ALTERED B CELL TOLL-LIKE RECEPTOR 9 RESPONSES AFTER THE ONSET OF PAEDIATRIC CHRONIC GRAFT-VERSUS HOST DISEASE

by

KEVIN SHE

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ABSTRACT

Chronic Graft-versus-Host disease (cGVHD) is a major complication after blood and marrow transplantation (BMT). B cells appear to play a role in cGVHD as evidenced in murine models and supported clinically by the success of B cell depletion treatment. Immunostimulatory microbial CpG-DNA responses are enhanced in splenocytes from mice with simulated Graft-versus Host Disease and we hypothesized that a similar response to CpG by B cells may be present in human cGVHD. Peripheral B cells from newly diagnosed cGVHD patients enrolled on the COG protocol ASCT0031 were divided into early (3–8 months post-BMT) and late (≥9 months post-BMT) onset groups and compared to time-matched control patients. A significantly greater percentage of B cells from cGVHD patients rapidly responded to phosphorothioate modified CpG compared to controls. There was a significant correlation between TLR9 expression and CpG response, also confirmed using entirely TLR9 dependent native phosphodiester CpG. There were no differences in response to peptidoglycan (TLR2, 6) or LPS (TLR4). Patients with hepatic involvement had stronger phosphodiester CpG response. These findings suggest that a larger pool of B cells from patients with cGVHD are specifically primed for TLR9 response, compared to patients who have not developed extensive cGVHD after allogeneic BMT, and may play a role in the pathophysiology or maintenance of cGVHD.

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ABBREVIATIONS

aGVHD	- Acute Graft-versus Host Disease
allo-BMT	- Allogeneic Blood and Marrow Transplant
APC	- Antigen Presenting Cell
ANA	- Anti-Nuclear Antibody
BMT	- Blood and Marrow Transplant
CD#	- Cluster of Differentiation
cGVHD	- Chronic Graft-versus Host Disease
CpG	- Cytosine - phosphate - Guanine dinucleotide motif
DC	- Dendritic Cell
DNA	- Deoxyribonucleic Acid
DNAPK-cs	- DNA Protein Kindase C subunit
EDTA	- Ethylene Diamine Tertraacetic Acid
FACS	- Fluorescence Activated Cell Sorting
GpC	- Guanine - phosphate - Cytosine dinucleotide motif
GVHD	- Graft-versus Host Disease
IFN-γ	- Interferon Gamma
IL-#	- Interleukin
IRAK	- IL-1R Associated Kinase
LRR	- Leucine Rich Repeat
MyD88	- Myeloid Differentiation Factor 88
NK Cell	- Natural Killer Cell
ODN	- Oligodeoxynucleotide
PAMP	- Pathogen Associated Molecular Pattern
PB	- Peripheral Blood
PBMC	- Peripheral Blood Mononuclear Cell
PCR	- Polymerase Chain Reaction
PD	- Phosphodiester (PO_4)
PS	- Phosphorothioate (PSO_3)
RBC	- Red Blood Cell (Erythrocyte)
RNA	- Ribonucleic Acid
RT-PCR	- Reverse Transcriptase Polymerase Chain Reaction
TIR	- Toll/IL-1R Domain
TIRAP	- TIR Domain Containing Adaptor Protein
TLR	- Toll-like Receptor
TNF-α	- Tumour Necrosis Factor Alpha
TRAF6	- Tumour Necrosis Factor Receptor Associated Factor 6

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1. CHAPTER ONE: INTRODUCTION

1.1 Allogeneic Blood and Marrow Transplant

Allogeneic blood and marrow transplantation (allo-BMT) is used as a treatment for an increasing number (Schrier et al., 2005; Wright-Kanuth and Smith, 2001) of malignant and non-malignant conditions. The most common among these are the various leukaemias (Buckner et al., 1970; Thomas et al., 1971) and there is an increasing range of non-malignant conditions that can be treated with allo-BMT including primary immunodeficiencies, autoimmune disorders, metabolic diseases, and anaemias. Primary immunodeficiencies where allo-BMT can be curative by reconstitution with a functional immune system include severe combined immunodeficiency (Gatti et al., 1968; Park et al., 1975) Wiskott-Aldrich syndrome (Bach et al., 1968), and X-linked lymphoproliferative syndrome (Pracher et al., 1994; Hoffmann et al., 1998). Autoimmune disorders such as systemic lupus erythematosus (Traynor et al., 2000), multiple sclerosis (La Nasa et al., 2004), and severe rheumatoid arthritis (Ikehara, 1998) can also be treated by ablating and replacing the dysfunctional immune system with that from a healthy donor. Metabolic disorders that involve inherited deficiencies in lysosomal enzymes are treated by substituting defective host cells with healthy donor haematopoietic cells. These disorders include Gaucher's disease (Rapperport and Gins, 1984), Hurler's syndrome (Hobbs et al., 1981), osteopetrosis (Coccia et al., 1980), or leukodystrophies such as adrenoleukodystrophy (Moser et al., 1984), metachromatic leukodystrophy (Bayever et al., 1985), and globoid leukodystrophy (Krabble's disease) (Krivit et al., 1995). Additionally, hereditary anaemias β-thalassemia (Thomas et al., 1982), sickle cell anaemia (Johnson et al., 1984), congenital hypoplastic anaemia (Blackfan-Diamond syndrome) (Iriondo et al., 1984), Fanconi's anaemia (Barrett et al., 1977), Shwachman-

Diamond syndrome (Barrios et al., 1991), as well as acquired anaemias such as aplastic anaemia (Storb et al., 1974), can be treated by allo-BMT.

Conventional allo-BMT is preceded by a conditioning regimen consisting of chemical and radiological ablation of existing haematopoietic cells in the bone marrow in part to suppress host immunity, overcome graft rejection (Billingham, 1966), and permit homeostatic expansion of newly transplanted donor cells (Laylor et al., 2005). This regimen is followed by the transfer of donor blood or marrow containing haematopoietic stem cells into the host which gives rise to red blood cells, platelets, and the myeloid and lymphoid white blood cells that serve immunological roles. Allogeneic donor cells are currently derived from one of four sources including bone marrow (Thomas et al., 1957, Humble, 1960), granulocyte colony stimulating factor (G-CSF) stimulated marrow (Masaoka et al., 1989), G-CSF-mobilized peripheral blood (Molineux et al., 1990), and haematopoietic precursor cell rich umbilical cord blood (Broxmeyer et al., 1989).

1.2 Graft-versus Host Disease

Both acute and chronic Graft-versus Host Disease (GVHD) remain major obstacles to successful transplant outcome and are major contributors to non-leukaemic relapse-related morbidity and mortality (Wingard et al., 1989). As allo-BMT is increasingly used as a treatment modality for numerous diseases, GVHD is progressively acknowledged as an important consideration. In 1966, Billingham set the general requirements for GVHD induction which are 1) the presence of immunologically competent cells within the graft, 2) host tissue that appears foreign to the graft and thus able to stimulate donor cells, and 3) a host immune system that is incapable of

generating an immune response to reject the graft before disease development (Billingham, 1966). Accordingly, if the reconstituted donor-derived immune system recognizes the host tissue as foreign, becomes activated, and is not rejected by residual host immune cells; these donor cells can initiate immune mediated damage to the host.

This thesis is focused on chronic GVHD (cGVHD), which was historically classified by a temporal criterion - that disease onset occurs later than 100 days, or approximately 14 weeks, post-BMT (Akpek, 2002). Although acute GVHD (aGVHD) tends to manifest relatively quickly after allo-BMT and cGVHD tends to occur after a relatively prolonged period, there is a significant overlap. aGVHD usually occurs within weeks after transplant with a peak incidence around 4 weeks although cases of initial aGVHD developing later than 15 weeks have been described (Valks et al., 2001). cGVHD most commonly arises between 16 and 28 weeks post transplant but can occur as early as 6 weeks post transplant. Skin involvement is common in both acute and chronic GVHD but the acute form commonly manifests as a generalized rash (Johnson and Farmer, 1998) while the chronic form is typically associated with lichenoid papules and sclerosis (Shulman et al., 1978). Aside from epithelial involvement, there are some differences in the sites of organ involvement between acute and chronic GVHD. Mucous membranes, biliary ducts, and intestinal tract crypts are the major tissues affected by aGVHD (Martin et al., 1987) whereas cGVHD, similar to autoimmune diseases such as systemic sclerosis or Sjögren's syndrome (Gratwohl et al., 1977), resembles histologically collagen vascular diseases that affect the oral mucosa, eyes, salivary glands, respiratory and gastrointestinal tracts, and the liver (Flowers et al., 1999). These injuries are commonly associated with the presence of fibrotic tissue indicative of chronic inflammation (Biedermann et al., 2002) as well as the presence of lymphocytic infiltrates in the affected organs (Hitchins et al., 1997). Additionally, the grading

system developed for diagnosing aGVHD when applied to cGVHD correlates poorly with clinical outcome (Glucksberg et al., 1974).

Based on these clinical differences and an increasing body of laboratory based investigations, distinct pathogenic mechanisms are suggested between the two diseases (Snover, 1984). aGVHD is primarily driven by cytokines (Piguet et al., 1987; Schots et al., 2003), with some direct T cell cytotoxicity (Clement et al., 1991). A three phase model for aGVHD induction has been proposed (Krenger et al., 1997) that begins during the host conditioning regimen required to ablate host marrow to suppress graft rejection (Billingham, 1966) and permit homeostatic expansion of newly transplanted donor cells (Laylor et al., 2005). The inflammatory responses to radiological and chemical insults facilitate donor T cell activation by host alloantigen as well as initiating a cascade of other inflammatory cytokines including interferon-γ (IFN-γ). The conditioning regimen also allows the translocation of microbial products such as lipopolysaccharide (LPS), a component of the gram negative bacterial cell wall, across the gastrointestinal mucosa into the bloodstream. It has been shown in murine models that macrophages primed by IFN-γ produce cytopathic levels of tumour necrosis factor-α (TNF-α) in response to LPS that contribute to a Th1-like storm of inflammatory cytokines (Cooke et al., 1998). This cascade can directly mediate tissue injury as well as contribute to the final stage by inducing the activation of cytotoxic allo-reactive T and natural killer cells (Krenger et al., 1997).

In contrast to aGVHD, both Th1- (Tanaka et al., 1994) and Th2-like (Kansu, 2004) cytokine profiles have been reported in human cGVHD. Additionally, it appears that cGVHD manifest from a more complex immune disregulation than aGVHD (Graze & Gale, 1979, Sherer &

Shoenfeld, 1998). cGVHD occurs in approximately 40% - 60% of all haematopoietic stem cell transplant patients with long term survival (Ratanatharathorn et al., 1998), with patients who have had previous histories of aGVHD being at higher risk. As yet, the complex pathophysiology of cGVHD is incompletely understood as there is a lack of highly satisfactory animal models and the development of uniform diagnostic criteria (Farag, 2004) is complicated by the highly pleiotropic nature of the clinical manifestations (Shulman et al., 1980). Virtually any organ can be affected although fibrosis of the skin, eyes, mouth, gastrointestinal tract, and liver, together with immunodeficiency, and the production of autoantibodies, is common (Parkman, 1993).

1.3 Clinical Manifestations of cGVHD

cGVHD generally presents as a complex array of symptoms and descriptive staging is divided into limited or extensive severity based on the cumulative extent of skin and organ involvement (Shulman et al., 1980). Skin involvement is present in virtually all cases of cGVHD and manifest as either early onset diffuse lichenoid lesions (Saurat et al., 1975; Touraine et al., 1975) or as more severe sclerodermatous lesions which typically show later chronological development (Chosidow et al., 1992). Lichenoid lesions are typically diffuse and affect the face, palms, and soles but can also form larger confluent areas and sometimes affect hair follicles and genital organs. Sclerodermatous lesions are typically shiny plaques that are indurated and sclerotic and are more centrally located, affecting the trunk, pelvis, and thighs. In severe cases the skin can become adherent to deeper tissue causing mechanical ulcers. Vitiligo (Aubin et al., 2000), the loss of pigment, and hyperpigmentation from inflammatory processes (Aractingi et al., 1996) have been reported in patients with cGVHD. Alopecia can occur in some patients who receive busulfan as part of their myeloablative regimen (Locatelli et al., 1993). Ocular involvement in

cGVHD in the form of dry eye syndrome is also common and is present in a majority of cases of cGVHD (Ogawa et al., 1999). This is another significant contributor to morbidity and in severe cases the end-stage can result in blindness (Lavid et al., 1995). The primary mediator of dry eye is lymphocytic infiltration of the lachrymal glands, notably by T cells with an activated phenotype and B cells expressing co-stimulatory molecules responsible for activating T cells, followed by fibrosis from the chronic inflammatory damage (Ogawa and Kuwana, 2003). Approximately half of cGVHD cases involve oral damage (Busca et al., 2005), typically characterized by activated T cell infiltrates in both the major and minor salivary glands. This leads to xerostomia which can exacerbate mechanical tissue damage and decreased immunoglobulin secretion which can further aggravate damage due to decreased protection against infection (Nagler and Nagler, 1999). Oral and gastrointestinal (GI) tract involvement can contribute to the weight loss seen in about half of patients with cGVHD. Like ocular and oral manifestations of cGVHD, complications of the GI tract involve activated lymphocytic infiltrates although it is unclear whether there are specific subsets of immune cells that preferentially target disparate organs as the constellation of organ involvement is variable (Nikolic et al., 2000). Presentation of symptoms of GI tract cGVHD is non-specific and includes abdominal pain, absorption dysfunction, bleeding, diarrhoea, gastroesophageal reflux, ileus, nausea, and vomiting (Goker et al., 2001). However, it is difficult to directly attribute many GI symptoms to cGVHD (Akpek et al., 2003). Clinical diagnosis of GI tract involvement is complicated as the entirety of the GI tract from the oesophagus to the colon may be affected (Patey-Mariaud de Serre et al., 2002) and approximately 40% of patients with positive histological diagnoses are clinically asymptomatic (Wakui et al., 1999). An additional obstacle in diagnosing GI involvement is that, given the involuted structure of the GI, mucosal biopsies are sampled at sub-optimal locations (Akpek et al., 2003). As there are unacceptable risks with the use of deep biopsies to detect inflammatory

processes present in the submucosa some manifestations have only been confirmed from autopsies and may remain under-diagnosed (Snover, 1990). Bronchiolitis obliterans, chronic fibrosis affecting the small airways of the lung resulting in obstructive lung disease, is characterized by lymphocytic infiltrates common to other cGVHD maladies. Bronchiolitis obliterans is, fortunately, a rare complication of cGVHD as the prognosis is uniformly poor and positive response to therapy is rare (Rosenberg et al., 1985; Epler, 1988).

Hepatic involvement in the form of cholestasis is also common in cGVHD (Shulman et al., 1988). Complete hepatic failure, however, is primarily mediated by viral hepatitis, especially hepatitis C (Bertheau et al., 1995; Strasser et al., 1999), in part due to immunodeficiency reported in patients with long term cGVHD. In addition to cGVHD prophylaxis that disrupt proper immune function, reconstitution of adaptive immune functions and populations, such as B cells and T cells, may be delayed (Kalwak et al., 2001; Perruche et al., 2005) in patients with cGVHD either as a result of damage to lymphocyte precursor cells or as a result of cGVHD prophylaxis and therapy. Regardless, patients with cGVHD are at extreme susceptibility to opportunistic infections (Meyers and Atkinson, 1983; Sullivan et al., 1986; Kulkarni et al., 2000) and antimicrobial prophylaxes against a variety of pathogens are crucial as infections accounts for a large percentage of deaths in patients with cGVHD (Parkman, 1993). In addition to being a major contributor to non-relapse mortality, infection can also instigate secondary organ dysfunction such as renal failure from cytomegalovirus infection in conjunction with cGVHD (Deconinck et al., 2005).

The production of autoantibodies against antigens including cell nucleus, smooth muscle, mitochondria, double-stranded DNA, cardiolipin (Lister et al., 1987; Rouquette-Galley et al.,

1988; Holmes et al., 1989), red blood cells (Fagiolo and Toriani-Terenzi, 2003; Zupanska et al., 2005), platelets (Anasetti et al., 1989), and others, have been reported in patients with cGVHD although there is a wide degree of disconcordance between studies. Cardiac involvement has been recognized as a rare target of cGVHD (Buja et al., 1976, Rackley et al., 2005), and while autoantibodies may be a factor, infiltrating lymphocytes are involved in this pathology (Saito et al., 2005). Skeletal muscle complications such as polymyositis have been associated with cGVHD and, similar to cardiac involvement, activated lymphocytic infiltrates appear to be an important mechanism (Urbano-Marquez et al., 1986). Neuromuscular complications such as myasthenia gravis have also been reported in association in cGVHD after allo-BMT (Adams et al., 1995) and have been attributed to both the presence of autoantibodies (Lefvert and Bjorkholm, 1987) and inflammatory mechanisms (Urbano-Marquez et al., 1986). Another rare manifestation of cGVHD is polyserositis, the inflammation of serous membranes (Toren and Nagler, 1997), which resembles autoantibody mediated familial recurrent polyserositis (Ben-Chetrit & Levy, 1990). Additionally, there are numerous other clinical presentations of cGVHD ranging from nail involvement (Sanli et al., 2004) to endocrine dysfunction that is secondary to conditioning regimen mediated glandular injury (Tauchmanova et al., 2002).

1.4 Pathophysiology of cGVHD

Despite the comprehensive clinical identity of chronic GVHD (cGVHD) and the recognition of the more rare manifestations, the pathophysiology of cGVHD is complex and remains incompletely understood. While there are acceptable animal models of acute GVHD (aGVHD) that closely resemble the clinical identity in humans, there is a lack of satisfactory animal models for cGVHD. The semi-allogeneic parental-to-F1 model using minor histocompatibility mismatch

has been used to investigate cGVHD although there is much contention regarding whether observations made in this animal model correlates with human cGVHD (Vogelsang and Hess, 1994).

Currently there are two primary theories of the aetiology of cGVHD. One of these is that cGVHD is the end stage of aGVHD where the alloreactivity of T cells has shifted to a Th2 response (Kataoka et al., 2001). However the alternative model, that cGVHD is a distinct entity where previous aGVHD merely induces a more permissive environment for cGVHD development (Lee et al., 2003), is better supported as the clinical manifestations of cGVHD suggest that it is a distinct autoimmune disorder. Indeed, there may be more than one form of cGVHD, although in all cases there is an intricate interplay between T cells, dendritic cells, B cells, and possibly other immune cell populations such as natural killer and natural killer T cells.

Murine models simulating cGVHD have identified several mechanisms that may mediate cGVHD in humans. T cells are an immune population that undergo the final stage of development and selection in the thymus and are capable of directly destroying target cells that they recognize. This population has been identified as a major mediator of cGVHD-like symptoms. In these models, the depletion of T cells from donor haematopoietic stem cells prior to transplantation reduces the incidence of cGVHD (Korngold and Sprent, 1978) although this is compromised by increased haematopoietic stem cell engraftment failure (Martin, 1993). Murine models that mimic thymic injury suggest that the failure to delete potentially host-reactive T cells permits the development of cGVHD-like symptoms (Zhao et al., 2003). Although important, the direct cytotoxic damage to organs by T cells is only part of the immune dysfunction that contributes to cGVHD. Thymic damage models that impair the negative

selection of T cells also show that the tolerogenic capacity of regulatory T cells can be overwhelmed as the generation of cytotoxic T cells becomes unregulated (Ghayur et al., 1988). In these models the loss of positive selection of cytotoxic CD8+ T cells that are capable of eliminating host-reactive B cells can also be impaired and are sufficient to permit persistent Th2-like humoral autoimmunity characteristic of cGVHD in humans (Shustov et al., 1998). Additionally, this humoral autoimmunity in mice depends on direct and specific interaction with alloreactive T cells (Morris et al., 1990).

Reciprocally, the ability of professional antigen presenting cells (APC) such as dendritic and B cells to present host antigen to donor T cells is instrumental for the development of symptoms similar to cGVHD (Reid et al., 2000). Activation of donor T cells in the context of cGVHD requires that the T cell can recognize host antigen as foreign and have that epitope be presented by a professional APC along with sufficient co-stimulatory signals. In transplant models, potent antibody blockade of the T cell co-stimulatory molecules CD80 and CD86 are sufficient to completely abrogate GVHD like symptoms (Lang et al., 2002). Similarly, experimental blockade of CD40:CD154 (CD40L), a reciprocal interaction between B cells and T cells, was able to induce the transformation of CD4+ T cells into an immunoregulatory population and confer protection against disease development (Taylor et al., 2002). Results from a murine parental-to-F1 minor histocompatibility (MiHC) model for simulating cGVHD reveal that dendritic cells and B cells capable of presenting Ag through class II MHC are required for disease induction (Shlomchick et al., 1999). Additionally, the lack of cGVHD-like symptoms in B cell depleted mice in a similar MiHC mismatch model further demonstrates the significance of B cells in disease development (Schultz et al., 1995). Recently, there is evidence from murine experiments that direct recognition of host antigen presented by host APC by donor T cells transitions into

donor T cell recognition of host antigen presented by donor APC (Tivol et al., 2005). During the transition, CD4+ T cells become dysregulated and are able to recognize both host antigen as well as antigen from the donor lending weight to the idea that loss of tolerance rather than direct recognition of alloantigen is the primary mechanism of cGVHD, consistent with the many autoimmune disease hallmarks of cGVHD. Although murine models are not ideal simulations of cGVHD in humans, the interactions between T cells, dendritic cells, and B cells have been suggested to play important roles.

T cells, dendritic cells, and B cells have been shown to be important in human cGVHD. T cells have been demonstrated as an important participant in human cGVHD as T cells with an activated phenotype are commonly present in affected organs (Urbano-Marquez et al., 1986; Nagler et al., 1999; Rackley et al., 2005). As in murine models, depletion of T cells from the source of haematopoietic stem cells is effective in decreasing cGVHD (Prentise et al., 1984) albeit at the expense of undesirable consequences including the increased risk of engraftment failure (Martin et al., 1985), leukaemic relapse (Ash et al., 1991), and infection (Li et al., 1994). It appears that thymic injury, either through increased age of the recipient, insult from the conditioning regimen prior to transplantation, damage from previous aGVHD, or as a consequence of cGVHD is also involved in permitting cGVHD in humans. The decline of both positive and negative selection of T cells may contribute to different manifestations of disease. The reduction in the positive selection of T cells, required for normal protective immunity, contributes to the immunodeficiency seen in cGVHD patients (Weinberg et al., 1995). Decreased thymic function may also have a detrimental impact on the ability to delete host-reactive T cells through negative selection (Atkinson et al., 1982). It has been observed that host-reactive Th2-type CD4+ T cells are capable of interacting with host-reactive B cells and drive

increased production of Th2-like cytokines and the production of autoantibodies (Flowers et al., 1999). Reciprocally, professional antigen presenting cells are also important in activating host-reactive T cells. In addition to the risk correlation of the persistence of host derived dendritic cells more than three months post transplantation with cGVHD development (Chan et al., 2003), co-stimulatory molecule expressing B cells are present in conjunction with T cells at sites of organs affected with cGVHD (Ogawa et al., 2003). Recently, the efficacy of B cell depleting antibodies in clinically treating some cases of complicated cGVHD (Ratanatharathorn et al., 2000; Ratanatharathorn et al., 2003) provides additional support for the importance of B cells in cGVHD.

1.5 B cells

B cells are the major contributor to humoral immunity which is characterized by antibody production that mediates pathogen and toxin neutralization, classical complement activation, and the opsonization signals to phagocytes for pathogen removal. Although first identified in birds and named after the avian organ in which they were found, the bursa of Fabricius, haematopoiesis and initial maturation of B cells in mammals occurs in the bone marrow and subsequent education occurs in the germinal centers of the spleen and lymphoid organs. B cell receptors (BCR) are generated through gene rearrangement. Upon activation, antibodies that recognize the same epitope as the BCR are synthesized and transported to the cell surface in vesicles to be secreted (Uhr, 1970). Newly generated B cells bearing self-reactive BCR are normally controlled through clonal deletion (Nemazee & Burki, 1989) or induced anergy (Goodnow et al., 1988). In some cases, self-reactive B cells can be instructed to rejoin their BCR gene segments to generate an alternate receptor (Radic et al., 1993; Tiegs et al., 1993).

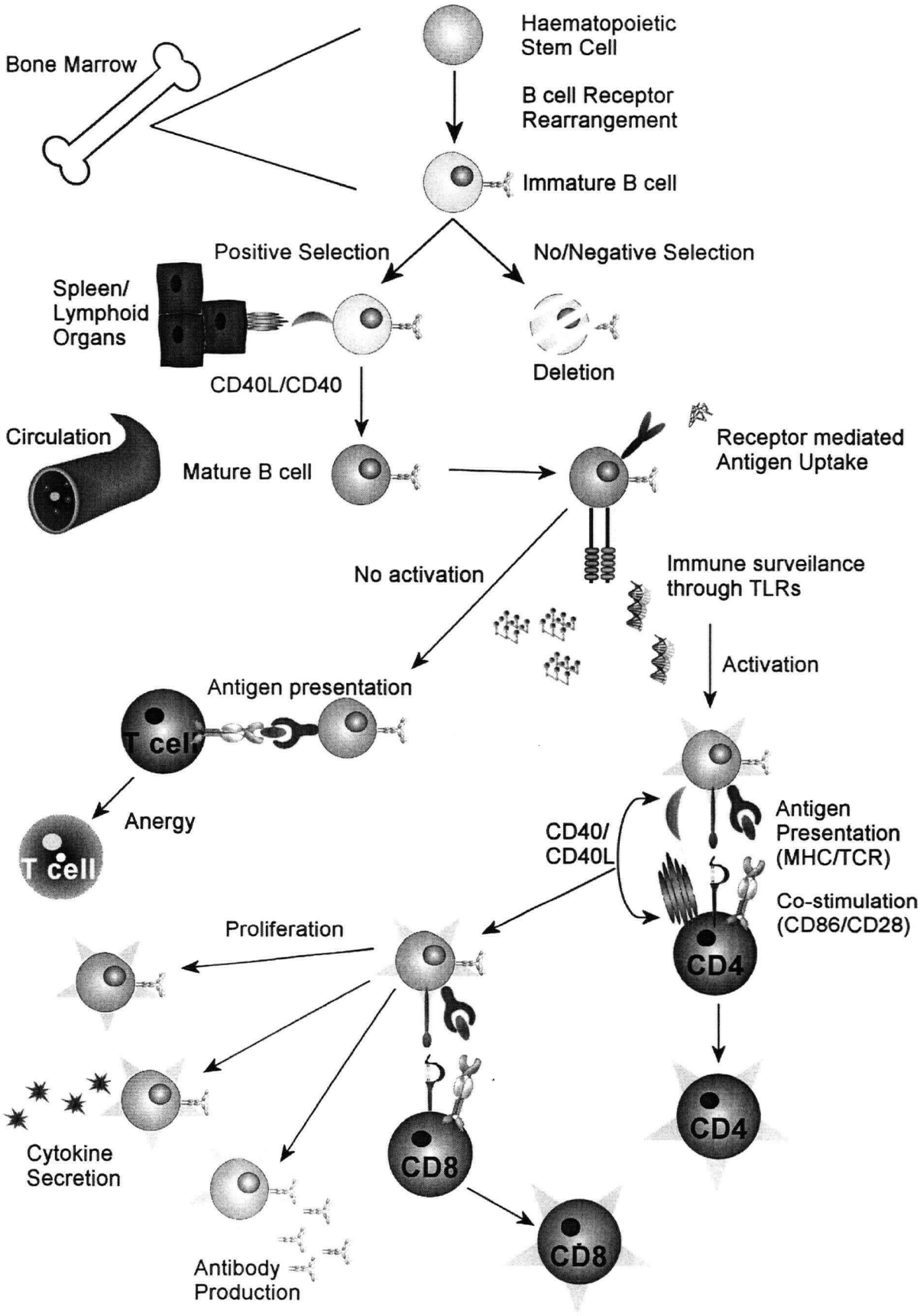
B cells are involved in modulating adaptive immunity through direct cell contact as well as through soluble cytokine signals. In addition to their association with humoral immunity, B cells can as effective as dendritic cells in directly priming CD4+ (Cassell and Schwartz, 1994) with processed external antigen through class II histocompatibility molecule. With the help of CD4+ T cells, B cells generally prime CD8+ T cells with intracellular antigens (Castiglioni et al., 2005) through class I histocompatibility molecule. However, it has been suggested that activation of B cells through CD40 ligation can mediate increased cross presentation of exogenous antigen through the class I pathway and directly activate CD8+ T cells (Ritchie et al., 2004) The initial priming and maintenance of a T cell response to antigen requires at least two signals; presentation of a recognizable antigen and the non-antigen specific ligation of co-stimulatory molecules (Bretscher, 1992). The B7 family of co-stimulatory molecules, which include CD80 and CD86, have been identified in murine models as essential for priming T cells and initiating cGVHD (Lang et al., 2002). These co-stimulatory molecules are expressed by activated, but not resting, B cells and have been well characterized as being key signals in inducing T cell activation by interaction with constitutively expressed CD28 counter-receptors on the T cell (Freeman et al., 1991). B7.1 (CD80) expression is delayed after activation and is only capable of activating antigen primed T cells (Hathcock et al., 1993) suggesting that its role is in the maintenance or expansion of T cell responses whereas B7.2 (CD86) is expressed rapidly and at robust levels after B cell activation and is important for initiating the activation of naïve T cells (Chen et al., 1994). CD40 is a constitutively expressed surface receptor on B cells (Banchereau et al., 1995) that is upregulated after cell activation (Torres and Clark, 1992). Unlike the B7 family of co-stimulatory molecules that primarily deliver activation signals to T cells, CD40 is a receptor that both positively regulates B cells as well as the T cell when ligated to CD154 (CD40L). CD154 is primarily expressed transiently on activated CD4+ T cells (Klauss et al., 1997) although activated

memory T cells can maintain robust expression for an extended period of time (Lee et al., 2002). Aside from expression on T cells, CD154 is also expressed in germinal center tissues (Berek et al., 1991) and is involved in B cell education. CD154 can also be expressed in certain situations by activated B cells, natural killer cells, monocytes, eosinophils, basophils, DCs, platelets, endothelial cells, and smooth muscle cells (Fiumara and Younes, 2001). Transient T cell dependent ligation of CD40 induces B cell activation, proliferation (Gauchat et al., 1993), and immunoglobulin isotype-switching and production (Durandy et al., 1993). Sustained stimulation, though, can temporarily arrest antibody secretion while maintaining cell activation and proliferation (Lee et al., 2002). In the germinal centers, CD40 signalling is involved in B cell selection by preventing spontaneous apoptosis (Holder et al., 1993). Hence, there are intimate reciprocal relationships between T and B cell activation. (Illustration 1)

1.6 Activated Professional Antigen Presenting Cells and cGVHD

There is strong evidence supporting the importance of activated antigen presenting cells (APC) in murine models that attempt to simulate chronic GVHD (cGVHD) (Lang et al., 2002, Taylor et al., 2002). When activated, APCs upregulate both the surface expression of MHC as well as of co-stimulatory molecules vital for T cell activation. APCs, while able to mediate long term and antigen-specific adaptive immunity, are also able to recognize and rapidly activate in response (Medzhitov et al., 1997) to pathogen-associated molecular patterns (PAMP) from microorganisms that are interpreted as 'danger signals' (Matzinger, 1994). It has been established that microorganisms can alter the post-BMT environment and increase the risk of complications such as, but not limited to, graft rejection (Johnston et al., 1999) and GVHD (Nestel et al., 1992). Microfloral decontamination of the gastrointestinal tract through the aggressive use of antibiotics

Illustration 1. Ontogeny and Function of B cells



prior to transplantation is effective in decreasing GVHD clinically (Vossen et al., 1990) and it can also be difficult to induce experimental GVHD in animals that have been raised in too fastidious environmental conditions. These findings suggest that microorganisms, or their products, may contribute to the initiation or maintenance of immune dysregulation responsible for cGVHD.

1.7 Professional Antigen Presenting Cell Activation via Toll-like Receptor Signalling

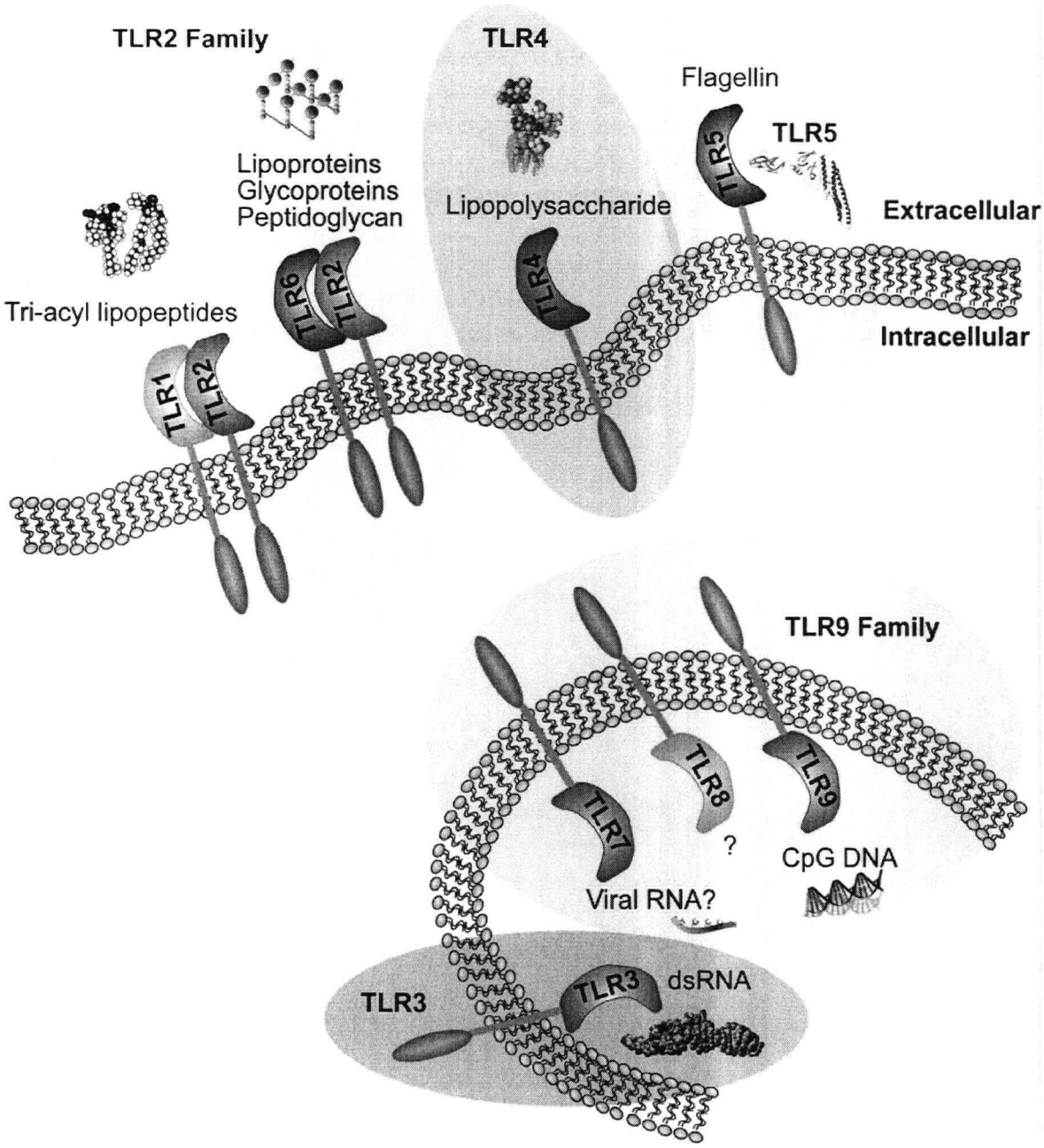
The vertebrate immune system utilizes the cooperation of fast acting innate immunity with specific and long-lasting adaptive immunity. While it has been understood that the adaptive immune system, which generates antigen receptors through gene rearrangement, can potentially recognize an incredibly wide range of antigens in a specific manner, the innate immune system has previously been regarded as relatively non-specific. However, the discovery and characterization of the Toll-like receptor (TLR) family revealed that the innate immune system has highly developed invariant germ-line encoded arrangements to discriminate between varying classes of potential pathogens (Janeway and Medzhitov, 2002). Moreover, there is increasing evidence suggesting that activation of the innate immune system is required for the induction of adaptive immunity and that the TLRs act as a bridge between the two (Akira et al., 2001; Pasare and Medzhitov, 2005).

Many different mammalian TLRs have been identified to date (Aderem and Ulevitch; 2000, Akira et al., 2001; Janeway and Medzhitov, 2002). TLRs are a major class of signalling receptors that recognize evolutionarily conserved microbial structures described as pathogen associated molecular patterns (PAMP) and signal in response to these danger signals. The first mammalian

TLR to be well characterized was TLR4 which specifically recognizes lipopolysaccharide (LPS), an evolutionarily conserved component of the gram-negative bacterial cell wall (Medzhitov et al., 1997; Poltorak et al., 1998), while other TLRs recognize different conserved microbial structures. To date, six primary subfamilies of TLRs have been identified based cumulatively on their amino acid sequence, genomic structure, and their ligands which have been confirmed through phylogenetic nucleotide sequence analysis (Roach et al., 2005). The TLR2 subfamily is composed of TLR1, 2, 6, 10, and 14 which recognize lipopeptides while the TLR9 subfamily is composed of TLR7, 8, and 9 which recognize nucleic acids. The TLR11 subfamily is composed of TLR11, which recognize uropathic bacterial products, 12, and 13. TLR3 which recognizes double stranded RNA, TLR4 which recognizes lipopolysaccharide, and TLR5 which recognizes flagellin are the lone members of their families. Interestingly, the cellular location of various TLRs corresponds somewhat to the molecular location of their ligands (Ahmad-Nehad et al., 2002) as surface expressed TLR1, 2, 4, 5, and 6 recognize cell wall components while intracellularly located TLRs 3, 7, and 9 recognize nucleic acid structures which reside within a cell or capsid. (Illustration 2) Discovery of new TLRs and identification of their ligands are proceeding at a rapid pace.

The TLR family members are type I transmembrane receptors with a leucine-rich repeat (LRR) extra-phospholipid bilayer domain that forms a horseshoe structure responsible for directly recognizing ligand and an interleukin-receptor (IL-R) -like cytoplasmic domain referred to as the Toll/IL-1R (TIR) domain which is responsible for coupling downstream signalling molecules (Slack et al., 2000). Upon successful ligation of their agonist, TIR couples to myeloid differentiation primary response gene 88 (MyD88). MyD88 in turn recruits IL-1R-associated kinase (IRAK) (Wesche et al., 1997) which undergoes phosphorylation and associates with

Illustration 2. Toll-like Receptor Families and Their Ligands

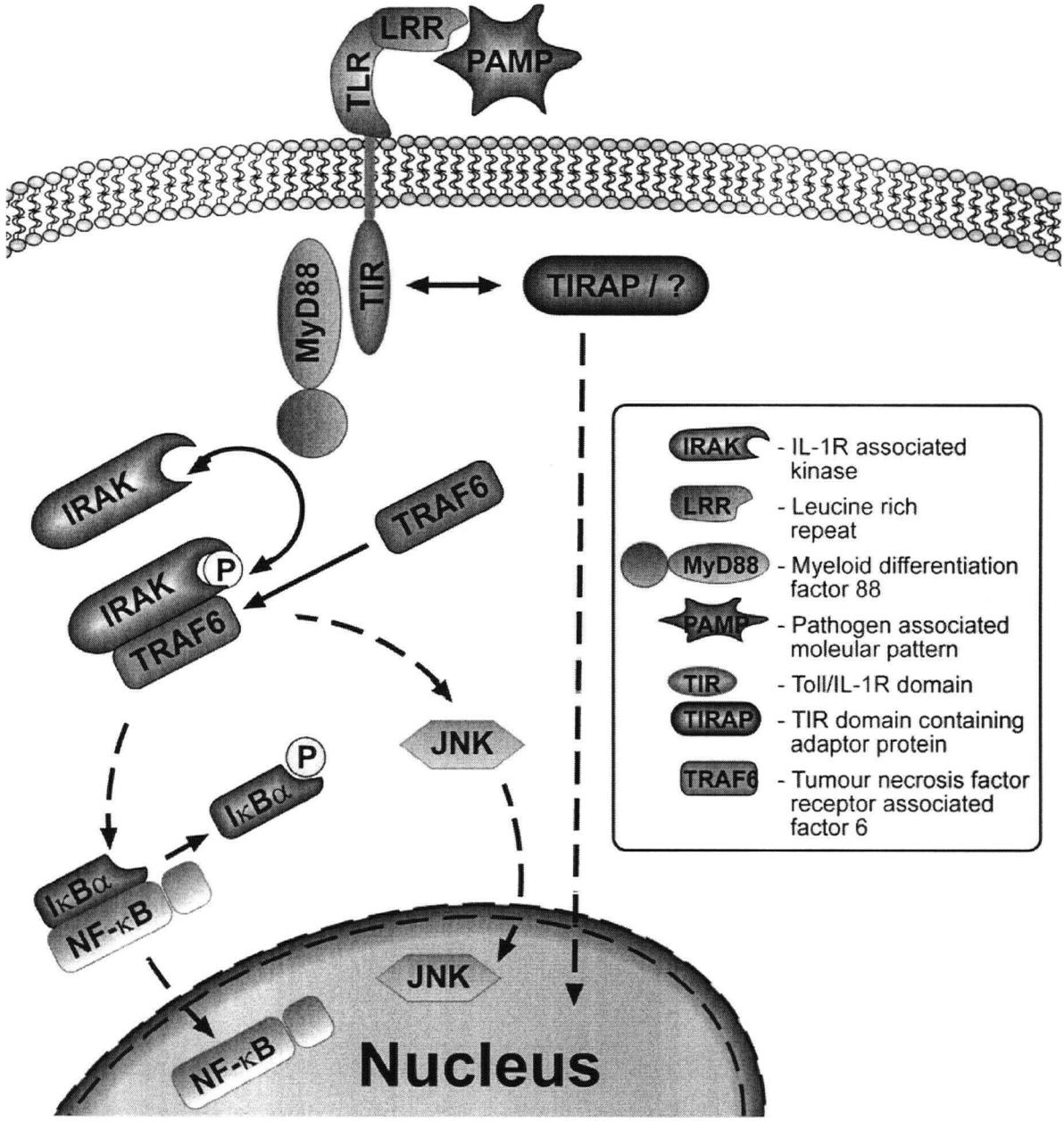


tumour necrosis factor receptor-associated factor 6 (TRAF6) (Muzio et al., 1998). This leads to the activation of subsequent signalling molecules including JNK, and ends in the activation of NF-κB which initiates the transcription of assorted gene products. The exact mechanism behind the diverse responses to agonist ligation of different TLRs is incompletely characterized but it likely involves complementary non-MyD88 dependent pathways. For example, in the case of stimulation of TLR4 with LPS, signalling through the TIR domain-containing adaptor protein (TIRAP or Mal) is also required in addition to the MyD88 signalling pathway to induce the full complement of LPS responses (Horng et al., 2001; Fitzgerald et al., 2001) (Illustration 3).

1.8 Toll-like Receptor 9

Different Toll-like receptors (TLR) specifically recognize different classes of conserved microbial structures ranging from cell wall components to nuclear elements. CpG motifs are immunostimulatory portions of microbial DNA and are recognized by TLR9. These immunostimulatory DNA sequences are characterized by short unmethylated CG nucleotide tandem repeats which are common in microbial DNA (Bird, 1987), but are methylated and statistically under-represented in vertebrate genomes, although potentially active sequences are present (Lipford et al., 1997). Indeed, methylation of immunostimulatory CpG motif-containing microbial DNA renders it immunologically inert (Krieg et al., 1995). However, while demethylation of vertebrate DNA confers immunostimulatory activity the increase in activity is far lower than would be expected from the frequency of known active CpG motifs present. This effect has been attributed to inhibitory guanine-rich sequences within vertebrate genomes (Stacey et al., 2003) although additional factors are likely involved. The nucleotide sequences flanking the CpG dinucleotide are important in determining the magnitude and quality of response

Illustration 3. Overview of Toll-like Receptor Signalling After Productive Receptor Ligation



(Liang et al., 1996) as well as which cell population can optimally respond to it (Krug et al., 2001). The optimal flanking sequences differ between vertebrate species (Bauer et al., 2001) consistent with the co-evolution of innate immune systems alongside species-specific microbial pathogens. Moreover, the recognition of immunostimulatory motifs by TLR9 depends not only on the primary nucleotide sequence but on secondary structure and its capacity to multimerize into quaternary complexes which are required to crosslink the receptor's LRR domain (Wu et al., 2004) and initiate the signalling cascade.

Activation of long lasting and specific adaptive immunity is contingent on the activation of innate immunity. Professional antigen presenting cells, such as plasmacytoid dendritic cells (DC) and B cells, are important in mediating the activation of adaptive immunity. In order to do so they must first recognize and respond to danger signals which is accomplished by surveillance through an array of different receptors which include the TLRs. Microbial CpG DNA, a danger signal, is recognized by TLR9 which is expressed primarily by plasmacytoid DC and B cells in humans. While there is detectable but low expression in monocytes (Stacey et al., 2003), there is debate regarding the ability of monocytes to directly respond to CpG DNA in a TLR9 dependent manner. There are reports that CpG motif containing oligodeoxynucleotides can activate human monocytes (Sester et al., 2000) although it is suggested that this activation may not be TLR9 dependent (Yamane et al., 2005). Likewise, natural killer cells express minimal amounts of TLR9 and there is debate regarding whether these cells directly respond to CpG in a TLR9 dependent manner (Roda et al., 2005) or if the activation is induced by CpG responding populations such as plasmacytoid dendritic cells (Romagnani et al., 2005). T cells do not express TLR9 nor respond directly to CpG although they may be modulated by signals from responder populations (Hornung et al., 2002). In DCs, CpG motifs induce maturation, the expression of

co-stimulatory molecules, and migration (Sparwasser et al., 1998). In B cells, CpG DNA induces cell activation (Krieg et al., 1995), cytokine secretion (Klinman et al., 1995), expression of co-stimulatory molecules, and upregulation of class II MHC (Hartman and Krieg, 2000) which facilitates the activation of adaptive immunity.

TLR9, unlike TLRs 1, 2, 4, 5, or 6, is intracellularly located (Ahmad-Nejad et al., 2002). DNA is non-specifically taken up into the cell (Hacker et al., 2000) and transported via early endosomes into tubular lysosomal compartments proximal to the endoplasmic reticulum (ER). The presence of DNA in the tubular lysosomes rapidly induces TLR9 protein to redistribute from the ER and aggregate at the lysosome (Latz et al., 2004). Following lysosomal acidification (Rutz et al., 2004), TLR9 binds directly to DNA and MyD88 rapidly co-accumulates contingent on the presence of DNA complexes that are capable of crosslinking the LRR domain of TLR9. Upon MyD88 phosphorylation by the TLR9 TIR domain, the signalling cascade proceeds to stimulate the transcription of cell activation genes. Human B cell responses to successful TLR9 ligation can manifest as the production of cytokines such as interferon-γ (IFN-γ) (Sun 1998), IL-6 (Klinman et al., 1996), IL-12 (Redford et al., 1998) and class switching to IgG_1, IgG_2, and IgG_3. Additional responses include polyclonal proliferation (Liang et al., 1996) and the expression of co-stimulatory molecules such as CD40, CD80, and CD86 and upregulation of class II MHC (Hartman and Krieg, 2000). However, there is a degree of heterogeneity in the population level responses by B cells to a particular synthetic immunostimulatory CpG sequence (Leifer et al., 2003) although variation in healthy individuals over multiple assays is reportedly low. The observed heterogeneity is suggested to be due to genetic polymorphisms in TLR9 (Lazarus et al., 2003; Kikuchi et al., 2005) or to environmental factors that may alter CpG response. Various

such factors can modulate TLR9 expression (An et al., 2002) and altered TLR9 expression affects CpG response (Bourke et al., 2003).

1.9 Toll-like Receptor 9, CpG, and cGVHD

Given the immunostimulatory properties of CpGs, synthetic immunostimulatory CpG has been investigated, in murine models, as an agent to enhance the graft-versus leukaemia effect by activating donor-derived immune cells against residual host-derived leukaemic cells. Administration of CpG to naïve and semi-allogeneic parental-to-F1 blood and marrow transplant mice prior to lethal challenge by leukaemic cells, as a model of graft-versus-leukaemia, provided protection from mortality (Blazar et al., 2001). However, the administration of CpG to the semi-allogeneic blood and marrow transplant mice alongside a donor lymphocyte infusion increased the severity of GVHD as measured by mean weight loss. Our group has previously shown using a similar murine model that splenocytes, which B cells comprise a significant population, from mice with experimentally induced GVHD after semi-allogeneic blood and marrow transplant are hyper-responsive to immunostimulatory CpG compared to syngeneic transplant controls that do not develop GVHD (Schultz et al., 2002). The hyper-responsiveness was characterized by an exaggerated mitogenic response to optimized murine immunostimulatory CpG as well as by elevated IL-6 production. The hyper-responsive events were significantly ameliorated by chloroquine, a weak base that is a member of the 4-aminoquinoline family that has been successfully adapted from malarial treatment to therapy for various autoimmune diseases such as rheumatoid arthritis (Fox, 1993) and systemic lupus erythematosus (Wallace, 1994). Moreover, 4-aminoquinoline treatment has been shown to inhibit GVHD in murine GVHD models (Schultz2 et al., 1995) and in the clinical setting (Gilman and Schultz, 2000). While the 4-

aminoquinolines are extremely high affinity DNA intercalators (Strekowski et al., 2002), their DNA binding properties are unrelated to their ability to inhibit CpG-TLR9 responses which rather depends on their ability to partition into acidic vesicles and disrupt pH gradients. This mechanism is indirectly supported by the inability of a panel of DNA binding agents to decrease CpG-TLR9 signalling while other agents that inhibit or disrupt vesicular acidification achieved partial inhibitory effects (MacFarlane and Manzel, 1998). It is possible that the disruption of the pH gradient alters the secondary folding structures of the oligonucleotides and prevents the formation of quaternary structures capable of crosslinking the LRR domain of TLR9. The efficacy of hydroxychloroquine, a 4-aminoquinoline, in treating human cGVHD (Schultz et al., 2000) and the efficacy of Rituximab, a monoclonal pan-B cell antibody used for ablating B cells *in vivo*, in treating complicated human GVHD (Ratanatharathorn et al., 2003) suggests an important role for B cell responses to immunostimulatory CpG in human cGVHD development or maintenance.

2. CHAPTER TWO: RATIONALE AND OBJECTIVES

Allogeneic blood and marrow transplantation (BMT) is an increasingly common treatment modality for an expanding list of malignant and non-malignant conditions. However, graft-versus host disease (GVHD) remains a major obstacle to successful transplant outcome and is progressively acknowledged as an important consideration. Although much is known about acute GVHD (aGVHD) due to the existence of animal models that translate well to the clinical situation, less is known about the pathophysiology of cGVHD. This is due in part to the difficulty in developing uniform standardized diagnostic criteria as manifestations of cGVHD are highly pleiotropic and in part due to the lack of highly satisfactory animal models.

Despite the limitations of animal models of cGVHD, they have previously been successfully used to identify productive avenues of study. The observed interactions between T cells, dendritic cells, and B cells in animal models of cGVHD have lead to directed examination of these lymphocyte populations in human cGVHD. Also, animal models have suggested the involvement of microbial products. Experiments in enhancing the graft-versus leukaemia response in a semi-allogeneic murine models first suggested that synthetic CpG, an analogue of immunostimulatory microbial DNA, increases cGVHD-like symptoms. Experiments in a similar semi-allogeneic model revealed that splenocytes, comprising mainly B cells, from mice with simulated cGVHD were hyper-responsive to CpG. This observation suggests that microbial nucleotide danger signals may be involved in the immune dysfunction associated with cGVHD. Cytomegalovirus infection is associated with the development of cGVHD (Lonnqvist et al., 1984) and DNA from this virus is potentially recognized by TLR9 (Delale et al., 2005).

The 4-amoniquinoline family of small molecules, adapted successfully to treat autoimmune disorders such as lupus erythematosus and rheumatoid arthritis, potently block the ability of TLR9 to recognize immunostimulatory CpG DNA and ameliorate the hyper-responsiveness observed in cGVHD mice. Additionally, 4-aminoquinoline treatment of mice prevents cGVHD symptoms and, importantly, has also shown success in treating human cGVHD.

The ablation of B cells in murine models prevents the development of cGVHD-like symptoms and suggests the importance of this lymphocyte population in disease development. Evidence of the importance of B cells in human cGVHD lies in the success of B cell ablation using Rituximab, a monoclonal antibody to pan-B cell marker CD20, in successfully treating some cases of complicated human cGVHD.

Given these observations, the goals of this thesis were:

1) To investigate whether CpG hyper-responsiveness, seen with simulated cGVHD in mouse splenocytes, is also present in human B cells after the diagnosis of cGVHD.
2) To determine if enhanced CpG responsiveness is directly related to altered TLR9 expression.
3) To determine if an enhanced CpG response is specific to TLR9 or due to an overall inflammatory response.
4) To determine if different B cell subpopulations present in patients with or without cGVHD account for CpG hyper-responsiveness.
5) To determine if the clinical severity of cGVHD is related to an altered CpG/TLR9 response.

3. CHAPTER THREE: MATERIALS AND METHODS

Cells and Tissue Culture

Up to 1 mL/Kg of peripheral blood (PB) was collected in Sodium-citrate heparin glass tubes from patients enrolled on Children's Oncology Group Study ASCT0031, a phase III trial that evaluated the efficacy of the 4-aminoquinoline hydroxychloroquine (Plaquenil) in treating newly diagnosed pediatric cGVHD, at the time of cGVHD diagnosis before receiving either standard or experimental treatment. Control samples were collected from patients who have undergone allo-BMT but have not been diagnosed with cGVHD at regular intervals (3, 6, 12, and 15 months) post-transplant. Both patient groups were on identical standard prophylactic therapy at the time of sample acquisition. Days post transplant upon cGVHD diagnosis was used to divide the patients into early onset (<9 months) or late onset (≥9 months) (Figure 1D) based on the 3 and 6 month and 12 and 15 month control patient groupings. Based on these divisions we selected the control time point of 6 months for the early onset and 12 months for the late onset cGVHD groups. PB was shipped or stored overnight at ambient temperature. Peripheral blood mononuclear cells (PBMCs) were harvested by layering PB over Ficoll-Paque™ Plus (Amersham Pharmacia Biotech AB, Upsala, Sweden) and centrifuged at 1500rpm for 20min in an IEC Centra GP8R centrifuge at 19°C. Mononuclear cells at the interface were removed and washed with PBS 2%FBS and resuspended in complete media at $2x10^6$ cells/mL. In all experiments involving oligonucleotides (ODN) stimulation, $1x10^6$ cells were incubated in complete RPMI media with or without 6μg/mL ODN for 48 hours prior to analysis. The ODNs used include phosphorothioate (PS) modified CpG 2006 (t*c*g* t*c*g* t*t*t* t*g*t* c*g*t* t*t*t* g*t*c* g*t*t*),

phosphodiester (PD) CpG 2006 (tcg tcg ttt tgt cgt ttt gtc gtt), control PS modified GpC 2137 (t*t*g* c*t*g* t*t*t* t*g*c* t*g*t* t*t*t* g*c*t* g*c*t*), control PD GpC 2137 (ttg ctg ttt tgc tgt ttt gct gct), PS end-capped PD CpG 2006 (t*cg tcg ttt tgt cgt ttt gtc gtt*), or PS end-capped PD GpC 2137 (t*tg ctg ttt tgc tgt ttt gct gct*). All ODN were obtained from Sigma-Aldrich (St. Louis, MO). All ODN tested negative for endotoxin contamination with Limulus Amebocyte Lysate PYROGENT® Plus (Cambrex Bio Science, Walkersville, MD). The activity of CpG 2006 used in this study has been previously extensively analyzed (Hartman and Krieg, 2000). In experiments involving peptidoglycan (PG) stimulation, $1x10^6$ cells were incubated with or without 10μg/mL PG (Fluka Production GmvH, Buchs, SG) for 48 hours prior to analysis. In experiments involving lipopolysaccharide (LPS) stimulation, $1x10^6$ cells were incubated with or without 1μg/mL LPS (Sigma Aldrich, St. Louis, MO). After the culture, cells were harvested by pipetting and adherent cells were further collected by pipetting after incubation in PBS-1 mM EDTA for 5 minutes at 37°C.

Immunophenotyping

Four color flow cytometry was performed on a FACSCalibur™ flow cytometer (Becton Dickinson). For analysis of cell surface molecules, cells were resuspended at $1x10^6$ cells/mL and $1x10^5$ cells/assay tubes were labelled with directly conjugated fluorescent antibodies at 4°C for 20 minutes. The samples were then washed and resuspended in phosphate-buffered saline - 1.5% paraformaldehyde and analyzed immediately. All antibodies were obtained from BD Pharmingen (San Diego, CA). The relevant labelled isotype control antibodies were included in all experiments to control for instrument changes over time. All cells with a staining intensity

higher than the upper limit obtained using the isotype control were considered positive. Analysis was performed using CellQuest software (Becton Dickinson) after gate exclusion of non-viable cells based on forward and side scatter, for specific cells by surface marker expression, and for other surface molecules (Illustration 4).

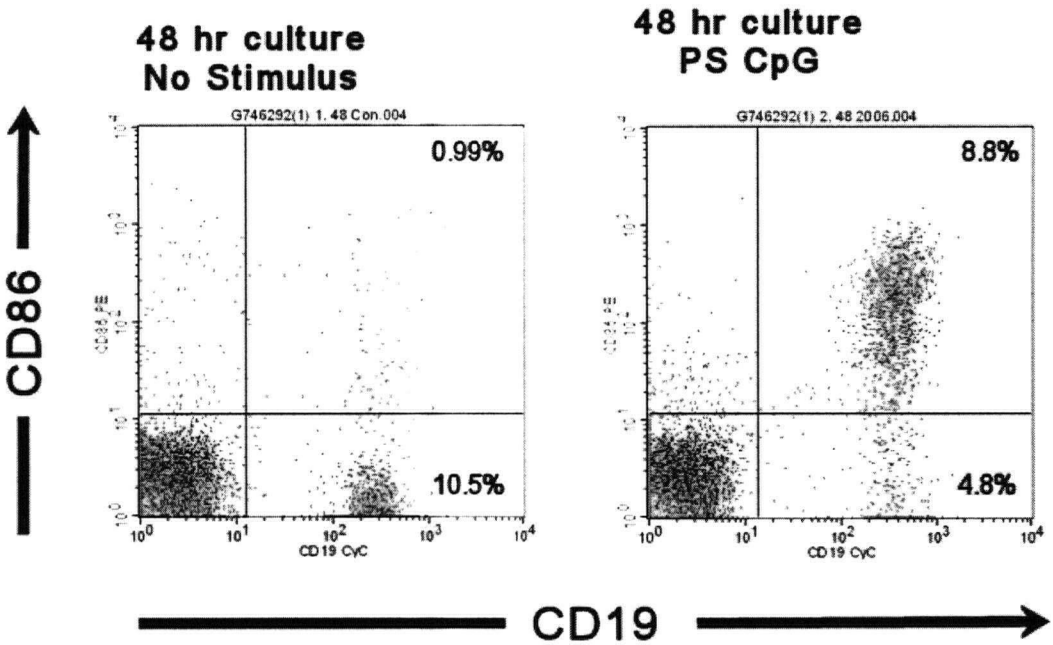
B cell and T cell Enrichment

PBMCs were isolated over Ficoll-Paque™ Plus as described above and resuspended at $5x10^7$ cells/mL in PBS 4% FBS. B cells and T cells were isolated using StemSep with human B cell Enrichment Cocktail and human T cell Enrichment Cocktail (Stem Cell Technologies Inc., Vancouver, Canada) respectively according to manufacturer's protocol. Purified cells were resuspended at $2x10^6$ cells/mL in complete RPMI media. Cells were >95% pure as determined by flow cytometry.

Proliferation Assays

Assays were set up in triplicate in complete media using round bottom 96-well plates. $1x10^5$ B cells were plated in each well. Cell stimulation was performed with the same reagents and concentrations as described above. Plates were incubated for 28 hours at 37°C and then ^{3}H-thymidine was added to each well at a final concentration of 1μCi/mL and incubation was continued for an additional 20 hours. DNA was then harvested using a Mach III M harvester (Tomtec, Hamden, CT) and ^{3}H-thymidine incorporation was counted on a 1450 Microbeta

Illustration 4. FACS Analysis of B Cell Surface Molecule Expression



% B cells CD86+
0.99% / (0.99% + 10.5%)

8.6%

Expression Differential
64.7% - 8.6%

56.1%

% B cells CD86+
8.8% / (8.8% + 4.8%)

64.7%

liquid scintillation counter (Wallac Trilux, Turku, Finaland) using Wallac 1450 Microbeta windows workstation software (version 2.70.004).

Quantitative RT-PCR

Total RNA was extracted from purified B cells using Trizol reagent (Gibco BRL, Rockville, MD). Following DNAse I treatment, cDNA was synthesized with 1μg of total RNA using oligo dT primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Real-time quantitative PCR was performed using the TaqMan system (Applied Biosystems, Foster City, CA). The expression level of TLR9 and internal reference TBP was measured by PCR using TaqMan probes labelled with FAM and VIC, respectively. Probes and primers were obtained from Applied Biosystems; TLR9 (Toll-like receptor 9) forward primer 5' - TGC AGC CGG AGA TGT TTG - 3', reverse primer 5' - CGC GGT AGC TCC GTG AAT - 3', probe 6FAM-CAG TCA ATG GCT CC-MGB NFQ used at a final concentration of 900 nM and 200 nM respectively. Control reagents were used as per the pre-developed TaqMan Assay Reagent for Human TBP (Applied Biosystems). Real-time PCR amplification and product detection was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as recommended by the manufacturer. The quantity of cDNA for each RNA sample was normalized to the quantity of TBP cDNA in each sample. Relative expression was determined using ΔΔCt (threshold cycle) method according to the manufacturer's protocol. Each assay includes a fixed standard, a no-template control, and a cDNA sample in triplicate.

Depletion of Adherent Cells

PBMC were incubated in complete RPMI in 50mL Falcon polystyrene culture plates at 37°C for 3 hours at 5% CO_2. Non-adherent cells were harvested by pipetting and analyzed by FACS for the absence of dendritic cells (Lin+, HLA-DR+, CD123+/CD11c+) and monocytes (CD14+).

Cell stimulation with agonistic monoclonal antibodies

Agonistic monoclonal antibodies (α human CD28 clone CD28.2, α human CD3 clone OKT3, (eBiosciences San Diego CA, USA), or α human IgM mouse F(ab')2 (Southern Biotechnology Associates Inc. Birmingham, AL USA)) were adsorbed onto polystyrene plates in 50 mM Tris HCl (pH 9.5) for 3 hours at 37°C and washed twice in complete RPMI media. Cells were plated at $2x10^6$ cells/mL and incubated for 48 hours at 37°C. Cells were harvested by pipetting and adherent cells were further collected after incubation in PBS-1 mM EDTA for 5 minutes at 37°C.

Assessment of Red Blood Cell Lytic Activity

PBMC from patients were cultured as described above. Evidence of red blood cell (RBC) lytic activity was determined if there was the lack of RBC by visual inspection in the harvested cell pellet after 48 hours of culture with stimulus contingent on the presence of RBC in the harvest cell pellet after 48 hours of culture without stimulus.

Assays were set up in triplicate in complete media using round bottom 96-well plates. $1x10^5$ RBC from non-transplant volunteers were plated in triplicate in 100µl of previously frozen supernatants conditioned by patient cells with or without stimulus and cultured for 72 hours at 37°C. Evidence of RBC lytic activity was determined by a decrease in pellet size compared to RBC plated in fresh complete media.

Statistical Analysis

Descriptive statistics were generated on all data using Prism version 4 for PC (Graphpad Software, San Diego, CA). Expression differential for cell surface molecule expression was defined as the difference in the percentage of positive cells after culture with stimulation and the percentage of positive cells after culture with no stimulation (% stimulated cells positive - % cultured, non-stimulated cells positive). The degree of dispersion from the mean was calculated as a standard deviation (σ). Significance of observed changes was determined using Mann-Whitney U t-test. The alpha (P) value was set at 0.05, making all p values <0.05 statistically significant.

4. CHAPTER 4: RESULTS - RESPONSES TO CpG

Hyper-responsiveness to CpG was observed in splenocytes from mice with simulated chronic GVHD (cGVHD) using a semi-allogeneic transplant model but not in splenocytes from syngeneic transplant control mice with no cGVHD-like symptoms. CpG responses were assessed in paediatric patients with newly diagnosed cGVHD prior to the initiation of standard or experimental therapy and were compared to post-transplant time-matched control paediatric patients who have not developed cGVHD.

4.1 Selection of Control Populations and Division of cGVHD Patients

To determine if there were altered responses to CpG after the onset of chronic GVHD (cGVHD), responses to stimulation as was assessed in patients upon the diagnosis of cGVHD and, at regular intervals, in patients who had undergone allogeneic blood and marrow transplant (allo-BMT) but had not developed cGVHD. CpG induced upregulation of co-stimulatory molecules CD80, CD86, and CD40 was assessed in B cells. Mononuclear cells were harvested from peripheral blood by density-gradient centrifugation and immediately cultured with or without phosphorothioate modified (PS) CpG 2006 for 48 hours. B cells were identified by FACS by sub-gating CD19+ cells from the live gate. Upregulation of surface molecules was assessed as the difference between the percentage of cells staining positive above isotype controls for the co-stimulatory molecule of interest after culture with stimulation and the percentage of positive B cells after culture in complete media without stimulation.

Evaluation of the upregulation of CD80 by CD19+ B cells in response to PS CpG 2006 in non-cGVHD control patients revealed that there were neither differences between the 3 and 6 month, nor the 12 and 15 month non-cGVHD controls. There was a trend towards increased B cell upregulation of CD80 in response to PS CpG 2006 in the 12 and 15 month non-cGVHD control groups compared to the 3 and 6 month control groups (Figure 1A). There were no differences in the upregulation of CD86 by B cells in response to PS CpG 2006 between the 3 and 6 month, nor the 12 and 15 month non-cGVHD controls but there was a significantly lesser percentage of B cells capable of upregulating CD86 in the 6 month non-cGVHD controls (n=9, 15.8% ±8.2% σ) compared to both the 12 month (n=8, 42.8% ± 4.6% σ, n=0.006) and 15 month non-cGVHD controls (n=5, 44.2% ±6.3% σ, p=0.02) (Figure 1B). There were no differences in B cell CD40 upregulation between all non-cGVHD control groups (Figure 1C). Based on these time post-transplant dependent gain in responses, cGVHD patients were divided into an early onset (between 3 - 8 months upon cGVHD diagnosis; n=36, 171 days [5.7 months] ± 50 days σ) which were compared to the 6 month post-transplant non-cGVHD controls and a late onset (≥ 9 months upon cGVHD diagnosis; n=15, 429 days [14.3 months] ± 181 days σ) group which were compared to 12 month post-transplant non-cGVHD controls (Figure 1D).

4.2 Differential B cell Response to Phosphorothioate-modified Immunostimulatory CpG 2006

Evaluation of the upregulation of CD80 by CD19+ B cells in response to phosphorothioate (PS) CpG 2006 stimulation revealed that a significantly greater percentage of B cells from the early (3 - 8 month) cGVHD patients (n=19, 6.7% ± 2.3% σ) compared to the 6 month non-cGVHD controls (n=9, 0.3% ±1.2% σ, p=0.02), but not from the late (≥ 9 month) cGVHD patients

Figure 1. Selection of Control Populations and Division of cGVHD Patients

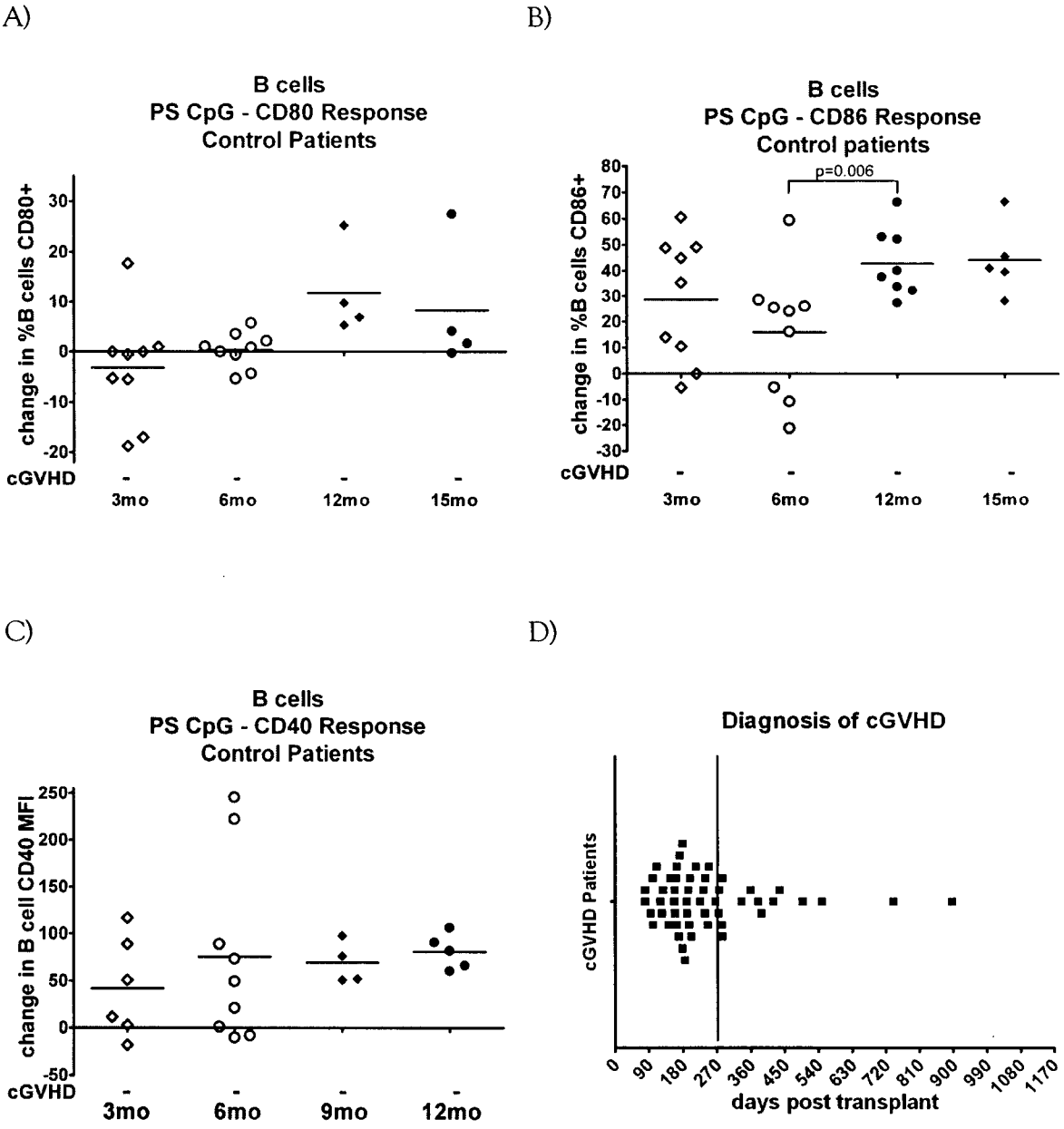


Figure 1. B cell upregulation of (A) CD80, (B) CD86, and (C) CD40 in peripheral blood mononuclear cell cultures, from non-cGVHD control patients, stimulated with PS CpG 2006 for 48 hours as determined by FACS. (D) Distribution of patients diagnosed with cGVHD based on the days post-transplant upon the diagnosis of cGVHD and entry into study which were subsequently divided into early onset (3 - 8 months) and .late onset (≥ 9 months) groups.

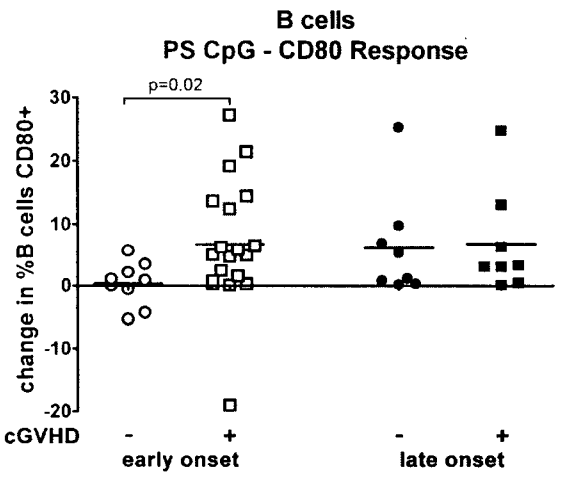
compared to the 12 month non-cGVHD controls, were capable of rapidly upregulating CD80 in response to PS CpG 2006 (Figure 2A). Similarly, a significantly greater percentage of B cells from the early cGVHD patients (n=19, =56.3% ±2.0 σ) compared to the 6 month non-cGVHD controls (n=9, 15.8% ± 8.2% σ, p=0.0004), but not from the late cGVHD patients compared to the 12 month non-cGVHD controls, were capable of rapidly upregulating CD86 in response to PS CpG 2006 (Figure 2B). There were no significant differences in the B cell upregulation of the surface density of CD40 as measured by mean-fluorescence intensity in response to stimulation with PS CpG 2006 for 48 hours between all groups (Figure 2C).

4.3 Homogenous Baseline B Cell Expression of Co-stimulatory Molecules Between All Groups

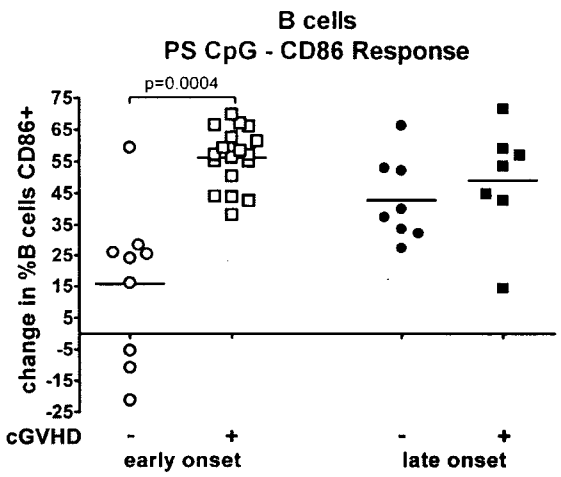
To determine if the differences observed between cGVHD and non-cGVHD control patients in the upregulation of co-stimulatory molecules by B cells in response to phosphorothioate (PS) CpG 2006 stimulation was due to differences in B cell phenotype before stimulation, we assessed co-stimulatory molecule expression in un-manipulated B cells and in B cells after culture for 48 hours without stimulation. There were no baseline differences in the percentage of B cells expressing CD80 (Figure 3A), CD86 (Figure 3B), or CD40 (Figure 3C) in un-manipulated whole blood between all groups. Culture of cells in complete media without stimulation did not significantly alter, from un-manipulated baseline levels, the percentage of B cells expressing CD80, CD86, or CD40 in all groups. There were no significant differences between all groups in the change in absolute number of B cells that entered culture and after 48 hours of culture without stimulus.

Figure 2. B cell Responses to Phosphorothioate-modified Immunostimulatory CpG 2006

A)



B)



C)

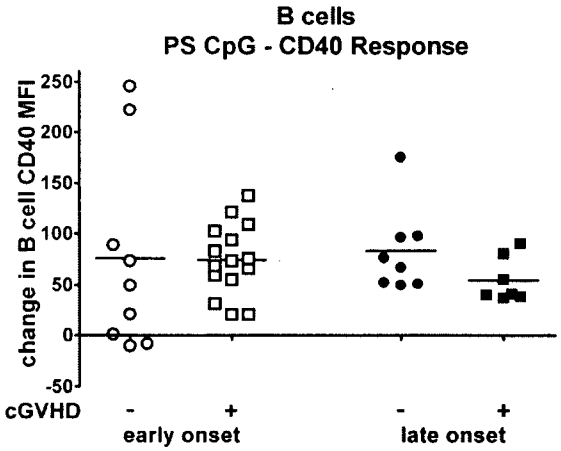
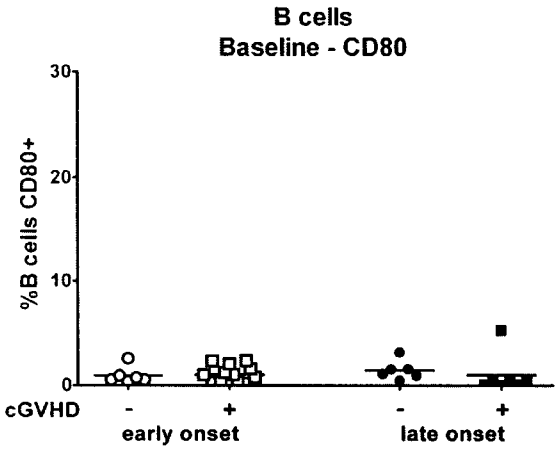


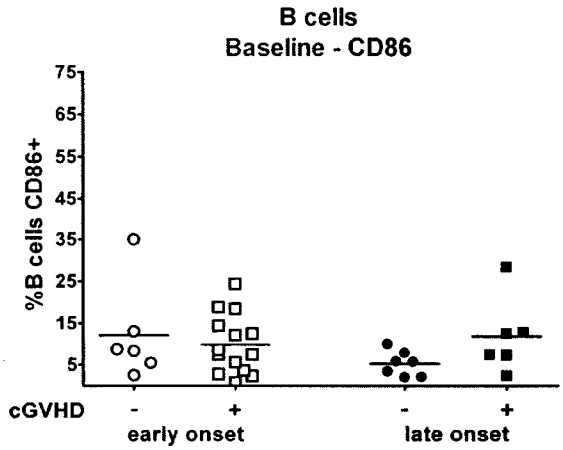
Figure 2. B cell upregulation of (A) CD80, (B) CD86, and (C) CD40 in peripheral blood mononuclear cell cultures stimulated with PS CpG 2006 for 48 hours from early onset (3 – 8 months) and late onset (≥ 9 months) cGVHD patients compared to 6 month and 12 month non-cGVHD controls, respectively as determined by FACS.

Figure 3. Baseline B Cell Expression of Co-stimulatory Molecules

A)



C)



C)

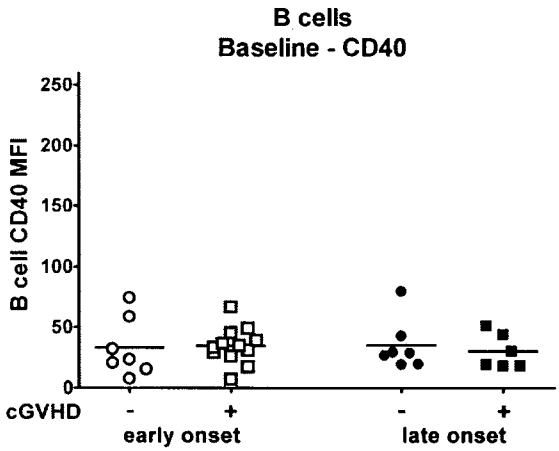


Figure 3. Baseline B cell expression of CD80 (A), CD86 (B), and CD40 (C) between early onset (3 -8 months) and late onset (≥ 9 months) cGVHD compared to 6 month and 12 month non-cGVHD control patients as determined by FACS.

4.4 CpG Responses - Conclusions

The limited accrual of patients into the Children's Oncology Group study ASCT0031 and the sharing of these patient samples with other basic biology studies prevented the grouping of cGVHD patient samples for their direct comparison with non-cGVHD control patient samples taken at 3, 6, 12, and 15 months post transplant. There was no difference in B cell response to phosphorothioate (PS) CpG 2006 between the 3 and 6 month controls and between the 12 and 15 month controls. However, there was a difference in response between the 6 month controls compared to both the 12 and 15 month controls. Based on this difference, all subsequent evaluations between control and cGVHD patients were divided into early onset (between 3-8 months upon cGVHD diagnosis) and late onset (≥9 months upon cGVHD diagnosis) groups based on the midpoint between the 6 month and 12 month time points. The early and late cGVHD time points were compared to the 6 and 12 month non-cGVHD controls, respectively.

A significantly greater percentage of B cells from early onset (3 - 8 months) cGVHD patients were capable of upregulating CD80 and CD86 in response to PS CpG 2006 stimulation compared to the 6 month non-cGVHD control patients. In contrast, there were no significant differences in PS CpG 2006 response between the late onset (≥ 9 month) cGVHD patients and the 12 month non-cGVHD controls. There were no differences in degree of CD40 upregulation in response to PS CpG 2006 between all groups.

The increased PS CpG 2006 response as it was measured was not due to differences in the pre-existing B cell phenotype between non-cGVHD control and cGVHD patients as there were no

differences in the percentage of peripheral blood B cells exhibiting an activated phenotype between cGVHD and non-cGVHD control patients.

5. CHAPTER 5: RESULTS - MECHANISM OF INCREASED CpG RESPONSE

The classical and primary mechanism of cellular recognition of immunostimulatory CpG-DNA is via Toll-like Receptor 9 (TLR9). TLR9 mRNA levels in B cells were assessed and compared to the B cell response to phosphorothioate (PS) CpG. Since it has been determined that the phosphorothioate backbone modification confers non-sequence specific and non-TLR9 mediated immunostimulatory activity, responses to native phosphodiester (PD) CpG, which has no extra-sequence or TLR9-specific activity, were assessed in patients with or without chronic GVHD (cGVHD) to confirm the observation that there is increased B cell response to PS CpG in paediatric patients who have developed cGVHD compared to post-transplant time-matched control paediatric patients who have not developed cGVHD.

5.1 CpG Response is Determined by TLR9 Expression

Toll-like receptor 9 (TLR) is the primary mediator of immune surveillance of microbial DNA. TLR9 expression can vary between individuals (Kikuchi et al., 2005), cell types (Hornung et al., 2002; Bernasconi et al., 2003) and this expression can also be modulated through cell activation (An et al., 2002). Additionally, these changes in TLR9 expression confer quantitatively altered responses to immunostimulatory CpG. However, since it was possible that an activated or otherwise altered cell activation state may augment or suppress TLR9 mediated signalling in response to CpG, TLR9 mRNA expression levels in B cells were assessed in a subset of patients.

The role of TLR9 in B cell response to phosphorothioate modified (PS) CpG 2006 was determined by comparing the degree of B cell upregulation of CD86 in response to PS CpG 2006 (Figure 2C) and the expression levels of TLR9 mRNA in B cells which had not been manipulated. TLR9 mRNA was quantified in B cells from patients prior to, or who did not require, cGVHD therapy when a sufficient volume of peripheral blood was available for B cell purification. There was a significant correlation ($n=12$, $r^2=0.65$, $p=0.002$) in PS CpG response and B cell TLR9 mRNA levels (Figure 4).

5.2 Phosphodiester CpG Stimulation is a More Stringent Assessment of TLR9 Response

Although the magnitude and quality of CpG response is sequence specific and this response is mediated through TLR9, it has been shown that phosphorothioate modified (PS) CpG can induce B cell activation through non-TLR9 and non-sequence specific mechanisms. These mechanisms include, but are not necessarily limited to, the involvement of DNA-Protein Kinase-c subunit (DNA-PKcs) (Dragoi et al., 2005), a protein normally associated with DNA repair, triggering the Akt pathway cascade leading to cell activation. It was observed that there was a heterogeneous B cell response to PS CpG stimulation in the non-cGVHD control patients, specifically, that there were time-post transplant dependent increases in CD86 upregulation (Figure 1B). Additionally, while there was a significantly increased percentage of B cells that upregulate CD86 from the early (3 - 8 month) cGVHD group compared to the 6 month non-cGVHD controls, there was no significant difference between the late (≥ 9 months) cGVHD group compared to the 12 month non-cGVHD controls in the percentage of B cells that upregulated CD86 (Figure 2B).

Figure 4. B Cell Expression of TLR9 mRNA and B Cell Response to Phosphorothioate-modified Immunostimulatory CpG 2006

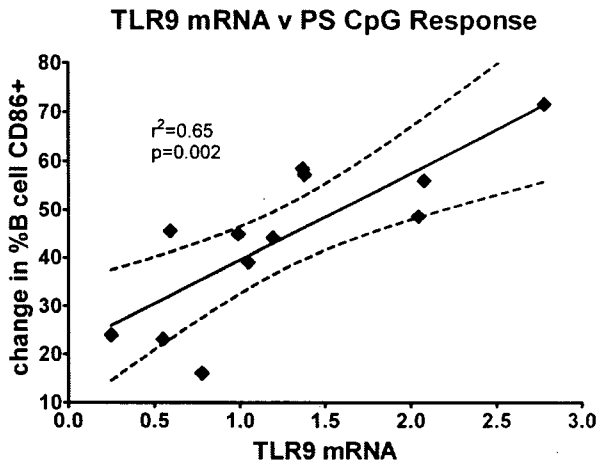


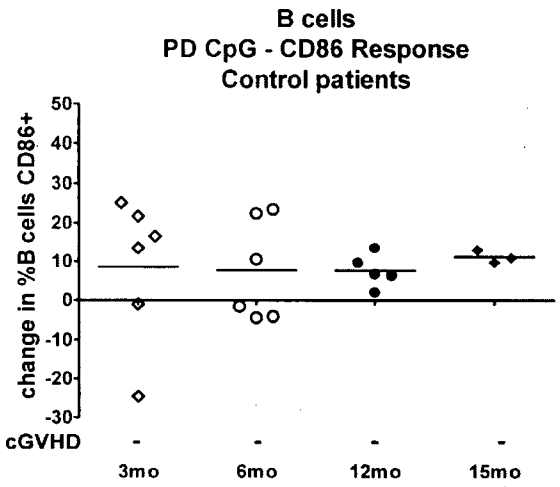
Figure 4. The degree of B cell CD86 upregulation in response to PS CpG 2006 (Figure 2C) significantly correlates with the relative expression of TLR9 mRNA compared to endogenous control protein TATA-box Binding Protein normalized to expression in B cell like KSEBV in *ex vivo* B cells from the same patients as determined by quantitative real-time RT PCR. Diamonds represent individual patients who have not initiated cGVHD therapy.

To determine if these phenomena were due to non-TLR9 specific mechanisms, peripheral blood mononuclear cells from patients were harvested and cultured in an identical manner to that described previously with the exception that the stimulating CpG oligodeoxynucleotide, while having the same sequence as PS CpG 2006, has an unmodified native phosphodiester (PD) backbone and more closely resembles the natural ligand of TLR9. In contrast to the heterogeneous B cell CD86 response to PS CpG 2006 between the non-cGVHD control groups, there were no differences in B cell CD86 response to native phosphodiester (PD) CpG 2006 response in the 3, 6, 12, and 15 months post-transplant non-cGVHD control groups (Figure 5A). Additionally, stimulation with native PD CpG 2006 induced a significantly greater percentage of B cells from the late ($\geq$ 9 month) cGVHD group to rapidly upregulate CD86 (n=3, 30.9% $\pm$ 0.8% σ) compared to the non-GVHD 12 month controls (n=5, 7.6% $\pm$ 1.9% σ, p=0.03) (Figure 5C). Stimulation of cells from the early (3 - 9 month) group with native PD CpG 2006, similar to stimulation with PS CpG 2006, induced a significantly greater percentage of B cells to upregulate CD80 (n=5, 2.5% $\pm$ 1.0% σ) compared to the 6 month non-cGVHD controls (n=5, -2.8% $\pm$ 2.1% σ, p=0.02) (Figure 5B) as well as CD86 (n=5, 36.2% $\pm$ 3.2% σ) compared to the 6 month non-cGVHD controls (n=6, 7.7% $\pm$ 5.3% σ, p=0.004) (Figure 5C). Using CD86 as a marker for B cell activation, a significantly greater percentage of B cells from all patients diagnosed with cGVHD were activated by PD CpG 2006 compared to any of the non-cGVHD control patients.

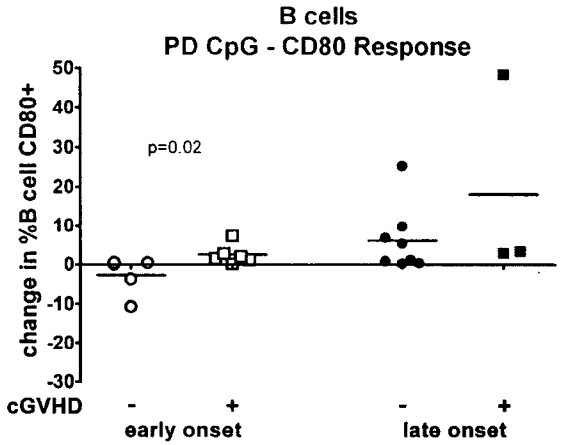
Since TLR9 recognizes DNA in a sequence specific manner the reverse sequence of oligonucleotide 2006, which possessed GpC repeats, should be immunologically inert. However,

Figure 5. B Cell Responses to Native Phosphodiester Immunostimulatory CpG 2006

A)



B)



C)

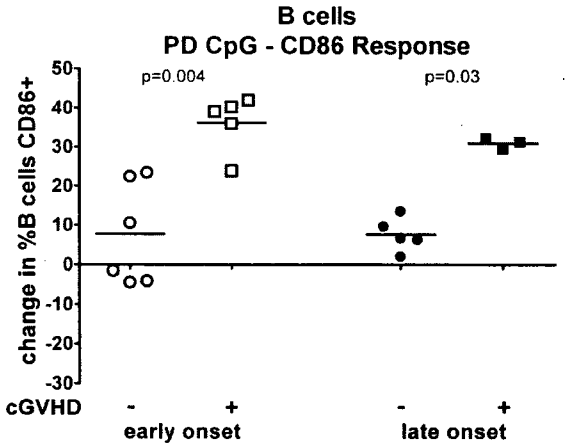


Figure 5. (A) B cell CD86 response to phosphodiester (PD) CpG 2006. (A) B cell CD80 and CD86 expression response to phosphodiester (PD) CpG 2006 in the early cGVHD group and (C) the late cGVHD group compared to the 6 month and 12 month non-cGVHD control groups respectively.

phosphorothioate (PS) modified GpC 2137, the reverse sequence of CpG 2006, exhibited significant but variable ability to activate not only B cells, but also cell populations which do not express TLR9 (data not shown). This observation prompted investigation into the activity of phosphodiester (PD) oligodeoxynucleotides. Unlike PS GpC 2137, there was negligible immunostimulatory activity with the immunologically inert control sequence PD GpC 2137 (Figure 6A) suggesting that PD DNA has little or no DNA-PKcs mediated activity and exerts immunogenicity primarily through TLR9. As PD oligodeoxynucleotides are not used for *in vivo* stimulation due to concerns regarding nuclease degradation, whether the decreased magnitude of response to PD CpG 2006 was due to nuclease activity was assessed. Cells from adult non-BMT volunteers prepared in an identical manner as patient cells and stimulated with PD CpG 2006 and with a PD oligonucleotide of the same sequence but with phosphorothioate backbone modifications only to the leading and trailing nucleotides of the oligonucleotide (EC PD CpG 2006). These modifications should prevent exonuclease activity, and there were no differences in response to PD CpG 2006 or EC PD CpG 2006 suggesting that the decreased responses to PD CpG 2006 were not a result of, at least, exonuclease activity (Figure 6B) as there was minimal and non-significant difference in response to the unmodified (n=3, 23.3% ± 1.3% σ) and modified (n=3, 21.1% ± 0.8% σ) oligonucleotides. There were also negligible immune effects from stimulation with PD GpC 2006 with phosphorothioate backbone modifications to the leading and trailing nucleotides.

Figure 6. Responses to Immunologically Inert Native Phosphodiester GpC 2137

A) B)

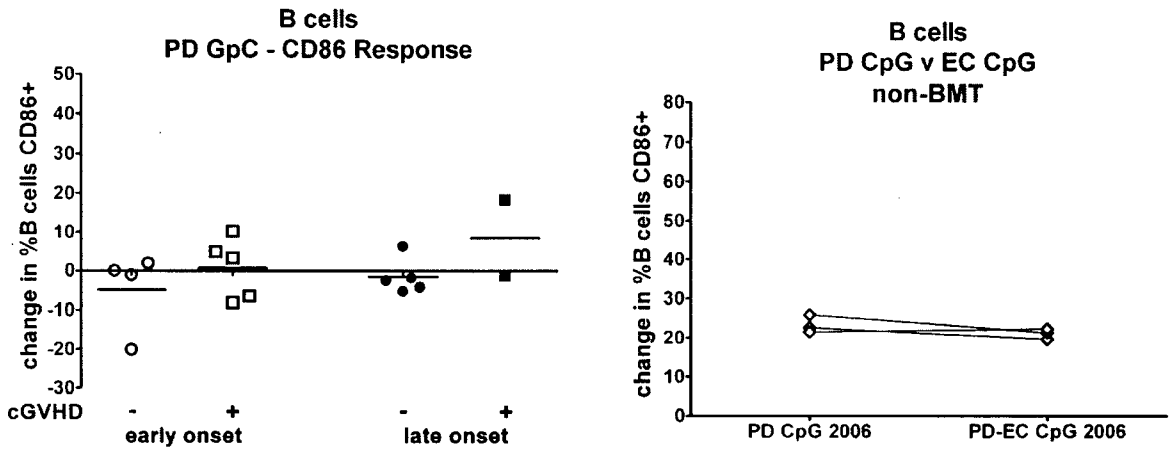


Figure 6 (A) B cell CD86 response to control oligonucleotide PD GpC 2137. (B) Comparison of B cell CD86 responses to PD CpG 2006 and PD-EC CpG 2006 with phosphorothioated 5' and 3' nucleotides in non-BMT volunteer (n=3).

5.3 B Cell Activation Through Other TLR Ligands

It was a possibility that the increased B cell responsiveness to CpG stimulation after the onset of cGVHD was due to a general upregulation of TLR receptors indicative of heightened 'danger signal' surveillance or simply a heightened global response to stimulation in general. To determine if this was the case, direct stimulation of a B cell expressing TLR and indirect stimulation of B cells through a non-B cell expressed TLR was performed. Peptidoglycan (PG), an evolutionarily conserved structural molecule of gram-positive bacterial cell wall, is recognized by the heterodimer of TLR2 and TLR6 which are expressed by B cells and reside on the cell surface. Lipopolysaccharide (LPS), a conserved structural molecule of gram negative bacterial cell wall, is recognized by TLR4 and was used as a negative control. While TLR4 is expressed by murine B cells, it is not present in human B cells. Additionally, human B cells do not have direct non-TLR mediated mechanisms to recognize LPS so B cell responses in a whole PBMC stimulation assay are secondary to stimulation signals from responder cell populations.

There were no differences in PG response between the 3, 6, 12, or 15 month post-transplant non-cGVHD controls or between the early and late cGVHD groups. The 6 month control was chosen as the comparison for the aggregated cGVHD group. There were no significant differences in B cell response to PG between the cGVHD group (n=5, 18.6% ± 7.0% σ) and the 6 month non-cGVHD controls (n=4, 12.1% ±11.8% σ) (Figure 7A). Likewise, there were no temporal differences in B cell responses to signals from other LPS responsive cell populations between the 3, 6, 12, or 15 month post-transplant non-cGVHD controls or between the early and late cGVHD groups. The 6 month control was chosen as the comparison for the aggregated

Figure 7. B Cell Responses to TLR Ligands Peptidoglycan and Lipopolysaccharide

A) B)

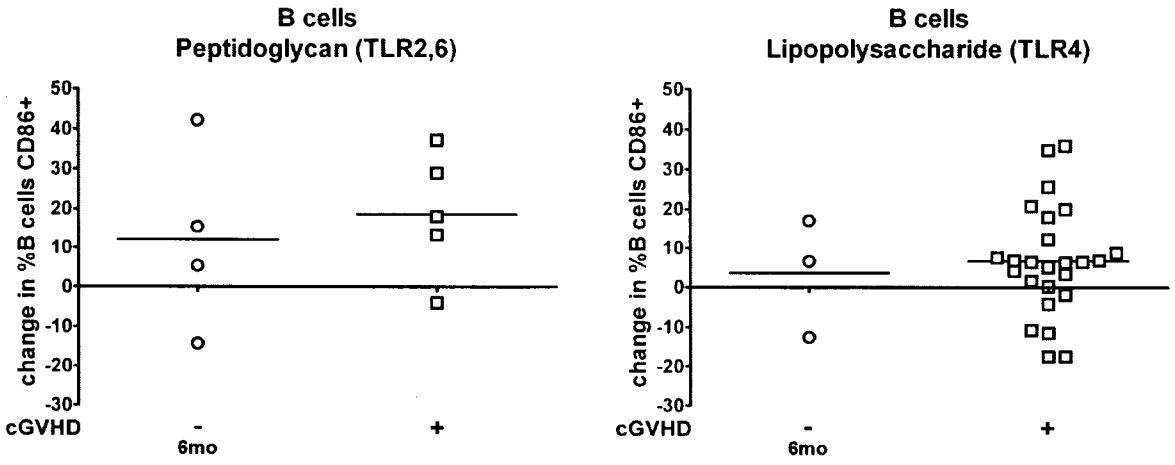


Figure 7 (A) B cell CD86 responses to peptidoglycan (PG) stimulation between cGVHD patients and 6 month non-cGVHD control patients. (B) B cell CD86 responses to lipopolysaccharide (LPS) between cGVHD patients and 6 month non-cGVHD control patients. There were no differences in response within neither the cGVHD control nor the cGVHD group regardless of time post transplant to PG and LPS. The 6 month non-cGVHD control group responses were chosen as the comparison group for the aggregated responses of cGVHD patients.

cGVHD group. There were no significant differences in B cell response to signals from other LPS responsive cell populations between the cGVHD group (n=26, 6.6% ± 2.8% σ) and the 6 month non-cGVHD controls (n=3, 3.6% ± 8.7% σ) (Figure 7B).

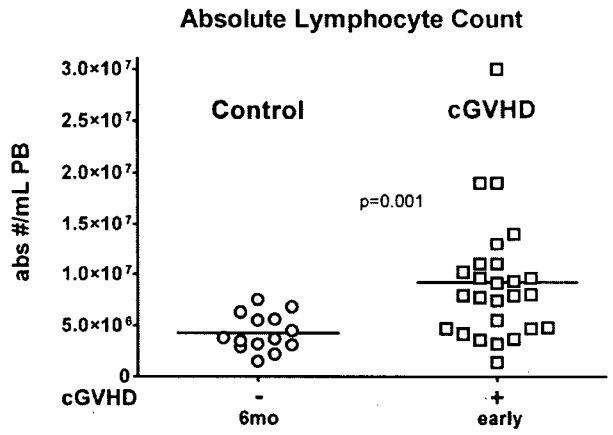
5.4 Characterized B Cell Subpopulations do not Appear to be Major Factors in Differences Observed in CpG Response

There was a significantly elevated (Figure 8A) absolute lymphocyte count (ALC) in the early (3 - 9 month) cGVHD group (n=26, $9.2x10^6 \pm 1.2x10^6$ cells/mL σ) compared to the 6 month non-cGVHD controls (n=14, $4.3x10^6 \pm 0.48x10^6$ cells/mL σ, p=0.001) although the late (≥ 9 month) cGVHD group was comparable to both the 12 and 15 month non-cGVHD controls. Evaluation of CD33+ monocytes, CD4+ and CD8+ T cells, the CD56+ subpopulation of natural killer cells, and CD19+ B cells revealed that the elevated ALC was secondary to increased B cell numbers. There was a trend towards increased numbers of B cells/mL of peripheral blood (Figure 8B) in the early onset cGVHD group (n=25, $6.8x10^5 \pm 1.9x10^5$ cells/mL σ) compared to the 6 month non-cGVHD controls (n=14, $2.2x10^5 \pm 0.6x10^5$ cells/mL σ, p=0.06). B cells/mL of peripheral blood was comparable between the late cGVHD group and both the 12 and 15 month non-cGVHD controls and with the early cGVHD group.

It has been suggested that CD27 positive memory B cells are capable of responding more rapidly to CpG stimulation (Bernasconi et al., 2003) compared to naïve and resting B cells. While the absolute numbers of CD27+ memory B cells were depressed in the 6 month non-cGVHD

Figure 8. Absolute Lymphocyte and B Cell Counts

A)



B)

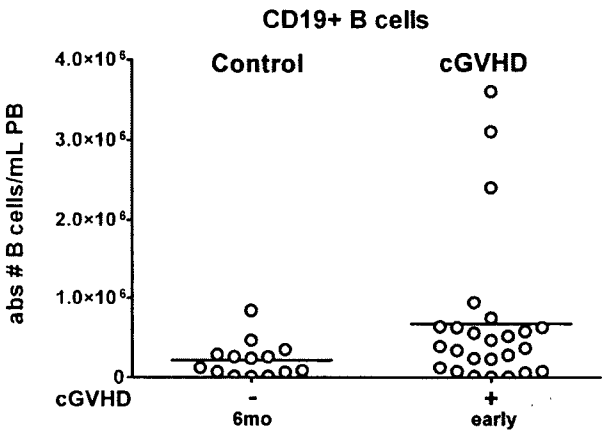


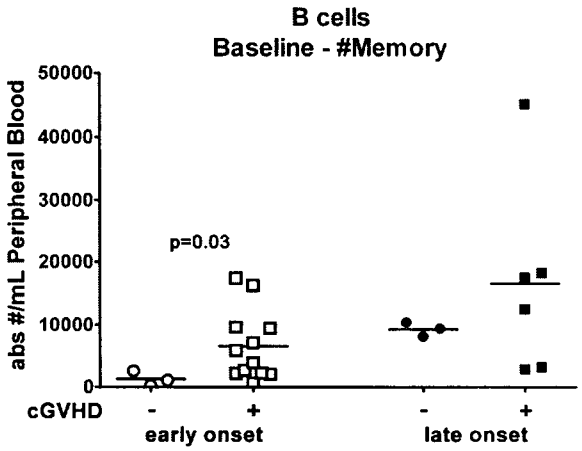
Figure 8. (A) Absolute lymphocyte counts/mL peripheral blood (PB) from 6 month controls and early cGVHD patients. (B) Absolute counts of CD19+/CD33- /mL PB from 6 month controls and early cGVHD patients.

controls, there were no significant differences when compared to the early cGVHD group (Figure 9A). There were significantly decreased absolute numbers of CD27+ B cells in the 6 month non-cGVHD controls (n=3, $1.3x10^3 \pm 0.7x10^3$ cells/mL σ) when compared to the 12 month non-cGVHD controls (n=3, $9.3x10^3 \pm 0.3x10^3$ cells/mL σ, p=0.03). However there were no differences in the percentage of B cells with a CD27+ memory phenotype across all groups (Figure 9B). Additionally, there was no difference in the change in the percentage of B cells with a CD27+ memory phenotype after stimulation with PS CpG 2006 compared to culture in complete media without stimulation (Figure 9C). In a very limited number of patients, the CD86 response to PS and PD CpG 2006 was assessed in B cells with a CD27+ memory phenotype (Figure 9D). Although the sample size is insufficient to infer with any certainty, CD27+ memory B cells from patients with cGVHD do not appear to overwhelmingly upregulate CD86 in response to PS CpG 2006 stimulation compared to CD27+ memory B cells from non-cGVHD control patients. CD27+ memory B cell CD86 responses to native PD CpG 2006 were also similar.

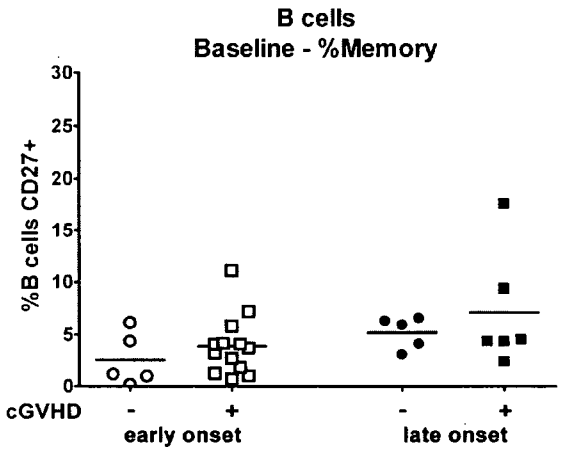
B-1 B cells are a distinct population of B cells with unique phenotypic, developmental, and functional characteristics identified in humans by their constitutive surface expression of CD5 (Kantor, 1991). A defining functional characteristic is their T cell-independent ability to produce low affinity but potentially autoreactive IgM antibodies which has made them of interest in autoimmune diseases (Becker et al., 1990). However, doubt has been cast as to whether B-1 B cells are responsible for the autoantibodies observed in cGVHD although this has only been established in a murine model simulating cGVHD (Reap et al., 1993). Like the memory B cells,

Figure 9. CD27+ Memory B Cells

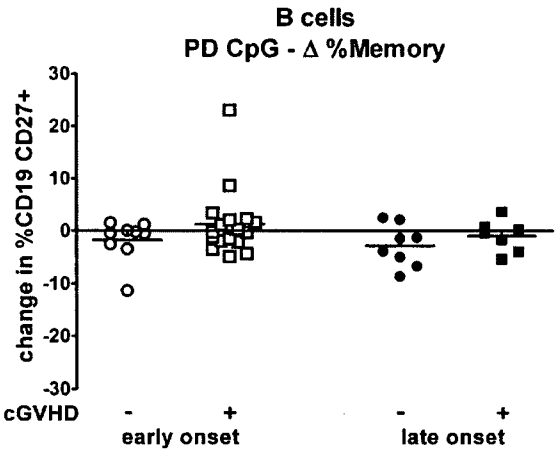
A)



B)



C)



D)

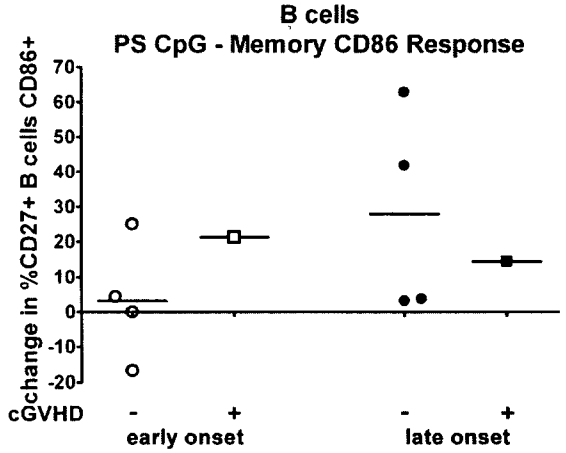


Figure 9 (A) Absolute counts of CD19+/CD33-/CD27+ memory B cells/mL of PB. (B) Percentage of B cells with a CD27+ memory B cell phenotype at baseline. (C) Change in the percentage of B cells with a CD27+ memory B cell phenotype after stimulation with phosphorothioate (PS) CpG for 48 hours. (D) CD27+ memory B cell CD86 response to PS CpG stimulation.

there were significantly decreased absolute numbers of CD5+ B-1 B cells (Figure 10A) in the 6 month non-cGVHD control group (n=5, $0.9x10^4 \pm 0.4x10^4$ cells/mL σ) compared the early cGVHD (n=12, $5.5x10^4 \pm 1.3x10^4$ cells/mL σ, p=0.02) while the late cGVHD group was comparable to the 12 month non-cGVHD controls. However there were no differences (Figure 10B) in the percentage of B cells with a C5+ B-1 phenotype across all groups. Additionally, there was no significant difference in the change in the percentage of B cells with a CD27+ memory phenotype after stimulation with PS CpG 2006 compared to culture in complete media without stimulation (Figure 10C). In a similar situation as for the analysis of CD27+ memory B cell response to CpG stimulation, while the sample size is insufficient to infer with any certainty, it does not appear that CD5+ B-1 B cells from patients diagnosed with cGVHD overwhelmingly upregulate CD86 in response to PS CpG 2006 stimulation compared to CD5+ B-1 B cells from non-cGVHD controls (Figure 10D). Likewise, the CD5+ B-1 B cell responses to native PD CpG 2006 were similar.

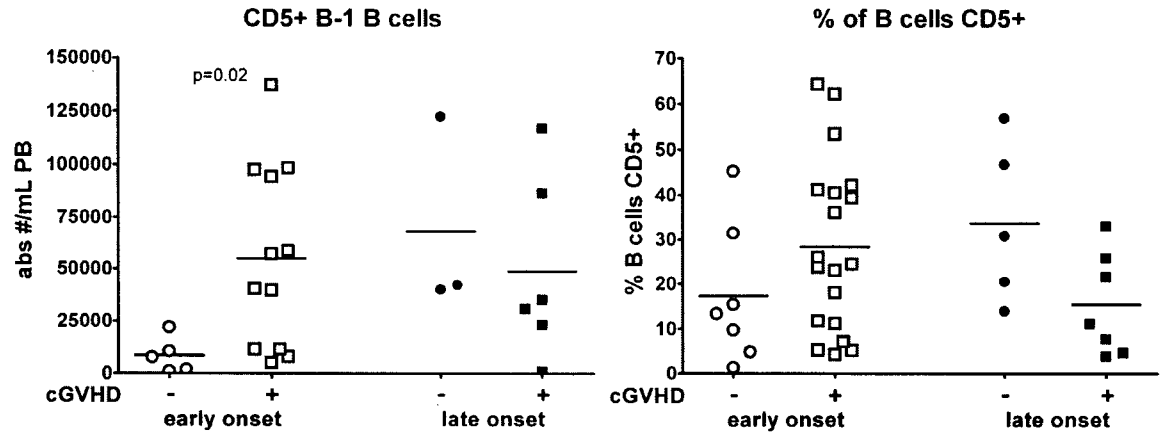
5.5 B Cell Co-stimulatory Molecule Expression, but not Proliferative Responses, to CpG Appear to be Independent of Other Lymphocyte Populations

Plasmacytoid dendritic cells constitute a minute proportion of circulating mononuclear cells (Tsai et al., 1989) and are responsive to immunostimulatory CpG (Sparwasser et al., 1998) and monocytes have also been suggested to respond to CpG (Sester et al., 2000) although whether this is through TLR9 is debatable (Roda 2005). To determine if monocytes and dendritic cells are indirectly modulating B cell CpG response, peripheral blood mononuclear cells (PBMC) from non-BMT adult volunteers were depleted of adherent cells, which are predominantly

Figure 10. CD5+ B-1 B Cells

A)

B)



C)

D)

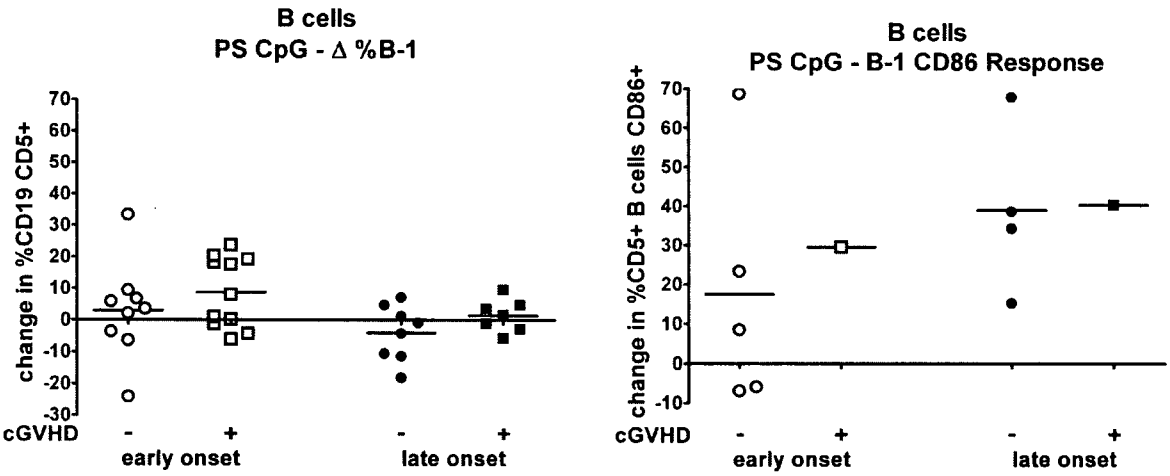


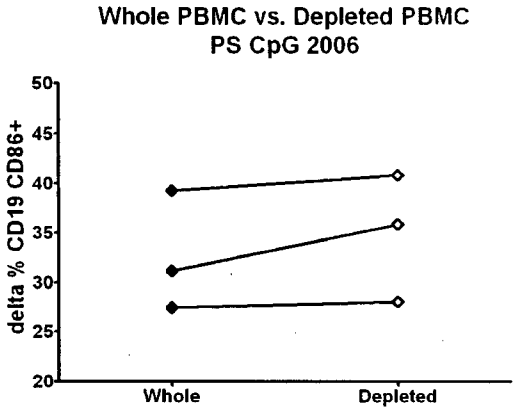
Figure 10 (A) Absolute counts of CD19+/CD33-/CD5+ B-1 B cells/mL of PB. (B) Percentage of B cells with a CD5+ B-1 B cell phenotype at baseline. (C) Change in the percentage of B cells with a CD5+ B-1 B cell phenotype after stimulation with PS CpG for 48 hours. (D) CD5+ B-1 B cell response to PS CpG stimulation.

monocytes and dendritic cells, and the remaining cells were stimulated as above with PS CpG 2006. There were no significant differences in B cell response to 48 hours of stimulation by PS CpG 2006 between un-manipulated whole cell cultures and cultures that were depleted of monocytes and dendritic cells (Figure 11A).

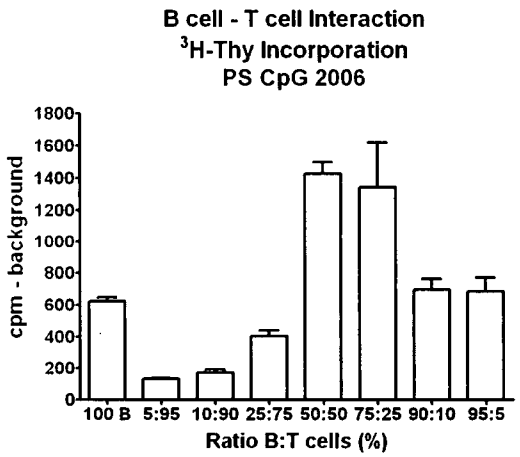
Splenocytes from mice with simulated cGVHD had an enhanced proliferative response to CpG compared to control mice without simulated cGVHD. To assess whether this phenomenon was also present in human cGVHD we assessed proliferative responses by ^{3}H-thymidine incorporation. However, there was heterogeneity in the ratio of T and B cells in patient PBMC harvested by density-gradient centrifugation. To determine whether this would confound the evaluation of proliferative response, T and B cells from healthy non-BMT adult volunteers were enriched by negative selection from PBMC and cultured alone or in varying ratios, stimulated with phosphorothioate (PS) CpG 2006, and proliferation was measured by ^{3}H-thymidine incorporation. As differing ratios of T and B cells resulted in non-linear proliferative responses (Figure 11B), assessment of proliferative responses to PS CpG 2006 by ^{3}H-thymidine incorporation was limited to B cells enriched by negative selection. B cell proliferative response was assessed only in patients from whom a sufficient volume of blood was available. Due to the small number of patients who qualified, we were only able to demonstrate a trend ($p=0.06$) toward increased proliferation of B cells from cGVHD patients ($n=5$, 7.4×10^{3} cpm $\pm$ 2.3×10^{3} cpm σ) compared to non-cGVHD controls ($n=5$, 1.6×10^{3} cpm $\pm$ 0.6×10^{3} cpm σ, $p=0.06$) in response to PS CpG 2006 (Figure 11C). The background proliferation of non-stimulated purified B cells was minimal and consistent between cGVHD and controls.

Figure 11. Lymphocyte Populations and Proliferation

A)



B)



C)

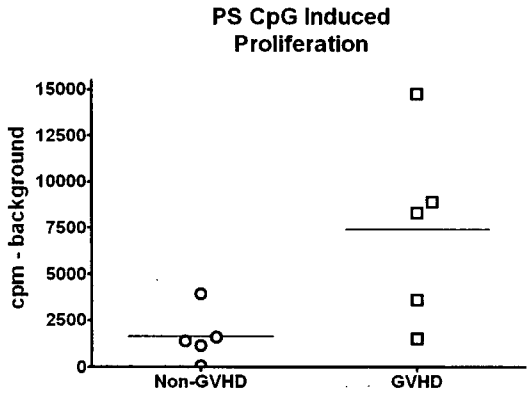


Figure 11 (A) Comparison of B cell CD86 response to 48 hours of stimulation with phosphodiester (PS) CpG in whole PBMC cultures and cultures depleted of adherent cells including dendritic cells and monocytes from non-BMT volunteers. (B) Proliferation in response to 48 hours of stimulation with PS CpG 2006 as measured by ^{3}H-thymidine incorporation in B cells, T cells, and varying ratios, but identical absolute numbers, of B and T cells from non-BMT volunteers. (C) Proliferation as measured by ^{3}H-thymidine incorporation in purified B cells from non-cGVHD control and cGVHD patients in response to 48 hours of stimulation with PS CpG 2006

5.6 Mechanism of Increased CpG Response - Conclusion

There was a significant correlation between phosphorothioate (PS) CpG 2006 responses, measured as the change in the percentage of B cells upregulating CD86 after stimulation and in culture without stimulation, and the relative expression level of TLR9 mRNA in baseline B cells.

Native phosphodiester (PD) ODN was a more stringent assay of TLR9 response. In addition to the negligible activity of control PD ODN GpC 2137 in contrast to the PS modified GpC 2137, there were significantly greater percentages of B cells from both the early (3 - 8 month) and late (≥9 month) cGVHD groups upregulating CD86 in response to PD CpG 2006 compared to the 6 month and 12 month non-cGVHD controls respectively. Also, there was no time-post transplant dependent increase in function in the non-cGVHD controls that was observed in response to PS CpG 2006.

There were no time-dependent differences in peptidoglycan and lipopolysaccharide responses in cGVHD or non-cGVHD control patients. There were also no differences in response to either TLR ligand between the 6 month post-BMT non-cGVHD control patients and the aggregate of the cGVHD patients suggesting that the B cell hyper-responsiveness after the onset of cGVHD may be limited to immunostimulatory nucleic acids.

Contrary to what has previously been reported (Foot et al., 1993; Kalwak et al., 2002; Abrahamsen et al., 2005), there was a significantly increased absolute lymphocyte count (ALC) per mL of peripheral blood in the early (3 - 9 month) cGVHD group compared to the 6 month non-cGVHD controls secondary to B cell numbers. ALC was comparable between the late (≥ 9

month) cGVHD group and both 12 and 15 month non-cGVHD control groups. B cell numbers in the early cGVHD group were increased, but not significantly, compared to the 6 month controls.

There were depressed numbers of CD27+ memory B cells/mL of peripheral blood in the 6 month non-cGVHD controls compared to the early onset cGVHD group. However, the percentage of B cells with a CD27+ memory phenotype was not significantly different between all groups. There were significantly decreased numbers of CD5+ B-1 B cells in the 6 month non-cGVHD controls but the percentage of B cells with a CD5+ B-1 phenotype was similarly comparable between all groups. There was no change in the percentage of either the CD27+ memory or CD5+ B-1 B cells compared to the entire B cell pool in response to PS CpG stimulation suggesting that these two populations do not preferentially expand in response to CpG stimulation. Due to very limited number of patients assayed for CD27+ memory B cell and CD5+ B-1 B cell upregulation of CD86 in response to CpG it is difficult to infer whether the differences in CpG response observed between non-cGVHD controls and cGVHD controls were due to these two B cell subpopulations although it does not appear to be the case.

Monocytes and dendritic cells in a mixed mononuclear cell culture do not appear to be inducing a bystander effect in B cell co-stimulatory molecule upregulation in response to CpG stimulation. The presence of T cells obscures the proliferative response to CpG in B cells as they may respond to signals produced by B cells in response to CpG. Purified B cells from cGVHD patients had an increased proliferative response to phosphorothioate (PS) CpG 2006 compared to non-cGVHD control patients although this increase was not statistically significant when analyzed using the Mann-Whitney U t test.

6. CHAPTER 6: RESULTS - HAEMOLYSIS

During the execution of the previous experiments it was noted that in some patients there were residual erythrocyte red blood cells (RBC) in the peripheral blood mononuclear cell (PBMC) preparations isolated by density gradient centrifugation. CpG stimulated samples from patients with cGVHD had a striking level of lysis of residual RBCs when compared to samples from patients without cGVHD. Autoimmune haemolytic anaemia has been associated with chronic GVHD (cGVHD) (Sevilla et al., 2001; Pihusch et al., 2002). Since it has been suggested that viral infections may be a triggering mechanism for autoimmune haemolytic anaemia (Musaji et al., 2005; Velduis et al., 2004) the susceptibility to CpG induced haemolysis in cGVHD patients may indicate a pathophysiology of haemolytic anaemia reported as a symptom of cGVHD.

6.1 Red Blood Cell Lysis Observed in CpG Stimulated Cultures in cGVHD Patients

Identification of the evidence for RBC lysis was contingent on identifying the presence of RBC after culture with no stimulation for 48 hours from the same patient. After stimulation for 48 hours with phosphorothioate (PS) CpG 2006, approximately half of PBMC cultures (60%) with residual RBC from patients with cGVHD and no cultures (0%) from non-cGVHD control patients had evidence of RBC lysis. Similar results were observed after stimulation with phosphodiester (PD) CpG 2006 with half of cultures (50%) with residual RBC from patients with cGVHD and no cultures (0%) from non-cGVHD control patients having evidence of RBC lysis. There were no differences between early or late onset cGVHD or between 3, 6, 12, and 15 month post-transplant non-cGVHD controls. By contrast, stimulation with peptidoglycan (PG) for 48 hours of all cultures (100%) with residual RBC from patients with cGVHD and almost all

cultures (93.8%) from non-cGVHD control patients resulted in the loss of RBC compared to cultures of the cells from the same patient in without stimulus. PBMCs from healthy non-BMT volunteers supplemented with $1x10^5$ autologous RBC and cultured in an identical manner revealed evidence of RBC lysis only in response to PG stimulation (Table 1).

Possible mechanisms for RBC lysis include a) haemophagocytosis by phagocytic populations seen in response to some infections (Fishman 2000) and autoimmune diseases (Sekigawa et al., 2001), b) RBC lysis by rosetting of RBC by T cells expressing CD2 (Plunkett et al., 1987), or c) soluble factors such as RBC reactive antibodies (Fagiolo et al., 2003; Zupanska et al., 2005) produced in response to Toll-like receptor (TLR) agonist ligation observed with other autoantibodies (Christensen et al., 2005).

To address the possibility of haemophagocytosis, air dried smear preparations of cells after culture with and without stimulus were assessed by light microscopy. There was no obvious evidence of haemophagocytic activity as determined by the presence of RBCs in macrophages (data not shown).

T cell involvement in the clearance of RBC was investigated by attempting to reproduce the haemolytic phenomenon in the blood products of healthy non-BMT adult volunteers. $1x10^6$ un-manipulated PBMCs or T- and B cells isolated by negative selection were co-cultured with $1x10^5$ autologous RBC and stimulated with PS CpG 2006, peptidoglycan (PG), αCD3 and αCD28 stimulating monoclonal antibodies, αIgM stimulating monoclonal antibody, or αIgM and PS CpG 2006 (Table 2). Stimulation with PS CpG alone was unable to induce RBC lysis in non-

Table 1. Frequency of Red Blood Cells Present in Patient Mononuclear Cell Preparations After Density Gradient Centrifugation and Frequency of Red Blood Cell Lysis.

Patients	Total Assessed	# Patients RBC Positive	% Patients RBC Positive	% of RBC Positive Cultures With Evidence of RBC Lysis in Response to		
				PS CpG	PD CpG	Peptidoglycan
all cGVHD	29	20*	65.50%	60%^	50%	100%^
all non-cGVHD	35	28*	77.10%	0%'	0%	93.8%^

* one patient did not have RBC associated with mononuclear cells, but 100,000 autologous RBC were added into cultures of 1,000,000 mononuclear cells
^ culture with autologous RBC added exhibited RBC lysis
' culture with autologous RBC added did not exhibit RBC lysis

Table 2. The Haemolytic Phenomenon Cän be Reproduced by Stimulating Cells from Non-BMT Volunteers

	Response to Stimulus				
Non-BMT Cells	PS CpG	Peptidoglycan	αCD3αCD28	αIgM	αIgM + PS CpG
PBMC	No	Lysis	Lysis	No	Lysis
T	No	Lysis	Lysis	No	No
B	No	Lysis	No	No	Lysis
n=2					

BMT volunteer whereas stimulation with PG induced RBC lysis in all samples. Stimulation of PBMC or purified T cells with αCD3 and αCD28, which generates highly activated T cells, was sufficient to induce autologous RBC lysis without PS CpG stimulation. Since cells from cGVHD patients that are capable of mediating RBC lysis after stimulation do not do so without stimulation, this suggests that highly activated T cells are not the primary mechanism of this observed phenomenon.

While IgM expressing B cells highly activated by αIgM stimulation was unable to induce RBC lysis, αIgM activated B cells stimulated with PS CpG 2006 were capable of inducing RBC lysis. Since activation through IgM alone was insufficient to produce self reactive factors, such as erythrocyte-reactive antibodies, suggests that B cell tolerance can be broken through stimulation through the TLR9 pathway.

To assess whether the experimentally induced haemolysis was mediated by soluble factors such as erythrocyte-reactive antibodies, conditioned supernatants were incubated with $1x10^5$ autologous RBC for 72 hours (Table 3). Supernatants were conditioned by stimulating PBMC from non-BMT volunteers for 48 hours with PS CpG 2006, αIgM, αIgM and PS CpG 2006, or PG. Fresh complete media and fresh complete media supplemented with 10μg/mL of PG were used as controls. Supernatant conditioned by PBMC stimulated with either αIgM or PS CpG 2006 alone was unable to mediate lysis but the supernatant from PBMC stimulated with both αIgM and PS CpG 2006 was able to mediate lysis. While complete media supplemented with PG was sufficient to induce RBC lysis, culture supernatant conditioned by PBMC stimulated with PG induced evidence of lysis at 48 hours instead of 72 hours. There were no differences between

Table 3. Red Blood Cell Lysis by Media Conditioned by Cells from Non-BMT Volunteers

Supernatant Condition by:				Lysis:
Complete medium				No
Conditioned medium	PBMC			No
Conditioned medium	PBMC	PS CpG		No
Conditioned medium	PBMC		αIgM	No
Conditioned medium	PBMC	PS CpG	αIgM	Yes
Conditioned medium	PBMC	Peptidoglycan		Yes
Complete medium		Peptidoglycan		Yes

n=2

fresh or frozen conditioned media. To determine if conditioned supernatant from patient samples had similar properties, $1x10^5$ RBC from non-BMT volunteers were plated with previously frozen supernatant and incubated for 72 hours (Table 4). While supernatant from PS CpG 2006 stimulated cultures from cGVHD patients with positive evidence of RBC lytic activity was capable of inducing third party RBC lysis, there was no lysis by supernatant from unstimulated cultures of cells from the same patient. Supernatant from cell cultures where there was the presence of RBC but no evidence of lysis was unable to induce lysis of third party RBC regardless of conditioning conditions. These results suggest that soluble factors such as erythrocyte-reactive antibodies may be mediating the observed RBC lysis in cultures stimulated with PS CpG in some cGVHD patients.

6.2 Haemolysis – Conclusion

Haemolysis of autologous red blood cells (RBC) after stimulation with phosphorothioate (PS) CpG or phosphodiester (PD) CpG 2006 was observed in some peripheral blood mononuclear cells (PBMC) cultures from cGVHD patients but not from non-cGVHD patients. Peptidoglycan alone was sufficient to cause RBC lysis although culture supernatants conditioned by PBMC stimulated with PG were more effective in clearing both autologous and allogeneic RBC. Conditioned supernatant from cGVHD patient stimulated cell cultures with positive evidence of RBC lysis was able to lyse third party RBC although conditioned supernatant from unstimulated cultures from the same patient did not. Although pro-inflammatory cytokines have been associated with anaemia (Means, 1995) it appears that this is mediated by the increased production of hepcidin by the liver (Andrews, 2004) rather than by directly inducing erythrocyte apoptosis.

Table 4. Red Blood Cell Lysis by Media Conditioned by Cells From Non-cGVHD Controls and cGVHD Patients

Supernatant Conditioned by:			Lysis:
non-cGVHD		No previous lysis	No
non-cGVHD	PS CpG	No previous lysis	No
cGVHD		No previous lysis	No
cGVHD	PS CpG	No previous lysis	No
cGVHD	PS CpG	Lysis	Yes
n=2			

To determine whether TLR9 signalling may indeed be involved in clinical haemolytic anaemia, supernatants from future samples with evidence of RBC lysis should be tested against a battery of ABO and non-ABO RBC antigens.

CHAPTER 7. CpG RESPONSES AND CLINICAL ASSESSMENT OF PATIENTS

There was a striking increased responsiveness to CpG, especially to native phosphodiester (PD) CpG, by B cells from patients diagnosed with chronic GVHD (cGVHD) compared to non-cGVHD control patients. CpG responses were compared to various clinical criteria of cGVHD. Additionally, CpG responses after the onset of therapy was assessed and compared to treatment outcome.

7.1 CpG Response and Clinical Assessment of Patients With cGVHD

There was no relationship between B cell response to phosphorothioate (PS) or phosphodiester (PD) CpG 2006 stimulation and the total number of organs affected by cGVHD. There was no relationship between PS CpG 2006 responses and the location of organ involvement when assessing cGVHD, classified for joint, skin, oral, hepatic, oesophageal, lower gastrointestinal tract, weight, pulmonary, or absolute eosinophils count (AEC) (Table 5). There appeared to be a possible association of stronger B cell response to native PD CpG 2006 in cGVHD patients with hepatic involvement as determined by ASCT0031 study protocol (Figure 12). The analysis was limited by the small number of patients available for evaluation. There have been numerous autoantibodies reported to be present in cGVHD including anti- cardiolipin and anti-nuclear antibodies (ANA) (Lister et al., 1987; Roquette-Galley et al., 1988; Holmes et al., 1989). There

Table 5. Affected Organs in cGVHD Patients and PS CpG Response

Average B cell CD86 Response to PS CpG							
	Not Affected			Affected			P
Joint	n=11	53.3% ±	4.8%	n=6	57.7% ±	3.5%	0.88
Skin	n=3	46.2% ±	16.2%	n=17	56.1% ±	2.3%	1.00
Oral	n=7	48.5% ±	6.5%	n=13	57.9% ±	2.6%	0.13
Hepatic	n=14	58.0% ±	2.4%	n=6	46.8% ±	7.5%	0.13
Oesophageal	n=14	53.8% ±	3.5%	n=3	59.9% ±	10.9%	0.35
Lower GI	n=14	56.0% ±	2.2%	n=6	51.5% ±	8.8%	0.84
Weight	n=12	59.5% ±	2.5%	n=6	47.6% ±	7.7%	0.10
Pulmonary	n=12	55.1% ±	4.2%	n=10	54.0% ±	3.5%	0.72
AEC >500	n=14	53.1% ±	3.8%	n=6	58.2% ±	4.0%	0.54

Figure 12. Phosphodiester CpG Responses and Hepatic Involvement

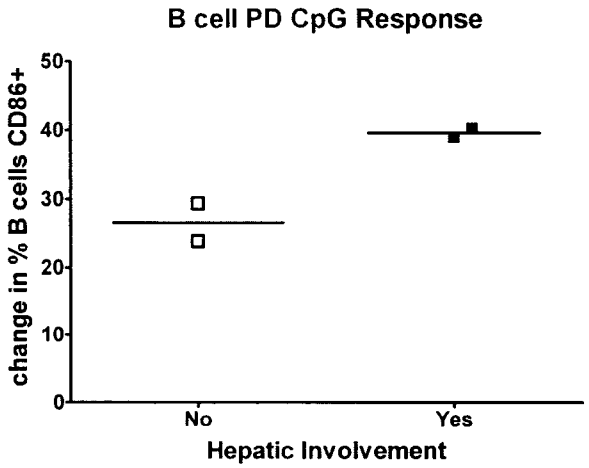


Figure 12. Possible increased B cell CD86 response to native PD CpG 2006 associated with the diagnosis of hepatic involvement in patients diagnosed with cGVHD.

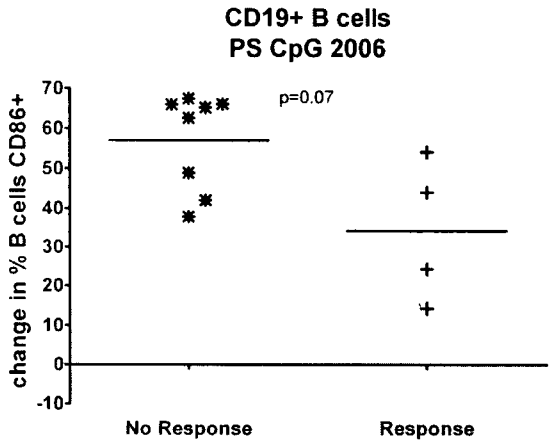
were no correlations between anti-cardiolipin autoantibody titre and PS or PD CpG 2006 responses. There were no clear correlations between ANA positivity to PS or PD CpG 2006 response. However, 6/7 cGVHD patients with identified RBC lysis in response to PS CpG stimulation were ANA positive whereas only 2/6 cGVHD patients identified as having no RBC lysis in response to PS CpG were ANA positive.

7.2 Altered CpG Response After Initiation of Treatment and Clinical Response to Treatment

A small subset of cGVHD patients was available for CpG response evaluation 2 months after the initiation of cGVHD treatment. B cell CD86 response to phosphorothioate (PS) modified CpG 2006 was assessed in an identical manner as previously. There was a trend towards decreased CpG response, as evaluated by B cell upregulation of CD86, in the patients who were evaluated as being in either a state of complete or partial remission (n=4, 34.2% ± 9.1% σ) compared to those patients with no response to therapy (n=8, 57.0% ± 4.3%, p=0.07) (Figure 13A). Since hydroxychloroquine is a potent inhibitor TLR9 recognition of immunostimulatory CpG, it was possible that the decreased CpG response in the responsive patients were due only to exposure to hydroxychloroquine. To evaluate this possible effect, the B cell CD86 response to PS modified CpG 2006 was evaluated at 2 months after the initiation of therapy in patients that received cyclosporine, prednisone, and hydroxychloroquine and compared to the responses in patients that received a placebo instead of hydroxychloroquine. There were no significant differences in PS modified CpG response between patients treated with placebo in addition to standard therapy compared to patients treated with hydroxychloroquine in addition to standard therapy (Figure 13B).

Figure 13. Clinical Response to Therapy and CpG Response

A)



B)

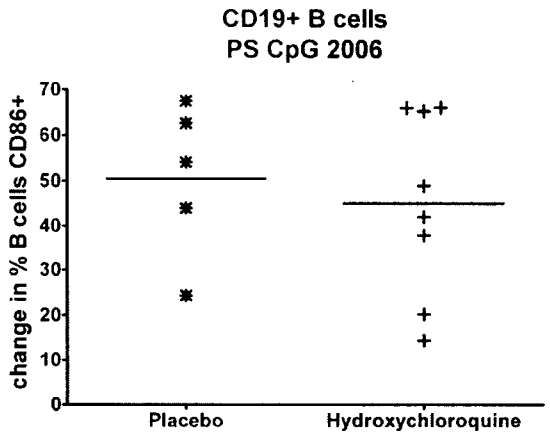


Figure 13. (A) cGVHD patients were evaluated for B cell CD86 upregulation in response to PS modified CpG stimulation 2 months after the initiation of treatment. Patients who were evaluated as responding to therapy at 9 months were compared to cGVHD patients who had no response to therapy at 9 months. (B) B cell CD86 upregulation response to PS modified CpG was evaluated in cGVHD patients 2 months after the start of treatment. Patients treated with hydroxychloroquine in addition to standard therapy were compared to cGVHD patients on standard therapy and placebo.

7.3 CpG Response and Clinical Manifestations - Conclusion

There was no clear correlation between the cGVHD severity, as measured by the number of organs diagnosed to be affected by cGVHD, and the patients' magnitude of CpG response. However, there was a significant correlation between a stronger B cell response to phosphodiester (PD) CpG and hepatic involvement. Additionally, there were no correlations between anti-cardiolipin autoantibody titres or anti-nuclear antibody (ANA) positivity and CpG response. However, 6/7 cGVHD patients with identified RBC lysis in response to phosphorothioate (PS) CpG were ANA positive compared to only 2/6 cGVHD patients identified as having no RBC lysis response to PS CpG. Although the number of patients evaluated was low, there appears to be a trend in diminished CpG response 2 months after the onset of therapy in those patients who subsequently responded to cGVHD therapy compared to those patients who were unresponsive to therapy. This altered CpG response was not associated with differences in treatment modality.

8. CHAPTER EIGHT: DISCUSSION

8.1 Increased Phosphorothioate CpG Response in cGVHD Patients

There was a significantly greater percentage of B cells from early onset (3 - 8 months) chronic GVHD (cGVHD) patients that rapidly responded to phosphorothioate (PS) CpG compared to the 6 month non-cGVHD controls.

Since there were an increased percentage of B cells from a subset of cGVHD patients capable of rapidly upregulating T cell activating co-stimulatory molecules such as CD80 and CD86 in response to CpG, it is tempting to speculate that there is an increased chance that a host-reactive B cell can be activated and potentially produce antibody. Likewise, presentation of host antigen without co-stimulatory signals normally induces a tolerogenic response in a T cell that recognizes it (Yuschenkoff et al., 1996); however, these inappropriately expressed co-stimulatory signals may prime the T cell instead (Nasir et al., 1994). We observed a robust upregulation of CD86 which is important in activating naïve T cells (Chen et al., 1994) and could be an important trigger for the initiation of cGVHD. Additionally, it is tempting to speculate that this could be a potential mechanism involved in replenishing the pool of activated host-reactive T cells that maintain cGVHD in the face of both endogenous immune regulation as well as exogenous therapeutic immunosuppression. Similarly, it is possible that the increased capacity for B cells to express CD80 could provide a potential mechanism for maintaining cGVHD by re-activating host-reactive but immune-regulated T cells as this co-stimulatory signal is primarily involved in activating T cells that have already been primed by antigen (Hathcock et al., 1993). It was unexpected that there were no differences in the upregulation by B cells of CD40 in response to

PS CpG 2006 stimulation. However, the magnitude of B cell CD40 upregulation was comparable to the B cell response from healthy non-BMT volunteers suggesting that the increased B cell CpG response after the diagnosis of cGVHD may be limited to particular cellular responses.

8.2 No Differences in Baseline B Cell Co-stimulatory Molecule Expression

There were no differences in the baseline expression of co-stimulatory molecules CD80, CD86, or CD40 by B cells from either patients diagnosed with chronic GVHD (cGVHD) or from non-cGVHD control patients.

It was expected that there would be a population of B cells with an activated phenotype in circulation in patients who have developed cGVHD. One possible explanation is that activated B cells may have been targeted to affected organs or lymphoid tissue and as such do not persist in circulation. Additionally, peripheral blood was shipped or stored in ambient temperature overnight during which phenotypic changes may have occurred.

8.3 TLR9 mRNA Expression Correlates with Phosphorothioate CpG Response

Toll-like Receptor 9 (TLR9) mRNA levels in non-manipulated B cells and B cell response to phosphorothioate (PS) CpG was assessed in a subset of patients and was found to significantly correlate.

This correlation appears to confirm that TLR9 ligation is a major pathway between PS CpG stimulation and B cell response. TLR9 has been implicated as important in autoimmune diseases, for example, its expression is essential for the production of anti-DNA antibodies commonly present in lupus erythematosus (Christensen et al., 2005). While memory B cells have been suggested to constitutively express robust levels of TLR9 mRNA and are able to more rapidly respond to immunostimulatory CpG, there was no detectable increase in the percentage of these cells in chronic GVHD (cGVHD) patients compared to non-cGVHD control patients. As a result, the hypothesis is that there may be a subset of B cells that have been provoked into enhancing surveillance of danger signals specific to immunostimulatory DNA and that this may contribute to the immune dysfunction that drives cGVHD. Consistent with the anti-leukaemic effect of cGVHD (Weiden et al., 1981), it is possible that this enhanced surveillance may be in response to leukaemic cells which can undergo aberrant genome-wide hypomethylation (Roman-Gomez et al., 2005) that disproportionally affects repetitive DNA sequences comparatively rich in CpG dinucleotides (Luning and Kazazian, 2000).

It is interesting that there was a concomitant upregulation of TLR9 expression and PS CpG 2006 response observed in patients diagnosed with cGVHD. This warrants investigation into possible mechanisms of this upregulation in the context of a post-BMT environment. Likely candidates to explore would be the CD40:CD154 and B cell receptor engagement pathways of B cell activation *in vivo* as these have been shown to be sufficient, in some circumstances, to modulate TLR9 expression (Bourke et al., 2003).

Another attractive approach to determine possible causes of TLR9 upregulation would be to perform microfloral and viral surveillance in patients following BMT, in particular for

Staphylococcus aureus, as its products have been shown to be able to upregulate TLR9 (Bourke et al., 2003), and for human cytomegalovirus, due to its clinical association with increased cGVHD (Larsson et al., 2004) among other complications.

8.4 Use of Phosphodiester CpG Presents a More Stringent Assessment of TLR9 Response

B cell responses to phosphodiester (PD) CpG were assessed in patients diagnosed with chronic GVHD (cGVHD) and in non-cGVHD control patients. In contrast to phosphorothioate (PS) CpG stimulation, a significantly greater percentage of B cells from both early (3 – 8 month) and late (≥ 9 month) onset cGVHD patients rapidly responded to PD CpG compared to appropriate time-matched non-cGVHD controls. Additionally, in contrast to PS CpG stimulation, there was no time-post transplant related increase in response to PD CpG in the non-cGVHD control patients.

PD CpG oligonucleotides (ODN) are more structurally related to the natural ligand of Toll-like Receptor 9 (TLR9) than are phosphorothioate modified (PS) CpG ODNs. This structural similarity affords a far more stringent agonist for the determination of TLR9 function *in vitro* as its immunogenicity aside from the TLR9 pathway is minimal. The reverse sequence of a CpG ODN, a GpC motif, theoretically should have no TLR9 mediated immunological activity. The increased specificity of PD CpG in assessing TLR9 function is highlighted by the high background activity of PS modified GpC ODN and the lack of activity by control PD GpC ODN with the same nucleotide sequence. This enhanced discrimination underscores that there is increased responsiveness to CpG by all patients with cGVHD regardless of time post transplant

of disease diagnosis and that the response to CpG does not increase with time post transplantation without disease development. Additionally, all non-GVHD control patient PD CpG responses were very similar to responses by healthy non-transplant volunteers suggesting that the difference in CpG responses are due to enhanced responses in cGVHD patients rather than suppressed responses in the non-cGVHD control patients.

While the CD80 and CD86 response to both PS and PD CpG were significantly greater in the early cGVHD group compared to the 6 month controls, only the CD86 response to PD CpG by the late group was significantly greater compared to the 12 month controls. The percentage of B cells rapidly upregulating CD80 in response to either PS or PD CPG is much lower than the percentage of B cells rapidly upregulating CD86. The lack of difference in CD80 response to PD CpG in the late onset group may be due to that the sample size was insufficient to observe statistical significance.

The expression of TLR9 has been implicated in lupus erythematosus, an autoimmune disease with similarities to cGVHD. In animal models of lupus, the presence of functional TLR9 is essential for the induction of autoantibody production. Additionally, it has been recently demonstrated that T cell dependent B cell antigen specific responses require the activation of B cell TLRs (Pasare and Medzhitov, 2005). Here, we have observed that the magnitude of CpG response is related to the expression level of TLR9 despite the non-TLR9 mediated activity of PS CpG. Assaying TLR9 responses using more stringent PD CpG reveals that increased sensitivity is not restricted to early onset cGVHD but is a general phenomenon in the vast majority of cases of cGVHD. This increased sensitivity suggests that TLR9 and the recognition of nucleic acid elements, perhaps even self DNA, may be an important facet or marker of autoimmunity.

8.5 Enhanced TLR Responses After cGVHD may be Restricted to the TLR9 Family

There does not appear to be generalized upregulation in Toll-like Receptor (TLR) 2 and TLR6 responses or heightened sensitivity to TLR4 expressing cell population responses by B cells associated with chronic GVHD (cGVHD). This suggests that the increased CpG response of cGVHD patients is limited to TLR9 rather than a generalized inflammation that enhances immune response. Additionally, the secondary response to TLR4 stimulation suggests that B cells from cGVHD patients are not merely more reactive in a general sense but that the heightened response has some specificity to TLR9 although it remains to be ascertained whether other members of the TLR9 family such as TLR7 and TLR8 are similarly affected.

8.6 Memory and B-1 B Cells do not Appear to be the Primary Cause of the Observed Increased CpG Response

Decreased B cell numbers in the 6 month control group did not contribute to the decreased CpG response observed as the response was determined on a percentage basis. However, of the patients assayed, eight in total could not be evaluated due to B cell insufficiency. Four were initial 3 month post-allogeneic blood and marrow transplant (allo-BMT) non-chronic GVHD (cGVHD) control patients but subsequent samples had sufficiently reconstituted B cell numbers for evaluation. One non-cGVHD control patient could not be evaluated at 6 months post-BMT but previously had sufficient B cell numbers at 3 months post-transplant. There were three initial cGVHD diagnosis patients who could not be evaluated due to B cell insufficiency. The consistent response to phosphodiester (PD) CpG in all non-cGVHD control patients and the consistent and relatively higher response by all cGVHD patients assayed suggest that these

patients who could not be evaluated did not artificially introduce the differences in CpG response between cGVHD and control patients.

However, the CpG response by these B cell subpopulations should be of interest in future studies as CD27+ memory B cells from patients diagnosed with cGVHD may be more responsive to phosphorothioate (PS) CpG than those from non-cGVHD control patients. CD5+ B cells, despite being shown not to be involved in autoantibody production in a murine model (Reap et al., 1993), remain of interest. In addition to the correlation between the expansion of the CD5 subpopulation of B cells and lupus erythematosus which is similar to cGVHD (Wouters et al., 2004), it has recently been suggested that surface expression of Toll-like Receptor 9 (TLR9) is elevated in CD5+ B-1 B cells compared to CD5- B cells (Dasari et al., 2005). Given this new development, it would be of interest to determine whether the localization of TLR9 may affect the degree or kind of CpG response.

8.7 B Cell CpG Response is Independent of Other Lymphocyte Populations

Although non-blood and marrow transplant (BMT) volunteer B cell responses to phosphorothioate (PS) CpG 2006 in monocyte and dendritic cell depleted cultures were not different from B cell responses to PS CpG 2006 in whole culture, it is still possible that immune dysregulations present in chronic GVHD (cGVHD) may allow other cell populations to modulate B cell responses to CpG. However, when assessing the proliferative response by purified B cells to PS CpG 2006 there was a trend in increased response by B cells from patients with cGVHD compared to B cells from non-cGVHD control patients. As the proliferative

response of B cells from non-cGVHD control patients was not lower than the response of B cells from non-BMT volunteers; it appears that the increased B cell response is intrinsic to the B cell.

Due to the small body volume of pediatric patients only a limited amount of blood can be obtained resulting in the inability to assess whether CpG response by the relatively rare dendritic cell population was altered in patients diagnosed with cGVHD as was observed in B cells. Additionally, the limited sample volume also restricted the number of patients in whom B cell proliferation could be assessed.

8.8 Observed *in vitro* Autologous Haemolysis in Response to CpG Stimulation may be Mediated by Soluble Factors Produced by B Cells

Autoimmune haemolytic anaemia has been associated with chronic GVHD (cGVHD) (Sevilla et al., 2001; Pihusch et al., 2002). Murine models (Musaji et al., 2005) and clinical reports (Veldhuis et al., 2004; Elhajj et al., 2004) have strongly suggested a role for viral infections as a triggering mechanism for autoimmune haemolytic anaemia. Expansion and activation of polyclonal B cells secreting IgG or IgM autoantibodies has been implicated in disease development but the mechanism that produces the breakdown of immunologic tolerance against erythrocyte red blood cell (RBC) self-antigens is not well defined. Polyclonal B cell activation via Toll-like Receptor 9 (TLR9) signalling may be one possible mechanism. However, the finding that RBC lysis after CpG stimulation is only observed in patients with cGVHD or in cells that have been highly activated artificially suggests that other immune dysfunction is required to permit TLR9 signalling mediated tolerance loss against self RBC antigen. Since TLR9 biases class switching of B cells towards IgG_1, IgG_2, or IgG_3 isotypes associated with haemolytic

anaemias (Semple and Freedman, 2005; Stahl and Sibrowski, 2005) and since culture supernatant from cultures previously demonstrated to be capable of haemolysis is capable of lysing allogeneic RBC, it is possible that this phenomenon may be complement mediated although this may also be complemented by antigen-dependent cellular cytotoxicity mechanisms as RBC lysis, when present, occurs more quickly in cell cultures.

8.9 No Clear Relationship Between Strength of CpG Response and Clinical Involvement in Patients

Although there was no clear correlation between the severity of chronic GVHD (cGVHD) and the magnitude of CpG response, there remains a clear hyper-responsiveness to CpG in patients diagnosed with cGVHD compared to non-cGVHD control patients. Toll-like Receptor 9 (TLR9) expression levels are increased in patients after the onset of cGVHD. The number of non-cGVHD control patients that subsequently developed cGVHD was lower than the expected 40 – 60%. None of the control patients assessed for CpG response subsequently developed cGVHD and had uniformly low PD CpG responses that were very similar to responses by healthy non-blood and marrow transplant volunteers. Despite the lack of an association with the magnitude of CpG response and the extent and severity of cGVHD, CpG response may be a useful diagnostic biomarker and perhaps an early detection for the risk of developing cGVHD as the presence of a B cell population highly expressing TLR9 may be an indication of a permissive condition for immune dysregulation.

Although the number of evaluable samples at 2 months after the initiation of therapy for cGVHD was limited for correlation with response, there was a trend toward a drop in the B cell

response to PS modified CpG at 2 months post-transplant in those patients who were subsequently evaluated as having a response to therapy at 9 months. These data suggest that the CpG response may be a useful biomarker for both the diagnosis and evaluation of response in cGVHD therapy.

9. CHAPTER NINE: SUMMARY

Currently there is a lack of murine models for chronic GVHD (cGVHD) that are highly satisfactory. However, animal models have been exploited to determine cell populations that are involved in human cGVHD. Likewise, an animal model has suggested a role for microbial DNA in cGVHD. Using synthetic microbial DNA analogues to stimulate B cells from patients post allo-blood and marrow transplant (BMT) reveals that there is an increased responsiveness to immunostimulatory CpG DNA upon the onset of cGVHD. Additionally, this response is dependent primarily on the expression levels of Toll-like Receptor 9 (TLR9). The observed increased responsiveness in cGVHD patients is increasingly clear when stimulating with a CpG DNA that more closely resembles the natural ligand of TLR9. Namely, there is an increased responsiveness in cGVHD patients as control patient responses were comparable to individuals who have no undergone allo-BMT.

Through these observations a previously undescribed immune population has been identified which may be important in cGVHD. These B cells expressing TLR9 at high levels may be a significant factor through which the immune dysregulation that results in cGVHD is initiated or maintained. Although there is no clear relationship between the magnitude of CpG response and the extent and severity of cGVHD as assessed clinically, the differences in phosphodiester CpG response may be a useful and reproducible diagnostic for patients who may be at risk of developing cGVHD. Additionally, the observed decrease in response to CpG in those patients who subsequently responded to therapy compared to patients with no response to therapy may be a useful biomarker for identifying at-risk patients who may require more extensive cGVHD therapy.

10. CHAPTER TEN: FUTURE DIRECTIONS

B cell CpG responses, especially when using native phosphodiester CpG, appear to be a possible marker of chronic GVHD (cGVHD). While it is unfortunate that the magnitude of CpG responses could not be associated with the general severity of the clinical manifestation of cGVHD as determined by the number of organs involved, there is an association between the magnitude of B cell PD CpG response and an independent but non-validated cGVHD severity scoring (data not shown). Additionally, the association of decreased CpG response shortly after the initiation of therapy and subsequent positive response needs to be validated.

Given the association of increased B cell Toll-like Receptor 9 (TLR9) expression and function in patients diagnosed with cGVHD, it would be of interest to perform similar assessments in other antigen presenting cell populations such as plasmacytoid dendritic cells and monocytes. As the current study was performed in a moderate number of paediatric patients, validation of these findings should be attempted in adult transplant recipients. Since larger blood volumes can be obtained from adult patients, additional tests should be manageable where the smaller blood volumes from paediatric patients available for this study were a limiting factor.

Since these experiments were retrospective in that they were assessments performed after the clinical diagnosis of cGVHD, the role of immunostimulatory nucleic acid structures and cGVHD remain unclear. However, the availability of transgenic TLR9 deficient mice presents an attractive tool to resolve this issue through transplantation experiments.

11. CHAPTER 11: REFERENCES

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