447.

Sato,S., Qian,J.H., Kokudo,S., Ikegami,R., Suda,T., Hamaoka,T., and Fujiwara,H. (1988). Studies on the induction of tolerance to alloantigens. III. Induction of antibodies directed against alloantigen-specific delayed-type hypersensitivity T cells by a single injection of allogeneic lymphocytes via portal venous route. J. Immunol. *140*, 717-722.

Sayegh,M.H. and Turka,L.A. (1998). The role of T-cell costimulatory activation pathways in transplant rejection. N. Engl. J. Med. *338*, 1813-1821.

Sayegh,M.H., Akalin,E., Hancock,W.W., Russell,M.E., Carpenter,C.B., Linsley,P.S., and Turka,L.A. (1995). CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. J. Exp. Med. *181*, 1869-1874.

Sayegh,M.H., Khoury,S.J., Hancock,W.W., Weiner,H.L., and Carpenter,C.B. (1992a). Induction of immunity and oral tolerance with polymorphic class II major histocompatibility complex allopeptides in the rat. Proc. Natl. Acad. Sci. U. S. A *89*, 7762-7766.

Sayegh,M.H., Zhang,Z.J., Hancock,W.W., Kwok,C.A., Carpenter,C.B., and Weiner,H.L. (1992b). Down-regulation of the immune response to histocompatibility antigens and prevention of sensitization by skin allografts by orally administered alloantigen. Transplantation *53*, 163-166.

Schilham,M.W., Fung-Leung,W.P., Rahemtulla,A., Kuendig,T., Zhang,L., Potter,J., Miller,R.G., Hengartner,H., and Mak,T.W. (1993). Alloreactive cytotoxic T cells can develop and function in mice lacking both CD4 and CD8. Eur. J. Immunol. *23*, 1299-1304.

Schroter,M., Lowin,B., Borner,C., and Tschopp,J. (1995). Regulation of Fas(Apo-1/CD95)- and perforin-mediated lytic pathways of primary cytotoxic T lymphocytes by the protooncogene bcl-2. Eur. J. Immunol. *25*, 3509-3513.

Schwarze,J., Cieslewicz,G., Joetham,A., Ikemura,T., Hamelmann,E., and Gelfand,E.W. (1999). CD8 T cells are essential in the development of respiratory syncytial virus-induced lung eosinophilia and airway hyperresponsiveness. J. Immunol. *162*, 4207-4211.

Seino,K., Kayagaki,N., Fukao,K., Okumura,K., and Yagita,H. (1997). Rejection of Fas ligand-expressing grafts. Transplant. Proc. *29*, 1092-1093.

Selvaggi,G., Ricordi,C., Podack,E.R., and Inverardi,L. (1996). The role of the perforin and Fas pathways of cytotoxicity in skin graft rejection. Transplantation *62*, 1912-1915.

Senesi,B., Curiale,V., Lione,L., Puddu,A., Pedemonte,A., Pastorino,A., and Viviani,G.L. (1998). Oral tolerance and allografts: can multiple oral administrations of low doses of

highly purified islets induce peripheral tolerance in pancreatic islet allografts? Transplant. Proc. *30*, 545-549.

Shapiro,R., Fung,J.J., Jain,A.B., Parks,P., Todo,S., and Starzl,T.E. (1990). The side effects of FK 506 in humans. Transplant. Proc. *22*, 35-36.

Sharma,D.P., Ramsay,A.J., Maguire,D.J., Rolph,M.S., and Ramshaw,I.A. (1996). Interleukin-4 mediates down regulation of antiviral cytokine expression and cytotoxic T-lymphocyte responses and exacerbates vaccinia virus infection in vivo. J. Virol. *70*, 7103-7107.

Sharma,V.K., Bologa,R.M., Li,B., Xu,G.P., Lagman,M., Hiscock,W., Mouradian,J., Wang,J., Serur,D., Rao,V.K., and Suthanthiran,M. (1996). Molecular executors of cell death--differential intrarenal expression of Fas ligand, Fas, granzyme B, and perforin during acute and/or chronic rejection of human renal allografts. Transplantation *62*, 1860-1866.

Shi,H.N., Ingui,C.J., Dodge,I., and Nagler-Anderson,C. (1998). A helminth-induced mucosal Th2 response alters nonresponsiveness to oral administration of a soluble antigen. J. Immunol. *160*, 2449-2455.

Siegling,A., Lehmann,M., Riedel,H., Platzer,C., Brock,J., Emmrich,F., and Volk,H.D. (1994). A nondepleting anti-rat CD4 monoclonal antibody that suppresses T helper 1-like but not T helper 2-like intragraft lymphokine secretion induces long-term survival of renal allografts. Transplantation *57*, 464-467.

Singh,V.K., Kalra,H.K., Yamaki,K., and Shinohara,T. (1992). Suppression of experimental autoimmune uveitis in rats by the oral administration of the uveitopathogenic S-antigen fragment or a cross-reactive homologous peptide. Cell Immunol. *139*, 81-90.

Sirak,J.H., Orosz,C.G., Roopenian,D.C., Wakely,E., and VanBuskirk,A.M. (1998). Cardiac allograft tolerance: failure to develop in interleukin-4-deficient mice correlates with unusual allosensitization patterns. Transplantation *65*, 1352-1356.

Spits,H., Yssel,H., Paliard,X., Kastelein,R., Figdor,C., and de Vries,J.E. (1988). IL-4 inhibits IL-2-mediated induction of human lymphokine-activated killer cells, but not the generation of antigen-specific cytotoxic T lymphocytes in mixed leukocyte cultures. J. Immunol. *141*, 29-36.

Spits,H., Yssel,H., Takebe,Y., Arai,N., Yokota,T., Lee,F., Arai,K., Banchereau,J., and de Vries,J.E. (1987). Recombinant interleukin 4 promotes the growth of human T cells. J. Immunol. *139*, 1142-1147.

Squiers,E.C., Salomon,D.R., Pickard,L.L., Howard,R.R., and Pfaff,W.W. (1990). Abrogation of the induction of portal venous tolerance in a cardiac transplant model resulting from Kupffer cell inhibition by gadolinium. Transplantation *50*, 171-173.

Steinmuller,D., Snider,M.E., Noble,R.L., and Waldschmidt,T.J. (1990). Dissociation of tissue destruction induced by cytolytic T cells in vivo and cytotoxicity as measured in vitro. Transplantation *50*, 663-668.

Stransky,B., Faria,A.M., and Vaz,N.M. (1998). Oral tolerance induction with altered forms of ovalbumin. Braz. J. Med. Biol. Res. *31*, 381-386.

Streilein,J.W. (1995). Unraveling immune privilege [comment]. Science *270*, 1158-1159.

Strobel,S. and Mowat,A.M. (1998). Immune responses to dietary antigens: oral tolerance. Immunol. Today *19*, 173-181.

Strober,W., Kelsall,B., and Marth,T. (1998). Oral tolerance. J. Clin. Immunol. *18*, 1-30.

Stuart,P.M., Griffith,T.S., Usui,N., Pepose,J., Yu,X., and Ferguson,T.A. (1997). CD95 ligand (FasL)-induced apoptosis is necessary for corneal allograft survival. J. Clin. Invest *99*, 396-402.

Suda,T. and Nagata,S. (1997). Why do defects in the Fas-Fas ligand system cause autoimmunity? J. Allergy Clin. Immunol. *100*, S97-101.

Suss,G. and Shortman,K. (1996). A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. J. Exp. Med. *183*, 1789-1796.

Swenson,K.M., Ke,B., Wang,T., Markowitz,J.S., Maggard,M.A., Spear,G.S., Imagawa,D.K., Goss,J.A., Busuttil,R.W., and Seu,P. (1998). Fas ligand gene transfer to renal allografts in rats: effects on allograft survival. Transplantation *65*, 155-160.

Tada,Y., Ho,A., Koh,D.R., and Mak,T.W. (1996). Collagen-induced arthritis in CD4- or CD8-deficient mice: CD8+ T cells play a role in initiation and regulate recovery phase of collagen-induced arthritis. J. Immunol. *156*, 4520-4526.

Takeda,Y., Gotoh,M., Dono,K., Grochowiecki,T.R., Kimura,F., Okuyama,M., Shimizu,J., Nagano,H., Umeshita,K., Sakon,M., Miyasaka,M., and Monden,M. (1999). Acceptance of islet allografts transplanted with Fas ligand expressing testicular allografts. Transplant. Proc. *31*, 624.

Takeuchi,T., Lowry,R.P., and Konieczny,B. (1992). Heart allografts in murine systems. The differential activation of Th2-like effector cells in peripheral tolerance. Transplantation *53*, 1281-1294.

Takeuchi,T., Ueki,T., Nishimatsu,H., Kajiwara,T., Ishida,T., Jishage,K., Ueda,O., Suzuki,H., Li,B., Moriyama,N., and Kitamura,T. (1999). Accelerated rejection of Fas ligand-expressing heart grafts. J. Immunol. *162*, 518-522.

Takeuchi,T., Ueki,T., Sunaga,S., Ikuta,K., Sasaki,Y., Li,B., Moriyama,N., Miyazaki,J., and Kawabe,K. (1997). Murine interleukin 4 transgenic heart allograft survival prolonged with down-regulation of the Th1 cytokine mRNA in grafts. Transplantation *64*, 152-157.

Thompson,S.J., Thompson,H.S., Harper,N., Day,M.J., Coad,A.J., Elson,C.J., and Staines,N.A. (1993). Prevention of pristane-induced arthritis by the oral administration of type II collagen. Immunology *79*, 152-157.

Thorbecke,G.J., Schwarcz,R., Leu,J., Huang,C., and Simmons,W.J. (1999). Modulation by cytokines of induction of oral tolerance to type II collagen. Arthritis Rheum. *42*, 110-118.

Tilney,N.L., Garovoy,M.R., Busch,G.J., Strom,T.B., Graves,M.J., and Carpenter,C.B. (1979). Rejected human renal allografts: recovery and characteristics of infiltrating cells and antibody. Transplantation *28*, 421-426.

Tomura,M., Nakatani,I., Murachi,M., Tai,X.G., Toyo-oka,K., and Fujiwara,H. (1997). Suppression of allograft responses induced by interleukin-6, which selectively modulates interferon-gamma but not interleukin-2 production. Transplantation *64*, 757-763.

Trauth,B.C., Klas,C., Peters,A.M., Matzku,S., Moller,P., Falk,W., Debatin,K.M., and Krammer,P.H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301-305.

Trenn,G., Takayama,H., Hu-Li,J., Paul,W.E., and Sitkovsky,M.V. (1988). B cell stimulatory factor 1 (IL-4) enhances the development of cytotoxic T cells from Lyt-2+ resting murine T lymphocytes. J. Immunol. *140*, 1101-1106.

Tschopp,J., Irmler,M., and Thome,M. (1998). Inhibition of fas death signals by FLIPs. Curr. Opin. Immunol. *10*, 552-558.

Turvey,S.E., Gonzalez-Nicolini,V., Kingsley,C.I., Larregina,A.T., Morris,P.J., Castro,M.G., Lowenstein,P.R., and Wood,K.J. (2000). Fas ligand-transfected myoblasts and islet cell transplantation. Transplantation *69*, 1972-1976.

van Emmerik,N.E., Daane,C.R., Knoop,C.J., Hesse,C., Vaessen,L.M., Balk,A.H., Mochtar,B., Claas,F.H., and Weimar,W. (1997a). The avidity of allospecific cytotoxic T lymphocytes (CTL) determines their cytokine production profile. Clin. Exp. Immunol. *110*, 447-453.

van Emmerik,N.E., Vaessen,L.M., Balk,A.H., Bos,E., Claas,F.H., and Weimar,W. (1996). Progressive accumulation of CTL with high avidity for donor antigens during the development of acute cardiac rejection. Transplantation *62*, 529-536.

Van Parijs,L., Biuckians,A., and Abbas,A.K. (1998). Functional roles of Fas and Bcl-2-regulated apoptosis of T lymphocytes. J. Immunol. *160*, 2065-2071.

Van Wilsem,E.J., Van Hoogstraten,I.M., Breve,J., Scheper,R.J., and Kraal,G. (1994). Dendritic cells of the oral mucosa and the induction of oral tolerance. A local affair. Immunology *83*, 128-132.

Vancott,J.L., Kweon,M., Fujihashi,K., Yamamoto,M., Marinaro,M., Kiyono,H., and McGhee,J.R. (1997). Helper T subsets and cytokines for mucosal immunity and tolerance. Behring Inst. Mitt. 44-52.

Varadhachary,A.S., Perdow,S.N., Hu,C., Ramanarayanan,M., and Salgame,P. (1997). Differential ability of T cell subsets to undergo activation-induced cell death. Proc. Natl. Acad. Sci. U. S. A *94*, 5778-5783.

Vella,J.P., Magee,C., Vos,L., Womer,K., Rennke,H., Carpenter,C.B., Hancock,W., and Sayegh,M.H. (1999). Cellular and humoral mechanisms of vascularized allograft rejection induced by indirect recognition of donor MHC allopeptides. Transplantation *67*, 1523-1532.

Vella,J.P., Vos,L., Carpenter,C.B., and Sayegh,M.H. (1997). Role of indirect allorecognition in experimental late acute rejection. Transplantation *64*, 1823-1828.

Vidal,K., Samarut,C., Magaud,J.P., Revillard,J.P., and Kaiserlian,D. (1993). Unexpected lack of reactivity of allogeneic anti-Ia monoclonal antibodies with MHC class II molecules expressed by mouse intestinal epithelial cells. J. Immunol. *151*, 4642-4650.

Vignes,C., Chiffoleau,E., Douillard,P., Josien,R., Peche,H., Heslan,J.M., Usal,C., Soulillou,J.P., and Cuturi,M.C. (2000). Anti-TCR-specific DNA vaccination demonstrates a role for a CD8+ T cell clone in the induction of allograft tolerance by donor-specific blood transfusion. J. Immunol. *165*, 96-101.

Villacres,M.C. and Bergmann,C.C. (1999). Enhanced cytotoxic T cell activity in IL-4-deficient mice. J. Immunol. *162*, 2663-2670.

Viney,J.L., Mowat,A.M., O'Malley,J.M., Williamson,E., and Fanger,N.A. (1998). Expanding dendritic cells in vivo enhances the induction of oral tolerance. J. Immunol. *160*, 5815-5825.

Vogel,K., Kantor,J., Wood,L., Rivera,R., and Schlom,J. (1998). Oral immunization with enterocoated microbeads induces antigen-specific cytolytic T-cell responses. Cell Immunol. *190*, 61-67.

von Willebrand,E. and Hayry,P. (1978). Composition and in vitro cytotoxicity of cellular infiltrates in rejecting human kidney allografts. Cell Immunol. *41*, 358-372.

Wagener,M.E., Konieczny,B.T., Dai,Z., Ring,G.H., and Lakkis,F.G. (2000). Alloantigen-driven T cell death mediated by Fas ligand and tumor necrosis factor-alpha is not essential for the induction of allograft acceptance. Transplantation *69*, 2428-2432.

Wallick,S.C., Figari,I.S., Morris,R.E., Levinson,A.D., and Palladino,M.A. (1990). Immunoregulatory role of transforming growth factor beta (TGF-beta) in development of killer cells: comparison of active and latent TGF-beta 1. J. Exp. Med. *172*, 1777-1784.

Waltenberger,J., Wanders,A., Fellstrom,B., Miyazono,K., Heldin,C.H., and Funa,K. (1993). Induction of transforming growth factor-beta during cardiac allograft rejection. J. Immunol. *151*, 1147-1157.

Wang,J., Nonomura,N., Ichimaru,N., Azuma,H., Hatori,M., Kokado,Y., Matsumiya,K., Miki,T., Takahara,S., and Okuyama,A. (1997). Expression of Fas and Fas ligand in renal grafts with acute and chronic rejection in the rat model. J. Interferon Cytokine Res. *17*, 369-373.

Weiner,H.L. (1994). Oral tolerance [comment]. Proc. Natl. Acad. Sci. U. S. A *91*, 10762-10765.

Weiner,H.L. (1997). Oral tolerance: immune mechanisms and treatment of autoimmune diseases. Immunol. Today *18*, 335-343.

Weiner,H.L., Friedman,A., Miller,A., Khoury,S.J., al Sabbagh,A., Santos,L., Sayegh,M., Nussenblatt,R.B., Trentham,D.E., and Hafler,D.A. (1994). Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu. Rev. Immunol. *12:809-37*, 809-837.

Weiner,H.L., Gonnella,P.A., Slavin,A., and Maron,R. (1997). Oral tolerance: cytokine milieu in the gut and modulation of tolerance by cytokines. Res. Immunol. *148*, 528-533.

Wever,P.C., Aten,J., Rentenaar,R.J., Hack,C.E., Koopman,G., Weening,J.J., and ten Berge,I.J. (1998b). Apoptotic tubular cell death during acute renal allograft rejection. Clin. Nephrol. *49*, 28-34.

Wever,P.C., Boonstra,J.G., Laterveer,J.C., Hack,C.E., van der Woude,F.J., Daha,M.R., and ten Berge,I.J. (1998a). Mechanisms of lymphocyte-mediated cytotoxicity in acute renal allograft rejection. Transplantation *66*, 259-264.

Whitacre,C.C., Gienapp,I.E., Orosz,C.G., and Bitar,D.M. (1991). Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. J. Immunol. *147*, 2155-2163.

Whitehead,B.F., Stoehr,C., Wu,C.J., Patterson,G., Burchard,E.G., Theodore,J., Clayberger,C., and Starnes,V.A. (1993). Cytokine gene expression in human lung transplant recipients. Transplantation *56*, 956-961.

Widmer,M.B. and Grabstein,K.H. (1987). Regulation of cytolytic T-lymphocyte generation by B-cell stimulatory factor. Nature *326*, 795-798.

Wildner,G., Hunig,T., and Thurau,S.R. (1996). Orally induced, peptide-specific gamma/delta TCR+ cells suppress experimental autoimmune uveitis. Eur. J. Immunol. *26*, 2140-2148.

Williams,N.S. and Engelhard,V.H. (1997). Perforin-dependent cytotoxic activity and lymphokine secretion by CD4+ T cells are regulated by CD8+ T cells. J. Immunol. *159*, 2091-2099.

Williamson,E., O'Malley,J.M., and Viney,J.L. (1999). Visualizing the T-cell response elicited by oral administration of soluble protein antigen. Immunology *97*, 565-572.

Witkin,S.S., Richards,J.M., Bongiovanni,A.M., and Yu,I.R. (1984). Inhibition of lymphocyte proliferation by sera from rectally inseminated male rabbits. Ann. N. Y. Acad. Sci. *437:503-7*, 503-507.

Wolf,J.L. and Bye,W.A. (1984). The membranous epithelial (M) cell and the mucosal immune system. Annu. Rev. Med. *35:95-112*, 95-112.

Wood,K.J., Dallman,M.J., and Morris,P.J. (1987). Blood transfusion induces specific cytotoxic cells. Transplant. Proc. *19*, 1458-1460.

Wood,K.J., Dallman,M.J., and Morris,P.J. (1989). Cytotoxic cells alone are not sufficient to mediate renal graft rejection. Transplant. Proc. *21*, 338-339.

Wood,K.J., Hopley,A., Dallman,M.J., and Morris,P.J. (1988). Lack of correlation between the induction of donor class I and class II major histocompatibility complex antigens and graft rejection. Transplantation *45*, 759-767.

Wu,X.M., Nakashima,M., and Watanabe,T. (1998). Selective suppression of antigen-specific Th2 cells by continuous micro-dose oral tolerance. Eur. J. Immunol. *28*, 134-142.

Xu,X.Y., Honjo,K., Devore,C.D., and Bucy,R.P. (1997). Immunosuppression by inhibition of cellular adhesion mediated by leukocyte function-associated antigen-1/intercellular adhesion molecule-1 in murine cardiac transplantation. Transplantation *63*, 876-885.

Yamagami,S., Kawashima,H., Tsuru,T., Yamagami,H., Kayagaki,N., Yagita,H., Okumura,K., and Gregerson,D.S. (1997). Role of Fas-Fas ligand interactions in the immunorejection of allogeneic mouse corneal transplants. Transplantation *64*, 1107-1111.

Yamamoto,M., Rennert,P., McGhee,J.R., Kweon,M.N., Yamamoto,S., Dohi,T., Otake,S., Bluethmann,H., Fujihashi,K., and Kiyono,H. (2000). Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. J. Immunol. *164*, 5184-5191.

Yamamoto,N., Einaga-Naito,K., Kuriyama,M., Kawada,Y., and Yoshida,R. (1998). Cellular basis of skin allograft rejection in mice: specific lysis of allogeneic skin components by non-T cells. Transplantation *65*, 818-825.

Yi,S., Feng,X., Wang,Y., Kay,T.W., Wang,Y., and O'Connell,P.J. (1999). CD4+ cells play a major role in xenogeneic human anti-pig cytotoxicity through the Fas/Fas ligand lytic pathway. Transplantation *67*, 435-443.

Yin,D. and Fathman,C.G. (1995). CD4-positive suppressor cells block allotransplant rejection. J. Immunol. *154*, 6339-6345.

Yoshimura,N. and Kahan,B.D. (1985). Suppressor cell activity of cells infiltrating rat renal allografts prolonged by perioperative administration of extracted histocompatibility antigen and cyclosporine. Transplantation *40*, 708-713.

Yoshimura,N., Matsui,S., Hamashima,T., Lee,C.J., Ohsaka,Y., and Oka,T. (1990). The effects of perioperative portal venous inoculation with donor lymphocytes on renal allograft survival in the rat. I. Specific prolongation of donor grafts and suppressor factor in the serum. Transplantation *49*, 167-171.

Yoshino,S. (1998). Treatment with an anti-IL-4 monoclonal antibody blocks suppression of collagen-induced arthritis in mice by oral administration of type II collagen. J. Immunol. *160*, 3067-3071.

Yoshino,S. and Ohsawa,M. (1997). Effect of a monoclonal antibody against interleukin-4 on the induction of oral tolerance in mice. Eur. J. Pharmacol. *336*, 203-209.

Yoshino,S. and Yoshino,J. (1998). Effect of a monoclonal antibody against interleukin-4 on suppression of antigen-induced arthritis in mice by oral administration of the inducing antigen. Cell Immunol. *187*, 139-144.

Yu,S., Tu,Y., and Flye,M.W. (1997). The shift of Th1/Th2 ratios induced by portal venous administration of UVB-treated donor spleen cells prolonged mouse cardiac allografts. Transplant. Proc. *29*, 1162.

Zavazava,N., Fandrich,F., Zhu,X., Freese,A., Behrens,D., and Yoo-Ott,K.A. (2000). Oral feeding of an immunodominant MHC donor-derived synthetic class I peptide prolongs graft survival of heterotopic cardiac allografts in a high-responder rat strain combination. J. Leukoc. Biol. *67*, 793-800.

Zhang,H.G., Fleck,M., Kern,E.R., Liu,D., Wang,Y., Hsu,H.C., Yang,P., Wang,Z., Curiel,D.T., Zhou,T., and Mountz,J.D. (2000). Antigen presenting cells expressing Fas ligand down-modulate chronic inflammatory disease in Fas ligand-deficient mice. J. Clin. Invest *105*, 813-821.

Zhang,H.G., Su,X., Liu,D., Liu,W., Yang,P., Wang,Z., Edwards,C.K., Bluethmann,H., Mountz,J.D., and Zhou,T. (1999). Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. J. Immunol. *162*, 1423-1430.

Zhang,L., Miller,R.G., and Zhang,J. (1996). Characterization of apoptosis-resistant antigen-specific T cells in vivo. J. Exp. Med. *183*, 2065-2073.

Zhang,X., Brunner,T., Carter,L., Dutton,R.W., Rogers,P., Bradley,L., Sato,T., Reed,J.C., Green,D., and Swain,S.L. (1997). Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. J. Exp. Med. *19;185*, 1837-1849.

Zheng,L., Fisher,G., Miller,R.E., Peschon,J., Lynch,D.H., and Lenardo,M.J. (1995). Induction of apoptosis in mature T cells by tumour necrosis factor. Nature *377*, 348-351.