Role of Peroxisome Proliferator-Activated Receptors in
Mechanisms of Rejection in Heart Transplantation

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Abstract

Peroxisome proliferator-activated receptors (PPARs) belong to a nuclear receptor superfamily; two major isoforms, PPARα and PPARγ, are primarily involved in lipid and glucose homeostasis. However, evidence also suggests roles for PPARs in regulating inflammation and atherosclerosis, and prompted investigation into the efficacy of PPAR agonists in parenchymal rejection (PR) and transplantation-associated arteriosclerosis (TxA). Four different PPAR agonists (fenofibrate and Wy14643 for PPARα; BRL69453 and 15-deoxy-Δ^{14,15} Prostaglandin-J for PPARγ) in an in vitro model of the alloresponse all demonstrated a robust and substantial attenuation of IFNγ, a cytokine that critically affects both rejection and TxA. This occurred in a dose-dependent manner, independent of known IFNγ-inducing cytokines. At the same times, PPAR activation increased the overall expression of chemokines but substantially decreased expression of two relevant chemokine receptors. Of the four agonists tested, fenofibrate, a largely PPARα-specific agonist, had the best profile of IFNγ production to chemokine and chemokine receptor expression. At early time points after cardiac transplantation, fenofibrate administration showed findings consistent with those seen in vitro, including a tendency to reduce IFNγ. However, long-term fenofibrate treatment significantly increased graft IFNγ expression and inflammatory cell infiltration, thereby augmenting PR without ameliorating TxA. Congenital deficiency of PPARα confirmed that the receptor plays a regulatory role in IFNγ expression but is not necessary for graft infiltration by inflammatory cells. The findings constitute the first examination of the efficacy of PPAR agonists in solid-organ transplantation and suggest that in order to fully realize the beneficial anti-inflammatory effects of fenofibrate, additional strategies must be employed to inhibit graft inflammatory cell infiltration. Finally, a novel immunologic research tool based on the boron neutron capture reaction is proposed.
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5.6: Influence of recipient PPARα deficiency on allograft activation markers: Although there are no statistically significant differences and thus no overall effect, the trends merit some mention. MHC-II and VCAM-1 tend to increase, while ICAM-1 tends to decrease. Sections were evaluated according to the following scheme: 0, no staining; 1 focal weak; 2 focal moderate, diffuse weak; 3 focal strong, diffuse moderate; 4 diffuse strong. Statistical significance (not affected by observer) was tested using the Mann-Whitney test (p<0.05). Allografts were harvested at 1 week post-transplantation.
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5.14: Influence of donor PPARα deficiency on allograft pathology: Deficiency of PPARα on donors results in edema, without thrombus formation (see photomicrograph of sample vessel). The pathological appearance of allografts with and without donor PPARα is not substantially different. Consistent with an increased graft infiltrate, PR modestly increases.

6.1: Use of the boron neutron capture reaction as an immunologic research tool: Mice genetically unable to produce T or B cells would receive a heterotopic cardiac transplant, followed by the injection of boron-loaded T cells. After defined times, neutron irradiation would be performed, inducing the boron neutron capture reaction in the adoptively transferred T cells.

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6.3: Schematic of hydrophilically enhanced carboranyl thymidine analogs: The lengths of the methylene chains of the two nucleosides tested are four (N4-20H) and five groups (N5-20H), respectively. These were chosen on their optimal efficiency compared to thymidine. Advantages of these compounds are their incorporation into nuclear material of a cell, thereby reducing risk of leakage and leading to a potentially more effective dose delivery, as well as specificity if loading is accomplished during in vitro stimulation. Potential disadvantages are limited boron incubation concentrations due to compound toxicity, and overall lack of experience in T cells. Readers are referred to the literature cited in the text for a more chemically accurate depiction of structure.

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6.8: Results of functional T cell assays using carboranyl thymidine analogs: There is no significant difference in IFNγ concentration between days following the addition of N4-2OH or N5-2OH to the cultures on any of the days tested (p ns). Proliferation was not measured out of concern for possible competition between thymidine substrates since the technique readily available in the laboratory for measuring proliferation depends on the incorporation of ³H-thymidine.
Chapter 1. Introduction

The last decades have seen significant improvement in immunosuppressive therapy, rendering heart transplantation a clinically feasible therapeutic modality. Currently, heart transplantation has been adopted as a treatment for end-stage cardiac diseases including coronary artery disease and cardiomyopathy. Rejection pathology may involve the parenchyma and/or vasculature of the transplanted heart. Parenchymal rejection (PR), which may occur from days to months (to sometimes even years) post-transplantation, is characterized histologically by a diffuse interstitial inflammatory infiltrate (mostly composed of T cells and macrophages) with associated myocyte necrosis, edema, and hemorrhage. Although responsive to treatment, PR remains an important cause of morbidity and mortality in heart transplant patients. Transplantation-associated arteriosclerosis (TxAA), which may be seen as early as several months after transplantation but more typically develops on the order of years, is characterized by concentric vascular lesions formed by the intimal proliferation of smooth muscle cells and associated extra-cellular matrix proteins, admixed with inflammatory mononuclear cells (including T cells and macrophages). Unlike PR, TxAA is not responsive to immunosuppressive therapy and has thus emerged as the major limitation to long-term survival in heart transplant recipients.

Peroxisome proliferator-activated receptors (PPARs) belong to a superfamily of nuclear hormone receptors that act as gene transcriptional regulators. Of the three isoforms that have been identified to date (α, β/δ, γ), two of them, the α and γ isoforms, are most well understood. Traditionally, PPARα is associated with the regulation of fatty-acid catabolism. PPARα agonists (e.g. fenofibrate/Tricor) are in clinical use for the treatment of hypertryglyceridemia. PPARγ is known to be essential for adipocyte differentiation and is also involved in glucose metabolism. PPARγ agonists (e.g. rosiglitazone/Avandia and pioglitazone/Actos) are in current clinical use as insulin sensitizing agents (acting as oral hypoglycemics) for patients with type II diabetes mellitus. Over the last several years, however, PPARs have also emerged as immunomodulatory agents. They are expressed in most cells of the vascular and immune systems, including vascular endothelial and smooth muscle cells, as well as macrophages and T cells. The majority of the literature on PPAR ligand-mediated activation suggests anti-inflammatory activity in the relevant cell types listed above. In addition, experimentation in rodent models of various chronic and inflammatory diseases, most notably atherosclerosis, shows therapeutic benefits independent of glucose and lipid profiles upon treatment with PPAR agonists. Based on the reported effects of PPAR activation both in vitro and in vivo, there was reason to hypothesize beneficial effects of PPAR agonists on rejection, namely PR and TxAA. Moreover, given the current broad use of several PPAR ligands in the clinical setting, a demonstration of benefit in transplant rejection could translate rapidly into clinical application.
Thus, the overall goal of this thesis was to investigate the role of PPARs in mechanisms of rejection in heart transplantation. This thesis is organized in the following order. Chapter 2 presents background information on mechanisms of rejection and PPARs, including general characteristics and evidence from in vitro and in vivo studies in the literature. It also introduces the boron neutron capture reaction that serves as the basis for a proposed novel immunologic research tool. Chapter 3 describes the in vitro experiments performed to assess the effect of PPAR agonists on an alloresponse (relative to mitogenic stimulation not dependent on major histocompatibility complex disparity), while chapter 4 describes the effect of PPARα agonist treatment in vivo in a murine heterotopic model of cardiac transplantation. The availability of mice congenitally deficient in PPARα allowed the examination of PPARα requirement both in vitro and in vivo, as described in chapter 5. A novel immunologic tool is presented in chapter 6, along with preliminary experiments, to explore the timing of transplant commitment to a particular outcome. Lastly, chapter 7 provides a summary of relevant conclusions and recommendations for future work.
Chapter 2. Background

This chapter contains background information on mechanisms of cardiac rejection and peroxisome proliferator-activator receptors, as well as the fundamental basis of the proposed novel immunologic research tool. Section 1 discusses mechanisms of cardiac rejection, including an overview of the pathology and evidence from both \textit{in vitro} and \textit{in vivo} studies. Section 2 reviews the general characteristics of the peroxisome proliferator-activated receptors and implications for the vasculature. Finally, section 3 introduces the boron neutron capture reaction and its current applications, as well as its proposed use as an immunologic research tool.

2.1 Mechanisms of cardiac rejection

Heart transplantation has been adopted as a treatment for end-stage cardiac diseases including cardiomyopathy and coronary artery disease. Since the first human heart transplant in the 1960s, more than 48,000 transplants have been performed worldwide [Magliato and Trento 2001]. In the United States alone, over 2,000 heart transplants are performed annually, leaving approximately 4,000 patients on the waiting list [UNOS 2000]. The following section describes the pathological features of the different forms of rejection and introduces fundamental concepts in the pathogenesis of rejection as a whole. Then, evidence from both \textit{in vitro} and \textit{in vivo} studies is discussed with respect to the elucidation of mechanisms involved in rejection.

2.1.1 General characteristics

Contrary to the relatively subtle changes in surgical techniques since the beginning of clinical cardiac transplantation [Magliato and Trento 2001], the last decades have seen significant expansion and improvement in immunosuppressive therapy, including cyclosporine, OKT3, tacrolimus, and mycophenolate mofetil among others. These powerful drugs have been a primary reason behind transplant survival rates of over 80% at one year post-transplant [Hosenpud et al 1996].

Hyperacute rejection, characterized by graft failure within 24 hours and by the histologic appearance of interstitial hemorrhage, edema and fibrin thrombi, occurs only in about 0.5% of cardiac transplants [Duquesnoy and Demetris 1995]. This condition is often fatal [Cotts and Johnson 2001] but if the patient lives long enough, is further characterized by myocyte and vascular degeneration with eventual necrosis and neutrophilic responses [Winters and Schoen 2001]. It is generally believed that circulating preformed antibodies are the cause of this type of rejection; however, occasional lesions exhibiting similar pathology, in the absence of apparent immune reactions, suggest that non-immune factors may also be involved [Platt 1995]. Immunosuppression, targeting the immune system’s cellular components, has no effect on hyperacute rejection.
Parenchymal rejection (PR), which may occur from days to months (to sometimes even years) post-transplantation, is generally responsive to immunosuppressive agents. As shown in figure 2.1, PR is characterized histologically by diffuse interstitial inflammatory infiltration (largely composed of T cells and macrophages) with associated myocyte necrosis, edema and hemorrhage [Billingham 1990]. Despite the responsiveness to cellular immune therapy, PR remains an important cause of morbidity and mortality in heart transplant recipients [Winters and Schoen 2001]. Study is ongoing to determine antigen-specific and nonspecific mechanisms involved in the development of the inflammatory infiltrate, as well as the resulting myocyte necrosis.

Occasionally, rejection may be primarily vascular and therefore characterized by endothelial swelling and necrosis, extensive infiltration of the blood vessel wall and deposition of fibrin plugs both along the vascular wall and the extravascular spaces [Platt 1995]. Given the unresponsiveness of acute vascular rejection to cellular immune therapy, it is hypothesized that recipient antibodies mediate this relatively uncommon type of rejection (termed acute vascular or humoral rejection) [Platt 1995].

**Figure 2.1:** Sample photomicrograph of parenchymal rejection (PR): Histologically, PR is characterized by a diffuse interstitial inflammatory infiltrate with associated myocyte necrosis, hemorrhage and edema. Despite the responsiveness to cellular therapy, PR remains an important cause of mortality in heart transplant recipients.

With improvement in immunosuppressive agents and concomitant decreases in acute rejection, transplantation-associated arteriosclerosis (TxAA) has emerged as the principal limitation to long-term survival of heart transplant recipients. At Stanford, where over 750 heart transplants have been performed and where the five and ten year survival rates compare favorably to international rates, TxAA accounts for approximately 30% of deaths after the first year of heart transplantation [Billingham 1995]. TxAA may be present as early as several months after transplantation but more typically develops on the order of years. As shown in figure 2.2, TxAA is characterized by concentric lesions formed by the intimal proliferation of smooth muscle cells and associated extra-cellular matrix proteins, admixed with inflammatory mononuclear cells [Billingham 1995].
Figure 2.2: Sample photomicrograph of transplantation-associated arteriosclerosis (TxAA): Histologically, TxAA is characterized by concentric lesions, formed by intimal proliferation of vascular smooth muscle cells and associated extra-cellular matrix proteins, admixed with inflammatory mononuclear cells. Despite improvements in immunosuppressive regimes, TxAA remains refractory to treatment and has emerged as major limitation to long-term survival of heart transplant recipients.

The proliferation of smooth muscle cells results in diffuse arterial occlusion that is difficult to detect via conventional angiographic means until relatively late in the process. This may be due to compensation in vessel enlarging and remodeling of missing branches [Aranda and Hill 2000]. Despite the difficulty in lesion visualization, it is estimated based on conventional angiographic data that approximately 40% of heart transplant patients will develop TxAA by 5 years post-transplant [Billingham 1995]. This may be an underestimate based on the sensitivity of angiography compared to intravascular ultrasound. A review of diagnostic methods for TxAA has found that, although its role in therapeutic decision-making remains to be firmly established, intravascular ultrasound is more sensitive than conventional angiography [Aranda and Hill 2000]. In fact, studies using intravascular ultrasound show that TxAA lesions are already detectable in 60% to 75% of the patients at 1 year post-transplant [Yeung et al 1995, Julius et al 2000].

The pathogenesis of TxAA is firmly grounded on the immunological differences between donor and recipient. This leads to the creation of an inflammatory reaction that result in the endothelial cell activation, smooth muscle cell proliferation, mononuclear cell infiltration and fibrosis typically seen in TxAA lesions. The suggestion that TxAA is a form of delayed type hypersensitivity was initially decades ago [Brent 1997] and still holds in current times [Libby and Pober 2001]. In this model, the offending agent is the presence of the allograft; delayed would refer to the slow development, while hypersensitivity refers to the inappropriate damage of tissues by the immune system. There is evidence that persistent graft parenchymal rejection is not required for the development of TxAA [Izuta et al 1995, Nagano et al 1998], although studies have found that episodes of parenchymal rejection may potentiate the development of TxAA [Hullet et al 1996, Nakagawa et al 1995].
Certainly, non-immunologic factors also contribute to the development of TxAA. In fact, some authors question whether TxAA is an immunologic or non-immunologic disease [Mehra and Uber 2003], while others go even further and contend that the failure is not due to an immunologic process but to failure of the microvessels to remain open and that TxAA is nothing more than a particular form of atherosclerosis [Labarrere et al 2003]. One other paradigm is to ascribe endothelial injury the central role in spurring the development of TxAA [Stoica et al 2002; Valantine 2003]. Ischemia-reperfusion injury, pre-existing atherosclerosis, dyslipidemia, infection and hypertension have all been identified as potential contributing factors [Libby and Pober 2001].

An in-depth understanding of the mechanisms of rejection is motivated by the hope that it will lead to the identification of novel therapeutic targets and development of effective agents to decrease the morbidity and mortality associated with heart transplantation. Study of the mechanisms of rejection may take place at both the in vitro and in vivo levels. The following sections describe the models used in this thesis, as well as selected findings from the literature.

2.1.2 In vitro models

The recognition of non-self, or allore cognition, is fundamental to the immunology of transplantation. The mainstay in vitro assay for measuring allogeneic responses in vitro is the mixed leukocyte reaction (MLR), where leukocytes from two individuals, or animals, are mixed together in culture. The proliferative response measures T cell reactivity against alloantigen. The MLR is a convenient, relatively straightforward assay to perform and may be relatively useful predictors of the in vivo response [Singer and Bach 1995].

The response seen in the MLR is based on differences in major histocompatibility (MHC) molecules. There are two types of MHC molecules: class I and class II. MHC class I molecules are expressed on all nucleated cells while MHC class II are expressed primarily on professional antigen presenting cells (APCs) such as B cells, dendritic cells and macrophages. Both MHC I and II present protein antigens, albeit originating in different cell compartments: proteins synthesized in the cytoplasm are classically presented via MHC I, while proteins originating from the outside of the cytoplasm and degraded within acidic endosomal vesicles are classically presented via MHC II.

The MHC classes are recognized by T cell receptors of different T cell subsets that are identified by the presence of two markers called CD4 and CD8. Typically, CD4 molecules associate with the non-variable region of MHC-II, while CD8 molecules associate with the non-variable region of MHC-I. The variable portion of the MHC molecule contains a processed antigen that is recognized by T cell receptors. Although it is generally believed that T cells are self-MHC restricted (i.e. reactive to antigen presented only in the context of their own MHC molecules), T cells may cross-react with foreign MHC, with or without antigen. Thus, antigen recognition may be direct, where T cells recognize alloantigen in the context of allogeneic APC, or indirect, where alloantigen recognition takes place in the context of self-MHC; the presence of two sets of APCs in a graft is unique to the transplantation setting [Auchincloss 1995].
If there are differences in MHC expression between the two populations of leukocytes in an MLR, a proliferative response will ensue within three to five days. In order to observe the response of the leukocytes separately, i.e. a one-way MLR, one population can be inactivated by either gamma irradiation or treatment with the antimitotic drugs, generally mitomycin C, so that it does not proliferate. The cells from this population are called "stimulators" and are analogous to the donor in the setting of a transplant, while the cells from the untreated population are called "responders" and are analogous to the transplant recipient. The proliferative response is typically measured by adding radioactively labeled thymidine to the culture for a specified amount of time to assess incorporation as a measure of new DNA synthesis. Cell-free supernatant from the culture may be tested for the presence of various molecules elaborated during proliferation. It should be noted that the activated T cell response is not only dependent on differences in MHC, but is also dependent on the existence of a functional T cell receptor and costimulatory molecules. For example, stimulators lacking the B7 costimulatory molecules induce hyporesponsiveness, or diminished proliferation, in murine T cells.

Figure 2.3 shows a schematic of a one-way MLR, where the stimulators (Balb/c) and responders (C57/BL6) are mismatched in both MHC-I and MHC-II.

The MLR was first described in 1964 by two independent groups who co-cultured blood leukocytes from two unrelated individuals and led to the discovery that the lymphocytes generated in mixed cultures were cytotoxic to stimulator cells [Brent 1997]. Since then the MLR has served to elucidate not only mechanisms of allore cognition, but also the interplay between the various chemical mediators produced by lymphocytes, namely cytokines (protein hormones with both local and systemic effects) and chemokines (chemotactic cytokines). For example, following the discovery of a naturally occurring interleukin (IL)-1 receptor antagonist, it was found that its addition to an MLR decreased production of the IL-8 chemokine [Lakacs et al. 1993]. On the other hand, addition of a competitive inhibitor of L-arginine-dependent nitric oxide synthase to an MLR increased levels of IL-8 [Orens et al. 1994].

Although in vitro assays may be useful and convenient, there are several limitations in the translation of the in vitro results to the in vivo setting of transplant rejection. One is that the responder lymphocytes are reacting to stimulator lymphocytes, rather than endothelial cells or other parenchymal cells as would be present in an allograft. This may result in the expression of different surface molecules and subsequently different signaling pathways. Also, notably absent from the MLR are the vascular smooth muscle cells, which play an important role in the development of the proliferative lesions that are characteristic of TxAA. Another is that in vitro, cells are placed in close proximity and static setting, while, for example, in a cardiac allograft, the responder (or host) cells would be coursing through the bloodstream. As a consequence, cellular interactions are enhanced; contact-dependent mechanisms may artificially dominate and/or lower quantities of proteins may be required for downstream effects. Finally, cells are generally not cultured for more than seven days, such that late effects, potentially important in the rejection process, may not be recapitulated.
Figure 2.3: A one-way mixed leukocyte response (MLR): Spleens are extracted from two allogeneically different mice, and after lysis of the red blood cells, leukocytes are isolated. In order to observe the response of the leukocytes separately, one population can be inactivated by either gamma irradiation or treatment with the antimitotic drugs, so that it does not proliferate. Cells from this population are called "stimulators" and are analogous to the transplant donors, while the cells from the untreated population are called "responders" and are analogous to the transplant recipient. The proliferative response is measured by adding radioactively labeled thymidine. Cell-free supernatant may be tested for the presence of various molecules elaborated during proliferation.
The literature abounds with examples of findings that do not translate from the \textit{in vitro} to \textit{in vivo} setting. One particularly interesting example is the antibody to CD3. \textit{In vitro}, it is commonly used to stimulate T cell responsiveness. Its addition to T cell cultures results in massive proliferation and cytokine elaboration. This would lead one to expect a similar effect \textit{in vivo}. However, \textit{in vivo}, it acts as a T cell suppressor. In fact, the common immunosuppressive agent, OKT3 is an anti-CD3 antibody. When administered to patients, it acts by one of two mechanisms; it either activates complement-mediated lysis of T cells or it renders T cells susceptible to phagocytosis by macrophages. Thus, the same substance may serve as an activator \textit{in vitro} and a suppressor \textit{in vivo}, completely opposite functions.

Therefore, while the MLR is most appropriate as an \textit{in vitro} model to understand the effect of a potentially therapeutic intervention on allore cognition (and its downstream consequences), for an accurate reflection on mechanisms of rejection, it needs to be evaluated in an \textit{in vivo} setting. The next subsection discusses the \textit{in vivo} model and results relevant to the development of PR and TxAA. Animal models are the next logical progression following cellular assays, although even with those, translation to the clinic is far from assured since even slight immunologic differences may lead to vastly different results.

\textbf{2.1.3 \textit{In vivo} models}

To evaluate the contribution of selected components of the immune system to cardiac rejection \textit{in vivo}, a murine heterotopic cardiac transplantation method has been developed; the heart is removed from the donor mouse following heparinization and ligation of both the vena cava and pulmonary veins; it is then transplanted into the abdomen of the recipient mouse via ligation of the donor aorta and pulmonary artery to the abdominal aorta and inferior vena cava of the recipient mouse, respectively \cite{Corry et al 1973}. The recipient thus has two hearts; its original heart performing the essential cardiac functions and the donor heart (in the abdomen) perfused with recipient blood. Since the donor heart is not involved in cardiac function, evaluation of cardiac output and other physiologically relevant parameters are not germane. Nevertheless, the heart is perfused at normal aortic systolic pressures and beats; cessation of allograft beat, as assessed by manual palpation, is defined as the point of allograft failure. Figure 2.4, on the next page shows a schematic, as well as photomicrographs, of this murine heterotopic cardiac transplantation model.

Development of phenotypes characteristic of either PR or TxAA may be achieved by manipulating the expression of MHC molecules on both donor and recipient. Transplants performed between donors and recipients that are mismatched in MHC-I and MHC-II (thus a total allomismatch) develop severe PR, constituting an acute rejection model. Transplants performed between donors and recipients that are mismatched in MHC-II function long-term without immunosuppression and result in vascular lesions that are characterized by endothelial cell activation, vascular smooth muscle proliferation, mononuclear cell infiltration and fibrin deposition, thereby constituting the TxAA model.
Figure 2.4: Murine heterotopic cardiac transplantation model: a) Schematic showing the native heart of the donor transplanted to the recipient, such that recipient has two hearts, its own native and the allograft; b) photomicrographs showing, on the left, the recipient mouse, and, on the right top and bottom panels, schematics of the ligations and allograft itself.
Mismatches in MHC classes have implications for the type of T cell mediating effector functions since typically CD4+ cells are restricted by MHC-II and CD8+ cells recognize MHC-I. Although exceptions have been noted, CD8+ cells are classically ascribed cytolytic activity, while CD4+ cells are classically ascribed the function of immune modulation. Administration of antibodies to CD4+ cells (anti-CD4) in strain combinations mismatched in both MHC class I and II should be analogous to an MHC-I mismatch while antibodies to CD8+ cells (anti-CD8) to an MHC-II mismatch. This analogy in fact holds true with respect to the histologic appearance of the graft following anti-CD4 and anti-CD8 administration separately, although interestingly, survival times do not follow the pattern and are variable, demonstrating that graft failure may not correlate to the histological appearance of the graft [Nagano et al 1997].

The next two subsections discuss the role of cytokines and chemokines, respectively, in allograft rejection. In order to increase the applicability of the following discussion to the work contained in this thesis, inclusion of the literature will emphasize those reports using the murine vascularized heteroropic cardiac allograft model as described above, as much as possible.

Role of cytokines

There is ample evidence in the literature that many different cytokines and/or their receptors may play important roles in mechanisms of transplant rejection [Piccotti et al 1997, Nickeison et al 1997, Benjamin et al 2002, Hidalgo and Halloran 2002, Walsh et al 2004]. Cytokine manipulation has varying results on cardiac allograft pathology, ranging from beneficial to harmful. For example, prolongation of murine cardiac allograft survival has been reported with selective blockade of IL-15 [Smith et al 2000] and the complete absence of tumor necrosis factor-α (TNFα) receptors on donors significantly reduced the development of TxAA [Suzuki et al 2003]. On the other hand, the adoptive transfer of IL-4 secreting T cells into immunodeficient mice bearing cardiac allografts had no effects on survival [VanBuskirk et al 1996]. Furthermore, the exogenous administration of IL-10 to murine cardiac allograft recipients augmented both PR and TxAA [Furukawa et al 1999]. IL-2 is a target of many clinical immunosuppressive agents but the complete absence of IL-2 does not prolong graft survival, suggesting the important of other growth factors [Walsh et al 2004].

However, of the multitude of cytokines, interferon-γ (IFNγ) has emerged as particularly important in the setting of cardiac rejection, presenting a potential therapeutic target as well as illustrating the mechanistic dichotomy between the development of PR and TxAA [Benjamin et al 2002, Hidalgo and Halloran 2002]. Several properties render IFNγ important in the development of immune responses. It is a potent activator of macrophages [Nathan et al 1983]. Activated macrophages have effector functions that include phagocytosis, destruction of foreign organisms and potentiation of inflammation through the secretion of additional pro-inflammatory cytokines. Macrophage-derived products modulate the tissue environment by inducing the deposition of connective tissue. Finally, IFNγ increases the expression of MHC [Skoskiewicz et al 1985].
Examination of the role of IFNγ in cardiac transplantation has been greatly facilitated by the use of mice genetically engineering to congenitally lack IFNγ (Dalton et al 1993) or IFNγ receptors (Huang et al 1993). The mice whose IFNγ gene has been knocked-out, denoted as GKO mice, allow identification of the physiological role of IFNγ in the transplant setting relative to wild type mice whose IFNγ is naturally regulated.

The role of IFNγ in acute or parenchymal rejection was evaluated via allomismatched heterotopic transplants of Balb/c (B/c) hearts into C57BL/6 (B6) wild-type (WT) and GKO recipients, respectively (Nagano et al 1998). At the time of harvest (ranging from 6 to 7 days), allografts of B6WT recipients exhibited severe parenchymal rejection, while those of GKO recipients exhibited coagulation necrosis and arterial thrombosis, in addition to parenchymal rejection. Given that thrombosis and coagulative necrosis are not typical features of parenchymal rejection, the presence in GKO recipients suggest activation of a pro-thrombotic pathway normally suppressed by IFNγ. Potential mechanisms include an upregulation of IL-6 (Stimu et al unpublished) or platelet factor 4 (Saïura et al 2002). Thus, results indicate that IFNγ is not required for the development of PR; on the contrary, its absence results in an accelerated rejection phenotype.

The role of IFNγ is vastly different in TxAA. Transplants performed across MHC-II mismatches result in moderate parenchymal rejection and early TxAA when the recipient is WT; however, when the recipient is GKO, there is no TxAA, despite similar levels of parenchymal rejection (Nagano et al 1998). The absence of IFNγ results in abrogation of TxAA, in the face of persisting parenchymal rejection. Conversely, as was shown in severe combined immunodeficient murine recipients of human arterial transplants, the addition of IFNγ, without the presence of leukocytes, can induce TxAA (Tellides et al 2000). Consistent with this finding, the adoptive transfer of IFNγ deficient T cells into recombination-activating gene (RAG)-1 deficient recipients of MHC-II mismatched grafts, does not induce TxAA, compared to wild type cells that do (Furukawa et al 2004). Thus, IFNγ is both necessary and sufficient for the development of TxAA. Given the central nature of the role of IFNγ in mechanisms of rejection, this particular cytokine represents a principal point of interest throughout this thesis.

Role of chemokines

Interest in chemokines has grown. Together with their receptors, chemokines have been the subject of numerous general reviews (Cascieri and Springer 2000, Murdoch and Finn 2000, Gerard and Rollins 2001, Mackay 2001, Moser and Loetscher 2000) as well as reviews detailing their roles in transplant rejection (Hancock et al 2000, Nelson and Kremsky 2001, Calvin and Thomson 2002, El-sawy et al 2002, Shimizu and Mitchell 2003). Structurally, chemokines may be separated into two major subgroups based on the spacing of the first cysteine pair in their structure; chemokines in which the cysteine residues are adjacent are termed CC chemokines, while those in which the residues are separated by a single amino acid are termed CXC chemokines. There also exist two additional, smaller, and less well studied subgroups; the C group (where the first cysteine is missing) and the CXC(C subgroup (where the cysteine residues are separated by three amino acids). Discussion in this thesis is limited to selected CC and CXC chemokines.
Although there is evidence for migration-unrelated functions, such as lymphocyte differentiation [Luther and Cyster 2001], monocyte adhesion [Gerszten et al 1999] and smooth muscle cell proliferation [Shimizu and Mitchell 2003], the main task of chemokines is leukocyte traffic control [Moser and Loetscher 2001]. CC chemokines include, among others, regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, macrophage chemoattractant protein-1 (MCP-1). CXC chemokines include interferon-γ-inducible protein 10 (IP-10) and monokine induced by interferon-γ (MIG), among others; these are particularly germane in TxAA since their expression is profoundly influenced by IFNγ.

Chemokine receptors are membrane-bound and coupled to G-proteins [Murdoch and Finn 2000]. They are named according to the subgroup of chemokines that serve as their ligands; hence there are the CC-receptors (CCRs) and the CXC-receptors (CXCR). There have been 11 CCRs and 6 CXCRs identified to date [Shimizu and Mitchell 2003]. Generally, interactions between chemokines and their receptors are promiscuous. For example, CCR1, CCR4 and CCR5 bind to RANTES, MIP-1α and MIP1-β, while CCR2 binds to MCP-1 and CXCR3 to IP-10 and MIG. As can be seen, each of these chemokine receptors may bind to a variety of ligands and are considered “shared receptors”. There are few chemokine receptors that display unique specificity to one ligand [Cassani and Springer 2000]. Cellular chemokine receptor expression has been recently summarized and similar promiscuity appears in vascular and immune cells; notably CCR1, CCR2 and CCR5 are expressed on T cells, monocytes and smooth muscle cells, while CCR2 is also expressed on endothelial cells [Shimizu and Mitchell 2003].

Mechanistic examination of chemokines in acutely rejecting murine cardiac allografts has thus far included IP-10 and MIG. While administration of monoclonal anti-IP-10 antibody prolongs graft survival, congenital deficiency of IP-10 prolongs survival only if expressed in the donor, rather than recipient, leading to the conclusion that it is the IP-10 derived from the donor that initiates development of acute allograft rejection [Hancock et al 2001]. Treatment of recipients with rabbit antiserum to MIG results in a decrease in T cell infiltration and a prolongation of graft survival [Miura et al 2001]. Eventually compensatory mechanisms overcome the prolongation in the anti-serum MIG treated mice [Miura et al 2001]; it is unclear whether the same effect is seen with administration of the monoclonal antibody to IP-10 [Hancock et al 2001].

With respect to chemokine receptors in the acutely rejecting model, examination has been more extensive and included CCR1, CCR2, CCR5 and CXCR3. Cardiac allografts from recipients congenitally deficient in CCR1 have significantly prolonged survival times; furthermore, treatment with cyclosporine-A induces permanent graft acceptance, pointing to an important role for CCR1 [Gao et al 2000]. Graft survival time also increases when recipients were congenitally deficient in CCR2, concomitant to a decrease in the number of macrophages in the graft and despite constant numbers of other graft infiltrating cells and [Furukawa et al unpublished]. A more dramatic increase in allograft survival time is seen with recipient congenital deficiency of CCR5, interestingly with equivalent amounts of inflammatory cell infiltration [Gao et al 2001]. However, most dramatic, is the
increase seen in CXCR3 deficient recipients, where further treatment with cyclosporin A results in permanent graft acceptance (with no TxAA); administration of anti-CXCR3 antibody to WT recipients also increases graft survival [Hancock et al 2000].

TxAA in murine cardiac allografts may be attenuated by targeting MIG [Yun et al 2002] but not by RANTES or MIP-1α [Gao et al 2001]. Antibody neutralization of MIG reduced intimal thickening and the development of TxAA lesions, in the face of a significantly reduced inflammatory infiltrate (specifically CD4+ T cells), pointing to a critical role of recipient CD4+ cells in the sustained production of MIG [Yun et al 2002]. Surprisingly, based on their potent T cell attractant properties, congenital deficiency of either RANTES or MIP-1α had no significant effect on TxAA [Gao et al 2001].

The receptors examined in the murine TxAA MHC-II mismatched model include most of those discussed in the acute rejection model, namely CCR1, CCR2 and CCR5. Cardiac allografts from untreated recipients with a congenital deficiency in CCR1 show a complete lack of TxAA development, as per histologic evidence, when harvested 100 days after transplant [Gao et al 2000]. Deletion of CCR2 in the recipient does not have as pronounced an effect, but still significantly reduces TxAA, with only transient early decreases in macrophage infiltration and no other changes in cellular infiltrate [Furukawa et al unpublished]. Finally, congenital deficiency in CCR5 results in permanent graft acceptance with no sign of TxAA [Gao et al 2001]. This result, taken together with the finding that congenital deficiency of RANTES and MIP-1α, both CCR5 ligands, points to the potential advantage in targeting receptors, rather than chemokines [Gao et al 2001]. In fact, a recent report documents beneficial effects from administration of an agent antagonistic to CCR1 and CCR5 [Yun et al 2004]. The effect of combining immunosuppressive therapy with chemokine or chemokine receptor targeting has yet to be explored in MHC-II mismatched cardiac allografts. Based on the success in abrogating TxAA in cardiac allografts in fully allomismatched recipients congenitally deficient in CXCR3 and receiving cyclosporine A, there may be some promise in this approach.

Factors accounting for the delay in mechanistic studies of chemokines and their receptors include the deficiency in readily available monoclonal antibodies and until relatively recently, the lack of congenitally deficient mouse [Hancock et al 2000]. This lack of readily available monoclonal antibodies resulted in the use of imperfectly characterized polyclonal antibodies, fusion proteins and synthetic chemokines in studies, leading to potentially confounding results, particularly given the close homology of chemokines and their receptors [Hancock et al 2000].

Caution is advised with respect to the application of results obtained from murine studies to humans, based on several factors including: differences in cellular receptor expression (e.g. in mice CCR1 is expressed in neutrophils, while it is not in humans); differences in specificity (e.g. in mice RANTES does not bind to CCR1, while in humans it does); and occasional lack of homology (e.g. IL-8 does not have a structural homolog in mice), along with dichotomous in vitro and in vivo data [Hancock et al 2001]. Nonetheless, the recent literature regarding chemokines and their receptors offer potential therapeutic targets for ameliorating graft rejection.
2.2 Peroxisome proliferator-activated receptors

This section begins by describing some of the general characteristics of the peroxisome proliferator-activated receptors (PPARs), particularly with respect to structure, activation and expression. Then, it reviews the literature regarding the role of PPARs in vascular immunology, first discussing evidence from in vitro studies and then turning to in vivo models. A detailed review of the clinical literature, which to a large degree focuses on the role of the PPARs in lipid and triglyceride homeostasis, is beyond the scope of this section. Suffice it to reiterate that PPAR ligands are in active clinical use. PPARα ligands (fenofibrate, or Tricor) for hypertryglyceridemia and PPARγ ligands (rosiglitazone, or Avandia and pioglitazone, or Actos) for diabetes mellitus type 2. As the range of the pleiotropic effects of PPAR expand and become established, particularly with respect to inflammation, the clinical arena for these ligands is certain to expand.

2.2.1 General characteristics

PPARs belong to a nuclear hormone receptor superfamily, that act as gene transcriptional regulators. To date, three isoforms are known, α, γ and β/δ. PPARα was the first isoform to be identified in 1990 [Issemann and Green 1990] and is typically associated with regulation of fatty-acid catabolism, while PPARγ is essential for adipocyte differentiation and may mediate insulin sensitization [Lee et al 2003]. The role of the widely expressed β/δ isoform remains to be elucidated, although recent evidence suggests a role in the control of triglyceride levels by sensing very low-density lipoproteins [Lee et al 2003]. This thesis focuses on the PPARα and PPARγ isoforms. According to a recently proposed unifying nomenclature for nuclear receptors, PPARα and PPARγ form group C of the subfamily 1 of the nuclear hormone superfamily, and thus may be identified in the literature as NR1C1 and NR1C3, respectively [Escher and Wahli 2000]. However, throughout this thesis, they are referred to as PPARα and PPARγ. The following sections first discuss PPAR structure and then their activation, including a description of both synthetic and natural ligands. Finally, a summary of the expression profiles in cells and tissues is given.

Structure

The structure of the PPARs is well known [Nolte et al 1998, Uppenberg et al 1998]. As shown in figure 2.5, there are four major domains. The known details of each of the four receptor domains has been recently summarized [Escher and Wahli 2000] and, unless otherwise noted, the statements about PPAR structure in the following paragraphs derive from this review.

The first is the A/B domain. Poorly conserved between PPAR isoforms, this N terminal domain varies in both sequence and length and contains a ligand-independent activation factor (AF), named AF-1. Studies have shown that phosphorylation at specific sites in this domain, e.g. serine residues, may modulate receptor activity, either intensifying or decreasing the effect of ligand activation, suggesting interdomain regulation.
The next domain is the C domain. Highly conserved, it is approximately 70 amino acids long and comprises the DNA-binding domain of the receptor. It folds into two “zinc finger”-like structures with α-helical DNA binding motifs. One of the fingers contains the “P-box” that is involved in recognizing and binding to part of the nucleotide sequence known as the PPAR Response Element (PPRE); the consensus sequence of the PPRE is 5'-AACT AGGNCA A AGGTCA-3' [Michalik and Wahli 1999]. Mutations in the P-box can completely abolish transcriptional activity. The other zinc finger contains the “D-box” that is involved in dimerization of the DNA-binding domain.

The third domain is the D domain, also called the “hinge” domain because it links the DBD domain to the next one. However, others have also noted that, in addition to its linking function, this domain is important for co-factor docking [Debril et al 2001], that in turn, is critical for PPAR activation, upon ligand binding.

The last domain is the C terminal, E/F or ligand-binding domain, which contains a ligand-dependent AF-2. It consists of thirteen α-helices and a small β-sheet, forming a large T-shaped hydrophobic binding pocket and interface for heterodimerization with the 9-cis retinoid X receptor (RXR), its obligate partner [Debril et al 2001]. The placement of the amino acid residues appears to be exquisitely important, such that a change in even only one amino acid may result in a fourfold lower affinity for a ligand. Overall, there are two main differences between the PPAR and other nuclear receptors; firstly, its binding pocket is much larger, such that less than half of the cavity is occupied by any of the ligands identified to date; and secondly, that it has an extra α-helix, contributing to the increased size of the pocket, and participating in the entry of the ligand [Michalik and Wahli 1999].

Activation

Figure 2.6 shows schematic of PPAR activation. In addition to binding by ligand, receptor activation requires heterodimerization with the RXR, as well as the release of several co-repressors and interactions with a host of co-activators. Specifically, upon ligand binding, the LBD undergoes a conformational change, particularly involving AF2, that allows the release of co-repressors, including the nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptor [Michalik and Wahli 1999] and interaction with the co-factor, steroid receptor coactivator-1, that in turn, interacts with the A/B domain and then recruits two other co-factors, the cAMP-responsive element-binding protein-binding protein (CBP)/p300 and the p300/CBP-associated factor, further contributing to histone acetyltransferase activity [Escher and Wahli 2000]. The conformational change also stabilizes the interaction between PPAR and RXR for binding of the PPAR:RXR complex to the PPRE [Debril et al 2001]. This, combined with the histone acetyltransferase activity of the co-factors, results in the interaction of the activated heterodimer with the promoter, stimulating the transcription of target genes. A functional consequence of this complex regulatory system is that receptor activation may potentially be influenced by ligand-specific interactions with any one of the co-activators and/or co-repressors.
Figure 2.5: PPAR structure: There are four domains in the receptor. The first is the A/B domain that contains a ligand-independent transactivating factor called activation factor (AF) 1. The second is the C domain that is responsible for binding to the DNA and is referred to as the DNA-binding domain. The third is the D domain that contains a hinge region. The fourth is the E/F domain (containing a ligand-dependent activating factor called AF-2) and is generally referred to as the ligand-binding domain.

<table>
<thead>
<tr>
<th>A/B</th>
<th>C</th>
<th>D</th>
<th>E/F</th>
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<tr>
<td>Ligand-independent (AF-1)</td>
<td>DNA-binding</td>
<td>Hinge</td>
<td>Ligand-binding (AF-2)</td>
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N terminal  C terminal

Figure 2.6: PPAR activation: Upon ligand binding, PPAR undergoes a conformational change that allows the release of co-repressors and interactions with co-activators. This change stabilizes the interaction between PPAR and RXR for binding of the PPAR:RXR complex to the PPRE and results in the interaction of the activated heterodimer with the promoter, thereby stimulating the transcription of target genes.
Several non-ligand dependent factors may be responsible for receptor regulation. One is the availability of co-factors; several co-factors exhibit tissue-restricted distribution and physiological regulation [Escher and Wahli 2000]. Another is the direct phosphorylation of residues on the receptor, as may occur in the presence of particular kinases [Debriel et al 2001]. Finally, the amount of retinoid acid receptor (RAR) present in a cell, given the ability of the RXR to also heterodimerize with RAR, such that an increase in RAR can increase the formation of RAR:RXR complexes and decrease the amount of RXR available to bind with the PPAR and vice-versa, as has been shown in adipocytes [Debriel et al 2000].

It has also been noted that the PPAR:RXR heterodimer can also be activated by ligand binding to the RXR such that, for example, synthetic ligands specific for RXR (namely LG1069 and LG100268) are under current investigation as alternative treatments to PPARγ ligands [Kersten et al 2000]. Nonetheless, the most significant and well studied activation results from the binding of ligands to the PPAR.

There are natural and synthetic ligands for PPARα. Naturally occurring long-chain polyunsaturated fatty acids, e.g. linoleic and arachidonic acids, show the highest affinity for PPARα; additional natural ligands include fatty acid derivatives, such as leukotriene B4 and 8(S)-hydroxy-eicosatetraenoic acid [Michalik and Wahli 1999], the latter having the highest affinity [Dussault and Forman 2000]. With respect to synthetic ligands, PPARα is the molecular target of a class of drugs called fibrates that are in clinical use for the treatment of hypertriglyceridemia. This class of drugs includes clofibrate, fenofibrate, bezafibrate, gemfibrozil and lastly WY14643, with reportedly the highest affinity [Dussault and Forman 2000]. Interestingly, it has been noted that, although their main effects are not mediated through this particular mechanism of action, two non-steroidal anti-inflammatory agents indomethacin and fenoprofen, may also bind to PPARα [Escher and Wahli 2000].

Similarly, PPARγ also has both natural and synthetic ligands. Natural ligands for PPARγ also include polyunsaturated fatty acids, as well as components of oxidized low-density lipoproteins such as the 9- and 13-hydroxy-octadecadienoic acids, and prostaglandins with their metabolites, particularly 15-deoxy-Δ12,14 prostaglandin J2 [Escher and Wahli 2000], one of the most potent [Michalik and Wahli 1999], although there is controversy surrounding its relevance as a PPARγ agonist [Maxey et al 2000; Dussault and Forman 2000; Rossi et al 2000]. Synthetic ligands for PPARγ come from family of agonists called thiazolidinediones, of which two of the agents (rosiglitazone, also known as BRL49653, and pioglitazone), are currently used clinically as insulin-sensitizing hypoglycemics. The first agent in this class, troglitazone, has been withdrawn from the market secondary to the incidence of hepatotoxicity [Rosen and Spiegelman 2000].

Although ligands are generally ascribed either PPARα or PPARγ specificity, there is evidence of overlap between receptors. For example, some fatty acids such as arachidonic and eicosapentaenoic acids bind similarly to both PPARs, while others, such as linolenic and palmitoleic acids, bind more strongly to PPARγ but also exhibit PPARα binding.
activity [Hwang 2000]. Potential endogenous ligands, such as dehydroepiandrosterone, bind to PPAR\( \alpha \) [Peters et al 1996], although a recent study also suggests interaction with PPAR\( \gamma \) [Kajita et al 2003]. Even in the realm of synthetic ligands, there may be overlap. At high doses, fibrates, as exhibited by the compound Wy14643, may activate PPAR\( \gamma \) in addition to PPAR\( \alpha \) [Escher and Wahli 2000]. The non-steroidal anti-inflammatory agents (indomethacin and fenoprofen) reported above to activate PPAR\( \alpha \) also activate PPAR\( \gamma \), albeit weakly [Escher and Wahli 2000].

Expression

PPARs have distinct, tissue-specific expression and distribution in human tissues [Escher and Wahli 2000]. PPAR\( \alpha \) is expressed primarily in the liver, heart and kidney, while PPAR\( \gamma \) is predominantly expressed in white adipose tissue. Accordingly, hepatocytes, cardiomyocytes and adipocytes express PPARs of various isoforms. Adult rodents show a similar pattern of expression, with the additional demonstration of PPAR\( \gamma \) in the spleen [Escher and Wahli 2000]. One notable exception is that expression of hepatic PPAR\( \alpha \) expression is much lower in the human compared to the rodent [Escher and Wahli 2000]; in fact, contrary to results using rodent hepatocytes, PPAR\( \alpha \) agonists do not induce peroxisome proliferation-associated genes in human hepatocytes [Lawrence et al 2001].

PPAR expression has also been identified in the vasculature and several cells in the immune system. Vascular endothelial cells express both PPAR\( \alpha \) [Inoue et al 1998] and PPAR\( \gamma \) [Marx et al 1999a]. Similarly, vascular smooth muscle cells also contain both PPAR\( \alpha \) [Staels et al 1998] and PPAR\( \gamma \) [Hijima et al 1998, Law et al 2000]. With respect to the immune system, monocytes/macrophages, the most well studied inflammatory cells, contain both PPAR\( \alpha \) [Chinetti et al 1998] and PPAR\( \gamma \) [Ricote et al 1998, Marx et al 1998b]. Following the same trend, T cells also express both PPAR\( \alpha \) [Jones et al 2002] and PPAR\( \gamma \) [Clark et al 2000, Harris and Phipps 2001, Padilla et al 2002]. Contrasting with the other immune cells discussed thus far, dendritic cells express the PPAR\( \gamma \) isoform but not PPAR\( \alpha \) [Faveeau et al 2000]. Thus, with the exception of dendritic cells, all of the vascular wall cells, as well as monocytes/macrophages and lymphocytes, express both PPAR isoforms.

2.2.2 In vitro effects

PPAR activity has been linked to relevant cell types in the vasculature, namely vascular endothelial cells and smooth muscle cells, as well as immune cells that play important roles in the vasculature, namely macrophages and T cells. The role of PPARs in the cardiovascular system is the subject of several reviews [Bishop-Bailey 2000, Buchan and Hassall 2000, Francis et al 2003]. This section discusses evidence of PPAR effects on vascular immunology, i.e. on cells of the vasculature, as well as on cells of the immune system that are particularly relevant to the development of vascular disease. Relevant information from other cell types is also included. Emphasis in this section is placed on results from in vitro studies, while the next section includes a discussion of relevant findings from in vivo models.
Vascular endothelial cells

Reports in the literature on the effects of PPARα agonists on endothelial cells suggest a broad range, from anti-inflammatory to pro-inflammatory. Anti-inflammatory effects of PPARα agonists include a reduction in the adhesion of monocytes by diminishing the expression of vascular cell adhesion molecule-1 (VCAM-1) upon either mitogenic or cytokine stimulation [Jackson et al 1999, Marx et al 1999b, Pasceri et al 2000, Rival et al 2002]. A mechanism of this inhibition is by the decrease in nuclear p65 amount, a protein critical in the nuclear factor-κB (NF-κB) pathway [Rival et al 2002]. On the other hand, when stimulated by oxidized phospholipids, however, PPARα agonists increase the synthesis of selected chemokines [Lee et al 2000]. The overall effect of PPARα activation is largely dependent on the setting, particularly the stimulation type and ligand. For example, PPARα increases phospholipid-stimulated expression of MCP-1 [Lee et al 2000], but has no effect if it is stimulated by cytokines [Marx et al 2000]. Another example is the effect of PPARα activation on the expression of plasminogen-activator-inhibitor-1 (PAI-1); modulation is ligand-dependent [Nilsson et al 1999].

PPARγ ligands exhibit a range of anti-inflammatory effects in vascular endothelial cells, again dependent on stimulation type and ligand. With respect to ICAM-1, studies show that PPARγ ligation may either inhibit [Pasceri et al 2000] or augment [Chen et al 1999] its expression. Similar variation in results exists for the expression of VCAM-1, with evidence showing both no effect [Marx et al 1999b, Jackson et al 1999] and a decrease [Pasceri et al 2000, Jackson et al 1999]. PPARγ ligands inhibit release of endothelin-1, a potent vasoconstrictor [Delerive et al 1999a, Saroh et al 1999]. The literature on the effect of PPARγ ligands on the expression of PAI-1 also varies with reported increases [Marx et al 1999a, Xin et al 1999], and decreases [Kato et al 1999]. PPARγ agonists may also reduce expression of endothelial cell growth receptors [Xin et al 1999], as well as the IFNγ-induced chemokines IP-10, MIG and ITAC [Marx et al 2000].

Vascular smooth muscle cells

PPARα ligands have significant anti-inflammatory effects on vascular smooth muscle cells without affecting migration. Specifically, PPARα ligands exhibit anti-inflammatory responses in vascular smooth muscle cells by repression of NF-κB signaling, thereby inhibiting IL-1 induced production of IL-6 [Staels et al 1998, Delerive et al 1999b]. PPARα ligands also prevent prostaglandin production by inhibiting expression of inducible cyclooxygenase (COX) or COX-2, as determined by specifically testing its promoter [Staels et al 1998]. The PPARα-mediated inhibition of IL-6 gene transcription occurs by interference with c-jun and p65 [Delerive et al 1999b]. Despite this activity, PPARα agonists do not influence smooth muscle cell migration as evidenced by the lack of expression of matrix metalloproteinase-9 (MMP-9) or tissue inhibitor of matrix metalloproteinasess (TIMP)-1 or TIMP-2 platelet-derived growth factor-BB [Marx et al 1998a], or on interferon regulatory factor-1 (IRF-1), a transcriptional factor that mediates vascular smooth muscle cell proliferation [Lin et al 2004].
PPARγ ligands, on the other hand, have significant effects on vascular smooth muscle cell migration without affecting inflammatory activity. The effects on vascular smooth muscle cells are demonstrated by the ability of several different PPARγ ligands to inhibit migration under several stimulation conditions [Marx et al 1998a, Benson et al 2000, Goetze et al 1999]. PPARγ ligands block several events, including downstream of mitogen-activated protein kinase (MAPK) cytoplasmic activation [Goetze et al 1999], downregulate growth-factor-induced expression of c-fos [Benson et al 2000] and decrease MMP-9 levels and activity [Marx et al 1998a]. Selected PPARγ ligands enhance IL-1 induced nitric oxide synthesis [Hattori et al 1999], increase IRF-1 [Lin et al 2004], and, via effects on cell calcium mobilization, may attenuate both proliferation [Asano et al 1999] and contractile ability [Song et al 1997]. With respect to inflammatory activity, unlike PPARα ligands, PPARγ ligands do not affect IL-1 induced production of IL-6 or COX-2 expression on vascular smooth muscle cells [Staels et al 1998].

Monocytes/macrophages

PPARα activation generally has anti-inflammatory effects on monocytes/macrophages, although there is some suggestion of potential pro-inflammatory effects. Synthetic ligands induce apoptosis in cytokine-activated macrophages activated [Chinetti et al 1998] and regulate the expression of AdipoR2, a receptor for an adipocyte-derived cytokine, adiponectin, recently implicated in regulation of macrophage phagocytic activity, as well as lipid accumulation [Chinetti et al 2004]. Ligand-activation also reduces nitrite accumulation, associated with a reduction in inducible nitric oxide synthase (iNOS) and induction of heme oxygenase 1 [Colville-Nash 1998]. Furthermore, PPARα activation down-regulates platelet-activating factor binding and receptor expression, suggesting, as a consequence, a reduction in the recruitment and adhesion of monocytes, in addition to a decrease in the production of inflammatory cytokines [Horton et al 2001]. However, using peritoneal macrophages and fluid, the addition of a synthetic PPARα activator actually enhanced monocyte migration, suggesting that PPARα inhibitors may also have pro-inflammatory properties [Hornung et al 2001]. Another potential pro-inflammatory effect of PPARα activation is from the upregulation of macrophage lipoprotein lipase expression, with the downstream effects of enhancing monocyte binding to the endothelium and inducing monocyte/macrophage activation, among a host of other effects [Li et al 2002].

PPARγ agonists are ascribed a multitude of negative regulatory effects on macrophage function. Specifically, activation with synthetic ligands reportedly decreases the expression of iNOS and MMP-9 [Ricote et al 1998, Colville-Nash 1998, Marx et al 1998b, Li et al 2000b, Chen et al 2003, Welch et al 2003]. PPARγ agonists also have anti-inflammatory actions by decreasing the production of several cytokines, including IL-1, IL-6, TNFα and IL-12 [Jiang et al 1998, Meier et al 2002, Chung et al 2000, Welch et al 2003]. It should be noted that the PPARγ mediated effects on cytokines are not without contention based on results showing a lack of effect with the synthetic ligands on IL-6 and TNFα [Thieringer et al 2000, Welch et al 2003]. This led to a suggestion that the effects seen with natural ligands are independent of PPARγ [Thieringer et al 2000] and subsequent studies supported this notion [Rossi et al 2000].
Interestingly, there is evidence in the literature that part of the effect of at least two commonly available non-steroidal anti-inflammatory drugs are mediated by PPARγ, based on a feedback control of COX-2 expression [Inoue et al 2000]. Although there is decreased migration upon incubation with PPARγ ligands [Hornung et al 2001], the effect on chemokine production varies, ranging from a decrease in MCP-1 [Zhang et al 2001] and CCR2 [Han et al 2000], to no effect on RANTES and an increase in IL-8 [Zhang et al 2001, Fu et al 2002]. Ligand-activation of PPARγ also induces apoptosis in macrophages [Chineti et al 1998] and upregulates lipoprotein lipase, adipor2 and thrombomodulin, a receptor that is involved in anti-coagulation [Li et al 2002, Kanekara et al 2003, Chinetti et al 2004]. PPARγ may act in part by antagonizing the activities of several transcription factors including activating protein-1 janus kinase STAT and NF-κB [Ricote et al 1998, Chung et al 2000, Chen et al 2003], as well as direct interaction with CREB-binding protein coactivator [Li et al 2000a].

T cells

Overall, PPARα agonists may have anti-inflammatory properties related to effects on T cells, as demonstrated by a decrease in the production of several inflammatory cytokines. Following T cell activation with anti-CD3, the production of IL-2, TNF-α and IFNγ is significantly decreased in T cells pre-treated with synthetic PPARα agonists [Marx et al 2002]. A similar effect on IL-2 and IFNγ is observed following stimulation with concanavalin-A [Cunard et al 2002a]. However, an inhibitory effect on IL-2 production is not observed upon T cell stimulation with phytohemagglutinin after pre-treatment with a synthetic PPARα ligand [Yang et al 2000], suggesting stimulation pattern dependence. Interestingly, synthetic ligands may have PPARα independent effects on cytokine concentration, such as an increase in IL-4 production [Cunard et al 2002a]. The effect of PPARα ligand-mediated activation is modest on cell proliferation [Cunard et al 2002a], and absent on cell viability [Marx et al 2002]. With respect to the mechanisms of inhibitory action, experiments show that PPARα ligand-activation may effectively antagonize the actions of NF-κB, a critical transcription factor mediating cytokine production [Jones et al 2002]. Following the identification of T-bet, a transcription factor that affects T cell development by its ability to transactivate IFNγ while repressing IL-2 gene expression [Szabo et al 2000], recent experiments point to a potential role for PPARα in the regulation of T-bet through p38 mitogen-activated protein kinase [Jones et al 2003].

The majority of PPARγ ligand-mediated effects reported to date are anti-inflammatory, although there are reports of potential pro-inflammatory actions. Treatment of T cells with PPARγ agonists decreases the stimulation-induced production of IFNγ, TNFα and IL-4 [Cunard et al 2002a, Marx et al 2002, Chung et al 2003, Wang et al 2003]. Furthermore, in cultures treated with PPARγ agonists, T cell production of IL-2 both consistently and significantly decreases [Clark et al 2000, Yang et al 2000, Cunard et al 2002a, Wang et al 2003]. The dose at which this decrease occurs correlates with a modest decrease in T cell proliferation [Cunard et al 2002a], while high doses of PPARγ agonists show more pronounced inhibition of proliferation [Clark et al 2000, Yang et al 2000] and
induce apoptosis [Harris and Phipps 2001]. Recent evidence demonstrates the ability of synthetic PPARγ agonists to enhance mitochondrial potential and actually promote cellular survival, when used at concentrations that optimally induce transcriptional activation, thereby suggesting a possible augmentation of immune responses, or pro-inflammatory effect [Wang et al 2002]. Although ascribed a PPARγ independent mechanism, consistent with this pro-inflammatory effect is the potential for a natural PPARγ ligand to induce production of the IL-8 chemokine [Harris et al 2002]. PPARγ regulation of cytokine production by stimulated T cells strongly correlates with the inhibition of transcription factors activating protein-1 (AP-1) and NF-κB [Wang et al 2001] and also involves a physical association between PPARγ and the nuclear factor of activated T cells transcription factor [Chung et al 2003].

Relevant evidence from other cell types

PPARγ may also play a role in immune regulation by affecting the maturation and immunogenicity of dendritic cells [Faveeuw et al 2000, Gosset et al 2001, Nencioni et al 2002]. Dendritic cells are important in the generation of primary immune responses given their ability to stimulate naïve T cells; upon encounter with antigen, dendritic cells undergo a maturation process that includes upregulation of co-stimulatory molecules and cytokine production [Steinman et al 1997]. PPARγ ligand activation alters co-stimulatory expression, upregulating B7-1, while down-regulating B7-2 [Gosset et al 2001, Nencioni et al 2002], and impairing the expression of CD40, as well as the dendritic cell hallmark molecule, CD1a [Nencioni et al 2002]. PPARγ agonists also decrease the dendritic cell production of cytokines, most notably IL-6, IL-10, IL-15, TNFa and IL-12 [Faveeuw et al 2000, Nencioni et al 2002] and the production of selected chemokines, [Gosset et al 2001]. Part of these inhibitory effects of PPARγ agonists may be due to the blocking of the RelB protein (a member of the NF-κB family), given the reduced expression of the nuclear localized RelB protein [Nencioni et al 2002].

Investigation into the mechanistic details of PPAR action continues and recent work, using rodent islet cells, has added to the understanding of signaling pathways [Weber et al 2003]. With respect to IFNγ, the protein factor that binds to the gamma activating sequence is the STAT1 factor and PPARγ ligand-activation attenuates IFNγ-induced STAT1 phosphorylation, nuclear localization and DNA binding [Weber et al 2003]. Furthermore, results show that the corresponding increase in the expression of heat shock protein 70 (potentially important for preventing the damaging actions of nitric oxide) does not affect the PPARγ ligand-mediated inhibition of STAT1 activation; the inability of a cell line overexpressing heat shock protein 70 to modulate STAT1 provides corroboration that the PPARγ-mediated effects on cytokine inhibition are not mediated via this heat shock protein [Weber et al 2003].

Further mechanistic insight is given by experimentation into the activation and degradation of PPARγ in adipocytes [Floyd and Stephens 2002]. Similar to other transcription factors and members of the nuclear receptor superfamily [Weissman AM 2001], the degradation of PPARγ is subject to regulation by the ubiquitin-proteasome pathway, thereby requiring covalent tagging of the substrate with a polyubiquitin chain.
and the subsequent recognition by the proteasome [Floyd and Stephens 2002]. Following IFNγ-mediated activation and serine phosphorylation, ubiquitin conjugation increases and PPARγ degradation ensues without requiring export from the nucleus [Floyd and Stephens 2002]. Inhibition of extracellular signal-regulated kinases (ERKs) 1 and 2 decreases the rate of degradation and prolongs the half-life of PPARγ proteins [Floyd and Stephens 2002]. Studies using adipocyte cell lines also show additional pathways involving PPAT in the cardiovascular system, namely ligand-activated decrease in the expression of PAI-1 and aromatase, a critical enzyme in estrogen biosynthesis [Tbana et al 2001, Rubin et al 2000]. PAI-1 promotes both thrombosis and fibrosis, such that its inhibition may be desirable and has resuscitated interest in the development of PAI-1 therapeutic strategies [Lyon and Hsueh 2003]. Estrogen has been ascribed many cardioprotective effects, such as arterial vasodilation, inhibition of cellular response to vascular injury and prevention of atherosclerosis [Mendelsohn 2003]. On the other hand, in a setting such as breast cancer, where adipocyte-derived estrogen may promote growth, PPARγ-induced decrease in its biosynthesis may be beneficial [Rubin et al 2000]. Thus, as shown in several vascular cell types, PPAR ligands may play both protective and harmful roles in the cardiovascular system.

Finally, caution is advised with respect to the comparison of rodent and human cellular results. For example, results show that two potent and synthetic PPARα agonists have differential induction of peroxisome proliferation-associated gene expression in rodent and human hepatocytes [Lawrence et al 2001]. There are several hypotheses to account for the difference, including differences in the tissue expression of PPARα or in the responsiveness of the PPRE [Lawrence et al 2001]. A human hepatic cell line, manipulated to overexpress the PPARα protein, does not significantly increase the induction of peroxisome proliferator-activated genes, implying that the failure of human PPARα in the induction of peroxisome proliferator-activated genes is independent of the level of receptor expression [Lawrence et al 2001]. The second hypothesis, of differing PPRE, however, is more consistent with the finding that different synthetic agonists have dramatically different affinities between rodent and human hepatocytes, as well as different peroxisome proliferator-activated expression profiles, such that the rodent and human PPARα may be distinguished pharmacologically [Lawrence et al 2001]. One potential reason for the difference in binding affinities is postulated to be differences in two amino acids in the ligand-binding domain of PPARα between the two species [Lawrence et al 2001]. Potential explanations for the differences in PPRE responsiveness resulting in a change in the induction of peroxisome proliferator-activated genes include changes in the PPRE, causing a loss of functionality, or a change in one or more of the transactivating factors (either coactivators or corepressors).

2.2.3 In vivo effects

The role of PPAR agonists in the cardiovascular system is under examination in a number of in vivo models, ranging from models of ischemia/reperfusion, to myocardial function, and to atherosclerosis. Furthermore, studies are elucidating PPAR effects in inflammation. Results from the literature on PPARs effects in vascular immunology (in vitro and in vivo) suggest a possible role for PPARs in mechanisms of transplant rejection.
Although ischemia/reperfusion injury does not cause allograft vasculopathy in syngeneic grafts, it can exacerbate existing vasculopathy [Furukawa et al 2003]. Protection against ischemia/reperfusion injury and myocardial infarction size may be achieved following activation of both PPARα and PPARγ [Tabernero et al 2002, Wayman et al 2002, Ito et al 2003, Yue et al 2003]. Mice congenitally lacking PPARα exhibit increased susceptibility to ischemic damages and are refractory to treatment with a PPARα agonist [Tabernero et al 2002, Yue et al 2003]. Administration of a potent PPARα agonist causes a reduction in infarction size in rodent models [Yue et al 2003, Wayman et al 2003]. Furthermore, PPARα agonist administration also improves endothelial vasodilatation [Tabernero et al 2002] and contractile function [Yue et al 2003]. Similar results are observed in rat models using the two clinically available PPARγ agonists [Yue et al 2001, Wayman et al 2002, Ito et al 2003]. Concomitant to the attenuation of ischemia/reperfusion injury were decreases in neutrophil and monocyte accumulation [Yue et al 2002, Ito et al 2003].

Increasing evidence suggests a potential role for PPARs in atherosclerosis. Although TxAA and traditional atherosclerosis differ in several ways with respect to lesion characteristics (concentric, lipid-poor, diffuse vs. eccentric, lipid-rich, focal), there are several similarities, including endothelial cell activation, vascular smooth muscle cell hyperplasia and extracellular matrix synthesis [Hansson 1995]. Both also have T cell and macrophage accumulation, and in fact, the immunologic basis of atherosclerosis is being increasingly appreciated [Ross 1999]. The role of lipid homeostasis is less relevant to the development of allograft vasculopathy than to typical atherosclerosis, such that potentially pro-atherosclerotic effects, such as the PPARγ ligand-mediated conversion of macrophages to foam cells in atherosclerotic plaques [Tontonoz et al 1998, Nagy et al 1998], are less germane. On the other hand, the inflammatory properties of lipids may affect the translation of anti-inflammatory findings from typical atherosclerosis to TxAA.

The potential benefit of PPAR agonist administration in murine models of atherosclerosis is under active investigation. One study examining the effect of fenofibrate, a PPARα agonist, on a murine model of atherosclerosis based on apolipoprotein E (apoE) congenital deficiency, shows that treatment increases plasma lipid levels and decreases both cholesterol content and MCP-1 expression [Duez et al 2002]. Administration of PPARα ligand in apoE mice that overexpress the human apoA-I, also results in a diminished aortic lesion area [Duez et al 2002]. Similar effects in lesion reduction may be seen with PPARγ agonists. In a study using mice that are congenitally deficient in LDL, administration of PPARγ activators strongly inhibited the development of atherosclerosis decreases in TNFα [Li et al 2000]. Similar reduction in lesion formation is seen with PPARγ agonists in apoE deficient mice [Chen et al 2001]. Moreover, using an atherosclerotic model of apoE deficient mice, results show that angiotensin II, a potent vasoconstrictor implicated in atherosclerosis, is associated with activation of NF-κB genes and downregulation of PPARs, as well as MCP-1, ICAM-1, VCAM-1, iNOS and COX-2 throughout the entire aorta [Tham et al 2002]. Overall, there is strong interest in the potential use of PPAR agonists in atherosclerosis [Plutzky 2000, Neve et al 2000, Rosen and Spiegelman 2000, Duval et al 2002, Barbier et al 2002, Puddu et al 2003].
With respect to inflammation and immunity, PPARα agonists have profound effects. Following immunization with myelin basic protein, mice treated with PPARα agonist have an impaired immunoglobulin G response and a decreased number of splenocytes, which, when restimulated ex-vivo, produce decreased quantities of IL-6, TNFα and IFNγ [Cunard et al 2002b]. It should be noted that the decrease in the number of splenocytes is attributed to effects independent of PPARα, based on the ability of the PPARα agonist to induce a similar degree of apoptosis in splenocytes from mice congenitally deficient in PPARα (PAKO) compared to wild type mice [Cunard et al 2002b]. Aortic explants from PAKO mice demonstrate dramatically increased production of IL-6, when stimulated by lipopolysaccharide (LPS), and conversely, treatment with PPARα agonist fenofibrate results in the reduction of LPS-stimulated aortic IL-6 secretion [Delepine et al 1999b]. Results also indicate that the inhibition of inflammatory responses involves direct protein interaction with p65 and c-Jun [Delepine et al 1999b].

The above findings naturally lead to the hypothesis that treatment using the same class of PPARα agonists can attenuate acute inflammation, as determined by the production of TNFα. However, in a mouse model of endotoxemia, mice treated with PPARα agonists have fivefold higher levels of LPS-induced TNFα in the plasma [Hill et al 1999]. In addition, the treated mice had significantly lower glucose levels and, more importantly, significantly lower lethal LPS dose [Hill et al 1999]. Experiments using PAKO mice confirm that this effect is indeed mediated via PPARα, although, interestingly, and for reasons unclear, administration of the PPARα agonist to the PAKO mice resulted in decreased TNFα levels and increased glucose levels compared to the wild type mice [Hill et al 1999]. There may be an issue of ligand specificity, with potential effects mediated by other PPARs. Overall, the results suggest that the cell and tissue specific anti-inflammatory activity of PPARα (demonstrated by in vitro and in vivo studies) may be overshadowed systemically [Hill et al 1999] via hitherto unrecognized pathways.

Activation of PPARγ in vivo also has profound immunomodulatory effects and potential benefits in inflammatory bowel disease and diabetes mellitus type 1, both of which are characterized by significant immune components. In a murine model of inflammatory bowel disease, PPARγ agonist administration of a classic molecule attenuated disease and concomitant inflammation [Su et al 1999]. Further investigation, using an intestinal cell line, shows that the PPARγ ligands reduce the nuclear protein binding to the NF-κ and render the IκB-a protein (that works by inactivating the NF-κB factor) resistant to degradation upon stimulation with IL-1β [Su et al 1999]. Treatment with a PPARγ agonist reduces the incidence of autoimmune diabetes progression in a mouse model, [Augstein et al 2003]. In this model, islet cells from the treated mice express significantly lower levels of IL-1β induced ICAM-1, while the T cell expression remains unchanged [Augstein et al 2003]. The CD4+ and CD8+ intracellular expression of IL-2, IFNγ, IL-4 and IL-10 is also not significantly unaffected by PPARγ treatment, although cluster analysis reveal that mice in the study segregate into groups of IFNγ-high and IFNγ-low producing populations, with most of the IFNγ-low cells being in the treated group [Augstein et al 2003].
PPAR activation also has several modulatory effects on hepatic function, in addition to peroxisomal proliferation. Treatment in mice with PPARα agonists may downregulate the activity of the 11β-hydroxysteroid dehydrogenase type 1 enzyme, that is important in the conversion of doxycortisol to cortisol [Hermanowski-Vosatka et al 2000]. Cortisol is a potent gluconeogenic hormone with a variety of anti-inflammatory actions, including a decrease in phospholipase A₂ activity (necessary for the generation of thromboxanes and prostaglandins), neutrophil migration and phagocytic activity, as well as fibroblast proliferation and proteolysosome secretion via lysosomal stabilization [Porterfield 2001], such that a decrease in cortisol has a potential pro-inflammatory effect. On the other hand, treatment with the same PPARα agonist suppresses fibrinogen gene expression and the levels of plasma fibrinogen, a potentially protective effect given the association between elevated fibrinogen levels and the incidence of cardiovascular disease [Kocks et al 2002]. Another PPAR-mediated protective effect may be seen in ethanol-induced liver injury in rats [Enomoto et al 2003]. Treatment with PPARγ agonists greatly diminishes the pathological parameters of hepatic steatosis and necrosis [Enomoto et al 2003]. Kupfer cells from the treated group also produce significantly lower concentrations of TNFα compared to wild type; in fact, the protective effect of PPARγ agonists is ascribed to the abrogation of Kupfer cell sensitization to LPS [Enomoto et al 2003].

Based on the reported effects of PPAR ligand-activation both in vitro and in vivo on vascular endothelial and smooth muscle cells, as well as on monocytes/macrophages and T cells, there is reason to hypothesize beneficial effects of PPAR ligand-activation on parenchymal rejection and transplantation-associated arteriosclerosis. Given the current broad use of several PPAR ligands in the clinical setting, a demonstration of benefit in transplantation could translate rapidly to clinical application. Therefore, investigation of the role of PPAR in rejection at both in vitro and in vivo levels is highly warranted and represents the overall goal of this thesis.

2.3 Boron neutron capture

Although it has been shown that persistent graft PR is not necessary for the development of TxAA [Izutani et al 1995, Nagano et al 1998], it is not known at what time point a graft becomes committed to a particular outcome. Currently, there are no readily available tools to determine the minimum amount of time required by alloreactive T cells (a measure of PR) to initiate the irreversible sequence of events leading to TxAA.

Early technologies have been hampered by several factors. The relationship between PR and TxAA was first investigated by using immunosuppressive agent [Hulet et al 1996, Nakagawa et al 1995]. These agents, however, have many confounding factors and do not allow the inhibition of specific immune cell subsets. The use of antibodies to specific T cell subsets [Nagano et al 1998] improved upon some of the drawbacks encountered with immunosuppressive agents. However, T cell depletion occurs mainly in the periphery such that there is no verification whether the T cells that have already infiltrated into the graft have also been removed.
A newer technique to investigate the role of T cells in rejection improved upon some of the drawbacks listed above. This technique involves the adoptive transfer of allospecific T cells into an allograft recipient that lacks the recombinase gene (RAG -/-) and thus cannot make mature T or B cells [Bingaman et al. 2000]. This method allows the transfer of selected T cell populations that lack specific mediators, thereby permitting the assessment of individual effector molecules in TxAA development. In fact, this adoptive transfer method has been recently and successfully employed to compare the effects of T cells from WT or GKO mice [Urakawa et al. 2004]. However, the method does not allow the removal of T cells after transfer to examine temporal issues related to subsequent effector function.

The ability to selectively and rapidly inactivate adoptively transferred cells at specific times after transfer could yield useful temporal information about requirements of antigenic stimulation and the relationship between PR and TxAA. After inactivation of the adoptively transferred cells, the graft could be allowed to function for varying times in order to assess various stages of lesion formation. Contributions to the general understanding of T cell dynamics in the development of TxAA can further the identification of prognostic factors and/or therapeutic targets for transplant patients. Moreover, the knowledge is of particular value in the accurate assessment of the efficacy of pharmacologic agents in a murine model of cardiac transplantation. This is particularly germane for agents where administration may have to be delayed for technical reasons or in situations where the timing of the desired pharmacologic effect of the agent may permit transient episodes of PR.

Manipulation of the boron neutron capture reaction may allow this type of detailed temporal information to be obtained. The following section describes the general characteristics of the boron neutron capture reaction. The current applications of this reaction are discussed; given that they are primarily clinical, emphasis is placed on the description and findings from clinical trials. Finally, boron neutron capture reaction is proposed as a novel immunologic research tool. Although it is proposed in the context of elucidating mechanisms of rejection of heart transplantation, it could have a number of applications in many areas of immunology research.

2.3.1 General characteristics

The boron neutron capture reaction is based on the very high probability of $^{10}$B (non-radioactive isotope of natural boron) capturing a thermal, or slow, neutron. The thermal neutron capture cross section of $^{10}$B is 3840 barns ($1 \text{ barn}=10^{-24} \text{ cm}^2$) and decreases with increasing neutron energy. When a $^{10}$B nucleus captures a thermal neutron, a compound $^{11}$B nucleus is formed. After $10^{-12}$ second, the compound nucleus disintegrates into an alpha ($\alpha$) and $^7\text{Li}$ particle, which goes into an excited state 94% of the time, and into the ground state 6% of the time. The $^7\text{Li}^*$ nucleus promptly deexcites by emitting a 0.48 MeV photon. Figure 2.7 schematically illustrates the possible events following capture of a thermal neutron by $^{10}$B. The average energy release in the reaction, weighted by the event probabilities, is 2.34 MeV, with 0.85 MeV carried by the $^7\text{Li}$ particle and 1.49 MeV carried by the $\alpha$ particle.
Figure 2.7: The boron neutron capture reaction: After a $^{10}$B nuclei captures a thermal neutron, compound $^{11}$B* nuclei are formed, which disintegrate into alpha ($\alpha$) and $^{7}$Li particles; the average energy released is 2.34 MeV (with 0.85 MeV carried by $^{7}$Li and 1.49 MeV carried by the $\alpha$).

Both the $^{7}$Li and $\alpha$ are heavy charged particles giving rise to closely spaced ionizations with a high linear energy transfer of about 200 keV/μm. The ranges of the $^{7}$Li and $\alpha$ particles in tissue are, on average, 5 and 9 μm, respectively. Assuming the diameter of a cell to be 10 μm and the intracellular $^{10}$B to capture a thermal neutron, the energy that is released is deposited within that cell (the 0.48 MeV photon emitted by the $^{7}$Li* nucleus does not contribute to the local energy deposition because it has a low linear energy transfer). The ionization density of both the particles has the potential to cause complex damage to DNA in association with adjacent intracellular structures, damaging the cells with little or no possibility of subsequent repair [Goodhead 1989].

Herein lies the basis of the use of the boron neutron capture reaction: by delivering $^{10}$B to selected cells, their death can be induced by irradiation with thermal neutrons. It must be emphasized that it is the combination of low-energy neutrons with $^{10}$B nuclei that results in the specific destruction of boron-containing cells. The $^{10}$B nucleus must be activated (via irradiation with low-energy neutrons) to cause the magnitude of energy deposition necessary for cell death. Alone, it has no significant deleterious effect. Natural boron, made of 20% $^{10}$B and 80% $^{11}$B, is ubiquitous in rocks, soil and water [Woods 1994] and is also present in foods such as vegetable [Nielsen 1986]. Moreover, supplementation with boron alone may improve osteoarthritic conditions [Travers et al 1990].

2.3.2 Current applications

The main application of the boron neutron capture reaction is as a therapy (boron neutron capture therapy, hence the acronym BNCT) for brain tumors. Clinical trials examining efficacy are in progress in the US [Busse et al 2002], Japan [Nakagawa et al 2002], Sweden [Capala et al 2002], Finland [Joenasu et al 2002], Czechoslovakia [Burian et al 2002], and the Netherlands [Witting et al 2002]. In clinical trials, patients are given a boron compound either orally or intra-vascularly and after some time, to allow accumulation of the boron in the tumor, irradiation regimens are begun. A schematic of this procedure is shown in figure 2.8. Generally, patients are adults, although children may be included [Uyama et al 2002], and neutron irradiation takes place after surgery, although intraoperative approaches are also being tested [Matsumura et al 2002].
2) neutrons

1) boron

Figure 2.8: Boron neutron capture therapy for brain tumors: In this type of radiation treatment, patients are first given a boron compound either orally or intra-vascularly, then the tumor is irradiated with neutrons, inducing the boron neutron capture reaction in boron-loaded cells. Traditionally, the tumor has three times the boron concentration of surrounding tissue, such that upon irradiation, the tumor will receive three times the dose given to surrounding tissues.

Thus far, results appear to be encouraging. Follow-up data on 17 patients with either glioblastoma or intracranial melanoma show that 76% experienced a sizable reduction in tumor enhancing volumes; division of the patients into two groups based on tumor volumes show that for volumes less than 30 cc 10/11 patients responded, while for volumes greater than 30 cc 3/6 patients responded [Busse et al 2002]. In a cohort of patients, including 44 patients with glioblastoma, 17 with anaplastic astrocytoma, and 2 with low-grade astrocytomas, five-year survival significantly improved in most groups, as defined by recursive partitioning analysis, compared with standard treatment as per the Japanese brain tumor registry [Ono et al 2002]. With respect to safety, no dose limiting toxicity has been observed in several Phase I trials of glioblastoma, including one group of 30 patients [Wittig et al 2002], another of 9 patients [Burian et al 2002] and another of 18 patients [Joesuhi et al 2002]. A Phase II trial with 27 glioblastoma patients (who had no incidence of acute BNCT-related toxicities) is underway [Capala et al 2002].

To date, there has been only one non-brain tumor clinical experience reported and this involves the treatment of patient with liver metastases secondary to colon carcinoma [Pinelli et al 2002]. Motivations for approaching this clinical problem are multi-fold and include the ineffectiveness of standard chemotherapy in significantly prolonging survival, the unavailability of external radiotherapy due to high risk of actinides hepatitis, and the high incidence of relapse even after local aggressive therapy due to the difficulty in nodule detection and localization [Pinelli et al 2002]. Following extensive neutron beam
modifications and pre-clinical investigations, clinical experience began with a 48 year old with diffuse liver metastases from a sigmoid adenocarcinoma; the boron-compound was slowly injected into the patient’s colic vein and after perfusion, the liver was removed, irradiated, and reconnected to the vascular and biliary stumps [Pinelli et al 2002]. The patient recovered from postoperative complications (including thrombotic occlusion of the left femoral vein, hepatic and renal insufficiency, and left foot drop) and at 7 months, is in very good general condition, with normal liver function tests and an absence of metastatic nodules in the liver [Pinelli et al 2002].

2.3.3 A potential new application

The potential design of an experiment would be as follows: two days after the heterotopic cardiac transplantation of hearts into Rag -/- mice, T cells would be purified from the spleens of WT mice and incubated in boronated medium (parameters to be determined from in vitro work). The boron-loaded T cells would then be injected into the tail veins of transplant recipients. After a set number of days (e.g. 2 to 4), irradiation would take place, inducing the boron neutron capture reaction in boron-loaded T cells. At 1, 2 and 8 weeks post transfer, the mice would be sacrificed and the grafts analyzed via biological techniques. Specific T cell effects can thus be restricted to defined windows of exposure. Development of TxAA requires 8 to 12 weeks, a short time frame that minimizes the potential of observing effects resulting from neutron irradiation alone.

Based on the binary nature of the boron neutron capture reaction, the research approach, as shown in figure 2.9, is two-pronged. On one hand, is the required boron concentration in the target tissue and on the other hand, the neutron flux parameters (based upon the concentration of boron in the target). High boron concentrations allow lower neutron intensities (lower flux or less time), while high neutron intensities allows for a lower boron concentration. An optimal balance must be reached between the two parameters in order to design a practical system. In this thesis, emphasis is placed on determination of the boron concentration achievable in the desired cell type, namely T cells.

**Figure 2.9:** Approach to boron neutron capture-based research: Parameters must be optimized so as to have a sufficient boron concentration in target tissue to induce cell death and appropriate neutron irradiation parameters so as not to harm surrounding tissue.
Results from BNCT research in tumors indicates that concentrations of 30 to 50 ppm may be adequate for induction of the boron neutron capture reaction in a therapeutic setting. In general, it is desirable to have as large a number of \(^{10}\text{B}\) nuclei as possible, since it would lower the number of thermal neutrons required and thereby minimize the energy deposited by the interaction of thermal neutrons with nuclei present in surrounding tissue, namely from the capture of thermal neutrons by hydrogen and nitrogen, written as \(^{1}\text{H}(n,\gamma)^{2}\text{H}\) and \(^{14}\text{N}(n,p)^{14}\text{C}\), respectively. While the former reaction does not contribute to the local deposition of energy, the latter may deposit up to 0.626 MeV within 1 mm as a result of the recoil proton and carbon nucleus.

With respect to neutron beam parameters, unlike traditional BNCT, where tumors are often several centimeters deep to the surface of the head and therefore require epithermal beams, the target in this application sits directly under the skin of the abdomen (i.e., just a few millimeters beneath the surface). Based on the shallow depth of the target area, a thermal neutron is likely required. The target dose is estimated to be 3000 rads based on the dose delivered to inactivate T cells in vitro for MLRs. Finally, the duration of the neutron irradiation should be such that a goal of about 90% cell kill should be reached, that is comparable to T cell depletion experiments, where the reported depletion ranges from 87% to 98% depending on the timing of the measurement with respect to the administration of the depleting antibodies [Nagano et al 1997].

It should be noted that the nuclear-related tools required to fully develop the above technique (i.e. a method for quantifying the boron concentration of cells both in vitro and in vivo, Monte Carlo simulation and a thermal beam) are in large part available. A method for quantifying boron concentration of cells in vitro has been developed [Binello 1999] and is based on prompt gamma neutron activation analysis (PGNAA). A fully equipped state-of-the-art PGNAA facility [Riley and Harling 1998] is also available for use at the MIT Reactor. A recent Nuclear Engineering PhD thesis [Kiger 2000] has optimized a technique of high-resolution quantitative autoradiography that allows measurement of the \(^{10}\text{B}\) microscopic distribution in thin tissue sections (such as those from the transplanted hearts) and simultaneous visualization of tissue histology. Monte Carlo simulation is a readily available calculational tool. A thermal beam is currently under construction at the MIT reactor.
Chapter 3. *In vitro* evaluation of PPAR agonists

3.1 Introduction

As discussed in chapter 2, studies in the literature have documented a number of effects of PPAR agonists on inflammatory reactions, mediated by cell types of both the immune and vascular systems. Important among the many markers of inflammation are the production of cytokines and chemokines, as well as the expression of their cognate receptors (particularly relevant for chemokines given the multiplicity of ligand-receptor interactions). Evaluation of the literature demonstrates that following treatment of immune cells with PPAR agonists *in vitro*, there is an overall reduction in the production of pro-inflammatory cytokines and variable effects on chemokines and their receptors [Cunard et al 2002a, Marx et al 2002, Wang et al 2003] and these findings suggest potential therapeutic benefit of PPAR agonists in inflammatory pathologies, in addition to their already clinically documented beneficial effects on lipid and glucose homeostasis.

Inflammation plays an important role in mechanisms of heart transplant rejection. As highlighted in chapter 2, IFNγ is ascribed a particularly important role; congenital deficiency of IFNγ on recipient mice of heterotopic cardiac transplants abrogates TxAA, while exacerbating PR [Nagano et al 1998]. Antibody targeting of selected chemokines and/or congenital deficiency has been found to decrease the severity of PR [Hancock et al 2001, Mura et al 2001] and attenuate the development of TxAA [Yun et al 2002]. Furthermore, congenital deficiency of selected chemokine receptors also ameliorates TxAA [Gao et al 2001]. The responsiveness of PR to standard immunosuppressive therapies and concomitant lack of available treatments for TxAA, motivated a search for readily available agents with modulatory effects on IFNγ (as well as on other cytokines, chemokines and their receptors), that could be extended to the transplant setting.

On the basis of their immunomodulatory properties, PPAR agonists may have beneficial effects in transplant rejection. A mainstay of the *in vitro* evaluation of potential therapeutic agents for transplantation is the primary mixed leukocyte reaction (MLR). In that model, lymphocyte stimulation is fundamentally based on the phenomenon of allorecognition, a process central to transplant rejection. The overall goal of the research presented in this chapter is the characterization of the immunomodulatory effects of several PPAR agonists (shown in figure 3.1) on the MLR, and, for comparison, on splenocyte stimulation independent of allorecognition, namely Concanavalin-A mitogenesis. This encompasses dose response assays on proliferation and cytokine production, as well as investigation into potential cell viability issues, and detailed analysis of mRNA expression of a variety of cytokines, chemokines and chemokine receptors.
PPARα agonists

Fenofibrate

Wy14643

PPARγ agonists

BRL49653

15-deoxy Δ12,14, Prostaglandin J₂

Figure 3.1: Schematics of the structures of the four PPAR agonists evaluated: Two agonists are directed towards PPARα, namely fenofibrate (FENO) and Wy14643 (WY), while the other two agonists are directed toward PPARγ, namely BRL49653 (BRL) and 15-deoxy Δ12,14 Prostaglandin J₂ (PGJ₂).
3.2 Methods

Mice

Two inbred strains of mice, C57/BL6 and Balb/c (Jackson Laboratories, Bar Harbor), approximately six to eight weeks old, were housed in the animal facility of Harvard Medical School on acidified water and chow ad libitum. Sentinel mice were co-housed in each room and were surveyed to ensure that that the facility was free of viral pathogens. Experiments were done according to protocols approved by the animal care committee.

Splenocyte isolation

Spleens were extracted and ground, using the barrel of a 5 ml syringe, against fine silver mesh screen in a sterile culture dish. RPMI medium (Invitrogen, Carlsbad) supplemented with 10% fetal bovine serum, non-essential amino acids, L-glutamine, HEPES buffer, sodium pyruvate, penicillin, streptomycin and mercaptoethanol (denoted as C-10) was added to the dish and the resulting solution was transferred to a 15 ml conical tube. The tubes were centrifuged for 5 minutes at 800 g. Supernatants were aspirated and cells were resuspended in 5 ml of tris ammonium chloride buffer (1:10 dilution of tris buffer and 0.83% NaCl) for 5 minutes at 37°C to lyse the red blood cells. C-10 medium was added to a volume of 15 ml and the tubes were centrifuged for 10 minutes at 800 g. Supernatants were aspirated, cells resuspended in 10 ml of C-10, and counted using a hemocytometer; viability was also assessed by trypan blue exclusion and uniformly exceeded 98%. Cells were resuspended at a concentration of 10^7 cells per ml and kept on ice until plating.

PPAR agonists

For PPARα agonists, the concentrations ranged from 0 to 100 μm for fenofibrate (Sigma, St Louis), and from 0 to 500 μm WY14643 (Biomol, Plymouth Meeting). For PPARγ agonists, the concentrations ranged from 0 to 50 μm for BRL49653 (Cayman Chemicals, Ann Arbor) and from 0 to 10 μm for 15-deoxyΔ12,14-Prostaglandin J3 (Cayman Chemicals, Ann Arbor). In order to achieve the final concentrations and ensure simultaneous exposure of cells to the agonists, solutions with prefinal concentrations of double the desired ones were prepared and serially diluted. This allowed the cells to be resuspended in C-10 with no drugs.

Proliferation assays

C57/BL6 splenocytes were stimulated both in the setting of a mixed leukocyte reaction (MLR; via co-culture with radiation-inactivated allomismatched splenocytes from Balb/c mice), and following the addition of Concanavalin-A (ConA). For both conditions, 100 μl of C-10 with PPAR agonists were first added in quadruplicate to 96-well plates, in the concentrations as described above. In the MLR, Balb/c splenocytes were inactivated by 3000 rads of gamma radiation and are referred to as “stimulators”. To each of the wells already plated with PPAR agonist-containing drug, 50 μl of C57/BL6 splenocytes
("responders") were added, for a total volume of 200 µl per well. Responder and
stimulator control sets were plated in quadruplicate by adding 50 µl of the respective cell
type and 50 µl of C-10 to the wells with the PPAR agonist-containing drug. For ConA
stimulation, the culture preparation was similar to the above except that instead of adding
50 µl of stimulator cells, 50 µl of ConA solution at 4 µg per ml was added, for a final
ConA concentration of 1 µg per ml. Furthermore, while responder alone wells were
plated in quadruplicate, as in the MLR, no stimulator alone wells were plated in the
ConA assays. To measure proliferation, 25 µl of ³H-thymidine (NEN, Boston) was added
to the plates every day up to 5 days; after six hours, the plates were placed in the freezer
to stop the reaction. Plates were subsequently thawed and ³H-thymidine incorporation
assessed by a standard beta scintillation counter.

Enzyme-linked immunosorbert assays

The concentration of IFNγ in the supernatants of the proliferation assays was determined
using enzyme-linked immunoassays (ELISAs) as per the manufacturer’s protocol
(Pharmingen, San Diego). Unless otherwise noted, all antibodies were from Pharmingen
and all incubations done at room temperature. Specifically, plates were coated by adding
50 µl per well of purified anti-IFNγ at 2 µg/ml diluted in carbonate buffer (0.1 M
NaHCO₃ and 0.1M NaCO₃, pH 9.0) and incubated overnight at 4°C. Plates were then
washed twice with PBS, that was supplemented with 1% Triton-X (Sigma, St. Louis).
Plates were blocked by the addition of 100 µl per well of borate buffered saline (0.17M
borate and 0.12 M NaCl in distilled water, pH 8) containing with 2% bovine serum
albumin. After two hours of incubation, plates were again washed two times and samples
were added, in addition to a standard curve made from murine recombinant IFNγ diluted
in C-10. Following another overnight incubation, plates were washed four times and 50
µl of biotinylated anti-IFNγ at a concentration of 1 µg/ml was added to each well. Plates
were incubated for 45 minutes and washed six times, followed by 50 µl of Avidin-HRP
(at 1:1000) 30 minutes. Plates were washed six times and developed by first adding 100
µl per well of citrate buffer (0.1M citrate) and then adding 50 µl of a solution where 2.17
mg of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (Sigma, St. Louis) and 5 ml of
30% H₂O₂ (Sigma, St. Louis) are dissolved per 5 ml of citrate buffer. The concentration
of IFNγ was indicated by development of a green color in the wells. Optical density was
measured using a plate reader and was converted to pg/ml using a calibration curve. Data
analysis of optical density was done using the “Softmax” software (Molecular Devices
Corporation).

Propidium iodide staining and flow cytometry

Spleens were isolated as stated above, plated in 12-well culture plates and stimulated with
ConA at a final concentration of 1 µg/ml with and without PPAR agonists at selected
concentrations. The same volume ratios were maintained as in proliferation assays. After
12 hours, cells were scraped from the bottom of the culture wells and aliquoted into
plastic tubes typically used for flow cytometric assays. Tubes were centrifuged for 10
minutes at 800 g in the Beckton Table Top centrifuge and supernatant was decanted. One
of the tubes containing the splenocytes cultured in medium alone was kept on ice (serving as the "unstained" control). To the other tubes (including one with untreated cells and the others treated with a PPAR agonist), 500 μl of a 1 mg/ml propidium iodide/PBS solution (Sigma, St. Louis) was added. After incubation in the propidium iodide solution for 5 minutes at room temperature, cells were immediately analyzed by flow cytometry.

RNA isolation

Splenocytes were isolated using the above technique and cultured in bulk for varying amounts of time with selected PPAR agonist concentrations. Cells were then scraped and transferred to 15 ml conical tubes, followed by centrifugation. Supernatants were aspirated and the cell pellets were homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad), per manufacturer's instructions. To ensure more complete homogenization, cells were repeatedly passed through a 1 ml syringe with a 20- or 21-gauge needle. The homogenized solution was transferred to a 1.5 ml microcentrifuge tube and 0.2 ml of chloroform per ml of TRIzol was added. Tubes were shaken vigorously for 15 minutes by hand to create an emulsification. After a five-minute incubation at room temperature, tubes were centrifuged for 20 minutes at 14,000 rpm in a microcentrifuge. The upper aqueous phase was transferred to another microcentrifuge tube and 0.5 ml of isopropanol per ml of TRIzol was added. The incubation was carried out for 15 minutes at room temperature. Samples were centrifuged for 10 minutes at 4°C and the supernatant was removed before the addition of 1 ml of 75% ethanol per ml of TRIzol. Samples were quickly vortexed and centrifuged for 5 minutes at 8,000 rpm. The supernatant was discarded and samples air dried for 5 to 10 minutes. The RNA pellet was dissolved in 50 to 100 μl of RNase-free DNase-free water and incubated for 10 minutes in a dry water bath at 55 to 60°C, before being placed on ice. RNA content was quantified by measuring absorbance at 260 nm. Aliquots with the desired RNA amounts were then prepared for storage at −70°C until analysis using the RNase protection assay.

RNase protection assays

The mRNA expression of stimulated splenocytes from bulk cultures was determined using the RNase protection assay (RPA), per the manufacturer's protocol (Pharmingen, San Diego). Specifically, the following murine multi-probe templates were used: mcK-1 (containing templates for IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, INFγ, L32, GAPDH), mcK-2b (containing probes for IL-12p35, IL-12-p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, INFγ, MIF, L32, GAPDH), mcK-5c (containing probes for lymphotactin, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, Eotaxin, L32, GAPDH) and mcR-5 (with probes for CCR1, CCR1b, CCR3, CCR4, CCR5, CCR2, L32, GAPDH). In brief, probes were synthesized, using α32P-UTP (NEN, Boston) in addition to RNasin, GACU pool, DTT and T7 RNA polymerase, incubating for 1 hour at 37°C. The reaction was terminated by the addition of DNase and subsequent incubation for 30 minutes at 37°C. A mixture of EDTA, tris-saturated chloroform-isoamyl alcohol-phenol and yeast tRNA was added to the probe and vortexed into an emulsion. After a brief centrifugation, the upper aqueous phase was transferred to a fresh microcentrifuge tube and the RNA was precipitated by the addition of ammonium acetate and 100% cold ethanol.
ethanol, and incubation at \(-70^\circ\text{C}\) for a minimum of 30 minutes. Following centrifugation for 15 minutes and a wash of the pellet with cold 90% ethanol, the probe was dried, dissolved in 50 \(\mu\)l of hybridization buffer and stored at \(-20^\circ\text{C}\) until use. Probe yield was quantitated using duplicate 1 ml samples in a scintillation counter, with lower acceptable activity being approximately \(3 \times 10^5\) cpm per ml. Given an acceptable probe yield, samples were dried and (following the addition of 8 \(\mu\)l of hybridization buffer and 2 \(\mu\)l of probe diluted at the optimal strength, per manufacturer specifications) hybridized for 12 to 16 hours in a dry heat bath ranging in temperature from 55 to 60\(^\circ\text{C}\). Samples were then digested using a mix of RNAs A and T1, and subsequently treated with proteinase K. A solution containing tris-saturated chloroform-isoamyl alcohol-phenol was added to each sample. After a quick vortex and centrifugation, the upper phase, containing the protected RNA fragments, was transferred to fresh tubes for precipitation with 100% cold ethanol in the presence of ammonium acetate. Samples were incubated for over 30 minutes at \(-70^\circ\text{C}\) and, following a 15-minute centrifugation, washed with cold 90% ethanol. After drying, samples were resuspended in loading buffer, denatured via incubation in a 90\(^\circ\text{C}\) dry water bath and loaded onto a 5% acrylamide-urea sequencing gel made using the Sequagel Sequencing Kit (National Diagnostics, Atlanta). \(^3\)P-labelled probes were also loaded, serving as band identification markers. Electrophoresis was performed at a constant power of 50W. The gel was dried under vacuum and exposed on a phosphor screen. Quantification of band intensities was performed using “ImaqeQuant” (Molecular Dynamics). The volume integrations of each band were normalized to the bands for GAPDH in the corresponding lane. Samples were run in triplicate and results are presented as the average and standard deviation of each group.

Data analysis

Three experiments were performed for each of the four agonists in both ConA and MLR cultures. Unless otherwise noted, results are expressed as the mean and standard deviation of the mean of a representative experiment plated in triplicate to quadruplicate. Statistical analysis was performed using Student’s t test, where \(p<0.05\) was considered statistically significant.

3.3 Results

This section first presents results of increasing PPAR agonist concentrations on proliferation and IFN\(\gamma\) production in splenocyte cultures following stimulation with ConA and via MLR. Cell viability was addressed with propidium iodide staining and flow cytometry. Finally, the effects of PPAR agonists on the mRNA expression of a variety of cytokines and chemokines, as well as chemokine receptors, are also presented.

Figure 3.2 shows lymphocyte proliferation and IFN\(\gamma\) production following stimulation with ConA and in the setting of an MLR. With ConA stimulation, proliferation peaks around day 2 and is higher in magnitude compared to the MLR, where proliferation peaks on day 4. IFN\(\gamma\) concentrations are higher and seen at day 2 in the ConA cultures, while in the MLR it is lower and not seen until day 3. Controls of non-stimulated responder cells and stimulator alone cells (in the MLR) were also performed (not shown for clarity).
a) ConA stimulation

![Graph showing CPM and IFNγ concentration over days 1 to 4 with peak proliferation and IFNγ levels at day 2 and 3 respectively.]

b) MLR stimulation

![Graph showing CPM and IFNγ concentration over days 2 to 5 with peak proliferation and IFNγ levels at day 4 and 5 respectively.]

**Figure 3.2:** Proliferation and IFNγ concentration as a function of time: a) ConA stimulation, proliferation peaks at day 2 and IFNγ levels become significantly elevated by day 2; b) MLR, proliferation peaks on day 4 and IFNγ levels become significant on day 3. Proliferation peaks earlier and is of higher magnitude in ConA cultures compared to MLRs. Furthermore, IFNγ production begins earlier and is also of higher magnitude in the ConA cultures compared to MLRs. Results are expressed as the mean and standard deviation of three experiments plated in triplicate to quadruplicate.

**Dose Response**

The effect of varying PPAR agonist dose on both proliferation and IFNγ production over 5 days was tested following stimulation with ConA, and in the setting of the MLR. Figures 3.3, 3.4, 3.5 and 3.6 show the dose response results for fenofibrate (FENO), Wy14643 (WY), BRL49653 (BRL), and 15-deoxy Δ12,14 PGJ2 (PGJ2), respectively. Results are expressed as the mean and standard deviation of one representative experiment from a set of three, plated in triplicate to quadruplicate. The thickened plot markers indicate the day of peak proliferation and the first day of elevated IFNγ production.
a) ConA stimulation

![Graph showing dose-dependent effects of FENO on proliferation and IFNγ production for ConA stimulation.](image)

**Figure 3.3:** Dose-dependent effects of FENO on proliferation and IFNγ production: a) ConA stimulation, proliferation assays show that FENO concentrations up to 20 μM have no significant effects on cell proliferation, while concentrations of 100 μM and above significantly decrease it; IFNγ production is significantly decreased at concentrations beginning with 20 μM; b) MLR, FENO concentration of 20 μM significantly decreases IFNγ without effects on proliferation, while higher concentrations diminish both proliferation and IFNγ production. These results indicate 20 μM as the “therapeutic” FENO concentration. Results are expressed as the mean and standard deviation of one representative experiment from a set of three, plated in triplicate to quadruplicate. The thickened plot markers (■) indicate the day of peak proliferation and the first day of elevated IFNγ production.
Figure 3.4: Dose-dependent effects of WY on proliferation and IFNγ production: a) ConA stimulation, proliferation assays show that WY concentrations up to 100 μM tend to increase cell proliferation, while concentrations of 250 μM and above significantly decrease it; IFNγ production is significantly decreased at concentrations beginning with 100 μM; b) MLR, WY concentration of 100 μM significantly decreases IFNγ without effects on proliferation, while higher concentrations diminish both proliferation and IFNγ production. These results indicate 100 μM as the “therapeutic” WY concentration. Results are expressed as the mean and standard deviation of one representative experiment from a set of three, plated in triplicate to quadruplicate. The thickened plot markers (■) indicate the day of peak proliferation and the first day of elevated IFNγ production.
a) ConA stimulation

Figure 3.5: Dose-dependent effects of BRL on proliferation and IFNγ production: a) ConA stimulation, proliferation assays show that BRL concentrations up to 50 μM have no significant effects on cell proliferation, IFNγ production is significantly decreased at concentrations beginning with 10 μM; b) MLR, BRL concentration of 10 μM and above significantly decrease IFNγ without significant effects on proliferation. These results indicate 10 μM as the “therapeutic” BRL concentration. The concentration range tested did not include any toxic doses, based on results from proliferative assays. Results are expressed as the mean and standard deviation of one representative experiment from a set of three, plated in triplicate to quadruplicate. The thickened plot markers (■) indicate the day of peak proliferation and the first day of elevated IFNγ production.
a) ConA stimulation

b) MLR stimulation

Figure 3.6: Dose-dependent effects of PGJ2 on proliferation and IFNγ production: a) ConA simulation, proliferation assays show that while PGJ2 concentrations up to 3 μM have no significant effects on cell proliferation, while 10 μM results in a marked decrease; IFNγ production is significantly decreased at 3 μM and above; b) MLR, similarly, PGJ2 concentrations of 3 μM significantly decrease IFNγ without significant effects on proliferation, while 10 μM decreases both proliferation IFNγ production. These results indicate 3 μM as the “therapeutic” PGJ2 concentration. Results are expressed as the mean and standard deviation of one representative experiment from a set of three, plated in triplicate to quadruplicate. The thickened plot markers (■) indicate the day of peak proliferation and the first day of elevated IFNγ production.
The PPARα agonists show different “therapeutic” and “supratherapeutic” doses. As seen in figure 3.3, at fenofibrate (FENO) concentration of 20 μM, there is a significant decrease in IFNγ production, despite comparable proliferation rates. At fenofibrate concentrations of 100 and 500 μM, no proliferation is measured and no IFNγ produced. Based on these results, 20 μM is a “therapeutic” concentration, while concentrations above 100 μM is toxic. As shown in figure 3.4, the concentrations of WY14643 (WY) had no significant effects on proliferation at 100 μM, despite significant decreases in IFNγ production at 100 μM. At 250 μM, peak proliferation decreased (as well as proliferation on all days of the MLR), while at 500 μM, no proliferation was observed.

Figure 3.5 shows the results obtained with PPARγ agonists. BRL49653 (BRL) had no significant effects on cell proliferation up to the highest dose tested. Significant reductions in IFNγ concentrations, however, were observed at 10 μM and above. Finally, experiments with the natural PPARγ ligand, (PGJ2), showed no inhibitory effects on proliferation up to 3 μM, at which concentration, there was a tendency to increase proliferation. However, at 3 μM a significant decrease is observed in IFNγ production. At a concentration of 10 μM, there is a lack of proliferative response, corresponding to lack of IFNγ production. These results indicate that 3 μM is a “therapeutic” concentration, while 10 μM is “supratherapeutic”.

Cell viability

To address potential cell viability issues associated with the use of PPAR agonists in culture, ConA-stimulated splenocytes were incubated in the selected “therapeutic” and “supratherapeutic” concentrations of all PPAR agonists for 2 and 12 hours and stained with propidium iodide. Flow cytometry results for the 2 hours incubation are presented in figure 3.7, while those for the 12 hours incubation are shown in figure 3.8. After two hours, there was no significant change in cell viability at any of the doses tested. After 12 hours, however, while the therapeutic doses continued to show no significant changes, while the “supratherapeutic” concentrations showed increased amounts of cell death, with FENO and PGJ2 the most dramatic of the three. Therefore, the previously identified “supratherapeutic” doses that suppressed proliferation and IFNγ production correspond to doses at which significant cell toxicity is observed.

It is stressed that the propidium iodide staining protocol used in this thesis assessed effects on cell viability rather than cell cycle. Cell cycle analysis requires cell fixation, membrane solubilization and RNA degradation, such that the levels of propidium iodide staining accurately reflect DNA measurement. Instead, the determination of cell viability requires the addition of only propidium iodide to the cells followed by flow cytometry (without cell fixation, nor membrane solubilization, nor RNA degradation), such that effects on cell cycle cannot be determined. Thus, in this live/dead version of propidium iodide staining, any positive staining implies increased membrane permeability, allowing the propidium to bind and denoting cell death.
Figure 3.7: Cell viability after two hours of incubation with four PPAR agonists: flow cytometric analysis of propidium iodide staining with a) FENO at 20 and 500 μM; b) WY 100 and 500 μM; c) BRL, 10 μM and 50 μM; d) PGJ2 at 3 and 10 μM. There are no significant effects on cell viability with any of the agonists. The filled areas represent the cells incubated with the designated agonist concentration, while the green line represents cells incubated with medium alone. An increase in the area under the M1 marker represents cells with increased membrane permeability allowing the dye to bind, and thereby denoting cell death.
Figure 3.8: Cell viability after twelve hours of incubation with four PPAR agonists: flow cytometric analysis of propidium iodide staining with a) FENO at 20 and 500 μM; b) WY 100 and 500 μM; c) BRL, 10 and 50 μM; d) PGJ2 at 3 and 10 μM. There are no significant effects on viability at the therapeutic concentrations, while there is increased cell death at the supratherapeutic levels. The filled areas represent the cells incubated with the designated agonist concentration, while the green line represents cells incubated with medium alone. An increase in the area under the M1 marker represents cells with increased membrane permeability allowing the dye to bind, and thereby denoting cell death.
mRNA expression

Having established effective *in vitro* doses of the four PPAR ligands with respect to diminished IFNγ production, the same concentrations were used to examine other potential effects of these four PPAR ligands. Included were analyses of the expression of other cytokines as a function of time in both ConA and MLR cultures. Figure 3.9 shows that in both cultures, IFNγ is the dominantly expressed. In ConA cultures, it peaks after one day of stimulation, while in the MLRs, the peak occurs after two days. The extent of expression at peak time is significantly higher in ConA cultures. By the last day of culture, the expression of IFNγ is comparable to that of other cytokines, e.g. IL-2, IL-4, IL-6 and IL-10.

There are also distinct effects on other cytokines. For example, IL-2 expression is several-fold higher in the ConA cultures compared to MLR (although, the proportion to IFNγ, is roughly similar), which may be a major contributing factor to the higher levels of proliferation observed in splenocytes stimulated with ConA. The expression of IL-12 and IL-18 up to day 2 of the ConA cultures and day 3 of the MLR cultures are approximately two-fold higher in the MLR compared ConA, suggesting that activation based on specific MHC differences potentiates the role of these two cytokines. With respect to IL-4, the ratio of its expression to that of IFNγ is dramatically increased in the MLRs.

The effect of adding the previously determined therapeutic concentrations of PPAR agonists to both cultures was determined for the day of peak IFNγ mRNA expression yields. The expression profiles of selected cytokines from the two panels tested are shown in figure 3.10. As expected, based on the extensive proliferation and cytokine production data presented in the previous figures, all PPAR agonists markedly and significantly decrease expression of IFNγ mRNA, to approximately 50% of the original value. However, there are no significant effects on IL12-p35, no decreases in IL-12p40 or IL-18. In fact, with the exception of WY, PPARα agonists statistically, yet to a small degree, increase IL-12p40 and IL-18 in both cultures.

In comparison to IFNγ, the effects on other cytokines are less marked. Thus, changes in IL-2 and IL-6 mRNA are less dramatic in comparison to the effect on IFNγ. Interestingly, PPARα agonists increased IL-2 mRNA expression in both ConA and MLRs, while PPARγ agonists had no significant effects in the ConA cultures and mixed effects on the MLR (BRL had no effect on IL-2, PGJ2 increased it). With respect to IL-6 mRNA, it was significantly decreased by PGJ2 in the ConA cultures, and significantly increased by WY in the MLRs. It must be emphasized that the initial level of IL-6 expression was quite low, less than 0.05 when normalized to GAPDH, and no agonist raised it above 0.05, rendering it a relatively insubstantial player in the culture. As a group, PPAR agonists had no significant effects on IL-4 mRNA expression under either stimulation, although they significantly increased IL-10 mRNA expression in the ConA cultures. In the MLR, however, only WY and PGJ2 increased IL-10 mRNA expression to a varying extent.
a) **ConA stimulation**

![Graph showing cytokine mRNA expression as a function of time for ConA stimulation.](image)

b) **MLR stimulation**

![Graph showing cytokine mRNA expression as a function of time for MLR stimulation.](image)

**Figure 3.9:** Cytokine mRNA expression as a function of time: a) ConA stimulation, IFNγ is the dominant cytokine, with a peak at day 1, followed by a return to baseline and a potential beginning of a second wave of cytokine production on day 3; b) MLR stimulation, IFNγ is also the dominant cytokine in this culture, with a peak occurring at day 2. As a technical note, cultures for the top and bottom of the figure were set up simultaneously, such that comparison between them shows that IFNγ at peak times is significantly higher for ConA mitogenic stimulation.
**a) ConA stimulation**

![Bar chart showing cytokine mRNA expression](image)

**b) MLR stimulation**

![Bar chart showing cytokine mRNA expression](image)

**Figure 3.10:** Effect of PPAR agonists on cytokine mRNA expression on the day of maximal IFN\(\gamma\) mRNA expression: a) ConA, day 1; b) MLR, day 2. The most marked effect of PPAR agonists as a group is decreased IFN\(\gamma\) expression. Statistical significance (p<0.05) relative to cultures without PPAR agonist treatment is denoted by an asterisk (*). Legend key is as follows: 0 for 0 \(\mu\)M (control, no PPAR agonist), F for FENO, W for WY, B for BRL, J for PGJ2.
Because reduced IFNγ could conceivably lead to augmented expression of IL-4 and IL-10, levels of IL-4 and IL-10 mRNA were examined throughout the culture time course. Figure 3.11 displays the results of this analysis. While the other PPAR agonists tend to increase IL-4, WY is the only one that consistently results in increased IL-4 mRNA expression. On day 4 of the MLR, WY continues to sustain a significant increase in the expression of IL-4 mRNA. With the exception of BRL, early effects of PPAR agonists enhance IL-10 mRNA expression. Following a transition on day 3, where agonists such as FENO begin to exhibit inhibitory effects on the expression of IL-10 mRNA, the last day of MLR culture shows that IL-10 mRNA expression is not significantly affected by WY or BRL but is significantly decreased by FENO and PGJ2. Looking at the decreasing trend exhibited by WY over the duration of the culture, it appears that at culture times past day 5, WY may result in a significant decrease in IL-10 mRNA expression.

Figure 3.11: Effect of PPAR agonists on IL-4 and IL-10 mRNA expression in the MLR over time: mRNA expression of a) IL-4 and b) IL-10 in the MLR changes in response to the addition of PPAR agonists to the cultures as a function of time. While the other cytokines tend to increase IL-4 mRNA, WY is the only one that consistently results in increased IL-4 mRNA expression. On day 4 of the MLR, WY continues to sustain a significant increase in the expression of IL-4 mRNA. Expression of IL-10 mRNA is not significantly affected by WY, but is significantly decreased by FENO and PGJ2, and not affected by BRL.
The expression of chemokine and their receptors as a function of time for both ConA and MLR cultures is shown in figures 3.12 and 3.13. Once again, as in the analysis of cytokine mRNA expression, there are several differences between the ConA and MLR profiles. In the ConA cultures, there is a steady decrease in chemokine expression, beginning at 12 hours; the chemokine with the highest expression is MIP-1β, with the next two being RANTES and MCP-1. The MLRs demonstrate a clear peak in expression at day 2 of all chemokine mRNA species except MCP-1, whose expression on days 1 and 2 is roughly the same. In the MLR, RANTES is by far the most dominant chemokine with mRNA expression levels two fold higher than the next two most abundantly expressed, MIP-1β and MIP-1α, in that order. In both cultures, the least expressed chemokine is IP-10. Chemokine receptor expression is also different between the two cultures. Overall, the level of mRNA expression is higher in the MLR cultures. In the MLRs, CCR5 appears to be the dominant receptor expressed on days 2 and 4, with CCR2 emerging as the second most abundant chemokine receptor by day 4. In the ConA cultures, CCR5 and CCR4 are the two most abundantly expressed receptors over all days, except 3, where CCR4 expression falls to levels of CCR1 and CCR2.

The effects of PPAR agonists on chemokines and chemokine receptors are shown in figures 3.14 and 3.15, respectively. In the ConA cultures, on the day of maximal mRNA expression (0.5 day), RANTES, MIP-1α, MIP-1β and IP-10 are all significantly decreased by addition of PPAR agonists, while MCP-1 remains unchanged. On day 2 of the MLR, however, mRNA expression of all the chemokines tested is not significantly affected by addition of the PPAR agonists. With respect to chemokine receptor mRNA expression, in the ConA cultures, PPARα agonists significantly increase CCR1 and CCR2, have no significant effect on CCR4, and demonstrate variable effects on CCR5. PPARγ agonists have no significant effect on CCR1 and CCR4, significantly decrease CCR5, and increase CCR2 with variable significance. In the MLR, there is great variability, particularly with respect to PPARα agonists: fenofibrate decreases CCR1, tends to increase CCR4 and CCR5 (without reaching statistical significance) and increases in CCR2; WY causes significant and marked increases in expression of all receptors tested. PPARγ agonists have no significant effects on CCR1, CCR2 or CCR4; as to CCR5, BRL has no effect, while PGJ2 significantly decreases. The relative control mRNA expression of chemokines and receptors is consistent with that in figure 3.13.

Finally, in light of the increase in RANTES mRNA on day 4 in the MLR, as well as the overall increase in chemokine receptor mRNA expression, the effect of PPAR agonists was assessed on day 4 of the MLR, as shown in figure 3.16. All of the PPAR agonists, except FENO, cause modest decreases in RANTES and dramatic increases in MIP-1α and MIP-1β (two- to four-fold) mRNA. FENO, on the other hand, results in only modest increases in RANTES and MIP-1β, and does not affect MIP-1α mRNA. The only other significant changes are the increases in IP-10 by WY and in MCP-1 by WY and PGJ2. With respect to receptors, all agonists have no effect on CCR1 and markedly decrease CCR2 and CCR5 mRNA. The effect on CCR4 is variable, ranging from a decrease (FENO) to no change (WY and BRL) to an increase (PGJ2). Relative expression of chemokines in control samples is consistent with that shown in figures 3.13 and 3.14.
a) ConA stimulation

![Graph showing chemokine mRNA expression as a function of time for ConA stimulation.]

b) MLR stimulation

![Graph showing chemokine mRNA expression as a function of time for MLR stimulation.]

**Figure 3.12:** Chemokine mRNA expression as a function of time: a) ConA, mRNA expression decreases as a function of time, with MIP-1β and RANTES as the chemokines with highest levels of mRNA expression at the peak time of 12 hours; b) MLR, chemokine mRNA expression peaks on day 2 (with the exception of MCP-1), with RANTES appearing to be the most dominant cytokine on the day of peak mRNA expression.
Figure 3.13: Chemokine receptor mRNA expression as a function of time: a) ConA stimulation, CCR5 and CCR4 are the two most abundantly expressed receptors over all days except the third and last day, where CCR4 levels falls to similar levels as CCR1 and CCR2; b) MLR stimulation, CCR5 appears to be the dominant receptor, with mRNA expression peaking on days 2 and 4; CCR2 emerging as the second most abundantly expressed chemokine receptor by the fourth and last day of culture.
a) ConA stimulation

![Graph showing chemokine mRNA expression](image)

b) MLR stimulation

![Graph showing chemokine mRNA expression](image)

**Figure 3.14:** Effect of PPAR agonists on chemokine mRNA expression: a) ConA, 12 hours, decrease in all chemokines except MCP-1 by all agonists (exception IP-10 by F); b) MLR, day 2, no significant changes by any of the agonists tested. Statistical significance (p<0.05) relative to cultures without PPAR agonist treatment is denoted by an asterisk (*). Legend key is as follows: 0 for 0 μM (control, no PPAR agonist), F for FENO, W for WY, B for BRL, J for PGJ2.
Figure 3.15: Effect of PPAR agonists on chemokine receptor mRNA expression: a) ConA, day 1, PPAR\(\alpha\) agonists increase CCR1 and CCR2, while PPAR\(\gamma\) agonists increase CCR2 and decrease CCR5; b) MLR, day 2, WY increases all chemokine receptors, while FENO increases CCR2 and PGJ2 decreases CCR5. Statistical significance (p<0.05) relative to cultures without PPAR agonist treatment is denoted by an asterisk (*). Legend key is as follows: 0 for 0 \(\mu\)M (control, no PPAR agonist), F for FENO, W for WY, B for BRL, J for PGJ2.
a) Chemokine mRNA expression

![Bar chart showing chemokine mRNA expression]

b) Chemokine receptor mRNA expression

![Bar chart showing chemokine receptor mRNA expression]

**Figure 3.16:** Late effects of PPAR agonists on chemokine and chemokine receptor mRNA expression on day 4 of the MLR: a) chemokines, marked increase in MIP-1α and MIP-1β by all agonists, except fenofibrate, which, increases RANTES, while other agonists decrease it; b) chemokine receptors, uniform decrease in CCR2 and CCR5. Statistical significance (p < 0.05) relative to cultures without PPAR agonist treatment is denoted by an asterisk (*). Legend key is as follows: 0 for 0 μM (control, no PPAR agonist), F for FENO, W for WY, B for BRL, J for PGJ2.
3.4 Discussion

For the first time the effects of four PPAR agonists, two PPARα agonists (fenofibrate and Wy14643) and two PPARγ agonists (BRL49653 and 15-deoxy Δ12,14 PGJ2), have been examined in an in vitro model of an alloresponse, namely the mixed leukocyte reaction. Experiments were also performed with ConA-stimulated splenocytes and comparison revealed critical differences, pointing to the need for specific testing in the alloresponse setting for potential translation to in vivo transplant rejection. Dose response studies identified the “therapeutic” dose, defined as the dose at which a decrease in IFNγ is achieved without a concomitant decrease in proliferation; flow cytometric analysis of propidium iodide staining confirmed cell viability at therapeutic doses. The effects of PPAR agonists at therapeutic concentrations on cytokine, chemokine and chemokine receptor expression profiles was determined, following a thorough examination of the same as a function of time. Based on effects on the in vitro alloresponse, implications for PPAR agonist therapy in transplant rejection are discussed in depth and a candidate for in vivo trials in transplant rejection is identified.

Dose response results using fenofibrate show that 20 μM significantly decreases IFNγ production without decreasing proliferation or affecting viability. This result is in line with the observations of functional effects without decreased cell viability in other assays, e.g. decreased VCAM-1 expression and monocyte adhesion [Rival et al 2002]. The decreased proliferation observed in the results of this chapter at 100 μM of fenofibrate suggests a possible detrimental effect on viability and hence a potential difference compared to the literature [Marx et al 2002, Rival et al 2002]. Two reasons could be responsible. One could be in the varying exposure times; studies in the literature treated the cells for only 2 hours [Marx et al 2002, Rival et al 2002], while in this thesis, there was continuous treatment. This hypothesis finds support in the propidium iodide results showing that even at the highest doses, PPAR treatment for only 2 hours does not diminish cell viability. Another potential explanation is that human and murine cells may have differential sensitivity to fenofibrate [Lawrence et al 2001].

Dose response results identified 100 μM of WY as sufficient to significantly decrease IFNγ, with no decrease in proliferation. These results are consistent with other studies that find no significant effects on cell proliferation or viability up to 100 μM [Yang et al 2000, Harris and Phipps 2000, Cumard et al 2002], at which concentration significant reduction in the levels of IFNγ are found in treated anti-CD3 stimulated human T cells [Marx et al 2002]. Results in this thesis suggest possible effects on proliferative capacity at 250 μM, more so in the MLR setting, compared to ConA stimulation, where the decrease in proliferation was not sustained by the third day of culture (further implying that studies done at that time point alone may not show decreases in proliferation). However, this was not investigated further, since the goal of this research was to find the lowest possible dose that would reach the desired endpoint; this is particularly important with PPAR agonists, since high doses can activate PPAR isoforms other than the ones classically ascribed to an agonist [Bishop-Bailey 2000].
With respect to BRL, dose response results show that at 10 μM, a significant decrease in IFNγ production is seen, while proliferation is not affected. This is consistent with experiments using human anti-CD3 stimulated T cells, which also showed decreased TNFα [Marx et al 2002]. The concentration of 10 μM has been widely used in the literature to demonstrate effects, including decreased vascular smooth muscle cell migration [Goetze et al 1999], and endothelial cell MHC-II expression [Kwak et al 2002]. It should be noted that the effects of BRL are affected by stimulation conditions and cell type. For example, treatment with 10 μM had no effect on IL-6 production by human smooth muscle cells [Staels et al 1998], yet significantly reduced the IL-6 produced by human monocytes [Meier et al 2002]. BRL doses above 10 μM may have PPARγ-independent effects [Welch et al 2003], supporting the choice of 10 μM as the “therapeutic” concentration.

Finally, using PGJ2, the decrease in proliferation and cell viability observed at 10 μM is in agreement with other results documenting a decrease in proliferation in murine and human T cells [Yang et al 2000, Kwak et al 2002], as well as the induction of apoptosis in murine T cells [Harris and Phipps 2001]. At lower concentrations (e.g. 3 μM), there was no effect on human endothelial cell or murine macrophage viability [Colville-Nash et al 1998, Rival et al 2002]. The decrease in proliferation observed around 3 μM by some [Harris and Phipps 2001, Clark et al 2000] may be specific to a particular assay, since in one of the same studies, stimulation with IL-2 increased the rate of T cell proliferation at these same concentrations [Clark et al 2000]. The ability of PPARγ agonists to induce apoptosis in murine DO.11-derived T cells were not recapitulated in C3H/10T1/2-derived T cells, suggesting strain-specific sensitivity [Harris and Phipps 2001]. One of the first studies reporting immunomodulatory effects in macrophages following PPARγ activation used 3 μM PGJ2 [Ricote et al 1998]. Therefore, the lack of an effect on proliferation or viability observed at 3 μM of PGJ2 is not inconsistent with the existing literature.

IFNγ is the only cytokine of the ones tested whose expression is uniformly and markedly decreased by all of the PPAR agonists used at the therapeutic concentrations described above. The decrease is to approximately 50% of initial levels. As can be seen from the graphs of cytokine expression profiles as a function of time, IFNγ is the dominant cytokine in culture. Consistent with the proliferation and protein concentration profiles shown in the dose response figures, IFNγ expression is different between ConA and MLR cultures. In the ConA cultures, it begins earlier and is higher than in the MLRs, explaining the earlier appearance of IFNγ protein, as well as the higher concentrations. It should be noted that for each panel of cytokines the ConA and MLR analyses were performed at the peak of IFNγ mRNA expression. The differences between peak values of IFNγ expression between the two panels of cytokine expression for the same culture condition underscore the sensitivity of mRNA expression to time.

Given the reported IFNγ-inducing abilities of both IL-12 and IL-18 [Sutterwala and Mossner 1999, Akira 2000, Affleck et al 2001], the finding that PPAR agonists diminish IFNγ without causing a concomitant decrease in either IL-12 or IL-18, suggests a direct effect of PPAR agonists on IFNγ. Recent evidence corroborates a direct effect of PPAR
activation on IFNγ, with one study demonstrating attenuation of IFNγ-induced STAT1 phosphorylation, nuclear localization and DNA binding [Weber et al 2003]. PPARα agonists, fenofibrate and WY, increase IL12-p40 only in the MLR and increase IL-18 in both ConA and MLR cultures. PPARγ agonists, BRL and PGJ2, have no effect on IL-12 and tend to increase IL-18. The tendency to increase IL-12 or IL-18 could possibly represent a feedback loop in the attempt to increase IFNγ. An increase in IL-12 could imply an increase in T cell proliferation and survival [Yoo et al 2002], while an increase in IL-18 has been associated with rejection when seen in association with elevated IFNγ [Affleck et al 2001]. However, the increase in both IL-12 and IL-18 mRNA expression is small, suggesting that PPAR activation of these pathways is not likely to play a large role in vivo.

Since inhibition of IFNγ expression could augment that of IL-4 and IL-10, the expression levels of these two cytokines were specifically examined. PPAR agonists do not significantly affect IL-4 expression on the days of peak IFNγ expression in either ConA or MLR culture but exert variable effects in the MLR over time, such that by day 4 of the MLR, WY markedly increases IL-4 expression. This is consistent with results from the literature that find increased concentrations of IL-4 on day 4 following WY pretreatment of murine ConA-stimulated T cells [Cunard et al 2002]. Given that IL-4 stimulates the generation of PPARγ ligands [Huang et al 1999], there is potential for this to occur with PPARα ligands as well. However, despite the identification of IL-4 as an inhibitor of the macrophage-activating effects of IFNγ [Medzhitov and Janeway 2000], a review of the data with respect to potential benefits on allografts has shown equivocal results, with some reports documenting increased rejection severity with increased concentrations of IL-4, and a number of others reporting a positive correlation between IL-4 and graft survival [Piccotti et al 1997]. This could imply that the increase in IL-4 by WY could make it a less than ideal candidate compared to the other agonists for testing in vivo.

With respect to IL-10, a number of in vivo studies show a stimulatory effect on IL-10 secretion upon treatment with PPARα and PPARγ agonists [Maruyama et al 2002, Zingarelli et al 2003, Hammad et al 2004]. This is consistent with the small, yet statistically significant increase in IL-10 expression seen with all agonists in the ConA culture on the day of peak IFNγ expression. In the MLR there is also a tendency to increase IL-10 by PPARα agonists (WY>FENO) with mixed results seen when using PPARγ agonists; PGJ2 showed increased IL-10, while BRL showed no significant effect. However, by day 4 of the MLR, fenofibrate and PGJ2 both decrease IL-10 expression. The decrease in IL-10 by fenofibrate and PGJ2, particularly at the late stage, may be of importance in light of reports that increased levels of IL-10 exacerbate allograft disease in vivo [Furukawa et al 1999], and suggest potential benefit of these two agonists in vivo. In any event, it is apparent that generally PPAR inhibition of IFNγ expression does not induce net IL-4 or IL-10 differentiation in lymphocyte stimulated cultures.

Under the specific culture conditions in this chapter, PPARα agonists statistically increase IL-2 in both cultures, while PPARγ agonists have no statistically significant effects in the ConA cultures, and variable effects on the MLR, with PGJ2 causing an
increase IL-2 and BRL a decrease. These effects are internally consistent with the tendency to observe increased proliferation in the dose response curves at the “therapeutic” concentrations. However, they sharply contrast with studies reporting a decrease in IL-2 production upon PPAR activation by WY in human T cells [Marx et al 2002] and murine splenocytes [Cunard et al 2002]. This could potentially represent another instance of differential effects or sensitivities between human and rodent cells, as has been previously discussed. The literature provides some corroboration in that while 100 μM of WY was sufficient to inhibit IL-2 in human cells, murine experiments showed no decrease until 250 μM [Cunard et al 2002]; in fact, average IL-2 concentration in cells treated with 100 μM of WY was higher than controls, although statistical significance was not reached. Experiments using splenocytes congenitally absent in PPARα may aid in the clarification of its role in IL-2, and are presented in Chapter 5. With respect to the PPARγ agonists, the results in this thesis using BRL trend in the same general direction as earlier literature reports [Yang et al 2000, Marx et al 2002]. The increase in IL-2 in the PGJ2-treated MLR cultures does contrast with results obtained in other stimulation assays with either human or murine cells [Clark et al 2000, Yang et al 2000, Marx et al 2002]. In the context of the paradigm of cytokine grouping [Goy et al 2003], it is possible that a feedback loop to counter the decrease in IFNγ may yield augmented IL-2. In any event, the result supports the conclusion that alloresponses are unique stimulatory settings not entirely analogous to ConA mitogenic stimulation [Ebert and McColl 2002].

Regarding IL-6, the results in this thesis are roughly consistent with reports in the literature. Overall, there were no dramatic changes in expression, with the only statistically significant changes associated with WY in the MLR and PGJ2 in the ConA cultures. The lack of effect of FENO is consistent with a study using endothelial cells where a decrease in IL-6 was not observed until 50 μM of drug [Xu et al 2001]. The increase in the MLR IL-6 expression by WY could represent an increasingly sensitive response to decreased IFNγ levels, similar to the significantly increased IL-6 seen in acutely rejecting cardiac allografts in recipients lacking in IFNγ [Stijn et al unpublished]; conversely, congenital deficiency of IL-6 in this setting leads to increased IFNγ [Xing et al 1998]. Alternatively, the increase in IL-6 by WY could represent a direct comparable to the increase in IL-6 in adenomas from mice treated with WY [Anderson et al 2001] and contrasting with the decrease seen in smooth muscle cells [Staels et al 1998]. The lack of effect on IL-6 when using PPARγ agonists (besides PGJ2 which attenuates IL-6 production) has been a recurrent theme [Thieringer et al 2000, Hinz et al 2003]. In fact, experiments have shown the ability of PGJ2 to directly interact with transcription factors independently of PPARγ [Rossi et al 2000]. The inability of PGJ2 to significantly decrease IL-6 production in the MLR may be another example of a feedback mechanism, or of the uniqueness of the alloresponse.

The differences between ConA and MLR cultures are more dramatically evidenced in the profiles of selected chemokines before and after treatment with PPAR agonists. In ConA cultures, chemokine mRNA expression decreases as a function of time, with MIP-1β being the most abundant, closely followed by RANTES and MCP-1. In the MLR, there is a clear peak at day 2, with RANTES being by far the most dominant chemokine (by over
a factor of 2), followed by MCP-1 and MIP-1α. The experiments were performed concurrently such that time points and relative transcript expressions correspond.

Based upon the uniform decrease in IFNγ, it was expected that a similarly uniform decrease would be seen in the IFNγ–inducible chemokines, such as RANTES and IP-10. In fact, in the ConA cultures, incubation with PPAR agonist results in a uniform decrease in both chemokines on the day of peak expression, a finding consistent with a number of other studies, albeit in different in vitro systems, with reported decreases in RANTES and IP-10 [Gosset et al 2001, Hirano et al 2003, Marx et al 2000]. However, this does not extend to the MLR. Peak chemokine expression occurred one day later than in ConA cultures. No decrease in IP-10 mRNA expression was observed for any agonist, where no decrease was observed and WY significantly increased expression. RANTES displayed the expected decrease with WY and PPARγ agonists, suggesting an eventual change to an IFNγ-dependent effect. Fenofibrate results in a small (~10%), yet statistically significant, increase in RANTES expression, possibly representing a kinetic issue (i.e. a delay in the change from an IFNγ-independent to an IFNγ-dependent pathway), or could be based on downstream signaling issues specific to fenofibrate. The lack of a decrease in RANTES and IP-10 with PPAR treatment renders unavailable a substantial benefit associated with a decrease in IFNγ. A number of reports have established these chemokines as important mediators in the cardiac allograft rejection [Fairchild et al 1997, Yun et al 2000, Hancock et al 2001, Morita et al 2001, Melker et al 2001, Yun et al 2001, Horiguchi et al 2002, Fahmy et al 2003].

To date, no studies have tested the effects of PPAR activation on either the expression or production of MIP-1α or MIP-1β. Recent evidence has identified these MIP chemokines (along with RANTES) as functioning in concert with IFNγ on target cells [Dorner et al 2002]. Consequently, decreased MIP expression would be expected based on the decrease in IFNγ. This is clearly observed on the day of maximal chemokine expression in the ConA cultures, where all agonists uniformly and significantly decrease expression of MIP-1α and MIP-1β. However, once again it does not apply to the MLR, where on the day of maximal mRNA expression, there is no significant effect on MIP expression. The suggestion of a direct stimulatory mechanism is clearly suggested on day 4 of the MLR where all of the agonists, except fenofibrate, result in a dramatic two to three-fold increase in MIP expression. A recent study reports increased secretion of a number of cytokines by T cells from immunized mice receiving exogenous MIP-1α or MIP-1β [Lalliard et al 2003]. This suggests that the increase in these chemokines elicited by PPAR agonists may have consequences on cytokines and could be one of the reasons why MLR increases in IL-2 and IL-6 were associated with PPAR activation by selected agonists such as WY and PGJ2 (these have the most marked increase in MIP-1α and MIP-1β). The effect on these chemokines could also explain some of the differences between ConA and MLR cultures, in that higher expression may lead to more pronounced chemokine-mediated effects. The properties of the MIP-1 proteins as both macrophage activators and lymphocyte chemoattractants [Melker et al 1999] have given them roles in the development of PR and TxAAs [Shimizu and Mitchell 2003]. Based on their function and implications for rejection, the PPAR agonist with the highest
probability of maximizing the cytokine benefits is the one that minimizes chemokine expression and subsequent cellular graft infiltration. Hence, of the agonists tested, fenofibrate emerges as the most likely candidate for success in vivo.

The importance of timing and stimulus type is once again exemplified in the PPAR effects on MCP-1 expression. While PPAR activation has no effect on either ConA or MLR associated early mRNA expression, by day 4 of the MLR, PPARα agonist WY resulted in a marked increase, while others continued to have no significant effects. Studies on human endothelial cells confirm an induction in MCP-1 by WY in when stimulated by oxidized phospholipids [I.e.e et al 2000] yet report suppression when stimulated by C-reactive protein [Pasceri et al 2001]. These raise the issue of whether suppression is a result of an effect on C-reactive protein, rather than on MCP-1 directly (not tested in the study). Unlike the in vitro results reported here where no effects were found, the literature suggests that fenofibrate has the potential to decrease MCP-1 expression in certain in vivo models [Duez et al 2002], possibly due to an effect on endothelial cells [Rival et al 2002]. Results of PPARγ activation on MCP-1 typically show a lack of effect with BRL and other thiazolidinediones, while significant effects with PGJ2 [Rival et al 2002. Ishibashi et al 2002], are potentially attributable to PPARγ-independent effects [Rossi et al 2000]. The ability of MCP-1 to directly stimulate vascular smooth muscle cell proliferation [Setzman et al 2002], as well as its more traditional role in monocyte attraction, activation and adhesion [Rollins 1997. Gerzsten et al 1999], has attracted interest in MCP-1 modulation for coronary artery diseases [Ikeda et al 2002] and has suggested that it may be an important factor in the process of rejection, particularly TxAA [Shimizu and Mitchell 2003]. This suggests that the PPARα ligand WY would possibly contribute to vascular smooth muscle cell proliferation in the transplant setting, thus exacerbating the overall process.

The multiplicity of chemokine ligand-receptor interactions suggests that targeting chemokine receptors (rather than chemokines) is a potentially more useful therapeutic strategy in the attenuation of rejection [Hancock et al 2000]. This prompted examination of PPAR effects on chemokine receptor mRNA expression. CCR1, CCR4 and CCR5 serve as receptors for RANTES, MIP-1α and MIP-1β, while CCR2 serves as the receptor for MCP-1 (details are reviewed in chapter 2). These are the first experiments to comprehensively evaluate the effects of PPAR activation on these receptors. On day 1 post ConA stimulation, PPARα agonists increase CCR1 and CCR2, have no effect on CCR4 and have variable effects on CCR5. PPARγ agonists have no effects on either CCR1 or CCR4, increase CCR2 and decrease CCR5. On day 2 of the MLR, PPARα agonists have variable effects on CCR1, CCR4 and CCR5 and increase CCR2, while PPARγ have no significant effects on CCR1, CCR2, CCR4 and variable effects on CCR5. By day 4, all PPAR agonists showed no effect on CCR1, variable effect on CCR4 and uniform decrease in CCR2 and CCR5. In general, MLRs exhibit higher levels of receptor expression, with the peak level of the most abundant chemokine receptor, CCR5, over a factor of two higher in the MLR compared to ConA. CCR4 is more comparable to CCR5 in ConA cultures for a majority of the days. CCR1 and CCR2 have the lowest levels of expression compared to other chemokine receptors in both cultures.
Potential PPAR-mediated effects on PPAR expression on cytokines and/or chemokine receptors chemokine receptors may offer explanations for some of the ConA and MLR results. As shown previously, ConA cultures have substantially higher expression of IL-2, and PPARα agonists increased it significantly. Reports have shown that IL-2 may upregulate CCR1 and CCR2 \cite{Loetscher et al. 1996}; consequently early upregulation of CCR1 and CCR2 with PPARα agonists may be an indirect result of IL-2 modulation. By day 4 of the MLR, IFNγ protein is present in high concentrations and can thereby mediate secondary effects, such as downregulation of CCR2 \cite{Penton-Rol et al. 1998}. Consequently, decreased IFNγ caused by PPAR agonists could lead to increased CCR2 expression. However, PPAR agonists uniformly decrease CCR2 expression, suggesting an IFNγ-independent effect. The downregulation of CCR2 upon treatment with PPAR agonists is consistent with the only two reports in the literature \cite{Han et al. 2000, Ishibashi et al. 2002}. Decreased CCR5 expression observed on day 4 of the MLR, on the other hand, may be an IFNγ-dependent effect. The association of CCR5 with IFNγ \cite{Sallusto et al. 2000, Liang et al. 2003} suggests that a decrease in IFNγ could lead to decreased CCR5, which is, in fact, observed. This is further supported by the varied response on day 2, when essentially no IFNγ protein is present. Effects on day 2 of the MLR may therefore be interpreted as IFNγ-independent and possibly directly mediated by PPAR agonists. Nevertheless, the conclusions herein are largely speculative without definitive promoter and transcription analysis.

The ConA vs MLR presented in this chapter highlight that type of splenocyte stimulation can affect regulation of chemokine receptors. For example, examination of the natural time course of chemokine receptor expression shows that in ConA cultures, CCR5 expression at early and late times is roughly unchanged, while in the MLR, the level of expression increases over two-fold during that interval. The difference between the first and last time points in the ConA cultures is that by the last time point, expression of the other chemokine receptors in the panel is diminished; despite the lack of a change in absolute mRNA expression, the relative mRNA expression compared to other receptors makes it the dominant receptor in the ConA, as well as the MLR, cultures. However, consideration of the absolute level of expression shows the comparative inability of ConA to upregulate CCR5 expression. This is consistent with studies in the literature that found upregulation of CCR5 in antigen-activated T cells, in contrast to stimulation with mitogens, phorbol esters, or anti-CD3 acting alone or in concert with anti-CD28 \cite{Ebert and McColl 2002}. An implication of this is that the varying experimental results achieved with PPAR agonists using different stimulation assays may yield varying results due to differences in baseline levels, or possibly effect of PPAR activation on the stimulus itself, rather than on the consequence of the stimulation. This is particularly applicable to studies where PPAR-modulated cytokines have downstream effects, raising the issue of whether the endpoint change is mediated directly by PPAR or by the stimulating cytokine (for example, TNFα-induced adhesion molecule expression). The use of cytokine neutralization experiments, in addition to signaling experiments, could such issues.
Regardless of cytokine-dependence, attenuation of CCR2 and CCR5 by PPAR agonists in the late stage MLR is of particular interest for modulating transplant rejection and suggests possible benefit of PPAR administration in vivo. Murine recipients of MHC-II mismatched allografts congenitally lacking CCR5 demonstrate diminished PR and abrogation of TxAA [Gao et al 2001] and a recent study documents similar benefits upon the administration of an agent antagonistic to CCR1, as well as CCR5 [Yun et al 2004]. Graft survival time also increases when recipients are congenitally deficient in CCR2, with a concomitant decrease in macrophage number in the graft [Furukawa et al unpublished]. Deletion of CCR2 in the recipient does not have as pronounced an effect on TxAA, but still significantly reduces it with transient early decreases in macrophage infiltration [Furukawa et al unpublished].

Given the apparent differences between ConA stimulation and MLRs, determination of PPAR-mediated effects potentially translated to the in vivo setting should likely be based on the MLR results alone. As a group, PPAR agonists displayed several “anti-rejection” effects, including a decrease in IFNγ, CCR5 and CCR2. With respect to other cytokines, PPARα agonists displayed some potentially pro-rejection effects such as a small, yet statistically significant increase in IL-2, IL12-p40 and IL-18, while PPARγ agonists had no effects on either IL12-p40 or IL-18 and displayed drug-specific effects on IL-2. More compelling, however, is examination of PPAR-mediated effects on chemokine production in the late stage of the MLR. Despite causing a 30% decrease in RANTES (potentially attenuating graft infiltration, thus “anti-rejection”), PPARγ agonists resulted in a 200 to 300% increase in MIP-1α and MIP-1β (potentially causing a severe augmentation of graft infiltration, thus “pro-rejection”). PPARα agonists had mixed results, with WY closely mimicking the PPARγ agonist behavior. FENO, on the other hand, did not decrease RANTES (actually increased by ~20%) but had significant enhancement of MIP-1 expression (increased MIP-1α by ~10% and had no effect on MIP-1β).

Therefore, fenofibrate minimizes the chemokine-mediated “pro-rejection” properties of WY and the PPARγ agonists, while continuing to take advantage of the “anti-rejection” properties of PPAR agonists. The extent of the additional “pro-rejection” properties displayed by fenofibrate, such as increased pro-inflammatory cytokines, are small compared to the extent of the chemokine effects of WY and the PPARγ agonists. Fenofibrate appears to allow minimization of cellular infiltration and subsequent maximization of the “anti-rejection” benefits from the decrease of IFNγ, CCR2 and CCR5. Furthermore, other potentially beneficial (or “anti-rejection”) effects, such as decreased VCAM-1 (appealing in light of the above chemokine-related issues), are reported to be associated only with PPARα and not PPARγ agonists [Marx et al 1999b]. Thus, fenofibrate emerges as the candidate most likely to be successful in the attenuation of rejection mechanisms in vivo.

In conclusion, the experiments presented in this chapter constitute the first evaluation of the effects of PPAR activation on an alloresponse, for possible translation to the in vivo setting of cardiac transplantation, and also included one of the most detailed analyses.
available to date of cytokine, chemokine, and chemokine receptor expression following antigen and mitogen mediated splenocyte stimulation. Dose response studies performed in both mixed leukocyte reactions, as well as following ConA-stimulation with two agonists per PPAR isoform, in order to discern potential drug-specific effects, identified the following therapeutic doses (defined as the doses at which a decrease in IFNγ is achieved without a concomitant decrease in proliferation): 20 µM for fenofibrate, 100 µM for WY14643, 10 µM for BRL49653 and 3 µM for 15-deoxy-A12,14-prostaglandin J2. Flow cytometric analysis of propidium iodide staining confirmed cell viability at these doses, at least up to 12 hours of continuous treatment. Normal cell proliferation at these doses also supports the contention that these doses of PPAR agonists do not affect cell viability.

Using these doses, effects of the PPAR agonists on cytokine, chemokine and chemokine receptor expression were investigated and discussed both in the context of internal networks, as well as findings from the literature. Comparison between results achieved in mixed leukocyte reactions and following ConA stimulation revealed critical differences and pointed to the need for specific testing of the alloresponse for potential translation of PPAR effects to in vivo transplantation rejection. Based on the results presented in this chapter, as well as ancillary results from the literature, fenofibrate has emerged as the most likely PPAR agonist to have beneficial effect in the attenuation of mechanisms of rejection in vivo. The next chapter presents the in vivo evaluation of fenofibrate in a murine heterotopic model of cardiac transplantation.
Chapter 4. *In vivo* evaluation of a PPARα agonist

4.1 Introduction

As discussed in the last chapter, PPAR agonists display several potential “anti-rejection” effects, including markedly decreased IFNγ, CCR2 and CCR5. Of the PPARα and PPARγ agonists tested, fenofibrate emerged as the most promising candidate for attenuation of rejection mechanisms *in vivo*. Despite some “pro-rejection” actions such as small, yet statistically significant, increases in a several cytokines (e.g. IL-2, IL12-p40, IL-18 and RANTES), fenofibrate, unlike the other PPAR agonists, did not markedly enhance the overall chemokine expression. Fenofibrate displayed the greatest potential for minimizing potential chemokine-mediated “pro-rejection” properties, while continuing to take advantage of the “anti-rejection” properties of PPAR agonists.

Fenofibrate has several other potential “anti-rejection” mechanisms of action. In light of the regulatory role of IFNγ in the expression of MHC-II [Boehm et al 1997], the fenofibrate-mediated decrease in IFNγ should result in decreased MHC-II expression and subsequently result in decreased CD4+ T cell activation. However, direct examination of PPARα ligands effects on MHC-II induction demonstrated no inhibition [Kwak et al 2002]. Moreover, fenofibrate may decrease the expression of ICAM-1 and VCAM-1 and subsequent monocyte binding to endothelial cells [Marx et al 1999b, Rival et al 2002, Deplanque et al 2003]. This may be particularly important in light of the potentially increased level of RANTES expression in the setting of fenofibrate administration, which may enhance the mononuclear cell infiltrate. Fibrates may also suppress both vascular endothelial and smooth muscle cell activation [Staels et al 1998, Xu et al 2001]. Finally, evidence from the clinical literature suggests that dyslipidemia may contribute to rejection [Behrendt et al 2000] and that fenofibrate is effective in decreasing lipid levels in heart transplant recipients [Keating and Omnord 2002]. Since PPARα activators may have species-specific effects [Lawrence et al 2001], these effects may not be fully recapitulated in murine studies. Nevertheless, there is evidence that hyperlipidemia may be associated with increased lesion frequency in rodent cardiac allografts [Tanaka et al 1994] and that fenofibrate may lower lipid levels in mice [Olivier et al 1988], potentially resulting in attenuation of lesions in murine cardiac allografts.

Therefore, based on the results of the previous chapter, it was reasonable to suggest that fenofibrate has the greatest likelihood of success in attenuating rejection mechanisms *in vivo*. Furthermore, results from the literature suggested additional potentially beneficial effects. However, the balance between potentially “pro-rejection” and “anti-rejection” effects remains to be determined by experimental testing, which is especially important given the uniqueness of the alloresponse setting. This chapter presents the first evaluation of fenofibrate, a PPARα agonist, on mechanisms of PR and TxAA in a murine model of cardiac transplantation.
4.2 Methods

Animals and Diet

Three inbred strains of mice, C57/BL6, Balb/c and B6.C-H-2^{bml2}KhEg (bml2 mice that are MHC-II mismatched with C57/BL6 but on the same background), approximately six to eight weeks old (Jackson Laboratories, Bar Harbor), were housed in the animal facility of Harvard Medical School on acidified water and chow ad libitum. Sentinel mice were co-housed in each room and were surveyed to ensure that that the facility was free of pathogens. Experiments were done according to protocols approved by the animal care committee. Recipients were started on a chow containing 0.2% w/w fenofibrate (Research Diets Incorporated, New Brunswick) three days after transplantation due to excessive bleeding. The fenofibrate diet was monitored by weekly measurement of food intake. The fenofibrate chow was continued until the time of organ harvest. In experiments on non-transplanted C57/BL6 mice, aimed at determining whether the effect of fenofibrate treatment was sustained over time, the fenofibrate chow was stopped one week before harvest and mice were returned to regular chow ad libitum.

Heterotopic Heart Transplantation

Heart transplantation of donor bml2 hearts into C57/BL6 recipients was performed according to the heterotopic technique described in the literature [Corry et al 1973, Furuikawa et al 2002] and in accordance to protocols approved by the Harvard Medical School Animal Care Committee. After administration of inhalation anesthesia, cold heparinized saline was injected into the inferior vena cava of the donor heart. Then, the inferior vena cava, as well as the superior venae cavae and pulmonary veins, were ligated. The donor heart was removed and placed in cold sterile saline. Preparation of the recipient initially entailed opening the abdominal cavity and identifying both abdominal aorta and inferior vena cava. After making careful incisions on those vessels, the donor aorta and pulmonary artery were ligated to the recipient abdominal aorta and inferior vena cava. The abdominal cavity was then closed and the animal kept under a warm lamp until recovery from anesthesia. For evaluation at 8 weeks, a total of 22 transplants were performed, with n=12 mice on regular chow (denoted as control) and n=10 mice on chow with fenofibrate (denoted as treated). Further study also involved a pilot study at 2 weeks, with an n=3 in the control group and n=4 in the treated group. At the time of harvest, the heterotopically transplanted heart was divided transversely in three sections, representing the base, middle and apex, each to be used for separate purposes as described below.

Plasma measurements

At the time of cardiac allograft removal, blood was obtained by cardiac puncture of the recipient’s native heart. It was immediately transferred to vacutainer tubes with 0.135 M of sodium citrate at a 1:10 dilution and centrifuged at 10,000 rpm for 5 minutes. Plasma was then transferred to 1.5 ml centrifuge tubes where it was frozen at –20°C until analysis. Samples were sent to the Clinical & Epidemiologic Research Laboratory at
Children's Hospital Boston for analysis of triglycerides, total cholesterol, glucose, fibrinogen, ALT and AST plasma concentrations. Due to limitations in sample volume, not all laboratory markers were obtained for all transplants and the number of mice is specifically noted.

Histological staining and scoring

The base of the heart and a liver section were fixed in 10% buffered formalin for staining with hematoxylin and eosin. The heart was also stained for elastic tissue and was scored for extent of PR and TxAA. The grading scheme for PR was modified from the International Society for Heart and Lung Transplantation scale and is described as follows: 0, no rejection; 1, mild interstitial infiltrate without necrosis; 2, focal interstitial with necrosis; 3, multifocal interstitial with necrosis; 4, widespread infiltrate with hemorrhage and/or vasculitis. The extent of TxAA was determined by average the scores of over 10 arteries where the scoring scheme is based on occlusion: 0, no occlusion; 1, <10%; 2, 10 to 25%; 3, 25-50%; 4, 50-100%. The liver was evaluated on the presence or absence of increased granularity and eosinophilia, representing increased peroxisome proliferation. The distinctness of the staining in the treated group led to a binary scoring of “control” and “PPAR liver”.

Immunohistochemical staining and scoring

The middle transverse section of the cardiac grafts and sections of the recipient liver were frozen in optimal cutting temperature (OCT) compound (Ames Co, Elkhart) and stored at -80°C. Heart sections were stained for expression of MHC-II, ICAM-1, VCAM-1, CD4, CD8 and CD11b, while the liver was stained for peroxisomal membrane protein 70 (PMP-70). Sections were cut at a thickness of 5 μm and stored at -20°C until staining was performed. Unless otherwise noted, all steps were preceded by three washes in phosphate-buffered saline for three minutes each. Slides were first air dried for approximately 20 minutes and then fixed with 4% paraformaldehyde for 5 minutes at room temperature. After washing three times for three minutes each with distilled water, sections were covered with 0.5% hydrogen peroxide (Sigma, St, Louis) for 15 minutes. Sections were then blocked with either rabbit or goat serum for 1 hour and with avidin and biotin for 15 minutes each. Primary antibodies (PharMingen, San Diego) ranging from in concentration from 1:40 to 1:200 were applied for times ranging from 30 minutes to 3 hours. If applicable, biotinylated secondary antibody (PharMingen, San Diego) was added. Chromogen was added and development times were established by microscopy to ensure that the isotype controls did not show staining. After development, slides were counterstained in hematoxylin for 1 minute and washed under running water for 15 minutes. Excess water was removed and Aqueous Quick Mount was applied, before covering sections with a coverslip. Heart sections were scored (3 blind observers) according to the following scheme: 0, no staining; 1 focal weak; 2 focal moderate, diffuse weak; 3 focal strong, diffuse moderate; 4 diffuse strong. For the liver, the scoring scheme was simplified to either absence or presence of peroxisomal proliferation, since typically the effect is observed throughout the liver.
RNase Protection Assay

The apex of the heart was frozen at -70°C until isolation of mRNA. Tissue was placed in 1 ml of TRIZOL (Invitrogen, Carlsbad) and homogenized using an electric homogenizer. The technique used for isolating the mRNA is fully described in chapter 3. Aliquots with 10 μg of mRNA were stored in RNase-free DNase-free water at -70°C. Analysis of mRNA expression was performed via the RNase protection assay (RPA) with a custom multi-template probe (for IFNγ, IL-4, IL-6, IL-10, IL-12p35, IL12-p40, IP-10, RANTES, CCR2 and CCR5). Quantification of band intensities was performed using the ImageQuant software (Molecular Dynamics). The volume integrations of each band were normalized to the bands for GAPDH in the corresponding lane. Samples were run in triplicate and results are presented as the average and standard deviation per group.

Mixed Leukocyte Reaction

The technique used for splenocyte isolation and culture in a mixed leukocyte reaction (MLR) is fully described in chapter 3. Briefly, splenocytes from transplant recipients (n=22) obtained at the time of organ harvest were restimulated in vitro with bm12 splenocytes, as well with third-party Balb/c stimulators. Experiments were also performed with splenocytes from non-transplanted C57/BL6 (n=6) recipients of fenofibrate to determine whether the effect observed in the transplant recipients would be sustained. Proliferation was assessed by incorporation of ³H-thymidine and supernatants collected for analysis described below.

Enzyme-Linked Immunosorbent Assays

The concentration of IFNγ in the supernatants of the proliferation assays was determined using enzyme-linked immunoassays (ELISAs), per the manufacturer’s protocol (Pharmingen, San Diego). Details of the procedure employed are fully described in chapter 3. Optical density of the solutions in the wells was measured using an ELISA plate reader and converted to pg/ml using a calibration curve prepared from recombinant IFNγ standards. Analysis of optical density and protein concentrations was done using “Softmax” (Molecular Devices Corporation).

Data Analysis

Results in bar and line graphs are presented as the mean and standard error of the mean, while results in scattered plots represent the mean only. Statistical significance for histological and immunohistochemical staining results, where scores ranged from 0 to 4, was determined using the Mann-Whitney test for non-parametric values with a two-tailed p-value criteria of less than 0.05. For immunohistochemical staining, the Kruskal-Wallis test, a one-way analysis of variance (ANOVA) for non-parametric values, was also performed to ensure that there was no statistically significant difference between the three, blinded observers. Statistical significance of other values was determined using the Student’s t-test for unpaired samples with two-tailed distribution and a p value less than 0.05. The “Prism” statistical software (Graphpad) was used for statistical analysis.
4.3 Results

Recipients of MHC-II mismatched murine cardiac allografts were sacrificed at both 2 and 8 weeks after transplantation and treatment with fenofibrate-laden chow (0.2% wt/wt). With the exception of findings common to both times, results are grouped and presented according to time of harvest.

Figure 4.1 shows representative photomicrographs of morphological changes in the livers of the murine allograft recipients at both time points. The livers of treated mice display increased granularity and hypereosinophilia on histological sections, as well as increased PMP-70 expression by immunohistochemical staining. It is emphasized that there were no histologic signs of liver damage but rather, the changes were a result of increased peroxisomal proliferation. This change is not likely to be observed clinically, since PPARα agonists do not induce peroxisomal proliferation in human hepatocytes [Lawrence et al 2001].

![Figure 4.1: Fenofibrate-induced effects on livers of murine cardiac transplant recipients: Appearance (via routine hematoxylin & eosin staining) and PMP-70 expression (via immunohistochemical staining). Fenofibrate treatment results in increased granularity and hypereosinophilia in sections stained with hematoxylin and eosin, and increased peroxisomal proliferation as shown by diffusely increased PMP-70 staining. These photomicrographs are representative of sections from livers harvested at both 2 and 8 weeks after transplantation.](image-url)
To check the early effects of fenofibrate, a small set of transplants (n=3 control and n=4 treated) with a 2-week survival time was performed. Physiological measurements confirmed the presence of fenofibrate in the treated group. Wet liver weight of treated mice, shown in figure 4.2, more than doubled (from 1.2 ± 0.2 to 2.8 ± 0.1 g; p < 0.05). Liver enzymes (ALT and AST) tended to increase with the increase in liver weight. Other plasma markers, shown in figure 4.3, varied without reaching statistical significance. Notably, fibrinogen tended to decrease, as did triglycerides and glucose, while cholesterol tended to increase. The lack of statistical significance may potentially be due to the low numbers of mice in this set of transplants.

Allografts were analyzed to determine the immunomodulatory effect of fenofibrate administration. Analysis of the mRNA expression of selected cytokines, chemokines and chemokine receptors tested, in figure 4.4, showed that fenofibrate tended to have a broad immunosuppressive effect. Allografts of treated mice demonstrated attenuated mRNA expression IFNγ, IL-6, IL-4, IL-10, RANTES, IP-10, CCR2 and CCR5.

The immunohistochemical appearance of the graft was examined to assess the effect of fenofibrate treatment on graft activation and infiltration. Fenofibrate had no substantial impact on graft activation; specifically, as shown in figure 4.5, there was no effect on MHC-II expression and only a tendency to decrease VCAM-1 and ICAM-1 expression. On the other hand, dramatic immunosuppressive effects were observed in terms of graft inflammatory infiltrate. Figure 4.6 shows that at 2 weeks, fenofibrate significantly reduced infiltration by CD4+ T cells and CD11b+ macrophages. Specifically, CD4+ cells decrease from 161 ± 24 to 22 ± 4 cells per high power field (p < 0.05) and CD11b+ from 167 ± 27 to 74 ± 10 cells per high power field (p < 0.05). CD8+ T cells also decreased (from 12 ± 3 to 4 ± 4 cells per high power field) without statistical significance.

In order to examine functional leukocyte effects of fenofibrate treatment, splenocytes from recipients were restimulated ex vivo with bm12 (same party), B/c (third party) and ConA (mitogenic). Figure 4.7 shows the effects on proliferation and IFNγ production. In broad terms, fenofibrate treatment does not significantly affect proliferation. The concentration of IFNγ is significantly decreased in all cultures on the third and fourth days. This decrease is sustained with both third party and mitogenic stimulation, but disappears by the last day of culture when leukocytes restimulated with bm12.

Physiological measurements once again confirmed the presence of fenofibrate-mediated effects. The mean wet liver weight figure 4.8 more than doubled in the treated group (from 1.6 ± 0.08 to 4.1 ± 0.33 g; p < 0.05). This correlated with significantly increased concentrations of the liver enzymes ALT (86 ± 10 to 148 ± 14 mg/dl; p<0.05) and AST (10 ± 2 to 68 ± 23 mg/dl; p < 0.05). Linear regression analysis revealed strong correlation between food intake and the AST liver enzymes (R²=0.86), and a moderate correlation with ALT (R²=0.39). Measurements of circulating levels of plasma metabolic markers,
presented in figure 4.9, further corroborated physiological effects; fenofibrate significantly decreased mean levels of triglycerides (41 ± 4 to 31 ± 3 mg/dl; p<0.05) and glucose (201 ± 11 to 159 ± 11 mg/dl; p<0.05) and significantly increased cholesterol from 83 ± 9 to 107 ± 6 mg/dl (p<0.05). There was no significant correlation between food intake and any metabolic markers (R² < 0.15). Fibrinogen was not significantly affected.

The immunosuppressive effect on mRNA expression of selected cytokines, chemokines and chemokine receptors seen at 2 weeks was not sustained. By 8 weeks, as shown in figure 4.11, fenofibrate actually augmented the mRNA expression of IFNγ, IL-4, IL-6 and IL12-p40, while not affecting that of IL-10 and IL12-p35. Of this panel of cytokines, IFNγ was the dominant cytokine in terms of expression level. Furthermore, as shown in figure 4.12, fenofibrate significantly increased IP-10 mRNA expression, but did not significantly affect the expression of RANTES, CCR2 and CCR5.

Fenofibrate also markedly augmented the allograft inflammatory cell infiltrate, as shown in figure 4.13. Specifically, fenofibrate significantly increased the CD4+ T cells (from 119 ± 12 to 139 ± 16 cells per high power field; p < 0.05) and dramatically increased the number of CD11b+ cells (from 35 ± 4 to 138 ± 24 cells per high power field; p < 0.05). There was no difference in the number of graft infiltrating CD8+ T cells (39 ± 5 cells per high power field; p<0.05).

To examine functional effects of *in vivo* fenofibrate administration, splenocytes from both control and treated mice were restimulated *ex vivo* with the same (bm12) and third (B/c) party stimulators. As shown in figure 4.14, fenofibrate does not significantly affect proliferation but consistently attenuates IFNγ production on the third day of culture (which is the first day that substantial protein concentrations are present). The effect on IFNγ, however, was transient and disappeared by day 4, raising the question of whether the disappearance was a result of diminished drug effect given the duration of the culture.

Thus, to test whether the effect was a function of time, splenocytes from wild-type mice (n=6) treated with fenofibrate for 8 weeks, were taken off the diet one week before harvest (rather than at the time of harvest). Figure 4.13 presents the results of the *ex vivo* restimulation with allomismatched B/c splenocytes. As expected, proliferation was not affected. However, IFNγ production was diminished for the entire duration of the assay. This suggests that the transience of the effect on IFNγ was not due to time, but rather represents a more fundamental alteration in leukocyte activity following transplantation.

Finally, the overall effect of long-term fenofibrate administration on allograft activation and pathology were examined. The results pertaining to allograft activation are shown in figure 4.14. Fenofibrate resulted in a small, but statistically significant decrease in the expression of MHC-II but did not affect the expression of the VCAM-1 and ICAM-1 adhesion molecules, such that there was essentially no net effect on markers of allograft activation. However, fenofibrate administration did have significant effects on allograft pathology, per figure 4.15. Long-term fenofibrate treatment significantly augmented PR, without ameliorating (or exacerbating) TxAA.
Figure 4.2: Effect of fenofibrate on liver weight and enzymes at 2 weeks: Liver weight increased by a factor of 2, while ALT and AST also increased, but not significantly. Statistical significance between the control (■, n=3) and treated (∇, n=4) groups was determined using Student’s t test (p<0.05); the bar indicates the mean value.

Figure 4.3: Effect of fenofibrate on plasma markers at 2 weeks: There was no statistically significant effect on any markers. However, it should be noted that fibrinogen and glucose demonstrated downward tendencies. Triglyceride levels remained unchanged and cholesterol levels tended to rise. Statistical significance between the control (■, n=3) and treated (∇, n=4) groups was determined using Student’s t test (p<0.05); the bar indicates the mean value.
Figure 4.4: Effect of fenofibrate on graft expression of selected cytokines, chemokines and chemokine receptors at 2 weeks: Fenofibrate reduces the expression of IFN\(\gamma\), as well as the other cytokines tested (IL-4, IL-6, IL-10). Accordingly, fenofibrate also substantially reduces IP-10. Despite the decrease in IFN\(\gamma\), however, fenofibrate does not affect the expression of RANTES. Finally, fenofibrate reduces expression of both CCR2 and CCR5. The lack of statistical significance may be attributable to the low numbers of transplants in this group. Results are normalized to the expression of GAPDH. Statistical significance between the control (\(\square\), n=3) and treated (\(\checkmark\), n=4) groups was calculated using the Student’s t-test (p < 0.05).
Figure 4.5: Effect of fenofibrate on the graft expression of activation markers at 2 weeks: Fenofibrate treatment had no effect on MHC-II. It tended to decrease VCAM-1 and ICAM-1. The Kruskal-Wallis test was used to ensure that there were no significant differences between the three, blinded observers. Statistical significance (*) between control (■, n=3) and treated (●, n=4) groups was determined using the Mann-Whitney test (p<0.05). Results are expressed as the mean and standard error of the mean.

Figure 4.6: Effect of fenofibrate on graft infiltrating cells at 2 weeks: Fenofibrate treatment significantly reduced the numbers of CD4+ and CD11b+ cells, while not affecting CD8+ cells. For each cell type, five fields per section were counted by two, blinded observers. Statistical significance (*) between the control (■, n=3) and treated (●, n=4) groups was calculated using the Student’s t-test.
Figure 4.7: Effect of fenofibrate on recipient splenocyte proliferation and IFN\(\gamma\) production ex vivo at 2 weeks: a, b) restimulated with bm12; c,d) stimulated with B/c; e,f) stimulated with ConA. In broad terms, there are no dramatic differences in proliferation. The concentration of IFN\(\gamma\) is significantly decreased in all cultures on days 3 and 4. Furthermore, IFN\(\gamma\) is also decreased on days 2 and 5 in the bm12 cultures, as well as on day 5 in the ConA culture. Results are presented as the mean and standard error of the mean of all recipients in triplicate to quadruplicate samples. Statistical significance (*) between the control (■, n=3) and treated (○, n=4) groups was calculated using the Student’s t-test (p<0.05).
Figure 4.8: **Effect of fenofibrate on liver weight and enzymes at 8 weeks:** Liver weight increased by a factor of 3, while ALT and AST also significantly increased. Statistical significance between control (■, n=12) and treated (□, n=10) groups was determined using Student’s t test (p<0.05), and the bar indicates the mean value.

Figure 4.9: **Effect of fenofibrate on plasma markers at 8 weeks:** Fenofibrate treatment significantly increases mean levels of total cholesterol, and significantly decreases the mean levels of both triglyceride and glucose. Fibrinogen levels are not significantly affected. Statistical significance between the control (■, n=12) and treated (□, n=10) groups was determined using Student’s t test (p<0.05); the bar indicates the mean value.
Figure 4.10: Effect of fenofibrate on the intragraft mRNA expression of selected cytokines at 8 weeks: Fenofibrate treatment statistically increased expression of IFNγ, IL-4, IL-6 and IL12-p40, while not affecting the expression of other cytokines (IL-10 and IL12-p35). In control allografts, IFNγ was the cytokine with the highest expression, while IL12-p35 is that with the lowest. IL-6, IL-10 and IL-12p40 had comparable expression, while IL-4 was approximately an order of magnitude lower. All mRNA expression levels are normalized to GAPDH. Statistical significance between control (■, n=9) and treated (□, n=9) groups was determined using Student’s t test (p<0.05); the bar indicates the mean value.
Figure 4.11: Effect of fenofibrate on intragraft expression of chemokines and chemokine receptors at 8 weeks: a) RANTES; b) IP-10; c) CCR2; d) CCR5. Fenofibrate statistically increased IP-10, while not affecting RANTES, CCR2 and CCR5. Statistical significance between control (■, n=9) and treated (□, n=9) groups was determined using Student’s t-test (p<0.05); the bar indicates the mean value.

Figure 4.12: Effect of fenofibrate on graft infiltrating cells at 8 weeks; there was a small, but statistically significant (p<0.05) increase in CD4+ cells, and dramatically increased the number of CD11b+ cells (p<0.05). There was no difference in the number of CD8+ cells. Five fields were counted per section (two blinded observers). Statistical significance (*) between the control (■, n=12) and treated (n=10) groups was calculated using the Student’s t-test.
Figure 4.13: Effect of fenofibrate on splenocyte proliferation and IFN\(_\gamma\) production *ex vivo* at 8 weeks: a, b) recipient splenocytes restimulated with bm12; c,d) recipient splenocytes stimulated B/c; e,f) comparison to results with splenocytes from C57/BL6 mice who were taken off the fenofibrate chow 1 week before harvest (denoted as "off 1 wk") and stimulated with B/c. Fenofibrate does not significantly affect proliferation on any day, while significantly reducing IFN\(_\gamma\) production on day 3. Please see text for more details. Statistical significance (*) was calculated using the Student’s t-test (p < 0.05). Results are presented as the mean and standard error of the mean of quadruplicate samples for the control (■, n=10), treated (□, n=9) and "off 1 week" (■, n=6) groups.
Figure 4.14: Effect of fenofibrate on allograft activation at 8 weeks: Representative photomicrographs of isotope control and examples of high expression show that the distribution of MHC-II tends to be diffuse, while the distribution of the VCAM-1 and ICAM-1 adhesion molecules tends to be localized to large vessels. Fenofibrate treatment results in a small but significant decrease in the expression of MHC-II. It does not significantly affect the expression of VCAM-1 or ICAM-1. Sections were evaluated (three, blinded observers) according to the following scheme: 0, no staining; 1 focal weak; 2 focal moderate, diffuse weak; 3 focal strong, diffuse moderate; 4 diffuse strong. The Kruskal-Wallis test was used to ensure that there were no significant differences between the three, blinded observers. Statistical significance (*) between control (■, n=12) and treated (□, n=10) groups was determined using the Mann-Whitney test (p<0.05). Results are expressed as the mean and standard error of the mean.
Figure 4.15: Effect of fenofibrate on allograft pathology at 8 weeks: Fenofibrate did not ameliorate TxAA, but significantly increased PR. Photomicrographs show representative vessels with TxAA scores ranging from 0 to 4. Statistical significance (*) between control (■, n=12) and treated (□, n=10) groups was determined using the Mann-Whitney test (p<0.05). Results are expressed as the mean and standard error of the mean.

4.4 Discussion

This chapter presents the first evaluation of fenofibrate treatment in a heterotopic murine cardiac transplant model where recipients and donors are mismatched across MHC-II. Fenofibrate administration resulted in distinct physiological and pharmacological effects on liver and plasma markers. Results show that at early times, fenofibrate had largely immunosuppressive effects and significantly reduced graft inflammatory cell infiltration. However, long-term fenofibrate treatment significantly augmented graft inflammatory cell infiltration and the expression of inflammatory molecules, thereby resulting in enhanced PR without amelioration of TxAA.

The choice of 0.2% wt/wt of fenofibrate is supported by a number of in vivo studies that used the same dose to achieve immunomodulatory effects [Hill et al 1999, Kockxx et al 1999, Murai et al 2002, Gervoys et al 2004]. In fact, this concentration is toward the high end of the range used in mice, with immunomodulatory and metabolic effects already present at a dose of 0.05% wt/wt of fenofibrate [Guerre-Milo et al 2000, Duez et al 2002]. Mice (with roughly comparable weights and food intake) on 0.2% wt/wt of fenofibrate-chow have plasma concentrations of up to 15 μM [Murai et al 2002]. This is comparable to the concentrations of fenofibric acid (active metabolite of fenofibrate) readily available in humans after fenofibrate administration [Masnatta et al 1996, Steed et al 2000, Lossner et al 2001] and to the dose required for IFNγ attenuation in vitro (as presented in the previous chapter).
The murine liver highly expresses PPARα and fenofibrate, as a PPARα agonist, induces peroxisomomal proliferation [Escher and Wahli 2000]. Increased liver weight is an established pharmacologic effect of PPARα ligand-activation [Fruchart et al 1998]. Results in this chapter are consistent with previously published increased liver weight following treatment with fenofibrate [Deplaque et al 2003]. Notably, the increase was observed within two weeks and subsequently continued to increase. Histological evaluation of the livers confirmed the presence of a physiological effect from the fenofibrate-laden chow and immunohistochemical staining of PMP-70 recapitulated results from the literature of PPARα activation [Lawrence et al 2001b]. The increases in ALT and, in particular, AST, strongly correlate with the total amount of fenofibrate consumed and likely reflect the increase in liver size (since there was no histologic evidence of liver damage). It should be noted that this effect is likely not present in humans, given that PPARα agonists do not induce peroxisome proliferation-associated genes in human hepatocytes [Lawrence et al 2001a].

The total increase in liver size may account for the lack of effect on fibrinogen. At 2 weeks, plasma fibrinogen displayed a strong tendency to decrease. However, by 8 weeks, fenofibrate had no significant effect on plasma levels of fibrinogen. This is contrary to observations from the clinical [Keating and Ormrod 2002] and rodent [Kockx et al 1999] literature. However, the clinical literature may not be particularly germane, given the differential expression and effects of PPARα activators on human hepatocytes [Fischer and Wahli 2001, Lawrence et al 2001a]. Insofar as the rodent literature, experiments were limited to two weeks in duration and the decreased fibrinogen expression was normalized to hepatocyte quantity [Kockx et al 1999]. By 8 weeks, liver weight is significantly higher in the treated mice and the lack of effect may be a result of having reached a threshold where the decrease in levels of fibrinogen made by hepatocytes is overwhelmed by the increase in hepatocyte size and number.

Fenofibrate decreased triglyceride levels, while elevating total cholesterol (the results at 8 weeks were statistically significant, while at 2 weeks, results constituted trends only likely due to small sample size). These findings are consistent with reports documenting that PPARα agonist administration decreases triglycerides and increases cholesterol levels in mice fed a standard diet [Olivier et al 1988, Fischer et al 2003]. As a clinical agent for dyslipidemia, the ability of fenofibrate to reduce triglyceride levels is firmly established [Keating and Ormrod 2001] and similar findings in mice fed a standard diet (and thus not exhibiting increased triglyceride levels) suggest that this is a particularly robust result that is not sensitive to negative feedback mechanisms. The effect of fenofibrate on total plasma cholesterol levels appears to be affected by whether baseline levels are associated with an underlying metabolic syndrome. In other words, when total cholesterol levels are elevated due to an underlying metabolic syndrome, fenofibrate treatment reduces total cholesterol in both clinical [Keating and Ormrod 2002] and experimental [Olivier et al 1988] settings. However, when total cholesterol levels are at baseline, experiments in murine models demonstrate that fenofibrate significantly increases total plasma cholesterol, by increasing HDL to a greater extent than reducing LDL [Olivier et al 1988].
Although hypoglycemic actions are traditionally ascribed to PPARγ agonists [Henry 1999], the present results show a significant reduction in glucose levels following PPARα agonist treatment. This effect has been previously observed in a murine model of endotoxemia [Hill et al. 1999] and potential mechanisms include a decrease in hepatic gluconeogenesis subsequent to decreased 11β-hydroxysteroid dehydrogenase-1 activity [Hennemann-Wosatka et al. 2000] and increased insulin sensitivity reducing adiposity [Guerre-Millo et al. 2000]. It should be noted that this regulation is a sexually dimorphic phenomenon, with females not exhibiting the same response to fenofibrate as their male counterparts [Yoon et al. 2002].

The effect of fenofibrate treatment on allograft expression of cytokines, chemokines and chemokine receptors at 2 weeks exhibited results roughly consistent with the in vitro results presented in the previous chapter, namely a decrease in IFNγ, CCR2 and CCR5, and increase in RANTES. However, by 8 weeks, fenofibrate displayed mixed results. The lack of effect on IL-10 and IL12-p35, together with an increase in IL12-p40, was hypothesized based on the results of the previous chapter. However, the increased mRNA expression of IFNγ, as well as IL-4 and IL-6, was contrary to expectations based of the previous chapter (where IFNγ was significantly and robustly reduced, while no effect was observed on IL-4 and IL-6). Similarly, while the tendency to increase RANTES was regarded as a potential effect, it was expected that fenofibrate would reduce the mRNA expression of CCR2 and CCR5 and not influence IP-10. Instead, CCR2 and CCR5 remained unchanged and IP-10 increased.

In the analysis of mRNA expression, results are normalized to the housekeeping gene and are thus proportional to cell number. While in splenocyte cultures, results expression closely corresponds to the numbers of lymphocytes, in the allografts, the correspondence is not as direct. The vast majority of the signal from the allograft RNA derives from myocytes rather than lymphocytes. Consequently, any potential increase in graft infiltration is not likely to be large enough to affect the housekeeping gene in proportion to the increased cytokine signal.

Consideration of this led to the hypothesis that the allograft expression of IFNγ (as well as other cytokines and chemokines) may be influenced by the extent of graft infiltration by inflammatory cells. It should be noted that masking of potentially anti-inflammatory activity of PPARα ligands by other effects has been previously reported; in a murine model of endotoxemia, fenofibrate decreased cellular TNFα production; yet mice on a fenofibrate diet had five-fold higher circulating TNFα levels upon stimulation with LPS and significantly lower lethal dose [Hill et al. 1999]. Accordingly, the increased mRNA expression of IFNγ (as well as IL-4, IL-6 and IP-10) seen in the 8-week allografts could be the result of an increased number of graft infiltrating cells, rather than an augmented expression of IFNγ by the leukocytes themselves.

Support for this hypothesis derives from the examination of graft infiltrating profiles, as well as functional effects of fenofibrate on recipient splenocytes upon restimulation ex vivo. At 2 weeks post transplantation and treatment, fenofibrate markedly diminishes graft infiltration by significantly reducing graft infiltrating CD4+ T cells and CD11b+
macrophages. This implies that the decreased cytokine and chemokine, as well as chemokine receptor mRNA expression at 2 weeks could be a result of diminished graft infiltration alone and requires the consideration of functional effects on leukocytes to determine the direct contribution of fenofibrate on a per cell basis. Examining this issue for IFNγ alone (given its particularly important role in allograft rejection as discussed in chapter 2) showed that, upon restimulation ex vivo with both same party (bm12), third party (B/c) stimulation, as well stimulation by a mitogenic agent (ConA), splenocytes from treated recipients produced significantly less IFNγ on the third day of culture (that corresponds to the first day of significant protein present in culture).

At 8 weeks, fenofibrate treatment significantly augmented the number of CD4+ T cells and CD11b+ macrophages infiltrating into the graft. The ability of CD4+ T cells to produce IFNγ upon stimulation is a fundamental property and there are a growing number of reports that document IFNγ production by antigen-presenting cells, including CD11b+ macrophages [Gessain and Belardelli 1998, Frucht et al 2002]. This implies that the increase in IFNγ could be a consequence of increased graft infiltration and once again, requires the consideration of functional fenofibrate-mediated effects on leukocytes. Upon restimulation ex vivo with either same party (bm12) or third party (B/c), splenocytes from 8-week recipients treated with fenofibrate produced significantly less IFNγ on the third day of culture (that corresponds to the first day when substantial concentrations of protein are present in culture).

Interestingly, the attenuation of IFNγ production in splenocytes from mice treated short term and restimulated ex vivo with same (bm12) party stimulators was not sustained for the entire duration of cultures. More evident is that lack of a sustained decrease in IFNγ production by splenocytes from 8-week treatment restimulated with both same (bm12) and third (B/c) party stimulators. This raised the question of whether this effect was a function of time, diminishing the ability of fenofibrate to influence IFNγ production. However, splenocytes from non-transplanted mice taken off the diet one week before harvest sustained the decrease in IFNγ for the entire duration of the culture. This suggests that time was not the issues but rather that an alteration in leukocytes activity following transplantation such that fenofibrate is less able to mediate its effects. One potential mechanism could be downregulation of T cell PPARα expression [Cimard et al 2002a, Jones et al 2002].

There are several potential mechanisms contributing to the overall increased graft infiltration in long-term allograft recipients treated with fenofibrate. At 2 weeks, despite an overall immunosuppressive effect, RANTES notably was not effect. This finding, taken together with the previously presented in vitro data showing a significant increase in RANTES expression from fenofibrate treatment (~20%), suggests that RANTES may be contributing to graft infiltration. Even a small increase in RANTES production can be significant due its particularly high level of expression in the alloresponse (dominant chemokine) and rejecting murine cardiac allografts (from this thesis). As graft inflammatory cells infiltration increases, net allograft expression of IFNγ also increases. This is likely responsible for the increased IP-10 expression seen at 8 weeks that, in turn, further contributes to the augmentation of graft infiltration.
Fenofibrate had time-dependent effects on graft expression of MHC-II, one of the markers of graft activation. At 2 weeks, graft expression of MHC-II was not influenced by fenofibrate, while at 8 weeks it decreased. The lack of an effect at 2 weeks is consistent with findings that fenofibrate had no effect on IFNγ-induced MHC-II promoter activity of the major transactivator factor CIITA in macrophages [Kwak et al. 2002]. By 8 weeks, however, despite an increase in IFNγ, there is a small, yet statistically significant decrease in MHC-II. This is contrary to expectations based on overall graft expression of IFNγ, but may be attributable to the increased expression of IL-4 in the graft, given the reported ability of IL-4 to induce the production of PPARγ ligands [Huang et al. 1999] and subsequent, profound attenuation of MHC-II by PPARγ activation at the transcriptional level by inhibiting promoter activity [Kwak et al. 2002].

There were no statistically significant changes at either time points in the expression of the adhesion molecules, VCAM-1 and ICAM-1, two other markers of graft activation. However, the observed trends are still worth discussing in light of the reported effects of PPARα activators on these molecules [Marx et al. 1999b, Rival et al. 2002, Deplanque et al. 2003], as well as their relevance to transplantation [Lee et al. 1999, Suzuki et al. 1999] and the responsiveness to IFNγ [Marx et al. 1999b]. It should be noted that, while the inhibitory effects on VCAM-1 are consistent and well documented [Marx et al. 1999b, Rival et al. 2002, Deplanque et al. 2003], the effects on ICAM-1 are somewhat controversial [Marx et al. 1999b, Deplanque et al. 2003].

At early times, fenofibrate tended to decrease the expression of both graft adhesion molecules, VCAM-1 and ICAM-1. This is consistent with the literature describing a decrease following fenofibrate treatment in vivo [Deplanque et al. 2003]. The decrease in VCAM-1 is also consistent with findings in vitro [Marx et al. 1999b, Rival et al. 2002]. The decrease in ICAM-1 may be reconciled with in vitro literature [Marx et al. 1999b] by considering that IFNγ expression was reduced in the treated grafts, leading to a decrease in ICAM-1 independent of PPARα activation.

By 8 weeks, there was essentially no difference in VCAM-1, while ICAM-1 tended to increase. These findings may be once again reconciled with the literature by considering the expression of IFNγ; contrary to expectations, IFNγ expression increased in long-term fenofibrate-treated allografts. This may account for the tendency of ICAM-1 to increase, given its responsiveness to IFNγ [Marx et al. 1999b], and may shed a different perspective on the effect on VCAM-1. According to its induction by IFNγ, VCAM-1 should have increased, such that the lack of an increase may actually be a reflection of the suppressive effect of fenofibrate on VCAM-1, thereby making it consistent with the literature [Marx et al. 1999b, Rival et al. 2002, Deplanque et al. 2003].

Given the central role of IFNγ in allograft rejection [Hidalgo and Halloran 2002] and the overall increase in allograft IFNγ expression, it is not surprising that long-term allografts of fenofibrate-treated recipients display increased PR, without amelioration of TxAA. Moreover, potential effects from the augmented mRNA expression of other cytokines and
chemokines on allograft pathology are also worth considering, particularly with respect to potential effects on PR and TxAA.

The augmented intragraft expression of IL4, IL-6, and IP-10 at 8 weeks following continual fenofibrate treatment of recipients is likely not beneficial for cardiac allografts, given the roles of these mediators in transplant rejection. Although controversial, especially in light of the generally accepted paradigm that IL-4 is a “protective” cytokine, a review of the literature highlights several reports indicating that increased graft expression of IL-4 is deleterious [Piccotti et al 1997]. IL-6, typically considered a “pro-inflammatory” cytokine, plays a role in the development of PR [Deng et al 2002] and increased concentrations are associated with an increased incidence of clinical TxAA [Gullestad et al 1999]. IP-10, particularly when donor derived, has a pivotal role in the development of PR; mice congenitally deficient in IP-10 showed reduced graft infiltration and injury [Hancock et al 2001]. Although persistent PR is not required for the development of TxAA [Izuani et al 1995, Nagano et al 1998], studies have found that episodes of PR may potentiate the development of TxAA [Hullet et al 1996, Nakagawa et al 1995].

The relevance of the increase in graft expression IL12-p40 merits discussion based on its high level of mRNA expression (same order of magnitude as IFNγ mRNA). Given that the formation of functional IL-12 requires an increase in IL12-p35 and IL12-p40 (to form the p35/p40 dimer or p75), the increase in IL12-p40 alone affect IFNγ expression [Puccetti et al 2002]. Several disease states are associated with increased IL12-p40 [Abdi 2002]. However, its role has not been specifically addressed in transplantation and thus discussion is limited to extrapolations from the literature. Along these lines, the finding that IL12-p40 inhibits T lymphocyte generation [Abdi and Hermann 1997], suggests potential benefit in the attenuation of the alloresponse. On the other hand, increased IL12-p40 is associated with pulmonary fibrosis, suggesting a potentially profibrotic and consequently deleterious for TxAA [Huaux et al 2002].

In conclusion, fenofibrate treatment reduces early graft expression of IFNγ, as well as other proinflammatory genes, with concomitant reductions in IP-10, CCR2 and CCR5. This results in a significantly diminished mononuclear cell infiltrate. However, the tendency to increase RANTES (despite the decrease in IFNγ), an effect that is consistent with the in vitro results presented in chapter 3, is likely one of the mechanisms contributing toward the eventual increase in graft infiltration (as the effect of RANTES becomes more dominant). The increase in long-term graft inflammatory cell infiltrate eventually results in an overall increase in the graft expression of IFNγ, although fenofibrate does continue to attenuate production of IFNγ on a per cell basis.

Moreover, the net effect is a significantly increased level of PR, without amelioration of TxAA. This suggests that in order to fully realize the benefit of diminished cytokine production by fenofibrate in the graft, concomitant administration of agents reducing graft infiltrate may be required. This may involve either agents directly attenuating recruitment by targeting the chemokine or chemokine receptors, or agents directed at
preventing adhesion and/or extravasation of circulating lymphocytes into the donor vasculature.
Chapter 5. Role of PPARα in vitro and in vivo

5.1 Introduction

Generally, PPAR ligands are ascribed a particular receptor specificity and receptors are ascribed specific activation and signaling pathways. However, there is growing evidence for “cross-talk” between various ligands and receptors. At selected doses, typical PPARα ligands may activate PPARγ in addition to PPAR, and vice versa [Escher and Wahli 2000]. This phenomenon may result from the large PPAR binding pocket, conducive to the accommodation of a variety of ligands [Escher and Wahli 2000]. Studies have also shown “cross-talk” between other nuclear receptors. For example, pituitary-dependent hormones specifically regulate PPARα expression in the liver [Jalouli et al 2003], while activation of the growth hormone receptor leads to activation of a signal transduced and activator of transcription (STAT) protein that can inhibit PPARγ-regulated transcription [Shipley and Wainman 2003].

The development of mice congenitally deficient in PPARα [Lee and Gonzalez 1996] potentially allows the elucidation of ligand and receptor specificity, as well as the accurate identification of processes regulated by PPARs. One of the primary PPARα targets is the liver, and studies have documented the specificity of PPARα-mediated effects, e.g. the reduction in the hepatic activity of 11β-hydroxysteroid dehydrogenase [Hermanowski Vosatka et al 2003] and expression of fibrinogen [Kockx et al 1999]. Recent evidence, using chimeric livers, has also shown that peroxisomal proliferation does not require PPARα in all hepatocytes [Weygand and Sandgren 2004]. Several experiments have examined PPARα-mediated cardiac actions. Myocardial pump function and male mortality were negatively affected in mice lacking PPARα and overexpressing lipoprotein lipase [Nothhammer et al 2003]. Congenital deficiency of PPARα protected against myocardial ischemic injury and improved endothelial vasodilatation [Tabernero et al 2002]. Finally, mice congenitally deficient in PPARα display an exaggerated response to lipopolysaccharide with significantly increased plasma levels of IL-6 [Defrere et al 1999] and TNFα [Hill et al 1999]. Demonstrating the relative a-specificity of fenofibrate, treatment with that agent in PPARα-deficient mice had no effect on IL-6 production [Gervois et al 2004].

This chapter utilizes mice congenitally deficient in PPARα to explore the specificity of the fenofibrate-mediated immunomodulatory effects in vitro (specifically with respect to IFNγ) and to identify potential regulatory roles of PPARα in the expression of selected cytokines, chemokines and chemokine receptors. It also examines the influence of congenital PPARα deficiency in vivo on both donors and recipients in murine heterotopic cardiac transplantation between strains disparate in MHC-I and MHC-II (a model of PR).
5.2 Methods

Animals and Diet

Inbred strains, 129Sv (129WT) and Balb/c, were purchased (Jackson Laboratories, Bar Harbor), while breeding pairs of mice congenitally deficient in PPARα (PAKO) on a 129Sv background were a generous gift from Dr. Jorge Plutzky. Mice were housed in the animal facility of Harvard Medical School on acidified water and chow ad libitum, as previously described, and experiments were done according to protocols approved by the animal care committee.

Mixed Leukocyte Reaction

The technique used for splenocyte isolation and culture in a mixed leukocyte reaction (MLR) is fully described in chapter 3. Experimental cultures were performed in vitro and used Balb/c splenocytes as stimulators with either 129WT or PAKO as responders. In the cultures testing fenofibrate specificity, samples were plated in quadruplicate and proliferation was measured on days 2 through 5. In the cultures testing for PPARα regulation, mRNA was isolated on days 2 through 5.

Enzyme-Linked Immunosorbent Assays

The concentration of IFNγ in the supernatants of the proliferation assays was determined using enzyme-linked immunoassays (ELISAs), per the manufacturer’s protocol (Pharmingen, San Diego). Details of the procedure are fully described in chapter 3. Optical density was measured using an ELISA plate reader and converted to pg/ml using a calibration curve prepared from recombinant IFNγ standards.

Propidium iodide staining

Details of this procedure have been described in chapter 3. Briefly, splenocytes from both 129WT and PAKO mice were incubated with 1 μg/ml of ConA for 12 hours, at which time point, cells were directly stained with propidium iodide (PI) and immediately analyzed by flow cytometry.

RNase Protection Assay

Details of this procedure are fully described in chapter 3. In brief, RNA from either cultures or allografts was isolated using TRIZOL (Invitrogen, Carlsbad). For in vitro samples, aliquots of 5 μg mRNA were prepared, while for the allograft samples, aliquots were prepared containing 10 μg of RNA. Aliquots were stored in RNase-free DNase-free water at −70°C and analysis of mRNA expression was performed via the RNase protection assay (RPA). Volume integrations of bands were normalized to GAPDH in the corresponding lane, using ImageQuant (Molecular Dynamics). Results of triplicate samples are presented as the average and standard deviation per group.
Heterotopic heart transplantation

As previously described, murine cardiac transplantation [Corry et al 1973, Furukawa et al 2002] was performed in accordance with protocols approved by the Harvard Medical School Animal Care Committee. In brief, the donor heart was removed after administration of inhalation anesthesia and injection of cold heparinized saline. The donor aorta and pulmonary artery were ligated to the recipient abdominal aorta and inferior vena cava, respectively. At the time of harvest, the allograft was divided transversely in three sections, representing the base, middle section and apex of the allograft, each to be used respectively for histological, immunohistochemical and RNA analysis. Transplants (n=11) were divided into four groups, with Balb/c (B/c) donors for both 129WT and PAKO recipients (2 groups), and 129WT and PAKO donors with B/c as recipients (2 groups). Allografts were harvested at 1 week post-transplant.

Histological staining and scoring

The base of the heart and liver sections were fixed in 10% buffered formalin for staining with hematoxylin and eosin. The heart was also stained for elastic tissue and was scored for extent of PR. The grading scheme for PR was modified from the International Society for Heart and Lung Transplantation scale and is described as follows: 0, no rejection; 1, mild interstitial infiltrate without necrosis; 2, focal interstitial with necrosis; 3, multifocal interstitial with necrosis; 4, widespread infiltrate with hemorrhage and/or vasculitis.

Immunohistochemical staining and scoring

The methodology used has been described in chapter 4. Briefly, the middle transverse section of the cardiac grafts and sections of the recipient liver were frozen at −80°C until analysis of MHC-II, ICAM-1, VCAM-1, CD4, CD8 and CD11b. Slides were air dried and fixed with paraformaldehyde. Sections were blocked with hydrogen peroxide, followed by blocking with either rabbit or goat serum, in addition with avidin and biotin. Primary antibody was applied and if applicable, a biotinylated secondary antibody was also used. Chromogen was added and development observed under a microscope until isotype controls showed early substrate reduction. Slides were then counterstained in hematoxylin and washed under running water. Heart sections were scored (3 blinded observers) according to the following scheme: 0, no staining; 1, focal weak; 2, focal moderate, diffuse weak; 3, focal strong, diffuse moderate; 4, diffuse strong.

Data Analysis

Results in bar and line graphs represented as the mean and standard error of the mean. Statistical significance for staining results was determined using the Mann-Whitney test for non-parametric value with a two-tailed, p < 0.05. For immunohistochemical staining, the Kruskal-Wallis test ensured that there was no statistically significant difference between the three, blinded observers. Statistical significance of other values was determined using the Student’s t-test for unpaired samples with two-tailed and a p value less than 0.05.
5.3 Results

In vitro

The *in vitro* evaluation of congenital deficiency of PPARα had two aims. The first was to establish if the effects of the fenofibrate doses previously employed to decrease IFNγ were largely attributable to pure PPARα activation. The second was to identify whether PPARα plays a regulatory role in the expression IFNγ and RANTES, as well as other cytokines, chemokines and chemokine receptors.

Figure 5.1 presents the evaluation of varying doses of fenofibrate on IFNγ production in the alloresponse using B/c stimulators and both WT and PAKO responders. At 20 μM (the dose previously identified as “therapeutic” based on the significantly attenuation of IFNγ, without effects on proliferation), fenofibrate significantly reduces the production of IFNγ in cultures with both 129WT and PAKO responders. This suggests “cross-talk” with either PPARγ or PPARδ, or a potentially drug-specific and PPAR-independent effect. At doses higher than 20 μM, fenofibrate reduces both proliferation and IFNγ production, indicating potential toxicity. To determine whether this was a result of toxicity, propidium iodide staining was performed at each of the fenofibrate doses. Figure 5.2 shows that doses of 4 and 20 μM do not affect cell viability, while doses of 100 and 500 μM result in significant cell death. PPARα deficiency results in no observable differences in fenofibrate toxicity at these concentrations.

The identification of PPARα regulatory roles was performed by examining the change in mRNA expression resulting from PPARα deficiency in responders compared to WT. Figure 5.3 shows that, when stimulated with B/c splenocytes, PAKO responders express higher levels of IFNγ compared to WT, thereby indicating a regulatory role in the expression of IFNγ. PPARα deficiency also increased expression of IL-6 and IL-12p40, also indicating PPARα regulation. The lack of an increase in IL-12p35, despite the increase in IL-12p40, implies that there is no increase in the expression of IL-12, further pointing to a direct regulatory role of PPARα in IFNγ expression. Figure 5.4 displays the influence of PPARα deficiency on chemokine and chemokine receptor expression (RANTES, IP-10, CCR2 or CCR5). At early times, PPARα had no significant effects on the expression. However, late in culture, when there is a significantly elevated level of IFNγ in culture, congenital deficiency of PPARα has significant effects. Based on traditional knowledge of IFNγ-mediated effects, elevation in IFNγ should increase RANTES. However, PPARα deficiency significantly decreases expression of RANTES, indicating a direct regulatory role of PPARα. This is consistent with the significant increase in RANTES expression observed with fenofibrate activation of PPARα. Taken together, these suggest a direct regulatory role of PPARα on RANTES expression that is dominant over the influence of IFNγ. The increased expression of IP-10, CCR2 and CCR5 are consistent with an elevation of IFNγ (again based on traditional knowledge of IFNγ-mediated effects) and thus does not constitute evidence of direct PPARα regulation.
In vivo

The *in vivo* evaluation of congenital PPARα deficiency involved the examination of the influence on PR in the heterotopic murine cardiac transplant model, particularly on graft infiltration. The effect on TxA2 could not be evaluated because of the background strain of the PAKO mice (129Sv) and consequent unavailability of an MHC-II mismatched strain. A limited number of mice were available per treatment arm; consequently, emphasis is placed on the examination of trends, in addition to statistical significance.

The first set of transplants examined the effect of congenital PPARα deficiency on the allograft recipient. Figure 5.5 shows that PAKO recipients have significantly more CD11b+ graft infiltrating cells (146±14 vs. 208±22 cells per high power field; p<0.05) and fewer CD8+ cells (17±4 vs. 3±1 cells per high power field; p<0.05), with the former result being more substantial due to the relative quantities of cell types. Infiltration by CD4+ cells is not significantly affected. There are no statistically significant effects on MHC-II, VCAM-1, or ICAM-1, as shown in figure 5.6; however, both MHC-II and VCAM-1 display a tendency to increase, while ICAM-1 tends to decrease. Examination of graft mRNA cytokine expression, presented in figure 5.7, reveals a tendency toward increased IFNγ expression, providing *in vivo* corroboration for PPARα regulation in IFNγ expression. Results of chemokine and chemokine receptor expression are shown in figure 5.8. There is a slight tendency for RANTES to decrease, which would be consistent with *in vitro* results. Furthermore, IP-10 and MCP-1 tend to increase. Pathology results, shown in figure 5.9, show that substantial differences arise from PPARα deficiency on the recipients; allografts from PAKO recipients display the formation of thrombi, in the absence of vascular edema, while those from WT recipients show edema without thrombus formation. This likely represents systemic effects (such as elevated fibrinogen levels) overwhelming local ones. Consistent with an increased graft infiltrate, PR modestly increases with PPARα recipient deficiency.

The second set of transplants examined the effect of PPARα deficiency on donors. PPARα donor deficiency affects augmented graft infiltration, as shown in figure 5.10, by increasing CD4+ (19±3 vs. 71±10 cells per high power field; p<0.05) and CD11b+ (142±23 vs. 204±15 cells per high power field; p<0.05) graft infiltrating cells, without affecting the number of CD8+ cells. As presented in figure 5.11, donor deficiency has mixed effects on graft activation; there is a decrease in MHC-II (trend) and ICAM-1 (significant), while VCAM-1 tends to increase. With respect to cytokine expression, presented in figure 5.12, PPARα deficiency on donors clearly tended to increase graft expression of IFNγ, once again providing *in vivo* confirmation of *in vitro* results. In addition, IL-4, IL-6 and IL-12 tend to increase. Most notable among the effects on chemokines and chemokine receptors, shown in figure 5.13, is a clear trend towards decreased RANTES expression, which would be consistent with the *in vitro* results. Pathology results are summarized in figure 5.14. Contrary to PPARα deficiency on recipients, PPARα deficiency on donors results in edema, without thrombus formation. The pathological appearance of allografts with and without donor PPARα deficiency is not substantially different. Consistent with an increased graft infiltrate, PR modestly increases.
Figure 5.1: Varying doses of fenofibrate *in vitro* with 129WT and PAKO responders: MLR with B/c stimulators and a, b) 129WT responders or c, d) PAKO responders. For both, the left panel represents proliferation, while the right panel represents IFNγ production. No effect is seen on either proliferation or IFNγ production at 4 μM. At 20 μM, however, fenofibrate significantly reduces the production of IFNγ in cultures with both 129WT and PAKO as responders. This indicates that fenofibrate either "cross-talks" with other PPARs (notably PPARγ or PPARδ) or has drug-specific and thus completely PPAR-independent effect. At doses higher than 20 μM, fenofibrate reduces both proliferation and IFNγ production, indicating potential toxicity (to be specifically addressed in the next figure). The bolded lines represent the day of peak proliferation and initial IFNγ production, respectively. Representative results are shown as the average and standard deviation of triplicate samples.
Figure 5.2: Cell viability after a 12 hour incubation with fenofibrate: flow cytometric analysis of PI staining of ConA-stimulated a) 129WT and b) PAKO splenocytes. The light-colored line represents control cultures (with no fenofibrate) while the filled area, represents splenocytes incubated with varying fenofibrate doses. A shift toward the M1 marker represents increased cell membrane permeability, allowing the propidium to bind, and thereby denoting cell death. Doses of 4 and 20 μM of fenofibrate did not affect cell viability in any culture, while doses of 100 and 500 μM resulted in significant cell death.
Figure 5.3: Influence of PPARα deficiency on cytokine mRNA expression in vitro: When stimulated by allomismatched B/c splenocytes, PAKO splenocytes express substantially higher levels of IFNγ compared to WT, indicating a regulatory role in the expression of IFNγ. Similarly, PPARα also significantly increases expression of IL-6 and significantly decreases expression of IL-12p40, thereby denoting regulatory role in the expression of both of these cytokines. The lack of an increase in IL-12p35 indicates that there is no increase IL-12 and corroborates a PPARα-dependent role in the regulation of IFNγ. Results are normalized to the expression of GAPDH and observed on the day of maximal cytokine expression (day 2 for IFNγ and day 1 for the remainder).
Figure 5.4: Influence of PPARα deficiency on chemokine expression *in vitro*: The early (and maximal) expression of RANTES, IP-10, CCR2 or CCR5 are not affected by PPARα deficiency. This is similar to the findings in chapter 3, where PPAR activation had no significant effects on chemokine expression. However, late in culture, PPARα deficiency significantly decreases expression of RANTES and increases IP-10, CCR2 and CCR5. The significantly elevated IFNγ levels and known IFNγ-mediated effector functions suggest that IP-10, CCR2, and CCR5 should also significantly increase. This is, in fact, observed and indicates that the effect is IFNγ-mediated and thus only indirectly influenced by PPARα. On the other hand, the decrease in RANTES (in the setting of increased IFNγ, which normally increases RANTES) suggests a direct PPARα regulatory role on RANTES expression. Results are normalized to the expression of GAPDH. Early and late refer to days 2 and 3, respectively.
Figure 5.5: Influence of recipient PPARα deficiency on allograft cellular infiltration:
The dominant graft infiltrating cell subtype is the CD11b+ macrophage and PPARα recipient deficiency results in significantly increased CD11b+ graft infiltration. Despite a small attenuation in CD8+ T cells, overall graft infiltration is augmented. This indicates that PPARα is not necessary for graft infiltration. Five fields were counted per section and statistical significance was tested using the Student’s t-test (p < 0.05). Allografts were harvested at 1 week post-transplantation.

Figure 5.6: Influence of recipient PPARα deficiency on allograft activation markers:
Although there are no statistically significant differences and thus no overall effect, the trends merit some mention. MHC-II and VCAM-1 tend to increase, while ICAM-1 tends to decrease. Sections were evaluated according to the following scheme: 0, no staining; 1 focal weak; 2 focal moderate, diffuse weak; 3 focal strong, diffuse moderate; 4 diffuse strong. Statistical significance (not affected by observer) was tested using the Mann-Whitney test (p<0.05). Allografts were harvested at 1 week post-transplantation.
Figure 5.7: Influence of recipient PPARα deficiency on cytokine expression: The most marked and noticeable trend is the increase in IFNγ mRNA expression. This is consistent with the in vitro results demonstrating a regulatory role for PPARα in IFNγ expression. The lack of statistical significance may be a result to the low number of samples in this set of transplants and the typical heterogeneity exhibited by transplant recipients. D and R refer to donor and recipient, respectively. Statistical significance was tested using Student’s t test (p<0.05). Results are normalized to the GAPDH housekeeping gene and are represented as the mean and standard error of the mean. Allografts were harvested at 1 week post-transplantation.
**Figure 5.8:** Influence of recipient PPARα deficiency on chemokine and chemokine receptor expression: There is a slight tendency to decrease RANTES, consistent with *in vitro* studies. In addition, there is a tendency to increase IP-10 and MCP-1. D and R refer to donor and recipient, respectively. Statistical significance was tested using Student's t test (p<0.05). The bar indicates the mean value and standard error of the mean. Results are normalized to GAPDH expression. Allografts were harvested at 1 week post-transplantation.

**Figure 5.9:** Influence of recipient PPARα deficiency on allograft pathology: PAKO recipients display the formation of thrombi, in the absence of vascular edema (see photomicrograph of sample vessel). The presence of PPARα, on the other hand, induces edema and does not result in thrombi formation. These results suggest effects of systemic factors, such as increased fibrinogen levels in PAKO mice, on local allograft pathology. Consistent with an increased graft infiltrate, PR is modestly increased in the PAKO recipients.
Figure 5.10: Influence of donor PPARα deficiency on allograft infiltration: PAKO donor allografts have significantly increased numbers of CD4+ and CD11b+ graft infiltrating cells (CD8+ cells are not affected). Interestingly, PPARα deficiency on recipients had no effects on CD4+ but rather influenced graft infiltration by CD8+ cells. Overall, graft infiltration by inflammatory cells is augmented. This also suggests that PPARα is not necessary for this process. Five fields were counted per section and statistical significance was tested using the Student’s t-test (p < 0.05). Allografts were harvested at 1 week post-transplantation.

Figure 5.11: Influence of donor PPARα deficiency on allograft activation: While VCAM-1 tends to increase, MHC-II tends to decrease and ICAM-1 is significantly attenuated in PAKO donors. Sections were evaluated as follows: 0, no staining; 1 focal weak; 2 focal moderate, diffuse weak; 3 focal strong, diffuse moderate; 4 diffuse strong. The Kruskal-Wallis test was used to ensure that there were no significant differences between observers. Statistical significance was determined using the Mann-Whitney test (p<0.05). Results are expressed as the mean and standard error of the mean. Allografts were harvested at 1 week post-transplantation.
Figure 5.12: Influence of donor PPARα deficiency on cytokine expression: PPARα donor deficiency tends to increase graft IFNγ expression, consistent with the in vitro findings supporting a regulatory role of PPARα in IFNγ expression. In addition, PPARα donor deficiency tends to decrease IL-10 and increase IL-4, IL-6 and IL-12. D and R refer to donor and recipient, respectively. Results are normalized to the GAPDH housekeeping gene and are represented as the mean and standard error of the mean. Statistical significance was tested using Student’s t test (p<0.05). Allografts were harvested at 1 week post-transplantation.
Figure 5.13: Influence of donor PPARα deficiency on chemokine and chemokine receptor expression: There is a tendency to decrease RANTES, consistent with in vitro studies. In addition, donor deficiency of PPARα tends to increase MIP-2 and decrease MCP-1, MIP-1α and MIP-1β. D and R refer to donor and recipient, respectively. Statistical significance was tested using Student’s t test (p<0.05). The bar indicates the mean value and standard error of the mean.

Figure 5.14: Influence of donor PPARα deficiency on allograft pathology: Deficiency of PPARα on donors results in edema, without thrombus formation (see photomicrograph of sample vessel). The pathological appearance of allografts with and without donor PPARα is not substantially different. Consistent with an increased graft infiltrate, PR modestly increases.
5.4 Discussion

This chapter utilized mice congenitally deficient in PPARα to explore the specificity of the fenofibrate-mediated immunomodulatory effects in vitro. It specifically addressed the production and expression of IFNγ, in addition to the expression of a number of other cytokines, chemokines and chemokine receptors. This chapter also investigated the influence of PPARα in vivo on parenchymal rejection, via murine heterotopic cardiac transplants between strains mismatched in both MHC-I and MHC-II, where either the donors or recipients were congenitally deficient in PPARα.

The findings in this chapter support PPARα-independent, as well as PPARα-dependent, effects on IFNγ. At 20 μM, fenofibrate reduces the in vitro production of IFNγ by splenocytes congenitally deficient in PPARα, without affecting cell proliferation or viability. This suggests the existence of cross-talk that could possibly exist at several levels; one is at the level of receptor, since there is evidence that at particular doses, ligands initially specific to one PPAR isoform, can also begin to activate others [Escher and Wahl 2000]. There could also be drug-specific effects or cross-talk downstream at the level of signaling molecules, for example on IκB, as with PGJ2 [Rossi et al 2000].

This raises the issue of whether the PPARα agonist effect on IFNγ production was mediated even in part by PPARα. Data in chapter 3 documented a significant (~50%) decrease in IFNγ expression and production upon PPARα activation by two different ligands (fenofibrate and WY14643). This leads to the hypothesis that if PPARα were involved in the inhibition (and/or regulation) of IFNγ, then deficiency in PPARα should lead to increased expression of IFNγ. Indeed, upon stimulation with allomismatched cells in the MLR, PPARα-deficient splenocytes expressed higher levels of IFNγ message, suggesting that, independently of possible fenofibrate-specific effects, PPARα does play a regulatory role in the expression of IFNγ.

Other cytokines significantly affected in vitro by congenital PPARα deficiency were IL-6 and IL-12p40. The increased expression of IL-6 is entirely consistent with previous reports documenting inhibition of IL-6 production by vascular smooth muscle cells upon PPARα activation by several ligands [Staels et al 1998] and an increase in IL-6 expression by cells from PAKO lipopolysaccharide-stimulated male mice [Delerive et al 1999]. Regarding IL-12p40, there is no prior literature available for comparison. Data in previous chapters demonstrated a small but significant augmentation IL-12p40 resulting from PPARα ligand-activation. If this effect were indeed mediated by PPARα, then congenital deficiency of PPARα should lead to a decrease in IL12-p40. This hypothesis finds support in the attenuated expression of IL12-p40 by the PAKO splenocytes. The other subunit of IL-12, namely II12-p35, is not significantly affected, such that functional IL-12 (which requires both subunits) is not increased. This further suggests that the PPARα-mediated regulation of IFNγ is direct and not through upstream cytokine signaling pathways.
Early in culture, PPARα deficiency has no significant effects on the mRNA expression of either chemokines or chemokine receptors. This is consistent with results in chapter 3 where PPARα ligand-activation also had no observable effects. However, late in culture, as in the setting of PPARα ligand-activation, significant effects are observed. Despite the four-fold increase in IFNγ, the expression of RANTES significantly decreases. This indicates that PPARα plays a regulatory role in the expression of IFNγ, and that furthermore this role overrides IFNγ-mediated pathways (which would have led to an increase in RANTES expression). These results are consistent with the increase in RANTES expression achieved with fenofibrate during the alloresponse (shown in chapter 3) and also suggest that the attenuation of RANTES expression observed with WY activation may have been a PPARα-independent or drug-specific effect (also presented in chapter 3). As expected, based on augmented IFNγ expression and downstream effects, the expression of IP-10 increased, as well as that of CCR2 and CCR5; thus, the effect of PPARα deficiency is mediated by IFNγ and only indirectly influenced by PPARα. Comparisons to the literature cannot be made because there is no literature available on PPAR effects on the alloresponse and, as shown in chapter 3, results using mitogenic stimulation do not necessarily translate to the alloresponse.

Congenital deficiency of PPARα on recipients (implying circulating leukocytes) influences the immunohistochemical appearance of the allografts. The dominant cell type in the acutely rejecting grafts is CD11b+, constituting approximately 75% of all graft infiltrating cells. Allografts from the PAKO recipients have a significantly increased number of CD11b+ cells (~30%), while graft infiltration of CD8+ cells is significantly attenuated (75%); however, given that the CD11+ cells are vastly more numerous, the overall effect is an increase in graft infiltration. This suggests that PPARα is not necessary for graft infiltration. Furthermore, augmented cell infiltration is consistent with the tendency of graft activation markers to increase (MHC-II and VCAM-1), although ICAM-1 tends to decrease, suggesting differential sensitivity to PPARα deficiency. Overall, the inflammatory state of the allografts is worsened in the absence of recipient PPARα.

Analysis of intragraft cytokine, and chemokine profiles, corroborates the presence of increased inflammation. Consistent with the above-discussed *in vitro* findings, PPARα deficiency on recipients (i.e. on the circulating lymphocytes) tends to increase intragraft expression of IFNγ. Based on the potent inflammatory activities of IFNγ, this may explain the tendency to increase MHC-II and VCAM-1 expression. The expression of ICAM-1 is unexplained based on IFNγ expression, but may be possibly be affected by mechanisms discussed below. The chemokines, IP-10 and MCP-1 tended to increase, potentially contributing to the increased inflammatory infiltrate.

Allograft pathology of recipients congenitally deficient in PPARα is substantially different compared to WT recipients. The allografts from PAKO recipients demonstrated widespread thrombi without vascular edema, compared to WT recipients that displayed the opposite, namely the presence of edema and absence of thrombi. This is similar to the results achieved with IFNγ knock-out recipients of completely allomismatched grafts [Nagano et al. 1998]. However, the mechanism is likely to be different since PAKO
recipients have increased levels of IFN$\gamma$. It is more likely that the thrombus formation is associated with increased systemic fibrinogen concentration that manifests itself in PAKO mice [Kockx et al. 1999]. This may, in turn, predispose recipients to an increased chance of clotting; in fact, fibrinogen has been associated as an independent risk factor for stroke and coronary artery disease [Olivier 1998]. This also suggests that in GKO recipients, compensatory mechanisms may result in the upregulation of systemic markers that dominate over the local environment. The PPAR literature offers precedent for this type of phenomenon; fenofibrate reduced lipopolysaccharide-stimulated TNF$\alpha$ production in aortic smooth muscle, yet the circulating values of TNF$\alpha$ were five-fold higher in mice treated with fenofibrate [Hill et al. 1999].

Congenital deficiency of PPAR$\alpha$ on donors (on graft endothelial and vascular wall cells) also influences the immunohistochemical appearance of the allografts. With respect to graft infiltration, PPAR$\alpha$ deficiency significantly changed not only the number, but also the relative proportions of infiltrating cells. The dominant cell type in the acutely rejecting grafts is CD11b$^+$; donor PPAR$\alpha$ deficiency significantly increases the number of CD11b$^+$ cells (~30%), as well as triples the number of CD4$^+$ cells. This also shifts the balance between CD4$^+$ and CD8$^+$ cells, which contribute roughly equally to the graft inflammatory milieu. Overall, these results indicate that PPAR$\alpha$ is not necessary for graft infiltration. With respect to graft activation, results are mixed; VCAM-1 tends to increase, while MHC-II tends to decrease and, notably, ICAM-1 is significantly attenuated (p<0.05). This demonstrates a clear difference between the VCAM-1 and ICAM-1 adhesion molecule sensitivity. Differential effects of these two adhesion molecules in pathological states are evident from the literature; for example, VCAM-1 but not ICAM-1 is critical in the initiation of traditional atherosclerosis [Cybulsky et al. 2001]; in the transplant setting, antibody treatment targeting ICAM-1 was associated with IL4 and IL-10, while antibody treatment targeting VCAM-1 was associated with an IFN$\gamma$ phenotype [Suzuki et al. 1999].

Analysis of intragraft cytokine profiles shows that, similar to PAKO recipients, congenital PPAR$\alpha$ deficiency on donors also tends to increase the expression of IFN$\gamma$. This may explain the tendency of VCAM-1 expression to increase; however, it is paradoxical to the significant decrease in ICAM-1 expression. As mentioned above, there may be a correlation to IL-10, which is the only cytokine tested that tended to decrease, thereby suggesting a possible connection. Recent evidence suggests that IL-10 may play a part in ICAM-1 regulation [Scuderi et al. 2003]. Another potential mechanism involves the retinoic acid receptor (RAR) to which PPARs must heterodimerize in order to bind to the PPRE (as described in chapter 2); RAR activation inhibits ICAM-1 expression at the transcriptional level [Aoudjit et al. 1994, Combe et al. 1995]. Deficiency of PPAR$\alpha$ may induce dysregulation of its required co-receptor, leading to increased activation and subsequent inhibition of ICAM-1. Regarding possible downstream consequences of ICAM-1 suppression, it should be noted that murine cardiac transplant studies using donors congenitally deficient in ICAM-1 did not affect either allograft survival in rejecting hearts or lesion development [Raisky et al. 2001], such that this is not a critical mediator of allograft rejection mechanisms.
Donor-deficiency of chemokines, however, may have significant effects on allograft survival; for example, using mice congenitally deficient in IP-10, investigators identified a critical role for IP-10 in the initiation and development of PR and allograft survival [Hancock et al 2001]. PPARα donor deficiency tended to increase the average expression of IP-10, which would suggest a worsening of PR. Such an increase in graft infiltration is, in fact, observed. Since grafts were all harvested at one week (without letting the allograft fail) the effect of PPARα on allograft survival was not formally investigated. Furthermore, PPARα donor deficiency clearly tended to decrease RANTES expression, confirming the previously described regulatory role of PPARα on RANTES.

Interestingly, contrary to the substantial differences elicited by congenital deficiency of PPARα on the recipient, congenital deficiency of PPARα on the donors do not markedly affect allograft pathology. The grade of PR is modestly increased. However, edema is present in both, with complete absence of thrombi. The lack of appearance of thrombi in the grafts from PAKO recipients supports the contribution of systemic factors, such as increased fibrinogen expression in PAKO hosts, to the local graft environment. Certainly, other graft-dominant hitherto-unrecognized effects may be playing critical roles. Part of the complexity of examining the effects of PPARα deficiency is the still evolving understanding of basic allograft immunobiology.
Chapter 6. A novel tool for examining mechanisms of rejection

6.1 Introduction

Despite the increasingly large amount of knowledge regarding mechanisms of rejection in heart transplantation, the time point at which a graft is committed to a particular outcome remains unknown. In other words, it is not known how long antigenic simulation needs to persist to induce the secondary cell recruitment that will ultimately develop into parenchymal rejection (PR) or transplantation-associated arteriosclerosis (TxAA) in a graft. This knowledge would not only be important regarding the relationship between PR and TxAA, but would also provide insight into potential pitfalls of experimental design. For example, in Chapter 4, the PPARα agonist was not administered to the recipients of heterotopically transplanted cardiac grafts until the third day post-transplant. No effect beneficial was observed. However, if the outcome of a graft were already determined by the second day after transplantation, then the lack of effect observed with PPARα agonist treatment may be a result of experimental technique, rather than a true absence of therapeutic benefit. If so, then any other experiments examining the effect of treatment with either PPAR agonists or other agents may suffer the same pitfall.

The studies examining the relationship between PR and TxAA performed to date have several important limitations. Initial studies used immunosuppressive agents [Hulett et al 1996, Nakagawa et al 1995]; however, these treatments suffer from additional potentially confounding factors and also do not allow the abrogation of specific cell subsets. An improvement on this approach used T cell antibodies [Nagano et al 1998]. However, the depletion of T cells occurred mainly in the periphery and not in the graft. An additional approach involved adoptive transfer [Furukawa et al 2004]. Allografts placed in mice genetically deficient in the production of the recombinase gene (and thus unable to produce mature T or B cells) survive indefinitely, regardless of the level of immunohistocompatibility complex matching. Purified T cells specific for the cardiac graft may be injected and their particular effects examined by sacrificing the mouse at the desired time points. While this approach allows control over the time of antigenic stimulation, it is not possible to selectively and effectively remove the transferred T cells to look for effects mediated through secondarily recruited cell populations.

As mentioned above, the ability to selectively and rapidly inactivate adoptively transferred cells at specific times after transfer could yield useful temporal information about requirements of antigenic stimulation and the relationship between PR and TxAA. After inactivation of the adoptively transferred cells, the graft could be allowed to function for varying times in order to assess various stages of lesion formation. There currently are no readily available tools to accomplish this.
Manipulation of the boron neutron capture reaction may allow this type of detailed temporal examination to take place. Readers are referred to chapter 2 for a more complete discussion of this reaction. In brief, $^{10}\text{B}$ has a high probability of capturing thermal neutrons and subsequently emitting two highly energetic particles that deposit their energy within a distance that is comparable to the diameter of a cell. Thus, cells that contain the boron may be inactivated by the intense energy deposition and subsequent damage following neutron irradiation.

A schematic of the use of the boron neutron capture reaction as a novel immunologic research tools is shown in Figure 6.1. In the proposed experiment, boron-loaded T cells would be adoptively transferred into a mouse that has lacks the recombinase gene and has received a heterotopic transplant. After a determined amount of time, neutron irradiation could take place, thereby inducing the boron neutron capture reaction and cell inactivation in the adoptively transferred boron-loaded graft-infiltrating T cells.

**Figure 6.1: Use of the boron neutron capture reaction as an immunologic research tool:** Mice genetically unable to produce T or B cells would receive a heterotopic cardiac transplant, followed by the injection of boron-loaded T cells. After defined times, neutron irradiation would be performed, inducing the boron neutron capture reaction in the adoptively transferred T cells.

Many of the design parameters for this application may be drawn from those established from other applications. Based on the shallow depth of the target area (thereby not contributing much to neutron moderation), it is estimated that a thermal beam is required for this application. Design parameters include a target dose of 3000 rads, based on the dose delivered in the inactivation of stimulator cells in the mixed leukocyte reaction, and a target deletion rate of 90%, based on the depletion rates achieved using T cell directed antibodies [Nagano et al 1998]. With respect to boron concentration in T cells, it should be noted that typical concentrations used for tumor cell killing ranges from 30 to 50 parts per million (ppm). This represents a likely target range of boron concentration, although
higher concentrations would certainly be desirable in that they would reduce neutron irradiation time and consequently reduce dose to surrounding healthy tissue.

The overall focus of the work presented in this chapter is the evaluation of potential boron compounds for using the boron neutron capture reaction to deplete adoptively transferred immune cell populations. This was done by testing the uptake of boron compounds by murine T cells, and by ensuring that the uptake would not have functional consequences, as measured by T cell proliferation and cytokine production; this is particularly important for this application, where the goal is to have fully functional T cells before their inactivation. Unlike other studies [Nguyen et al 1993], no attempt is made to determine the subcellular distribution of the boron; only to measure boron association with the cells that is termed “uptake” herein.

The first boron compound that was tested was K₂B₁₂H₁₂ (KBH), a compound containing the polyhedral closed-form borane ion, B₆H₁₂₅, a schematic of which is shown in figure 6.2. The structure of KBH was confirmed by an X-ray diffraction study [Wunderlich and Lipscomb 1960]. The B₁₂H₂⁻ ion occupies a volume equivalent to that of a benzene molecule spinning on a twofold axis and has high resonance stabilization [Muetterties and Knoth 1968]. Advantages of KBH include its ease of availability; it is commercially produced and may be acquired with either unenriched or enriched boron. Previous experimental have shown high uptake by human monocytic cells [Binello 1999]. Disadvantages include a relatively low overall fraction of boron retention, as well as concerns for possible leakage. Given the lack of specificity of the compound, the postulated mechanism of uptake is pinocytosis. This predisposes the cellular distribution to be cytoplasmic and/or endosomal, requiring potentially higher boron concentrations.

The other compounds tested were boronated nucleosides. A small library of thymidine derivatives, containing o-carboranylalkyl groups linked at the N-3 position via alkyl spacers of 2 to 7 methylene units, has been recently synthesized [Al-Madhoun et al 2002]. The library also included hydrophilically enhanced carboranyl thymidine analogs [Al-Madhoun et al 2002], the structure of which is shown in figure 6.3. Biological evaluation has focused on their phosphorylation rates by two enzymes that are pyrimidine nucleoside specific, thymidine kinase 1 and thymidine kinase 2, both of which are pyrimidine nucleoside specific; studies found that enzymatic activities of carboranyl thymidine analogs ranged from 10% to 40% those of regular thymidine [Al-Madhoun et al 2002]. The highest efficiencies relative to thymidine, based on enzymatic kinetic parameters, were found with boronated nucleosides having tether lengths of four and five methylene groups [Al-Madhoun et al 2002], and these represent the boronated nucleosides tested in this thesis. Advantages include their metabolic potential for incorporation into cellular nuclear material, thereby vastly reducing the risk of leakage and potentially increasing the efficacy of dose delivery, as well as providing high degree of specificity when stimulated in vitro. Disadvantages include the potentially low boron incubation concentrations, limited by compound toxicity, as well as limited availability (or not commercially available) and experience in cellular uptake.
Figure 6.2: **Schematic of the boron cage in $K_2B_{12}H_{12}$ (KBH):** Advantages of this boron compound include ease of availability and previously reported high uptake in monocytic cell line. Disadvantages include likely endosomal or cytoplasmic distribution and unclear pathway of uptake, leading to concerns of leakage.

Figure 6.3: **Schematic of hydrophilically enhanced carboranyl thymidine analogs:** The lengths of the methylene chains of the two nucleosides tested are four (N4-2OH) and five groups (N5-2OH), respectively. These were chosen on their optimal efficiency compared to thymidine. Advantages of these compounds are their incorporation into nuclear material of a cell, thereby reducing risk of leakage and leading to a potentially more effective dose delivery, as well as specificity if loading is accomplished during *in vitro* stimulation. Potential disadvantages are limited boron incubation concentrations due to compound toxicity, and overall lack of experience in T cells. Readers are referred to the literature cited in the text for a more chemically accurate depiction of structure.
6.2 Methods

The first step involved the isolation of murine T cells isolation, as described in the following subsection. Optimal parameters for testing uptake were determined for each compound, as described in the subsequent subsection. Finally, functional assays were performed, including measurement of proliferative capacity and production of cytokines.

T cell isolation

Spleens were extracted from C57/BL6 mice (Jackson Laboratories, Bar Harbor) and ground, using the barrel of a 5 ml syringe, against fine silver mesh screen in a sterile culture dish. C-10 medium (described in previous chapters) was added to the dish and the resulting solution was transferred to a 15 ml conical tube. The tubes were then centrifuged in a Beckton table top centrifuge for 5 minutes at 800g. Supernatant was aspirated and cells were resuspended in 5 ml of tris ammonium chloride buffer (1:10 dilution of tris buffer and 0.83% NaCl) for 5 minutes at 37°C to lyse erythrocytes. C-10 medium was added to a volume of 15 ml and centrifuged for 10 minutes at 1200 rpm Supernatant was aspirated, cells resuspended in 10 ml of C-10, and the suspension passed through a nylon mesh of 45 μm and cells were counted with a hemocytometer.

T cells were then isolated from the splenocytes using a magnetic cell sorting system (Miltenyi Biotec, Auburn), with beads directed against the CD90 murine marker (also known as Thy1.2) and developed for a positive selection of the CD90+ T cells. Specifically, cells were washed with buffer, made of phosphate buffered saline pH 7.2, supplemented with 5% bovine serum albumin (Sigma, St. Louis). Following supernatant aspiration, cells were suspended at a volume of 90 μl per 10⁷ cells. Then, 10 μl of beads per 10⁷ cells were added and mixed well by repetitive pipetting. The solution was incubated for 15 minutes at 4°C. The cells were washed by adding 10 to 20 times the volume of beads (i.e. 100 to 200 μl per 10⁷ cells), centrifuging for 10 minutes at 800 g, aspirating the supernatant completely and resuspending the cell pellet in 1 ml of buffer per 10⁸ cells. An LS column (Miltenyi Biotec, Auburn) was placed in the magnetic field of an appropriate MACS separator and rinsed with 3 ml of buffer. The cell suspension was added to the column and the negative cells were allowed to pass through. The column was rinsed three times with 3 ml, before removal from the separator and placement on a collection tube. Then 5 ml of buffer were loaded onto the column and the positive fraction was firmly flushed out using the specially provided column plunger. To ensure that a maximum yield of cells would be obtained, an additional 5 ml of buffer were loaded and again flushed out. Cells were counted for final yield and resuspended at 10⁷ cells per ml in C-10.

To determine the purity of the T cell isolation, three aliquots of 5x10⁵ cells were taken and distributed in three plastic tubes. Cells were centrifuged for 5 minutes at 1200 rpm and supernatant was decanted. To each tube, 100 μl of 1% paraformaldehyde was added. Incubation was carried out for 5 minutes at 4°C. Then, phosphate buffered saline (PBS) was added and cells were washed. One tube was then left on ice, as the “unstained” control, while the other two received 12.5 μl of phycoerythrin-labelled antibodies.
(Pharmingen, San Diego), one against CD3, a T cell marker, and the other, hamster IgG, serving as the “isotype control”. Tubes were incubated for 30 minutes, in the dark, at 4°C. Cells were then washed again with PBS, resuspended, and covered with foil and stored at 4°C until analysis by flow cytometry.

Boron uptake

In order to determine appropriate boron uptake incubation times, T cell proliferative activity was measured in response to a powerful stimulus, namely antibody to CD3, or anti-CD3 (Pharmingen, San Diego). The wells in the 96-well cell culture plates designated for anti-CD3, were pre-coated in quadruplicate with 1 μg/ml of anti-CD3 and incubated at 37°C for over 5 hours. The antibody was aspirated and 100 μl of C-10 was added. Then 100 μl of cells from a suspension at 10⁷ cells per ml was added to each of the wells. To measure proliferative capacity, 25 μl of ³H-thymidine was added to the plates every day, and after six hours, the plates were placed in the freezer to stop the reaction. This was repeated for 5 days, and on the last days, plates were analyzed using a standard beta scintillation counter. Results showed an initially low proliferation rate at day 1, followed by peak at day 2, with subsequent rapid decrease by day 3 and resting levels by days 4 and 5. The proliferation rate at the peak was over 100-fold that observed at resting levels. All T cell stimulations were performed with 1 μg/ml of anti-CD3.

Bulk cultures were used for the determination of uptake. The two boron compounds were prepared and incubated with T cells. For testing KBH (Strem Chemicals, Newburyport), cells were incubated in medium containing 1 mg/ml and reincubated in boron-free medium for two to three days. For testing the boronated nucleosides (Dr. W. Tjarks, Ohio State University), cells were pulsed for 12 hours at a compound concentration of 50 μM ending on days two and three post-stimulation. Cell pellets were washed with PBS and supernatants were saved analysis.

The boron concentration was measured both in the cells and supernatants using prompt gamma neutron activation analysis (PGNAA), a technique where sample irradiation and spectroscopic analysis occur simultaneously. Measurements were made at the PGNAA facility at the MIT Research Reactor, using a focused diffracted neutron beam with a neutron flux at the sample position of 1x10⁷ n/cm²s and a high purity germanium detector positioned at 90⁰ from samples. Gamma rays were detected from the boron capture (478 keV) and hydrogen capture (2.2 MeV) reactions.

The count rates of the gamma rays coming from both reactions was determined by measuring the area under the appropriate gamma peak and dividing by irradiation time. The background counts for the gamma rays resulting from boron capture were measured by counting an empty teflon vial, while the background rate for the gamma rays resulting from the hydrogen capture reaction were measured by counting a vial with deionized water at a volume comparable to those of the cell solutions. Following the determination of both count rates and the subtraction of the appropriate backgrounds, the ¹⁰B/²H (B/H) ratio was calculated and the final ¹⁰B concentration was calculated against a calibration curve generated from boric acid standards. The use of the B/H ratio reduces effects of
inconsistent sample positioning in the beam and variations in the thermal neutron flux measurement. A calibration curve, relating B/H ratio to boron concentration (ppm) was constructed each time, using boric acid standards prepared by those responsible for maintenance of the PGNAA facility. Measurement error was calculated according to standard counting statistics formulas [Knoll 1989]. Based on the relatively high concentrations measured in the samples, there was no need to calculate the lower limits of detection for any of the samples.

With respect to the measurement of boron in cells, the B/H ratio was scaled to take into account the volume the cell pellet, as per a protocol developed using a monocytic cell line [Binello 1999]. The protocol is based on the fact that the B/H ratio obtained for a cell suspension will give the concentration of the entire suspension, including the PBS used for the final resuspension (since the hydrogen is in the denominator), rather than the cell pellet. Thus, the microcentrifuge tubes that are used to store the cells and suspension are weighed three times, i.e. when they are empty, when they contain the cell pellet, and after the pellet has been resuspended into a cell suspension. The boron concentration is multiplied by the volume of the cell pellet and divided by the volume of the cell suspension, thereby yielding the boron concentration of the cell pellet, or cellular “uptake”. In other words, (μg of 10B/g of solution)*(g of solution/g pellet)=μg/g of pellet. Scaling the ratio is allowed because the suspension is homogeneous. In order to decrease the amount of error with respect to weighing and effect of a potential drop of liquid not aspirated from the cell pellet, large amounts of cells were used to generate the cell pellet, i.e. approximately 30 million cells. Finally, it should be noted that all PGNAA counting errors were kept well below 10%, in order to keep a propagated total error below 10%.

Functional assays

The first of two functional assays tested the effect of boron uptake on proliferative capacity. This was only tested with KBH. As previously described, proliferation was measured by the addition of 3H-thymidine to cell cultures for 6 hours, ending on days 1 through 5. At the end of the pulse, plates were either read or frozen, until they could be read using a scintillation counter. Proliferation was not tested with the boronated nucleosides, out of concern for possible competition of thymidine substrates and/or interference with uptake.

The production of IFNγ was performed using enzyme-linked immunoassays (ELISAs) as per the manufacturer’s protocol (Pharmingen, San Diego) and is fully described in chapter 3. Briefly, plates were coated with purified antibody against IFNγ, and subsequent to overnight incubation, blocked and loaded with both samples and standards. Following another overnight incubation, a secondary biotinylated antibody that binds to IFNγ was added. Subsequent incubation with avidin-conjugated horse-radish peroxidase then allows chromogenic development by addition of an appropriate substrate (in this case, ABTS). Optical density was measured and IFNγ concentrations were determined using a calibration curve of known standards. Results were analyzed using “Softmax” software (Molecular Devices Corporation).
6.3 Results

T cell isolation

Figure 6.4 shows a representative result of over 95% T cell purity, following isolation via the magnetic cell sorting system and analysis using flow cytometry. The isolation technique was robust and yielded an average of 90% purity, as determined the fraction of cells positive for the CD3 T cell marker.

![Flow Cytometry Image]

**Figure 6.4:** Representative T cell purity as determined by flow cytometry: On the left, the filled area corresponds to unstained cells while the solid line cells stained with the isotype control (hamster IgG). The curve on the right, toward the M1 marker, corresponds to cells positively staining for the T cell marker, anti-CD3. Flow cytometric analysis showed that the fraction of positively staining cells, corresponding to the purity of isolation, was approximately 95%.

Boron uptake

Results of the KBH uptake by T cells are shown in figure 6.5. The average boron concentration achievable in T cell upon incubation with KBH was approximately 300 ppm (scaled to enrichment). The average boron concentration in supernatants from day 2 and 3 samples was less than 5 ppm, indicating retention against a concentration gradient and no significant cell death.

Results showing the uptake of the two hydrophilically enhanced carboranyl thymidine analogs are shown in figure 6.6. The uptake of N4-2OH was 80 ± 10 ppm and 290 ± 40 ppm on days 2 and 3, respectively, while the uptake of N5-2OH was 140 ± 20 ppm and 250 ± 30 ppm also on the respective days 2 and 3. The overall average uptake was 200 ppm over both days for both compounds. The boron concentration in the supernatants was minimal, ranging from 5 to 15% that measured in the cells, indicating retention of the boron against a concentration and consistent with the degree of cell apoptosis that may normally occur in immune cell cultures undergoing stimulation.
Figure 6.5: **Uptake of KBH by murine T cells:** Average boron concentration in cells and supernatants (denoted as sup) were 300 ppm and 5 ppm, respectively. There was no significant difference between the two days tested (p ns). The low concentrations in the supernatants indicate retention against a gradient.

Figure 6.6: **Uptake of two carboranyl thymidine analogs by murine T cells:** The average boron uptake of the N4-2OH and N5-2OH boronated nucleosides was approximately 200 ppm for both of the boron compounds over both days. The boron concentration in the supernatants ranged from 5% to 15% of that measured in cells, which is comparable to the normally occurring level of apoptosis in culture and indicate retention against a concentration gradient.
Functional Assays

Representative results of the functional T cells assays using KBH are presented in figure 6.7, expressed as the average and standard deviation of samples cultured in quadruplicate. Proliferation following stimulation with anti-CD3 peaked at day 2 and then went to the resting level (it should be noted that although the proliferation assay was carried out for five days, the fourth and fifth day are not shown on the graph because they were similar to the third day). There was no significant difference in proliferation between control and KBH-treated T cells on any of the days tested (p ns). Similarly, the concentration of IFNγ in the supernatants of the proliferation cultures on days 2 and 3 also did not differ significantly (p ns) upon treatment of the T cells with KBH. The average concentration of IFNγ in control cells was 12,600 ± 2,900 pg/ml over both days, compared to the average of 13,500 ± 5,000 pg/ml in KBH-treated cells.

With respect to T cell function, addition of the boronated nucleosides did not have significant effects as determined by the production of IFNγ, as shown in figure 6.8. Results are expressed as the average and standard deviation of samples cultured in quadruplicate. It is hereby noted that proliferative capacity was not evaluated out of concern for possible competition since the technique readily available in the laboratory for measuring proliferation depends on the incorporation of 3H-thymidine. However, it should be noted that it is unlikely for cells to incorporate the boronated thymidine unless they are proliferating. Following anti-CD3 stimulation, murine T cells produced an average of 12,700 ± 1,700 pg/ml of IFNγ over both days, between which there was no significant difference over the days tested (p > 0.5). The addition of N4-2OH resulted in an average IFNγ concentration of 12,100 ± 1,800 pg/ml, while addition of the N5-2OH compound yielded an average of 12,600 ± 11,200 pg/ml, both not significantly different (p > 0.5) compared to the controls pulsed with medium alone.

![Graph](image.png)

**Figure 6.7:** Results of functional T cell assays using KBH: There is no significant difference in either proliferation or IFNγ concentration between days following treatment with KBH on any of the days tested (p ns).
6.4 Discussion

A novel tool has been presented to investigate the temporal relationship between PR and TxA, as well as requirements of antigenic stimulation, in the attempt to determine the time point at which a heart transplant is committed to a particular pathological outcome. Existing immunological techniques have limitations that preclude the ability to remove the graft-infiltrating T cells and allow the mouse to survive in order to determine the effect on the lesion formation. Manipulation of the boron neutron capture reaction may allow this type of investigation to be performed. This method represents a novel application of the boron neutron capture reaction as an immunologic research tool rather than solely as a therapeutic modality.

The focus of the work presented in this chapter is examination of boron uptake by T cells. Boron uptake by murine T cells in vitro is required for potentially using the boron neutron capture reaction as an immunologic tool in the context of heart transplantation research. Boron compound evaluation also involved the examination of possible effects of uptake on T cell function, namely proliferative capacity and production of IFNγ. These functional assays are particularly important for this application of the boron neutron capture reaction where the goal is to have fully functional T cells present in the transplanted heart for specified times before their inactivation via the boron neutron capture reaction induced upon neutron irradiation.

Figure 6.8: Results of functional T cell assays using carboranyl thymidine analogs: There is no significant difference in IFNγ concentration between days or following the addition of N4-20H or N5-20H to the cultures on any of the days tested (p ns). Proliferation was not measured out of concern for possible competition between thymidine substrates since the technique readily available in the laboratory for measuring proliferation depends on the incorporation of ³H-thymidine.
Several compounds were screened, including KBH and two boronated nucleosides, i.e. hydrophilically enhanced carboranyl thymidine analogs, with tether lengths of 4 and 5 methylene groups, i.e. N4-20H and N5-20H. Uptake of both compounds was high. The average uptake of KBH was approximately 300 ppm, consistent with experiments using a human monocytic cell line [Binello 1999]. The uptake of the boronated nucleosides was roughly similar, with the averages ranging from 200 to 300 ppm, a factor of ten higher than the concentrations typically observed in clinical studies of boron neutron capture therapy. Supernatant concentrations were minimal with all of the compounds, indicating retention against a concentration gradient and no significant cell death. Functional assays demonstrated no adverse effects on T cells. Treatment of T cells with KBH had no significant effects on T cell proliferation, and neither KBH nor the boronated nucleosides had significant effects on IFNγ production. The effect of the boronated nucleosides on proliferative capacity was not tested due to concerns of possible competition given that the measurement technique is based on the incorporation of 3H-thymidine.

In light of the comparable uptake and lack of significant effects on T cell function, the preferred compounds for this application are the boronated nucleosides. The advantages of these compounds are reduced risk of leakage and potentially increased effectiveness of dose delivery, as well as uptake specificity following in vitro stimulation, all resulting from their metabolic incorporation into cellular DNA. Overall, their excellent uptake, taken together with their advantages, make them ideal compounds for this novel application of the boron neutron capture reaction. Thus, consideration of the high uptake levels achieved in vitro, taken together with the lack of adverse effects on cell function and design goals, suggests that boronated nucleosides have a high likelihood for success. One potential biological effect of T cell inactivation in vivo is decreased local macrophage activity. However, the extent of this effect is dependent on the dynamics of T cell function in the graft and is not yet fully understood, despite active investigation.

Further development of this immunologic research tool is warranted. This could entail the use of high-resolution quantitative autoradiograph [Kiger 2000] of frozen heart tissue sections after in vivo administration of boron-loaded T cells to ensure that the T cell concentrations achieved in vitro are retained in vivo. This is particularly important given the likelihood of T cell proliferation in vivo, thereby potentially diluting initial boron concentrations. Given positive results, effort could then directed toward testing the proposed tool in mice. Finally, it is emphasized that if successfully developed, this application of the boron neutron capture reaction could have wide application in immunology research.
Chapter 7: Summary and recommendations for future work

Over the last several years, PPARs have emerged as immunomodulatory agents. They are expressed in cells of the vascular and immune systems, including vascular endothelial and smooth muscle cells, as well as monocytes/macrophages and T cells. Based on the reported anti-inflammatory and anti-atherosclerotic effects of PPAR activation both in vitro and in vivo, there was reason to hypothesize beneficial effects of PPAR agonists on parenchymal rejection and transplantation-associated arteriosclerosis. Moreover, given the current broad use of several PPAR ligands in the clinical setting, a demonstration of benefit in transplant rejection could translate rapidly to clinical application, strengthening the motivation for pursuing study. Thus, the overall goal of this thesis was to investigate the role of PPARs in mechanisms of rejection in heart transplantation.

Evaluation of PPARs in mechanisms of rejection began in vitro. For the first time the effects of four PPAR agonists, two PPARα agonists (fenofibrate and Wy14643) and two PPARγ agonists (BRL49653 and 15-deoxy Δ12,14-PGJ2), were examined in the mixed leukocyte reaction (MLR), serving as a surrogate for the alloresponse. All agonists had the ability to significantly decrease production of IFNγ, without affecting proliferation or viability. Dose response studies identified the “therapeutic” dose for each PPAR agonist. A thorough examination of cytokine, chemokine and chemokine receptor expression profiles as a function of time in the alloresponse and following ConA simulation was performed. Comparison of these profiles revealed critical differences between ConA and MLR stimulation, pointing to the need for specific testing in the alloresponse setting for potential translation to in vivo transplant rejection. Therefore, only the highlights of the results obtained in the alloresponse are summarized here. Future work could be aimed at defining signaling pathways involved in the differences.

There were multiple (and occasionally unpredictable) effects of PPAR agonists on the cytokines, chemokines and chemokine receptors expressed in the alloresponse. IFNγ is typically the dominant cytokine expressed and PPAR activation uniformly decreased expression by ~50%. The lack of a concomitant decrease in either IL-12 or IL-18, suggests a direct effect of PPAR agonists on IFNγ, which has been recently substantiated by data showing PPAR effects on STAT1 phosphorylation, nuclear localization and DNA binding. Based on known roles of IFNγ on chemokine expression, a decreased chemokine profile was expected. Initially, PPAR activation had no significant effects on any of the chemokines or receptors tested. Later in culture, when high concentrations of IFNγ were present in culture, PPAR agonists diminished CCR2 and CCR5. Given the implications of these two receptors in mechanisms of rejection, the decrease in CCR2 and CCR5 is beneficial. However, PPAR ligands (with the exception of fenofibrate) significantly augmented chemokine production, thereby rendering unavailable a substantial benefit associated with a decrease in IFNγ.
Thus, in vitro, PPAR agonists display several “anti-rejection” effects, including marked decreases in IFN\(\gamma\), CCR5 and CCR2. Of the PPAR\(\alpha\) and PPAR\(\gamma\) agonists tested, fenofibrate emerged as the most promising candidate for attenuation of rejection mechanisms in vivo. Unlike the other PPAR agonists, it did not markedly enhance the overall chemokine expression. Fenofibrate displayed the greatest potential of minimizing chemokine-mediated “pro-rejection” properties, while continuing to take advantage of the “anti-rejection” properties of PPAR agonists. Moreover, reports from the literature suggested that fenofibrate could decrease the expression of VCAM-1 and subsequent monocyte binding to endothelial cells, as well as ICAM-1. The literature also suggested that fibrates could suppress both vascular endothelial and smooth muscle cell activation.

Fenofibrate treatment was consequently administered to MMHC-II mismatched recipients of heterotopic murine cardiac allografts. After two weeks, fenofibrate administration reduced the intragraft expression of IFN\(\gamma\), as well as other proinflammatory genes, with concomitant reductions in IP-10, CCR2 and CCR5. Graft infiltration by mononuclear cells was markedly attenuated as a result of the fenofibrate. At this early time point (2 weeks), the in vivo results closely recapitulated the in vitro results described above.

However, by 8 weeks, graft infiltration was substantially increased, likely a result of sustained RANTES production. Fenofibrate continued to attenuate production of IFN\(\gamma\) on a per cell basis. However, the increased number of cells in the graft led to paradoxically increased levels of IFN\(\gamma\) in the graft. Final pathological analysis of the grafts reflected this result; there was no change in transplantation-associated arteriosclerosis (roughly proportional to IFN\(\gamma\)), in the face of increased parenchymal rejection (roughly proportional to graft cellular infiltration). This suggests that in order to fully realize the benefit of diminished IFN\(\gamma\) by fenofibrate in grafts, concomitant administration of agents reducing graft infiltrate will be required. Future work may involve the co-administration either of agents attenuating recruitment by targeting the chemokine or chemokine receptors, or of agents directed at preventing adhesion and extravasation of circulating lymphocytes into the donor vasculature.

The availability of mice congenitally deficient in PPAR\(\alpha\) (PAKO) allowed the examination of PPAR\(\alpha\) requirement both in vitro and in vivo. Experiments specifically addressed whether, in the setting of congenital PPAR\(\alpha\) deficiency, fenofibrate could affect the production and expression of IFN\(\gamma\), in addition to other cytokines, chemokines and chemokine receptors. The influence of PPAR\(\alpha\) in vivo on parenchymal rejection was investigated via murine heterotopic cardiac transplants between strains mismatched in both MHC-I and MHC-II, in combinations where either the donor or the recipient were congenitally deficient in PPAR\(\alpha\).

In vitro experiments with PAKO leukocytes support a role for both PPAR\(\alpha\)-independent and PPAR\(\alpha\)-dependent effects in the MLR. At 20 \(\mu\)M, fenofibrate reduces the in vitro production of IFN\(\gamma\) by splenocytes congenitally deficient in PPAR\(\alpha\), without affecting cell proliferation or viability. This suggested the existence of “cross-talk” at several levels and raised the question of whether the effect on IFN\(\gamma\) was mediated even in part by...
PPARα. Using mice congenitally deficient in PPARα, allogeneic stimulation led to higher levels of IFNγ expression, suggesting that PPARα does indeed play a regulatory role in the expression of IFNγ. With the accumulation of IFNγ in culture, PPARα also influenced chemokine and chemokine receptor expression. As expected, based on augmented IFNγ and downstream effects, the expression of IP-10, CCR2 and CCR5 increased. However, RANTES expression decreased, suggesting a regulatory role of PPARα in RANTES production.

Transplants performed using mice congenitally deficient in PPARα as either recipients or donors showed significantly increased graft infiltration by CD11b+ cells. Analysis of intragraft cytokine, and chemokine profiles demonstrated increased intragraft expression of IFNγ and decreased expression of RANTES, implying PPARα regulation for both of these proteins. Cardiac allograft pathology of PAKO recipients distinct and demonstrated widespread formation of thrombi without vascular edema (compared to WT recipients that displayed edema, in the absence of thrombi). It is likely that thrombus formation is associated with increased systemic fibrinogen concentration in PAKO mice. In comparison, congenital donor deficiency of PPARα did not markedly affect allograft pathology, with both WT and PAKO donors displaying edema and no thrombi. Future work should be aimed at increasing the number of transplants per arm, in order to discriminate subtle changes in expression profiles, potentially contributing to mechanisms of rejection.

Finally, one technical issue was considered with respect to fenofibrate treatment of transplanted mice. Drug administration was not started until day 3 post transplant due to concerns over coagulation. However, it is not known when a graft becomes committed to a particular outcome. If the outcome of a graft were already determined by the second day after transplantation, then the lack of fenofibrate effect in vivo could be a result of experimental technique, rather than a true absence of therapeutic benefit. There are no readily available tools allowing the removal of T cells at determined times after infiltration into the graft and the survival of the murine transplant recipients, in order to assess the effect on lesion formation.

A novel tool based on the boron neutron capture reaction was presented and design goals for this application were delineated. Experimental work addressed the potential of achieving high boron uptake by T cells, without adversely affecting production of IFNγ. Optimal compounds tested were boronated nucleosides, with average uptake ranging from 200 to 300 ppm (a factor of ten higher than the concentrations typically observed in clinical studies of boron neutron capture therapy), without significant effects on IFNγ production. Advantages of the boronated nucleosides include the reduced risk of leakage and potentially increased effectiveness of dose delivery (both resulting from their metabolic incorporation into cellular DNA). Future work should involve high-resolution quantitative autoradiography on frozen sections of cardiac grafts after the adoptive transfer of boron-loaded T cells, to ensure that the uptake achieved in vitro is retained following injection in vitro. A positive result would then justify moving to an in vivo proof of concept in allograft rejection. If successful, this technique could have wide application in immunological research.
References


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