The expression of Toll-Like Receptors in B-Chronic Lymphocytic Leukaemia

Nigel James Oakes

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Research carried out in collaboration with the Royal Cornwall Hospital Trust, Cornwall, UK

Faculty of Health and Life Sciences,
University of the West of England, Bristol

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ABSTRACT

Chronic Lymphocytic Leukaemia (CLL) is the most common form of Leukaemia in the Western world and has a highly variable clinical course. Continuing advances in the range of therapeutic options available to clinicians require reliable prognostic indicators that can be used to group patients accurately according to their risk of disease progression, thereby allowing meaningful comparisons of treatments.

The expression of Toll-Like Receptors (TLR) on cells involved with the disease process in CLL was studied to establish links between levels of expression and the disease process. The expression levels of 5 different TLR were measured on a variety of haemic cells and compared with the TLR expression levels seen on their normal counterparts.

Flow cytometric analysis was used to establish the expression levels of TLR 1,2,3,4, and 9 on peripheral blood Monocytes, T Lymphocytes and B Lymphocytes from 129 patients. These results were compared with the TLR expression on corresponding cells from an equal number of age and sex matched controls. Further studies were performed which established the detrimental effect that storage of samples has on TLR expression, and also to compare TLR expression in patients who exhibited a positive Direct Antiglobulin Test (DAGT), with those that were negative for the DAGT.

Results from the study show that both T and B lymphocytes from CLL patients showed statistically significantly different levels of TLR expression when compared

with lymphocytes from age and sex matched controls. TLR expression levels on monocytes were similar in both patient and control groups. When comparing TLR expression between patients who were DAGT positive and those that were negative, a statistically significant difference was found in TLR9 expression on T lymphocytes.

These findings have established that that there are statistically significant differences in TLR expression on lymphocytes when comparing CLL patients with age and sex matched controls. It also establishes the differences in TLR expression levels seen in DAGT positive and DAGT negative patients. From findings made during this study, it is hypothesised that there may be a link between differential TLR expression and the autoimmune disease frequently reported in CLL.

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Dedication

This thesis is dedicated to the memory of my father, Major the Reverend Dennis Oakes, a soldier of Christ.

Requiescat in pace

1 Background

Chronic Lymphocytic Leukaemia (CLL) is the most common form of leukaemia worldwide and has been recognised as a distinct clinical entity for over 100 years (Perez and Winer 2011; Linet *et al.* 2007). The disease has an age adjusted rate in the UK of 4.2 per 100,000 per year (Oscier *et al.* 2012), has the strongest familial tendency of any malignancy (Goldin and Caporaso 2007) and has an extremely variable clinical course, with survival from months to decades (Rožková *et al.* 2010). For many years CLL was regarded as an incurable disease of the elderly, worthy only of symptom palliation (Tam and Keating 2010), however, new therapies and the possibility of stem cell transplantation have led to a resurgence of interest in reassessing the value of early intervention in high risk patients. Therapeutic interventions in those patients most at risk of progression at an early stage may have a positive impact on survival, particularly in younger patients who are more able to tolerate intensive treatment (Tam *et al.* 2008). The current standard of care is to initiate treatment when a patient has progressive or symptomatic disease (Hallek 2008). Continuing advances in the range of therapeutic options available to clinicians requires reliable prognostic indicators that can be used to group patients accurately according to their risk of disease progression, thereby allowing meaningful comparisons of treatments (Oscier, Fegan and Hillmen 2004). There remains however much about the disease to be discovered and evaluated (Hallek 2008).

1.1 Leukaemia

Leukaemia is a disease that results from the neoplastic proliferation of haemopoietic myeloid or lymphoid cells. It arises from a mutation in a single stem cell, the progeny of which form a clone of leukaemic cells. Leukaemias are broadly divided into i) acute leukaemias, which, if untreated lead to death in weeks or months and ii) chronic leukaemias, which, if untreated, lead to death in months or years. They are further subdivided into lymphoid, myeloid and biphenotypic leukaemias, the latter showing both lymphoid and myeloid differentiation (Bain 2010). Leukaemias are classified according to World Health Organisation (WHO) schemes laid down in the WHO classification of Tumours of Haematopoietic and Lymphoid tissues guide (Swerdlow *et al.* 2008). A simplified table of this classification system, showing the cellular origins of leukaemic cells is shown in table 1.

Type of Leukaemia	Cellular origin
Common acute lymphoblastic leukaemia (c-ALL)	Lymphoid progenitor cell
Pre-B acute lymphoblastic leukaemia (pre B-ALL)	Pre-B cell
B-cell acute lymphoblastic leukaemia (B-ALL)	B cell
Acute myeloid leukaemia (AML)	Myeloid precursor cell
Chronic lymphocytic leukaemia (CLL)	Mantle cell/follicular/mature B cell
Chronic myeloid leukaemia (CML)	Myeloid progenitor cell
Multiple myeloma (MM)	Plasma cell
Hairy cell leukaemia (HCL)	Mature B cell

Table1: Leukaemia classification and cellular origin (Taken from Bain 2010)

Acute leukaemias are characterised by a defect in maturation, which leads to an imbalance between proliferation and maturation. This in turn leads to a continuing

expansion of the clone where immature cells predominate. Chronic leukaemias by comparison are characterised by an expanding pool of proliferating cells that retain their capacity to differentiate to maturity (Greaves 1997; Bain 2010).

1.2 Chronic Lymphocytic Leukaemia (CLL)

CLL has been recognised as a distinct clinical entity for over 100 years. Originally identified by Turk in 1903, a fuller clinical description did not appear for some years after when it was cited as being an accumulative disease of immunologically incompetent lymphocytes (Dameshek 1967). Disease classification systems did not however begin to systematically distinguish between different forms of leukaemia until the late 1960's (Linet *et al.* 2007).

1.2.1 Aetiology of CLL

CLL is the most common form of leukaemia worldwide (Parker and Strout 2011). The incidence is 4.2 per 100,000 per year, with this incidence increasing to >30 per 100,000 at an age >80 years, with 2,750 new diagnoses per year in the United Kingdom (Eichhorst *et al.* 2011). The disease has a male to female ratio of 2:1 and is primarily a disease of the elderly, with a median age at presentation of 72 years; approximately 11% of patients are less than 55 years old at diagnosis (Howlader *et al.* 2012). Survival time is extremely variable and ranges from 2 to 20 years from diagnosis, with a median survival of 10 years (Ghobrial *et al.* 2004). An intriguing feature of the disease is whilst some patients can survive for decades without the requirement for treatment; others can die within a year or two of presentation from a drug resistant form of the disease (Lin *et al.* 2002). There are dramatic differences in the prevalence of CLL in different ethnic groups, it being virtually absent in Japan

and Africa, whist those of Jewish origin show a two-fold greater risk of developing the disease over those from other western countries (Caligaris-Cappio and Hamblin 1999; Landgren and Kyle 2007).

There is much speculation as to a link between certain occupations and the risk of developing CLL, in particular exposure to ionising radiation has long been thought to have no link to disease development (UNSCEAR 2008), but more recent studies have challenged this position (Rericha *et al.* 2006; Silver *et al.* 2007; Schubauer-Berigan *et al.* 2007). For reasons that remain unclear, an increased incidence of CLL is seen in farmers, rubber manufacturing workers and individuals working with asbestos (Linet *et al.* 2007). CLL has also been linked to occupational exposure to a number of chemical agents such as butadiene (Graff *et al.* 2005), carbon tetrachloride and chlorinated hydrocarbons (Seidler *et al.* 2007) and occupations where contact with such chemical is commonplace, such as drycleaners (Ji and Hemminki 2006), cleaners/janitors (Blair *et al.* 2000) and woodworkers (Flodin *et al.* 1988). Some studies have shown an association between tobacco smoking and CLL (Brown *et al.* 1990), although this is disputed by other workers who report no overall increase in risk for cigarette smoking and CLL (Morton *et al.* 2005).

A family history of CLL is one of the strongest risk factors for disease development (Goldin *et al.* 2004), CLL exhibiting one of the strongest familial tendencies of any malignancy (Goldin and Caporaso 2007). The genetic predisposition towards familial CLL is poorly understood but is the subject of intense research (Caporaso *et al.* 2007).

1.2.2 Clinical course

CLL follows an extremely variable clinical course with overall survival times ranging from months to decades (Abbot 2006). At least 20% of patients have either no or minimal signs and symptoms during their entire clinical course and have a survival similar to age-matched controls (Byrd, Stilgenbauer and Flinn 2004). Other patients show rapidly deteriorating blood cell counts and organomegaly and as such suffer from symptoms either at, or soon after diagnosis. The most common symptoms at diagnosis include anaemia, lymphadenopathy, hepatomegaly, splenomegaly and thrombocytopenia (Montserrat *et al.* 2006). Early stage CLL is generally not treated, as initiation of therapy for early stage patients has not been shown to prolong survival (Dighiero, Maloum and Bichoffe 1998; Parker and Strout 2011), whilst late stage disease is treated with chemotherapy and the use of pharmacologically active agents attached to monoclonal antibodies. Patients with slowly progressing disease may require no treatment during the course of their lives (Chiorazzi, Rai and Ferrarini 2005). More recently however highly effective and potentially curative approaches, such as allogeneic stem cell transplantation have been developed. The therapeutic options available to clinicians vary markedly with regard to efficacy, toxicity and cost and risk stratified algorithms of therapy are becoming increasingly necessary (Byrd, Stilgenbauer and Flinn 2004; Oscier *et al.* 2012).

1.2.3 Laboratory diagnosis of CLL

Most patients are diagnosed with CLL as an incidental finding following a routine full blood count that returns an elevated white cell count and/or a persistent lymphocytosis. Morphological examination of a blood film invariably reveals the presence of characteristic ‘smear’ or ‘smudge’ cells, as shown in figure 1; these are

clonal B cells that have altered morphology as a consequence of cellular fragility. Immunophenotyping of the mononuclear cell population will reveal a characteristic phenotype and can yield useful information for prognosis and on which to base treatment options (Bain 2010).

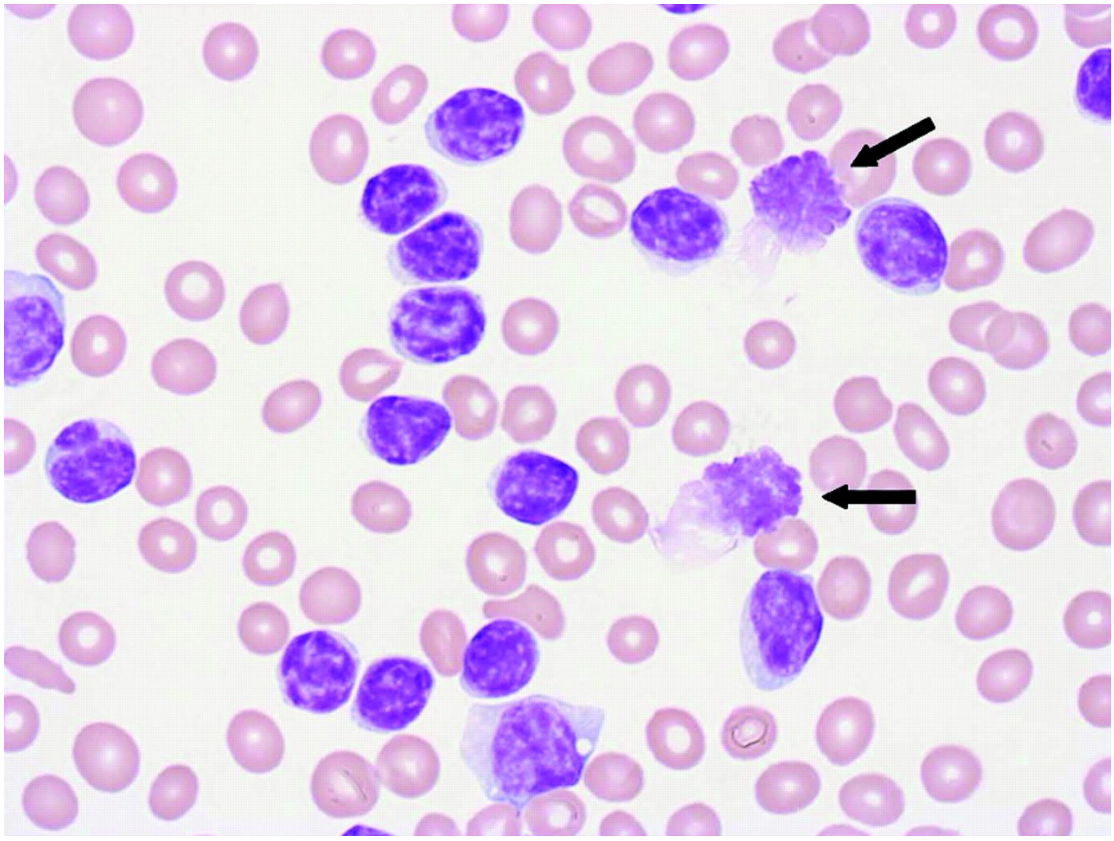


Figure 1: Rowmanowsky stained Peripheral blood film magnified x600 showing characteristic 'smear' cells (arrowed).

WHO classification state that a formal diagnosis of CLL is reached based on the combination of lymphocyte morphology, the presence of >5 $x10^9$/l circulating clonal B cells persisting for >3 months and a characteristic immunophenotype as described below (Swerdlow *et al.* 2008, Bene *et al.* 2011; Oscier *et al.* 2012):

i) Surface immunoglobulin expression of low intensity IgM or IgM/IgD, with either Kappa or Lambda light chain restriction.

ii) Expression of pan B-cell antigens CD19, CD20 and CD23

iii) Co-expression of CD5 on B cell clone

Depending on stage of disease at diagnosis the patient may at this point undergo bone marrow biopsy and cytogenetic testing. Bone marrow biopsy sampling is infrequently performed and although not required by British Committee for Standadisation in Haematology guidelines, some workers maintain that since it allows for the degree of marrow infiltration by the malignant clone to be assessed, it can be of use as an indicator of survival and disease prognosis (Cheson *et al.* 1996). Whilst bone marrow biopsy is not essential for the diagnosis of CLL it is sometimes used to define complete response and is also indicated in determining the cause of cytopenias pre-treatment (Oscier *et al.* 2012). The four patterns of marrow infiltration found in CLL are summarised in table 2:

Pattern of infiltration	**Degree of marrow infiltration**
Interstitial	33% of patients: normal haemopoietic cells are replaced in small quantities by mature lymphocytes but fat cells and bone marrow structure are preserved
Nodular	10% of patients: nodules of mature lymphocytes lacking clear centres are present in the bone marrow in greater quantities than normal lymphoid follicles. Fat cells still present
Mixed interstitial/nodular	25% of patients: a combination of both interstitial and nodular patterns of infiltration
Diffuse	25% of patients: a diffuse lymphoid infiltration is seen with massive replacement of normal haemopoietic cells and fat cells

Table 2: Bone marrow infiltration patterns identified in CLL patients with relative proportions of patients with corresponding degree of infiltration at diagnosis. (After Bain 2010)

1.2.4 Genetic changes

Clonal chromosomal changes are detected in up to 80% of patients with CLL (Dierlamm *et al.* 1997; Parker and Strout 2011), the most common being an interstitial deletion in 13q14, which is seen in approximately 50% of cases, followed by del 11q22 (20%), trisomy 12 (15%), del 6q21 (10%) and del 17p13 (5-10%) (Stilenbauer *et al.* 1993: Nguyen-Khan 2010). Despite extensive work over the past few years the relationship between many of these aberrations and disease activity is incompletely understood. The observation that the most common (13q14) chromosome abnormality observed in CLL is associated with a favourable prognosis, whilst the less common abnormalities result in adverse clinical outcomes, is similarly poorly understood (Dohner *et al.* 2000; Parker and Strout 2011).

Mutations in the gene controlling the production of the variable region of the immunoglobulin heavy chain (V_H) are of particular interest as up to 50% of CLL patients display (V_H) gene mutations. In normal B cell development the variable regions of both immunoglobulin heavy and light chains undergo somatic hypermutation as they pass through germinal centres. Studies on the (V_H) genes in CLL suggest that patients with unmutated (V_H) genes tend to have advanced stage disease and an unfavourable prognosis. Since the assessment of (V_H) mutation status is technically complex, there is much interest in the detection of surrogate markers such as ZAP-70 for this phenomenon (Parker and Strout 2011). ZAP-70 has been demonstrated to be an independent predictor of outcome and may be a better predictor of time to treatment initiation than immunoglobulin heavy chain mutation status (Rassenti *et al.* 2008).

1.2.5 Biology of CLL

The CLL B cell clone is an accumulation of mature long-lived B cells, which express a variety of surface markers which distinguish them from normal B cells (Caligaris-Cappio and Hamblin 1996, Nordgren and Joshi 2010). It is thought likely that these cells have arisen from one progenitor cell, as indicated by the presence of identical key markers, but uncertainty remains as to the genetic origin of the clone. Whilst many clonal cells express genetic abnormalities, phenotypically identical leukaemic cells are found with different genotypes in the same patient (Jurlander 1998; Nordgren and Joshi 2010). These genetic abnormalities may be pathogenic or purely secondary phenomena. Of equal importance to disease development is the potential loss of homeostasis in B cell ontogeny which may not be related to specific genetic abnormalities. It is therefore important to have an appreciation of normal B cell development in order to understand the possible ways in which malignancy may arise.

1.2.6 B cell development

B cell development begins when lymphoid stem cells in the bone marrow differentiate into the earliest distinctive B-Cell lineage, the progenitor B cell (pro B cell). Each stage in the development of B cells is accompanied, and often defined by, rearrangements of the B cell immunoglobulin (Ig) genes and is under the tight control of cytokines and the developmental microenvironment.

Pro B cells

Pro B cells are the most primitive recognisable cells in the B cell lineage (Stiles, Terr and Parslow 1997; LeBien and Tedder 2008). They express the surface proteins CD10 and CD19 and the nuclear proteins terminal deoxynucleotidyl transferase (TdT)

and recombinant activating genes 1 and 2 (RAG-1 and RAG-2) (Oettenger *et al.* 1990; Nordgren and Joshi 2010). Both RAG-1 and RAG-2 play an important role in immunoglobulin gene rearrangement as they have the ability to recognise and cleave DNA at specific sequences. Antibody diversity is further increased by the insertion of N regions, by TdT, at the point of joining between variable (V), diversity (D) and joining (J) segments (Spanopoulou *et al.* 1995, Montecino-Rodriguez and Dorshkind 2012). The first immunoglobulin rearrangements in the B cell take place in the heavy chain genes, once these have occurred the cell can be classified as a pre B cell; Pro B cells which fail to fail to make a functional VDJ_H rearrangement undergo apoptosis (LeBien 2008).

The bone marrow stromal environment plays a critical role in the development of pro B cells to pre B cells (Ansel and Cyster 2001). Pro B cells are able to bind to stromal cells through very late antigen 4 (VLA-4), which binds with vascular cell adhesion molecule 1 (VCAM-1/CD106) on the stromal cell. This interaction promotes the binding of another receptor pair, c-Kit on the pro B cell with stem cell factor, which in turn triggers the expression of interleukin 7 (IL-7) receptors on the pro B cell surface. IL-7 released by the stromal cells binds to the IL-7 receptors (IL-7R), inducing the pro B cell to mature into a pre B cell. Experimental models with mice bred with disruptions in the genes controlling IL-7 production show severe impairment of B cell development (Van Freeden-Jeffry *et al.* 1995).

Pre B cells

Pre B cells are found almost exclusively in the bone marrow and are representative of a transient phase in B cell development that lasts approximately 2 days (Stiles, Terr and Parslow. 1997; Montecino-Rodriguez and Dorshkind 2012). IL-7 secreted by

stromal cells, drive the maturation process by inducing down-regulation of the adhesion marker VLA-4 (Cd49d/CD29) on the pre B cell surface. The pre B cell can now detach from the stromal cell as direct contact is no longer required for growth. However, IL-7 released from the stromal cells is still required for growth and maturation. Rearrangement of the light chain genes begins in the pre B cell once heavy chain rearrangements are complete (Levine *et al.* 2000). TdT is no longer expressed and as a result there is no insertion of N regions into light chain genes (Le Bien 2000). Rearrangement continues until a functional light chain (either kappa or lambda) is produced. Once produced, the light chain associates with the existing heavy chain unit and together they are transported to the cell surface and membrane bound immunoglobulin. Expression of RAG-1 and RAG-2 is downregulated and the cell therefore loses the capacity for further light chain rearrangement. Successful assembly of one heavy and one light chain prevents any further gene rearrangements. This process, termed allelic exclusion, gives rise to the phenomenon of clonal restriction. Further division of the lymphocyte after this stage results in daughter cells which all express identical heavy and light chains, giving rise to a population of clonal B cells with specificity for a particular antigen.

Immature B lymphocytes

Once the B cell starts to express surface immunoglobulin, with functional heavy and light chains expressing B cell receptors (BCR), it is classified as an immature B lymphocyte. At this stage it will stay in the bone marrow for up to 3 days. At this point the immature B cell undergoes negative selection. B cells that can cross-link IgM receptors or bind to self antigen on the cell surface will undergo apoptosis. A large number of B cells are also lost at this stage owing to non-functional

immunoglobulin production (Thompson 1998; LeBien and Tedder 2008). It has been estimated that of the 2 x10^7 B cells that are developed each day, only 1-3% will enter the mature B cell pool. This fail safe mechanism is essential for the deletion of self reactive clones, which would otherwise recognise self proteins as foreign and increase the risk of autoimmune disease. Cells which remain following this checking phase will undergo further re-arrangement of heavy chain genes, which results in expression of IgD, at which point they are regarded as fully mature and enter the peripheral blood stream (Levine *et al.* 2000).

Mature B cells

Mature B cells express a number of surface antigens such as CD23, lymphocyte function associated antigen-1 (LFA-1) intracellular adhesion molecule-1 (ICAM-1/CD54) and major histocompatbility antigen complex II (MHC II) amongst others. Expression of these key surface antigens allows for interaction with antigen presenting cells such as dendritic cells and T lymphocytes. This interaction is of paramount importance in the generation of the immune response (Nordgren and Joshi 2010). The key stages in B cell development are summarised in figure 2 below.

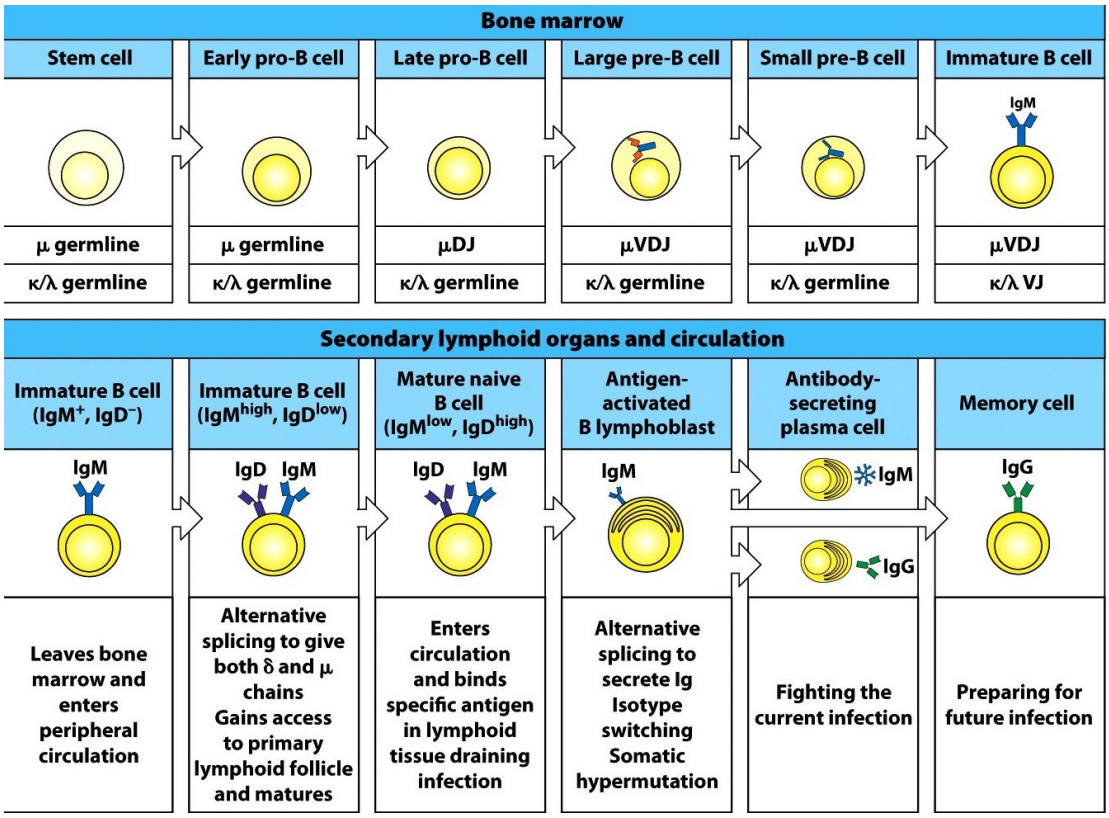


Figure 2: B cell development From: Kuby Immunology, 7/e, by Judy Owen (Haverford College) , Jenni Punt (Haverford College) , Sharon Stranford (Mount Holyoke College), Copyright 2013 by W.H. Freeman and Company. Used by Permission of the publisher.

1.2.7 Origins of the CLL cell

CLL B cells have a number of features that are unique to the malignant cell and some which they share with normal B cells in the developmental pathway. CLL B cells have low or undetectable levels of surface immunoglobulin, which are polyreactive and show autoreactive antibody activity, often acting as a rheumatoid factor (RF), specifically recognising the Fc region of IgG class immunoglobulin (Parham 2000; Seifert *et al.* 2012).

Autoantibody activity may also be directed against haemopoietic antigens expressed on the surface of red blood cells and platelets, which can lead to autoimmune haemolytic anaemia (AIHA) and autoimmune thrombocytopenic purpura (AITP) respectively (Caligaris-Cappio and Hamblin 1996; Dearden *et al.* 2008).

CLL B cells accumulate in the peripheral blood and bone marrow in the G_0 phase of the cell cycle, these apparently resting cells express membrane makers of cellular activation, such as CD80 and CD86 which are normally expressed by activated normal B cells (Caligaris-Cappio el at 2001; Seifert *et al.* 2012). Under normal conditions CLL B cells appear to be anergic and have a reduced capacity to act as APC's, whilst normal B cells are highly effective at this role (Caligaris-Cappio and Hamblin 1996). If, however, the CLL B cells are stimulated using monoclonal antibodies directed at activation ligands such as CD40, they can differentiate into effective APC's, suggesting a restricted dialogue between CLL B and functional T cells during an normal immune response (Scrivener *et al.* 2003).

The cell surface marker CD5 is expressed on almost all malignant CLL B cells, but is also expressed on normal T cells and a subset of normal B cells (Lydyard *et al.* 1999, Nordgren and Joshi 2010). Normal B cells that express CD5 are termed B1 cells, and represent the most prominent B cells found in early foetal life, the numbers of these cells decreasing with age. In the adult, circulating B1 cells are normally found in the follicular mantle zone of the lymph nodes, but a limited number are also found in the bone marrow. Normal B1 cells produce polyreactive autoantibodies, including those directed against blood group antigens and are also the main source of IgM derived autoantibodies (Lydyard *et al.* 1999; Nordgreand Joshi 2010). Patients with Rheumatoid Arthritis have been found to have increased levels of B1 cells (Plater-Zyberk *et al.* 1985). It is unclear what controls the levels of normal B1 cells in the peripheral blood, but children with DiGeorge syndrome, i.e. those lacking a functional thymus, have decreased levels of B1 cells, suggesting a role for the thymus in maintaining B1 cell numbers in the periphery (Kourtis *et al.* 1997). Early studies on Ig

V_H genes in CLL suggested that most malignant B cells had unmutated V_H genes (Kipps *et al.* 1989), later studies on larger groups of patients however reported that up to 50% of CLL patients V_H genes were in fact mutated, indicating that they would have passed through the germinal centre (Caligaris-Cappio 2001; Nordgren and Joshi 2010,). As the nature of the B cell has become clearer, attempts to identify its origin have combined the evidence outlined above. Initially, much work focused on the similarities between the CLL CD5+ B cell and the normal CD5+ B1 cell, these are summarised in table 3.

Feature	**CD5+ B cell**	**CD5+ normal B1 cell**
Polyreactive autoantibodies	Yes	Yes
Somatic hypermutation	Yes/No (50% of patients)	No
Formation of mouse erythrocyte rosettes	Yes	Yes
Bc1-2 expression	Yes	No
Low surface immunoglobulin expression	Yes	No

Table 3: The similarities and differences between CD5+ CLL B cells and the normal subset of B cells that naturally express CD5

A number of cellular and molecular similarities between the two cell lines have emerged. CLL CD5+ B cells and normal B1 cells both produce polyreactive IgM autoantibodies directed against RF and haemopoietic cell antigens (Caligaris-Cappio 1996; Montecino-Rodriguez and Dorshkind 2012). Both are able to produce these antibodies in the absence of somatic mutation, suggesting that neither subset has passed through the germinal centre. In addition, both lines express the surface marker CD20 and both are able to form rosettes with mouse erythrocytes. The normal B1 B

cell is considered as a possible candidate for the origin of the CLL clone. Since normal CD5+ B1 cells are located in the mantle zone of the secondary lymphoid follicles, it is suggested that it is from here that CLL B cells may arise (Soderberg 1998). One possible theory suggests that CLL is a malignancy of a mantle based subpopulation of anergic self reactive CD5+ B cells devoted to the production of naturally occurring polyreactive autoantibody (Caligaris-Cappio 2001).

There are however striking differences between the 2 cell types. Whilst CLL B cells express low to undetectable levels of surface immunoglobulin, normal B1 cells express normal levels. CLL B lymphocytes appear to be stuck in the G_0 phase of the cell cycle and yet are able to express cell surface markers and cytokines that are indicative of an activated B cell. B1 cells do not follow this pattern. The B1 cell cycle correlates with expression of cell surface markers and there is no difference between their activated and resting states. Normal B1 cells express low levels of Bcl-2, whilst CLL B cells express high levels. Normal B1 cells do not demonstrate somatic hypermutation, yet 50 % of CLL B cells do (Hamblin *et al.* 1999). Additionally, the production of autoantibodies in CLL is believed to result from the residual normal B cell population (Oscier 1999; Zent and Kay 2010).

Attempts to identify a normal cellular counterpart for the CLL B cell have so far failed. Such a counterpart would need to encompass all aspects of the disease, such as autoimmunity, hypogammaglobulinaemia, low surface immunoglobulin expression, CD5 expression and dysregulation of T cell function (Scrivener *et al.* 2003).

1.2.8 Morphology of CLL cells

CLL B cells appear as small, mature, unstimulated peripheral blood lymphocytes, which are easily damaged on preparation of a blood film, giving rise to the characteristic 'smear' or 'smudge' cell frequently observed during morphological examination (Hamblin *et al.* 1999). On examination of peripheral blood films, the malignant cells can be distinguished by their heavily clumped basophilic chromatin within the nucleus and the presence of a small agranular pale blue cytoplasm (Dierlamm *et al.* 1997). Approximately 15% of patients have larger B cells with a more prominent nucleolus, cleaved nucleus or lymphoplasmaytoid features. Examination of peripheral blood and bone marrow aspirate from the same CLL patient identifies an identical infiltrating population from both sources (Oscier 1999).

1.2.9 Immunophenotype

Morphological examination by itself is insufficient to make a laboratory diagnosis of CLL, and the detection of cell surface marker antigens by flow cytometry is considered essential (Bain 2010). One of the key distinguishing features of the CLL cell is low to undetectable levels of surface immunoglobulin (Dohner and Stilgenbauer 2001). In the majority of cases of CLL the surface immunoglobulin that is expressed is either class IgM, IgD or both. There is invariably restricted expression of either Kappa or Lambda light chains, this being indicative of the clonal nature of the malignant cell population (Jurlander 1998). CLL B cells have been shown to express the interaction and activation markers CD80 and CD86 (Caligaris-Cappio 2001). Expression of the B cell receptor (BCR) is altered in CLL patients. The BCR consists of surface immunoglobulin, non-covalently linked with CD79, CD79b and CD5. In most cases of CLL the extracellular domain CD79b is absent (Alfarano *et al.* 1999). However, CD79b has been shown to be functionally normal in CLL patients,

regardless of whether it is expressed at the cell surface or not (Rassenti and Kipps 2000). Normal B cells utilise CD7b expression, post activation, to downregulate BCR expression, suggesting that CLL B cells may be activated in some way (Scrivener at al 2002). There is a characteristic expression of a number of surface markers in CLL which are summarised in table 4.

Marker	Molecular weight	Normal expression	Role	Expression on CLL cells
CD5	67kDa	T cells, B1 B cells	BCR activation and differentiation	+
CD19	95kDa	B cells	T/B cell interaction	+
CD20	33-37kDa	B cells	B cell activation and signal transduction	+
CD21	145kDa	Mature B and T cells (Low) Immature T cells (high)	Antigen presentation	+
CD22	135kDa	B cells	Accessory signalling through BCR	-
CD23	45kDa	B cell, macrophage/monocytes)	B cell activation marker	+
CD79b	33-40kDa	B cells	Downregulation of BCR	-
CD80/CD86	60kDa/80kDa	B cells, dendritic cells, some T cells	T/B cell interaction	+
Surface Immunoglobulin	Highly variable	B cells	Antigen binding	+/-
Bcl-2	25kDa	Apoptosis-protected cells	Protection against apoptosis	+

Table 4: Key features of B cell antigens and differential expression on CLL B cells

1.3 Disease classification

1.3.1 Classification of disease stage and prognosis

The aim of any pathological classification system is to group cases that have fundamental similarities and are likely to have some common causational feature and pathogenesis. The French, American, British (FAB) classification of acute leukaemias was first published in 1976 in the hope of providing a universal system of classification for leukaemias which would in turn improve the accuracy of diagnosis (Bennett *et al.* 1976). The classification was subsequently expanded, modified and clarified over the next 15 years (Bain 2010). The FAB group also published during this time a classification for chronic lymphoid leukaemias (Bennett *et al.* 1989).

The classification systems for haematological malignancies have changed dramatically over the years (Smith *et al.* 2011). The currently used system is the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues 4th edition (Swerdlow *et al.* 2008), which was established by the WHO in 2001 and was modified in collaboration with the European Association for Haematopathology and the Society for Hematopathology in 2008 (Vardiman *et al.* 2009). The aim of this system is to separate and characterize leukaemias and lymphomas into clinically and biologically relevant entities, it also includes genetic, cytochemical, immunophenotypic and clinical information which assist in the construction of diagnostic algorithms.

There are presently two staging methods for CLL available to clinicians, the Rai system and the Binet system, which may be used alone or in conjunction with one another. The Rai staging system incorporates a grading of clinical signs and

laboratory findings at the time of diagnosis, which segregates patients into five stages (0 – IV). The system is summarised in table 5.

CLINICAL STAGING	CLASSIFICATION	MEAN SURVIVAL (MONTHS)
STAGE 0	Peripheral blood and marrow lymphocytosis (absolute blood lymphocyte count $\geq 15 \times 10^9$/l, 40% or more lymphocytes in marrow)	>120
STAGE I	Peripheral blood and marrow lymphocytosis with lymph node enlargement)	95
STAGE II	Peripheral blood and marrow lymphocytosis with splenomegaly and/or hepatomegaly (with or without lymph node enlargement)	72
STAGE III	Peripheral blood and marrow lymphocytosis with haemoglobin <110g/l (unless haemolytic anaemia present) with or without lymph node, spleen or liver enlargement)	30
STAGE IV	Peripheral blood and bone marrow lymphocytosis with platelet count <100 $\times 10^9$/l (with or without lymph node, spleen and liver enlargement)	30

Table 5: The Rai-Binet staging system showing the clinical stage of CLL and classification of symptoms at each stage as defined by Rai (Rai *et al.* 1975)

Rai's staging system identifies patients at stage 0 of the disease who do not receive chemo or radio-therapeutic treatment, but have regular monitoring of disease progression. In stages I and II, treatment is not generally required unless there are significant symptoms relating to advancing disease, and in whom a reduction in tumour mass may be clinically indicated. Patients with stage III or IV disease receive treatment aimed at achieving complete remission, or if this is not possible, a good partial remission. Rai showed that patients who present with more severe symptoms and a higher lymphocyte count at time of diagnosis tend to have a poorer prognosis,

with those patients presenting with stage 0 – II disease living longer than those with stage III or IV disease (Rai *et al.* 1975).

A modified staging system was proposed by Binet in 1981 in which more emphasis is placed on isolating patients defined as 'high risk' by the presence of anaemia or thrombocytopenia. Binets system is summarised in table 4.

CLINCIAL STAGING	CLASSIFICATION	MEAN SURVIVAL (MONTHS)
STAGE A	Haemoglobin ≥100g/l. Platelets ≥ 100 $x10^9$/l Less than 3 enlarged areas*	>120
STAGE B	Haemoglobin ≥100g/l. Platelets ≥ 100 $x10^9$/l 3 or more enlarged areas*	61
STAGE C	Haemoglobin < 100g/l and/or platelets < 100 $x10^9$/l. Any number of enlarged areas*	32

* incorporating cervical, axillary, inguinal lymph nodes, spleen and liver

Table 6 The modified Binet staging system showing clinical stage of CLL and classification of symptoms at each stage as defined by Binet (Binet *et al.* 1981)

It was postulated by Binet that it is possible to combine the two systems of classification, Rai stages 0 – III being assigned to Binet stage A, Rai stage IV being grouped with Binet stage C and the remaining patients from Rai stage II being separated into stag A or B depending on physical signs and symptoms (Binet *et al.* 1981).

Binets staging system has been subsequently modified with stage A disease being further sub-divided into stage A' and stage A" (Digheiro *et al.* 1991). Stage A' is

characterised by a lymphocyte count < 30 x10^9/l and a haemoglobin >120g/l. Approximately 80% of stage A patients fall into this category. Stage A" is categorised by either a lymphocyte count of > 30 x10^9/l or a haemoglobin < 120g/l. The remaining 20% of stage A patients falling into this category.

The use of these staging systems has helped simplify the classification of CLL patients by separating them into low, intermediate and high risk (Rai *et al.* 1975). Whilst both the Rai and Binet systems are the mainstay of staging there has always been controversy as to their usefulness in identifying groups of patients who may or may not benefit from therapy (Hallek 2008), and interest has focused on alternative, independent prognostic factors and indicators for the need for treatment (Shanafelt *et al.* 2009). The search for alternate staging systems lead to the publication of a CLL scoring system where points are allocated to patients dependant on the expression of key immunological markers associated with CLL, the final score indicating the likelihood of a positive diagnosis (Matutes *et al.* 1994). The Matutes scoring system for the diagnosis of CLL is based on the common marker profile of the strong expression of CD5 and CD23, negativity of FMC7, the weak to moderate expression of surface immunoglobulin and the negative or weak expression of CD79b. A score of 4/5 or 5/5 is strongly supportive of a diagnosis of CLL; if the score falls below this the diagnosis is less certain (Oscier *et al.* 2012). This scoring system is summarised in table 7.

Membrane marker	1 point	0 points
Surface Immunoglobulin expression strength	Weak	Moderate/Strong
CD5 expression	Positive	Negative
CD23 expression	Positive	Negative
FMC7 expression	Negative	Positive
CD79b expression	Weak/Negative	Moderate/Strong

Score 4-5: Typical CLL
Score 3-4: Atypical CLL
Score <3: Non-CLL

Table 7: The scoring system for CLL. Points are allocated to patients depending on presence or absence of surface antigens on malignant cell population. Total score aids in differentiation between typical CLL, atypical CLL and other forms of leukaemia (Matutes *et al.* 1994).

1.3.2 Newer prognostic factors

Although the use of these staging and scoring systems for the classification of CLL is now well established, they are not completely satisfactory and have been subject to regular modification (Chiorassi, Rai and Ferrarini 2005). Consideration of clinical and therapeutic factors, notably performance status and presence of adverse prognostic factors, is assuming increasing importance in the development of staging systems (Auer, Gribben and Cotter 2007).

Progress in the identification of molecular and cellular markers that have value in predicting disease progression and detection of minimal residual disease after therapy has lead to the discovery of a number of potential markers of prognostic importance

such as CD38 and the 70kDa Zeta associated protein (ZAP-70) (Razzenti, Lang-Huynh and Toy 2004, Razzenti *et al.* 2008; Parker and Strout 2011). This is important, as more than 80% of cases of CLL are diagnosed at early disease stage. These developments have however created uncertainty for clinicians who hope to incorporate the use of these markers into standard clinical practice (Binet, Caligaris-Cappio and Catovsky 2006). It is recognised that further research is needed to better understand if intrinsic differences in cell biology and analysis of the expression of molecular and cellular markers in CLL can be of prognostic use (Montserrat *et al.* 2006; Parker and Strout 2011).

There is also a need in the field of CLL treatment to identify factors that define outcome and refine disease categories (Caporaso *et al.* 2007). The Rai and Binet clinical staging systems are used to define disease extent and prognosis but new biological prognostic factors have become increasingly important, especially in early stage CLL. New prognostic factors continue to appear in the literature i.e. smudge cells, CAT scan abnormalities and new molecular and genetic markers (Nowakowski *et al.* 2007; Halek 2008). Any new prognostic information that can be proven to be of worth will independently add information to the many other traditional factors that are documented to influence CLL prognosis such as morphology, cytogenetics, lymphocyte doubling time and serum markers of rapid cell turnover, including elevated thymidine kinase and β-2 microglobulin (Binet, Caligaris-Cappio and Catovsky 2006).

CLL is a heterogeneous disease, which may change both within the individual and amongst the population; it can also however assume a steady state and remain

unchanged until death. A comparison between patients diagnosed with CLL in the period 1960 – 1979 against those diagnosed 1980 – 1989 shows significant differences. In the later cohort of patients, diagnosis was made at a later age, more patients were diagnosed in low risk groups and survival time was more than double (Rozman, Bosch and Montserrat 1997). As newer diagnostic tools have become available, their application has greatly influenced changes in both the accuracy and the speed of diagnosis. In particular the increased use of molecular techniques has introduced an opportunity to study the disease and its prognosis. It has been demonstrated that telomerase, an enzyme that mediates the repair and preservation of telomeres within chromosomes and thus prevent the ageing process normal in somatic cells, has significantly higher activity in B cells in CLL patients compared to normal controls. This enhances the ability of CLL cells to survive longer and continually repair DNA, not only by preventing the erosion of telomeres, but by actually restoring telomere length (Bechter *et al.* 1998). It is envisioned that the measurement of telomerase activity may in the future become and important variable in the new generation of prognostic factors, allowing for better and more accurate diagnosis and prognosis.

1.4 Toll Like Receptors

In the 1980's researchers in Germany working on the development of the fruit fly *Drosophila* found that the fly could not develop correct dorsal-ventral axis without the protein Toll, which is a trans-membrane signal receptor protein. The word Toll (meaning 'weird', 'odd' or 'really good' in German slang) refers to the bizarrely scrambled anatomy which results from the absence of this protein. In humans there are homologous proteins which appear to perform a vital role in innate immunity;

hence these became known as ‘Toll like receptors’ (Werling and Jungi 2003, Zhang and Ghosh 2001).

Toll like receptors (TLR) are pattern recognition receptors that trigger innate immunity, signalling via TLR playing a critical role in defence against pathogens and innate activation of the adaptive immune response (Akira and Takeda 2004).

TLR are membrane spanning proteins that consist of an exterior region, a membrane spanning region and an interior domain. To date 13 TLR have been discovered in humans and functions determined for 11 of them (Lu 2010). The current information relating to TLR is summarised in table 8. Each TLR detects a distinct repertoire of highly conserved pathogen molecules; the complete set can detect a broad variety of viruses, bacteria, protozoa and fungi. The ligands that bind to TLR’s are invariably an indispensable component of the pathogen in question, thus pathogens do not have the option of mutating to forms that lack the essential building blocks recognised by TLR.

When a pathogen activates a TLR by binding to the extracellular domain a signal transduction pathway is activated which results in the induction of an innate immune response. This response includes promotion of the expression of genes that contribute to inflammation, induction of changes to antigen presenting cells (APC) to make them more efficient at antigen presentation and it also causes the synthesis and export of extracellular signalling molecules that affect a variety of cells involved with the immune response such as leucocytes (Underhill 2003). Activated pathways involved in this response include the NF-κB pathway (IκBα phosphorylation, translocation of NF-κB p65 to the nucleus), mitogen activated protein kinases p38, Jun-N-terminal kinase (JNK), and the interferon pathway, these pathways are summarised in figure 3:

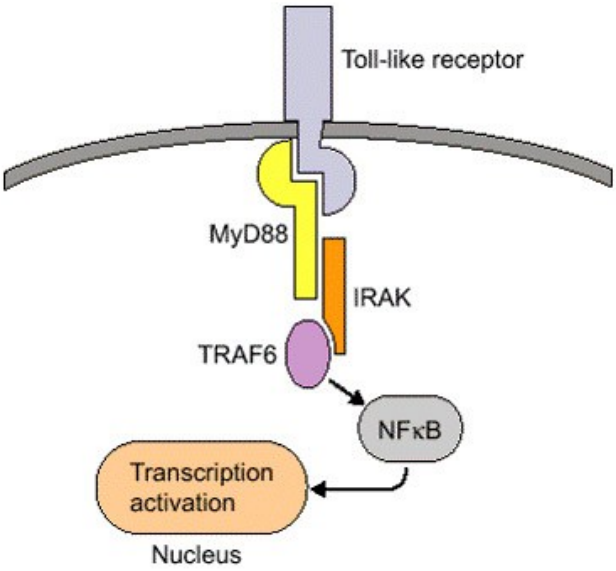


Figure 3: TLR structure showing signal transduction pathway From: Kuby Immunology, 7/e, by Judy Owen (Haverford College) , Jenni Punt (Haverford College) , Sharon Stranford (Mount Holyoke College), Copyright 2013 by W.H. Freeman and Company. Used by Permission of the publisher.

1.4.1 TLR function

The 13 different TLR currently identified in humans share similarities in their structure and function but respond differently to microbial components ((Kawai and Akira 2011)

Bacterial cell wall components are recognised by five TLR (1,2,4,5 and 6) and are termed extracellular TLR due to their expression on the cell surface and their extracellular domain, by contrast, TLR 3,7,8 and 9 are located in the cytoplasm and depend on the capacity of pathogens to penetrate the cell membrane . These internal TLR recognise both double and single stranded RNA, unmethylated DNA sequences and other motifs found predominantly in the bacterial genome. Together the extracellular and intracellular TLR's confer a germ line-encoded repertoire specific for ligands of bacterial and viral origin.

The TLR signalling pathway consists of a myeloid differentiation factor 88 (MyD88) dependant pathway which is common to all TLR and a MyD88 independent pathway selective to TLR 3 and 4.

Activation of MyD88 initiates a signalling cascade, which leads to the downstream activation of kinases and the translocation of the central transcription factors (NF)-kB and interferon regulatory factor (IRF)-3. Following this, MyD88 associates with Toll/interleukin (IL)-1 receptor (TIR) domain-containing adapter protein to form a complex that then recruits IL-1 receptor-associated kinase and subsequently tumor necrosis factor (TNF) receptor associated factor (TRAF)-6. This results in activation of the ikB kinase (IKK) complex.

In MyD88-independent signalling, the adaptor molecule TIR domain-containing adaptor-inducing interferon (TRIF) is recruited to the intracellular part of TLR3 directly or to TLR4 via TRIF-related adaptor molecule (TRAM), which consequently leads to activation of both tank-binding kinase 1 (TBK-1) and TRAF-6, this being a crucial checkpoint for the induction of a NF-kB controlled immune response or an IRF-3 controlled response with thyp 1 IFN activation pattern.

Activation of NF-kB is then one of the central signalling pathways after recognition of TLR ligands. This activation promotes phagocytosis of pathogens and inflammatory responses to phagosome contents. Additionally TLR 2 and 4 are able to interact directly with phagosomes and thus become activated at a very early stage of pathogen contact. In addition to triggering phagocytic and maturation signals,

activation of TLR also enhances co-stimulatory expression of accessory molecules such as CD80 and CD86 which provides a second signal for a full immune response.

Receptor	Ligand(s)	Ligand location	Location	Cell types
TLR1	Lipopeptides, soluble factors	Bacteria, mycobacteria	Cell surface	Monocytes, T & B lymphocytes, dendritic cells
TLR2	Glycolipids, lipoproteins, peptidoglycans	Gram positive bacteria, fungi	Cell surface	Monocytes, T & B lymphocytes, dendritic cells, mast cells
TLR3	Double stranded RNA	Viruses	Cell compartment	B lymphocytes, monocytes, dendritic cells
TLR4	Lipopolysacchride, heat shock protein, fibrinogen	Gram negative bacteria	Cell surface	Monocytes, T lymphocytes, dendritic cells, mast cells, intestitial epithelium
TLR5	Flagellin	Bacteria	Cell surface	Monocytes, dendritic cells, intestinal epithelium
TLR6	Diacyl lipopeptides	Gram positive bacteria, fungi	Cell surface	Monocytes, mast cells
TLR7	Single stranded RNA	Viruses, synthetic compounds	Cell compartment	Monocytes, dendritic cells, B lymphocytes
TLR8	Single stranded RNA	Viruses, synthetic compounds	Cell compartment	Monocytes, dendritic cells, B lymphocytes
TLR9	CpG-containing DNA	Bacteria	Cell compartment	Monocytes, dendritic cells, T&B lymphocytes
TLR10	Not determined	Unknown	Cell Surface	Monocytes, B cells
TLR11	Profilin	Uropathogenic bacteria	Unknown	Monocytes, liver/kidney cells, bladder epithelium
TLR12	Not determined	Unknown	Unknown	Unknown
TLR13	Not determined	Unknown		Unknown

Table 8: Human TLR distribution, specificity and function (adapted from Kingston & Mills 2011)

TLR control the activation of innate immunity through the induction of antimicrobial activity and the production of inflammatory cytokines (Yamamoto *et al.* 2002). They also control the generation of adaptive immunity through the induction of antigen-presenting (MHC class II) and co-stimulatory molecules such as CD80 and CD86 and specific cytokines such as Interleukin 6, on APC (Pasare and Medzhitov 2005). Gene targeting studies examining TLR and their cognate downstream signalling molecules provides evidence that the expression and activation of TLR *in vivo* contributes to host defence and the generation of specific antibodies following vaccination (Krutzik *et al.* 2005).

Despite the advances in understanding of the role TLR plays in host defence and the specific signalling events initiated following TLR activation, factors that regulate TLR expression and function are poorly understood (Lancaster *et al.* 2005). However a number of specific molecules are known to be involved in the TLR signalling pathway. These include adapter molecules such as MyD88, MyD88 adapter-like (Mal), also known as Toll/IL-1R (TIR) domain-containing adaptor protein (TIRAP), and TIR domain-containing adapter inducing interferon (TRIF), also known as TICAM1. Other key signalling proteins include IL-1 receptor associated kinases (IRAKs) such as IRAK1, 2, and 4, transforming growth factor kinase (TAK-1), IκB kinases (IKKs), and TRAFs (TNF receptor associated factors) (Zhang *et al.* 2004).

It is also believed that cytokines exert influence over the development of host immunity and have been shown to modulate the expression and activation of TLR. Viral infection of human macrophages for example induces expression of a number of

TLR, a process which is dependant on the production of type 1 interferons (Miettinen 2001). Other studies have shown that differential TLR expression and activation is regulated by activation via type 1 and type 2 cytokines (Krutzik *et al.* 2005).

Three key discoveries have confirmed the central role that TLR perform in innate immunity. Firstly the observation that the mutations in Toll which play a role in fly development also make the fly highly susceptible to lethal pathogens. This discovery demonstrates the importance of pathogen triggered immune response in invertebrate organisms.

Secondly it was discovered that human proteins with cytoplasmic domain homology with Toll activated the expression of immune response genes when transfected into a human experimental cell line. This demonstrated conservation of an immune response pathway between invertebrates and humans. And thirdly, studies with mutant mice homozygous for the *lps* locus were resistant to lipopolysacchride (LPS) also known as endotoxin, which is found in the cell walls of gram negative bacteria. Mutant strains of mice were susceptible to septic shock from endotoxins released from gram negative bacteria, the mouse *lps* gene encodes for a TLR, thus providing unequivocal evidence that TLR's play a central role in normal immunophysiology (Salaun, Romero and Lebecque 2007).

1.4.2 Toll like receptors and CLL

A rapid cellular response to pathogenic organisms is central to the maintenance of a fully functional immune system, this role is carried out by pathogen associated pattern recognition receptors (PAMP's), amongst which group the TLR have been identified as having prime importance (Akira, Uematsu and Takeuchi 2006).

TLR signalling plays an important role in the biology of B cells. It is postulated that they may be involved in the regulation of the B cell differentiation process (Hayashi, Akira and Nobrega 2005). TLR stimulation is also required as signal for the activation of naïve B cells (Ruprecht and Lanzavecchia 2006). In naïve B cells, TLR are expressed at low levels, but the expression of some TLR is induced upon BCR triggering, the effect of which is that some memory B cells express TLR at high levels. It has been suggested that TLR expression by memory B cells is crucial for the maintenance of long lived memory B cells (Bernasconi, Traggiai and Lanzaveccia 2002). TLR have also been reported to induce BCR independent B cell activation, and increase the immunogenicity of B cells by upregulating costimulatory molecules (Mansson *et al.* 2006). A role has also been identified for TLR in T lymphocyte function in the T helper cell related control of inflammation (McGettrick and O'Neill 2007).

Given that B CLL cells share some of the characteristics of their normal counterparts, investigation of TLR expression on the two groups of cells and possible links to prognosis and autoimmune phenomena is of great interest. The possibility of using TLR for targeted drug delivery has been proposed. CLL is especially amenable to TLR agonist therapy, as it is an immunologically susceptible tumour with strong TLR expression (Kanzler *et al.* 2007). It has also been suggested that the stimulation of TLR differentially expressed on B CLL cells could increase immunogenicity of tumour cells and thus potentially contribute to the induction of a leukaemia-specific immune response, which in turn would have great significance for possible therapeutic advances (Spaner and Masellis 2007). Interest in the role of TLR in CLL therapy has generated interest in their expression in both normal and malignant cell

populations; studies have indicated that B CLL cells display similar patterns of TLR as normal B cells but with different levels of expression (Grandjenette *et al.* 2007). To date however, there has not been a concise study of TLR expression patterns comparing B CLL and normal B cells (Rožková *et al.* 2010).

1.5 Project aims and hypothesis

It has long been accepted that improved understanding of prognostic factors in CLL should accelerate the development of risk adapted treatment strategies that also take into account more traditional prognostic indicators (Montserrat 2002, Parker 2011). A major review of the aetiology of CLL in 2007 concluded that the timely assessment of new leads in the study of the disease was due (Linet *et al.* 2007).

Chronic lymphocytic leukaemia is frequently associated with immune disturbances (Hodgson *et al.* 2011), the pathogenesis of the disease appearing to involve dysfunctional regulation of humoral and cellular immunology with subsequent development of genetic aberrations (Dearden *et al.* 2008). Since TLR are thought to bridge these two elements of the immune system, the aim of the project was to collect data on the expression of TLR on a variety of cells in CLL patients and age/gender matched controls, in an attempt to establish links between TLR expression and autoimmune involvement.

The hypothesis of this study is that there is a link between TLR expression levels on lymphocytes & monocytes and the disease process in CLL. In particular it is hypothesised that the development of autoimmunity in CLL is directly attributable to

TLR function and as such there will be a differential expression of TLR between patient and age/sex matched controls.

This hypothesis will be tested by assessing the expression of TLR on leucocyte subtypes in both patient and matched control groups to compare differences; this will be followed by a comparison of TLR expression patterns in patients exhibiting traits of autoimmunity, as demonstrated by a positive direct antiglobulin test (DAG). From these studies it is expected that a TLR based mechanism for autoimmunity in CLL will be revealed.

2 Materials

All flow cytometric consumables including flow sample tubes, sheath reagent and wash/dilution fluids were obtained from Becton Dickinson (Oxford UK). Blood collection equipment, including needles and Vacutainer brand sample tubes were also obtained from BD.

All monoclonal antibodies used in the first line diagnostic CLL panel were obtained from Dako (Cambridge UK), as were the fixation and permeability solutions and the Isotype negative controls.

The anti-TLR monoclonal antibodies were sourced from the following companies: Anti-TLR 1, 3 and 4 from RandD systems (Minneapolis USA), Anti TLR 2 and 9 from Insight Biotechnology (Wembley UK).

Patient and control samples were obtained from blood samples collected at the Royal Cornwall Hospital Haematology (RCH) outpatients department. Between January 2007 and May 2010 a total of 129 patients (82 males, 47 females), age 50-94 years, (mean age 71.5 years) with newly diagnosed CLL presenting at the Haematology department of the RCH, were recruited for the study. None of the study group had any history of malignancy prior to presentation. An equal number of age and sex matched case controls, with no previous history or current evidence of haematological malignancy, were recruited from patients presenting at RCH for pre-operative or general health screening during the same time frame. All controls were screened using the same diagnostic tests as the patient group, in order to be included in the study they

were required to have normal blood film morphology and Immunophenotyping (i.e. a Matutes CLL score of 0).

Only patients with a confirmed diagnosis of B-CLL, determined by morphology and immunophenotype, and having a Matutes CLL score of 3-5 were included in the study. The data from those patients subsequently found to fall outside these diagnostic criteria were excluded from further analysis.

A summary of key demographic details are attached as appendix i and summarised in table 9.

Demographic factor	Results from this study	National average figures for CLL
Average age	71.5 (Range 50-94)	72
Male: female ratio	1.75:1 (82♂:47♀)	2:1
Patients <55 years old	6.2%	11%

Table 9: Summary of key patient demographic data (National average figures from Oscier et al 2012)

All peripheral blood samples were collected by experienced phlebotomy or medical staff, using a standardised procedure. 4ml of peripheral blood was drawn from veins in the antecubital area of the forearm using Vacutainer evacuated blood collection tubes, containing the anticoagulant Di-potassium ethylenediamine tetra acetic acid. Samples were transported to the laboratory at room temperature and prepared for flow cytometry within 2 hours of phlebotomy.

3 Methods

The methods used in the study are detailed below; these were adopted following an extensive developmental period, details of which are recorded in the method development chapter.

3.1 Sample analysis

Patients were routinely diagnosed with CLL by the Haematology laboratory at RCH using the series of diagnostic tests described below, a diagnosis of CLL being based on a combination of lymphocyte morphology, the presence of >5 $x10^9$/l circulating clonal B cells and a characteristic immunophenotype (Eichhorst *et al.* 2011; Oscier *et al.* 2012)

3.1.1 Full blood count

A full blood count (FBC) was produced using the Advia 2010 automated blood count system (Bayer, New York, USA), which enumerates a number of parameters associated with the cellular components of the subjects peripheral blood including white blood cell count (WBC), haemoglobin (Hb), platelet count (Plt) and the numbers and proportions of white cells to produce a differential count (Dif). Other parameters were also simultaneously measured using this test, such as red cell and platelet volume, concentration and distribution. Commercial controls were used at regular intervals to ensure the accuracy and precision of the results and there was participation in a formal national external quality assurance scheme throughout the study. Samples with FBC parameters falling outside of normal ranges were referred for manual blood film analysis under a light microscope. If blood film analysis revealed the presence of characteristic ‘smear’ cells or pleomorphic lymphocytes,

suggesting the presence of a malignant haematological clone, the sample was referred for confirmatory testing by Immunophenotyping using multiparametric flow cytometry.

3.1.2 Immunophenotyping

A panel of monoclonal antibodies (shown in table 10 below) was used on samples and the matched control to confirm a diagnosis of CLL and to provide information to generate a CLL score. The panel of antibodies was chosen to allow assessment of disease stage and included markers to allow exclusion of other lymphoid malignancies such as Hairy cell leukaemia (CD25 and CD103), acute lymphoblastic leukaemia (CD10) and B cell lymphomas (FMC7).

Monoclonal antibody	Antigen description	Clinical utility	Expression in B-CLL
CD45	Leucocyte common antigen. 180-220 kDa antigen expressed at variable strength on all haemopoietic cells	Useful in distinguishing mature lymphoid neoplasms from acute Leukaemias and normal cell populations	Strong expression on malignant B lymphocyte clone
CD19	Pan B cell antigen. >120 kDa involved in B cell development, activation and differentiation	Indicates B cell lineage. May demonstrate abnormal intensity in B cell neoplasms	Strong expression on B lymphocyte clone
CD79b	Expressed with variable strength on mature B cells. 33-45 kDa receptor, expression varies with malignant state	Indicates B cell lineage. Intensity differs between subtypes of mature B cell neoplasms	Negative or weak expression in typical CLL
FMC7	Expressed with variable strength on mature B cells. 105 kDa B cell antigen	Strength of expression useful in distinguishing CLL from lymphoma	Negative or weak expression in typical CLL
CD3	Pan T cell antigen. 25 -28 kDa antigen involved in TCR signal transduction	Distinguishing T cell population	Negative on B lymphocyte clone
CD38	B cell prognostic marker. 45 kDa cell adhesion regulator	Poor prognostic indicator in CLL. Bright intensity staining indicates plasmacytic differentiation	Positivity on B lymphocyte clone associated with poor prognosis
CD5	Pan T cell antigen co-expressed on malignant B cells. 58 kDa receptor involved in T/B cell receptor signalling	Expressed on malignant B cells in conjunction with pan B cell markers. Useful in identifying malignant B cells	Positive on malignant B cell clone
CD10	Common acute lymphoblastic antigen. 100 kDa membrane bound peptide cleaving metallopeptidase.	Frequently present in ALL. Distinguishes acute from chronic B cell neoplasms	Negative
CD23	45 kDa B cell antigen expressed on B CLL lymphocytes	Distinguishes CD5+ B cell lymphoid neoplasms	Positive on B cell clone in typical CLL
CD103	150 kDa antigen expressed in Hairy Cell Leukaemia	Characterises Hairy Cell Leukaemia and Marginal Zone Lymphoma	Negative
CD25	55 kDa IL-2 receptor expressed on activated T and B lymphocytes.	Present in Hairy Cell Leukaemia	Negative
Anti Kappa/Anti Lambda light chains	Light chains associated with surface immunoglobulins of B cells. Strength of expression weaker on B CLL lymphocytes	Establishes light chain restriction and clonal nature of B cell neoplasms. Strength of expression useful in scoring CLL	Weak expression in typical CLL. Restricted to one light chain in CLL.

Table 10: Diagnostic monoclonal antibody panel used to confirm diagnosis of CLL and construct CLL score. ***Modified from Craig and Foon, 2008***

Following a diagnosis of CLL being made using the above criteria a number of additional investigations were routinely performed on each patient including bone marrow biopsy (where appropriate) to assess degree of infiltration, cytogenetic analysis to identify common risk factors and histology which is considered important to obtain a definitive diagnosis (Dronca *et al.* 2010). Bone marrow biopsy and histology were performed locally and analysed by the hospital histopathology department and cytogenetic analysis was performed by a regional cytogenetics centre at Bristol. These investigations did not form part of the study and were performed as part of a standard diagnostic protocol. The results of these investigations were however used to confirm a diagnosis of CLL. Additionally a direct antiglobulin test (DAG) was performed as part of the study to assess autoimmune status, this test also being one of those recommended in diagnostic guidelines (Oscier *et al.* 2012). The DAG results were recorded and analysed as part of the study.

Using the results of tests detailed above, coupled with physical findings on examination by a medical consultant, a diagnosis of either typical or atypical CLL was made for each patient and a CLL stage value assigned based on the surface antigen expression criteria summarised in table 7 (page 23). Patients with a CLL score 3-5 and co-expression of CD5 and CD19 on the B lymphocytes were included in the study, any patients who did not satisfy the criteria for a diagnosis of CLL were withdrawn from the study at this stage, and all associated sample data disregarded. All matched control subjects were also tested with this panel of monoclonal antibodies to exclude any that had an incidental diagnosis of CLL. Only control subjects with a 0

CLL score and no evidence of a CD5/CD19 co-positive B cell population were included in the study.

The distribution of CLL scores among the patient group are summarised below (full results attached as appendix i),

CLL Score	Number of patients	Classification
5	62	Typical CLL
4	59	Typical CLL
3	8	Atypical CLL

Table 11: Distribution of CLL scores amongst patient group in this study

3.1.3 Toll like receptor analysis

The TLR chosen for analysis in this study were selected to represent those commonly found on T and B lymphocytes on both cell membrane (TLR 1,2 and 4) and those expressed in the internal compartment (TLR 3 and 9). The TLR chosen for investigation was restricted to those for which a commercial antibody was readily available. The presence and importance of expression of these particular TLR on lymphocytes has been previously reported (Rožková *et al.* 2010) although on a limited number of subjects. All patient and matched control samples were stained using a panel of TLR antibodies in association with key lymphocyte and monocyte lineage markers (CD3, CD14 and CD19) which were used to identify target cell populations.

3.1.4 Flow cytometry protocol (CLL phenotype)

Samples of EDTA anticoagulated whole blood were manually aliquoted into 100 microlitre volumes and stained with a 10 microlitres mixture of appropriate directly conjugated monoclonal antibody (as detailed in table 12) and incubated for 15 minutes in the dark at room temperature. All monoclonal antibodies used in the diagnostic panel had previously been titred to find the optimal concentration for flow cytometric analysis and the volume adjusted to allow for a standard volume of 10 microlitres of each monoclonal antibody to be used in each test.

Tube number	Monoclonal antibody combination
1	CD14 (FITC) CD45 (PE)
2	CD5 (PE) CD19 (FITC), CD10 (APC)
3	CD5 (PE) CD19 FITC, CD79b (APC)
4	CD23 (PE),CD19 (FITC), FMC7 (APC)
5	CD3 (PE), CD4 (FITC), CD8 (APC)
6	CD25 (FITC),CD103 (PE)
7	CD38 (FITC), CD19 (PE)
8	Kappa (FITC), Lambda (PE), CD19 (APC)

Table 12: Diagnostic flow cytometry panel (flourochrome conjugates shown in parentheses)

Following this incubation step the red cells in each sample were lysed by the manual addition of 2ml of a lysing solution comprising of 16% Ammonium chloride per tube, the resultant solution was hand mixed and incubated for a further 15 minutes at room temperature in the dark to prevent flourochrome quenching. Samples were finally manually washed by the addition of 2 mls of a Phosphate Buffered solution of

isotonic saline (pH 7.4) to remove the lysing solution, red cell debris and any unbound antibody, the supernatant was removed following centrifugation at 320g for 45 seconds, and the remaining cells resuspended in 2 ml of BD Facsflow flow cytometry fluid (pH 7.2) prior to immediate analysis using a FACSCalibur (BD New York, USA).

For each sample, a standard 50,000 events were collected. The flow speed of the instrument was set to a low rate to avoid the false positive events that are frequently associated with high cell count samples (Bartle 2011).

Standard CD45 vs. side scatter/CD14 gating strategies were employed to identify the lymphocyte and monocyte populations in each sample in preparation for analysis, an isotype negative control was also analysed alongside each sample in line with international guidelines (Béné *et al.* 2011). For all monoclonal antibodies, both the percent positive cells and the mean fluorescent intensity were recorded to allow enumeration of cell subsets and strength of antigen expression per cell.

3.1.5 Flow cytometry protocol (TLR phenotype)

Since TLR 1, 2 and 4 monoclonal antibodies were available as pre-conjugated reagents and the target antigen in each case was expressed on the cell surface, the protocol for sample preparation was identical to that used for the CLL phenotype described in section 3.1.4. The antibody and flourochrome combinations used in this portion of the study are summarised in table 13.

Preparation of cells for the analysis of TLR 9 required an additional fixation and permeabalization stage as the target antigen is expressed intracellularly.

The Intracellular Fixation & Permeabalization technique is designed for use in intracellular staining and flow cytometric analysis and has been formulated to reduce non-specific staining of fluorochrome-labelled antibodies and increase fluorescence signal to noise ratios. In the first step live cells are fixed with a fixation Buffer, containing a 4% solution of formaldehyde which cross- links proteins. The second step uses a permeabalization Buffer which contains 0.1% saponin, which creates holes in the membrane thereby allowing the intracellular staining antibodies to enter the cell effectively.

In order to prepare cells for analysis, 100 microlitres aliquots of EDTA anticoagulated whole blood were pre-treated with Dako intrastain (Dako, Cambridge UK). 100 microlitres of intrastain solution A (containing formaldehyde) was added to the cells followed by a 15 minute incubation in the dark at room temperature. This step allowed for stabilisation of the cell membrane and was followed by the addition of 2mls of PBS (to quench the reaction), centrifugation at 320g for 5 minutes and discard of the supernatant containing the excess solution A. At this stage there followed the addition of 100 microlitres intrastain solution B (containing saponin) to the centrifuged cells, along with the appropriate monoclonal antibody mix. Subsequent incubation washing and analysis was the same in every respect to the protocol used for the analysis of the CLL phenotype described in section 3.1.4.

The protocol for the preparation of cells for the analysis of TLR 3 was similar to that used for TLR 9 but with the inclusion of an additional flourochrome conjugation stage, as anti TLR 3 was only available as an unconjugated monoclonal antibody. Following incubation with solution A, in addition to the anti TLR 3 monoclonal, 10

microlitres of anti-mouse FITC flourochrome (R &D systems Minneapolis USA), was included. Subsequent preparation was the same as that used in the TLR 9 preparation protocol described above.

3.1.6 Detailed protocols for preparation of TLR panel

Protocol 1: Preparation for TLR 1, 2 & 4 analysis

For each TLR, 3 tubes were prepared for each patient and control, into each was placed 100 microlitres of fresh whole blood collected in EDTA.
10 microlitres of the appropriate pre-conjugated anti TLR monoclonal antibody was added to the each tube and mixed well by gentle vortexing.

To the first tube 10 microlitres of pre-conjugated CD3 was added.
To the second tube 10 microlitres of pre-conjugated CD19 was added.

In each case the flourochrome conjugate of the second antibody added was different to that on the anti-TLR antibody.

Samples were incubated for 15 minutes in the dark at room temperature.
After this incubation step 2ml of a lysing solution comprising of 16% Ammonium chloride was added to each tube and the mixture was incubated for a further 15 minutes at room temperature in the dark.

Following this preparation the samples were analysed using a multiparametric fluorescent activated cytometer.

Protocol 2: Preparation for TLR 9 analysis

2 tubes were prepared for each patient and control, into each was placed 100 microlitres of fresh whole blood collected into EDTA. To this blood 100 microlitres of DAKO intrastain fixation solution A (DAKO, Ely, UK) was added and the sample left to incubate in the dark at room temperature for 15 minutes. The cells were then washed twice using 2ml of isotonic Phosphate Buffered Saline (PBS pH 7.2) and centrifuged at 320g for 5 minutes.

Following this fixation step, 10 microlitres of pre-conjugated anti TLR 9 monoclonal antibody and 100 microlitres of intrastain permeabalization solution B (DAKO) were added.

At this point to the first tube 10 microlitres of pre-conjugated CD3 was added.
To the second tube 10 microlitres of pre-conjugated CD19 was added.

In each case the flourochrome of the second antibody added was different to that on the anti TLR antibody.

The solutions were then incubated for a further 15 minutes in the dark at room temperature. Cells were then washed twice in 2 ml PBS pH 7.2 and centrifuged at 320g for 5 minutes, following which they were resuspended in 1ml PBS pH 7.2. Following this preparation the samples were analysed using a multiparametric fluorescent activated cytometer.

Protocol 3: Preparation for TLR 3 analysis

2 tubes were prepared for each patient and control, into each was placed 100 microlitres of fresh whole blood collected into EDTA. To this blood 100 microlitres of intrastain fixation solution A (DAKO) was added and the sample left to incubate in the dark at room temperature for 15 minutes. The cells were then washed twice using 2ml of Phosphate Buffered Saline (PBS) and centrifuged at 320g for 5 minutes.

Following this fixation step, 10 microlitres of unconjugated anti TLR 3 monoclonal antibody, 10 microlitres of anti-goat FITC flourochrome (R&D Systems, Oxford UK) and 100 microlitres of intrastain permeabalization solution B (DAKO) were added.

At this point to the first tube 10 microlitres of pre-conjugated CD3 was added.
To the second tube 10 microlitres of pre-conjugated CD19 was added.

In each case the flourochrome of the second antibody added was different to that on the anti TLR antibody.
The solutions were then incubated for a further 15 minutes in the dark at room temperature. Cells were then washed twice in 2 ml PBS pH 7.2 and centrifuged at 320g for 5 minutes, following which they were resuspended in 1ml PBS pH 7.2.
Following this preparation the samples were analysed using a multiparametric fluorescent activated cytometer.

Thus for each patient and matched control a total of twenty tubes were prepared and analysed using a multiparametric fluorescent activated cytometer.

Tube number	TLR monoclonal antibody	T/B lymphocyte monoclonal antibody
1	TLR1 (PE)	CD3 (FITC)
2	TLR 2 (PE)	CD3 (FITC)
3	TLR 3 (FITC)	CD3 (PE)
4	TLR 4 (FITC)	CD3 (PE)
5	TLR 9 (PE)	CD3 (FITC)
6	TLR1 (PE)	CD19 (FITC)
7	TLR 2 (PE)	CD19 (FITC)
8	TLR3 (FITC)	CD19 (PE)
9	TLR 4 (FITC)	CD19 (PE)
10	TLR 9 (PE)	CD19 (FITC)

Table 13: TLR flow cytometry panel (flourochrome conjugates shown in parentheses).

For each sample and matched control blood, the following data was recorded

i) % positivity for each TLR

ii) % co-positivity with T lymphocytes (expressing CD3) for each TLR

iii) % co-positivity with B lymphocytes (expressing CD19) for each TLR

iv) % co-positivity with monocytes for each TLR (using CD14 gated population data from CLL Immunophenotyping protocol)

3.1.7 Choice of flourochromes

A range of fluorescent molecules are available to label monoclonal antibodies and the choice of flourochrome for these experiments was made with due regard to standardisation and performance. The 2 flourochromes used were Fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE). FITC has the major advantage that it has a high extinction coefficient and quantum efficiency and an absorption maximum very close to the emission lines from a 488 nanometre Argon-ion laser, such as the main laser fitted to the FACSCalibur. The emission line from activated FITC is around 520nm in the green spectrum. FITC is also widely available linked to a variety of monoclonal antibodies and other probes. For the second label, PE was the obvious choice as it too is excited at 488 nm, meaning that only one laser is required for analysing the cell populations. The emission line for PE is at 576nm in the orange spectrum, meaning that it can be easily distinguished from both the laser source and the green fluorescence of FITC.

Instrument standardisation was performed prior to analysis of each batch of samples by running commercial calibration beads (Calibrite beads, Becton Dickinson, New York) with known ranges of size, granularity and fluorescence characteristics for each flourochrome used in the experiment. The standardisation procedure was carried out to confirm that the instrument was operating under standard parameters and that it met minimum performance requirements with respect to optics (including light-scatter and

fluorescent detectors) and electronics, as well as expected fluorescence staining patterns.

3.2 Gating strategy

As with the CLL flow cytometry protocol described earlier, samples were gated using a forward scatter (FSC) vs. side scatter (SSC) strategy to identify the monocyte and lymphocyte populations as appropriate. When evaluating cells in this way the Forward scatter axis provides information about the size of the cell, the degree of scatter being directly proportional to the size of the cell. The Side scatter axis provides information about the degree of complexity of the inner aspect of the cell such as the shape of the nucleus or the presence of cytoplasmic granules. This information is used to identify cell populations of interest and electronically 'gate' the cell population pending further investigation. Lymphocytes typically have low forward light scatter and low side scatter whereas monocytes form a discreet population on the scattergram due to the fact that they have a larger forward light and side scatter characteristic. These characteristics are summarised in figure 2:

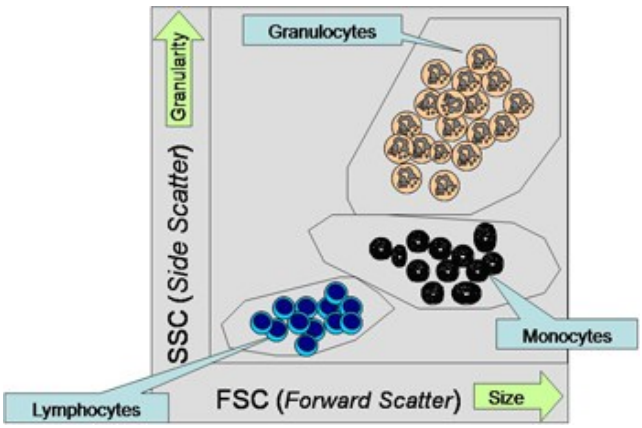


Figure 4: Cell population discrimination using physical parameters

The lymphocyte and monocyte populations identified by this strategy were checked for purity by backgating to the CD45 vs. CD14 tube used in the CLL Immunophenotyping panel.

3.3 TLR expression Data analysis

For each sample analysed, 3 separate graphs were generated in order to enumerate the TLR expression on the different cell populations of interest. The TLR expression on the monocytes was calculated by separating the monocyte population of the FSC vs. SSC dot plot by addition of on electronic gate. This gated region (R1) was then analysed and a histogram generated that showed positivity for the particular TLR. A second electronic gate was applied to the positive population on this histogram to allow accurate measurement of the positive population and assessment of the intensity of fluorescence.

TLR expression on the lymphocyte sub-populations involved firstly applying an electronic gate to the entire lymphocyte population and analysing this data on a 2 way dot-plot, plotting the TLR activity on one axis and the CD3 or CD19 activity, as appropriate, on the other axis. In this way co-positivity for TLR expression on the appropriate lymphocyte population could be accurately measured.

The process of identifying, gating and analysing the cell population of interest is demonstrated best via specific examples. Figure 5a shows an example of the dot plot obtained by plotting the side scatter characteristics of the leucocytes against the forward scatter attributes. In this example the lymphocyte population has been identified by the physical characteristics of their small size and lack of complexity of internal architecture, relative to the other leucocytes present in the sample. An electronic gate (labelled as R1) has been applied to this population and all subsequent analysis would be carried out exclusively on this population of cells. Due to the fact that the monocyte population can display similar physical characteristics to larger and more granular lymphocytes, the target population was analysed to ensure that the cells contained within this gate did not express any CD14 activity, CD14 is a marker specific for monocytes and aids in the gating process as it gives confidence that no monocytes were included in the gated population.

Having successfully isolated the lymphocyte population, the next stage was to identify the T and B lymphocyte populations in order to assess TLR expression on both separately. In order to achieve this, each TLR was analysed in conjunction with CD3 (a pan T cell marker) and CD19 (a pan B cell marker). Figure 5b shows an example of a dot plot that shows CD3 expression against TLR 4. In this example, the dot plot is divided into quadrants that indicate positivity or co-expression for each marker. In this way it is possible to identify and electronically gate, cells that are co-positive for CD3, indicating that they are T cells, and also positive for the TLR being investigated.

The electronically gated region can now be analysed for TLR expression via a histogram plot which will yield information regarding the mean (or median)

fluorescent intensity as shown in figure 5c. In this example the TLR being studied (TLR3) is being expressed at a relatively low MFI compared with the isotype negative control (shown as an overlay on the histogram).

The same process of identification, isolation and analysis is applied to the study of the B cell population. Figure 5d shows the identification of the B cell population (following separation of the total lymphocyte population as described above). In this example the B cells are identified due to the expression of CD19 on their cell surface. The dot plot shows this expression again that of TLR2, an electronic gate was then placed around the CD19/TLR2 co-expressing cell population.

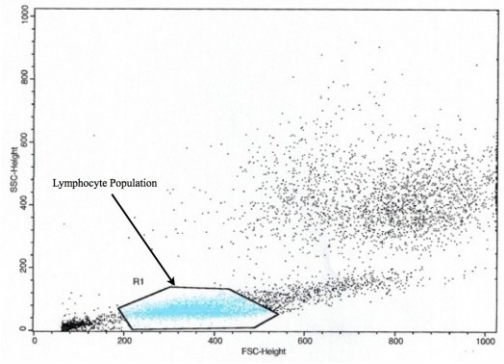
The final stage in the process is to display level of TLR expression on the gated B cell population via a histogram as shown in figure 5e.

It is useful to note the stronger expression levels of TLR in this example, reflected in a high MFI, when compared with those obtained in figure 5c.

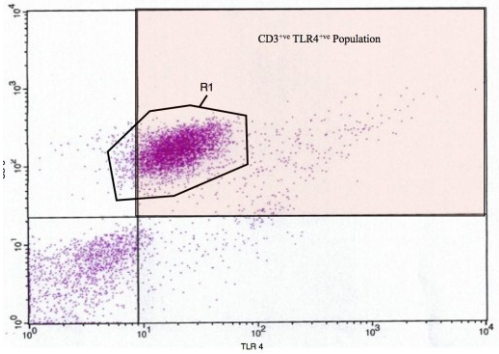
Just as it is important to exclude any monocyte data from the lymphocyte gate, it is equally important to ensure that the monocyte population is identified and isolated efficiently. This was achieved in a manner similar to that used for the lymphocytes, in that the leucocyte population for each sample was analysed using forward and size scatter characteristics. Using this physical information a gate was placed around those cells that showed characteristics consistent with monocytes, this approximates to a population of cells that are slightly larger than the lymphocytes but with a similarly low granularity. An example of this gating is shown in figure 5f.

In this example the monocyte population is gated as region 1. The next stage is to check that this population represents the monocyte population exclusively; this is achieved by analysing the expression of the pan-monocyte marker CD14 on these cells surface. This is best achieved by plotting the CD14 expression of these cells against the pan leucocyte marker CD45 since leucocyte populations show differential expression of CD45. The resultant dot plot clearly identifies the strongly CD14 positive monocyte population, allowing a second gate (R2) to be applied which uniquely isolates the monocyte population as shown in figure 5g.

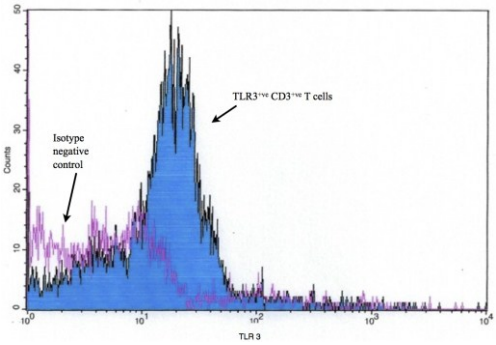
This pure monocyte population can then be analysed to show individual TLR expression on a histogram, as shown in figure 5h which illustrates TLR9 expression on the monocyte population isolated by the above method.



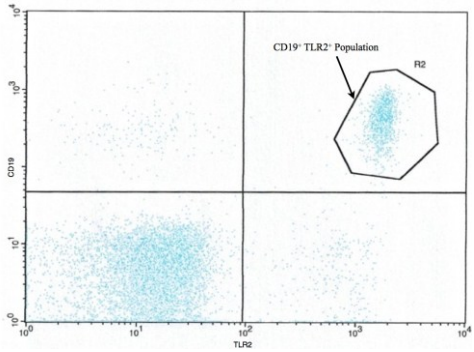
a: Gated lymphocyte population (R1)



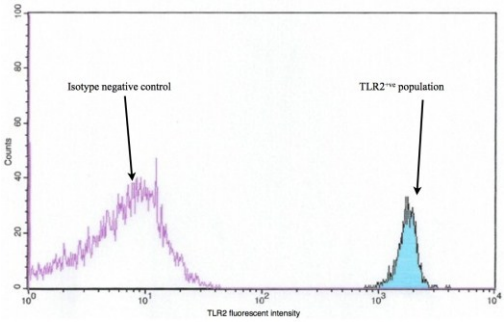
b: CD3/TLR4 co-positive population



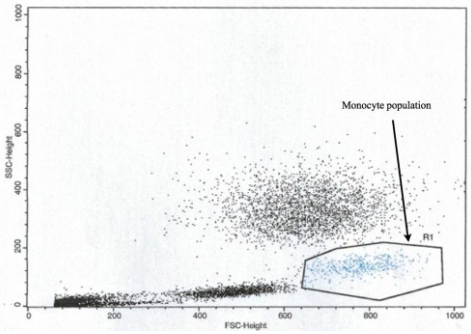
c: TLR3 fluorescence intensity



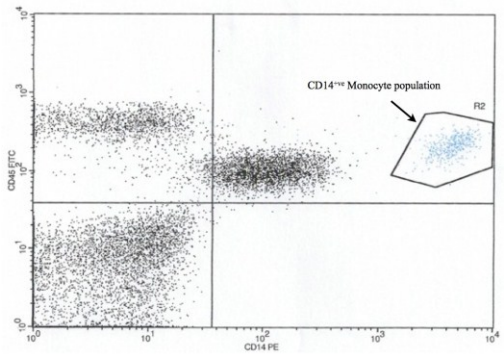
d: CD19/TLR2 co-positive population



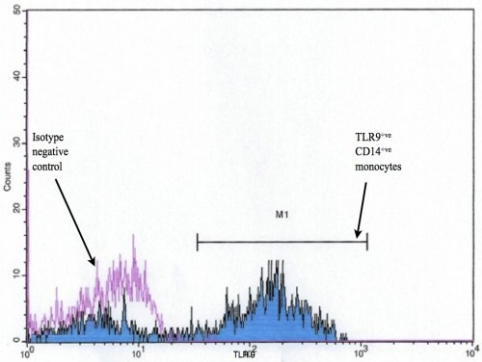
e: TLR2 fluorescence intensity



f: Gated monocyte population (R1)



g: Dot plot showing CD14 population (R2)



h: CD14/TLR9 co-positive population

Figure 5a-h: Representative dot plots, histograms and gating strategies used to identify target population for TLR quantification

3.4 Preparation and analysis of time delay study samples

In order to determine the effect of sample storage prior to staining and analysis, samples of EDTA anticoagulated whole blood from 5 patients and matched controls were stored after the initial staining and analysis. Initial staining and analysis was carried out as soon as possible after the patient was bled, this sample analysis was recorded as having taken place at time 0. A fresh aliquot was removed from the blood collection tube at 4h, 8h, 12h and 24h post collection. Upon removal each aliquot was stained and analysed immediately. Samples were stored at room temperature in the dark between testing and in each case the preparation and testing protocols were identical to those described for all other patients and controls.

3.5 Direct antiglobulin test (DAGT)

The direct antiglobulin test is used to test for the presence of IgG autoantibodies that can specifically bind to antigen on red blood cells. In the presence of such autoantibodies the patients RBC's become coated with IgG autoantibodies with subsequent binding of compliment proteins which leads to RBC destruction. The DAGT is used to detect the presence of IgG autoantibodies bound to the RBC surface. Washed RBC's are incubated with anti-human globulin, if Immunoglobulin or compliment factors have been fixed onto the RBC surface *in-vivo* then the anti-human globulin will agglutinate the RBC's by binding onto the RBC surface and bridging the gap between adjacent cells causing them to clump together, this agglutination is macroscopically visible.

A 100 microlitre aliquot of well mixed EDTA anticoagulated whole blood was added to a sterile 15ml glass test tube (Elkay, Coventry UK) containing 2 mls of PBS (pH

7.6). This mixture was capped and thoroughly mixed by gentle inversion. The sample was then centrifuged at 320g for 60 seconds using a Centra CE4 benchtop centrifuge (Centra corp, Baltimore USA). Following centrifugation the supernatant was aspirated and the cell pellet resuspended by gentle mechanical disruption. 1 ml of PBS ($_{P}$H 7.6) was added to the cells to produce an approximately 3% cell suspension. From this cell suspension a 100 microlitre aliquot was pipetted into a sterile 15ml glass tube and one drop of anti-human globulin solution (NBS reagents, Bristol UK) was added. This mixture was gently agitated by hand for 10 seconds to ensure thorough mixing of cells and reagent. The sample was then centrifuged at 320g for 15 seconds to produce a loose pellet of cells. This pellet was dispersed by gentle agitation of the tube, following which the contents were visually inspected to assess the degree of red cell agglutination as shown in figure 6. Samples which exhibited agglutination of the RBC'S as seen by macroscopic examination were recorded as having a positive DAG.

Figure 6: Positive and negative agglutination in a DAG test

3.6 Statistical analysis of TLR data

In order to compare the expression of TLR on CLL patients and matched controls, statistical analysis was carried out, however in order to apply the appropriate statistical testing the data was assessed for parametric/non-parametric distribution using Levene's test.

The two sets of data were firstly analysed using the t-test to compare the mean expression of TLR in the two groups. This test is however only accurate if it can be assumed that the data is both continuous and parametric (i.e., normally distributed). Whilst it was reasonable to assume that the data was indeed continuous, simple inspection of a bar chart of the TLR distribution results showed that in some instances that data was non-parametric. In order to quantify this phenomenon a statistical test of the parametric nature of the data needed to be applied.

3.6.1 Student t-test

This test compares the TLR expression in the two groups in order to check if there is a significant difference in the mean values. In statistical terms the test checks the probability that the two sets of data could have come from the same population

In order to achieve a check of the homogeneity of the variance, Levene's test was applied to the data as part of the t-test. This normality check looks at both the skewness and kurtosis of the data in order to check if the data is normally distributed and produces a numerical value which indicates the degree of departure from normality and from which a decision can be made as to the appropriateness of using the t-test.

An important part of Levene's testing is the facility to produce bar chart histograms; these have been used to display graphically the levels of TLR expression against frequency with which that level is expressed within the group. These graphs provide

an excellent visual check for skewness and kurtosis and can be used to confirm the validity of employing the t-test. As an example, the histograms for TLR 2 expression on B cells are shown in figure 7 below. In this instance the histogram was deemed by the SPSS software to show normal distribution, and a simple visual appreciation of the chart confirms this. Conversely the data for TLR 3 were deemed by the SPSS software to not meet normality distribution criteria and as such the t-test was not applied to this set. Inspection of the histogram chart shown in figure 5 below confirms that the data is indeed heavily skewed and therefore not normally distributed.

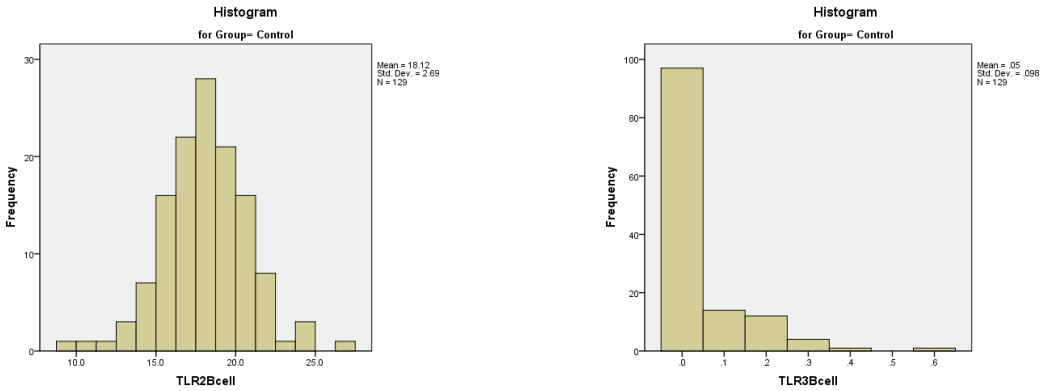


Figure 7: Normal parametric distribution of TLR 2 expression on B cells in control samples vs. non-parametric distribution of TLR 3 on B cells in control samples (n=129). TLR expression measured using flow cytometry on PBMC's co-stained with CD19.

3.6.2 Mann-Whitney U test

Where the Levene's test shows a violation of the assumption of homogeneity and variance, meaning that the t-test was not reliable, an alternative test (Mann-Whitney U test) was applied. This technique is used to test for differences between the two groups on a continuous measure, but instead of comparing the means of the two groups, as in the t-test, the Mann-Whitney U test compares medians. Levels of TLR

expression are converted to ranks across the two groups of data and the test evaluates whether the ranks for the two groups differ significantly. Since the expression levels are converted to ranks, the actual distribution of the levels does not matter. This test produces two important figures, the P value which determines the *significance* of the difference between the two groups and the Z value which is a *measure* of the difference between the two groups. The P value can be interpreted to relate to different degrees of significance, ranging from significant, through highly significant to very highly significant. The Z value increases proportionally to the difference between the two groups, hence a larger Z value is more likely to equate to a greater significant difference.

3.6.3 The *p* value

The *p* value generated as part of both the Student and Mann-Whitney tests in this study is used in the context of null hypothesis testing in order to quantify the statistical significance of the data. In this setting the null hypothesis has been constructed to assume that there is no difference in TLR expression between patient and age/sex matched control groups or between DAG positive and DAG negative patients. In essence, the null hypothesis is assumed true until statistical evidence proves otherwise, the *p* value giving a measure of the significance of differences between the two groups being compared. The smaller the *p* value, the larger the significance of the differences between the 2 groups will be as *p* value decreases proportionate to the likelihood that the null hypothesis is incorrect (Pezzullo 2013)

For the purposes of this study standard significance levels were adopted, the significance level being set at 5%, thus *p* values were set as follows:

$p > 0.05$ = Not significant (i.e. no presumption against null hypothesis)

$p \leq 0.05$ = Significant

$p \leq 0.01$ = Highly significant

$p \leq 0.001$ = Very highly significant

4 Technique development

4.1 Study design

A prospective observational cohort study of 129 patients with newly diagnosed CLL were analysed for expression of TLR 1, 2, 3, 4 and 9 using flow cytometry. Expression levels of TLR on three cell types (T lymphocytes, B lymphocytes and monocytes) were compared with age and sex matched controls. TLR expression levels on the different cell populations were also compared between those patients who exhibited signs of autoimmunity and those that did not.

4.2 Ethics and governance

Research and audit within the National Health Service is covered by the provisions of the National Information Governance Board (NIGB), which all laboratory staff are required to adhere to as a requirement of ongoing state registration. This legislation arose from section 251 of the NHS act 2006 and was originally enacted under section 60 of the health and social care act 2001 (NIGB 2012).

Care was taken in the study design to ensure that all patient and control identifiable information was link anonomised with the data key being held by an impartial third party individual. Patient care was in no way compromised and all tests were performed on residual clinical material destined for discard. The data collection, confidentiality and consent issued were specifically addressed in the governance process.

Approval was sought and granted from the Local Research Ethics Committee (REC reference 71.6.06) and from the University ethics committee. Approval was also sought and granted from the Royal Cornwall Hospital (RCH) Research and

Development directorate (R and D reference SPE.HAE.05). Throughout the study 6 monthly submissions were made to the National Research Register and annually to the Department of Health via the hospital annual research report. As a requirement of R and D approval, Good Clinical Practice (GCP) training was undertaken to ensure a good understanding of research governance issues and to comply with the Department of Health research governance framework for health and social care (2005). All paperwork pertaining to ethical and governance approval is attached as appendix ii

4.3 Reagent and technique development

Multiparametric flow cytometry is the most commonly used technique for diagnosis and monitoring of haematological malignancies, in particular the leukaemias (Béné *et al.* 2011; Peters and Ansari 2011) and was therefore the technique of choice. One of the primary considerations of the study was to develop a protocol that could be adopted using available instrumentation and commercially available reagents. At the start of the study the only anti-TLR antibodies available on the open market were those directed against TLR 1,2,3,4 and 9. It was recognised that analysis of these markers represented a restricted panel of the known TLR but since there was little published data at the time on their expression in CLL it was felt that it was still worthwhile investigating their distribution in the disease and control groups.

Although well established protocols were in use for the CLL diagnostic flow cytometry panel, the TLR analysis protocols were developed for exclusive use in this study. Since two of the TLR being investigated (TLR3 and TLR9) were expressed intracellularly, a fixation and permeabalization stage was added to the analysis protocol. Additionally, anti TLR3 antibody was only commercially available

unconjugated which necessitated the development of an indirect staining protocol to allow the addition of a flourochrome conjugate.

Analysis of a preliminary sample group (n=10) demonstrated broad heterogeneity of TLR expression, therefore co-staining with additional monoclonal antibodies to identify the monocyte and lymphocyte sub-populations was introduced in order to examine the differential TLR expression on these cell types. The value of this step is confirmed by works reporting a central role for the T cell in B cell malignancies (Scrivener *et al.* 2002).

The effect of sample storage on TLR expression was also investigated to establish if it were possible to collect and store samples to enable batch analysis. To assess the impact of storage prior to staining and analysis, EDTA anticoagulated whole blood samples were stored at room temperature in the dark following initial analysis and removed for retesting at 4, 8, 12 and 24 hours post collection.

4.4 Time delayed analysis results

The level of B cell expression of TLR1 was measured at 5 different time intervals on 5 patients and their matched controls and the difference in expression recorded.
The data from the time delay analysis studies are attached as appendix iii and summarised in figure 8 below.

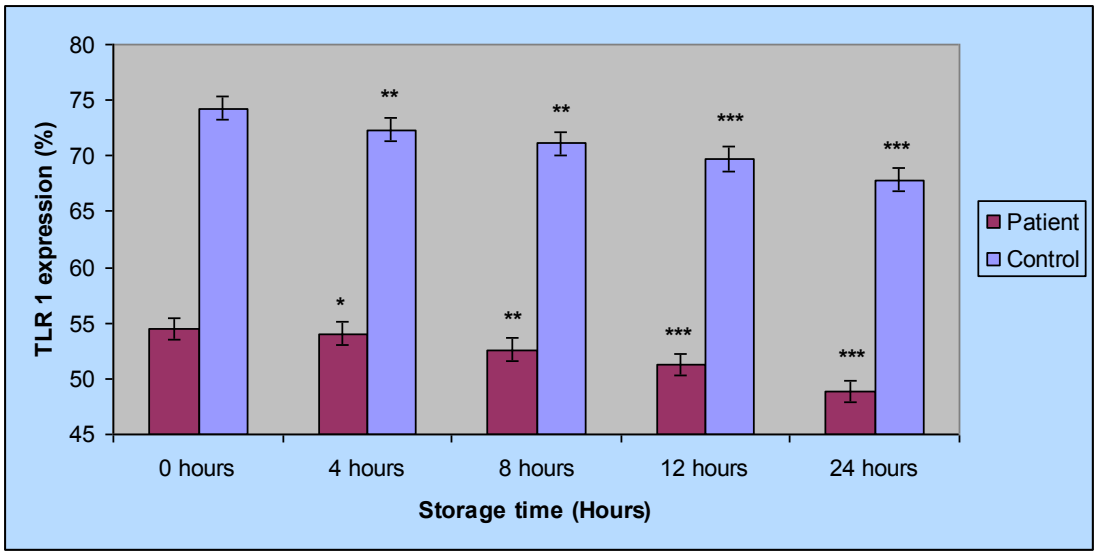


Figure 8: Effect of storage on B cell TLR 1 expression in patient and age/sex matched controls. Data represents mean TLR1 expression (n=5 patients, n=5 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells. Error bar represent standard error of mean. T-test results show significance of loss of expression relative to time 0:
* p <0.05, ** p <0.01, *** p <0.000

4.5 Summary of time delayed analysis results

In both the patient and control groups there was a significant change in the level of TLR expression over the 24 hour period. In all but one instance this change manifested as a fall in expression levels.

Statistical analysis of the significance of the change in TLR expression relative to time 0 was carried out using the Student t test, the results of this analysis are included in figure 8 above. Examination of this data revealed that there was a significant loss of TLR activity after 4 hours storage in the patient group, the loss of activity in the control group was more pronounced and was statistically highly significant within the same time period, the effect being more noticeable owing to the higher levels of TLR1 expression in the control group. After 8 hours storage the loss of TLR activity was more pronounced again and statistically highly significant in both patient and

control groups. Loss of TLR activity continued over the remainder of the time-delay study and after 12 hours of storage the loss of TLR activity relative to time 0 was statistically very highly significant for both patient and control groups. It was therefore concluded that there was significant loss of TLR activity on both patient and control groups after relatively short periods of storage.

This being the case, all subsequent analysis was carried out immediately on receipt of the sample (time 0) in order to standardise the measurement of expression levels. The time delay data beyond time 0 for patients 1-5 and their matched controls was not included in the study, only the time 0 data was used.

4.6 Flow cytometry

Since the analysis of TLR was a novel application for the instrument being used, standardised procedures were used in order to ensure robust and reproducible data. Accordingly, instrument standardisation was performed each day to confirm that the flow cytometer was operating under standard parameters and that it met minimum performance requirements. This standardisation included checks on the optics (including light-scatter detectors and fluorescence detectors) as well as the electronics (including the photomultiplier tube voltages and spectral compensation) as well as expected staining patterns of the monoclonal antibody reagents being used. The flow cytometer was aligned and calibrated using a commercial calibration check bead solution (BD Facscomp) at the start of each day.

To ensure all required parameters on the target cell population would be detected and recorded, the detectors recording physical data (i.e. cell size and granularity) and fluorescence activity were checked daily. In order to achieve this detector, voltages were set using unstained lysed blood samples collected and stored in identical conditions to the test bloods. Gates and gain settings were set using single stained controls for each flourochrome. In order to establish if samples obtained from the patient group and those from the control group required different set-up characteristics, unstained cell samples were prepared from the preliminary sample group (n=10) and their matched controls. From this it was established that while absolute cell numbers differed between patient and control groups, the physical characteristics were essentially similar. This is not an unexpected finding since B-CLL is essentially a disease of morphologically mature lymphocytes (Cheson *et al.* 1996, Bain 2010).

Subsequent to this initial set up, the instrument was checked on a daily basis by running a commercial standard calibration material (BD Facscomp) which allowed for adjustment of minor changes in electronic settings. The adjustments made as a result of running this standard were typically very minor and were achieved by altering the voltage applied to the photomultiplier tubes which detect fluorescence on the target cell.

Isotype controls were also performed to establish background levels of non-specific staining for each isotype and antibody flourochrome and to define the position of negatively staining cells by setting cut off points for the fluorescence markers. Mouse Immunoglobulin IgG1 and IgG2 conjugated with the flourochromes Fluorescein

isothiocyanate (FITC), Phycoerythrin (PE) and Allophycocyanin (APC) as appropriate were used.

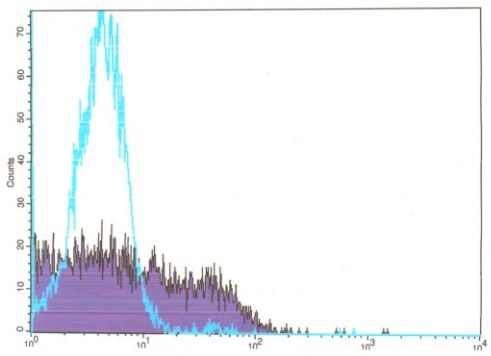
No data on TLR antigen distribution on monocytes or lymphocytes was readily available at the start of the study (in 2006). Accordingly a series of test protocols were run to match antigen to appropriate flourochrome. Antibody staining combinations were optimised so that the least abundant antigen in each sample was paired with the brightest staining flourochrome. The effect of the fixation and permeabalization stage (necessary for TLR 3 and TLR9 analysis), on prior staining of surface antigens CD3 and CD19 was also assessed, different flourochromes being used to establish optimal pairings.

Experimentation with both patient and control samples established what was believed to be optimal antibody/flurochrome pairings for both intracellular and extracellular TLR antigen identification and quantification. Antigen and flourochrome combinations are summarised in table 12 of the methods section (page 45). As there was very limited published information on analysis of TLR by flow cytometry, TLR expression was assessed using single anti-TLR antibody in each tube to simplify the process.

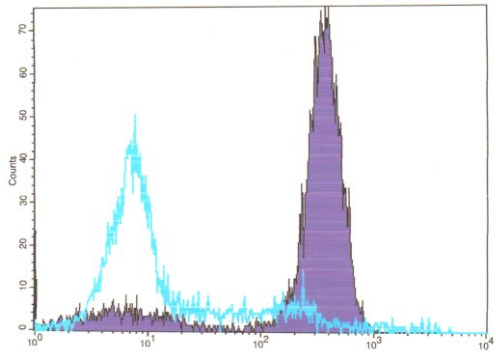
4.7 Antibody titration

Titration of all anti TLR monoclonal antibody reagents was undertaken to allow for optimal separation between positive and negative data using the minimum of reagent. The lymphocyte population was chosen as the target cell population for titration studies as it could be easily identified using physical data (FSC vs. SSC) and there

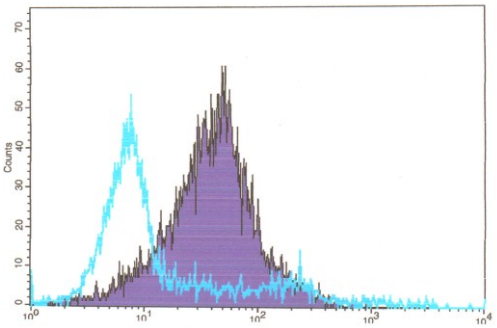
was good evidence to suggest that lymphocytes would express TLR antigen at a reasonably high level (Rožková *et al.* 2010). As no recommended antibody concentration was provided with the TLR reagents, a high concentration was chosen as the start point (10µg of antibody added to 100 microlitres of whole blood adjusted to contain approximately 500,000 cells) and dilutions made from this point. Threefold serial dilutions were made from the starting point of 10µg down to 0.005µg of antibody and these were added to cell count adjusted whole blood samples from the control group. Each sample was then analysed using flow cytometry and the mean geometric staining intensity calculated for each dilution, these were then compared and the optimal dilution ascertained by choosing the one that exhibited best separation between positive and negative populations. This process is illustrated in figure 9 which summarises the titration process undertaken to ascertain optimal dilution of TLR1. During the course of the study it became necessary to use a number of different batches of each reagent and on each occasion a new batch was started the optimal antibody titre was calculated, although in these instances a dilution approximating to that optimal for the previous batch was chosen as the starting point to prevent unnecessary reagent wastage.



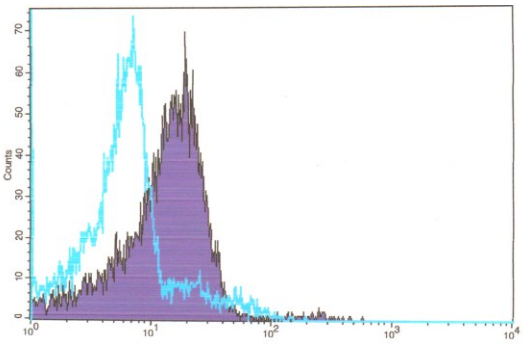
a)



b)



c)



d)

Figures 9a-9d: Histograms to demonstrate effect of serial dilution of TLR1 antibody used to obtain optimal staining of target antigen. TLR1 expression shown as solid purple histogram and isotype control as open blue histogram. TLR1 monoclonal antibody was titrated as follows: a)100µl, b)10µl, c 5µl, d)1µl

5 Results

5.1 CLL diagnosis

Representative flow cytometry data from a patient with typical B-CLL (with a CLL score of 5), a patient with non-typical B-CLL (with a CLL score of 3) and a patient with Non-CLL (with a CLL score of 0) is shown in figures 10-12 below. Only patients with a confirmed diagnosis of B-CLL, with a CLL score of 3-5 were included in the study.

Over the course of the study 312 potential subjects with suspected CLL were screened for inclusion, of these 183 were rejected from the study as they did not meet inclusion criteria. The main reasons for rejection included: diagnosis of CLL not made, insufficient sample retrieved for analysis and inability to find a matched control in a timely fashion.

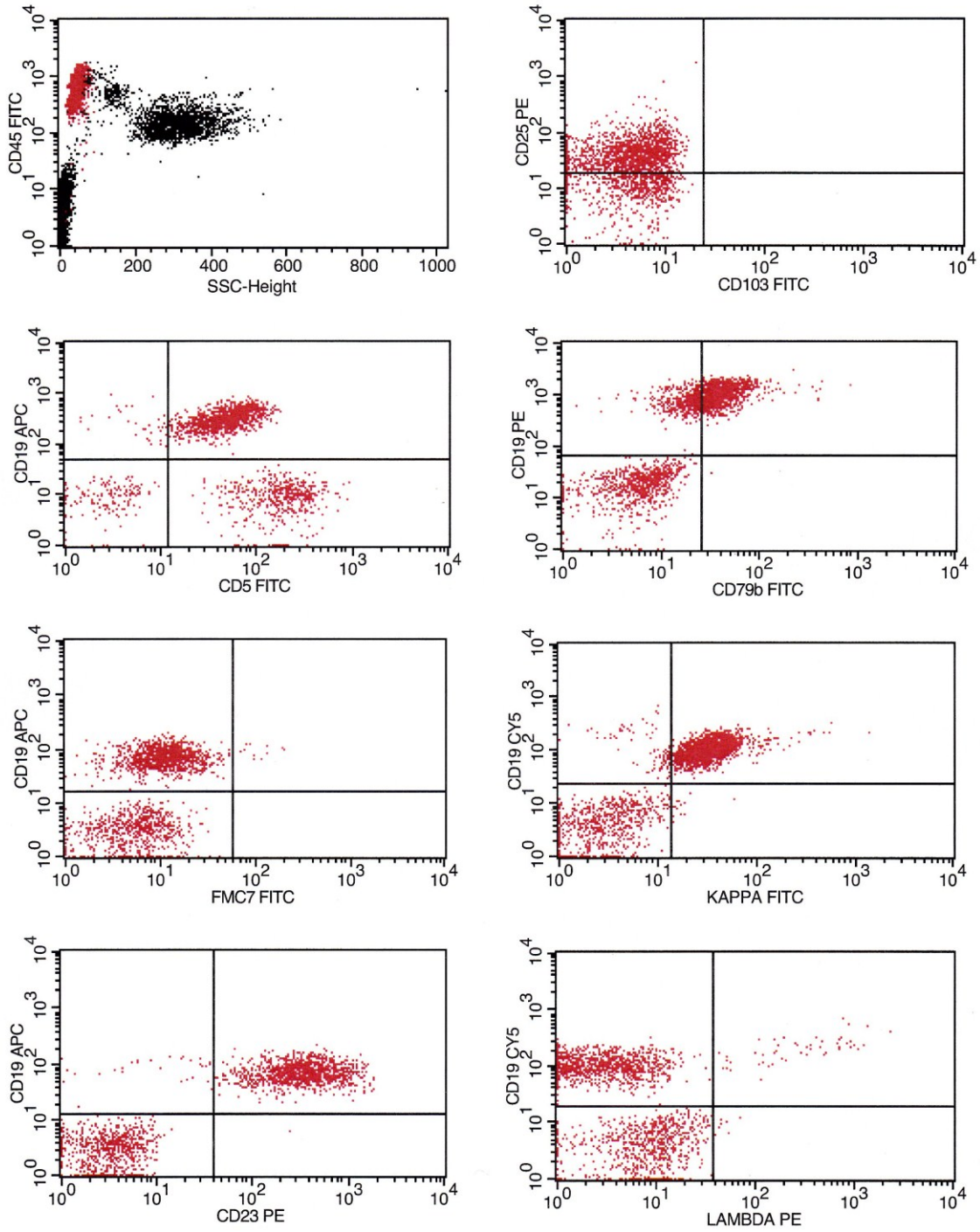


Figure 10. Representative flow cytometry data showing typical CLL phenotype, with CLL score of 5 based on CD5/19 co-positivity, CD23 positivity, weak CD79b expression, negative FMC7 expression and kappa light chain restriction with weak expression. Dot plots constructed from flow cytometric analysis of peripheral blood following preparation and staining with a standard panel of monoclonal antibodies.

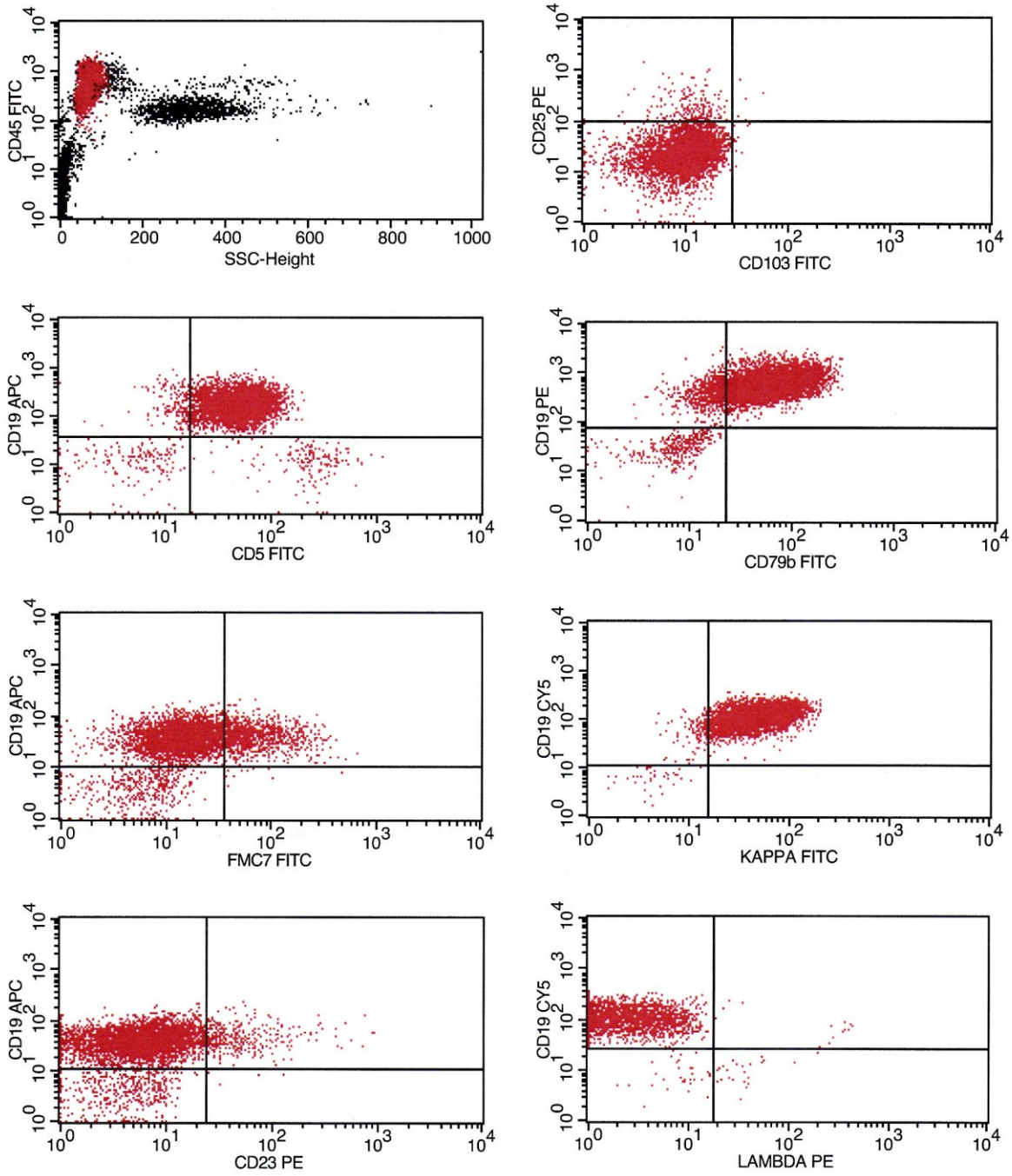


Figure 11. Representative flow cytometry data showing atypical CLL phenotype, with CLL score of 3 based on CD5/19 co-positivity, weak FMC7 expression and kappa light chain restriction with weak expression. Dot plots constructed from flow cytometric analysis of peripheral blood following preparation and staining with a standard panel of monoclonal antibodies.

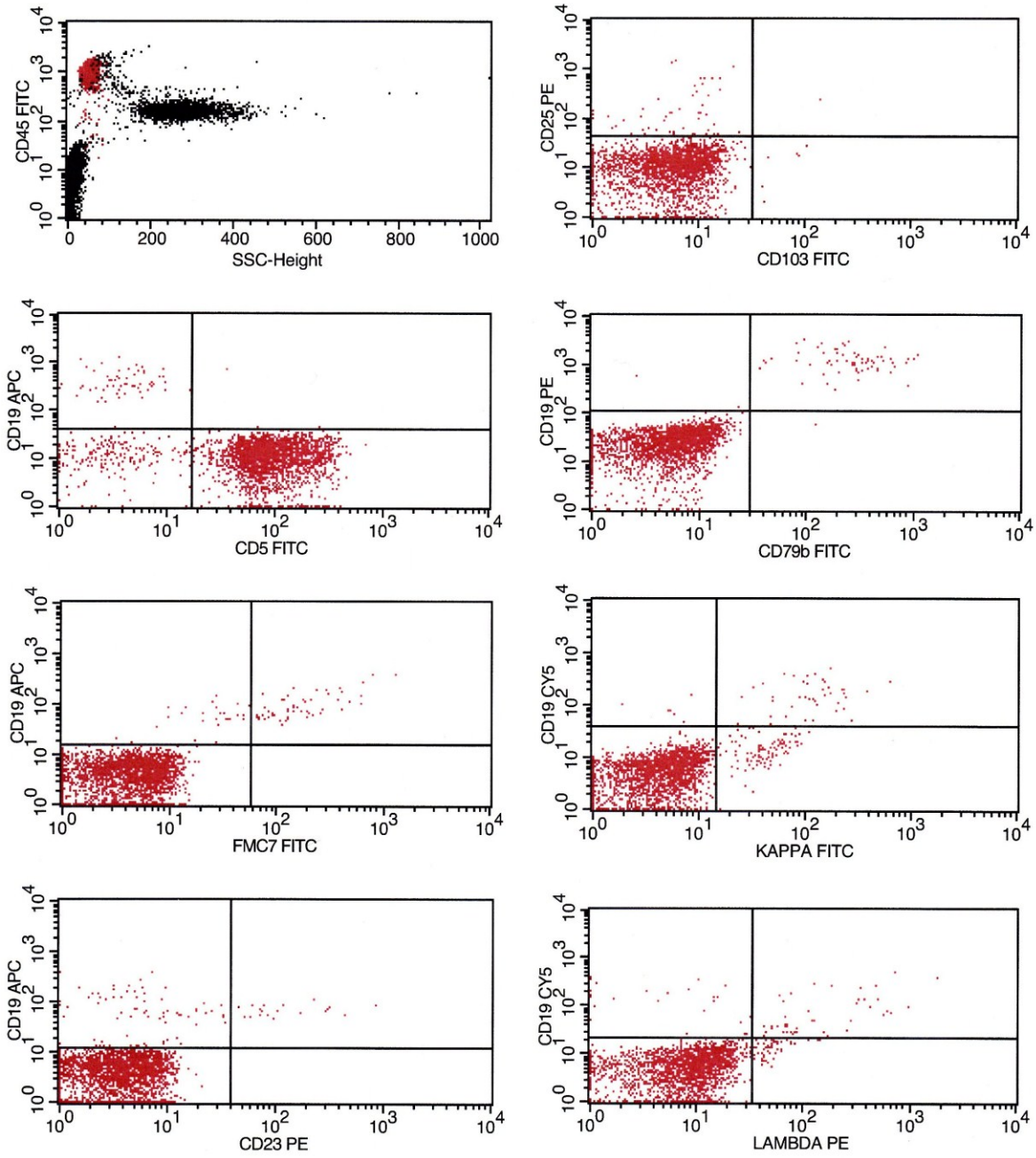


Figure 12. Representative flow cytometry data showing non-CLL phenotype, with CLL score of 0. Dot plots constructed from flow cytometric analysis of peripheral blood following preparation and staining with a standard panel of monoclonal antibodies.

5.2 TLR expression

5.2.1 TLR expression results figures

Patient demographics, diagnostic flow cytometric data, CLL score, DAG status and TLR expression levels on B lymphocytes, T lymphocytes and monocytes for both the patient and control groups are listed in appendix i.

In all instances the percentage positivity for each TLR is quoted, this figure was derived as an assessment of the number of cells that showed a significant change in geometric median fluorescent intensity (GMFI) between the TLR and the Isotype control. This method of recording and displaying results was decided upon as it corresponded to recent international guidelines on the publishing of flow cytometry data (Alvarez *et al.* 2010).

Figures 13-15 below summarise in the form of bar charts, the mean expression for each of the TLR measured in both groups, this information is displayed graphically to allow visual comparison of expression in the two groups, the standard error for both patient and control groups is shown as a Y error bar, the p values are also shown.

Figures 16-18 display the same data in the form of box and whisker plots. These plots show a better representation of the spread of the degree of TLR expression.

A more detailed breakdown of the results is given in table 14 which also lists the standard deviation, median levels and 95% confidence intervals calculated from the mean for each TLR in the two groups.

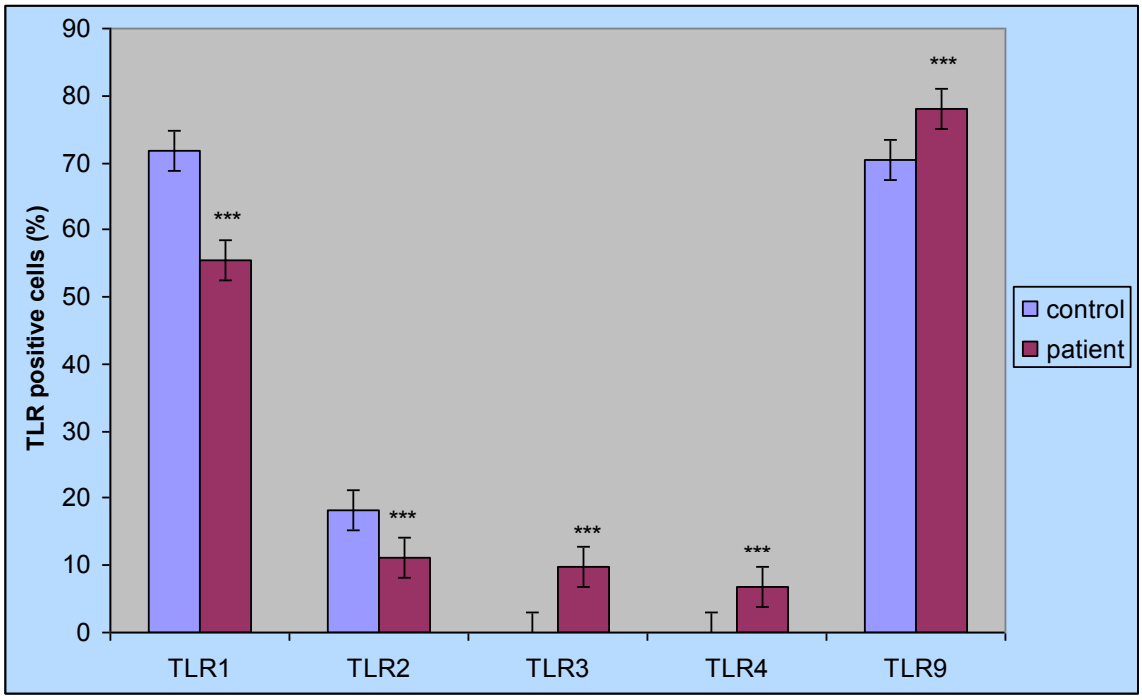


Figure 13: TLR expression on B cells in patient and age/sex matched controls. Data represents mean ±SEM TLR expression (n=129 patients, n=129 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells. T-test results show significance of difference between patient and control groups, *** p <0.005

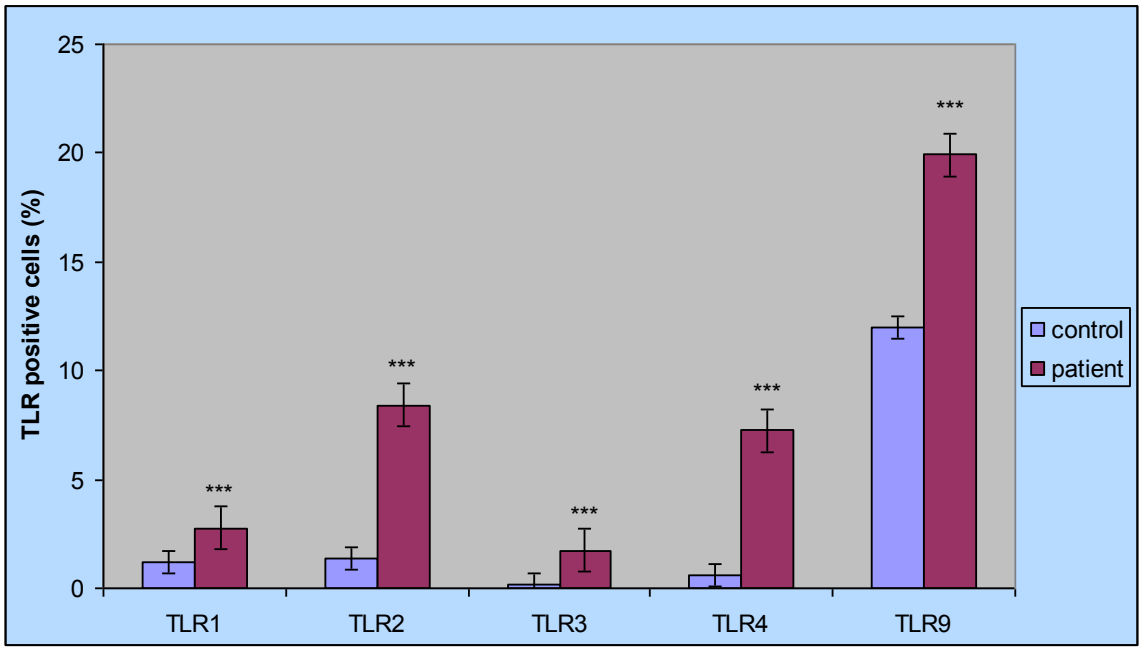


Figure 14: TLR expression on T cells in patient and age/sex matched controls. Data represents mean ±SEM TLR expression (n=129 patients, n=129 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD3 positive T cells. T-test results show significance of difference between patient and control groups, *** p <0.005

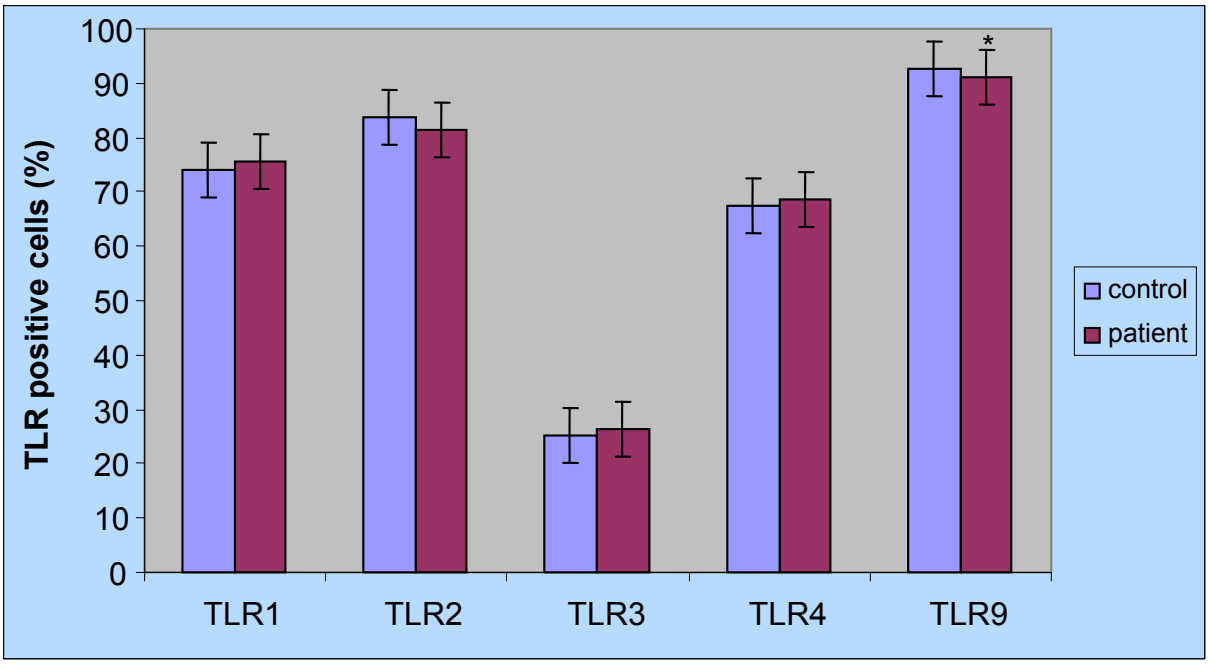


Figure 15: TLR expression on Monocytes in patient and age/sex matched controls. Data represents mean ±SEM TLR expression (n=129 patients, n=129 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD14 positive monocytes. (* p=<0.05)

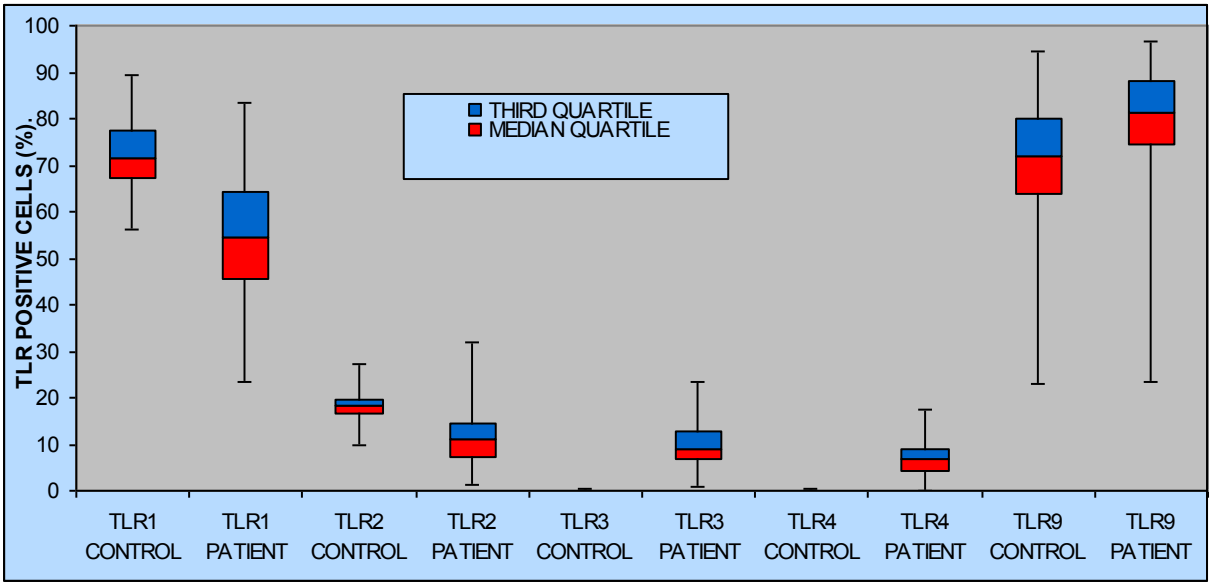


Figure 16: Box and whisker plot showing TLR expression on B cells. Data represents TLR expression (n=129 patients, n=129 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells.

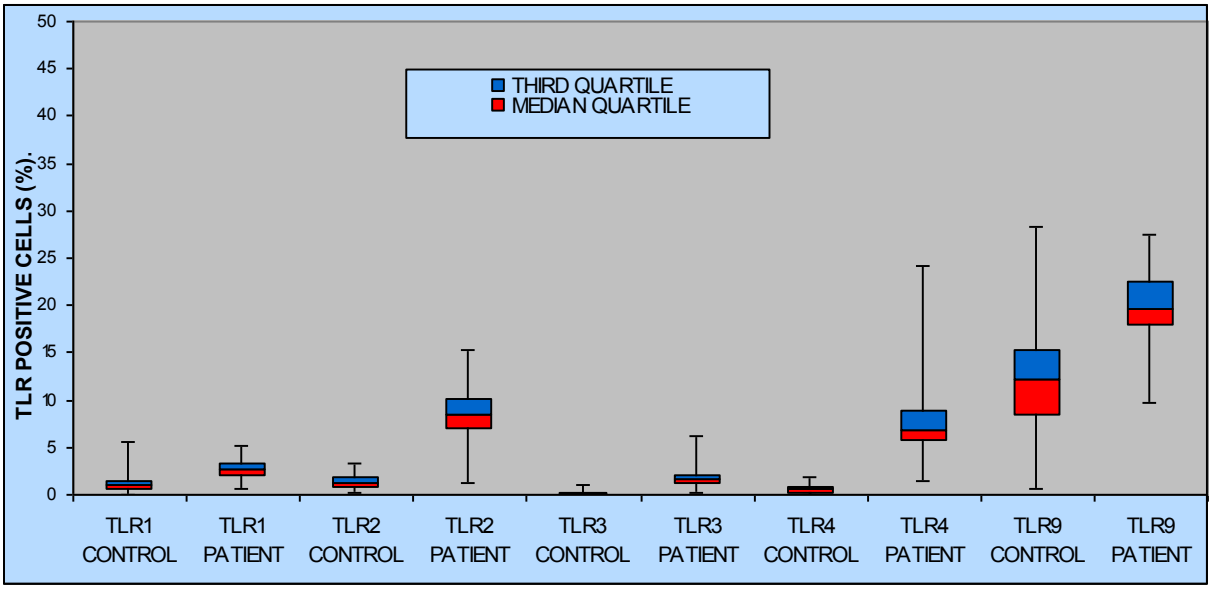


Figure 17: Box and whisker plot showing TLR expression on T cells. Data represents TLR expression (n=129 patients, n=129 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD3 positive T cells.

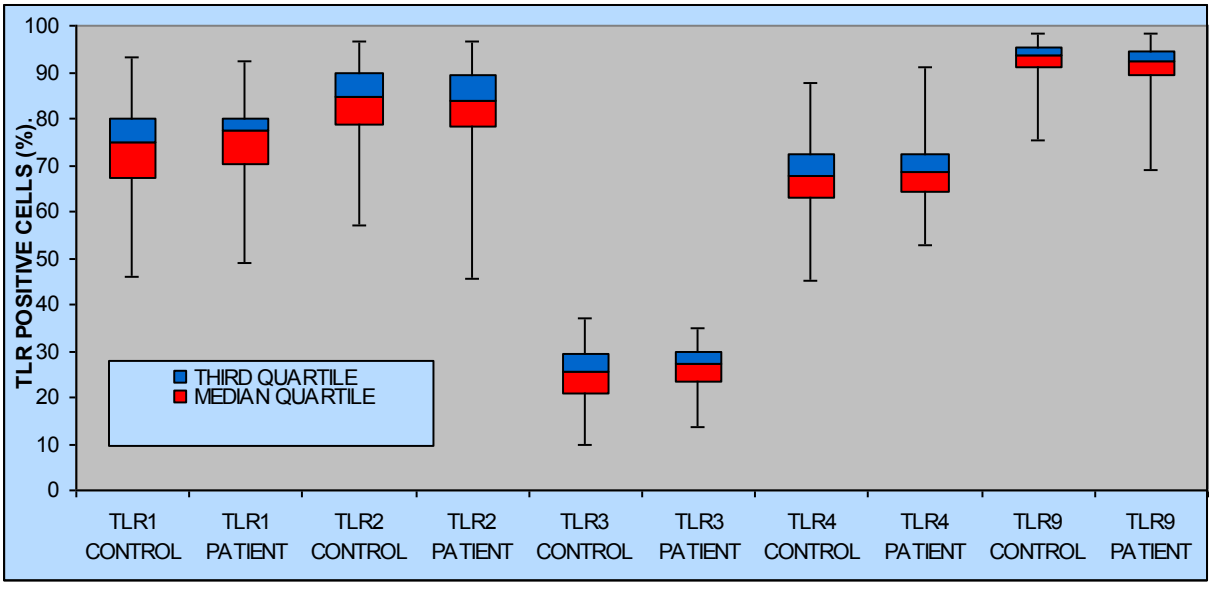


Figure 18: Box and whisker plot showing TLR expression on Monocytes. Data represents TLR expression (n=129 patients, n=129 controls. Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD14 positive monocytes.

	Type of Cell	Mean	S.D.	Median	95% C.I.
Patient	TRL1 B Cells	55.36	11.31	54.40	53.40-57.34
	TRL2 B Cells	11.24	5.49	11.10	10.28-12.19
	TRL3 B Cells	9.68	4.36	9.00	8.93-10.44
	TRL4 B Cells	6.82	3.86	6.60	6.14-7.49
	TRL9 B Cells	78.08	14.24	81.40	75.60-80.56
Control	TRL1 B Cells	71.85	7.17	71.70	70.60-73.10
	TRL2 B Cells	18.12	2.69	18.30	17.65-18.59
	TRL3 B Cells	0.047	0.098	0.00	0.029-0.064
	TRL4 B Cells	0.055	0.117	0.00	0.035-0.075
	TRL9 B Cells	70.48	14.00	72.00	68.04-72.92
Patient	TRL1 T Cells	2.76	0.89	2.70	2.60-2.91
	TRL2 T Cells	8.43	2.76	8.40	7.95-8.91
	TRL3 T Cells	1.74	0.76	1.70	1.61-1.88
	TRL4 T Cells	7.26	2.97	6.90	6.75-7.78
	TRL9 T Cells	19.91	3.70	19.70	19.27-20.56
Control	TRL1 T Cells	1.19	0.73	1.10	1.07-1.32
	TRL2 T Cells	1.39	0.74	1.20	1.26-1.52
	TRL3 T Cells	0.184	0.244	0.10	0.142-0.227
	TRL4 T Cells	0.591	0.394	0.60	0.522-0.659
	TRL9 T Cells	11.98	5.21	12.10	11.07-12.88
Patient	TRL1 Mono	75.52	7.84	77.60	74.15-76.88
	TRL2 Mono	81.40	10.08	84.00	79.64-83.15
	TRL3 Mono	26.41	4.83	27.40	25.57-27.25
	TRL4 Mono	68.74	7.25	68.50	67.48-70.01
	TRL9 Mono	91.09	4.66	92.20	90.28-91.90
Control	TRL1 Mono	74.13	10.11	74.70	72.37-75.89
	TRL2 Mono	83.77	7.93	84.80	82.39-85.15
	TRL3 Mono	25.17	5.67	25.50	24.18-26.15
	TRL4 Mono	67.58	7.77	67.50	66.22-68.93
	TRL9 Mono	92.73	3.98	93.70	92.03-93.42

Table 14: Statistical descriptives table for TLR expression in patient and control groups (95% confidence interval calculated from mean level of TLR expression)

5.2.2 Visual analysis of results charts

The results bar charts give an excellent visual representation of the difference in TLR expression between the patient and control groups. This is most strikingly apparent in the T cell expression chart, where it is immediately apparent that all TLR are expressed at higher levels on cells from the patient group than on those from the control group. Examination of the differences in expression within the groups reveals an equally striking difference, with TLR 9 being expressed at a level over ten times greater than that shown by the weaker expressed TLR. The results for the monocyte TLR expression are by contrast broadly similar in the patient and control groups with the patient group showing slightly higher expression for three of the TLR and slightly lower levels for the remaining two.

Examination of the standard error bars for Monocyte TLR expression shows a tight clustering of results, indicating that there is a reasonably small amount of intra-sample variation in the levels of TLR expression. Overall, the level of TLR expression in the Monocyte groups are statistically significantly higher than those seen on T cells, the highest level of TLR expression on the monocytes (TLR9) being 5 times higher than that seen on the highest expressing T cells. The chart for B cell TLR expression reveals the greatest variation in TLR expression, both within, and between, the two groups. The most striking difference is seen in TLR3 and TLR4 expression which are virtually absent in the control groups but show enhances expression in the patient group. Overall expression levels for TLR1 and 9 on B cells mimic those seen on the monocytes in that they are relatively strongly expressed, whereas TLR 2, 3 and 4 are expressed at levels closer to those seen on T cells. This is particularly noticeable in TLR 3 and 4 which are virtually absent on both T and B cells in the control group but are statistically significantly raised on patient group cells.

5.2.3 SPSS statistical analysis of TLR expression

Data was analysed used SPSS version 19 software (IBM Corporation) for both t-test and Mann-Whitney U test, as appropriate. The results from this analysis are attached as appendix viii.

5.2.4 Summary of results of t-test statistical analysis

Table 15 below lists the statistical test results from the data sets that satisfied the tests of normality applied during t-test analysis and were therefore analysed using this technique.

TLR/Cell type	T-test	Degrees of freedom	P Value	Significance
TLR 1 B cell	13.979	216.53	<0.005	Very high
TLR 2 B cell	12.781	186.07	<0.005	Very high
TLR 9 T cell	-14.109	231.03	<0.005	Very high
TLR 1 Mono	-1.236	241.08	0.217	Not significant
TLR 3 Mono	-1.894	249.70	0.059	Not significant
TLR 4 Mono	-1.249	256	0.213	Not significant

Table 15: Summary of results of statistical analysis using t-testing

5.2.5 Summary of results of Mann-Whitney U test statistical analysis

The data sets that did not satisfy the tests of normality applied as part of the t-testing were analysed by the Mann-Whitney U test, the results are summarised in table 16 below.

TLR/Cell type	Z-test	P Value	Significance
TLR 3 B cell	-14.270	<0.005	Very high
TLR 4 B cell	-14.270	<0.005	Very high
TLR 9 B cell	-5.322	<0.005	Very high
TLR 1 T cell	-11.763	<0.005	Very high
TLR 2 T cell	-13.597	<0.005	Very high
TLR 3 T cell	-13.707	<0.005	Very high
TLR 4 T cell	-13.884	<0.005	Very high
TLR 2 Mono	-1.695	0.090	Not significant
TLR 9 Mono	-3.620	0.072	Not significant

Table 16: Summary of results of statistical analysis using Mann-Whitney U testing

5.2.6 Summary of results of statistical analysis

The statistical methods employed to analyse test data revealed that in the case of both T and B lymphocytes, for each of the TLR investigated there was a very highly significant statistical difference between the level of expression on the patient and control groups (P=<0.005 in all instances).

In the case of monocytes however, none of the TLR showed a statistically significant difference between the two groups, (P range = 0.059 – 0.217).

5.2.7 Summary of B cell TLR expression

Whilst the P values obtained from the two statistical methods applied to the data show that all of the TLR expressions were very highly statistically significantly different between the groups, examination of the T-test value for the normally distributed data and the Z value for the non-normally distributed data, reveals the magnitude of these differences. In the case of the B cells the most striking difference is in the expression of TLR 3 and 4 (Z test statistic for both = -14.270), this is borne out by comparing the means of the patient group (TLR 3 = 9.68%, TLR 4 = 6.81%), with those of the control groups (TLR 3 = 0.05%, TLR 4 0.06%). In both instances, TLR 3 and 4 are virtually absent in control subjects but show significant expression in the patient group. This finding is mirrored in TLR 9 expression, which is also significantly raised in the patient group (Z test statistic = -5.322). Conversely TLR 1 and 2 are expressed at a significantly lower level in the patient group.

5.2.8 Summary of T cell TLR expression

Examination of the T test (applied for TLR 9) and Z test (applied for TLR 1, 2, 3 and 4), figures reveal that there is a large magnitude of difference in each case. This difference is reflected in a universal increase in TLR expression in the patient group compared to the control group. Once again TLR 3 and 4 show the greatest difference between the two groups (Z values -13.707 and -13.884 respectively), with TLR 9 showing a broadly similar magnitude of difference (T-test statistic = -14.109).

5.2.9 Summary of monocyte TLR expression

Neither TLR 1, 2, 3 or 4 showed any statistically significant difference in expression levels between the two groups, TLR 2 being expressed at a slightly lower (though not significant) level in the patient group and TLR 1, 3 and 4 being slightly (though again not significantly) raised in the patient group.

TLR9 in contrast was expressed at a statistically significantly lower level in the patient group, the Z statistic value (-3.620) does however indicate that the magnitude of this difference is not as large as those seen in the T and B cell TLR 9 increases in the patient group.

5.3 TLR expression vs. other parameters

The relationship between TLR expression and a number of other measured haematological parameters was investigated to establish if there was any correlation between expression levels and the parameter in question. In order to achieve this scatter plots were created which provided a simple graphical model comparing the

expression of the each of the 5 TLR on T cells, B cells and monocytes against the following:

i) Total white cell count

ii) % co-positive CD5/CD19 cells

iii) CLL score

iv) Absolute lymphocyte count

v) % lymphocytes

Plots were prepared comparing each TLR, on B cells, T cells and Monocytes against each of the parameters listed above. It was not considered necessary to reproduce each of these plots and accordingly representative plots for each of these graphs are shown below.

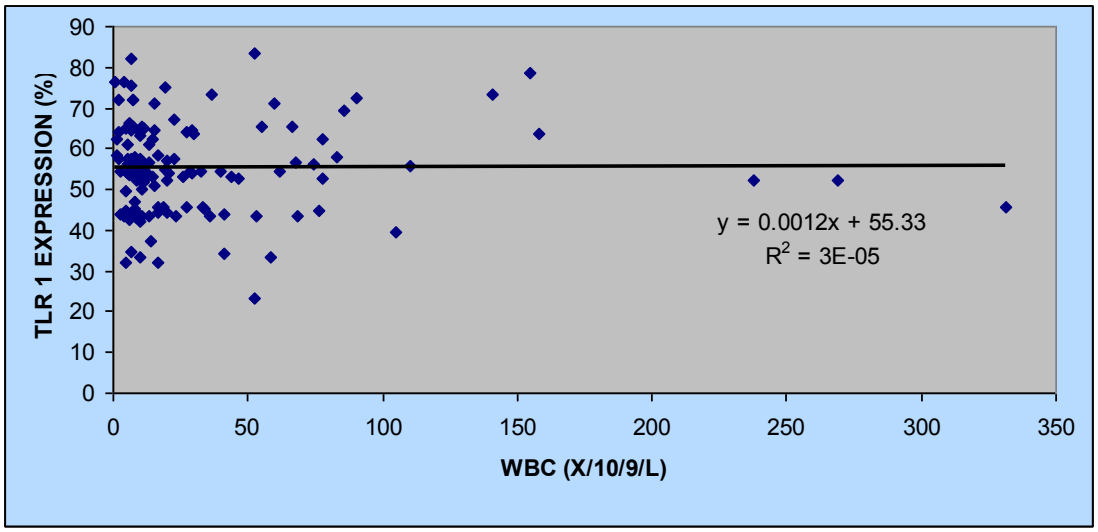


Figure 19: TLR1 expression on B cells in patient group vs. WBC. Data represents individual patient TLR1 expression (n=129) vs. patient WBC. Results for TLR expression obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells, WBC obtained from Advia 2120 analyser. Trendline analysis shows poor correlation between parameters.

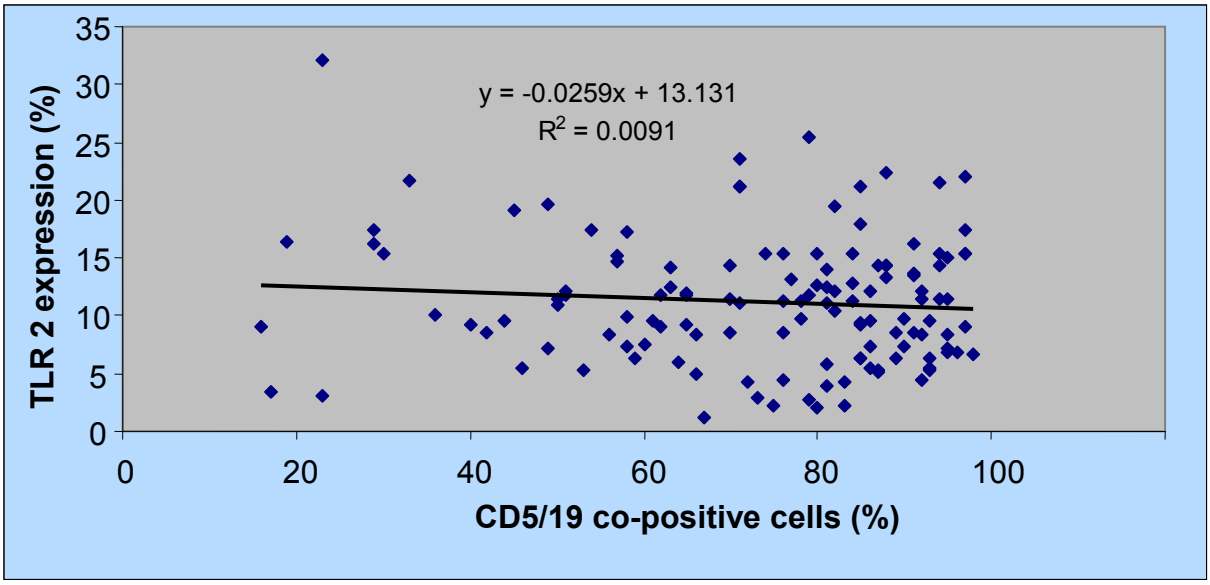


Figure 20: TLR2 expression on B cells in patient group vs. CD5/19 co-positive cell percentages. Data represents individual patient TLR2 expression (n=129) vs. % CD5/19 co-positive cells. Results for TLR expression obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells. Results for CD5/19 co-positive cells obtained using flow cytometric analysis of PBMCs. Trendline analysis shows poor correlation between parameters.

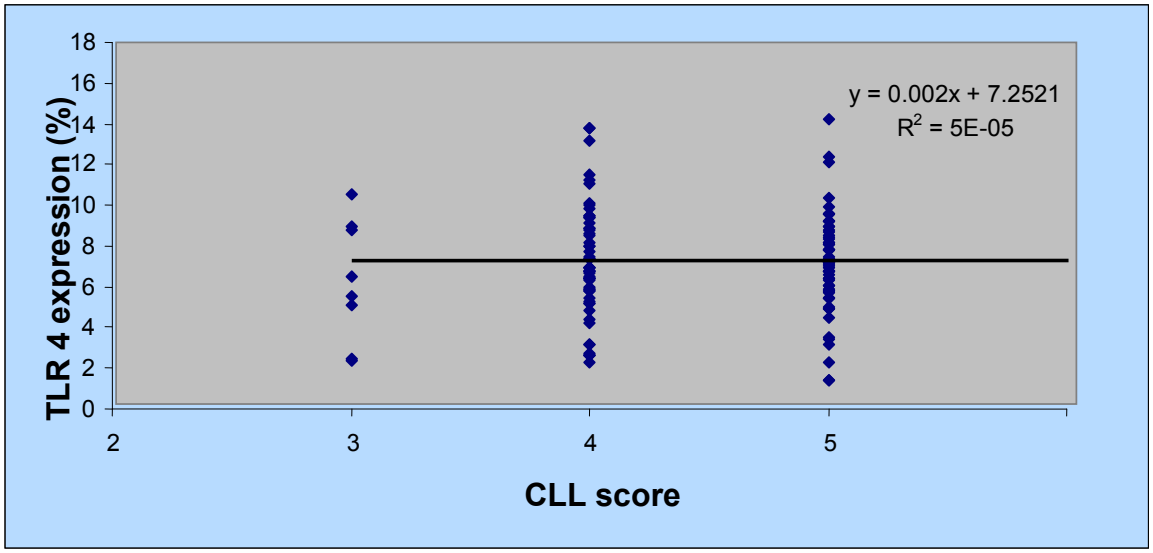


Figure 21: TLR4 expression on T cells in patient group vs. CLL score. Data represents individual patient TLR4 expression (n=129) vs. CLL score. Results for TLR expression obtained using flow cytometric analysis of PBMCs co-stained and gated on CD3 positive T cells. Trendline analysis shows poor correlation between parameters.

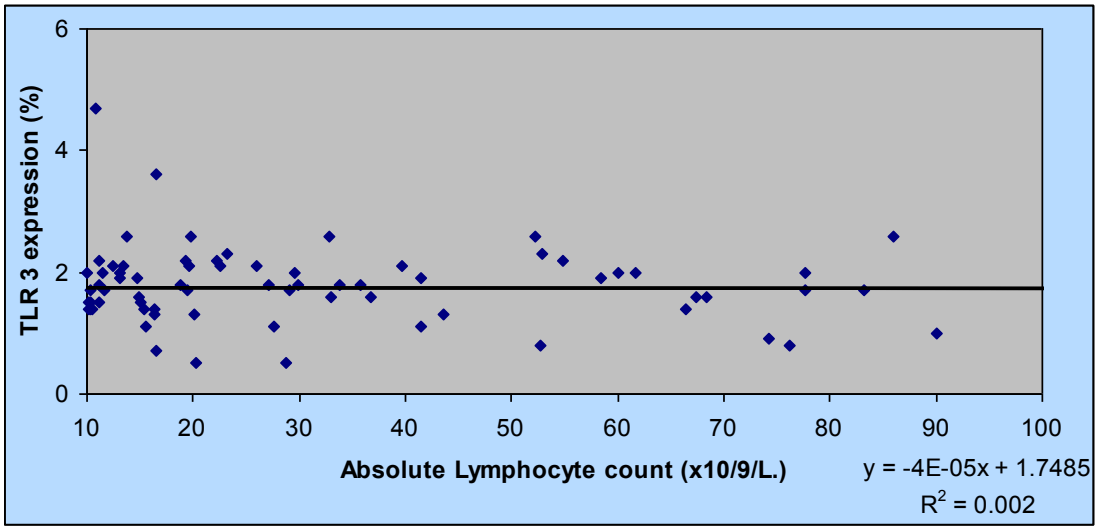


Figure 22: TLR3 expression on T cells in patient group vs. absolute lymphocyte count. Data represents individual patient TLR3 expression (n=129) vs. absolute lymphocyte count. Results for TLR expression obtained using flow cytometric analysis of PBMCs co-stained and gated on CD3 positive T cells. Trendline analysis shows poor correlation between parameters.

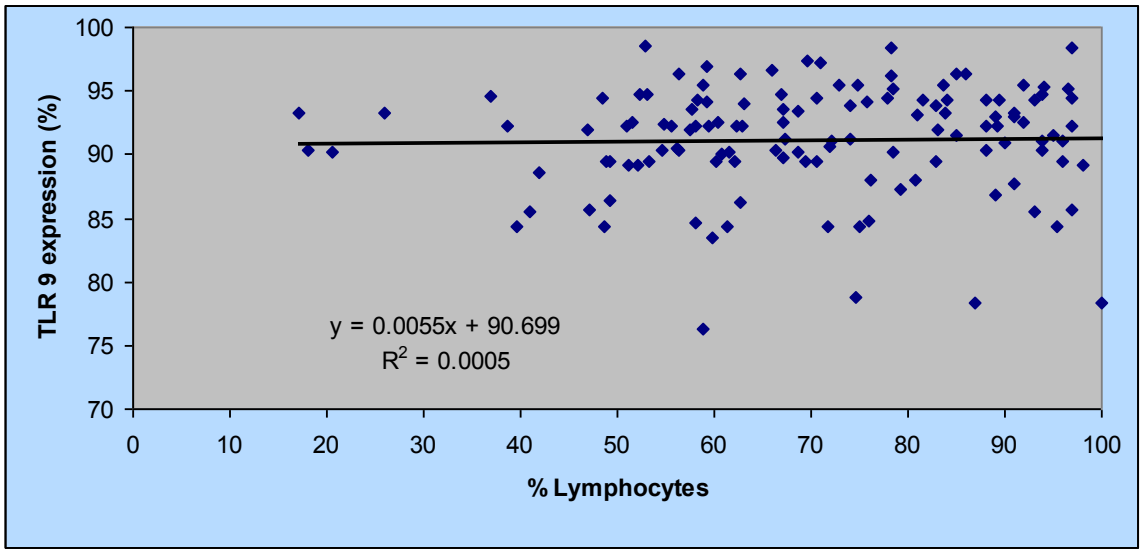


Figure 23: TLR9 expression on monocytes in patient group vs. % lymphocyte count. Data represents individual patient TLR9 expression (n=129) vs. % lymphocyte count. Results for TLR expression obtained using flow cytometric analysis of PBMCs co-stained and gated on CD14 positive monocytes. Trendline analysis shows poor correlation between parameters.

5.3.1 Summary of results of TLR expression vs. other parameters

Visual inspection of the scatter plots for each of the parameters displayed above failed to identify any obvious correlation between TLR expression and the various

parameters investigated. Furthermore the trendline analysis for each of these plots showed poor correlation in each instance.

5.4 Direct antiglobulin test results

Of the 129 patients included in the study, 28 (21.7%) had a positive DAG when presentation bloods were tested. In the matched control group only 2 patients had a weakly positive DAG. It was therefore decided that whilst further statistical analysis of the control group was unnecessary it would be useful to further investigate the patient group to see if there was any association between DAG positivity and TLR expression.

In order to achieve this comparison, those patients with a positive DAG were listed along with the levels of expression for each individual TLR, these expression levels were then compared with the expression levels in the DAG negative patient group and analysed statistically to see if there were any significant differences between TLR expression in the DAG positive and DAG negative groups. It was deemed prudent to examine individual TLR expression levels between the two groups since the earlier parts of the study revealed statistically significant variation in individual levels of TLR expression. Additionally the data was separated to show differences in TLR expression between leucocyte sub-populations. A further advantage of studying the data in this way was that DAG positivity was compared to individual TLR expression within lymphocyte sub-populations. In this manner it was hoped that a link to disease state may be found, thus providing a possible mechanism for the autoimmune phenomena frequently observed in CLL.

The results of the comparison of TLR expression between DAG positive and DAG negative patients for each of the leucocyte sub-populations are shown in figures 24-26 below. As with the previously displayed comparison bar charts standard error bars and p values where applicable are shown.

Figures 27-29 display the same data in the form of box and whisker plots. These plots show a better representation of the spread of the degree of TLR expression.

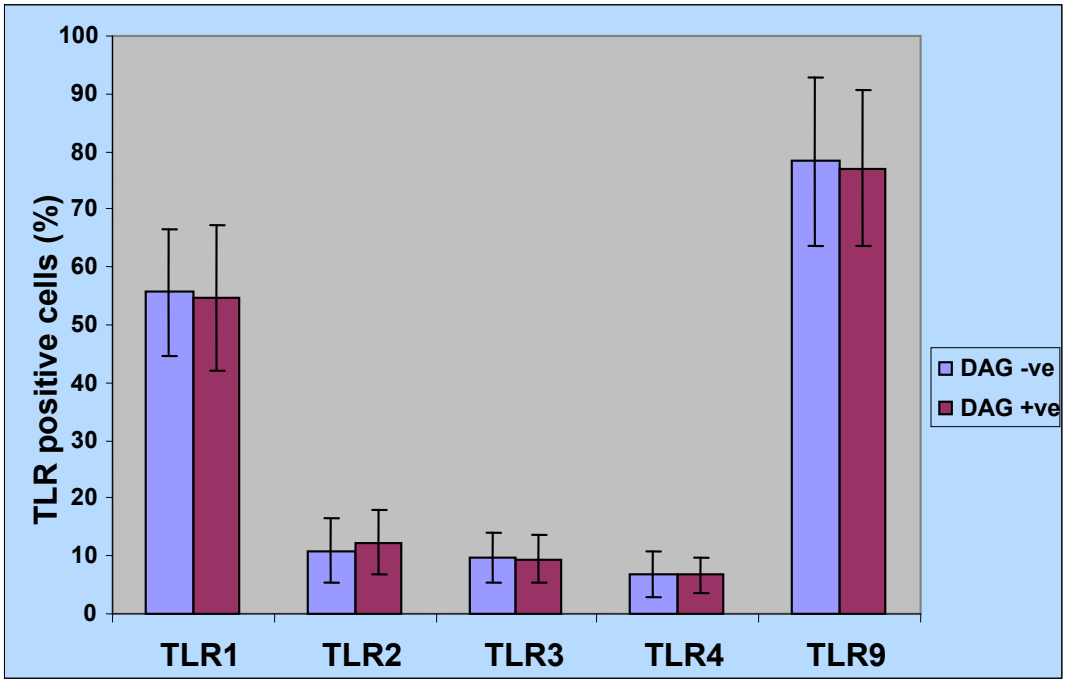


Figure 24: TLR expression on B cells comparing DAG positive and DAG negative patients. Data represents mean ±SEM TLR expression (n=28 patients, n=28 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells.

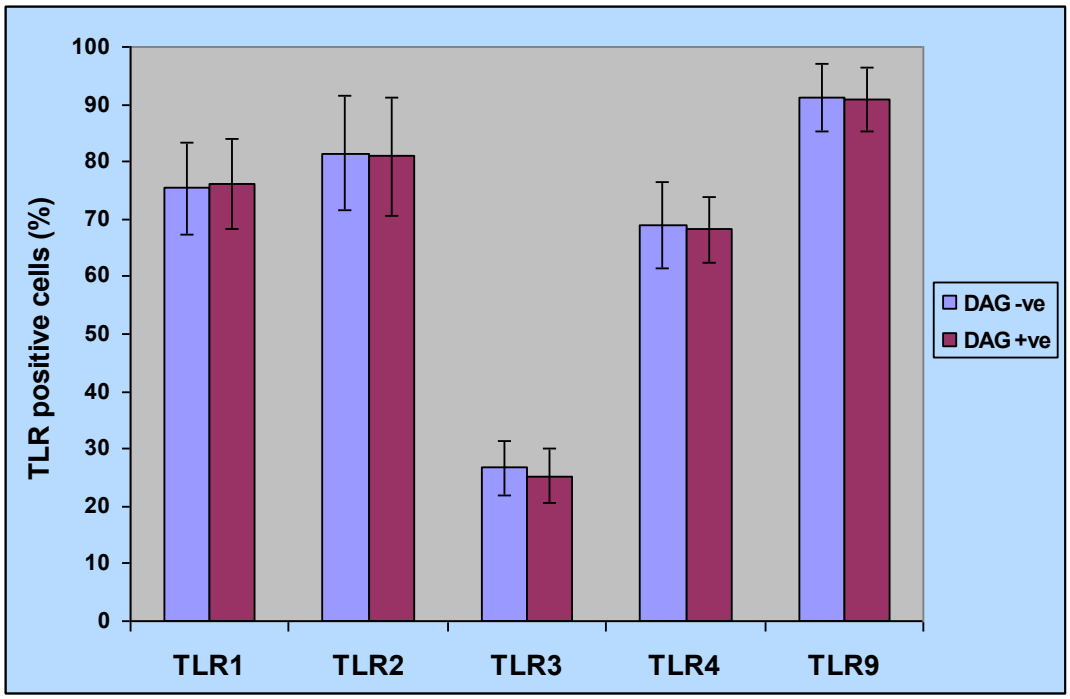


Figure 25: TLR expression on monocytes comparing DAG positive and DAG negative patients. Data represents mean ±SEM TLR expression (n=28 patients, n=28 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD14 positive monocytes.

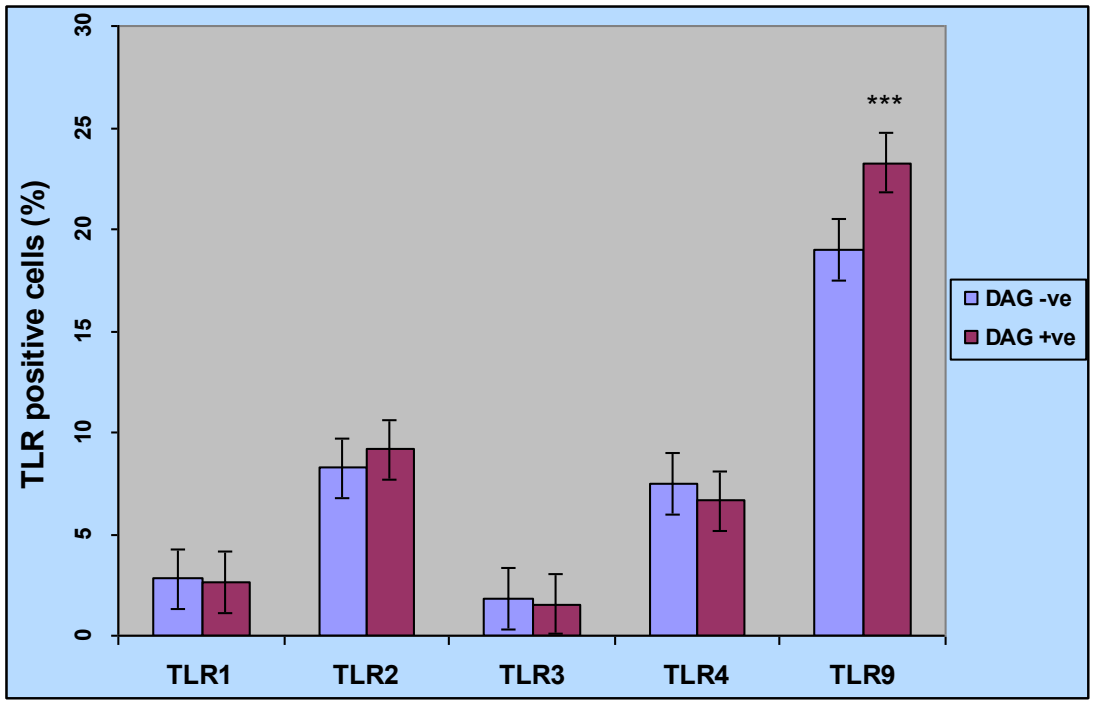


Figure 26: TLR expression on T cells comparing DAG positive and DAG negative patients. Data represents mean ±SEM TLR expression (n=28 patients, n=28 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD3 positive T cells. Z test results show significance of difference between patient and control groups, ***p <0.00

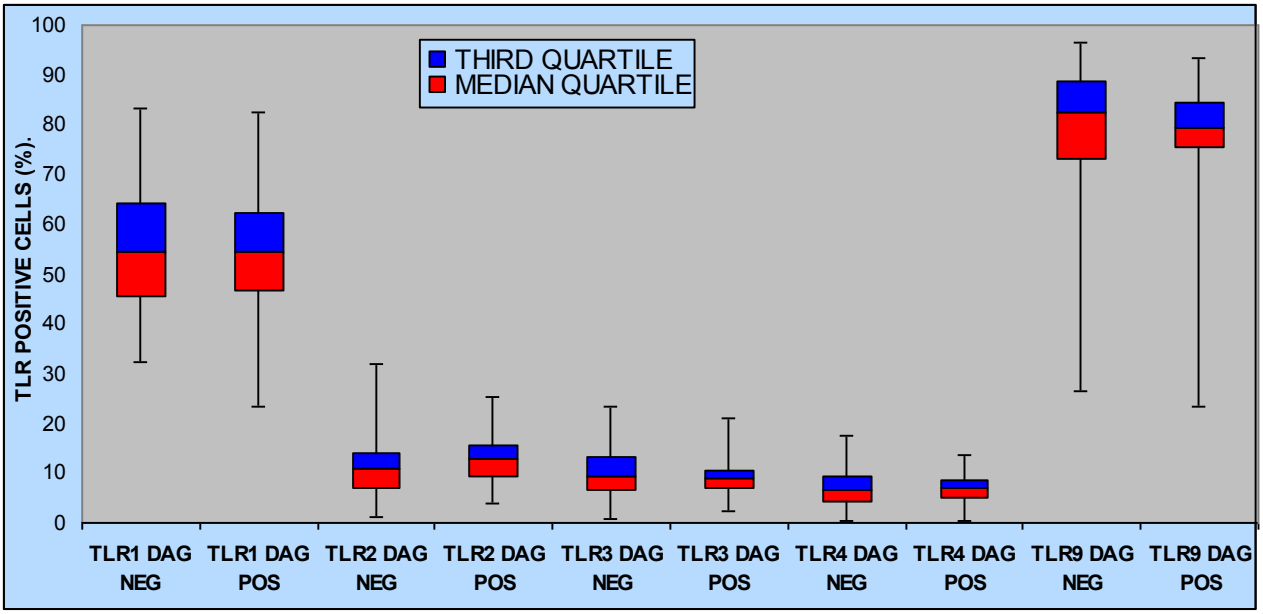


Figure 27: TLR expression on B cells comparing DAG positive and DAG negative patients. (n=28 patients, n=28 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD19 positive B cells

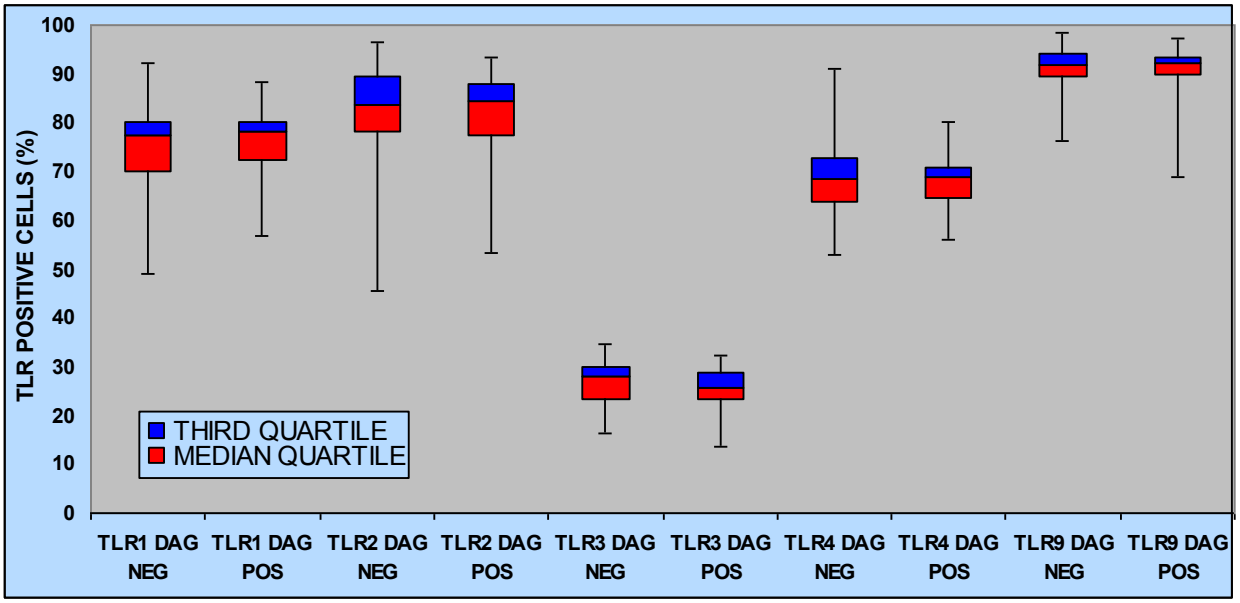


Figure 28: TLR expression on Monocytes comparing DAG positive and DAG negative patients. (n=28 patients, n=28 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD14 positive monocytes.

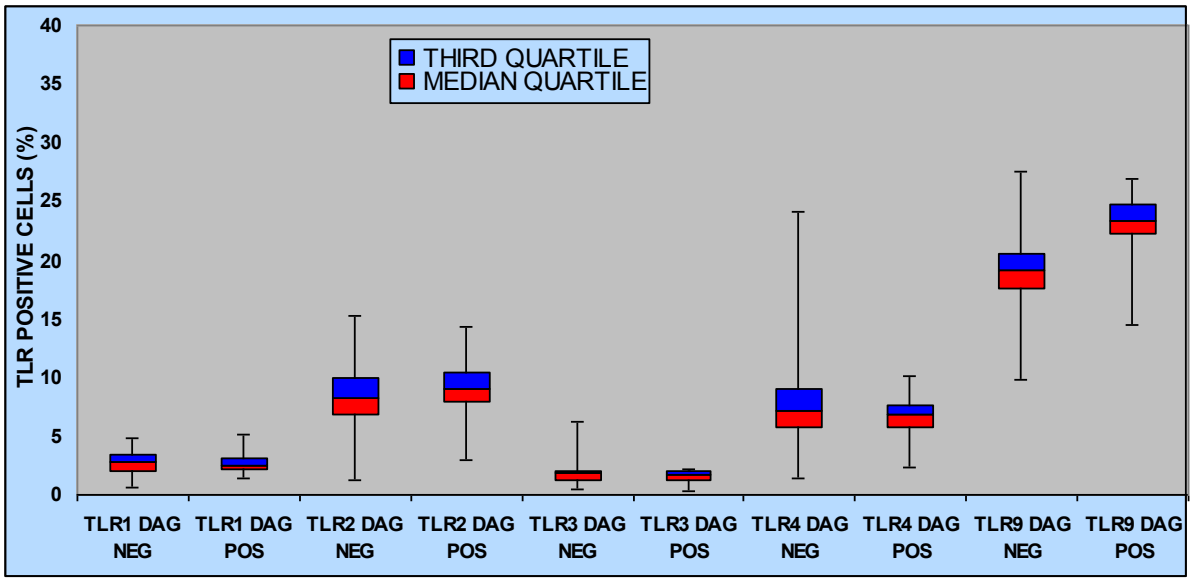


Figure 29: TLR expression on T cells comparing DAG positive and DAG negative patients. (n=28 patients, n=28 controls). Results were obtained using flow cytometric analysis of PBMCs co-stained and gated on CD3 positive T cells.

Visual inspection of these charts show that for the main part there is little or no appreciable difference in TLR expression between the DAG and the DAG negative patients on the B cells and monocytes. There does however appear to be some differences in expression levels on the T cells, this is most pronounced in TLR 9 which seems to be expressed a good deal stronger on the DAG positive patients. In order to establish the significance of this difference, further statistical analysis was carried out as detailed in the following section.

5.4.1 Statistical analysis of DAG results

In order to compare the expression of TLR on DAG positive and DAG negative patients statistical analysis was carried out using the t-test and where appropriate the Mann-Whitney U test.

Levine's test was once again applied to the data as part of the t test as a normality check and where there was a violation of the assumption of homogeneity and variance then the Mann-Whitney U test was applied.

Data was analysed using SPSS version 19 software for both t-test and Mann Whitney U test, as appropriate. The results from this analysis are attached as appendix iv and summarised in table 17 below.

Type of Cell	Normality Y/N	T-Test	Degrees of Freedom	Z Test	P Value
TRL1 B Cells	Y	0.397	127	-	0.692
TRL2 B Cells	N	-	-	-1.466	0.143
TRL3 B Cells	N	-	-	-0.331	0.740
TRL4 B Cells	Y	0.227	127	-	0.821
TRL9 B Cells	N	-	-	-1.046	0.296
TRL1 T Cells	N	-	-	-0.975	0.329
TRL2 T Cells	Y	-1.555	127	-	0.122
TRL3 T Cells	N	-	-	-0.884	0.377
TRL4 T Cells	N	-	-	-1.309	0.191
TRL9 T Cells	N	-	-	-5.872	0.000***
TRL1 Mono	Y	-0.503	127	-	0.616
TRL2 Mono	N	-	-	-.209	0.835
TRL3 Mono	Y	1.368	127	-	0.174
TRL4 Mono	N	-	-	-0.303	0.762
TRL9 Mono	N	-	-	-0.266	0.790

*** Denotes significance <0.000.

Table 17: Comparison of DAG positive vs. DAG negative patient TLR expression

5.4.2 Summary of results of statistical analysis of DAG results

It is immediately apparent when studying the above data that the only notable difference in TLR expression between DAG positive and DAG negative patients is in TLR 9 expression on T cells. The DAG positive patient group have been shown to have a statistically very highly significant greater level of TLR 9 expression on T cells when compared to the DAG negative patient group.

6 Discussion

The TLR expression patterns observed in this study in both patient and control groups are in broad concordance with previously published data (Babu *et al.* 2006, Rožková *et al.* 2010, Muzio, Fonte and Caligaris-Cappio 2012) the exception being the increased expression of TLR 3 and 4 observed in the patient group, this finding being in contradiction to that reported elsewhere (Grandjenette *et al.* 2007). The incidence of DAG positivity in the patient group of this study (21.7%) is higher than that reported in a large prospective trial of CLL patients (14% in UK LRF CLL4 trial) (Dearden *et al.* 2008). Direct comparisons of the data generated in this study and previously reported data are not however straightforward, as techniques, data set size and participant inclusion criteria vary greatly between studies.

The results of this study suggest that there is a statistically significant difference in TLR expression on both T and B lymphocytes between CLL patients and matched controls.

Given the well established role of the TLR as sensors of pathogen presence and triggers for antigen presenting cell (APC) maturation, it should be possible to link these changes to the role the lymphocytes play in the CLL disease process.

6.1 Patient demographics

One hundred and twenty nine patients with a confirmed diagnosis of CLL presenting at the Haematology department at the Royal Cornwall Hospital between January 2007 and July 2010 were included in the study. The age of the patients ranged from 50 -94

years with an average age of 71.5 years. There were 47 female patients in the study (36.4%) and 82 males (63.6%), a ratio of 1.75: 1. These figures compare favourably with published data which suggests a median age at presentation of 72 years and a male to female ratio of 2:1 (Howlander *et al.* 2012, Oscier *et al.* 2012). 25.6% of patients included in the study were less than 60 years old at diagnosis; this is slightly lower than the national average, of approximately 30% and probably reflects the population demographics of the patient group in Cornwall which has an over 60's population of 29.8% of the total population compared with the national average of 20.7% (Cornwall County Council demographic data).

The absolute leucocyte count of patients at presentation ranged from 5.8 $x10^9$/L to 400.5 $x10^9$/L and the percentage of cells co-expressing the surface antigens CD5 and CD19 which are characteristic of the disease, ranged from 16% to 98%. Of the 129 patients included in the study 28 had a positive DAG at presentation (21.7%). These results reflect the extremely variable nature of the disease and underline the importance of continued research to assist in an understanding of the disease process and the development of treatment strategies.

6.2 Role of T cells in B CLL

Activated APC's are known to stimulate naïve T cells, thus providing a role for TLR in forming a bridge between innate and adaptive immunity, it has also been reported that TLR play a pivotal role in the stimulation and maturation of B cells, which in turn leads to cell activation and the maintenance of serological memory (Bernasconi, Traggiai and Lanzaveccia 2002).

However, given the statistically significant differences observed during this study in TLR expression in the T cells, it is worth considering their role in what had traditionally been regarded as a predominantly B cell disease. A number of previous studies have focused on the role of the T cell in B CLL (Caligaris-Cappio and Hamblin 1999, Bannerji 2000, Caligaris-Cappio 2001). Since the immune system relies on a wide array of specific and critical interactions between many different cell types (including T and B lymphocytes), it is entirely possible that dysfunction in one cell type may play an important contributory role in the development and progression of B CLL (Robey and Allison 1995). The T cell undergoes a large number of interactions with the B cell both during lymphocyte development and in the immune response, the two are therefore intrinsically linked, dysfunction of the one potentially affecting development and response of the other. Previous studies have concluded that neither T or B lymphocytes can respond effectively without the full functional co-operation of the other (Scrivener *et al.* 2001). It can therefore be implied that in B CLL, a disease caused by uncontrolled B cell proliferation, T cell dysfunction may play some role.

Research into the role of the T cell in B CLL has focused primarily on 5 key areas:

1) CD4 and CD8 subpopulations and total T cell numbers
2) T cell responses to mitogen
3) T cell colony formation
4) T cell cytokine expression
5) T cell phenotype alteration and functional abnormalities.

6.2.1 CD4 and CD8 subpopulations and total T cell numbers

Whilst B cell numbers are by definition raised in B CLL, it is noteworthy that T cell numbers are invariably raised as well (Zaknoen and Kay 1990). Additionally, the ratio between CD4 positive T helper cells and CD8 positive T Supressor/cytotoxic cells is altered, with an absolute increase in CD8 positive cells being previously reported (Porakishvili *et al.* 2001). It is thought that this increase in linked to disease stage as the CD8 count rises as the disease progresses (Herrman *et al.* 1982). There are also differences in the expression of CD4 and CD8 antigen on the T cell surface of B CLL patients when compared to normal controls, CLL patients showing lower levels of both on individual T cells (Huang and Crispe 1992). Of particular relevance to this study is the finding that these low expression CD4 CD8 cells (termed $CD4^{lo}$ $CD8^{lo}$ cells) are also found as expanded populations in both human and murine autoimmune diseases (Huang and Crispe 1992). Similar work has suggested that such cells may show specificity for self antigens and may derive from a unique T cell lineage that is independent of clonal selection and which therefore escapes the mechanism responsible for the deletion of auto-reactive cell lines (von Boehmer 1992).

6.2.2 T cell response to mitogen

Studies investigating T cell responses to common mitogens such as phytohaemaggutinin (PHA) and pokeweek mitogen (PWM) have shown that there is a reduced proliferation and activation response by T cells in B CLL patients (Zaknoen and Kay 1990, Prieto *et al.* 1993). T cells recovered from B CLL patients also show a reduced ability to co-operate in the production of Immunoglobulin when cultured with normal B cells. (Callery *et al.* 1980). This suggests that there is an intrinsic T cell dysfunction which persists after removal of the malignant B cells and cannot be

corrected by normal B cells. Further studies found that the converse situation also held true, in that normal B cells are found to be unable to differentiate when cultured with T cells recovered from T CLL patients (Lauria *et al.* 1983).

Several theories have been postulated to explain the phenomenon of reduced T cell response to mitogen in B CLL, the most plausible of which predicts the presence of a soluble factor present in the serum of CLL patients which affects T cell response, to date however there is conflicting evidence, with no clearly defined mechanism (Scrivener *et al.* 2003).

6.2.3 T cell colony formation

T cells from CLL patients have been shown to form colonies *in vitro* less efficiently that those from normal subjects (Jehn *et al.* 1990). This abnormality correlates with disease stage, with T cells from recovered from stage 0 patients showing a greater degree of colony formation that those from later stage patients (Foa and Lauria 1982).

CLL T cells also show a reduced response in xenogenic graft-host transplantation reactions (Stark *et al.* 1999). In these experiments T cells were isolated from both CLL patients and normal controls and introduced to immunosupressed rats, the reaction to the injection was assessed after 4 days by measuring the size of the lesion at the injection site. T cells from normal control were found to cause a measurable rejection reaction in 97% of the tests. By comparison only 37.1% of T cells recovered from early stage CLL patients and 13.3% of T cells recovered from patients with advanced stage CLL caused a reaction, implying a dysfunctional T cell status in B CLL which worsens with advancing disease.

6.2.4 T cell cytokine secretion

Several studies have demonstrated that T cells from CLL patients show a reduced normal cytokine expression, in particular marked decreases in IL-4 and IL-2, after PHA and PWM stimulation have been described (Hill *et al.* 1999, Kay *et al.* 2001). Whilst the significance of these findings is debatable, it is interesting to note that B cells from CLL patients have been shown to have both increased numbers of IL-2 receptors on their cell surface and also the ability to secrete high levels of soluble IL-2 receptor into the serum (Zaknoen and Kay 1990). It has also been reported that T cells from CLL patients produced increased levels of Interferon-γ (IFN-γ), and that this increase could contribute to the survival of CLL B cells, which in turn have been shown to have an increased expression of IFN-γ receptors on their cell surface. It is thought that the increased uptake of these cytokines prolong survival of the CLL B cells by inhibiting apoptosis (Zaki *et al.* 2000).

6.2.5 T cell surface antigen expression

Studies on the phenotypic expression of cell surface antigens on T cells in B CLL have reported a significant number of differences when compared to T cells from control subjects (Scrivener *et al.* 2001, Johnstone 2013). There is also evidence to suggest that there may be a degree of host response by T cells against specific leukaemic antigens expressed on the malignant B cells (Porakishvili *et al.* 2001) and also that these T cells exhibit arrested development in the mid to late G1 phase of the cell cycle (Zaki *et al.* 2000).

6.2.6 Summary of role of T cell in B CLL

From the above it is clear that there is strong evidence to suggest that there is profound T cell dysfunction and dysregulation in B CLL. There are two theories to explain these findings, the first is immunosenescence and the second is anergy.

Immunosenescence, also termed replicative senescence, is described as a generalised, age related decline in immune responses found primarily in the elderly (Globerson and Effros 2000). This phenomenon leads to an increased susceptibility to infectious disease, cancer and autoimmunity (Hodgson *et al.* 2011). As lymphocytes age, they reach a point at which they cannot replicate and as a result their immune responses begin to diminish, this effect is however accompanied by restructuring of the immune system to enhance functionality in other areas (Globerson and Effros 2000). As CLL is typically a disease of the elderly, changes in T cell function and marker expression would not be unexpected.

The second theory to explain T cell dysregulation concerns clonal anergy. Complete activation of T cells requires 2 separate signals, one received through the T cell receptor as antigen in bound and the second through a costimulatory signal as the T cell interacts with the cell presenting the antigen (Lechler *et al.* 2001). If this second signal is missing or reduced, the T cell is only able to make a partial response and enters a state of unresponsiveness known as clonal anergy. Experimental evidence suggests that the first signal (caused by TCR engagement by antigen), leads to progression of the cell cycle into G_1 at which point further cell cycle progression can be blocked by cell cycle inhibitors. There is evidence to suggest that the critical second signal is transduced via a specific T cell surface antigen (CD28), which reacts

with co-receptors expressed on B cells (CD80 and CD86) to up-regulate interleukin 2 production, which in turn downregulates cell cycle inhibitors so the T cell can progress through the rest of the cycle. If the CD80 and CD86 co-receptors expressed on B cells are missing or reduced, the second signal is not received and the T cell remains in a partially activated state, unable to respond to further signals, and hence anergy ensues (Jenkins and Schwartz 1987). Given the differential expression of TLR on B cells in CLL, is it not unreasonable to assume this is a possible mechanism for T cell anergy in the disease.

Other evidence suggests that at some point in the development of the disease there may be a host T cell response directed against leukaemia-related antigens, demonstrated by the presence of clonal T cells (Serrano *et al.* 1997). These clonal T cells may have arisen from early T cells that attempted to respond to the malignant clone, leading to continuous low grade T cell activation with resultant anergy.

There is also experimental evidence to suggest that malignant B cells may have an immunosuppressive effect on the normal T cell complement (Scrivener *et al.* 2001). In a normally functioning immune system, T cells are more abundant than B cells, whereas in B-CLL, the number of B cells is dramatically increased which leads to a relative dilution of T cell numbers. When T cells are cultured with CLL B cells *in vitro*, the T cells show a reduced ability to co-operate in the production of immunoglobulins, this provides a possible explanation for the hypogammaglobulinaemia observed in many CLL patients (Callery *et al.* 1980).

It has also been reported that B cells may suppress T cell function in CLL by absorbing or utilising the cytokines essential for T cell function, suggesting reversed immune control (Zaknoen and Kay 1990).

Of particular relevance to this study however is the evidence that CLL B cells have the ability to force T cells to up-regulate surface markers that are not normally expressed (Cerutti *et al.* 2001). Experimental evidence suggesting that the upregulation of T cell markers caused by CLL B cells actively induces migration of T cells towards malignant B cell clones, where the T cells induce the B cells to proliferate further via chemokine production. This mechanism would induce a cycle of attraction and proliferation that would allow the clone to accumulate (Ghia *et al.* 2002).

There is then, much evidence to suggest that in B CLL the T cells express abnormal surface markers that may in fact contribute to the promulgation of the disease and explain some of the commonly found clinical phenomena. These differences result in a T cell population that do not respond to stimulation in the same way as normal T cells and which fail to interact normally with B cells. Since effective T/B cell interaction is central to immune function, this presents major problems for maintaining a functional immune response.

The findings of this study support this evidence, in that TLR expression is similarly disordered in the disease state.

6.3 Role of TLR in T cells

The role of TLR in the induction of an innate immune response (and subsequent induction of an adaptive response) via direct TLR activation on antigen presenting cells, has been well documented. (Muzio *et al.* 2009, Gonzalez-Navajas *et al.* 2010).

There is however an accumulation of evidence that suggests that T cell activation via TLR stimulation can lead to direct initiation of an adaptive immune response. This mechanism is distinct from the APC activation pathway (Macleod and Wetzler 2007). Much work in this field has centred on the role of T regulatory cells (Tregs), which are naturally arising T cells involved in the maintenance of immunological self tolerance. Tregs inhibit the proliferation of other T cell populations and as such play an important role in the inhibition of the development of autoimmune disease (Paust and Cantor 2005).

Tregs express TLR at levels similar to those seen on other T cells, although Tregs have been shown to express a wider range of TLR (Dai, Liu and Li 2009) and are thought to control inflammatory reactions to bacteria and opportunistic pathogens via upregulation of activation markers to enhance survival and proliferation. They perform this role independent of APC's (Carmalho *et al.* 2003). Tregs also play a pivotal role in the maintenance of self tolerance (Nyirenda *et al.* 2009). Autoreactive T cells are present in the normal T cell repertoire; these are however usually suppressed by the presence of Tregs. It has been postulated that Tregs become ineffective in CLL and this may result in activation of autoreactive T cells and the development of the autoimmune phenomena frequently seen in CLL patients (Hodgson *et al.* 2011).

Activation of TLR expressed on Tregs has been found to have a profound influence on cellular activity, and can either enhance or diminish their suppressive activity. This may influence a number of processes such as response to infection, immune surveillance, transplant rejection or (of particular relevance to this study), the induction of autoimmunity (Nyirenda *et al.* 2009). Work on mouse models has also shown that activation of TLR expressed on T helper (T_h) cells can affect the cells phenotype and their ability to provoke inflammatory responses (Sun *et al.* 2011). Other workers have also reported that inflammatory mediators can directly regulate TLR expression on T cells, which impacts directly on the role of the T cell in activation of the innate response (Flo *et al.* 2001) and that TLR signalling directly affects Treg expansion and function (Van Maren *et al.* 2008).

TLR activation can also affect cytotoxic T cell (T_c) responses following vaccination. Experimental evidence suggests that vaccination with virus like particle agonists produces a variety of responses dependant on the TLR targeted. It was found that stimulation of TLR 2 and 4 did not appreciably increase T_c response, whereas stimulation of TLR 3, 5 and 7 induced a moderate response. TLR 9 stimulation by comparison was found to induce a dramatically increased T_c response (Schwarz *et al.* 2003).

The most compelling evidence of the importance of differential TLR expression on T cells and the impact that this has on the immune response comes from studies on individuals infected with parasitic filariasis. An impaired T and B cell response has

been reported to be characteristic of filarial infection (Nutman and Kumaraswami 2001), this is the result of the development of an immune system evasion strategy by the parasite and plays a central role in the establishment of parasitic infection (Maizels *et al.* 2004). Studies on the effect on T cell TLR expression following infection by the filarial parasite *Wuchereria bancrofti* revealed that expression of TLR 1, 2 and 4 was significantly lower in infected patients compared with uninfected individuals. Interestingly, the level of TLR 9 expression remained unchanged by infection (Babu *et al.* 2006). It has been postulated that down regulation of TLR allows the parasite to become established in the host, as crucial host inflammatory responses are prevented from occurring. It had been previously thought that monocytes were responsible for the majority of inflammatory responses in filarial infection (Taylor *et al.* 2001), but there is an accumulation of evidence that T cells may also play a significant role (Babu *et al.* 2006). Although Filariasis and CLL are two very different clinical conditions, the fact that there is an alteration of TLR expression on T cells in both disease states highlights the important role TLR play in maintaining normal T cell function. Loss of T cell function, whether due to parasitic infection or by development of a malignancy, leads in each case to impairment of the immune response.

There is then an important role for the TLR in T cell function, particularly in the induction of inflammatory responses and the immune response to invading pathogens (McGettrick and O'Neill 2007). Given that the expression of TLR varies greatly between CLL patient and matched control groups, and that differential TLR expression has been shown to directly translate to functional differences, it is reasonable to assume that T cell responses in CLL may be significantly altered.

6.4 Role of B cells in CLL

The pathogenesis of B-CLL appears to involve dysfunctional regulation of both humoral and cellular immunity with subsequent development of genetic abnormalities (Shim *et al.* 2007). The CLL B cell originates in the bone marrow, where it is believed to originate from a single progenitor cell which becomes malignant when its replication and survival escape from normal regulatory mechanisms via a resistance to the normal processes of cell death and apoptosis. Leukaemic cell accumulation therefore appears to be the result of defective apoptosis rather than uncontrolled proliferation (Caligaris-Cappio 1996). The anti-apoptotic protein Bcl-2 is over expressed in 90% of the B cells from CLL patients (Faderl *et al.* 2002) and it has been suggested that this not only inhibits apoptosis and prolong survival, but that it also leads to an accumulation of malignant cells stuck in the G_0 phase of the cell cycle. Various other Interleukins and cell surface antigens such as IL-10 and CD6 have also been shown to accumulate on the surface of CLL B cells and it is thought that their presence may also contribute to protection from apoptosis (Osorio, Jondal and Aguilar-Santelise 1998).

As the leukaemic clone grows within the bone marrow, normal marrow function becomes compromised, although due to the insidious nature of the disease this may occur very slowly. With clonal expansion, normal haemopoiesis is compromised as the clone takes up an increasing amount of pace in the marrow, this leads to the cytopenias which manifest as the characteristic presenting signs and symptoms of CLL. Reduced erythrocyte production, coupled with the autoimmune haemolysis commonly associated with CLL, results in anaemia, decreased levels of functional

leucocytes and immunoglobulin leads to recurrent infections and thrombocytopenia increases the risk of bruising and bleeding (Caligaris-Cappio and Hamblin 1999).

B cells perform a number of functions that are crucial to the efficient functioning of the immune system namely: antibody production, expression of costimulatory molecules, production of inflammatory cytokines and secretion of microbial destruction factors (Cascalho and Platt 2006, Rožková *et al.* 2010). B-CLL is often accompanied by a multitude of immune system abnormalities, which when combined constitute a significant immunodeficiency for the patient. These dysfunctions can be directly related to the presence of clonal B cells, which are produced at the expense of their normally functioning counterparts (Bartik, Welker and Kay 1998).

Gene profiling studies comparing B cells from CLL patients with those from normal controls have found a large number of genes are differentially expressed (Kienle *et al.* 2005). In particular genes which encoded for proteins known to be involved in both the innate and the adaptive immune response appear to be significantly down regulated, whilst those involved in intracellular processing and response to interleukins were upregulated. Based on these expression patterns it is surmised that these are mechanisms for the prolongation of survival and evasion of the normal immune response. There is also evidence to suggest that CLL B cells have down regulated surface molecules that are critical to T cell interaction, thus precluding a normal immune response and providing further opportunity for prolonged B cell survival (Novak *et al.* 2002).

6.5 Role of TLR in B cells

B cell TLR activation results in the up-regulation of activation markers, proliferation, cytokine secretion, terminal differentiation and immunoglobulin secretion (Bekeredjian-Ding and Jego 2009). Engagement of TLR results in the activation of a number of pathways such as myeloid differentiation primary response protein 88 (myD88) activation of nuclear-factor kB (NF-kB), mitogen-activated protein kinase and protein kinase B (PKB) and Toll/Interleukin-1 receptor mediated interferon β production (Muzio, Fonte and Caligaris-Cappio 2012).

When a naïve B cell first encounters a pathogen it responds by secretion of both antigen specific and non-specific Immunoglobulin of the class IgM. It has been reported that TLR engagement in B cells could be instrumental in the initiation and amplification of this response (Chiron *et al.* 2008). The endosomal location of TLR 9 means that the pathogen must be disintegrated and endocytosed prior to being presented to this TLR. This raises the issue of spatial and temporal segregation of TLR ligand availability, which directly translates into different levels of B cell activation. This mechanism has been shown to be dependant of the presence of a co-stimulus, such as B cell receptor ligation with anti-human immunoglobulin or pathogen surface proteins (Bekeredjan-Ding and Jego 2009).

TLR-mediated B cell activation is limited in some degree by the nature of naïve B cells, as they are quite difficult to stimulate, TLR stimulation alone only inducing limited activation (Jiang, Lederman and Harding 2007). Full activation of B cells requires a combination of 3 signals; BCR triggering, T cell assistance and TLR

stimulation (Ruprecht and Lanzavecchia 2006). The absence of TLR stimulation has been shown to prevent strong activation and plasma cell differentiation. Further studies have indicated that co-operating immune cells provide co-stimulatory signals to B cells that enhance TLR signalling, dendritic cells in particular help control the humoral memory response to viruses through TLR expression and subsequent secretion of Interferons. TLR activation can therefore regulate the humoral response at several points (Jego *et al.* 2003).

It has also been reported that in naïve B cells, most TLR are expressed at low levels but their expression increases after BCR triggering and memory B cells acquire the capacity to respond to specific TLR agonists. Thus, BCR-independent stimulation of TLR-expressing B cells leads to the polyclonal activation of the memory B cells pool and contributes to the maintenance of serological memory (Bernasconi, Traggiai and Lanzavecchia 2002). In vitro studies have also confirmed that direct activation of B cell TLR induces proliferation of memory B cells (Ruprecht and Lanzavecchia 2006).

The expression of TLR on B cells has been shown to be shaped by the local environment, TLR 2, 3 and 9 expression and responsiveness to their respective ligands being increased in B cells isolated from tonsils when compared with those isolated from peripheral blood. According to one study, Naïve B cells are barely responsive to TLR stimulation and express low levels of TLR, whereas memory B cells are more reactive and more prone to proliferate and differentiate upon TLR activation (Poeck *et al.* 2004).

Further evidence of the important role that TLR play in antibody responses come from studies on immunodeficiency disorders that are the result of impaired TLR signalling (Picard, Casanova and Puel 2011, Warner and Núñez 2013) The disorders in question are:

(i) Interleukin-1 receptor-associated kinase 4 (IRAK4) deficiency

(ii) MyD88 deficiency

(iii) NF-kB essential modulator (NEMO) deficiency

Clinically these immunodeficiencies are mainly associated with severe, childhood infections with gram positive bacteria such as *S. aureus and S. pneumoniae.* Clinical case studies on these conditions describe humoral immune defects with defective antibody responses to certain vaccines and a failure to maintain protective levels of antigen specific antibody titres (Ku, Picard and Erdos 2007).

CLL is characterised by constitutive activation of the B-cell receptor (BCR) signalling pathway which is aberrantly active in the disease, leading to increased cell survival and proliferation (Woyach *et al.* 2014). Several mechanisms have been reported to provide CLL cells with a survival advantage; one such mechanism involves the activation of NF-κB (Nuclear factor kappa light chain enhancer of activated B cells) which plays an important role in the survival and proliferation of both normal and neoplastic B cells (Liu *et al.* 2011). A broad array of, mostly extracellular, stimuli have been reported to activate NF-κB to various degrees in CLL (Gilmore 2006). These stimuli bind to cell surface receptors, including TLR, causing downstream activation of NF-κB which enters the nuclei to activate target genes (Liu *et al.* 2011).

Since signalling through the BCR appears to be a major contributor to the pathogenesis of CLL, it is relevant to this study that TLR act as co-stimulatory signals for B cells. TLR dependent signals may be implicated in the regulation of B cell immune responses, either by inducing TLR tolerance or by subverting the mechanisms that silence autoreactive B cells, thus promoting autoreactivity (Arvaniti, Ntoufa and Papakonstantinou 2011). CLL cells are known to express a similar profile of TLR to that seen on activated B cells (Rožková *et al.* 2010).The vast majority of adaptors and effectors of the NF-κB pathway are intermediately to highly expressed, whilst inhibitors of the TLR pathway are generally low to undetectable, indicating that the TLR signalling framework is competent in CLL (Arvaniti, Ntoufa and Papakonstantinou 2011).

TLR mediated stimulation is therefore relevant to CLL development and evolution and has been implicated in malignant transformation, tumour progression and immune evasion processes (Isaza-Correa *et al.* 2014). It is reasonable then to infer that TLR are important regulators of a broad variety of B cell functions and play distinct roles in different B cell subsets, at different stages of differentiation or depending of distribution within the body. TLR can therefore be thought of as controlling differing roles depending on the immunological context in which they are employed. (Bekeredjian-Ding and Jego 2009). In the context of CLL this is particularly relevant, as it has been suggested that TLR stimulation of B cells increases the immunogenicity of tumour cells and potentially contributes to the induction of a leukaemia-specific immune response, which is distinct from the normal immune response observed in healthy donors (Grandjenette *et al.* 2007). There is also evidence to suggest that direct

TLR activation decreases the viability of B-CLL cells and increases their susceptibility to apoptosis in culture (Jahrsdorfer *et al.* 2005).

6.6 Autoimmunity in CLL

The two major disease-specific complications of CLL are infection and autoimmunity. Both of these can be related to underlying alterations in immune function, since the disease is characterised by a dysregulated immune system, as discussed in the preceding sections. The introduction of new therapeutic regimens have lead to improved overall survival for CLL and since patients live longer, disease-specific complications become more common (Dearden *et al.* 2008). It has also been suggested that more intensive treatment regimens and the use of immunosuppressive drugs contribute towards the increased incidence of autoimmunity seen in CLL (Diehl and Ketchum 2008).

Autoimmune complications are common in CLL, occurring in between 10 and 25% of patients at some time during their disease course (Hamblin 2006), and manifest in three distinct autoimmune diseases: Warm autoimmune haemolytic anaemia (AIHA), which occurs in 11% of patients, Idiopathic thrombocytopenic purpura (ITP), which is found in 2-3% of patients and Pure red cell aplasia (PRA), found in 6% of patients (Diehl 1998). More recent reviews of a large group of CLL patients have established that approximately 50% of patients exhibit autoimmune disease during or immediately after therapy (Hodgson *et al.* 2011, Zent and Kay 2010). Additional studies concluded that autoimmunity is associated with advanced clinical stage, high lymphocyte count and short lymphocyte doubling time. There was however no

significant differences found between overall survival times of those patients with and those without autoimmune disease (Moreno *et al.* 2010).

The autoantibodies found in the serum of CLL patients are largely polyclonal in nature and are invariably different in both specificity and isotype from those secreted directly by the malignant cell population. It is therefore reasonable to infer that the residual non-malignant B cell population must be responsible for autoantibody production; this is in contrast to other haematological malignancy conditions such as lymphoma where autoantibodies are produced by the malignant cells (Hodgson *et al.* 2011). A possible mechanism to explain this phenomenon is aberrant antigen expression by the malignant B cell population, it is thought that they may well express cryptic antigens to which the immune system is not normally exposed and for which there is no immunological tolerance. Since these cryptic antigens are essentially recognised by the immune system as 'foreign' an immune response will be initiated which manifests as an autoimmune condition (Hall *et al.* 2007).

Given the dramatic differences seen in TLR expression on both T and B cells between the patient and control groups in this study, it is important to consider the role of both T and B lymphocytes cell in autoimmune disease.

6.6.1 T cell induced autoimmunity

Within the T cell compartment, tolerance is maintained by the deletion of immature T cells that recognise self-antigens. This mechanism for removal of potentially self reactive cells occurs primarily in the thymus, although several accessory mechanisms

are also operative in the peripheral blood system, including induction of functional anergy, deletion by apoptosis and the suppressive actions of Tregs (Abbas *et al.* 2004). T cell derived autoimmunity is thought to be caused by germ line mutations or targeted deletions of genes which disrupts one or other of the pathways of tolerance, deletion or mutation of the growth factor IL-2 for example interferes with the generation of CD4 positive Treg which is key to the induction of T cell anergy, absence of these Tregs is directly attributable to autoimmune disease (Refaeli *et al.* 1998).

The fact that there are multiple mechanisms for removal of self-reactive T cells suggests that there is a high degree of co-operation between them to maintain self tolerance and any disruption may alter the balance between tolerance and autoimmunity (Abbas *et al.* 2004). As previously discussed, the differential expression of TLR can be linked to functional differences in cells of the immune system. These differences may provide a possible explanation for the high incidence of autoimmune disease seen in the patient group.

6.7 Role of B cells in autoimmunity

TLR signalling in B cells has been shown to exert a regulatory function that suppresses autoreactive T cells and can help self limit autoimmune disease (Lampropoulou *et al.* 2010). However, it has also been reported that inappropriate engagement of TLR on B cells can trigger an autoimmune response (Pasare and Menzhitov 2005). One of the major mechanisms that contributes towards autoimmunity in CLL is that B CLL cells will often act as aberrant antigen presenting cells (APC), which is linked to the development of AIHA (Galleti *et al.* 2008). The

mechanism responsible for this phenomenon is that CD5 positive B cells in CLL patients will process and present Rhesus (Rh) antigen to Th cells. It has been found that CLL patients with AIHA have a discreet population of activated Th cells that react specifically with Rhesus (Rh) blood group system epitopes on red blood cells. Positive selection of CD5 positive CLL B cells proved that it was these malignant cells that process and present purified Rh protein to the autoreactive Th cells (Hall *et al.* 2005). The leukaemic clone of B cells found in CLL commonly express IgM class antibodies on their surface; AIHA is however typically mediated by IgG class autoantibodies, suggesting that the control of self reactive immunoglobulin production is lost in CLL, as the majority of IgM produced will be of malignant clonal origin. It has been proved experimentally that the self-reactive repertoires of class IgM and IgA antibody are indeed disturbed by the production of monoclonal immunoglobulin produced by CLL B cells with the resultant production of autoimmune disease (Stahl *et al.* 2001). Thus the autoantibodies responsible for autoimmune disease in CLL appear to be produced by polyclonal 'bystander' B lymphocytes as a consequence of immune dysregulation associated with the malignant cell clone (Barcellini *et al.* 2002).

It has been suggested that some of the T cell defects found in CLL may be caused by immuosupression by the B cells (Scrivener *et al.* 2003). In a normally functioning immune system, T cells are more numerous than B cells, particularly in the peripheral blood. In B-CLL the number of B cells increases dramatically which has a relative dilution effect on the T cell compartment. As a result on this increase in B cell numbers, there is a corresponding increase in the markers expressed on their surface and factors secreted by them. These can have both direct and indirect effects on T cell

function, and may well account for the T cell dysfunction well described in the disease, in particular, there appears to be a reduced cooperation between the 2 cell types in immunoglobulin production which results in the hypogammaglobulinaemia commonly seen in CLL patients (Scrivener *et al.* 2003). B cells may also suppress T cells by either absorbing or utilising cytokines essential for T cell function. In particular IL2 levels are reduced in CLL, suggesting a reduced immune control by B cells over T cells (Zaknoen and Kay 1990). Other methods of immune control by the B cells include an ability to force T cells to upregulate surface markers to levels not normally expressed, for example experimental culturing of T cells in the presence of CLL B cells caused the T cells to upregulate the surface protein CD30, which in turn prevents the upregulation of another surface protein (CD40) on the surface of non-malignant B cells, the expression of which prevents immunoglobulin production (Cerutti *et al.* 2001).

There is then an accumulation of evidence that suggests that the differences in the surface expression of TLR on the T cells in B CLL may be a functional consequence of the malignant B cell clone itself, which may in turn affect non-malignant B cells.

6.8 Non haematological autoimmunity

There are also reports of autoimmune conditions associated with CLL that affect non-haematological tissues, in one study up to 16% of CLL patients had a positive marker for non-haematological autoimmune disease, such as a positive anti nuclear antibody or rheumatoid factor. These are however comparatively rare and in the majority of instances are thought to have been precipitated by therapy (Barcellini *et al.* 2006).

6.9 Role of TLR in autoimmunity

TLR have the capacity to stimulate both innate and adaptive immunity, they also have the potential to break immunological tolerance and induce autoimmune disease such as arthritis and diabetes (Lang *et al.* 2005). This is however balanced by the fact that TLR stimulation can also suppress autoimmune pathogenesis, indicating a dual role for TLR in autoimmune diseases (Lampropoulou *et al.* 2008). Studies on TLR 9 have indicated that polymorphisms leading to reduced TLR 9 expression increases predisposition to the autoimmune condition Systemic Lupus Erythematosus (SLE) (Tao *et al.* 2007).

In addition to their role in pathogen recognition, TLR also recognise a number of self proteins and endogenous nucleic acids. It has been suggested that inappropriate activation of TLR may lead to tissue injury and autoimmune disease (Papadimitraki, Bertsias and Boumpas 2007). There is also evidence that B cells can under certain conditions promote autoimmune disease by the production of autoantibodies, and as has been discussed above, by serving as antigen presenting cells for autoreactive T cells. Most normal individuals have a number of autoreactive B cells, these are however tolerant of self-antigens, and additional events must be initiated in order to promote alteration or loss of this tolerance with resultant initiation of autoimmune disease. Experimental evidence shows that TLR mediated signalling can indeed directly break B cell tolerance to self antigens and lead to overt autoimmune disease (Meyer-Bahlburg and Rawlings 2008).

Compelling evidence of a role for TLR in the development of autoimmune disease has come from studies on patients with SLE. In these patients anti-nuclear antibodies are a typical clinical feature and are considered a central diagnostic criterion (Terhorst *et al.* 2010). It has been postulated that recognition of nuclear components may contribute to the origin and perpetuation of the disease and that these substrates may also act as TLR stimulators that in turn activate autoreactive B cells (Ding *et al.* 2006, Giltiay, Chappell and Clark 2014). Interestingly much of the research in this field has focused on the role of TLR 9, there being a body of evidence that suggests polymorphisms in TLR 9 correlate well with the incidence of lupus in some populations (Tao *et al.* 2007, Klonowska-Szymczyk *et al.* 2014). Other workers investigating the role individual TLR play in the development of SLE have however reported that under different circumstances TLR signalling may either exacerbate or protect against SLE associated pathology (Rahman and Isenberg 2008).

Given that there is a well established link between TLR signalling and autoimmunity in SLE, it is postulated that a similar mechanism may be responsible for the autoimmune phenomena associated with CLL.

6.10 TLR9 expression and autoimmunity

Studies on TLR 9 function have determined that it recognises unmethylated CpG oligodeoxynucleotides, which are short single stranded DNA molecules that contain a cytosine base followed by a guanine base (the p referring to the phosphodiester backbone of DNA). Unmethylated CpG motifs have been shown to act as immunostimulants (Weiner *et al.* 1997), and are abundant in microbial genomes but rare in vertebrates (Bauer and Wagner 2002).

TLR-9 activation leads to a pro-inflammatory reaction which results in the production of cytokines including IFN-1 and IL-12. It is however hypothesised that TLR 9 can also bind to the body's own DNA and RNA from apoptotic cells initiating an immune response. Since this material is intracellular it is hypothesised that they become visible to the immune system when accumulate in the plasma membrane during apoptotic cell death (Casciola-Rosen, Anhalt and Rosen 1994, Graham and Utz 2005), subsequent uptake and processing by antigen presenting cells (APC's) leading to a loss of tolerance. Self ligands that can inappropriately stimulate TLR have been termed damage associated molecular patterns (DAMP) and are recognised to have the potential to activate self reactive lymphocytes and induce an autoimmune state. However it is thought that in an intact immune system, TLR stimulation alone will not be sufficient to overcome self-tolerance (Fischer and Ehlers 2008). There is then in patients with a compromised immune system, as is the case with those with B-CLL, the potential for autoimmunity.

In order for an autoimmune state to exist it is necessary that immune complexes form in the body and for this to happen it is necessary to have both autoantigens and autoantibodies present. The autoantigens as already premised, are released from

apoptotic cells and the autoantibodies are probably produced by self-reactive B cells, which constitute a reasonably proportion of the naïve B cell repertoire in most individuals (Wardermann *et al.* 2003). Defects in early B cell tolerance may lead to an even greater percentage of self-reactive B cells in patients prone to development of autoimmune disease (Yurasov *et al.* 2005), patients with B-CLL would fit into this category for reasons already discussed. The role of TLR 9 stimulation and autoantibody production in other disease such as SLE has been well documented (Christensen *et al.* 2006), the importance of TLR 9 expression being demonstrated by the apparent inability of TLR 9-deficient animal models to generate anti DNA antibodies.

Studies on individuals infected with helminthic parasites have determined that TLR 9 expression on T cells correlates with cell activation and that low TLR 9 expression prevents polyclonal activation during primary immune responses, conversely a high level of TLR 9 expression was found to facilitate polyclonal activation of the immune system (Ayash-Rashkovsky, Bentwich and Borkow 2005). This finding is significant to this study as it provides a link with autoimmunity since the potential for immune complex formation, and hence an autoimmune condition, is higher in individuals with increased TLR 9 expression. There are a number of other risk factors for the development of autoimmunity, such as genetic predisposition, environmental factors and compromised tolerance checkpoints (Fischer and Ehlers 2008), all of which are also implicated in the disease process in B-CLL. It is therefore likely that both B-CLL and autoimmunity may be linked by both the disease process and common risk factors.

6.11 The role of TLR in CLL

In the body the local environment seems to shape the TLR repertoire expressed on cells, TLR 2, 3 and 9 expression and responsiveness to their respective ligands is increased in B cells isolated from tonsils when compared with those isolated from peripheral blood. According to one study, Naïve B cells are barely responsive to TLR stimulation and express low levels of TLR, whereas memory B cells are more reactive and more prone to proliferate and differentiate upon TLR activation (Poeck *et al.* 2004).

The role of TLR in triggering an immediate immune response following direct recognition of molecular patterns found in microbial components is well documented (Akira, Uematsu and Takeuchi 2006, Kawai and Akira 2011), as it their role in bridging the innate and adaptive immune systems by acting as costimulatory signals for B cells to induce maturation, proliferation and antibody production after pathogen recognition (Pasare and Medzhitov 2005, Lanzavecchia and Sallusto 2007). However there is little published information on which TLR are expressed by CLL cells and relatively little is known regarding their function in the disease (Muzio *et al.* 2009).

Those studies that have set out to characterise TLR expression on CLL B cells have concluded that they are expressed in a similar pattern to that found in memory B cells (Ruprecht and Lanzavecchia 2006), and that their expression appears to be unrelated to disease stage or other prognostic factors (Muzio *et al.* 2009). Other workers have also reported that TLR are expressed at low levels on naïve B cells and are upregulated upon BCR triggering (Rožková *et al.* 2010). There is however growing evidence that TLR 9 shows the greatest variation in expression between CLL and

healthy individuals (Longo *et al.* 2007, Muzio *et al.* 2009). Given that TLR 9 has been reported to be upregulated upon antigen stimulation of normal B cells (Isaza-Correa *et al.* 2014) this provides evidence for a link between upregulation of TLR 9 and clonal stimulation of both normal B cells and malignant clones. It has also been suggested that enhanced expression of TLR 9 on memory cells allows for polyclonal activation of the entire pool of memory cells allowing the maintenance of serological memory and also the increase of the immunogenicity of B cells via the upregulation of costimulatory molecules (Rožková *et al.* 2010).

Given that TLR plays such a crucial role in the homeostasis of B cells, the differential expression of TLR on CLL cells compared to their normal counterparts may help explain the complex changes seen in the characteristics and function of leukaemic cells.

6.12 Limitations of study

This study is limited, of necessity in a number of areas, notably those relating to subject group, techniques and overall scope.

As with any research project, the sample size is governed by the availability of suitable candidates for inclusion, the time scale over which data is collected and the resources available for analysis. Over the course of the study a reasonable proportion of prospective candidates were rejected as they did not meet the criteria for inclusion i.e. a confirmed diagnosis of CLL and availability of age/gender matched control. Although strict inclusion criteria excluded participants, it is believed that this has

contributed to the robust nature of the study and by extension adds value to any conclusions drawn from it. With hindsight it may have been prudent to collect additional data from rejected candidates, as without a clear idea of what the results would show, valuable information may have been missed. When performing the statistical analysis of the data it also become evident that it would have been prudent to calculate the optimal sample size by performing some basic statistical power calculations, prior to commencing the study, as the adopted strategy of simply collecting as many samples as possible over a set time period lacks subtlety and weakens the project with respect to statistical robustness.

With respect to the techniques used to analyse the test subjects it is somewhat simplistic to have used a single analytical strategy. Confirmatory analysis using different techniques would have given a greater degree of confidence in the results generated and may have circumvented some of the technical difficulties encountered in the early stages of project development. This potential weakness is noted in particular with respect to the studies on the DAG testing phase of the investigation. DAG positivity is a relatively blunt tool with which to investigate the phenomena of autoimmunity and it is realised that thoroughness is lacking in this element of the study. The study as it stands only provides a snapshot of TLR expression in newly diagnosed CLL patients; it would perhaps have been prudent to plan to extend the study longitudinally. In particular it would have been beneficial to perform a serial study of TLR expression in a cohort of B CLL patients from diagnosis, through treatment and on to eventual outcome. This would have yielded valuable information on the change in TLR expression related to therapy and disease progression and

would also allow retrospective comparisons to be made between the pattern of expression at diagnosis and in a variety of outcomes.

The scope of this study is also limited, in that it is restricted to CLL. Given that over the course of the investigation a large number of patients presented with a plethora of other haematological malignancies, it may have been reasonable to investigate TLR expression on patients with a range of conditions or from different demographic groups.

Notwithstanding the above, the study although limited, has established ranges for TLR expression in a reasonable sample size of both patients and age/sex matched control. Statistically significant differences have been found between these 2 groups and also between patients expressing evidence of autoimmune disease and those that did not. Given the original hypothesis that there will be differential expression of TLR between patient and control groups, it is fair to say that this has been proven. What is however less certain is the contention in the original hypothesis that there is a link between TLR expression and the disease process in CLL. Whilst it has been established that DAG positivity and a high degree of TLR 9 expression are linked, there is insufficient evidence to conclude that TLR9 is directly responsible for this phenomenon; hence although there is a proven association, this element of the hypothesis remains unproven.

7 Future developments

There remain a number of additional studies that would compliment and enhance this project, as financial and ethical restrictions have meant that not all of the promising avenues of research could be investigated. In particular it would be beneficial to perform a serial study of TLR expression in a cohort of B CLL patients from diagnosis, through treatment and on to eventual outcome. This would yield valuable information on the change in TLR expression related to therapy and disease progression and would also allow retrospective comparisons to be made between the pattern of expression at diagnosis and a variety of outcomes. This latter area of study may prove beneficial to researchers involved in the search for newer prognostic factors, there being obvious clinical applications for such markers.

During the course of the study a number of additional anti TLR monoclonal antibodies have become commercially available, future studies would be well served by including these in a panel of reagents as they may well yield additional diagnostic and prognostic information. It would also be instructive to perform cell culture and stimulation studies on purified B cell populations from both patient and control groups in an attempt to explore the dynamics of TLR expression under a variety of conditions.

Although this study was restricted to patient groups with B CLL, the techniques that have been developed for elucidation of TLR expression and the gating strategies employed could equally be applied to the study of many different malignant haematological diseases, the acute leukaemias being an obvious choice, there are

however a potentially huge number of disease states that would bear close examination using this model.

The increasing use of monoclonal antibodies as therapeutic agents means that differential expression of cell surface antigens, such as TLR, will have to be monitored to establish the appropriateness and efficacy of such treatments. A natural extension to this study would involve investigation of patients on anti TLR drugs in order to monitor disease progression and provide a method for assessing areas of interest such as minimal residual disease and the risk of disease relapse when patients are in remission.

Although all of the above areas would require significant development of the techniques described in this study, the basic principles employed are sound and provide a robust foundation on which future studies can be based.

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Appendices

Name	Patient No.	DOB	Gender	WBC	CD5/19 %	Score	Abs lymph	Lymph %	DAG
EL	1	23/07/1926	MALE	90.7	94	4	77.6	86	N
MT	2	02/09/1937	MALE	12	82	5	8.1	67.1	P
RH	3	30/08/1949	MALE	22.6	88	5	15.1	67	P
UB	4	15/04/1930	FEMALE	14.6	50	5	8.47	58	N
CC	5	16/09/1941	MALE	24.2	87	5	22.3	92	N
JD	6	31/03/1934	MALE	28.4	84	5	19.7	69.4	P
PM	7	05/09/1944	MALE	14.8	56	4	7.6	51	P
DB	8	22/03/1940	FEMALE	19.9	65	4	15.6	78.3	N
GB	9	08/12/1951	MALE	23.6	81	4	19.8	84	N
MH	10	28/09/1938	MALE	54.9	97	5	33.77	61.5	N
JW	11	12/03/1946	MALE	10	61	5	5.76	57.5	P
JM	12	08/11/1936	FEMALE	130.6	84	3	157.89	95	N
DR	13	01/03/1934	FEMALE	9.7	49	4	5.48	56.3	N
DD	14	31/07/1941	MALE	9.1	81	4	4.73	52.3	N
AL	15	15/03/1946	FEMALE	17.2	58	4	10.18	59.3	N
LB	16	06/06/1926	MALE	35.7	93	5	29.5	82.8	P
JR	17	01/11/1945	MALE	12.7	70	5	7.9	62.2	N
BL	18	24/11/1943	MALE	16.4	76	5	10.3	63	N
AS	19	16/04/1940	FEMALE	19.3	58	5	14.67	76	P
JT	20	21/05/1942	MALE	13.6	51	4	5.03	37	N
CS	21	20/05/1941	FEMALE	66.4	91	4	61.75	93	N
WS	22	08/03/1935	MALE	32.5	88	5	68.3	71	N
SJ	23	13/06/1936	FEMALE	9.4	16	5	4.87	51.6	N
WH	24	01/03/1938	FEMALE	23.4	51	5	18.87	80.7	N
IB	25	27/09/1960	MALE	15.3	65	4	9.66	62.9	P
JA	26	21/06/1932	FEMALE	16.8	42	4	10.49	62.6	N
MY	27	27/01/1940	MALE	65.7	94	5	58.44	89	P
ST	28	19/07/1953	MALE	17	71	5	11.67	68.6	P
JL	29	23/06/1930	FEMALE	13.5	57	5	6.38	47.2	P
GH	30	04/04/1938	MALE	12.9	57	4	6.08	47	N
JS	31	15/02/1934	MALE	42.7	92	4	39.71	93	N
JB	32	14/10/1943	MALE	14.7	80	5	9.88	67.4	N
CB	33	28/12/1927	FEMALE	28.9	88	5	27.1	93.8	N
JS	34	22/09/1922	FEMALE	6.6	58	5	2.75	41.9	N
WD	35	25/08/1925	MALE	76.9	91	5	74.3	96.6	P
PT	36	13/12/1948	MALE	28.9	86	4	22.6	78.4	P
LC	37	06/04/1939	FEMALE	6.6	81	5	1.13	17.2	P
HP	38	19/02/1944	MALE	80.9	95	5	77.7	96	N
JB	39	17/07/1930	FEMALE	16.4	33	4	9382	60.1	N
MW	40	21/09/1939	MALE	12.9	65	4	6.63	51.2	N
WS	41	13/02/1943	MALE	47.4	89	5	43.6	92	N
GL	42	12/05/1932	MALE	19.6	91	5	15.37	78.3	N
DH	43	11/02/1938	MALE	21.4	85	4	14.94	69.6	P
AK	44	23/03/1939	MALE	66.3	97	3	60	91	N
TM	45	09/03/1935	MALE	44.1	90	5	35.69	80.9	N
MB	46	23/08/1925	MALE	13.8	76	5	10.21	74	N

CM	47	01/06/1932	MALE	14	85	4	8.44	75.8	N
LW	48	01/05/1943	FEMALE	247.8	89	5	237.89	96	N
BP	49	02/02/1947	MALE	116.5	96	5	104.85	90	N
BB	50	10/11/1929	FEMALE	18.3	79	4	11.16	60.4	N
JB	51	09/11/1938	MALE	12.8	23	4	7.03	54.9	N
DJ	52	11/02/1930	MALE	9.4	29	4	6.49	68.7	P
MK	53	05/03/1941	FEMALE	341.4	97	5	331.16	97	N
NS	54	26/06/1931	MALE	9.1	72	5	4.39	48.4	P
ZR	55	14/10/1938	FEMALE	10.5	19	5	5.87	56.2	N
PR	56	05/08/1944	FEMALE	11.1	82	3	7.33	65.9	N
SB	57	04/10/1916	FEMALE	10.8	71	4	6.36	58.9	N
CV	58	05/05/1941	MALE	75.4	95	4	66.35	88	N
CN	59	02/05/1950	MALE	5.8	86	5	2.12	56.3	N
TC	60	27/11/1951	MALE	33.3	91	5	25.96	77.9	N
OF	61	10/04/1954	MALE	8.2	46	5	4.86	59.2	N
JNF	62	05/07/1937	FEMALE	277	83	4	268.69	97	N
SH	63	26/07/1956	FEMALE	13.6	44	4	7.83	58.1	P
KV	64	12/08/1930	MALE	9.5	36	4	1.7	18	N
AW	65	26/07/1921	FEMALE	91.4	81	5	83.16	91	P
MD	66	30/04/1941	FEMALE	8.4	45	5	10.28	74.8	N
PW	67	26/03/1930	FEMALE	17.6	77	3	8.65	49.2	N
MB	68	29/11/1917	MALE	9.8	60	5	4.01	41	N
CM	69	06/07/1934	MALE	59.3	95	5	52.16	88	N
AS	70	03/05/1924	MALE	10.8	30	4	1.7	38.7	N
JH	71	01/11/1932	FEMALE	13.2	66	4	7.69	58.2	N
GH	72	16/11/1942	MALE	15.6	85	4	8.25	52.9	N
DW	73	04/02/1928	MALE	17.7	78	5	11.08	62.7	N
JM	74	19/04/1960	FEMALE	18.3	70	5	13.16	71.8	N
KP	75	10/04/1951	FEMALE	15.8	66	4	11.46	72.8	N
SW	76	17/05/1930	MALE	20.1	71	4	13.49	67.2	N
SF	77	06/07/1945	FEMALE	22.3	86	5	16.56	74.1	P
PK	78	18/05/1951	MALE	11.9	71	4	7.24	60.7	N
PD	79	15/09/1944	MALE	29.4	94	4	27.55	93.8	N
JT	80	05/03/1940	FEMALE	11.2	49	4	6.67	59.5	N
NR	81	25/02/1943	MALE	49.6	93	3	41.48	83.7	N
TT	82	11/09/1928	FEMALE	7.6	29	5	4.03	53.2	P
MC	83	12/12/1923	FEMALE	11.6	63	4	6.44	55.5	P
JB	84	03/07/1942	MALE	159.2	59	4	154.42	97	N
CE	85	06/04/1959	FEMALE	85.1	87	5	76.17	89.5	N
MY	86	25/01/1940	MALE	5.9	80	4	0.79	20.6	N
JR	87	29/09/1946	MALE	33.4	86	4	33.03	83	N
MH	88	06/04/1948	MALE	27.4	80	5	29.92	85	N
SH	89	14/04/1936	FEMALE	64.5	90	4	54.83	85	N
DR	90	01/03/1934	FEMALE	9.8	40	4	5.2	53.1	N
JJ	91	02/05/1947	FEMALE	14.2	23	5	1.4	26	N
FT	92	01/09/1937	MALE	10.2	64	5	5.31	52.1	N
JS	93	19/06/1959	FEMALE	7.5	17	5	2.97	39.6	N
EW	94	19/04/1936	FEMALE	19	54	4	10.39	54.7	N
DB	95	04/01/1946	MALE	14.7	73	4	7.14	48.6	N
FH	96	01/11/1920	MALE	25.3	87	4	12.47	49.3	N
PT	97	13/12/1948	MALE	85.9	92	4	85.9	100	N
BM	98	01/03/1952	MALE	15	74	3	9.36	62.4	P
JP	99	24/06/1938	FEMALE	12	50	4	5.87	48.9	N
PE	100	01/04/1948	MALE	21.8	88	5	13.04	59.8	P

AG	101	28/11/1948	MALE	33.4	85	5	29.06	87	N
MJ	102	17/11/1935	FEMALE	58.5	93	4	52.65	90	P
JF	103	19/03/1943	MALE	26.7	85	4	20.32	76.1	N
EB	104	15/11/1944	FEMALE	145.3	97	4	140.94	97	N
AE	105	14/09/1924	MALE	26.7	81	5	19.28	72.2	N
MR	106	18/03/1933	MALE	41.3	62	4	36.8	89.1	N
JH	107	14/02/1945	FEMALE	125.4	95	3	110.35	88	N
JN	108	19/08/1941	MALE	13.9	62	5	9.81	70.6	N
SP	109	17/02/1982	MALE	44.2	82	5	41.5	93.9	N
HS	110	24/08/1929	MALE	14.1	75	4	9.94	70.5	N
MM	11	27/02/1937	MALE	14.9	85	4	11.13	74.7	N
WK	112	19/06/1921	MALE	39.6	98	5	32.79	82.8	N
EW	113	20/01/1941	MALE	23.8	92	5	19.4	81.5	N
MM	114	16/04/1947	MALE	68.8	95	4	67.42	98	N
MS	115	17/12/1942	MALE	30.9	76	5	23.18	75	N
DB	116	10/10/1934	MALE	14.1	79	4	9.35	66.3	N
WM	117	23/09/1931	MALE	21.1	93	5	20.13	95.4	N
EB	118	24/10/1949	MALE	15.5	79	4	8.93	57.6	P
SC	119	19/03/1945	MALE	12.3	84	4	8.27	67.2	N
RR	120	30/12/1926	MALE	95.8	97	4	90.05	94	N
DT	121	29/06/1943	MALE	21	67	3	16.49	78.5	N
DD	122	05/10/1926	MALE	59.2	94	5	52.87	89.3	N
VW	123	11/01/1943	FEMALE	34.3	70	5	28.74	83.8	N
AP	124	29/08/1954	MALE	22.7	76	4	16.34	72	P
FH	125	16/05/1933	FEMALE	17.7	83	5	10.85	61.3	N
AH	126	30/04/1929	MALE	15.1	63	5	13.74	91	N
RH	127	30/12/1938	FEMALE	20.7	92	4	16.39	79.2	N
DT	128	22/03/1928	MALE	15.5	53	4	9.13	58.9	N
JB	129	15/12/1926	FEMALE	47.1	78	5	46.21	98.1	P

Royal Cornwall Hospitals NHS

NHS Trust

Research and Development Directorate

R&D Director	01872 256420
RDSU Co-ordinator	01872 256417
R&D Senior Manager	01872 256421
Clinical Trials Co-ordinator	01872 256423
R&D Personal Assistant	01872 256428
Research Information & Data Officer	01872 256427

The Knowledge Spa
Royal Cornwall Hospitals NHS Trust
Truro
Cornwall
TR1 3HD

17 October 2006

Mr Nigel Oakes
Chief Biomedical Scientist
Haematology Laboratory
RCHT

Dear Mr Oakes

Re: **2006.SPE.HAE.05 An investigation into the expression of toll like receptors in patients with chronic lymphocytic leukaemia and their associations with disease stage, prognosis and associated autoimmune disease.**

I am pleased to confirm Professor Anthony Woolf, Research & Development Director has granted Trust approval for the above study.

Please keep the R&D Directorate informed of your progress to allow accurate submissions to the National Research Register and to the Department of Health in our Annual Report. Please inform me of any changes or deviations from the research protocol, as this may need ethical and Trust approval again.

You must conduct the research in accordance with Good Clinical Practice and the Department of Health Research Governance Framework for Health & Social Care (2005). For further information, please contact the Research & Development Directorate or visit http://www.dh.gov.uk

Good luck with your project.

Yours sincerely.

Cathryn Love-Rouse
Research and Development Senior Manager

Copy to: Sharon Northey, Research Information & Data Officer

Fully Accredited

Cornwall Local Research Ethics Committee (LREC)
The Knowledge Spa
Royal Cornwall Hospitals NHS Trust
Truro, Cornwall TR1 3HD
Tel: 01872 256422
E-mail amanda.datson@rcht.cornwall.nhs.uk

October 5th 2006

Mr Nigel Oakes
Haematology
RCHT

Dear Mr Oakes

Full title of project: Chronic Lymphocytic Leukaemia Patients'Study
REC reference: 71.6.06

Thank you for seeking the Committee's advice about the above project.

You provided the following documents for consideration:

E-mail 26/5/06

These documents have been considered by the Chair, who has advised that the project is not one that is required to be ethically reviewed under the terms of the Governance Arrangements for Research Ethics Committees in the UK. He is satisfied for you to continue your research providing permission has been sought to use the samples.

Although review by a Research Ethics Committee is not required, you should check with the R&D Department for RCHT whether management approval is required before the project starts.

Yours sincerely

Mrs Amanda Datson
Cornwall REC Co-ordinator

Copy to: Cathryn Love-Rouse, R&D Department,RCHT

	0 hours	4 hours	8 hours	12 hours	24 hours
patient 1	52.7	52.2	50.4	48.9	45.5
patient 2	46.8	46.6	45.3	44.5	42.6
patient 3	51.1	51.3	50.6	49.5	46.4
patient 4	54.4	53.7	52.4	50.2	48.6
patient 5	67.3	66.4	64.4	63.2	61.1
MEAN	54.46	54.04	52.62	51.26	48.84
SEM	3.4	3.3	3.2	3.1	3.2
control 1	77.4	75.6	75	72.2	71.8
control 2	69.3	68.3	66.7	64.6	64.3
control 3	63.5	60.3	58.3	60.3	56.3
control 4	79.8	77.2	77.1	75	72.5
control 5	81.5	80.4	78.4	76.6	74.4
MEAN	74.3	72.36	71.1	69.74	67.86
SEM	3.4	3.6	3.8	3.1	3.4

Appendix 3:Time delay study results
% TLR 1 expression on B lymphocytes shown at time 0 and at 4hrs, 8 hrs, 12 hrs and 24 hrs post collection.

APPENDIX IV) Statistical analysis of TLR expression data (SPSS analysis)

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	Control or Patient	N	Percent	N	Percent	N	Percent
TLR1Bcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR2Bcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR3Bcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR4Bcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR9Bcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TRL1Tcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR2Tcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR3Tcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR4Tcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR9Tcell	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR1Mono	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR2Mono	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR3Mono	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR4Mono	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%
TLR9Mono	Control	129	100.0%	0	.0%	129	100.0%
	Patient	129	100.0%	0	.0%	129	100.0%

Descriptives

	Control or Patient			Statistic	Std. Error
TLR1Bcell	Control	Mean		71.852	.6312
		95% Confidence Interval for Mean	Lower Bound	70.603	
			Upper Bound	73.101	
		5% Trimmed Mean		71.818	
		Median		71.700	
		Variance		51.401	
		Std. Deviation		7.1694	
		Minimum		56.0	
		Maximum		89.3	
		Range		33.3	
		Interquartile Range		10.1	
		Skewness		.044	.213
		Kurtosis		-.546	.423
	Patient	Mean		55.367	.9961
		95% Confidence Interval for Mean	Lower Bound	53.397	
			Upper Bound	57.338	
		5% Trimmed Mean		55.399	
		Median		54.400	
		Variance		127.984	
		Std. Deviation		11.3130	
		Minimum		23.2	
		Maximum		83.2	
		Range		60.0	
		Interquartile Range		18.6	
		Skewness		.008	.213
		Kurtosis		-.030	.423
TLR2Bcell	Control	Mean		18.118	.2368
		95% Confidence Interval for Mean	Lower Bound	17.649	
			Upper Bound	18.586	
		5% Trimmed Mean		18.112	
		Median		18.300	
		Variance		7.236	
		Std. Deviation		2.6901	
		Minimum		9.7	
		Maximum		27.2	
		Range		17.5	
		Interquartile Range		3.1	

		Skewness		.077	.213
		Kurtosis		1.148	.423
	Patient	Mean		11.236	.4836
		95% Confidence Interval for Mean	Lower Bound	10.279	
			Upper Bound	12.192	
		5% Trimmed Mean		11.008	
		Median		11.100	
		Variance		30.166	
		Std. Deviation		5.4923	
		Minimum		1.2	
		Maximum		32.1	
		Range		30.9	
		Interquartile Range		7.3	
		Skewness		.723	.213
		Kurtosis		.860	.423
TLR3Bcell	Control	Mean		.047	.0086
		95% Confidence Interval for Mean	Lower Bound	.029	
			Upper Bound	.064	
		5% Trimmed Mean		.032	
		Median		.000	
		Variance		.010	
		Std. Deviation		.0977	
		Minimum		.0	
		Maximum		.6	
		Range		.6	
		Interquartile Range		.1	
		Skewness		2.680	.213
		Kurtosis		8.916	.423
	Patient	Mean		9.684	.3838
		95% Confidence Interval for Mean	Lower Bound	8.925	
			Upper Bound	10.444	
		5% Trimmed Mean		9.529	
		Median		9.000	
		Variance		19.004	
		Std. Deviation		4.3593	
		Minimum		.7	
		Maximum		23.2	
		Range		22.5	
		Interquartile Range		6.3	
		Skewness		.527	.213

		Kurtosis		.160	.423
TLR4Bcell	Control	Mean		.055	.0103
		95% Confidence Interval for Mean	Lower Bound	.035	
			Upper Bound	.075	
		5% Trimmed Mean		.038	
		Median		.000	
		Variance		.014	
		Std. Deviation		.1166	
		Minimum		.0	
		Maximum		.5	
		Range		.5	
		Interquartile Range		.0	
		Skewness		2.176	.213
		Kurtosis		3.992	.423
	Patient	Mean		6.815	.3399
		95% Confidence Interval for Mean	Lower Bound	6.142	
			Upper Bound	7.487	
		5% Trimmed Mean		6.677	
		Median		6.600	
		Variance		14.907	
		Std. Deviation		3.8610	
		Minimum		.2	
		Maximum		17.5	
		Range		17.3	
		Interquartile Range		4.8	
		Skewness		.430	.213
		Kurtosis		-.089	.423
TLR9Bcell	Control	Mean		70.481	1.2331
		95% Confidence Interval for Mean	Lower Bound	68.042	
			Upper Bound	72.921	
		5% Trimmed Mean		71.292	
		Median		72.000	
		Variance		196.135	
		Std. Deviation		14.0048	
		Minimum		23.0	
		Maximum		94.4	
		Range		71.4	
		Interquartile Range		16.9	
		Skewness		-.931	.213
		Kurtosis		1.312	.423

	Patient	Mean		78.077	1.2534
		95% Confidence Interval for Mean	Lower Bound	75.597	
			Upper Bound	80.557	
		5% Trimmed Mean		79.757	
		Median		81.400	
		Variance		202.652	
		Std. Deviation		14.2356	
		Minimum		23.2	
		Maximum		96.4	
		Range		73.2	
		Interquartile Range		13.8	
		Skewness		-1.824	.213
		Kurtosis		3.884	.423
TRL1Tcell	Control	Mean		1.192	.0645
		95% Confidence Interval for Mean	Lower Bound	1.065	
			Upper Bound	1.320	
		5% Trimmed Mean		1.135	
		Median		1.100	
		Variance		.537	
		Std. Deviation		.7328	
		Minimum		.1	
		Maximum		5.5	
		Range		5.4	
		Interquartile Range		.9	
		Skewness		2.023	.213
		Kurtosis		8.627	.423
	Patient	Mean		2.759	.0786
		95% Confidence Interval for Mean	Lower Bound	2.603	
			Upper Bound	2.914	
		5% Trimmed Mean		2.744	
		Median		2.700	
		Variance		.796	
		Std. Deviation		.8924	
		Minimum		.7	
		Maximum		5.2	
		Range		4.5	
		Interquartile Range		1.2	
		Skewness		.309	.213
		Kurtosis		-.141	.423
TLR2Tcell	Control	Mean		1.387	.0655

		95% Confidence Interval for Mean	Lower Bound	1.257	
			Upper Bound	1.517	
		5% Trimmed Mean		1.353	
		Median		1.200	
		Variance		.554	
		Std. Deviation		.7444	
		Minimum		.2	
		Maximum		3.3	
		Range		3.1	
		Interquartile Range		1.0	
		Skewness		.649	.213
		Kurtosis		-.168	.423
	Patient	Mean		8.430	.2426
		95% Confidence Interval for Mean	Lower Bound	7.950	
			Upper Bound	8.910	
		5% Trimmed Mean		8.459	
		Median		8.400	
		Variance		7.593	
		Std. Deviation		2.7555	
		Minimum		1.2	
		Maximum		15.3	
		Range		14.1	
		Interquartile Range		3.1	
		Skewness		-.231	.213
		Kurtosis		.414	.423
TLR3Tcell	Control	Mean		.184	.0214
		95% Confidence Interval for Mean	Lower Bound	.142	
			Upper Bound	.227	
		5% Trimmed Mean		.151	
		Median		.100	
		Variance		.059	
		Std. Deviation		.2435	
		Minimum		.0	
		Maximum		1.1	
		Range		1.1	
		Interquartile Range		.3	
		Skewness		1.846	.213
		Kurtosis		3.619	.423
	Patient	Mean		1.744	.0666
		95% Confidence Interval for	Lower Bound	1.612	

		Mean	Upper Bound	1.876	
		5% Trimmed Mean		1.697	
		Median		1.700	
		Variance		.573	
		Std. Deviation		.7569	
		Minimum		.3	
		Maximum		6.2	
		Range		5.9	
		Interquartile Range		.7	
		Skewness		1.982	.213
		Kurtosis		9.909	.423
TLR4Tcell	Control	Mean		.591	.0347
		95% Confidence Interval for	Lower Bound	.522	
		Mean	Upper Bound	.659	
		5% Trimmed Mean		.578	
		Median		.600	
		Variance		.155	
		Std. Deviation		.3938	
		Minimum		.0	
		Maximum		1.8	
		Range		1.8	
		Interquartile Range		.6	
		Skewness		.300	.213
		Kurtosis		-.468	.423
	Patient	Mean		7.263	.2615
		95% Confidence Interval for	Lower Bound	6.745	
		Mean	Upper Bound	7.780	
		5% Trimmed Mean		7.119	
		Median		6.900	
		Variance		8.820	
		Std. Deviation		2.9699	
		Minimum		1.4	
		Maximum		24.1	
		Range		22.7	
		Interquartile Range		3.1	
		Skewness		1.457	.213
		Kurtosis		7.304	.423
TLR9Tcell	Control	Mean		11.978	.4583
		95% Confidence Interval for	Lower Bound	11.071	
		Mean	Upper Bound	12.884	

		5% Trimmed Mean		11.856	
		Median		12.100	
		Variance		27.098	
		Std. Deviation		5.2056	
		Minimum		.6	
		Maximum		28.4	
		Range		27.8	
		Interquartile Range		6.9	
		Skewness		.422	.213
		Kurtosis		.495	.423
	Patient	Mean		19.911	.3258
		95% Confidence Interval for	Lower Bound	19.266	
		Mean	Upper Bound	20.555	
		5% Trimmed Mean		20.038	
		Median		19.700	
		Variance		13.690	
		Std. Deviation		3.7000	
		Minimum		9.8	
		Maximum		27.5	
		Range		17.7	
		Interquartile Range		4.6	
		Skewness		-.399	.213
		Kurtosis		.289	.423
TLR1Mono	Control	Mean		74.126	.8898
		95% Confidence Interval for	Lower Bound	72.365	
		Mean	Upper Bound	75.886	
		5% Trimmed Mean		74.349	
		Median		74.700	
		Variance		102.135	
		Std. Deviation		10.1062	
		Minimum		45.8	
		Maximum		93.2	
		Range		47.4	
		Interquartile Range		13.5	
		Skewness		-.302	.213
		Kurtosis		-.284	.423
	Patient	Mean		75.518	.6902
		95% Confidence Interval for	Lower Bound	74.152	
		Mean	Upper Bound	76.883	
		5% Trimmed Mean		75.803	

		Median		77.600	
		Variance		61.444	
		Std. Deviation		7.8386	
		Minimum		48.9	
		Maximum		92.2	
		Range		43.3	
		Interquartile Range		9.9	
		Skewness		-.592	.213
		Kurtosis		.493	.423
TLR2Mono	Control	Mean		83.772	.6983
		95% Confidence Interval for Mean	Lower Bound	82.390	
			Upper Bound	85.154	
		5% Trimmed Mean		84.266	
		Median		84.800	
		Variance		62.899	
		Std. Deviation		7.9309	
		Minimum		56.9	
		Maximum		96.4	
		Range		39.5	
		Interquartile Range		10.9	
		Skewness		-.887	.213
		Kurtosis		.894	.423
	Patient	Mean		81.398	.8878
		95% Confidence Interval for Mean	Lower Bound	79.641	
			Upper Bound	83.154	
		5% Trimmed Mean		82.238	
		Median		84.000	
		Variance		101.679	
		Std. Deviation		10.0836	
		Minimum		45.5	
		Maximum		96.4	
		Range		50.9	
		Interquartile Range		11.0	
		Skewness		-1.288	.213
		Kurtosis		1.569	.423
TLR3Mono	Control	Mean		25.166	.4994
		95% Confidence Interval for Mean	Lower Bound	24.178	
			Upper Bound	26.154	
		5% Trimmed Mean		25.227	
		Median		25.500	

		Variance		32.171	
		Std. Deviation		5.6719	
		Minimum		9.6	
		Maximum		37.2	
		Range		27.6	
		Interquartile Range		8.9	
		Skewness		-.160	.213
		Kurtosis		-.580	.423
	Patient	Mean		26.409	.4255
		95% Confidence Interval for Mean	Lower Bound	25.567	
			Upper Bound	27.250	
		5% Trimmed Mean		26.530	
		Median		27.400	
		Variance		23.351	
		Std. Deviation		4.8323	
		Minimum		13.7	
		Maximum		34.7	
		Range		21.0	
		Interquartile Range		6.4	
		Skewness		-.511	.213
		Kurtosis		-.574	.423
TLR4Mono	Control	Mean		67.576	.6841
		95% Confidence Interval for Mean	Lower Bound	66.222	
			Upper Bound	68.929	
		5% Trimmed Mean		67.470	
		Median		67.500	
		Variance		60.363	
		Std. Deviation		7.7693	
		Minimum		45.2	
		Maximum		87.5	
		Range		42.3	
		Interquartile Range		9.4	
		Skewness		.093	.213
		Kurtosis		.055	.423
	Patient	Mean		68.744	.6380
		95% Confidence Interval for Mean	Lower Bound	67.482	
			Upper Bound	70.007	
		5% Trimmed Mean		68.603	
		Median		68.500	
		Variance		52.510	

		Std. Deviation		7.2464	
		Minimum		52.9	
		Maximum		91.0	
		Range		38.1	
		Interquartile Range		8.1	
		Skewness		.292	.213
		Kurtosis		.423	.423
TLR9Mono	Control	Mean		92.726	.3504
		95% Confidence Interval for Mean	Lower Bound	92.033	
			Upper Bound	93.420	
		5% Trimmed Mean		93.070	
		Median		93.700	
		Variance		15.835	
		Std. Deviation		3.9794	
		Minimum		75.5	
		Maximum		98.5	
		Range		23.0	
		Interquartile Range		4.2	
		Skewness		-1.611	.213
		Kurtosis		3.298	.423
	Patient	Mean		91.089	.4100
		95% Confidence Interval for Mean	Lower Bound	90.278	
			Upper Bound	91.900	
		5% Trimmed Mean		91.458	
		Median		92.200	
		Variance		21.685	
		Std. Deviation		4.6568	
		Minimum		68.8	
		Maximum		98.5	
		Range		29.7	
		Interquartile Range		4.8	
		Skewness		-1.574	.213
		Kurtosis		4.208	.423

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Control or Patient	Statistic	df	Sig.	Statistic	df	Sig.
TLR1Bcell	Control	.044	129	.200*	.990	129	.438
	Patient	.081	129	.039	.985	129	.158
TLR2Bcell	Control	.063	129	.200*	.986	129	.229
	Patient	.066	129	.200*	.967	129	.003
TLR3Bcell	Control	.435	129	.000	.546	129	.000
	Patient	.081	129	.038	.978	129	.037
TLR4Bcell	Control	.457	129	.000	.540	129	.000
	Patient	.058	129	.200*	.974	129	.015
TLR9Bcell	Control	.107	129	.001	.948	129	.000
	Patient	.155	129	.000	.829	129	.000
TRL1Tcell	Control	.124	129	.000	.864	129	.000
	Patient	.099	129	.004	.981	129	.069
TLR2Tcell	Control	.126	129	.000	.953	129	.000
	Patient	.079	129	.047	.977	129	.030
TLR3Tcell	Control	.224	129	.000	.753	129	.000
	Patient	.134	129	.000	.861	129	.000
TLR4Tcell	Control	.105	129	.001	.960	129	.001
	Patient	.092	129	.010	.908	129	.000
TLR9Tcell	Control	.051	129	.200*	.986	129	.219
	Patient	.093	129	.008	.975	129	.016
TLR1Mono	Control	.065	129	.200*	.984	129	.132
	Patient	.109	129	.001	.974	129	.015
TLR2Mono	Control	.090	129	.012	.944	129	.000
	Patient	.135	129	.000	.895	129	.000
TLR3Mono	Control	.067	129	.200*	.987	129	.245
	Patient	.116	129	.000	.954	129	.000
TLR4Mono	Control	.052	129	.200*	.991	129	.545
	Patient	.079	129	.048	.982	129	.077
TLR9Mono	Control	.161	129	.000	.866	129	.000
	Patient	.134	129	.000	.892	129	.000

a. Lilliefors Significance Correction

*. This is a lower bound of the true significance.

Group Statistics

	Control or Patient	N	Mean	Std. Deviation	Std. Error Mean
TLR1Bcell	Control	129	71.852	7.1694	.6312
	Patient	129	55.367	11.3130	.9961
TLR2Bcell	Control	129	18.118	2.6901	.2368
	Patient	129	11.236	5.4923	.4836
TLR9Tcell	Control	129	11.978	5.2056	.4583
	Patient	129	19.911	3.7000	.3258
TLR1Mono	Control	129	74.126	10.1062	.8898
	Patient	129	75.518	7.8386	.6902
TLR3Mono	Control	129	25.166	5.6719	.4994
	Patient	129	26.409	4.8323	.4255
TLR4Mono	Control	129	67.576	7.7693	.6841
	Patient	129	68.744	7.2464	.6380

Independent Samples Test

		Levene's Test for Equality of Variances	
		F	Sig.
TLR1Bcell	Equal variances assumed	16.391	.000
	Equal variances not assumed		
TLR2Bcell	Equal variances assumed	44.584	.000
	Equal variances not assumed		
TLR9Tcell	Equal variances assumed	11.906	.001
	Equal variances not assumed		
TLR1Mono	Equal variances assumed	9.098	.003
	Equal variances not assumed		
TLR3Mono	Equal variances assumed	4.373	.037
	Equal variances not assumed		
TLR4Mono	Equal variances assumed	.918	.339
	Equal variances not assumed		

Independent Samples Test

		t-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
TLR1Bcell	Equal variances assumed	13.979	256	.000	16.4845
	Equal variances not assumed	13.979	216.534	.000	16.4845
TLR2Bcell	Equal variances assumed	12.781	256	.000	6.8822
	Equal variances not assumed	12.781	186.070	.000	6.8822
TLR9Tcell	Equal variances assumed	-14.109	256	.000	-7.9333
	Equal variances not assumed	-14.109	231.033	.000	-7.9333
TLR1Mono	Equal variances assumed	-1.236	256	.217	-1.3922
	Equal variances not assumed	-1.236	241.082	.218	-1.3922
TLR3Mono	Equal variances assumed	-1.894	256	.059	-1.2426
	Equal variances not assumed	-1.894	249.699	.059	-1.2426
TLR4Mono	Equal variances assumed	-1.249	256	.213	-1.1682
	Equal variances not assumed	-1.249	254.767	.213	-1.1682

Independent Samples Test

		t-test for Equality of Means		
		Std. Error Difference	95% Confidence Interval of the Difference	
			Lower	Upper
TLR1Bcell	Equal variances assumed	1.1792	14.1623	18.8067
	Equal variances not assumed	1.1792	14.1603	18.8087
TLR2Bcell	Equal variances assumed	.5385	5.8218	7.9425
	Equal variances not assumed	.5385	5.8199	7.9444
TLR9Tcell	Equal variances assumed	.5623	-9.0407	-6.8260
	Equal variances not assumed	.5623	-9.0412	-6.8254
TLR1Mono	Equal variances assumed	1.1261	-3.6098	.8253

	Equal variances not assumed	1.1261	-3.6105	.8260
TLR3Mono	Equal variances assumed	.6560	-2.5346	.0493
	Equal variances not assumed	.6560	-2.5347	.0495
TLR4Mono	Equal variances assumed	.9354	-3.0103	.6739
	Equal variances not assumed	.9354	-3.0103	.6739

Mann-Whitney Test

Ranks

	Control or Patient	N	Mean Rank	Sum of Ranks
TLR3Bcell	Control	129	65.00	8385.00
	Patient	129	194.00	25026.00
	Total	258		
TLR4Bcell	Control	129	65.17	8407.00
	Patient	129	193.83	25004.00
	Total	258		
TLR9Bcell	Control	129	104.78	13516.00
	Patient	129	154.22	19895.00
	Total	258		
TRL1Tcell	Control	129	74.89	9660.50
	Patient	129	184.11	23750.50
	Total	258		
TLR2Tcell	Control	129	66.34	8558.50
	Patient	129	192.66	24852.50
	Total	258		
TLR3Tcell	Control	129	66.19	8538.00
	Patient	129	192.81	24873.00
	Total	258		
TLR4Tcell	Control	129	65.04	8390.00
	Patient	129	193.96	25021.00
	Total	258		
TLR2Mono	Control	129	137.37	17721.00
	Patient	129	121.63	15690.00
	Total	258		
TLR9Mono	Control	129	146.31	18874.50
	Patient	129	112.69	14536.50

Ranks

	Control or Patient	N	Mean Rank	Sum of Ranks
TLR3Bcell	Control	129	65.00	8385.00
	Patient	129	194.00	25026.00
	Total	258		
TLR4Bcell	Control	129	65.17	8407.00
	Patient	129	193.83	25004.00
	Total	258		
TLR9Bcell	Control	129	104.78	13516.00
	Patient	129	154.22	19895.00
	Total	258		
TRL1Tcell	Control	129	74.89	9660.50
	Patient	129	184.11	23750.50
	Total	258		
TLR2Tcell	Control	129	66.34	8558.50
	Patient	129	192.66	24852.50
	Total	258		
TLR3Tcell	Control	129	66.19	8538.00
	Patient	129	192.81	24873.00
	Total	258		
TLR4Tcell	Control	129	65.04	8390.00
	Patient	129	193.96	25021.00
	Total	258		
TLR2Mono	Control	129	137.37	17721.00
	Patient	129	121.63	15690.00
	Total	258		
TLR9Mono	Control	129	146.31	18874.50
	Patient	129	112.69	14536.50
	Total	258		

Test Statistics[a]

	TLR3Bcell	TLR4Bcell	TLR9Bcell	TRL1Tcell	TLR2Tcell	TLR3Tcell
Mann-Whitney U	.000	22.000	5131.000	1275.500	173.500	153.000
Wilcoxon W	8385.000	8407.000	13516.000	9660.500	8558.500	8538.000
Z	-14.270	-14.270	-5.322	-11.763	-13.597	-13.707
Asymp. Sig. (2-tailed)	.000	.000	.000	.000	.000	.000

Test Statistics[a]

	TLR4Tcell	TLR2Mono	TLR9Mono
Mann-Whitney U	5.000	7305.000	6151.500
Wilcoxon W	8390.000	15690.000	14536.500
Z	-13.884	-1.695	-3.620
Asymp. Sig. (2-tailed)	.000	.090	.000