

**THE IMMUNE RESPONSE TO CYTOMEGALOVIRUS AND  
EPSTEIN-BARR VIRUS IN SYSTEMIC LUPUS ERYTHEMATOSUS**

**by**

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## **Abstract**

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown aetiology. Both genetic and environmental factors are known to contribute to disease development. Pathogenesis involves the production of autoantibodies, and the formation of immune complexes, leading to inflammation and destruction of autologous tissue. SLE is a heterogeneous disease both longitudinally and between affected individuals, and is characterised by periods of exacerbation, known as flares, and periods of remission. The ubiquitous human herpes viruses, cytomegalovirus (HCMV), and Epstein-Barr virus (EBV) have been associated with disease by a variety of mechanisms.

Data compiled here suggests SLE patients have elevated IgG responses to HCMV and EBV, but unlike healthy controls these responses do not accumulate with age. No association has been found between the carriage of these viruses, or the magnitude of response against these viruses, and any clinical measurements of disease activity. EBV load is 5.4 times higher in SLE patients than controls. Azathioprine treatment is associated with a 4.4 fold rise in EBV load, no other drugs show associations with EBV load. Among SLE patients EBV load is inversely correlated with CD8<sup>+</sup> T-cell IFN $\gamma$  responses, suggesting impaired T-cell responses are the cause of elevated load. HCMV seropositivity is associated with a 7-year delay in development of disease among SLE patients, and a reduction in plasma IFN $\alpha$  concentration.

## **Dedication**

This thesis is dedicated to Mr and Mrs Perks, for your unwavering faith in my abilities, or at least giving that impression. Also for Ted, for your constant support, and tolerance when times got tough. I am eternally grateful to you all. I'd never have made it to the end without your help.

Finally, for Sally Nall, for your unfailing enthusiasm in science, and helping me remember why I started this.

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# **INTRODUCTION AND REVIEW OF THE LITERATURE**

## **SLE**

Systemic Lupus Erythematosus (SLE) is a heterogenous autoimmune disease with unknown aetiology. As with other autoimmune diseases, pathogenesis is driven by immune targeting of autologous tissue. Disease activity and severity vary, both between individuals and longitudinally. Patients experience periods of activation, known as flares, and periods of remission. Despite advances in treatment the mortality rate of SLE patients remains up to three times higher than matched controls [1]. A European study of 1000 patients from 1990 to 2000 showed a 10-year survival of 90% [2], a huge improvement on the 5-year survival rate of 69% reported for 1949 to 1960 [3]. However, this reduction may also be due to changing criteria for SLE diagnosis[4]. SLE patients show a bimodal pattern of mortality. In individuals diagnosed less than 5 years ago, the primary causes are sepsis and disease activity. In those diagnosed over 5 years ago the main cause of mortality is atherosclerosis and associated cardiovascular disease[5].

SLE is a heterogenous disease in terms of symptoms and severity, both in cross section and longitudinally. This heterogeneity makes diagnosis a challenge, patients diagnosed since 2000 exhibited the earliest symptoms 9 months before diagnosis. Significant progress has been made in the time between onset of symptoms and diagnosis, which has improved from an average of 59 months in patients diagnosed before 1980 [6]. Because of this discrepancy, only the year, not the month or day, the patient was diagnosed is considered relevant in calculating disease duration. Diagnosis is based on a patient fulfilling a number of immunological and clinical features, and the ruling out of other conditions, which can mimic symptoms. Any organ and multiple organ systems can be involved. Typical symptoms can

include rash, fatigue, arthritis and arthralgia. More severe cases can include nephritis and neurological symptoms [5].

## **Epidemiology**

The prevalence of SLE varies between populations. In the US and Europe, incidence varies from 1 to 7.6 per 100,000 per year [7]. Prevalence has been reported to be as high as 206/100000 in Afro-Caribbean females [8]. Prevalence and severity generally are lower among patients of European descent, and greatest among African Americans and Afro-Caribbeans [9]. Prevalence and incidence depend on the method used to assess disease, and the method used to collect data. Community based studies give different rates to hospital based studies, as hospital based studies are less likely to identify those with very mild disease activity.

## **Risk factors**

Both genetic and environmental risk factors play a role in aetiology, as indicated by concordance rates of 25% in monozygotic twins and 2% in dizygotic twins [5]. Autoantibodies responsible for pathogenesis in SLE are also found in healthy people. It is not known why some individuals with these autoantibodies develop autoimmune disease and others do not. It is likely that a number of factors contribute, and that when a threshold number of factors have accumulated, autoimmunity becomes clinically relevant.

Environmental factors are also thought to contribute to SLE pathogenesis. Concordance rates indicate that genetics alone cannot be responsible for disease development.

## **Genetics**

### **HLA**

The first genetic association found with SLE was in the genes encoding the Human Leukocyte antigens (HLA). HLA molecules are the molecules used by cells to present peptides to T-cells for immune surveillance. They present to the immune system peptides within the cell and from the extracellular environment. T-cells can only recognise peptide in the context of MHC molecules, unlike B-cells and antibodies, which are capable of recognising free peptide. HLA molecules are divided into two sub-types, HLA class I is ubiquitously expressed on all nucleated cells and presents a selection of intracellular peptides to CD8+ T-cells. There are three different types of HLA I; HLA-A, HLA-B and HLA-C. The maternal and paternal allele of each of these three are presented on the surface of all nucleated cells, therefore there are up to 6 types of HLA class I molecule on the surface of a cell, each presenting intracellular peptides to circulating CD8+ T-cells. If a cell is infected with a virus, viral peptides are presented to the specific CD8+ T-cell, which will then kill the infected cell. The second type of MHC molecule; HLA II molecules, are found only on specialised immune cell types such as B-cells and dendritic cells. These cells are professional antigen presenting cells or APC. APCs take up exogenous antigen, both self and foreign, process it, and present via HLA II to CD4+ T-cells. HLA class II molecules, present these extracellular peptides to CD4+ T-cells. The HLA class II family is also comprised of three types; HLA-DR, HLA-DP and HLA-DQ. HLA molecules, of classes I, and II, are hugely variable in their composition and ability to bind peptides. Peptides are said to be 'HLA restricted' if they only bind to one allele of HLA molecule. Both HLA class I, and HLA class II genes have been shown to be associated with SLE. Certain HLA types are more common among SLE patients than

controls, in particular HLA-DRB1\*0301 and HLA-DRB1\*1501 and HLA-DQA1\*0501-HLA-DQB1\*0201 [10].

Certain HLA types have also been associated with specific autoantibodies [11]. For example, patients carrying HLA DRB1\*0402 are more likely to produce anti-cardiolipin antibodies, than other SLE patients. These associations between HLA type and SLE are indicative of T-cell involvement in development of pathogenesis.

### **Complement deficiencies**

SLE is a disease in which the deposition, and accumulation of immune complexes leads to inflammation and damage. As such, any genetic abnormality that impairs the clearance of immune complexes can predispose to SLE. This is particularly true with the early complement proteins, which are necessary to start the complement cascade, which allows complex clearance. Mis-sense mutations in the first complement protein common to all three types of cascade, C3, almost invariably lead to SLE [12].

### **Sex**

In common with other autoimmune diseases SLE shows a strong sex bias; prevalence is 10 times higher in females than males. Among autoimmune disease, only Sjogren's syndrome shows a stronger female bias. This sex bias is strongest during a childbearing years; juvenile SLE has a female to male ratio of 5.6: 1, and male patients are on average younger than female [13].

It has been suggested that the different susceptibility of males and females is due to the number of X chromosomes an individual carries. Individuals with Klinefelter's syndrome,

with the karyotype 47 XXY, appear to be at similar risk of developing SLE as 46 XX females [14]. DNA methylation of the inactive X-chromosome, allowing TLR activation may be involved in the increased risk. Female sex bias amongst SLE patients is strongest during the years in which females are fertile, leading to suggestions of an association with female hormones, such as oestrogen [15].

## **Environmental influences**

### **UV light**

Among the most widely studied environmental influences in the development of SLE is ultraviolet (UV) light. SLE flares are often preceded by sun exposure, amongst SLE patients living north of the arctic circle, flares are least common in January [16]. Furthermore, a photosensitive rash is amongst the American College of Rheumatology (ACR) criteria for defining SLE. Experimentally it has been shown that UVA and UVB exposure triggers skin lesions consistent with SLE disease, and the use of UVA and UVB sunscreens has a protective effect [17, 18]. Furthermore, use of a tanning bed by a previously healthy individual was linked to the development of SLE [19].

SLE-prone mice are more susceptible to DNA damage by UVA than non-SLE-prone mice [20], and male BXSB mice are susceptible to premature death on UV exposure [21].

Whole body UV irradiation of NZLB/W F1 mice results in antibodies binding to epidermal cell nuclei and the production of anti-DNA/DNA immune complexes [22]. It is possible that UV light is exacerbating disease by inhibiting DNA methylation. UV radiation de-methylates CD4<sup>+</sup> T-cell DNA causing autoreactivity and/via LFA-1 overexpression [23]. It is also possible that UV exposure increases apoptosis. UV irradiation of keratinocytes from SLE patients in-vitro increased the production of SLE associated antigens in apoptotic blebs [24].

### **Infectious agents**

Another widely studied environmental influence is the herpes virus Epstein-Barr virus (EBV), which is more commonly found in SLE patients than healthy individuals [25]. This will be discussed in more detail in the relevant sections.

### **HRT**

Risk of moderate flare is increased 1.34 times in menopausal women with lupus receiving hormone replacement therapy containing conjugated oestrogens and progesterone [26]. However, there was no effect on the frequency of severe flares in the 12 month period studied.

### **Drug induced SLE**

Some drugs have been implicated in the development of lupus-like autoimmunity. Procainamide and hydralazine have been associated with the development of a Lupus-like syndrome. Patients presenting with this condition usually have skin and joint involvement. Neurological or renal involvement is rare [Dubois 6<sup>th</sup> ed 2002;885-916]

### **Pathogenesis**

SLE is the result of type III hypersensitivity, in which antibody complexes are inappropriately formed and inefficiently cleared. In SLE antibodies are produced against a large range of nuclear and cytoplasmic antigens. Antibody complexes are deposited in tissues, causing inflammation and tissue damage. Some of these antigens are targeted in other autoimmune diseases, whereas others are considered to be specific for SLE, such as antibodies against double stranded DNA (dsDNA). Patients frequently have multiple auto-antibody specificities which accumulate over time, both before and after diagnosis [27].

Clinical SLE is often preceded by a period of benign autoimmunity in which autoantibodies are present but the threshold for pathogenesis has not been reached. Autoantibodies are detectable for years before disease development and are also detectable in individuals who never go on to develop an autoimmune disease. The production of autoantibodies can precede the identification of disease by years. In a prospective study of US military personnel, of 115 individuals who went on to develop SLE, 88% had at least one autoantibody present before diagnosis. Autoantibodies were detected up to 9.4 years before diagnosis, with a mean time of 3.3 years. These times are thought to be underestimates, as many of the first serum samples available for these individuals were already positive [27]. A later study of SLE patients in Sweden found that autoantibodies were detectable an average of 5.6 years before onset of symptoms, and 8.7 years before diagnosis [28]. This study also revealed that there are difference in the times between the appearance of certain autoantibodies and the development of disease. Anti-Ro and anti-La antibodies show the longest interval between detection and disease of  $3.68 \pm 0.34$  and  $3.61 \pm 0.38$  respectively. Anti-snRNP antibodies have an average interval of just  $0.8 \pm 0.32$  years. It is thought that autoantibody specificities accumulate in SLE patients both before and after diagnosis [27], possibly initiating with just one specificity, then slowly diversifying.

## **Humoral Abnormalities**

SLE patients, along with abnormal cellular immune function, have altered humoral responses. They have altered serum concentrations, and altered production capacity of a number of important cytokines. Cytokines are soluble mediators of immune function, produced by immune cells, acting in an autocrine or paracrine manner.

## **IFN $\alpha$**

IFN $\alpha$  is one of the most studied cytokines in SLE patients; it has been shown to be important in mouse models and in humans. B6/lpr mice, deficient in IFN $\alpha$  receptor, show improvement in clinical disease. Also, between 20% and 80% of patients treated with IFN $\alpha$  develop autoantibodies and some also develop clinical autoimmune diseases, including SLE. IFN $\alpha$  is a type I interferon. The type I interferons (IFN-I) are a cytokine family comprised of 13 different isoforms of IFN $\alpha$ , along with IFN $\beta$ , IFN $\epsilon$ , IFN $\kappa$  and IFN $\omega$ . IFN $\alpha$  can be produced by all leukocytes but is mainly produced by plasmacytoid dendritic cells (pDC), which comprise 0.2-0.6% of leukocytes. It is produced in response to hypomethylated CpG DNA (Dubois, ed4 p210) and immune complexes (Dubois, ed4 p27,30-32), via these molecules binding to Toll-like Receptor (TLR) 9. IFN $\alpha$  acts via the Jak/STAT1 pathway to modulate the responses of target cells causing the maturation of dendritic cells (Dubois, ed4 p65). It also leads to monocytes maturation, increasing their antigen presenting ability (Dubois, ed4 p38), and causes increased NK and T-cell mediated cytotoxicity (Dubois, ed4 p78-80). IFN $\alpha$  also signals directly to CD8<sup>+</sup> T-cells, acting as a third signal in combination with TCR and CD28 signalling. The addition of the third signal from IFN $\alpha$  increases proliferative ability, IFN $\gamma$  production and cytolytic capacity [29].

SLE patients have up-regulated expression of a set of genes known as the IFN $\alpha$  signature, which includes *PRKR*, *IFIT1*, *LY6E*, *OAS1*, *IDG15*, *MX1* and *IFI44* [30, 31].

SLE patients with the worst disease activity have higher serum IFN $\alpha$  than those with milder disease and controls. IFN $\alpha$  serum concentration in SLE patients correlates with anti-dsDNA

antibody titre and complement activation; However, SLE patients in general do not have higher serum IFN $\alpha$ . Seemingly in contradiction to these findings, the pDC of SLE patients have impaired ability to produce IFN $\alpha$  on direct, pDC specific, TLR-9 ligation. One in three SLE patients are unable to produce any IFN $\alpha$  with this type of stimulation. It is thought that the pDCs in SLE patients have become exhausted through overstimulation. Repeated stimulation of TLR-9 in healthy pDCs leads to exhaustion of IFN $\alpha$  production, which is reversible after 24 hours without stimulation. The PBMCs in SLE patients in this study had up-regulated IFN $\alpha$ -inducible genes, suggesting that they had been exposed to IFN $\alpha$  but the pDCs were unable to produce any more, due to overstimulation. The same study also showed that the level of immune complexes in SLE patient sera, correlated with IFN $\alpha$  production by healthy donor PBMC in co-culture. Furthermore, those patients whose sera caused large amounts of IFN $\alpha$  production from healthy PBMC were the same patients whose own pDC appeared to be exhausted [32].

Despite the many reports of up-regulation of the IFN $\alpha$  signature in SLE, and its association with disease activity, it appears it is not a good marker of longitudinal changes in SLE disease activity, so is of limited use clinically [31].

## **IFN $\gamma$**

Interferon  $\gamma$  (IFN $\gamma$ ) is the sole member of the type II interferon family. It is produced primarily by NK cells but is also produced by Th1 CD4<sup>+</sup> cells, and CD8<sup>+</sup> T-cells. It is a pro-inflammatory cytokine, which activates monocytes and induces TNF production, via STAT1 signalling (Dubois 4<sup>th</sup> edition 179). In mouse models of SLE, IFN $\gamma$  is crucial for the development of nephritis (Dubois 4<sup>th</sup> edition 168 185) and for the production of pathogenic

IgGIIa antibodies (Dubois 4<sup>th</sup> edition 168-170 180-183). Deletion of the IFN $\gamma$  receptor gene in NZB/W F1 and MRL/lpr mice increased survival and improved renal disease. Reduction of serum IFN $\gamma$  using the IFN $\gamma$ R fusion protein in MRL/lpr mice resulted in clinical benefit. A role for IFN $\gamma$  in human SLE is not as clear, expression level of CXCL9 mRNA; a gene product induced by IFN $\gamma$  is not upregulated in SLE patients. However, it has been shown that on stimulation with anti-CD3 and anti-CD28 antibodies, PBMC from SLE patients produce significantly more IFN $\gamma$  [33]

## **Cellular abnormalities**

The purpose of the immune system is to protect the body from damaging pathogens, and to destroy cancerous cells. It is important however, that the immune system does not damage self-tissue, so it must be able to distinguish between self and foreign antigens. T-cells are educated in discriminating between self and non-self in the thymus, after emigrating from the bone marrow. In the thymus new T-cells encounter a range of self-antigens. Those that react too strongly to self-antigens are deleted, a process known as central tolerance. It was thought that this was the only mechanism the body used to avoid autoimmune attack, however self-reactive B-cells and T-cells have been found circulating in the peripheral blood of healthy controls and in various autoimmune conditions [34]. In healthy control these cells are anergic, and do not cause autoimmune damage. In autoimmune conditions the peripheral tolerance required to keep these cells anergic has been overcome, and many cell types contribute to autoimmune damage. Here, the cellular abnormalities, which may be contributing to loss of tolerance in SLE patients, will be discussed.

### **Peripheral Blood Mononuclear cells (PBMC) abnormalities**

Cytopenias including thrombocytopenia, anaemia and lymphopenia are common in patients with SLE. Both autoimmune destruction, and disease treatment are thought to be responsible.

### **B-cell abnormalities**

As B-cells are responsible for the production of pathogenic autoantibodies in SLE, it is important to consider any alterations of frequency or function of this type of cell. B-cells comprise around 5-15% of circulating lymphocytes. They are derived from haemopoietic stem cells, via pro-B cells and pre-B-cells in bone marrow. B-cells are defined by the presence of surface immunoglobulin, of which there are five types. Most peripheral B-cells bear immunoglobulin M and D (IgM and IgD). A smaller percentage bears IgG, IgE and IgA immunoglobulins. On any particular B-cell, all immunoglobulins are identical, and define the cell's specificity. On encountering the antigen specific for its immunoglobulin, the B-cell undergoes maturation and develops into a long-lived memory cell, or an antibody producing plasma cell. Memory B-cells in SLE are hyper responsive to stimulation, possibly as a result of the altered cytokine profile, and therefore may be more likely to overcome tolerance [35].

The contribution of B-cells to SLE pathogenesis can be broadly grouped into two categories; firstly, by the production of pathogenic, immune complex forming autoantibodies, discussed earlier; and secondly by their ability to modulate the activity of other immune cells such as T-cells, by antigen presentation, or the production of immunoregulatory cytokines and chemokines.

## **T-Cell abnormalities**

T-cells are a group of lymphocytes defined by their expression of the T-cell antigen receptor (TCR). T-cells, like B-cells, are also derived from HSCs but mature in the thymus. There are two types of TCR, both disulphide bond linked heterodimers, called  $\alpha\beta$  TCR and  $\gamma\delta$  TCR, in reference to their polypeptide chain components.  $\alpha\beta$  T-cells are by far the more common cell type, comprising 90-95% of peripheral T-cell. TCRs are found in association with the CD3 complex, and a set of 5 other polypeptides, which combined make up the TCR-CD3 complex. This complex recognises antigen presented by APCs expressing MHC class I or II, and transmits the signal intracellularly.  $\alpha\beta$  T-cells are furtherer categorised by their expression of CD4 or CD8 molecules. Most  $\alpha\beta$  T-cells express one or the other of these molecules, but a small proportion express neither or both. CD4<sup>+</sup> T-cells recognise peptide presented by MHC class II molecules, CD8<sup>+</sup> T-cells recognise peptide presented by MHC class I molecules. CD8<sup>+</sup> T-cells are mostly cytotoxic, and kill infected cells or tumour cells, and are often referred to as T<sub>c</sub>. CD4<sup>+</sup> T-cells generally function by recognising pathogens and stimulating other cell types to destroy the infected cells, and are therefore often known as helper T-cells or T<sub>h</sub>. T-cells are further subdivided by the cytokines expressed, and by their activatory or inhibitory subtype.

T-cells have been implicated in the pathogenesis of SLE. Firstly, the selective deletion of T-cells in SLE prone mouse models abrogates disease, and T-cell deficient mice cannot develop SLE. In humans, SLE is strongly linked to HLA-type, indicating antigen presentation has a role in disease induction. Furthermore, plasma cells producing pathogenic autoantibodies show evidence of having had assistance from T-cells, producing large quantities of high avidity IgG antibodies that have undergone somatic hypermutation, a process dependant on T-

cell help. Finally, a number of groups have detected autoreactive T-cells in the peripheral blood of SLE patients [36]. As with autoreactive B-cells, these are also found in healthy controls. However, auto reactive. T-cells in SLE patients can support antibody production by B-cells ex-vivo. T-cells have also been found infiltrated into SLE kidneys in patients with lupus nephritis [37].

SLE patients have increased CD4:CD8 ratio and decreased HLA-DR expression on treatment with steroids or cyclosporine [38][39]. SLE patient CD3+ CD8+ and CD3+ CD4+ cells are more susceptible to apoptosis than cells of healthy individuals. When healthy PBMC were cultured with SLE serum rather than healthy control serum, they also underwent more apoptosis, an effect which was significantly reduced by the addition of an anti-IL-10 antibody [39].

SLE patients have reduced numbers of T-cell receptor excision circles (TREC) [40]. TRECs are recognised as markers of thymic output, cells bearing this intracellular marker are recent thymic emigrants. Another study found reduced TREC levels in patients with active disease, but not inactive disease, suggesting SLE disease activity modulated TREC levels [41].

### **Th17**

SLE patients have increased levels of IL-17 in plasma and serum [42]. This cytokine, produced by Th17 cells, is proinflammatory and recruits and activates neutrophils. It also works with BAFF (which is also up-regulated in SLE) to promote the survival of B-cells and their differentiation into plasma cells. SLE patients also have more CD4+ cells capable of producing IL-17 (Th17), despite having similar percentages of IFN $\gamma$  producing cells to healthy controls. IFN $\gamma$  usually suppresses the differentiation of Th17 cells, so it is suggested

that the balance between Th1 IFN $\gamma$  producing cells and TH17 IL17 producing cells is dysregulated in SLE.

### **CD8+**

SLE patients have been shown to have a higher proportion of CD8<sup>+</sup> CD28<sup>-</sup> T-cells [43]. This T-cell type is thought to be suppressive, both by cell/cell contact and via cytokine production. One of the cytokines they produce is IL10. Cells of this subset in SLE are not capable of producing as much IL10 as healthy controls [44, 45]. Reduction in CD28 expression is also a marker of cells having undergone stimulation. CD28 is downregulated on stimulation. Higher frequencies of cells which have downregulated this marker, indicates more highly stimulated T-cell pool.

### **T-reg**

T-regulatory cells (T-reg) are a group of T-cells commonly characterised by surface expression of CD4<sup>+</sup> CD25<sup>high</sup> CD127<sup>-</sup>, and intracellular expression of the transcription factor FoxP3<sup>+</sup>. Unlike other T-cells, they are suppressive, downregulating the inflammatory effects of other T-cells, and play a vital role in maintaining peripheral tolerance. It is therefore possible that in autoimmune conditions, T-reg function may be impaired, or frequency reduced. Studies reporting the frequencies of T-reg in SLE give conflicting results, some reporting reduced frequencies, others reporting no alterations [46]. Inconsistent methods of defining phenotype, and the heterogeneous nature of SLE disease and disease activity scoring techniques may account for these inconsistencies. Some studies rely only on the surface phenotype to define a T-reg, without considering the expression of Fox-P3. Examining functional capacity of T-regs in SLE patients has also given conflicting information, with

some studies describing altered suppressive capacity in SLE relative to healthy controls and other describing no such difference. In-vivo studies have shown that Fox-P3 levels are not consistent within lymphocytes, so a cell isolated based on its expression of Fox-P3 directly ex-vivo may not be expressing Fox-P3 and may not be acting as a regulatory cell type in functional assays [46]. Other suppressive T-cell populations may be involved in SLE pathogenesis, for example NKG2D+ CD4+ T-cells are inversely correlated with disease activity[47].

### **NK cell abnormalities**

SLE patients also have abnormalities in the innate immune compartment. These abnormalities contribute to disease by altering antigen presentation or abnormal signalling to the adaptive immune system.

Natural killer cells are innate immune cells, derived from hematopoietic stem cells. They comprise up to 15% of peripheral blood lymphocytes. They are identified by their surface expression of CD16, which is a Fcγ III receptor, and CD56, an adhesion molecule of the Ig superfamily. NK cells are activated by recognition of infected cells or tumour cells, and kill their targets using perforin and granzyme. Activation of NK cells is dependant on the balance of activatory and inhibitory signals they receive.

SLE patients have fewer than normal NK cells, both in terms of absolute numbers and as a percentage of the total. This was more significant in patients with lupus nephritis or thrombocytopenia. NK cells isolated from SLE patients have reduced cytotoxic capacity, due to a down-regulation of perforin and granzyme. Differentiation of HSCs into NKs is also

defective [48]. NK cells, along with a subset of T-cells, express killer immunoglobulin-like receptors (KIRs). These molecules bind to MHC class I molecules, and modulate NK activation. KIR binding can cause or suppress NK activation, depending on whether the KIR is activatory or inhibitory. Inhibitory KIRs bind MHCI presenting autologous peptide prevents NK activation, and therefore stops NKs killing healthy cells. MHC I is down-regulated by virus infection of cells, to prevent T-cell recognition of the infection. This leaves the cell vulnerable to NK-mediated killing, as the inhibitory KIRs are not stimulated. KIRs are highly polymorphic and have numerous alleles. SLE patients are more likely to have two or more activatory KIR genes than healthy controls. Frequency of activatory KIR genes is also higher in other autoimmune conditions [49]

## **Clinical evaluation and treatment**

### **Evaluation**

As SLE is such a heterogeneous disease it is vital to have a standardised method for the assessment of disease severity. Standardisation allows comparisons to be made between affected individuals, and longitudinally. This is an important consideration when assessing the efficacy of treatment and for the comparison of patients at different centres. There are two parameters that can be examined in assessing disease in SLE patients: Disease activity and damage. Both disease activity and damage are difficult to score directly; due to the multisystem nature of the disease, even within a given system, disease can be very heterogeneous. A number of indirect scoring systems have been developed to assess disease severity, not all of which have been validated. Disease activity is reversible, and the score

reflects the severity of disease on the day of assessment. Damage is not reversible, and is cumulative, reflecting the scarring that has occurred as a result of disease activity.

One of the systems used to grade disease activity is the BILAG scoring system. This is a physician completed system, developed in 1984 by the British Isles Lupus Assessment Group, since when, it has undergone numerous modifications. One of these modifications was validated in 1986 and became known as the 'Classic BILAG'. The scoring system is split into eight different systems, containing 86 items for assessment. The eight systems are: general, mucocutaneous, neurology, musculoskeletal, cardiorespiratory, vasculitis, renal and haematology. Each of these systems is given a grade to reflect the severity of disease in that system in the month of assessment compared to the previous month. An A grade reflects the most severe activity, progressing down to D grade indicating inactive disease, and E grade showing the system has never been involved. This method of assessing disease activity will be used throughout this work.

Disease damage is assessed using The Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI). This is a measure of chronic damage. It is a physician completed scoring system giving a numerical value of between 0 and 46, which reflects the level of accumulated damage. The system includes 41 items, covering 12 systems, with each item being weighed based on severity. Unlike SLEDAI scores can not reduce over time, but increases to reflect damage accumulation [50].

Markers of disease activity used clinically include the complement components 3 and 4 (C3, C4). The complement system is a collection of membrane bound and soluble proteins which, via sequential activation known as the complement cascade, aid in the defence against pathogens and mediate the function of other immune cells [12]. There are three known

complement cascades; the classic, alternative and lectin pathways. C3 activation is necessary for the activation of all three complement cascades. Individuals homozygous for a C4 null allele are likely to develop SLE; C4 deficiency is linked to other autoimmune conditions. This feature of C4 is shared with all members of the classical pathway including C3 [51] [12]. As C3 and C4 are required for complement activation, excessive activation of the immune system via autoantibody binding can cause exhaustion of the supply of these proteins. Therefore, low or declining levels of C3 and C4 are indicative of worsening activity, and are one of the clinical parameter used in disease monitoring.

C- reactive protein (CRP), named due to its ability to precipitate the C-fraction of the cell wall of *Streptococcus pneumoniae*, is used as a marker of systemic inflammation [52]. Serum levels rise non-specifically in response to tissue injury and infection. CRP binding, to antigens containing phosphocholine, triggers the classical complement pathway via opsonisation and the recruitment of C1q. It is measured routinely in SLE and a number of other conditions as a marker of disease activity [53].

The presence of antibodies capable of binding dsDNA is specific for SLE, as opposed to other autoantibodies, which are found in other autoimmune conditions. Their identification in tissues of patients with SLE mediated nephritis has lead to the suggestion that they are pathogenic, also titre correlates well with disease activity, so is widely used as a biomarker [54]. Furthermore they are found in up to 95% of untreated SLE patients[54]. Lymphocyte counts are also recorded as part of routine disease monitoring. Both SLE disease and its immunosuppressive treatment cause lymphocytosis.

## **Treatment**

SLE treatment, like disease itself, is heterogeneous. It is generally treated using a variety of immunosuppressive agents, steroids and anti-malarial drugs. One of the most commonly prescribed is prednisilone, a steroidal anti-inflammatory drug given varying doses, depending on severity of symptoms.

The anti-malarial drug hydroxychloroquine is also widely used, particularly for skin and joint involvement, and fatigue. It is the only drug that has been shown to inhibit the accumulation of SLE induced damage [55]. Its mechanism of action remains unclear. The immunosuppressants azathioprine and cyclosporine are also widely utilised, often as alternatives to corticosteroids, in order to avoid the side effects associated with corticosteroid usage [56]. Azathioprine is a purine analogue, used in solid organ transplants, haematological malignancies and inflammatory bowel disease in addition to SLE and other rheumatological diseases. It is thought to function by its incorporation into replicating DNA [57]. Azathioprine also functions by inhibiting CD28 signalling, causing CD28 ligation to result in apoptosis rather than co-stimulation [58]. Cyclosporine A selectively inhibits T-cell responses by inhibiting the secretion of a number of cytokines including  $IL-2$  [59]. Biological treatments for SLE include rituximab, and anti-CD20 monoclonal antibody, which binds the CD20 molecule, found on most stages of B-cell differentiation, depleting B-cell numbers. It has beneficial effects on CD3, CD4 and dsDNA titres [60]. In severe refractory SLE, autologous stem cell transplant has been used, resulting in around 30% complete response but 11% 1 year transplant related mortality [61]. Another study of 17 SLE patients showed 7 year overall survival of  $82.4 \pm 9.2$  years [62].

## **HCMV**

### **History and Discovery**

Human Cytomegalovirus (HCMV) was first identified as a result of the pathogenic consequences of congenital infections. The presence of features, now known to be a result of Cytomegalovirus infection, were noted in premature fetuses and stillborn infants in the late 19<sup>th</sup> and early 20<sup>th</sup> century (Fields Virology ed 4, 147 and 241-179). They described an intranuclear inclusion surrounded by a clear zone in multiple organs of infants thought to have died from other causes. It was noted that these cellular features were similar to those produced by herpesviruses. By 1926 it had been shown that the cytomegalic cells were due to a filtrable, heat-inactivated agent (Fields Virology ed 4, 71). In 1953 Minder et al had shown, using electron microscopy, what appeared to be virus particles in the cytoplasm of cells with included nuclei (Fields Virology ed 4, 190). HCMV was first isolated in the 1950s by three separate laboratories (Fields Virology ed 4, 244, 260, 302), allowing in-vitro study of the virus.

### **Phylogeny**

HCMV is a member of the order *Herpesvirales*, a group containing 17 genera and 90 different species, with host specificities encompassing the majority of vertebrate orders. This wide spread amongst different classes of host suggests these viruses are of an ancient origin, and that the viruses have been evolving with their hosts over millions of years. HCMV is one of eight Herpesviruses to infect humans, it is also known as human herpes virus 5, and is a member of the subfamily, *betaherpesvirinae*. This category, it shares with human herpes viruses 6 and 7 [63].

## **Structure**

All *Herpesvirales* share the same physical structures and use double-stranded DNA to carry their genetic information. Their DNA is packaged in an icosahedral capsid, which is surrounded by a protein rich matrix called the tegument. A glycolipid membrane then encases the entire virus.

## **Genetics**

At 235kbp, the genome of HCMV is one of the largest and most complex viral genomes. It is linear in structure and is G+C rich. One of the best studied viruses, AD169, reveals 208 non-overlapping ORFs of greater than 80 codons. However this virus is derived from a strain grown under conditions designed to reduce virulence for use as a live vaccine. As such it has undergone 25 years of serial passage in-vitro, and has accumulated errors and modifications which distinguish it from clinical isolates [63].

## **Epidemiology and transmission**

### **Prevalence**

HCMV is a very common virus; it is found in 30-90% of the world's population, prevalence is inversely correlated with socioeconomic status. It is more common in less developed countries. In common with the infection protocol of all herpes viruses, infection is followed by lifelong carriage in the healthy host. However, unlike some other herpes viruses, initial infection is usually asymptomatic, although it can cause a mononucleosis-like illness on occasion. HCMV is thought to be responsible for 7% of infectious mononucleosis cases [64].

HCMV persists in the myeloid cell line, in monocytes, but may also be capable of infecting other cell lines in vitro [65] [66]. The virus is also thought to persist in CD34+ cells within the bone marrow [66] [67]. In vitro culture of the virus is usually achieved in fibroblasts; other cell types are permissive but have limited ability to produce virus.

As initial HCMV infection rarely has symptoms, it is difficult to determine at what age an individual acquired the virus. The virus is normally acquired in infancy, when it is transmitted to neonates via breast milk. Unlike other human Herpesviruses, it can be transmitted in utero, via the placenta. It can also be transmitted to the neonate during labour [68].

As the prevalence of the virus increases with the age of the population studied, it is clear that the virus can be acquired at any age. Seroconversion rates also depend on the population studied, in particular, those who are in contact with young children have higher rates. Virus can be transmitted in any body fluid; young children frequently shed the virus. Seroconversion rates among the parents of non-shedding children in the USA between 1986 and 1989 were 3%, compared to parents of children who were shedding having a rate of 30%. Among the adult population in general, the annual seroconversion rate is 0.6%. This figure is based on the results of a German study of 13160 female blood donors between 30 and 35, between 1992 and 2002 [69]. Prevalence in this study varied from around 30% among 18-20 year olds to over 80% in those aged over 60. HCMV infection was consistently more common in females than males at all ages, and peak of seroconversion rate was between 31 and 35. In Europe, seroconversion rates among pregnant women are between 0.7 and 4% [70]

## **Immunology**

### **Immune control in healthy people**

HCMV specific T-cells are identifiable at an average of 7 days after the identification of HCMV DNA in blood [71]. Phosphoprotein 65 (pp65) is a viral peptide recognised by the CD4+ T-cells of 63% of seropositive donors. 83% of seropositive donors have a CD8+ T-cell response against this antigen [72]. Anti-HCMV IgM appears on primary infection but can persist in some individuals. IgG appears later. IgG in individuals also expressing IgM, is usually of low avidity, indicative of more recent infection as the response has not undergone affinity maturation or somatic hypermutation.

### **Immune evasion**

Lifelong persistence requires strict immune surveillance, co-ordinated between the humoral and cellular immune system, to stop reactivation. Conversely, the virus needs to persist, so alters the immune response in order to do so. A balance is struck, such that the virus persists at very low levels, without causing disease.

Cytomegalovirus has evolved a number of methods of evading immune destruction. It evades T-cell mediated immunity by downregulating surface expression of MHC-class II and MHC class I molecules, and expression of HLA-E [73]. HCMV infection of DCs results in impaired maturation and surface expression of MHCII [74]. The absence or reduction of MHC- class II should make the virus susceptible to NK mediated killing, as MHC-II engages inhibitory receptors on NK cells, and prevents activation. However NK cell killing or tolerance depends on the balance of activatory or inhibitory signals it receives. HCMV is also able to modulate the expression of the NKG2D ligands- ULBP1, ULBP2, ULBP3, ULBP4, ULBP5, ULBP6, MicA and MicB [75]. This set of molecules is expressed by infected or

damaged cells, to allow NK cell recognition via NKG2D, and ultimately, destruction of the cell. HCMV can reduce the surface expression of this set of molecules, allowing it to go undetected by NK cells. The virus normally achieves this by retaining the ligands intracellularly, often in the endoplasmic reticulum [75].

### **Immune modulation**

HCMV infection has a major impact on the composition of peripheral lymphoid subsets in healthy carriers. HCMV positive individuals have a significantly decreased ratio of CD4<sup>+</sup> T-cells to CD8<sup>+</sup> T-cells, due to an increase in CD8<sup>+</sup> LFA<sup>hi</sup> and CD8<sup>+</sup> LFA<sup>hi</sup> CD45RA<sup>+</sup> memory T-cells. This is accompanied by a 40% reduction in naïve CD8<sup>+</sup> T-cells. A reduction in the proportion of naïve CD8<sup>+</sup> T-cells is a normal consequence of aging; HCMV accelerates the changes HCMV positivity also increases the numbers of CD4<sup>+</sup> effector memory cells [69]. HCMV may contribute to an ‘immune risk phenotype’ and an associated increased risk of morbidity and mortality with increasing age.

HCMV infection is also associated with shortening of telomeres in T-cells, a characteristic caused by progressive shortening with each cell division and associated with immune senescence and increased morbidity [76].

### **Differentiation markers**

CD28 is a 44kDa homodimeric co-stimulatory molecule found on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. CD28 ligation, in combination with specific antigen/MHC interaction with TCR, causes T-cell activation via recruitment of protein kinase cθ, allowing proliferation and cytokine secretion. Its ligands are B7 family members, B7-1 and B7-2 (CD80 and CD86), which are expressed on antigen presenting cells. CD80 is constitutively presented on APCs,

whereas CD86 expression is upregulated on activation of APCs. CTLA-4 is a homologue of CD28, which has inhibitory activity. It also binds CD80 and CD86 but with higher affinity. CMV infection is associated with the presence of higher frequencies of CD28- CD4+ and CD8+ T-cells [77].

CD27 is a 55kDa transmembrane glycoprotein receptor. It is member of the TNF-receptor superfamily, and is a co-stimulatory molecule found on the majority of peripheral blood T-cells, NK cells and on 33% of peripheral B-cells. Its ligand, CD70 is expressed on activated B and T-cells. CD27 stimulation is necessary for the survival and proliferation of low affinity T-cells, by reducing the necessary stimulation threshold [78]. It is up-regulated following antigen exposure. CD27 is expressed at lower levels on CD4+ and CD8+ T-cells in HCMV positive individuals.

CD57 is an 110kDa glycoprotein expressed on subsets of NK, B-cells and T-cells. CMV positive individuals have significantly higher expression of CD57 on CD4+ T-cells [79]. It is commonly used as a marker of antigen experience.

## **Pathology**

HCMV is able to reactivate and replicate in hosts with impaired immune control. This occurs in the case of AIDS patients, those given immunosuppressant drugs, in utero and in those acquiring the virus from a HCMV positive organ without pre-existing anti-HCMV defences [80]. It has also been shown to reactivate in astronauts, possibly due to the stresses of space [81]. In general, in immunocompromised patients and pregnant women, primary infection is more severe than either a secondary infection or a reactivation of latent virus. This may be

because the individual has not developed the immune response required to control the infection.

### **Pathogenesis in utero**

Congenital HCMV infections occur when the mother experiences primary infection, secondary infection or reactivation of latent virus at any stage of gestation. Such infections are capable of causing significant damage to the baby and can cause mental retardation and other developmental problems. The risk of transmission to the foetus in primary maternal HCMV infection is 30.1% in the first trimester, rising to 72.2% in the last trimester. However, only 10% of newborns suffer severe symptoms, frequencies are higher among those infected early in pregnancy compared to those infected later [82]. Transmission via breast milk to low birth weight and premature infants can cause symptomatic infections.

### **Pathogenesis immunocompromised adults**

HCMV reactivation and primary infection are also a significant cause of morbidity and mortality among patients undergoing solid organ or bone marrow transplants. In the 1970s, 96% of kidney transplant recipients experienced HCMV reactivation, resulting in significant morbidity [80]. By 2008, 56% of patients were experiencing at least 1 reactivation in the year following transplant, an improvement likely due to the increasing use of anti-viral prophylaxis [83]. The outcome is also linked to seropositivity of donor and recipient. Morbidity is most severe if a HCMV seronegative individual receives a transplant from a seropositive individual, as the patient has no pre-existing immunity.

## **EBV**

### **History and discovery**

Epstein-Barr virus (EBV) was first identified by Tony Epstein and Yvonne Barr, by using electron microscopy to examine cell lines derived from Burkitt's lymphoma (Fields ed 4 124). This type of tumour is endemic to equatorial Africa, and was studied and treated by Denis Burkitt in the 1950s. Burkitt was first to note that the geographical distribution of this tumour suggested an infectious causative agent. EBV was the first virus to be associated with a tumour. It was later found, unexpectedly, that this virus was common in all human populations, not just in equatorial Africa, and could therefore not be sufficient for tumour formation.

### **Phylogeny**

Epstein-Barr virus (EBV) is a human  $\gamma$ -herpesvirus, it is present in over 90% of the adult population. As with HCMV, prevalence is highest in populations with a lower socioeconomic status, and increases with age. EBV is a lymphocryptovirus, a group defined by their ability to persist in the lymphocytes of seropositive hosts. The lymphocryptoviruses of other species are EBV homologues, and are found in old and new world primates. All are members of the  $\gamma$ -herpesviridae subfamily. Of the human herpes viruses, EBV is most closely related to Kaposi's sarcoma herpes virus (KSHV), also a  $\gamma$ -herpesvirus which infects B-cells and shows similar replication mechanism to EBV [84].

## **Structure and important components**

EBV and HCMV share a common structure, in which a double stranded linear DNA core is enclosed by the icosahedral capsid, and then surrounded by the tegument and the membrane.

EBV contains a linear double stranded DNA genome, composed of 184-kbp.

Epstein-Barr virus nuclear antigen-1 is the only antigen to be expressed in all EBV related malignancies. It is a transcriptional regulator of latent replication and, essential for the maintenance of the viral episome within the host. EBNA-1 specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells have been identified, despite earlier reports suggesting it was capable of evading T-cell responses [85]. Anti-EBNA-1 antibodies appear 3-4 weeks after infection, and are used as a marker of previous infection.

EBV viral capsid antigen (VCA) is a structural protein expressed only during lytic infection [86]. Antibodies appear soon after infection and are maintained for the lifetime of the host.

The presence of IgG and IgM antibodies against EBV VCA is used diagnostically.

*BALF-5* is an EBV encoded DNA polymerase. It encodes an early lytic cycle antigen[87].

This gene is used for the identification of EBV genome in PBMC.

## **In Vitro culture**

The ability of EBV to replicate in a specific cell type in-vitro is determined by the presence of the EBV receptor CR2. This molecule is found on most B-cell types, but not on plasma cells.

EBV has two forms of replication, lytic infection, and latent infection, which can be further categorised into Latency I, II, III and 0. Each replication method involves the expression of different sets of genes.

## **Epidemiology and transmission**

Primary EBV infection usually occurs asymptotically in infancy, and is acquired from the parent via saliva. If infection is delayed until adolescence, it can result in a self-limiting condition called infectious mononucleosis in up to 25% of cases. Infectious mononucleosis can also occur on infection of infants, but is rare. Symptoms include lymphadenopathy, fever and malaise. Symptoms are caused by T-cell activation, and cytokine production. Infectious mononucleosis is characterised by lymphocytosis, in which up to 80% of circulating lymphocytes can be CD8+. This expansion is antigen driven, and the majority of these cells are antigen specific. With the decline of symptoms, the T-cell pool contracts [84]. Virus is then maintained in a latent state, at greatly reduced levels, for the lifetime of the host. Reactivation can occur in cases of immunosuppression. Reactivation can also occur as a result of stress, as measured by serum cortisol and epinephrine concentrations. More frequent reactivation occurs in those with the highest concentrations of these hormones [88]. Reactivation also occurs in apparently healthy controls. A small study of 14 healthy people found that 12 of them had at least one asymptomatic reactivation within a year. Reactivation was associated with periods of significantly lower T-cell responses, as measured by IFN $\gamma$  production in response to lymphoblastoid cell lines [89].

Virus generally transmitted via saliva, it is shed from epithelial cells in the oropharynx. Virus is present in the saliva of most infected individuals [90]. Initial infection is also thought to occur in epithelial cells within the oropharynx. This is followed by transmission to B-cells, causing B-cell expansion, which serves to increase the number of virus particles. EBV can also be transmitted by sexual intercourse, as virus can be detected, although at low levels, in

cervical and semen samples, and viral isolates were more frequently identical amongst sexual partners than non-sexual contacts [90]. Risk of acquiring virus also increases with number of sexual partners [91] [92].

EBV seropositivity is less common in those with higher socioeconomic status; infection is associated with lower paternal education, and smoking [93] and in females. Seroprevalence is also higher amongst individuals with a larger number of sexual partners [90].

## **Immunology**

Control of EBV is primarily mediated by T-cells; both CD4+ and CD8+ cells are involved. The magnitude of T-cell response is generally smaller than anti-HCMV response. EBV specific cells have effector phenotype, but are not as highly differentiated and HCMV specific T-cells [84]. Frequency of anti-EBV T-cells is greatest during the recovery phase from primary infection, the increase in T-cell frequency correlated with the rate of reduction of EBV load [94].

## **Associations between SLE and HCMV**

### **IgG**

SLE patients have been shown to have higher titres of anti-HCMV IgG than both healthy controls and patients with other autoimmune conditions. The higher antibody titre appears to be specific for HCMV, as higher titre antibodies are not found against Hepatitis-B virus or Epstein-Barr virus. It has also been shown that SLE patients have a higher prevalence of antibodies against pp65 by pp65 specific ELISA, and titres around double those of normal individuals. This finding was specific for SLE patients, as increased titres were not found in patients with other autoimmune diseases [87]. In healthy individuals HCMV seropositivity is associated with increased titres of autoantibodies, against RNP, Sm and U1-70k ( $p < 0.0001$ ,  $p = 0.002$  and  $p < 0.0001$  respectively) despite total IgG titres being similar. U1-70k is one of the snRNP proteins, and is often the first, of this group against which autoantibodies are generated. Another study shows that U1-RNP is more frequently targeted in SLE patients with high anti-HCMV titres than those with low anti-HCMV titres [95]. Other sn RNP specificities then accumulate over time, and are found in 30% of SLE patients. If responses to this antigen are more common in HCMV positive individuals, those with genetic predisposition and HCMV seropositivity may be more likely to develop SLE than HCMV negative people.

Anti-RNP responses are also more common in HCMV positive SLE patients than HCMV negative SLE patients [96]. MRL/mpj autoimmune prone mice, when vaccinated with an adenovirus vector encoding HCMV gB developed autoantibodies against U1-70k. Three other non-autoimmune mouse strains also develop autoantibodies against U1-70kDa, suggesting this is not just an autoimmune phenomenon. All mouse strains also developed anti-RNP and

anti-sm antibodies on vaccination with gB, whereas those vaccinated with PBS or HeLa extract did not [97]. However, 4 years later the same group vaccinated HCMV negative healthy humans with recombinant soluble gB, and found no difference in pre and post-vaccination U1-70kDa responses. Furthermore no individual vaccinated had more than a 2-fold increase in anti-Sm and anti-RNP antibodies with vaccination. The purpose of this study however, was to assess the safety of a potential HCMV gB based vaccine, not to assess the potential of HCMV to cause autoimmunity. It does indicate that gB responses alone are insufficient to cause autoimmunity, and are probably insufficient to induce the production of autoantibodies. Instead, it is likely that genetic predisposition plays a role in disease development. A/J and B6 mice were immunised with an adenovirus vector expressing gB. A/J mice developed responses to U1-70kDa rapidly, and also accumulated deposits of IgG in their kidneys. B6 mice developed neither feature. A/J mice also exhibited a divergence of their antibody responses to other closely associated snRNP antigens, as is seen in the development of human SLE [98]. GB and U1-70kDa share some structural homology, meaning this generation of autoantibodies could be as a result of molecular mimicry. This concept, first suggested by Damian et al in 1964 [97], suggests that antibodies can bind different antigens, with very similar structures, and that on encountering either antigen, the host develops a response against both. This notion has been suggested numerous times in studies of the association between pathogens and autoimmune diseases, and is a very attractive mechanism. It means that a host, by responding to a pathogen, can also be generating antibodies against self. It can also help to explain why autoantibodies are not deleted, as they are still potentially useful against pathogens. Potential molecular mimicry between autoantigens and herpesvirus antigens has been suggested for a number of antibodies.

Pp65, being the most abundant antigen in a HCMV particle, is a major target for the host immune system. SLE prone NZB/W F1 mice immunised with a plasmid encoding pp65 developed earlier onset of autoantibody production. They developed autoantibodies against dsDNA and other nuclear antigens, at roughly 4 and 2 times the titres of their pre-vaccination levels respectively ( $p=0.007$  and  $p=0.047$  respectively). Titres of anti-dsDNA did not correlate with the increased titres generated against pp65, indicating the increased titres found are not due to cross reactivity within the ELISA. Mice vaccinated with empty plasmid did not produce autoantibodies at detectable levels. Pp66 immunised mice also developed more severe glomerulonephritis than those vaccinated with empty plasmid ( $p=0.001$ ) but did not show earlier mortality [98].

Zhu et al showed that infection of keratinocytes *ex vivo* with HCMV induced the surface expression of 60kDa/Ro antigen [95]. This antigen is targeted in 30-40% of SLE patients. Surface expression of this normally intracellular antigen, may make it more immunogenic in HCMV positive individuals.

## **IgM**

Patients positive for anti-HCMV IgM have worse disease activity than IgM negative patients. This suggests a number of possibilities. Firstly, that HCMV reactivation is triggering flares. Secondly, that HCMV is reactivated during flares, or finally, that some other event is causing both a rise in IgM and a worsening of disease activity. However, these findings relied entirely on the detection of IgM antibodies and did not detect virus. It is therefore possible that non-specific antibody binding is responsible. It is plausible that false positive anti-

HCMV IgM is due to the presence of autoantibodies, which are found at higher levels in patients with worse disease activity. In a cohort of pregnant SLE patients, those with false-positive anti-HCMV-IgM antibodies were shown to have poorer pregnancy outcome [99]. In this case, false positives were defined by the apparent presence of anti-HCMV IgM when HCMV was undetectable in amniotic fluid or urine, and HCMV-IgG was of high avidity. False positive IgM antibodies may therefore be markers of more severe disease activity.

### **Epidemiological association**

Reports on prevalence of HCMV in SLE are variable, as are reports of HCMV prevalence in healthy individuals. A UK study in 1997 of 97 patients and 97 controls, found a prevalence of 90.87% in SLE, and a prevalence of 43.3% among the controls[100]. However, this study did not match controls by ethnicity.

### **Experimental evidence**

UVB exposure in combination with HCMV infection causes the surface expression of 52kDaRo antigens [101], allowing recognition of autoantigen by antibody.

### **Case studies**

Along with experimental and epidemiological evidence, numerous case studies have been published suggesting a concurrent HCMV activation and SLE exacerbation. HCMV has been associated with the onset and with exacerbation of SLE disease [96, 102-104]. There are also two cases of HCMV induced mortality in SLE [105], and fatal cavity lung masses in patients caused by HCMV [106].

The relationship between HCMV infection and SLE may be caused by IFN $\alpha$  production. As Herpesvirus infection induces TLR9 stimulation and IFN $\alpha$  production, it is possible that impaired control of these viruses leads to excessive IFN $\alpha$  production, and the following upregulation of the IFN $\alpha$  signature genes, resulting in increased antigen processing, increased T cell stimulation and ultimately increased antibody production by overstimulated or inappropriately stimulated B cells.

## **Associations between SLE and EBV**

Epstein-Barr virus has been associated with a number of autoimmune diseases, including SLE. Prevalence of the virus has been shown to be higher in SLE patients than in healthy matched controls. Of 196 SLE patients, only 1 lacked IgG antibodies against purified EBV viral capsid antigen (VCA) by ELISA. Of the 392 controls, matched by age, sex and self-declared ethnicity, 22 did not have antibodies against EBV-VCA (99.5% vs. 94.4%,  $P=0.014$ ). A subset of these patients were examined for EBV load by PCR, this also showed higher virus prevalence in SLE patients. All 32 patients tested were positive by this method whereas only 23 of 32 controls were positive (100% vs. 72%,  $p<0.002$ ). No differences were seen in the prevalence of HCMV, HSV-1, HSV-2 or VZV [107]. As EBV is so common in the adult population, large numbers of patients and controls are needed to detect differences in seropositivity rates. This issue can be avoided by using a younger cohort, in which seropositivity in a normal population is lower, so differences are detectable with a smaller cohort. It has been shown that the presence of IgG antibodies against EBNA-1 is more common in paediatric SLE patients than in healthy matched controls (100% vs. 69%,  $p<0.001$ ) [25].

EBV load is 40 fold higher in SLE than in controls, suggesting impaired control of virus replication [108]. This could also explain the higher virus prevalence in SLE when examined by PCR, as samples with lower viral loads may be below the sensitivity limit of the assay. Anti-viral responses by T-cells have previously been examined using HLA-tetramers and by ex-vivo peptide and lysate stimulation, followed by identification of any cytokines produced.

It has been shown that SLE patients (n=7) have a tendency towards higher proportion of cells responding to the HLA-A2 restricted peptide derived from BMLF-1 (p=0.07). These authors also showed that among those patients with responses, fewer were capable of producing large amounts of IFN $\gamma$  in response to stimulation of PBMC with the same peptide in ELISPOT assay [109]. It is suggested that SLE patients have increased numbers of cells, with impaired function, leaving the patient unable to adequately control the virus. These findings are unrelated to either disease activity or treatment, however with such limited patient numbers, it is difficult to assess the significance of these results.

It is possible that EBV presence can accelerate autoimmunity by expressing a molecule which results in excessive signals to autoreactive B-cells, allowing their proliferation and survival. LMP1 is an EBV encoded mimic of cellular CD40, this viral protein is only detectable in PBMCs during disease flares. Unlike CD40, LMP1 is constitutively active and signals more intensely than its cellular counterpart. Mice were bred to express chimeric molecules, comprised of the transmembrane and extracellular domains of CD40 and the intracellular signalling domain of LMP1. The transmembrane and extracellular domains of CD40 were retained to allow the signalling to be switched on and off, as intact LMP-1 is constitutively active. In these SLE-prone mice, it was shown that the excessive signalling provided by LMP-1, caused the mice to have enlarged lymphoid organs with more germinal centres and activated memory cells than their non-transgenic, SLE prone, littermates. Furthermore, transgenic mice had elevated levels of autoantibodies compared to their littermates and signs of kidney pathology. It is suggested that the combination of an SLE-prone background, with EBV induced excessive B-cell signalling accelerates autoimmune disease [110].

As with HCMV antigens, EBV antigens may be triggering or exacerbating SLE by molecular mimicry. BALB/c mice injected with recombinant EBNA-1 peptide developed autoantibodies against dsDNA. They did not develop as fast as anti-EBNA-1 antibodies, leading to the suggestion that they developed as a consequence of epitope spreading, which resulted in antibodies being able to bind both EBNA-1 and dsDNA [111]. EBNA-1 contains a peptide with the sequence PPPGRRP; this sequence is very similar to a sequence found in the autoantibody Sm B'- PPPGMRPP. EBNA-1 has also been shown to be a molecular mimic of 60 kDa Ro, animals immunised with either peptide develop antibodies against both antigens [112].

There are also numerous reports of a temporal association of EBV infection and SLE diagnosis [104, 113, 114].

The magnitude of EBV-specific response is increased in SLE patients, compared to matched controls. It has been suggested that the functional ability of these cells is impaired, specifically, in their ability to produce IFN $\gamma$ [115].

## **Research aims**

HCMV infection and carriage is asymptomatic in the context of an intact immune system. EBV persistence is also asymptomatic in healthy individuals. Impairment of immune function by disease or chemotherapeutic agents can lead to impaired control of the viruses and pathogenesis. It is therefore important to consider the result these infections in Systemic Lupus Erythematosus: a condition in which both disease, and its treatment modify the function of the immune system.

There are a number of possible relationships between HCMV and EBV, and SLE, the aim of this thesis was to examine the following four hypotheses.

Hypothesis 1. EBV or HCMV are necessary for development of disease.

If this were the case, we could expect to see 100% prevalence of the associate virus in SLE patients, and a significantly lower prevalence in matched healthy controls.

Hypothesis 2. HCMV and/or EBV reactivate more frequently in SLE as a result of the more pro-inflammatory cytokine environment.

In this case, we would expect to see elevated virus load, reflecting an accumulation of virus over time, and elevated T-cell and B-cell responses in order to control the viruses.

Hypothesis 3. HCMV and EBV are inadequately controlled in SLE patients, as a result of their impaired immune function.

Were this hypothesis true, we could expect to see reduced frequency or magnitude of T-cell and B-cell responses to the virus, but higher virus loads.

Hypothesis 4. HCMV or EBV are impacting on disease by causing the release of IFN $\alpha$ , exacerbating disease activity or hastening the onset of disease in those infected.

Disease activity would be more severe and disease onset would be at a younger age in those infected, were this hypothesis to be supported.

These hypothesis are not mutually exclusive, the true nature of the relationship between HCMV and EBV infection with SLE is likely to be complex and multifactorial.

## **METHODS**

## Patients and Controls

Patients fulfilling 4 of 11 ACR criteria were recruited into the study at the Wellcome Trust Clinical Research Facility. All patients and controls gave informed written consent. SLE patients donated blood samples on recruitment; further samples were taken at subsequent clinic visits, with a minimum interval of three months. 86 SLE patients were recruited, giving 253 samples. Healthy controls (n=48) were selected based on their lack of family history of autoimmune disorder; they donated blood samples on one occasion only. Healthy controls and SLE patients were matched for age ( $43.23 \pm 1.858$  vs.  $40.73 \pm 1.272$ , mean  $\pm$  SEM,  $p=0.4132$ ) sex (9.3% male vs. 9.2% male). Patients and controls were identified as HCMV+ if the first sample tested positive by in-house developed ELISA for anti-HCMV IgG. Clinically, the presence of anti-CMV IgG is considered definitive of previous HCMV infection[99]. Patients and controls were considered EBV positive if any two of the 4 methods used (EBV IgG ELISA, EBV VCA, EBV load, anti-EBV IFN $\gamma$  response) returned a positive result, in the first sample given. This was necessary as not all samples were tested by all available methods, and correlation between methods was unreliable. Samples with inconclusive viral positivity were not included in comparisons between virus positive and virus negative populations, but were included in comparisons between controls and SLE patients. This study has been approved by the Birmingham, East, North and Solihull Research Ethics Committee.

## **Sample preparation**

Blood samples of 20ml were taken by venepuncture into heparinised tubes from patients and controls. Samples were mixed 1:1 with RPMI (Sigma-Aldrich) and layered onto 15ml Ficol. Samples were spun at 1800rpm for 30 minutes and the resulting PBMC layers and plasma/RPMI mixture collected. PBMC were then washed in RPMI, and either used fresh for intracellular staining or frozen for DNA extraction and tetramer staining. PBMCs were stored at  $<10 \times 10^6/\text{ml}$  in FCS +10% DMSO at  $-80^\circ\text{C}$  in liquid nitrogen. Two aliquots of plasma/RPMI mix were retained per sample and frozen at  $-80^\circ\text{C}$  for serological analysis.

## **HCMV IgG**

Maxisorb plates (NUNC ebioscience) were coated overnight at  $4^\circ\text{C}$  with 50 $\mu\text{l}$  of lysed uninfected fibroblasts or HCMV infected lysed fibroblasts at 5ng/ $\mu\text{l}$ . Plates were then washed three times with PBS (0.05% Tween20). Plasma/RPMI was diluted to 1/450 and incubated on the plate for 30 minutes at room temperature. After washing three times with PBS (0.05% tween 20), anti-human IgG monoclonal antibody conjugated to HRP was added (eBioscience) for another 30 minutes at room temperature. Plates were washed another three times then antibody substrate added for 10 minutes. The reaction was stopped using 100 $\mu\text{l}$  1M HCl and optical density (OD) read at 450nm. The OD of binding to uninfected fibroblast lysate was subtracted from the OD binding to infected lysate to give a measure of binding to HCMV specific proteins. Arbitrary units of antibody binding were assigned to samples by comparison to a standard, using a sigmoid dose response curve fit, with a maximum of 1000

units. A cut off of 10 units was assigned, having two points of the standard curve below it, and 4 points above, and being at the bottom of the linear section of the sigmoid curve.

### **HCMV IgM**

Plasma anti-HCMV IgM was measured by ELISA, using a kit from IBL international designed to detect the presence of IgM antibodies against HCMV antigens. Samples were diluted 1/50 in the provided diluent, to account for the previous 1/2 dilution in RPMI. Tests were then run according to manufacturer's instructions. Results were calculated as a proportion of the provided reference control, and are therefore only semi-quantitative. Both equivocal and clear positive samples were considered positive, samples giving an OD reading of >90% of cut off control were considered positive. As samples are diluted 1:1 in RPMI, guidelines for seropositivity cannot be considered completely accurate.

### **EBV IgG ELISA**

Samples were tested using ELISA kits supplied by IBL international, designed to test for the presence of IgG antibodies against EBNA-1 antigen. Samples were diluted to half the recommended dilution to account for the previous 1:1 dilution in RPMI, the according to the manufacturers instructions. Both clear positive and equivocal samples were considered positive, therefore samples were considered positive at >12U/ml. Again, as samples are diluted 1:1 in RPMI, supplied guidelines for seropositivity can not be considered completely accurate.

### **EBV IgM ELISA**

Relative anti-EBV EBNA-1 IgM titres were determined using ELISA assays form IBL international designed to test for the presence of IgM antibodies against EBNA-1. Samples were run according to manufacturers instruction with the exception of the dilution factor,

being half that prescribed. Samples were also pre-incubated with anti-rheumatoid factor serum to remove confounding antibodies, which could lead to false positive results. Both clear positive and equivocal samples were considered positive, samples were considered positive at >8U/ml. As samples are diluted 1:1 in RPMI, guidelines for seropositivity cannot be considered completely accurate.

### **EBV IgG Immunofluorescence**

IgG antibodies specific for viral capsid antigen (VCA) were detected by immunofluorescence. EBV positive (P3HR1) and EBV negative (BJab) cells were applied to glass microscope slides at  $10 \times 10^5$  per 20 $\mu$ l well and air-dried at 37°C for up to 14 hours. Cells were then fixed in ice-cold acetone for 10 minutes, and stored at -20 until needed. Plasma/RPMI was diluted 1/10 in PBS supplemented with 10% heat inactivated goat serum (HINGS). 20 $\mu$ l was applied to cells and incubated for 1hr at 37°C. Slides were washed for 10 minutes in PBS and stained with anti-human IgG  $\gamma$  chain specific FITC conjugated antibody for 30 minutes at 37°C (Sigma). Slides were then washed and mounted using 0.05% DABCO glycerol mounting media and examined at 400x magnification. Positive samples were identified as those causing homogenous staining of around 1/10 B-cells at a dilution of 1/10. Samples showing strong autoantibody binding were diluted to 1/40, 1/80 and 1/160. Those in which autoantibody staining remained at the lowest dilution, were excluded from this set of results as autoantibody staining interfered with identification of anti-VCA staining.

### **Tetanus**

Anti-tetanus toxoid IgG was measured in the first supplied sample using an ELISA kit supplied by Demeditec. The assay was sensitive to 0.004IU/ml (WHO 76/589). Assays were

performed according to manufacturers instructions, with the exception of the sample dilution factor. Samples were diluted by half the factor suggested.

## **IFN $\alpha$ ELISA**

Plasma IFN $\alpha$  concentrations were measured using kits supplied by R and D Systems, using the sandwich principle to quantify IFN $\alpha$  in media. Plasma/RPMI mix was applied neat to the wells. Plasma IFN $\alpha$  levels were calculated based on this 1:1 dilution.

## **T-cell assays**

### **HCMV lysate production**

Human foetal foreskin fibroblasts were cultured in DMEM supplemented with 10% FCS, 2mM L-glutamine and 1% penicillin/streptomycin. Fibroblasts were grown to 70% confluence then infected with AD169 at 4:1 MOI. Supernatant was collected every 8 days, pooled, and ultra-centrifuged at 12000 rpm for two hours. Fibroblasts were harvested after they had detached themselves from the flask. Supernatant pellet and harvested fibroblasts were added together and exposed to multiple rounds of freezing in liquid nitrogen, and thawing at room temperature. The mixture was then sonicated to break up large particles, and irradiated to destroy any remaining live virus.

### **Intracellular staining**

Freshly isolated PBMC were stimulated with 10 $\mu$ l HCMV lysate, HCMV peptide mixes, and EBV peptide mixes (see tables 2 and 3) for 18 hours, the last 17 being in the presence of BFA, in 500 $\mu$ l RPMI supplemented with 10% FCS and 1% penicillin/streptomycin. Staphylococcus enterotoxin B (SEB) was used as a positive control. Cells were then washed in MACS buffer and stained for surface markers for 30 minutes at 4 $^{\circ}$ c in 100 $\mu$ l MACS buffer

(see table 1). See appendix 2. for gating strategy. Following another wash, cells were fixed using 100µl 0.4 % PFA. Cells were then washed and permeabilised using 100µl 0.5% saponin and intracellularly stained for IFN $\gamma$ . Excess antibody was removed by washing with MACS buffer and cells were analysed within 2 hours by flow cytometry using LSRII (BD Bioscience).

Cells were gated using forward scatter height and area to identify single cells, single PBMC were identified by their forward and side scatter properties. Single PBMCs were gated based on the presence of CD3 to identify T-cells, then on the presence of CD4 or CD8. Cells bearing both CD4 and CD8, and those bearing neither, were excluded. CD4<sup>+</sup> and CD8<sup>+</sup> were then categorised by the presence or absence of CD28, CD27 and CD57.

<b>Specificity</b>	<b>Colour</b>	<b>Supplier</b>
CD8	AmCy	BD
CD4	PeCy7	e-biosciences
CD57	PE	biolegend
CD28	PerCPCy5.5	e-biosciences
CD27	APC-Cy7	e-biosciences
CD3	APC	invitrogen
IFN $\gamma$	FITC	BD

***Table 1: Antibody staining panel for intracellular staining***

HLA	Code	EBV lytic Peptide	Code	EBV Latent Peptide
A2			FLY	LMP-2
A2.01	GLC	BMLF-1		
A2.01	YVL	BRLF-1		
A2.01			CLG	LMP-1
A24	DYC	BRLF-1		
A24			TYG	LMP-2
B27	ARY			
B27.02/.04/.05			RRI	EBNA-3C
B27.05			HRC	EBNA-3B
B35.01	EPL	BZLF-1		
B35.01			HPV	EBNA-1
B35.01			YPL	EBNA-3A
B38			YHL	EBNA-2
B44.03			EGG	EBNA-3C
B45	AEN			
B7			RPP	EBNA-3A
B8	RAK	BMLF-1		
B8			FLR	EBNA-3A
B8			QAK	EBNA-3A

***Table 2: EBV lytic and latency peptide panels, used for stimulation of CD8+ T-cells.***

**IE-1 peptide mix**

HLA restriction	CD4/CD8	Peptide sequence
A68	CD8	ATTFLQTMLRK
B40	CD8	KEVNSQLSL
B8	CD8	QIKVRVDMV
B44	CD8	DELRRKMMY
B8	CD8	ELRRKMMYM
B8	CD8	ELKRKMIYM
B27	CD8	KRKMMYMCY
B55	CD8	FPKTTNGCSQA
A1/B18	CD8	CVETMCNEY
B7	CD8	CRVLCCYVL
A2	CD8	YVLEETSVML
B27	CD8	RRIEEICMK
B44	CD8	EEAIVAYTL
A2	CD8	VLEETSVML

**pp65 peptide mix**

HLA restriction	CD4/CD8	Peptide sequence
A68	CD8	DTPVLPHETR
B35	CD8	QPSLILVSQY
A68	CD8	YTPDSTPCHR
B35	CD8	CPSQEPMSIY
A24	CD8	VYALPLKML
B35	CD8	IPSINVHHY
A68	CD8	FVFPTKDVALR
B35	CD8	FPTKDVAL
A24	CD8	QYVKVYLESF
B7	CD8	RIPHERNGFTVL
B35	CD8	QAIRETVELR
A24	CD8	QYDPVAALF
A1	CD8	YSEHPTFTSQY(HPTFTSQY)
B7	CD8	TPRVTGGGAM
A2	CD8	NLVPMVATV
A2	CD8	RIFAELEGV
DR7	CD4	PDVYYTSAFVFP
DR4	CD4	IIKPGKISHIMLDVA
DR11	CD4	PQYSEHPTFTSQYRI
DR11	CD4	FTSQYRIQGKLEYRHT
DR ?	CD4	AGILARNLVPMVATV
DR52	CD4	KYQEFFWDANDIYRI

**pp50/gB/gH peptide mix**

HLA restriction (and protein)	CD4/CD8	Sequence
A1 (pp50)	CD8	VTEHDTLLY
DR7 (gB)	CD4	DYSNTHSTRYV
DR7 (gB)	CD4	VFETSGGLVFWQGI
DR4 (gB)	CD4	CMLTITTARSKYPYH
DR11 (gH)	CD4	HELLVLVKKAQL

**Table 3: HCMV peptide mixes used to stimulate CD4+ and CD8+ T-cells.**

### **Tetramer staining**

PBMC were defrosted in PBS and vital dye was added to identify dead cells for exclusion. Cells were washed and transferred to MACS buffer. Tetramers were added, based on the patient or control's HLA type for 15 minutes at 37°C. Cells were washed again in MACS buffer and antibodies added against surface phenotypic molecules for 30 minutes at 4°C. Cells were washed once more and examined using flow cytometry via LSR II (Beckman Coulter). Cells were gated using the same method as that employed for intracellular staining, however a vital dye stain was added so dead cells could be excluded.

HLA type	HCMV		EBV	
	code	protein	code	protein
A1	VTE	pp50	-	-
A1	YSE	pp65	-	--
A2	NLV	pp65	GLC	BMLF-1 (lytic)
A2	VLE	IE-1	CLG	LMP-1 (latent)
B7	RPH	pp65	RPP	EBNA-3A (latent)
B7	TPR	pp65		
B8	ELR	IE-1	RAK	BMLF-1 (lytic)
B8	ELK	IE-1	FLR	EBNA-3A (latent)
B8	QIK	IE-1		

*Table 4: HLA tetramer specificities.*

## DNA extraction

DNA extraction was performed using QIAamp DNA mini kits from QUIAGEN, according to manufacturers instructions. DNA was extracted from  $1 \times 10^6$  PBMC pellets, which had been stored at  $-80^\circ\text{C}$  since collection. DNA was stored in 50 $\mu\text{l}$  water at  $-80$  until needed.

## HLA typing

Patient and control DNA samples were used to identify those expressing HLA A1, A2, B7 and B8, so that only the relevant tetramers were applied to samples. A method developed by Bunce et al was adapted for this purpose [116]. PCR products were examined by gel

electrophoresis on Tris-Borate-EDTA gel. Lanes showing bands at the correct molecular weight for the specific primers were considered positive.

## EBV load PCR

The number of copies of EBV genome per million PBMCs was measured using EBV BALF5, a gene encoding EBV polymerase. Three master mixes were made for each sample, containing 2 $\mu$ M forward and reverse POL primers, 5 $\mu$ M POL probe, 3 $\mu$ M  $\beta$ 2m forward primer, 4 $\mu$ M  $\beta$ 2m reverse primer and 5 $\mu$ M  $\beta$ 2m probe, to give a total volume of 7.5 $\mu$ l. To this mix was added 12.5 $\mu$ l Taqman Universal 2x primer mix and 5 $\mu$ l DNA sample. Samples were run in 96 well plates, using an ABI7500 (Applied Biosystems). Primers are shown in table 5

<b>BALF5</b>	
Forward primer	<b>CTT TGG CGC GGA TCC TC</b>
Reverse primer	<b>AGT CCT TCT TGG CTA GTC TGT TGA C</b>
Probe	<b>(FAM) CAT CAA GAA GCT GCT GGC GGC C (TAMARA)</b>
<b><math>\beta</math>2m</b>	
Forward primer	<b>GGA ATT GAT TTG GGA GAG CAT C</b>
Reverse primer	<b>CAG GTC CTG GCT CTA CAA TTT ACT AA</b>
Probe	<b>(VIC) AGT GTG ACT GGG CAG ATC ATC CAC CTT C (BHQ)</b>

*Table 5: Primers and probes for quantification of EBV.*

## **Ethical considerations**

All laboratory data and clinical data were compiled and organised using Microsoft Excel. Patients were identifiable in this study only by an anonymous reference number. Date of birth was used for confirmation of identity. Controls were identified only by the use of an anonymous reference number. Controls were given the opportunity to provide their age, sex and self-declared ethnicity. Control recruitment was from amongst staff at Queen Elizabeth Hospital Birmingham UK and City hospital Birmingham UK. Controls were also recruited using the University of Birmingham's Health research bus, a mobile research facility, for this study located in Birmingham city centre.

## **Clinical information**

Clinical information and patient information were collected using Patients Informatics Consult Service (PICS) software, using the patient's anonymous reference number, and date of birth. Disease activity, damage index, CRP, C3, C4, dsDNA, medication and lymphocyte count were collected for the day of blood donation. Total disease activity was calculated by assigning any A values a score of 12, any B values a score of 5, and any C values a score of 1. The total disease activity score is the sum of all systems. Any missing data values were entered as the mean of previous and subsequent clinic visit, as missing data were not available at submission. See appendix 1. for full disease activity and treatment details.

## **Statistics**

Data were analysed and presented using Graphpad Prism. Correlations were all assessed using Spearman's rank. Other data were all compared using the Mann-Whitney test when data was not normally distributed (as assessed using D'Agostino and Pearson omnibus normality test), of T-test when data was normally distributed.  $\chi^2$  analysis was used to

compare differences between populations. Kruskal-Wallis in combination with Dunns test was used for comparisons of more than two groups. All tests were two tailed.



## **Results 1: Serology**

## **Introduction**

When considering the association between HCMV and EBV and SLE, it is important to consider how common the virus is within the disease population, compared to a matched non-disease control population. If the virus is more prevalent in the disease population it is possible that the virus is contributing to aetiology, or the disease population is more susceptible to virus infection. If the virus is found in 100% of the disease population it is possible that the virus is necessary for disease, and if the virus is less common in disease, it is unlikely to be a major contributing factor, or may be less readily acquired by those with disease.

It is also important to consider the magnitude of anti viral responses, as differences may reflect alterations in ability to control virus reactivation. SLE patients are immunosuppressed, due to disease and treatment, it is therefore important to assess whether viral control is adequate.

HCMV prevalence is higher in older populations, the sero-conversion rate has been estimated to be 0.55% per year, and can occur at any age [70]. As infection is generally asymptomatic it is difficult to determine when an individual has become infected. Incidence in younger people compared to older members of the same population can give clues regarding age of seroconversion in different groups. This is not an ideal method but is used here as a surrogate in place of a longitudinal study. If incidence is more common in younger members of one population, than younger members of a different population, it suggests that the first population has a tendency to acquire the virus at a younger age.

If differences in prevalence or anti-viral response do occur between populations, it is necessary to know whether the difference is specific for the type of virus being studied, and its pattern of persistent infection over the lifetime of the host. For this purpose, the immune responses to tetanus toxoid, a temporary immune challenging antigen, have been used. Tetanus vaccine responses are almost ubiquitous, and tetanus vaccination is not contraindicated in SLE.

Both HCMV and EBV are only clinically relevant in pregnancy and immunocompromised hosts. In these situations, HCMV antigen and DNA are detectable. As it is difficult to detect antigen or DNA in healthy controls, for the purpose of this study, the determination of virus carriage is made using the detection of antibodies. HCMV seropositivity is determined by the presence of anti-HCMV IgG. Anti-CMV IgM antibodies are made early in infection but persist in some individuals. IgG avidity is used in pregnant women to determine whether the presence of anti-HCMV IgM represents a primary infection or a virus reactivation [117]. Low avidity IgG antibodies are found early in infection, so the presence of these combined with the presence of IgM antibodies, represents a recent infection. IgG avidity increases over time, so the presence of high avidity antibodies indicates a less recent infection. EBV IgG and IgM serological responses are also considered in this chapter. EBV DNA is detectable in healthy controls, and will be examined in chapter three.

This chapter also examines IFN $\alpha$  concentrations in plasma, and how this relates to viral infection. SLE patients have up-regulated expression of IFN $\alpha$  inducible genes, and higher IFN $\alpha$  concentrations are associated with the worst disease activity. HCMV and EBV infection of plasmacytoid dendritic cells causes the release of IFN $\alpha$  [118]. Although it has

been shown that CMV seropositivity does not affect concentration of IFN $\alpha$  in plasma in healthy individuals[119] it is possible that HCMV infection is associated with IFN $\alpha$  in SLE.

## Results

EBV seropositivity has, for the purpose of this study, been defined as the identification of IgG antibodies against VCA, or the presence on IgG antibodies against EBNA-1. HCMV seropositivity has been defined as the presence of IgG antibodies against HCMV infected, lysed fibroblasts.

	Control			SLE			P value
	n	% Negative	% Positive	n	% Negative	% Positive	
CMV IgG ELISA	48	39.58	60.42	72	51.39	48.61	0.2041
CMV IgM ELISA	23	91.30	8.70	52	61.54	38.46	0.009
EBV IgG ELISA	35	11.43	88.57	61	18.03	81.97	0.391
EBV IgM ELISA	35	65.71	34.29	60	78.33	21.67	0.1779
EBV IgG VCA	25	4.00	96.00	27	3.70	96.30	0.9557

**Table 6: Percentages of controls and SLE patients HCMV and EBV positive by each method used, at the first sample donation. Borderline and clear positive samples have been included as positive in commercially available assays. Samples showing fluorescence at 1/10 have been considered positive.**

There is no significant difference in the prevalence of EBV infection as measured by the presence of anti-EBV IgG, between SLE patients and healthy controls, using either method employed (Table 6). Anti EBV IgG was measured by ELISA, measuring titre of anti-EBNA-1 IgG, and by immunofluorescence, identifying the presence of IgG antibodies against VCA. IgG antibody responses to EBV by ELISA and IFA are not of similar frequency. In SLE patients and controls there was not an exact correlation between the presence of anti VCA

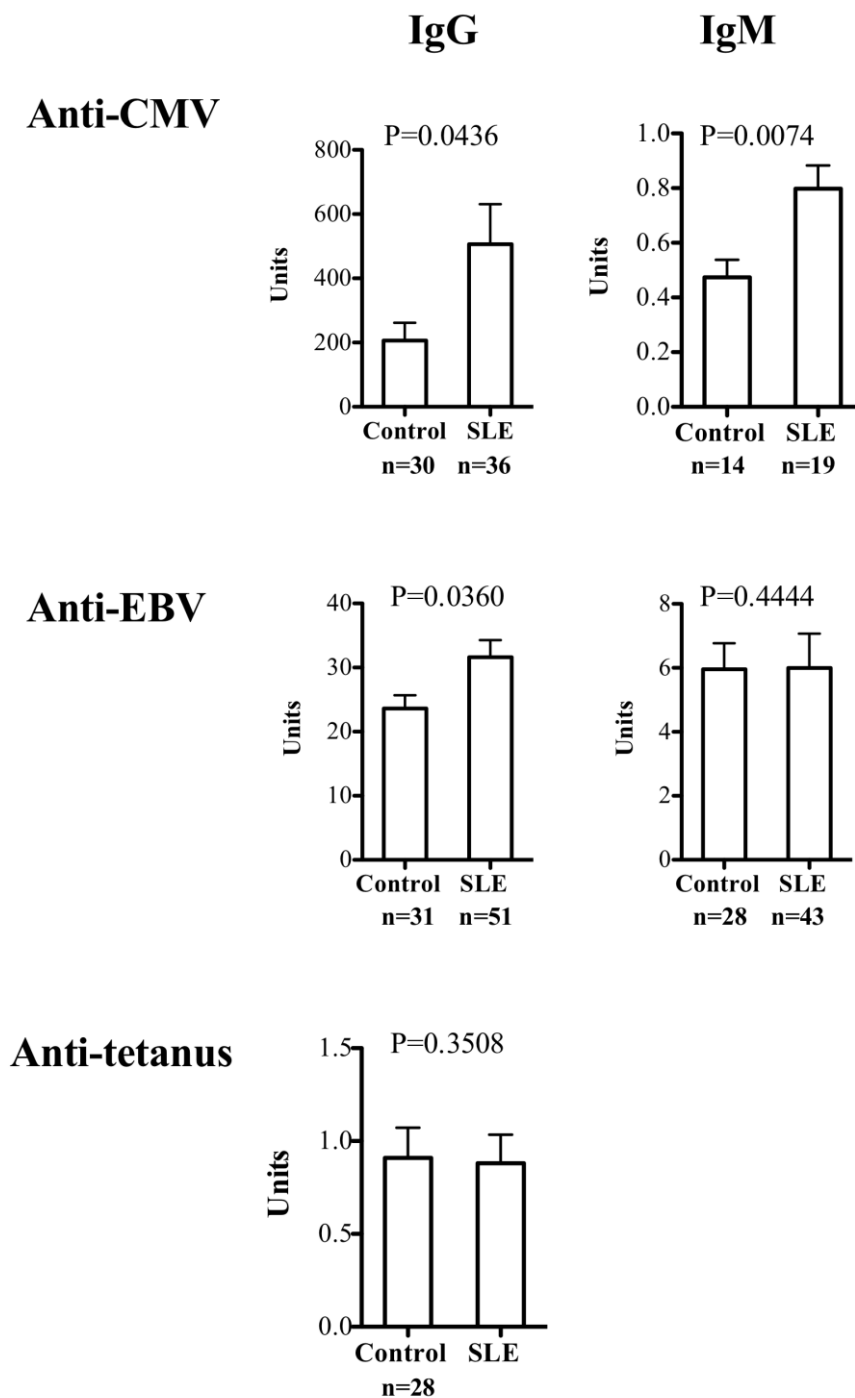
antibodies and anti EBNA-1 antibodies. 22% of SLE patients tested by both methods carried anti-VCA-IgG, but no anti-EBNA-1 IgG. 13% of controls shared this response. No members of either population had anti-EBNA-1 responses without anti-VCA responses ( $p=0.5098$  by  $\chi^2$ ). Detection of anti VCA antibodies by IFA is more sensitive than detection of anti-EBNA-1 antibodies by ELISA. Five patients' samples contained too high a concentration of autoantibodies to allow detection of the presence or absence of anti-VCA antibodies. Autoantibody binding to all B-cells made the identification of binding to VCA antigens on around 1/10 EBV infected B-cells difficult. Alternative samples, from later donations, were available which allowed detection of anti-VCA antibodies in all but two patients. These two were excluded from analysis for this method.

There was no significant difference in the frequency of detection of anti EBNA-1 IgM antibodies in SLE patients and controls. The frequency of anti EBNA-1 IgM antibodies in SLE patients was not significantly different from expected data supplied by manufacturer (84.1% negative of 88 samples, vs 78.33% negative SLE patients  $p=0.3732$   $\chi^2$ ). Control samples did show significantly higher seropositivity than the data supplied by the manufacturer ( $p=0.0243$ ).

There was no difference in the frequency of detection of anti-HCMV IgG antibodies between SLE patients and controls. The prevalence of HCMV was similar to that previously reported [120]. It was not possible with this size of population to identify any differences in seroprevalence between males and females. The percentage of SLE patients testing positive for anti HCMV IgM antibodies is significantly higher than that found in controls.

As HCMV prevalence is thought to increase with age, patients and controls were split into two populations, based on being older or younger than the median age of 43. Younger SLE

patients had similar seropositivity rates to younger healthy controls (47% vs. 37.5% respectively,  $p=0.5052$ ), whereas older SLE patients were less likely to be HCMV seropositive (50% vs. 89% respectively,  $p=0.0043$ ). Healthy controls have higher seropositivity rates in the older group than the younger group (89% vs. 37.5% respectively,  $p<0.0001$ ), whereas SLE patients have similar prevalence in young and older groups (47% vs. 50% respectively,  $p=0.826$ ). EBV seropositivity by either method does not show any association with age in either population.

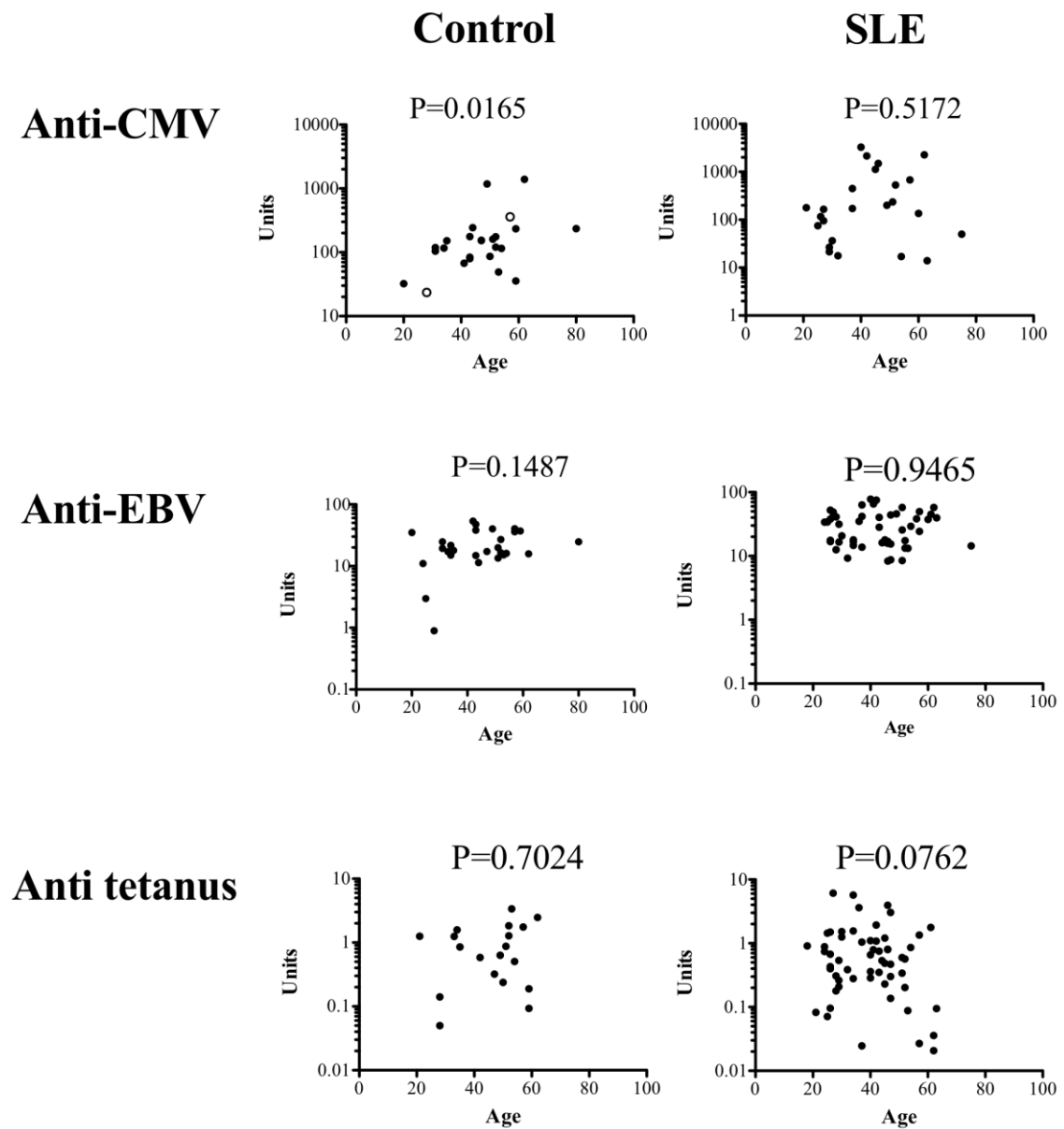


**Figure 1:** IgG and IgM titre in response to HCMV, EBV and Tetanus ELISAs. Only IgG seropositive individuals have been included for HCMV and EBV. Tetanus data is representative of the whole cohort. IgG antibody titres are shown as arbitrary units from a quantitative scale. IgM antibody titres are shown as arbitrary units from a semi-quantitative scale. Bars represent mean values  $\pm$  SEM.

Anti-HCMV IgG titres are elevated in SLE compared to healthy controls (Figure 1  $p=0.0436$ ). IgG binding to autoantigens has been excluded by the use of an uninfected control. IgG binding to uninfected fibroblasts is elevated in SLE patients (Data not shown  $p=0.0009$ ), but has been accounted for in the results. Anti-HCMV IgM titres are also elevated in SLE patients. The anti-HCMV IgM assay does not account for the presence of autoantibodies, but anti-HCMV IgM binding to uninfected fibroblasts is not elevated (data not shown  $p=0.26$ ).

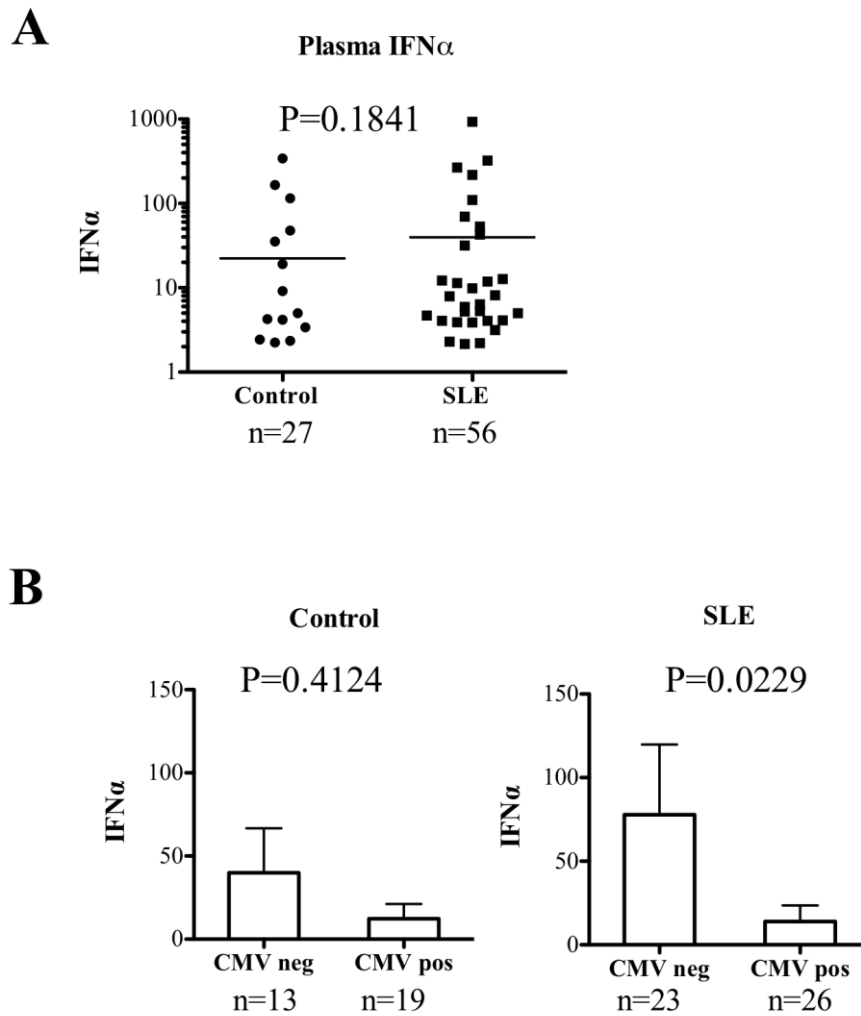
Anti-EBV IgG titres are elevated compared to controls. Anti-EBV IgM titres are similar in SLE patients and healthy controls. The assay used to determine EBV titres, uses EBNA-1 peptide as antigen, therefore risk of non-specific autoantibody binding is minimal. The magnitude of IgG response to tetanus toxoid is similar in SLE patients and healthy controls.

77% of SLE patients had protective antibody titres against tetanus as defined by the presence of  $>0.1\text{IU/ml}$  (WHO 76/589). 85% of controls had protective antibody titres by the same criteria ( $p=\text{ns}$ ).



**Figure 2: Association of IgG titre with age.** Each point represents an individual's age and IgG titre at the first sample donation. IgG titres are arbitrary units from a quantitative scale. Only positive individuals have been included for HCMV and EBV.

As seroprevalence increases with age in controls but not SLE patients, we wished to further investigate the relationship between age and antiviral responses. In controls, anti-HCMV titre correlates with age (Figure 2,  $p=0.0165$ ). This is not the case in SLE patients. IgG titres are increased in older controls ( $>43$ ), compared to younger controls ( $p=0.0272$  t-test), but there is no difference in titre in SLE between older and younger patients. Anti-HCMV IgM titre is not correlated with age in controls or SLE patients (data not shown). Anti-EBV IgG titres are not correlated with age in SLE patients or controls. Anti-EBV IgM titre is not correlated with age in controls, but is negatively correlated with age in SLE patients (data not shown  $p=0.0246$ ). There is no correlation between anti-CMV IgG titres and anti-EBV IgG titre in controls or SLE patients (data not shown  $p=0.8933$  and  $p=0.6680$  respectively).



**Figure 3: IFN $\alpha$  concentrations in plasma.** (A) Plasma IFN $\alpha$  in controls and SLE patients. Bar represents mean value. (B) Control and SLE plasma IFN $\alpha$  concentration in HCMV negative and HCMV positive individuals. Bars represent mean  $\pm$  SEM. IFN $\alpha$  concentration is shown in pg/ml.

There is, as shown previously, no difference in IFN $\alpha$  concentration in SLE and control plasma (Figure 3). Among SLE patients, HCMV positivity is associated with reduced plasma concentrations of IFN $\alpha$  ( $p=0.029$ ). HCMV seropositivity does not affect plasma IFN $\alpha$  levels in healthy controls ( $p=0.4124$ ). There was no association between EBV positivity by any

method and plasma IFN $\alpha$  concentrations (data not shown). There was also no relationship between the magnitude of anti-HCMV or anti-EBV response and IFN $\alpha$  concentration. IFN $\alpha$  levels are not associated with the age of SLE patient or healthy controls, nor with age of diagnosis or time since diagnosis. 56% of SLE patients have detectable levels of IFN $\alpha$  ( $>0$ pg/ml). 41% of controls had detectable levels of IFN $\alpha$  using the same criteria ( $p=ns$ ).

## Discussion

SLE patients have previously been reported to have increased EBV seroprevalence relative to controls, in both paediatric and adult populations [25, 108]. This is not the case in this study, by both ELISA and IFA, SLE patients showed similar prevalence to controls. However, the presence of anti-EBNA-1 antibodies as detected by ELISA, does not always correspond with the presence of anti-VCA antibodies by IFA. Anti-VCA-antibodies have been detected, in the absence of anti-EBNA-1 antibodies. This serological pattern was also identified in 11.8% of samples in a study in 2009 [86]. Anti-VCA antibodies normally develop before anti-EBNA-1 antibodies, and maintain a higher titre [121]. Therefore, those that are positive only by VCA may have been recently infected, or they may have not yet developed a high enough anti EBNA-1 titre to be detected.

While HCMV seroprevalence is similar between SLE patents and controls when entire populations are considered, there are significant differences in prevalence in the older half of the population. Controls' seroprevalence increases with age, as does anti-HCMV IgG titre. The increase in seroprevalence in older people has been reported previously [120]. This is not the case in SLE, both seroprevalence and titres are similar in the older and younger patients. This indicates that SLE patients are not acquiring the virus at a similar rate to controls, possibly because they acquired it at a younger age. However, in this study younger patients (<43) had similar seroprevalence to younger control. The only difference exists in those older patients. It is possible that SLE patients do not acquire the virus as efficiently as healthy controls. This may be due to social factors, disease activity or disease treatment. It has been impossible in this study to control for social factors, and no differences were discovered between those who acquired the virus and those who didn't in terms of disease activity or

treatment. It may be that the immune environment in SLE patients is not conducive to infection, either because of disease characteristics or because of treatment (to be discussed in more detail later).

It has not been possible to identify any association with age and EBV seroprevalence, likely due to the very low frequency of EBV negative individuals. A larger cohort would be necessary to test any association.

The only antibody specificity to show a difference in prevalence between SLE and controls is anti-HCMV IgM. Increased positive IgM antibodies may be representative of increased CMV reactivation in SLE patients compared to healthy controls. The presence of anti-HCMV IgM antibodies in SLE patients has been associated with worse disease activity, and it has been suggested that virus reactivation exacerbates disease [122]. However, SLE patients have an increased rate of false IgM positivity, defined by the lack of anti-HCMV IgG, and the presence of anti-HCMV IgM antibodies. Of 21 SLE patients negative by IgG, six had apparent IgM antibodies. No IgG negative controls had IgM antibodies. Patients with autoimmune diseases do have increased rates of false positive IgM antibodies. The presence of false positive IgM in pregnancy, among patients with autoimmune diseases, was associated with worse outcome, including earlier delivery and lower birth weight [99]. It is possible that the elevated level of IgM binding to HCMV assays in SLE patients is the result of autoantibody binding, levels of which are elevated in patients with worse disease activity. The alternative explanation for the presence of IgM antibodies without IgG antibodies is that they are experiencing a primary infection, or have delayed class switching. A small study in 1996 of immunocompromised patients found that those undergoing a primary infection had significantly delayed maturation of the IgG response and maintained IgG response longer than immunocompetent individuals [123]. Determination of IgG avidity would have been useful

in clarifying this situation. However, given the available data, it seems unlikely that 28.6% of patients are experiencing a primary infection. It is more plausible that the elevated IgM seropositivity in SLE is a result of false positives, possibly due to autoantibody binding to the plate. Indeed, when only anti-HCMV IgG positive individuals are included, there is no difference in the prevalence of anti-HCMV IgM between SLE patients and controls ( $p=0.1804 \chi^2$ ). False positives are more common in SLE patients, for diseases such as HIV [124] syphilis, and SARS [125], due to cross-reaction of autoantibodies in laboratory assays. There was no difference in anti-EBV IgM seropositivity between SLE patients and controls, suggesting there are no differences in the frequency of EBV reactivation in these populations. Controls showed higher seropositivity than predicted by the kit manufacturer, probably due to differences in the population being studied. EBV seropositivity is less common in those with higher socioeconomic status, infection is associated with lower paternal education, smoking and in females [93]. Using different groups of healthy people can therefore give very different results. It is also possible that by diluting plasma in RPMI 1:1 then using half the dilution factor suggested by the manufacturer, that interfering factors were introduced to the assays causing elevated antibody binding.

Anti-HCMV IgG titre is elevated in SLE patients compared to controls, this may be indicative of elevated HCMV load, requiring additional humoral control. Anti HCMV IgG responses may be elevated due to antibody binding to self-peptides expressed as a result of HCMV infection. HCMV infection causes a number of changes to occur within the cell and the expression of a number of antigens. Antibodies may be binding antigens expressed as a result of the intracellular changes that occur on infection. Infection of fibroblasts with HCMV causes the expression of 60kDa Ro/SSA antigens, although these are not expressed on the cell

surface, lysing of infected cells would cause these antigens to be available for binding in ELISA [101]. 35% of SLE patients have antibodies against this antigen [126].

Anti-EBV EBNA-1 IgG titre is also elevated in SLE patients compared to controls, indicating elevated requirement for virus control, possibly due to increased EBV load or impaired cellular control. These aspects will be discussed in chapters 3 and 2 respectively. The EBV assay measures binding to peptides, minimising the potential for false positives, although, the peptide used, EBNA-1, has been shown to be a molecular mimic of the autoantigen Sm. Sm autoantibodies present in SLE patients, these may be responsible for the elevated binding. Anti-EBV IgG antibodies were also found among patients with rheumatoid arthritis, population with similar treatment regimen to SLE patients [127].

Anti-tetanus IgG responses are not elevated in SLE patients; increased HCMV and EBV responses are therefore not the result of hypergammaglobulinemia.

Increasing anti-HCMV IgG titres with age in controls may reflect increasing virus loads, requiring increased immune control. It is also possible that this increase in titre is the result of an accumulation of herpes specific responses, either due to constant immune stimulation or as a result of a small accumulation with every period of virus reactivation. Anti-HCMV IgG titre may therefore reflect the time since infection, but as we are unable to determine date of primary infection, it is not possible to confirm this hypothesis.

EBV responses do not increase with age in this population. There was also no association found between anti-EBV response and sex, although with only 1/10 SLE patients being male, the difference would have to be massive to be detected. A study in Texas showed that anti-EBV IgG responses are higher in older people, and in females, however this study involved 2706 people, giving it considerably more power [128].

As the anti-VCA-IgG antibody assay was not quantitative, it was not possible to examine an association between anti-VCA titre and anti EBNA-1 titre. Anti-tetanus IgG titre does not increase with age in controls or SLE patients. Tetanus infection, unlike HCMV infection, is not a lifelong challenge to the host, and it is possible therefore, that it does not require an increase in humoral control. EBV however, is a persistent virus, requiring consistent control, but unlike HCMV anti-EBV IgG titre does not increase suggesting the increase is HCMV peculiar to HCMV.

Increased anti-HCMV IgM titres in SLE patients may be explained by the presence of autoantibodies. These would bind to non-HCMV peptides on the ELISA plate, giving a falsely elevated result. The other antibody assays used are not susceptible to this interference, as they are either coated with peptide alone, or autoantibody binding is accounted for by use of an uninfected control.

The decrease in anti-EBV IgM response in older people may reflect reduced frequency of reactivation in older patients. However, since a number of SLE patients are likely to have false positive IgM results, it may be that the frequency of false positivity reduces with age. This may reflect changes in the pattern of disease activity, as patients get older.

Plasma IFN $\alpha$  concentration is not elevated in SLE patients relative to healthy controls as shown previously. SLE patients have lower serum IFN $\alpha$  levels if HCMV seropositive, compared to seronegative. This data does not obviously fit with the fact that HCMV infection of pDCs causes the release of IFN $\alpha$  [129]. However, it is well known that HCMV makes use of multiple immunomodulatory mechanisms, the ability to impair production of an inflammatory cytokine would be of benefit to HCMV. IFN $\alpha$  causes the up-regulation of anti-

viral function in a number of immune cell types. It causes the upregulation of MHC I molecules, increasing T-cell recognition of infected cells, enhances NK killing, and supports the differentiation of monocytes into antigen presenting DCs [129]. It has also been suggested that IFN $\alpha$  causes the differentiation of naïve CD4<sup>+</sup> T-cells into IGNg producing T-cells [130]. There is no evidence that HCMV downregulates IFN $\alpha$  expression, but given the viruses other immunomodulatory abilities, it is not implausible.

Alternatively, HCMV infection may be overstimulating pDCs causing them to become anergic and unable to produce more IFN $\alpha$ . Repeated stimulation of pDCs via toll-like receptor ligation resulted in reduction of IFN $\alpha$  production capacity. However, the ability to produce IFN $\alpha$  was regained after 24 hours without stimulation.

Conversely, SLE patients with the highest IFN $\alpha$  concentrations may be protected from HCMV infection.

Serological responses in SLE patients are similar to, or exceed those found among controls, it is therefore likely that these responses are adequate to control virus. This indicates that neither disease itself nor its treatment has a detrimental effect on the humoral immune response to HCMV or EBV.

## **Results 2: Cellular responses**

## Introduction

T-cell responses are fundamental in the control of herpes viruses, inadequate responses, such as those found post allogeneic stem cell transplant and in HIV patients, lead to virus reactivation and pathogenesis. Post transplant, HCMV specific cell counts of  $10^6$  to  $20 \times 10^6$  cells/L are protective against HCMV disease [131]. Adoptive transfer of HCMV specific lymphocytes, generated from the stem cell donor, is used post transplant to prevent and treat viral reactivation [129]. In HIV patients, frequency of HCMV specific cytotoxic CD8 T-cells correlates with protection from HCMV disease [129].

Expansions of HCMV specific CD8+ T-cells are found in older people, as determined by both tetramer staining and IFN $\gamma$  production [129]. These cells remain functional, suggesting the increase in frequency is not driven by an accumulation of anergic cells [132]. HCMV specific CD4+ T-cells account for around 1-2% of peripheral CD4+ T-cells in seropositive people, although some individuals have much higher frequencies. HCMV specific T-cells frequently target pp65, and are either CD45RA+ CD45RO+, or an effector memory phenotype. HCMV specific CD4-T-cells are capable of producing IFN $\gamma$  and TNF $\alpha$ , identifying them as part of the Th1 subset [133]. A small study by Bitmansour et al showed that healthy carriers generally have 1-3 dominant clonotypes of CD4+ HCMV specific T-cells, as defined by TCR V $\beta$  usage. This population accounted for up to 50% of the total HCMV response, and up to 4% of peripheral blood CD4+ T-cells [129]. A decline in CMV specific cell frequency post-renal transplant is predictive of HCMV disease [134], indicating the necessity of adequate CD4+ T-cell responses.

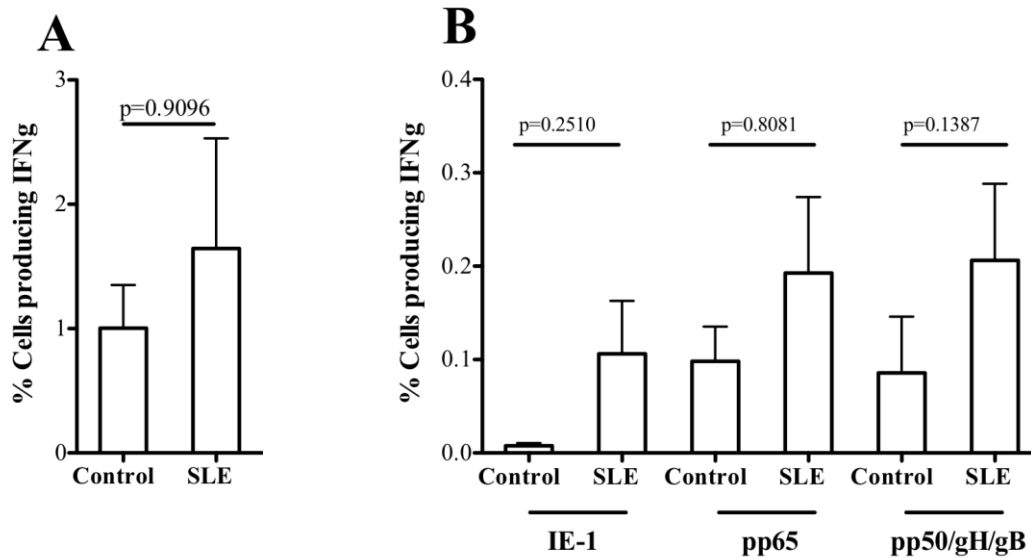
T-cells are also crucial for the control of replication of EBV after primary infection, as demonstrated by the occurrence of lymphoproliferative disorders in those with impaired T-cell responses. (Rickinson, A.B., Kieff, E., 2007. Epstein-Barr virus. In: Knipe, D.M), Field's Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 2575–2627).

Low numbers of EBV specific CD4<sup>+</sup> T-cells are a risk factor for EBV associated disease in patients treated with immunosuppressive medication [129]. Anti-EBV T-cell responses are primarily directed against EBNA3A, LMP2 and EBNA1, with frequencies of up to 1% of CD8<sup>+</sup> T-cells [84]. Lytic responses reach up to 3% of peripheral CD8<sup>+</sup> T-cells, and typically target BZLF1 and BRLF1. Anti-EBV responses are considerably smaller than HCMV specific responses [135] [129].

Peptide stimulation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells by their respective HCMV peptide targets can result in the production of IFN $\gamma$  and TNF $\alpha$ . CD4<sup>+</sup> T-cells can also be stimulated using a mixture generated by lysing HCMV infected fibroblasts. This method requires the presence of antigen presenting cells, which take up the antigen, process it, and present it to CD4<sup>+</sup> T-cells. Peptide stimulation involves peptides, which activate T-cells, without the requirement for processing by APCs.

T-cell responses are thought to be more important than humoral responses for control of herpesviruses [128]. T-cells detect intracellular pathogen, by the presentation of viral antigens on infected cell surfaces, and are therefore much more efficient at controlling virus.

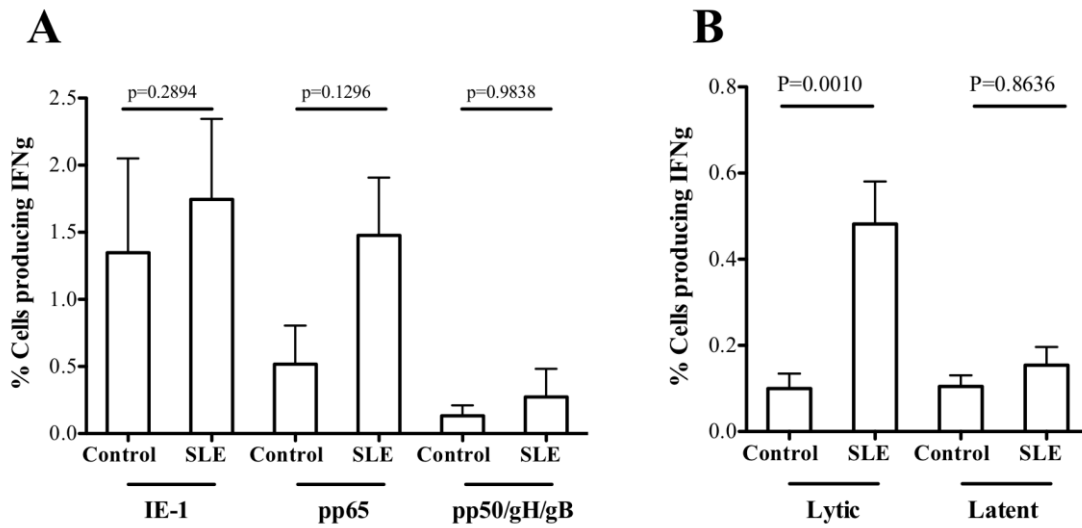
## Results



**Figure 4: Percentage of CD3+ CD4+ T-cells producing IFN $\gamma$  in response to stimulation with; A, HCMV infected lysed human fibroblasts (control n=14, SLE n=22), B, three HCMV peptide mixes (control n=14, SLE n=15). Only HCMV seropositive individuals have been included. Bars represent mean  $\pm$  SEM.**

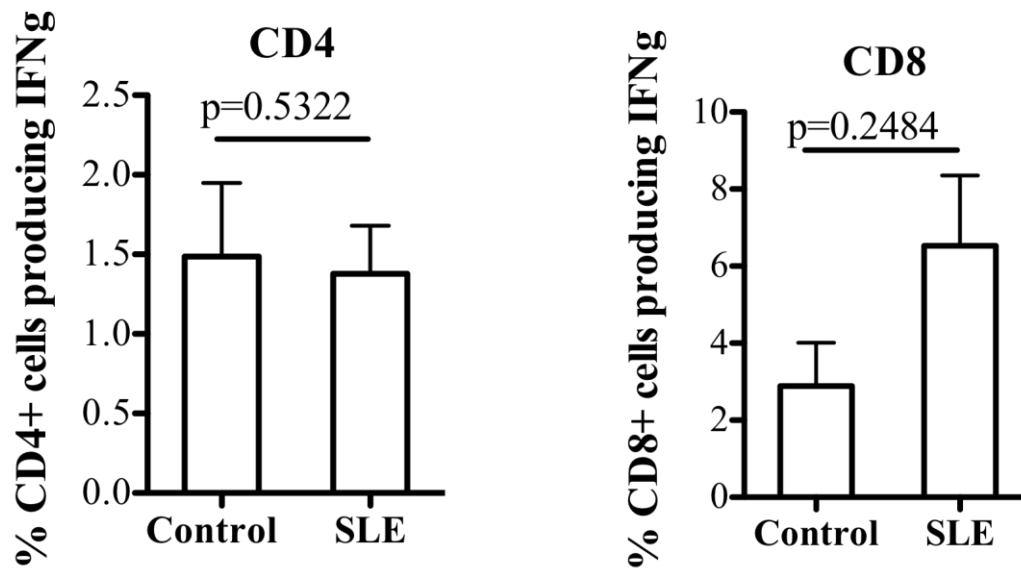
A similar proportion of CD3+ CD4+ PBMCs from SLE patients and healthy controls produce IFN $\gamma$  in response to in-vitro stimulation with HCMV lysate or HCMV peptide pools (Figure 4). Absolute numbers of CD3+ CD4+ T-cells are not known for controls so can not be compared directly with those of SLE patients. However, absolute numbers of HCMV specific CD3+ CD4+ cells are likely to be lower, as 38.1% of SLE patients measured here were lymphopenic ( $<1 \times 10^9/L$ ), and median lymphocyte count was  $1 \times 10^9/L$ . cell Generally, patients and controls with negative IgG responses do not produce IFN $\gamma$  in response to HCMV lysate stimulation. Mean IFN $\gamma$  production on response to HCMV lysate in sero-negatives was 0.013% and 0.005% in controls and SLE patients respectively, compared to 1.095% and

1.096% of sero-positives. Similar results were found on stimulation with HCMV peptide mixes. Six of 24 seropositive SLE patients, capable of producing IFN $\gamma$  in response to HCMV peptide stimulation, did not produce any IFN $\gamma$  on stimulation with HCMV lysate. All of 20 healthy sero-positive controls tested were able to produce IFN $\gamma$  on stimulation with HCMV lysate ( $p=0.0161$  by  $\chi^2$ ). There was no association between ability to produce IFN $\gamma$  in response to HCMV lysate and treatment by any specific drug, or with disease activity. In both SLE patients and healthy controls, the percentage of CD3+ CD4+ cells producing IFN $\gamma$  is lower in response to peptide stimulation than HCMV lysate stimulation. The frequency of IFN $\gamma$  response to HCMV lysate was similar in seronegative SLE patients and seronegative controls ( mean  $\pm$  SE,  $0.019\pm0.013$  vs.  $0.013\pm0.007$  respectively,  $p=0.04808$ ).



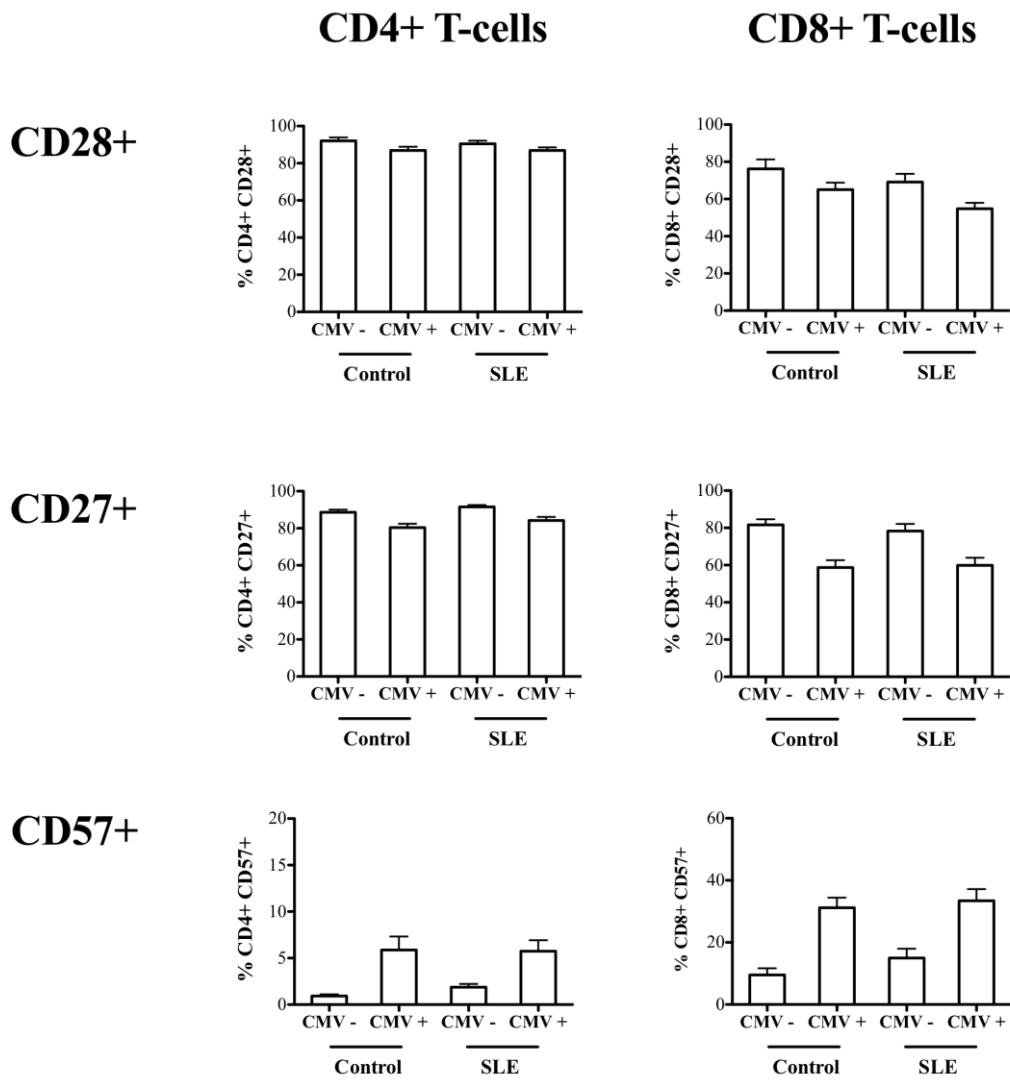
**Figure 5: Proportion of CD3+ CD8+ T-cells producing IFN $\gamma$  in response to stimulation with A, HCMV peptide pools (control n=16, SLE n=17) and B, EBV peptide pools (control n=24, SLE n=34). Only seropositive patients and controls have been included for each virus. Bars represent mean  $\pm$  SEM.**

A similar proportion of CD3+CD8+ T-cells produce IFN $\gamma$  in response to ex-vivo stimulation with HCMV peptide mixes in vitro in SLE patients and controls (Figure 5). As with CD3+ CD4+ T-cells, absolute numbers of cells responding to HCMV in vivo is likely to be lower in SLE patients, due to lymphopenia. SLE patients have an increased frequency of CD3+ CD8+ T-cells responding to EBV lytic peptides by producing IFN $\gamma$  (0.4820%  $\pm$ 0.09873 versus 0.09980%  $\pm$ 0.03461 respectively, mean  $\pm$  SEM. p=0.01). There is no difference in the frequency of cells producing IFN $\gamma$  in response to latency peptides (p=0.8636). SLE patients have significantly more CD3+ CD8+ cells producing IFN $\gamma$  in response to lytic peptides, than to latency peptides (p=0.0023). This is not the case in controls (p=0.3963).



**Figure 6: Proportion of CD4+ and CD8+ cells producing IFN $\gamma$  in response to stimulation with SEB (control n=14, SLE n=35).**

There is no difference between the proportion of CD4+ or CD8+ T-cells producing IFN $\gamma$  in response to SEB stimulation (Figure 6). Neither are there any differences in the percentages of individuals able to generate a response to SEB.



**Figure 7:** Expression of differentiation markers on T-cell pool. Cells were analysed after 18 hours incubation in 5% FCS RPMI with no stimulation. The proportion of CD4+ or CD8+ cells expressing CD28, CD27 and CD57 differentiation markers is shown. SLE patients and healthy controls have been grouped based on HCMV seropositivity. Bars represent mean  $\pm$  SEM. Statistics are shown in table 7. Control CMV- n=12, control CMV+ n=23, SLE CMV- n=25, SLE CMV+ n=24.

			CD4+			CD8+		
	Populations compared	Bars compared	CD27+	CD28+	CD57+	CD27+	CD28+	CD57+
Control	CMV+ vs CMV-	1 vs. 2	**	ns	***	***	ns	***
SLE	CMV+ vs CMV-	3 vs. 4	***	*	**	**	ns	***
CMV -	Control vs SLE	1 vs. 3	ns	ns	*	ns	ns	ns
CMV+	Control vs SLE	2 vs. 4	ns	ns	ns	ns	ns	ns

**Table 7: Significance of data shown in figure 7 by Mann-Whitney. \*\*\* represents  $p < 0.001$ , \*\* represents  $p < 0.01$ , \* represents  $p < 0.05$ .**

SLE					Control		
		Correlation	Linear regression	$r^2$	Correlation	Linear regression	$r^2$
CD28	4+	<b>0.0446*</b>	0.073	0.0866	<b>0.0084*</b>	<b>0.0303*</b>	0.1561
	8+	0.5069	0.4566	0.0155	<b>0.0001*</b>	<b>0.0001*</b>	0.4915
CD27	4+	0.1323	0.6941	0.0031	<b>0.0001*</b>	<b>0.0021*</b>	0.2743
	8+	<b>0.0011*</b>	<b>0.0004*</b>	0.2229	<b>0.0001*</b>	<b>0.0001*</b>	0.4627
CD57	4+	0.075	0.7444	0.0022	<b>0.0002*</b>	<b>0.0223*</b>	0.1621
	8+	<b>0.0029*</b>	<b>0.0016*</b>	0.1881	<b>0.0001*</b>	<b>0.0001*</b>	0.3954

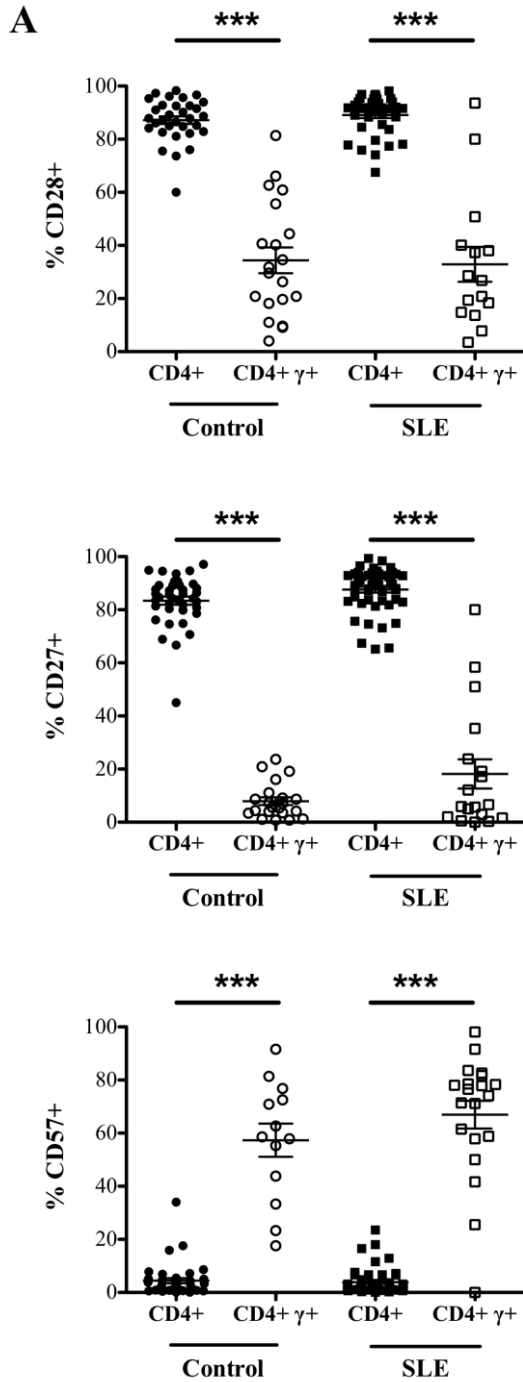
**Table 8: The association between the expression of phenotypic markers and age of participants. Correlation by spearman's rank, and  $p$  value and  $r^2$  of linear regression are shown.**

There were no differences in the percentage of CD4+ or CD8+ T-cells expressing CD27, CD28 or CD57 between SLE patients and healthy controls when entire populations are examined (data not shown  $p = ns$ ). Seropositivity in both controls and SLE patients is associated with a reduction in percentage of T cells expressing CD27, and an increase in

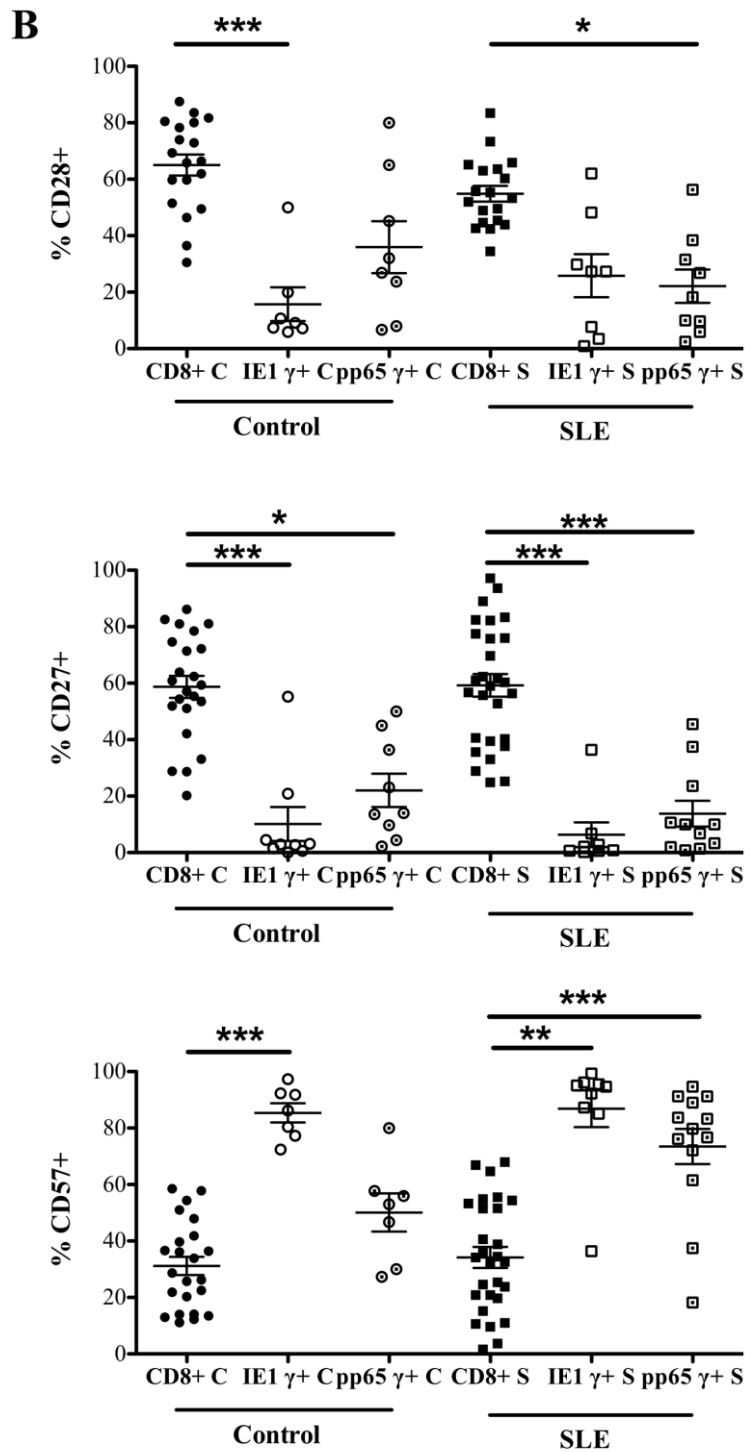
surface expression of CD57 on CD4+ and CD8+ T-cells (Figure 7 and Table 7). There is also significantly lower expression of CD28 in HCMV positive SLE patients compared to HCMV negative SLE patients. Among HCMV positive individuals, there is no difference in expression of any of the markers frequencies between controls and SLE patients. Among HCMV negative individuals, SLE patients have significantly higher expression of CD57 on CD4+ cells, than controls. HCMV has a marked effect on the expression of CD27 and CD57 on T-cells from controls and SLE patients. SLE patients' overall immune repertoire is very similar to that of controls, and is similarly affected by CMV seropositivity.

There was a trend towards SLE patents having a lower CD4+ to CD8+ (i.e. fewer CD4+ cells per CD8+ cells) ratio compared to controls but the result did not reach significance ( $p=0.0563$ ). There were no significant differences in CD4+ CD8+ ratio between younger and older patients and controls. Furthermore, an inverted CD4+ CD8+ ratio was not associated with any alterations in disease activity ( $p=0.51$ ).

There is enormous variation in expression of the phenotypic markers considered, on both CD4+ and CD8+ cells, among SLE patients and controls. These variations were strongly associated with the age of the participant among controls. Among controls, in CD4+ and CD8+ T-cells there are correlations between CD28, CD27 and CD57 expression and age (**Table 8**). There are also significant associations by linear regression among controls with expression of these phenotypic markers. The relationship between age and expression of these markers is less clear among SLE patients.

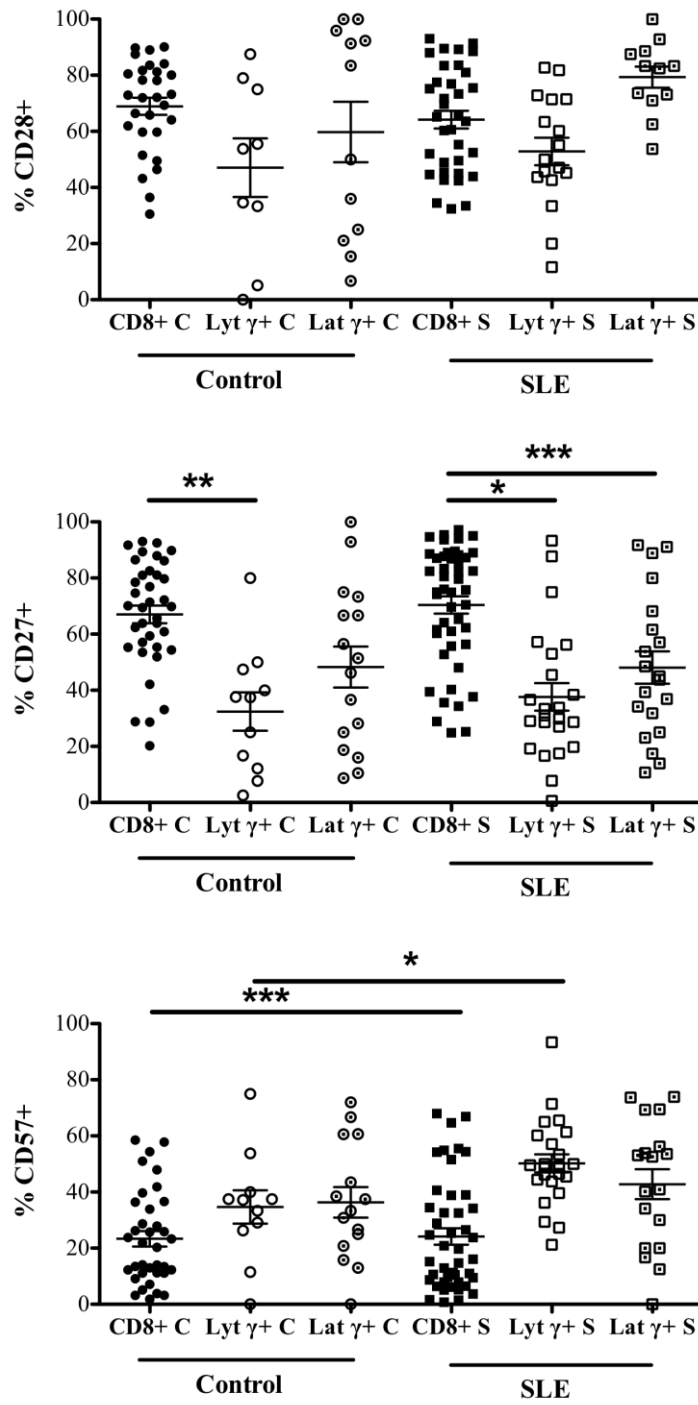


**Figure 8a:** Percentage of CD4<sup>+</sup> T-cells expressing CD28, CD27 and CD57 differentiation markers on stimulation with HCMV lysate. Percentage expression on total CD4<sup>+</sup> cell pool and on CD4<sup>+</sup> cells producing IFN $\gamma$ . Production of IFN $\gamma$  was in response to 18h stimulation with HCMV infected, lysed fibroblasts. Only individuals producing IFN $\gamma$  in response to CMV lysate have been included. Bars represent mean values  $\pm$  SEM. Kruskal-Wallis analysis and Dunn

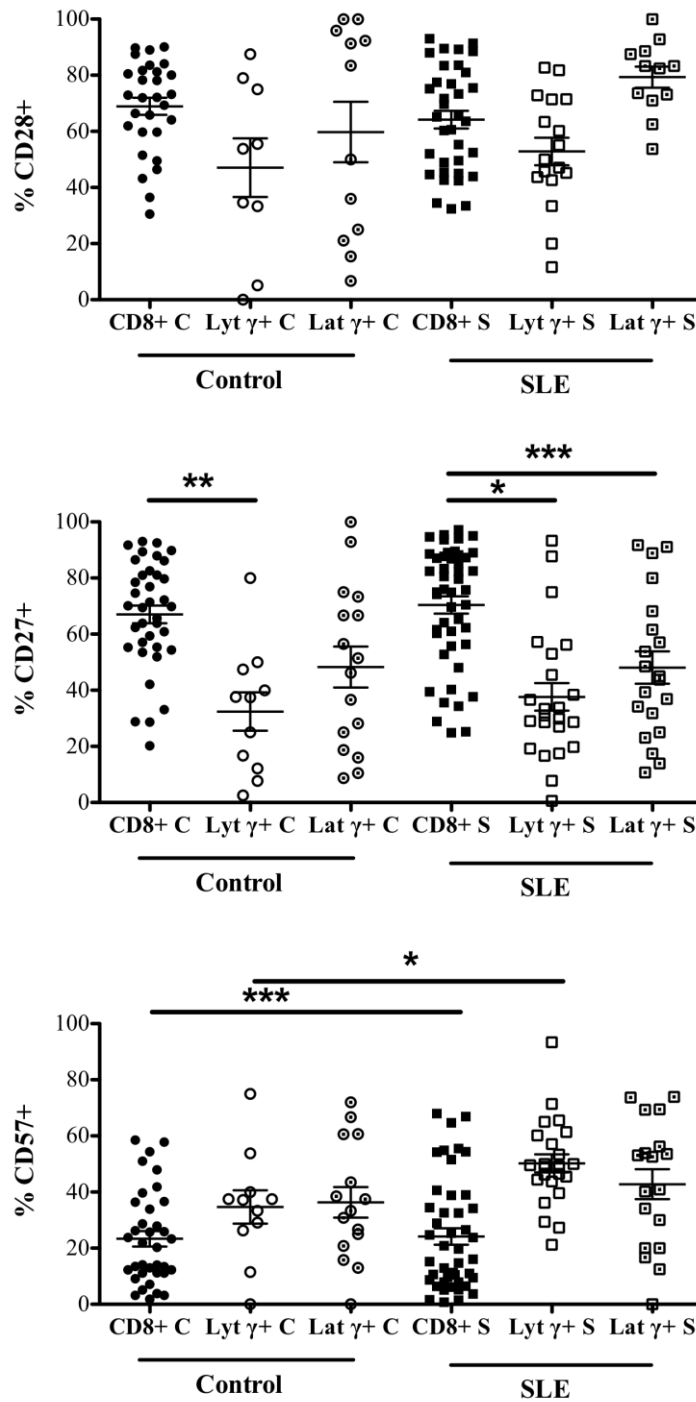


**Figure 8b:** Percentage of CD8+ T-cells expressing CD28, CD27 and CD57 differentiation markers. Percentage expression on CD8+ cells and on CD8+ cells producing IFN $\gamma$ . Production of IFN $\gamma$  was in response to 18h stimulation with HCMV IE-1, and pp65 peptide mixes. Bars represent mean values. Kruskal-Wallis analysis and Dunns

C



**C**

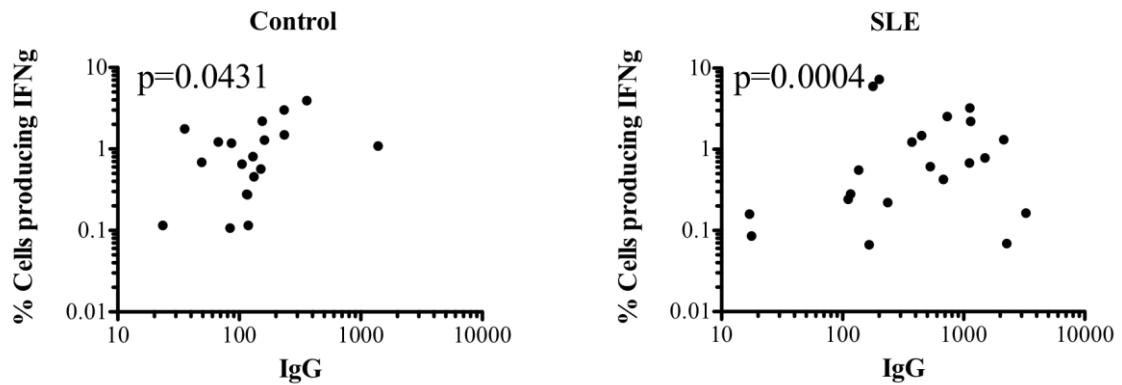


**Figure 8c:** Percentage of CD8+ T-cells expressing CD28, CD27 and CD57 differentiation markers. Percentage expression on CD8+ cells and on CD8+ cells producing IFN $\gamma$ . Production of IFN $\gamma$  was in response to 18h stimulation with EBV lytic and latent peptide mixes. Bars represent mean values. Kruskal-Wallis analysis and Dunns

CD4<sup>+</sup> T-cells producing IFN $\gamma$  in response to HCMV lysate have reduced surface expression of CD28 and CD27, and increased surface expression of CD57 compared to the total CD4<sup>+</sup> T-cell population (Figure 8a). This is true in SLE patients and healthy controls and the degree of reduction/ increase is similar in SLE patients and controls.

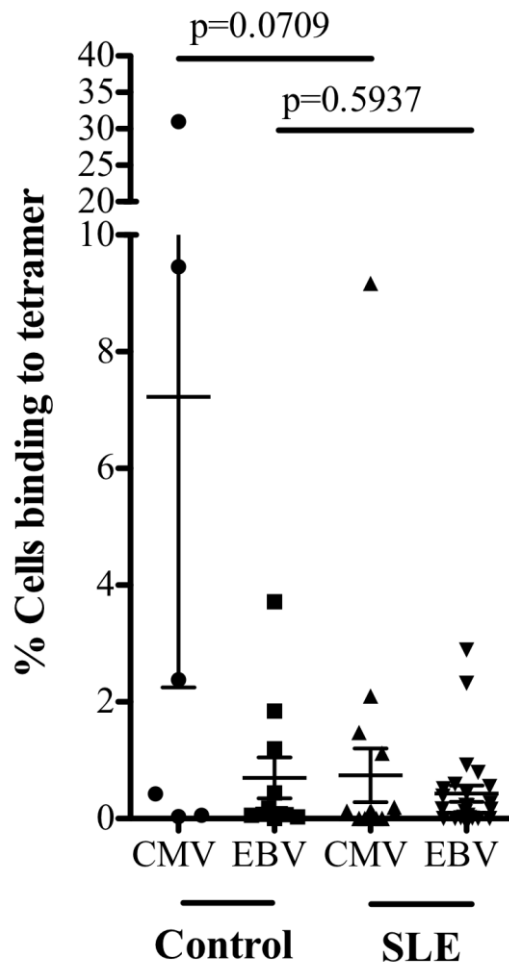
As with CD4<sup>+</sup> T-cells, HCMV specific cells express lower levels of surface CD27. CMV specific CD8<sup>+</sup> cells also express lower levels of CD28 and increased levels of CD57, although this does not reach significance for all examples (Figure 8b). There are no other significant differences in expression of these surface markers between SLE patients and healthy controls, nor any significant differences in expression levels between cells responding to IE-1 or pp65, although there is a trend towards cells responding to IE-1 having a more differentiated phenotype than those responding to pp65, as determined by expression levels of CD27, CD28 and CD57.

CD8<sup>+</sup> T-cells producing IFN $\gamma$  in response to EBV peptide pools have reduced expression of CD27 (Figure 8c), but no significant reduction in surface expression of CD28. CD57 expression on CD8<sup>+</sup> T-cells responding to EBV lytic peptide is significantly higher for SLE patients than healthy controls (Figure 8c,  $p=0.0264$ ). CD57 expression is also higher on CD8<sup>+</sup> cells from SLE patients than controls. These are the only differences between expression of these markers between SLE patients and controls. The phenotypic alterations associated with CMV specificity are of much greater magnitude than those associated with EBV seropositivity, especially the alterations in CD28 expression.



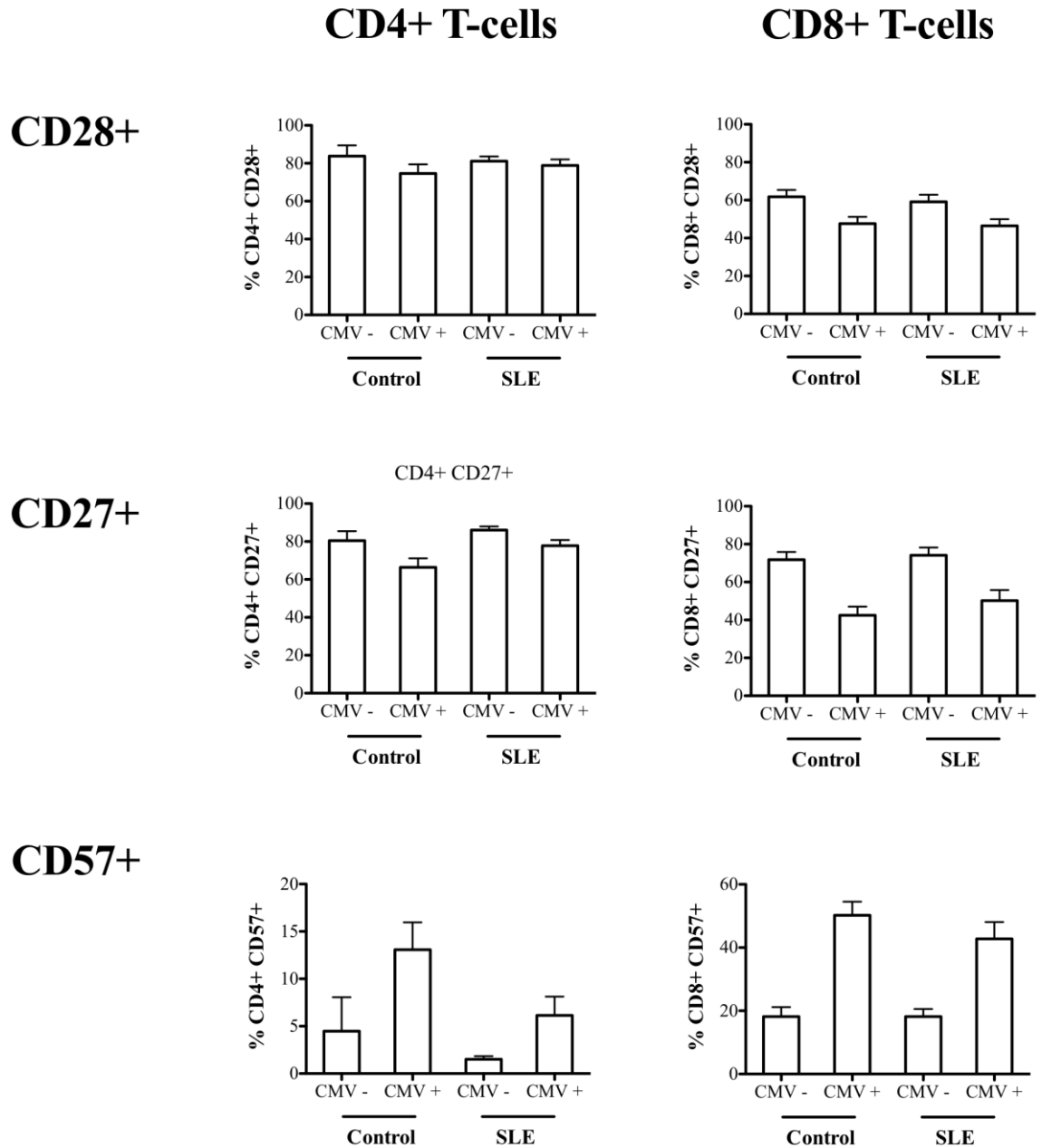
**Fig 9: Association between cells IFN $\gamma$  production and IgG titre in controls (n=19) and SLE patients (n=28). The proportion of CD4+ T-cells producing IFN $\gamma$  in response to 18h stimulation with HCMV infected, lysed fibroblasts compared to anti-HCMV IgG titre. Only HCMV seropositive individuals have been included. Correlation by Spearman's rank**

There is a significant correlation between anti-HCMV IgG titre and the percentage of cells producing IFN $\gamma$  in response to stimulation with HCMV lysate (Figure 9), in controls and SLE patients (p=0.0431 and 0.0004 respectively, Spearman's rank)



**Figure 10: Percentage of CD8+ T-cells binding to tetramer. Cells from seropositive individuals were stained with tetramers appropriate to their HLA type. Results for cells stained individually have been pooled to give a total percentage of cells responding in each individual. Bars represent mean  $\pm$  SEM.**

There is no difference in the percentage of CD8+ T-cells specific for the HCMV or EBV peptides tested (Figure 10). There is however a trend towards higher frequencies of T-cells responding to HCMV in SLE patients than controls.

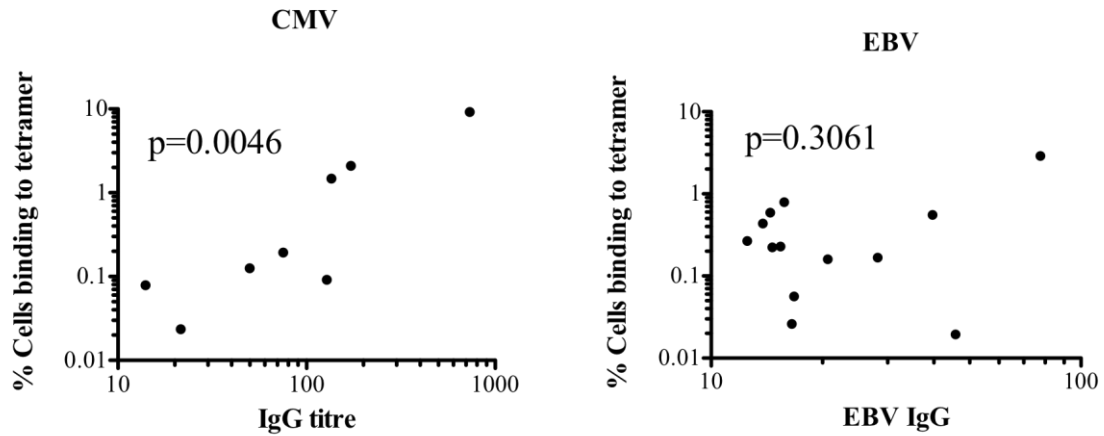


**Figure 11: Expression of differentiation markers T-cells.** The proportion of CD4+ or CD8+ cells expressing CD28, CD27 and CD57 differentiation markers. PBMC were stored in liquid nitrogen for up to 1 year, defrosted and then analysed. SLE patients and healthy controls have been grouped based on HCMV seropositivity. Bars represent mean  $\pm$  SEM. Control CMV - n=7, control CMV + n=10, SLE CMV- n=23, SLE CMV+ n=16.

			CD4+			CD8+		
	Populations compared	Bars compared	CD27+	CD28+	CD57+	CD27+	CD28+	CD57+
Control	CMV+ vs CMV-	1 vs. 2	ns	ns	*	***	*	***
SLE	CMV+ vs CMV-	3 vs. 4	*	ns	**	**	*	***
CMV -	Control vs SLE	1 vs. 3	ns	ns	ns	ns	ns	ns
CMV+	Control vs SLE	2 vs. 4	*	ns	ns	ns	ns	ns

**Table 9: Significance of data shown in figure 11 by Mann-Whitney. \*\*\* represents  $p<0.001$ , \*\* represents  $p<0.01$ , \* represents  $p<0.05$ .**

Phenotypic analysis of CD4+ and CD8+ cells that have previously been frozen (Figure 8), gives different results to analysis using fresh cells incubated overnight in 10% FCS (Figure 11, Table 9). In cells that have been previously frozen the most profound effects are noticeable in the CD8+ T-cell pool. Here, in common with results from fresh cells, CMV seropositivity is associated with a reduction in surface expression of CD28 and an increase in expression of CD57, in SLE patients and controls. In addition, frozen cells show a significant reduction in expression of CD28 on CD8+ cells from HCMV seropositive SLE patients and controls. In the CD8+ pool there are no differences in surface expression of any of the markers used between SLE patients and controls. CD4+ T-cells show a CMV associated increase in CD57 expression in SLE patients and controls, but no reduction on CD28 expression. CD27 expression is higher amongst HCMV positive SLE patients than controls, but is still expressed at reduced level compared to CMV+ SLE patients.



**Figure 12:** Association between percentage of cell binding tetramer and IgG titre in SLE patients. Only seropositive SLE patients have been included. P value represents Spearman's rank.

There is a significant positive correlation between anti-HCMV IgG titre and the proportion of CD8+ T-cells binding to tetramer in SLE patients (Figure 12). No such association exists for anti-EBV response. It was not possible to analyse similar data for healthy controls, as insufficient numbers were available.

## **Discussion**

### **IFN $\gamma$ responses to EBV lytic peptides are elevated in SLE patients**

Frequency of CD8<sup>+</sup> T-cells responding to EBV lytic peptides is elevated in SLE patients compared to controls, this may indicate more frequent experience of lytic peptides caused by more frequent virus reactivation. Responses are not elevated in response to latency peptides; therefore the increase is specific for lytic peptides.

Elevated frequencies of CD8<sup>+</sup> cells responding to EBV have also been found in patients with rheumatoid arthritis, following stimulation with a mixture of lytic and latency peptides [127].

Percentages of T-cells producing IFN $\gamma$  in response to peptide are lower than those generated in response to HCMV lysate as HCMV lysate contains all HCMV peptides. The peptide mixes are a limited set of peptides, which are in turn restricted by a limited set of HLA types, which the tested individual may, or may not possess.

The percentage of CD8<sup>+</sup>T-cells responding to stimulation with the third peptide mix is very low as only one CD8 specific peptide is included in the pool. The proportion of CD4<sup>+</sup> T-cells responding to HCMV peptide stimulation is lower than the proportion of CD8<sup>+</sup> cells responding. This may be due to the CD8<sup>+</sup> T-cells being more important in defence against HCMV, but it may also reflect the lower proportion of CD4 specific epitopes available.

The proportion of CD3<sup>+</sup> CD8<sup>+</sup> T-cells specific for CMV is greater than those specific for EBV, as measured by IFN $\gamma$  production. This indicates that HCMV requires a greater degree of immune control than EBV.

It was not possible in this study to directly compare the frequencies of HCMV specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. Gamadia et al showed that functional CD8<sup>+</sup> T-cells, as determined by ability to produce IFN $\gamma$ , correlates with functional CD4<sup>+</sup> responses in healthy controls. This

suggests both CD4<sup>+</sup> and CD8<sup>+</sup> responses are controlled by the same factors, and that both are crucial in virus management [133].

### **IFN $\gamma$ production is impaired in a subset of SLE patients**

It is more common for HCMV seropositive SLE patients to not produce IFN $\gamma$  in response to HCMV lysate stimulation. Six SLE patients are unable to produce IFN $\gamma$  in response to lysate but do in response to HCMV or EBV peptide. SLE patients' PBMCs also respond to SEB at a similar frequency to control and produce a similar sized response. They are therefore capable of IFN $\gamma$  production on appropriate stimulation, but are not producing it in response to HCMV lysate. HCMV lysate stimulation differs from peptide stimulation in that it requires processing by antigen presenting cells. As T-cells are stimulated with lysate in the presence of autologous APCs within the PBMC mix, it is possible that APCs in these SLE patients are not adequately processing or presenting peptide to generate T-cell stimulation. It is also possible that there is some impairment of IFN $\gamma$  production by CD4<sup>+</sup> T-cells, which CD8<sup>+</sup> T-cells do not share. It has not been possible to identify any characteristics of disease, or treatment regimen, associated with this inability to respond to HCMV lysate, but it is plausible that this impairment is due to treatment.

The quantity of IFN $\gamma$  produced per responsive cell was not recorded; it is possible that SLE patients have reduced intensity of IFN $\gamma$  production per cell, compared to controls.

Frequency of IFN $\gamma$  producing HCMV specific CD8<sup>+</sup> T-cells is elevated in corticosteroid treated patients who have undergone kidney transplant [133].

The lack of elevated IFN $\gamma$  production in response to HCMV lysate by HCMV seronegative SLE patients, compared to seronegative controls, suggests that CD4<sup>+</sup> T-cells are not responding to the self antigens in the stimulation mix.

### **Tetramer responses do not correlate with IFN $\gamma$ production**

The elevated anti-EBV IgG and elevated IFN $\gamma$  production in response to EBV peptide would suggest that both humoral and cellular responses to EBV are exaggerated in SLE patients. However, the proportion of T-cells binding tetramer is not elevated. The lack of any significant differences may be due to the restricted numbers of patients and controls tested, and the limited panel of tetramers used. As tetramers are specific for particular HLA restricted peptides, they are not necessarily representative of the magnitude or specificity of the entire T-cell response.

### **Cell surface differentiation markers are similar in SLE and controls**

There is no difference in the percentage of CD4<sup>+</sup> or CD8<sup>+</sup> T-cells expressing the differentiation markers CD28, CD27 and CD57, between SLE patients and healthy controls. Using this limited method of assessing the differentiation status of cells, it appears that the overall immune repertoire is similar in SLE and healthy controls[136]. The degree to which HCMV infection alters the expression of surface markers associated with differentiation status, is similar in SLE patients and healthy controls. This suggests HCMV has a similar immunomodulatory capacity in SLE patients as controls. CD4<sup>+</sup> CD28<sup>-</sup> T-cells have been associated with HCMV infection in SLE previously [43].

CD57 expression is elevated on unfrozen un-stimulated CD8<sup>+</sup> T-cells. CD57 is a known marker of antigen experience, being up-regulated on antigen stimulation. It is plausible that the inflammatory environment and excessive antigenic stimulation in SLE causes increased expression of this molecule on the T-cell pool. T-cells producing IFN $\gamma$  in response to IE-1 stimulation also have increased expression of CD57 compared to controls, this may be due to

normal up-regulation in response to HCMV infection in combination with the increased background expression found in SLE patients.

Phenotyping of T-cell pools using unfrozen cells incubated for 16 hours gave different results to when cells that had been previously frozen were used. On previously frozen cells only CD8<sup>+</sup> T-cells showed phenotypic changes associated with CMV infection. It is impossible to know with this data whether cells that have been frozen immediately after isolation, or cells that have been cultured overnight, give the most accurate representation of in-vivo phenotype, or whether CD4<sup>+</sup> or CD8<sup>+</sup> cells are being modified to a greater extent. The most accurate in-vitro method would be to examine cells immediately after separation, but even this method cannot accurately recreate the conditions a peripheral PBMC experiences in vivo.

The expression of differentiation markers on CMV and EBV specific cells in healthy people has been widely studied, with similar results to those found here. What has not been shown before however, is the expression level of differentiation markers of HCMV and EBV specific cells in SLE patients. PBMCs show very similar modifications in differentiation marker expression in SLE as in normal individuals, suggesting they have similar responses to, and similar effects of, herpesvirus infection.

It is worth considering the variation in level of surface expression of CD28, CD27 and CD57. Even in individuals with no known immune dysfunction there is huge variation in expression level. The variation in expression is most likely due to the large variation in age of participants. The correlation between loss of CD28 and CD27, and gain of CD57 with age, is less clear in SLE patients than controls. A study by Du et al, found that in healthy controls, TREC frequency correlated with age in healthy controls but not in SLE patients[40]. Both phenotyping and quantification of TRECs can be considered markers of previous stimulation,

both indicate there is no accumulation of cells, which have undergone increasing stimulation with age. Other previous work has shown that the decline of CD8<sup>+</sup> CD28 expression is already present in younger SLE patients, and is not dependant on age [43]. This may be due to younger SLE patients having already undergone significant levels of stimulation. The maintenance of expression of these markers in older SLE patients may also reflect the reduced seroconversion of patients, compared to controls.

The association between CD28 expression and the other characteristics investigated, is less powerful than the associations with CD27 and CD57. This may be related to the reduced numbers of controls and SLE patients' PBMCs stained with this antibody.

### **T-cell IFN $\gamma$ production correlates with IgG titre**

The correlation in SLE patients and controls between anti-CMV IgG titre and frequency of T-cell responses to CMV indicate that both branches of the immune system are co-ordinated in their function. It is possible that like IgG titre, anti-CMV T-cell responses accumulate with age, and may reflect time since infection.

It has been shown previously that magnitude of CD8<sup>+</sup> response does not correlate with the frequency of anti HCMV IgG antibodies. However, the same study showed that the diversity of the T-cell response, as measured by V $\beta$  usage, is inversely correlated with anti-HCMV IgG titre. This suggests the diversity, not the magnitude is responsible for virus control, and that those with large clonal expansions, and theoretically large viral loads, also have large humoral responses.

### **Results 3: EBV load**

## **Introduction**

EBV load varies enormously between healthy individuals. Load is 500x to 1000x higher during acute infection than in long term carriage, and high load in IM is associated with more severe symptoms [129]. Elevated EBV loads are also found in cases of asymptomatic primary infection, at similar levels to those in symptomatic infection. Cases of asymptomatic infection are, by their very nature, rarely identified, so it not known how commonly this occurs [129]. Post-IM, and presumably post asymptomatic infection, EBV load in blood reduces with a half-life of 1.5 days for the first 2 weeks, then more slowly after this date [94]. Reduction in load in the oropharynx, as demonstrated by continued virus shedding, takes considerably longer [129].

EBV load will be examined with reference to anti-EBV responses in controls and SLE patients. It has previously been reported that EBV load is 40x higher in SLE patients than in healthy controls, so this will be examined in relation to humoral and cellular responses.

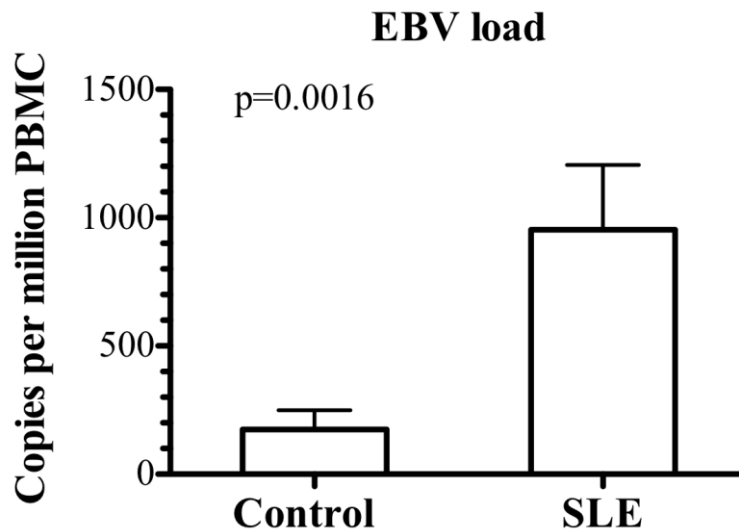
In healthy controls, periods of EBV reactivation are associated with periods of reduced frequency of anti-EBV CD8+ responses [89], implying T-cell frequency or function is responsible for EBV control. Decline of EBV load post IM is also accompanied by an increase in frequency of anti-EBV CD8+ T-cells. Number of EBV specific T-cells also correlates with rate of reduction in EBV load [94].

## **CMV/EBV interaction**

There is evidence to suggest that HCMV infection can have effects on the immune response to EBV infection in healthy controls. In HCMV negative, EBV positive individuals, anti-EBV T cell responses increase with age, in those also carrying HCMV there is no such increase [129].

EBV load correlates with HCMV load in bronchoalveolar lavage samples from patients post lung-transplant, suggesting factors controlling increase in load for one virus, also have an impact on the load of the other[129]. Experimentally, superinfection of EBV positive cell lines results in EBV reactivation [129].

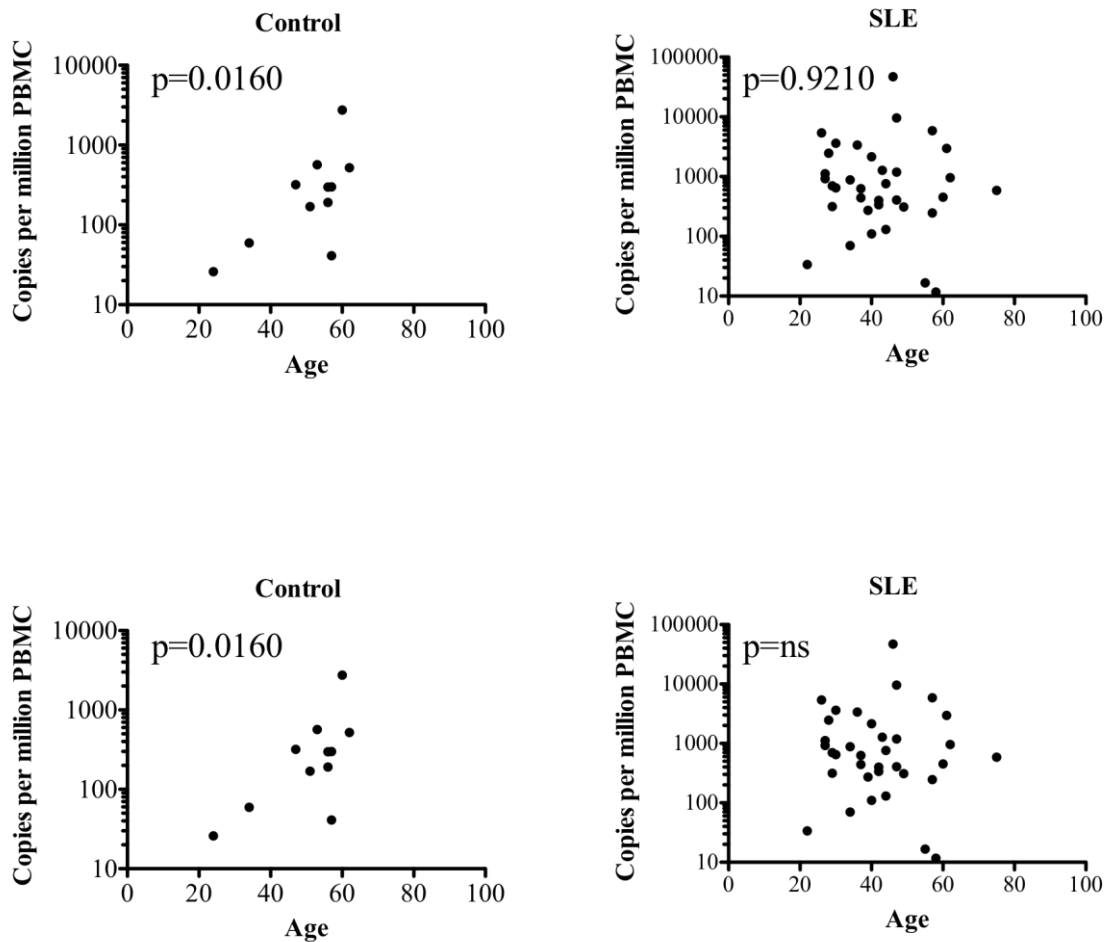
## Results



**Figure 13: EBV load.** Copies of virus genome per million PBMC in controls (n=37) and SLE patients (n=51). Bars represent mean  $\pm$  SEM. Only EBV+ individuals are represented.

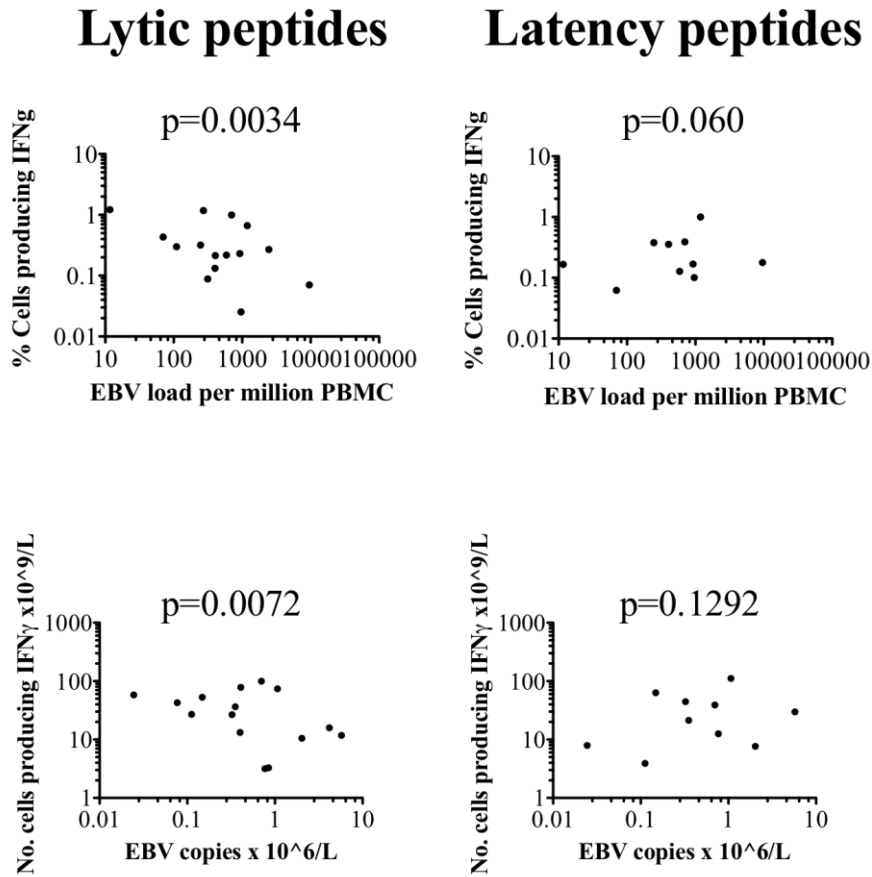
EBV+ SLE patients have significantly higher viral loads than controls, with mean loads of 952.9 and 174.8 copies of virus genome per million PMC respectively (Figure 13). SLE patients are also more likely to have a detectable virus load, with 16 of 38 positive controls having a detectable load and 34 of 51 positive SLE patients having detectable load ( $p=0.0209\chi^2$ ). Increased detectable load overall is caused by an increase in detectable load in the younger population. More SLE patients under the age of 43 have a detectable load than controls under the age of 43 ( $p=0.0091\chi^2$ ). There is no difference in the frequency of virus detection between older SLE patients and older controls. Nine controls with no detectable load had detectable serological responses. 16 SLE patients with no detectable EBV load were

seropositive by anti-EBNA-1 ELISA anti-VCA IFA ( $p=ns$ ). There was a trend towards seropositive controls with undetectable load being younger than seropositive controls with detectable load but it did not reach significance ( $p=0.082$ ).



**Figure 14: Association between EBV load and age. Each point represents an individual's EBV load per million PBMC, and their age at first donation. All controls ( $n=25$ ) and SLE patients ( $n=58$ ) have been included.**

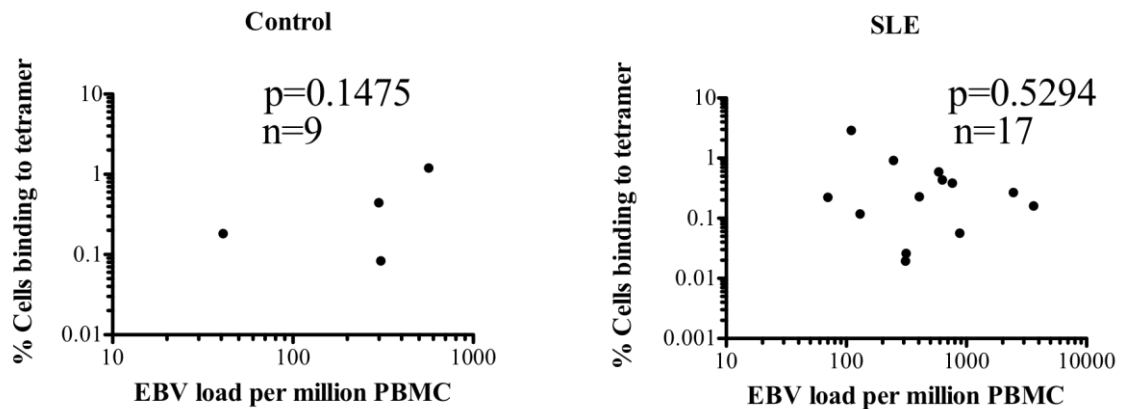
To determine whether the reduced detection of EBV genome in younger controls was due to load being below detection threshold or due to reduced prevalence we examined the correlation between age and EBV load. Among the control population, there is a correlation between age, and copies of virus genome per million PBMC (Figure 14  $p=0.0160$  Spearman's rank). No such correlation exists in SLE patients ( $p=0.9210$ ). There is also a correlation between virus load per B-cell and age in controls (data not shown  $p=0.004$ ), there is no such association in SLE patients, nor is there an association between age and virus load per ml of blood. There is no difference in EBV load between males and females (data not shown).



**Figure 15:** Association between EBV load and the number of cells specific for lytic and latent peptide mixes in SLE patients. Both frequency ( $n=41$ ) and absolute numbers ( $n=40$ ) of cells are shown. Specificity is determined by the production of IFN $\gamma$  in response to stimulation with each peptide. Correlation by spearman's rank.

There is a negative correlation between the percentage of CD8+ T-cells producing IFN $\gamma$  in response to lytic peptide mixes, and the viral load per million PBMC in SLE patients (Figure 15  $p=0.0034$ ). No similar correlation occurs in healthy controls, although numbers are limited. There is also a negative correlation between absolute numbers of CD8+ T-cells responding to lytic peptides and absolute viral load, no such association for latent responses exists ( $p=0.0072$  vs  $p=0.1292$  respectively). Among healthy controls there is a trend towards a negative correlation between EBV load and percentage of cells producing IFN $\gamma$  in response to latency peptides ( $p=0.0611$ ), but not lytic peptide ( $p=0.4727$ ).

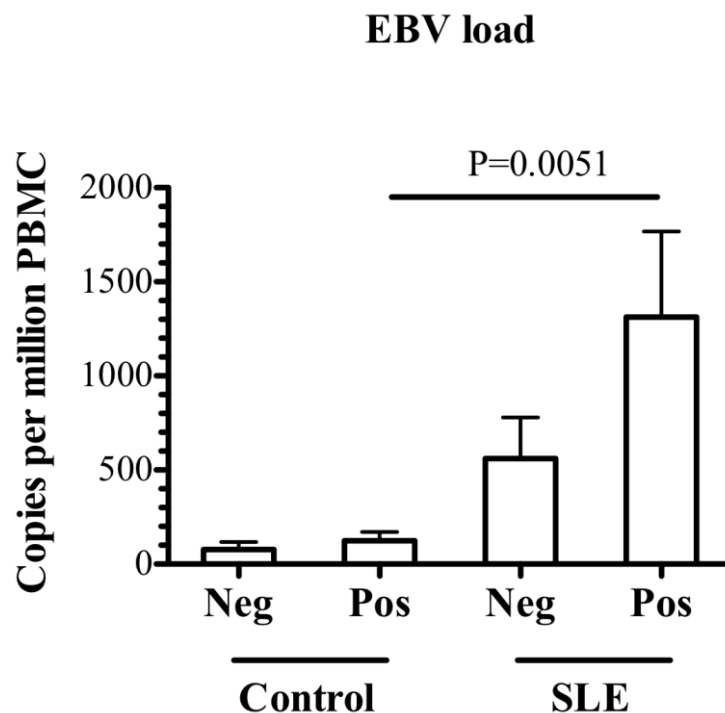
There is no correlation between anti-EBNA-1 IgG titre and viral load in SLE patients or healthy controls. There is also no association between anti-EBV IgM titre and EBV load.



**Figure 16: Association between EBV load and tetramer responses. Each point represents EBV genome copies per million PBMC and the percentage of CD8+ T-cells binding to tetramer. The first donation of SLE patients only is represented. Patients with values of 0**

*will not appear on the graph due to the scale but have been included in p value calculations.*

There is no correlation between frequency or absolute number of tetramer responses and EBV load, in SLE patients or healthy controls (Figure 16).



**Figure 17: EBV load in HCMV+ and HCMV- groups.** Controls and SLE patients were divided into HCMV+ and HCMV- categories. EBV load is shown as copies of virus genome per million PBMC. Bars represent mean  $\pm$  SEM. Control CMV- n=11, control CMV+ n=18, SLE CMV- n=21, SLE CMV+ n=25.

There is a significant difference in the viral load of SLE patients, compared to controls, but this difference is limited to those who are HCMV seropositive (Figure 17). There is no

difference in EBV load of HCMV positive and negative controls, or HCMV positive or negative SLE patients.

## **Discussion**

### **EBV load is elevated in SLE patients**

The increased frequency of detection of virus in younger SLE patients is probably due to increased virus load. Younger controls, who are sero-positive and have therefore encountered the virus, do not have a high enough viral load for detection. A more sensitive technique for detection of virus in PBMC would be useful. It is also possible that SLE patients acquire the virus at a younger age than controls, allowing an accumulation of the virus with age as shown in figure 14. It has been previously demonstrated that EBV is higher in SLE patients by up to 40 fold. A seven-fold increase in EBV load in PBMC has also been detected in patients with rheumatoid arthritis [127].

It is worth noting that EBV load is not as sensitive a test as immunofluorescence or ELISA. EBV load may be elevated in SLE patients due to more frequent viral reactivation. HCMV and EBV are also reactivated in the synovial fluid of patients with rheumatoid arthritis [129].

If the relationship between EBV and the human immune system were representative of all herpesviruses, HCMV may have a similar relationship with its hosts. Accumulation of virus load over time could explain the increase in HCMV specific immune responses. Were the virus to accumulate over time in any given individual, EBV load could be used as a marker of time since infection. Unfortunately it has not been possible to determine HCMV load in SLE patients or controls. It could be predicted that, like EBV, HCMV load would be higher in SLE patients, and inversely correlated with the proportion of immune repertoire devoted to its control.

### **EBV load is inversely correlated with CD8+ T-cell responses in SLE patients**

Elevated EBV loads are found in SLE patients with low frequencies and numbers of CD8+ EBV specific T-cells. This suggests reduced T-cell control allows an increase in EBV load. The same correlation does not occur in controls as SLE patients, indicating this is not a universal phenomenon, but a result of impaired T-cell responses. However, fewer controls were assessed than SLE patients, and there was a trend towards an inverse correlation between EBV and latent peptide responses in controls.

The lack of any correlation between EBV load and tetramer positive cell frequency is likely due to tetramers only recognising a very restricted panel of antigen specificities. Indeed, there was also no correlation between tetramer frequency and frequency of cells producing IFN $\gamma$  in response to stimulation. A further factor may be the reduced numbers of patients and controls samples analysed using tetramers.

A small study in 2011 of 16 older adults found that those with detectable HCMV load, by nested PCR from monocytes, had significantly higher frequencies of pp65 specific CD8+ T-cells, but found no association between virus detection and IgG responses[137]. Among rheumatoid arthritis patients, EBV load was positively correlated with frequency of CD8+ EBV specific T-cells [127].

### **EBV load is not associated with current antibody responses**

There is no such correlation between EBV load and current anti-EBV EBNA-1 IgG, suggesting that this response is not impaired in SLE. Either IgG is not vital in the control of replication, or IgG and EBV load are measuring different aspects of the host/virus relationship. Anti-EBNA-1 IgG response reflects the current state of immune challenge EBV represents. As such, if the challenge is not currently elevated in SLE, the IgG titre will not be elevated. However, EBV load represents years of accumulation of virus, and as such may represent the lifetimes immunological challenge the virus has represented.

Anti-EBNA-1 antibodies have also been shown to bind dsDNA, so it is possible that anti-dsDNA antibodies are altering the results, and hiding any association. There is no correlation between anti-EBNA-1 IgG and EBV load in controls, but this population is smaller. Furthermore, when patients with the highest level of anti-dsDNA binding were excluded, there was still no correlation between EBV load and anti-EBNA- IgG ( $p=0.1712$  data not shown).

### **EBV load is only elevated in CMV positive SLE patients**

HCMV seropositivity does not affect EBV load in controls, suggesting the immunomodulatory activities of HCMV do not affect host ability to control EBV in healthy individuals.

The elevated EBV load found in SLE patients is limited to CMV positive individuals. This suggests HCMV may have some contributing effect to the elevation in load. However, among SLE patients, EBV load is not significantly higher in CMV positives, as would be expected if HCMV were responsible for the increased load.



## **Results 4: Clinical Associations**

## **Introduction**

One of the most important issues regarding the relationship between herpes viruses and SLE, is whether these viruses have any effect on disease activity. Were they found to have a role in exacerbation of disease; it may be clinically useful to give antiviral medication. Primary infection or periodic reactivation of viruses could, in theory cause an exacerbation of disease by provoking non-specific or bystander immune responses against self, or specific responses caused by molecular mimicry. Conversely, SLE and its associated immune suppression due to disease activity and treatment, could impair the ability of the immune system to control the viruses, resulting in more frequent reactivations.

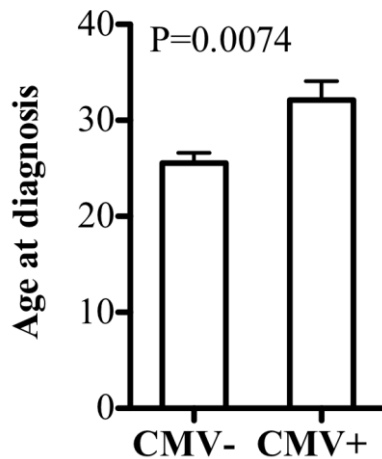
This chapter will consider the effects HCMV and EBV seropositivity have on disease activity, and other parameters used clinically, to measure disease severity. Current disease activity is measured in this study using the BILAG scoring system [50]. A damage index is used to reflect the severity of past disease activity. CRP levels are examined as a measure of systemic inflammation. C3 and C4 levels also give clues to current disease activity by showing how much of these complement proteins have been used up in immune responses. A drop in complement level therefore indicates an increase in disease activity. An increase in anti-dsDNA IgG titre is predictive of flare.

The stability of IgG response to HCMV, and EBV load in SLE patients will also be examined longitudinally and compared to changes in disease activity or medication.

Finally, patient characteristics such as age, sex, time since diagnosis and the age at which disease was diagnosed, are examined in relation to the carriage of HCMV or EBV.

Treatment data was collected and patients were categorised based on the drug taken at that visit. Only the presence or absence of each drug was recorded, not the dose, nor any alterations in dose.

## Results

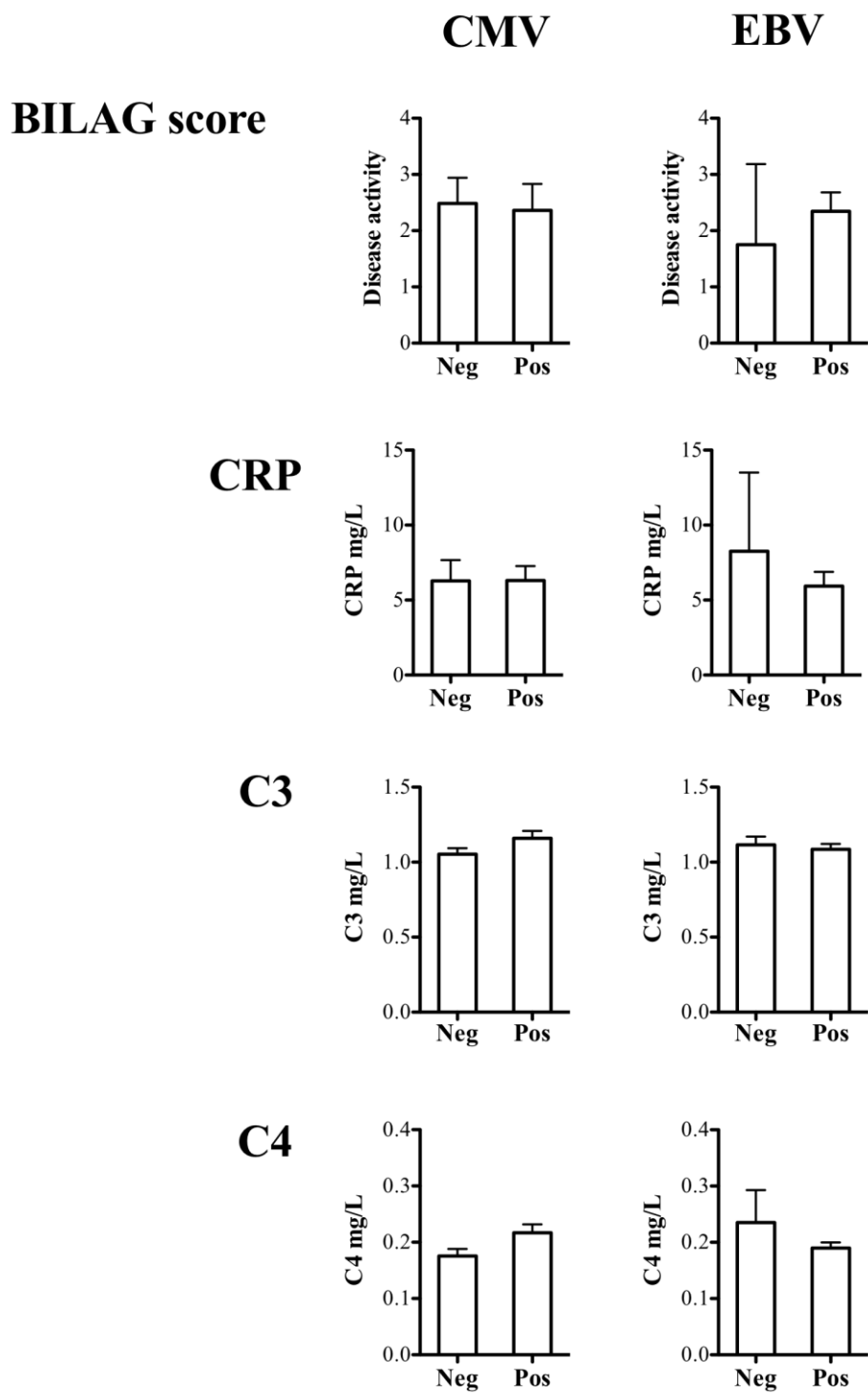


**Figure 18: Age at diagnosis.** All SLE patients were categorised as HCMV negative ( $n=32$ ) or HCMV positive ( $n=39$ ). Mean age at diagnosis is represented  $\pm$  SEM.

HCMV negative patients are diagnosed at a mean age of  $25.56 \pm 1.060$ , whereas HCMV positive patients are diagnosed 7 years later, at  $32.13 \pm 1.968$  respectively (Figure 18). Conversely, those diagnosed at a younger age are less likely to be seropositive than those that developed the virus later. Median age of diagnosis for all patients was 26 years, those diagnosed younger than this were less likely to be seropositive than those diagnosed after 26 ( $p=0.0484$ ). 100% of patients diagnosed at or older than 40 years are seropositive ( $n=11$   $p=0.0007$  when compared to prevalence among  $<40$ ). No relationship was found between HCMV seropositivity and the number of years since diagnosis. There is no significant difference in age of HCMV positive and HCMV negative individuals ( $p=0.1176$ ).

In healthy controls HCMV sero-positives are significantly older than seronegatives ( $p=0.0085$ ). There is also no difference in the age at which EBV seronegative and EBV

seropositive patients were diagnosed, or their age at blood donation. There are no differences in clinical data between patients diagnosed younger and older than mean diagnosis age of 30. The youngest diagnosis was at age 14, the oldest at 61, therefore if HCMV infection is resulting in a delay of onset, it is of no benefit to patients once a diagnosis has been made.



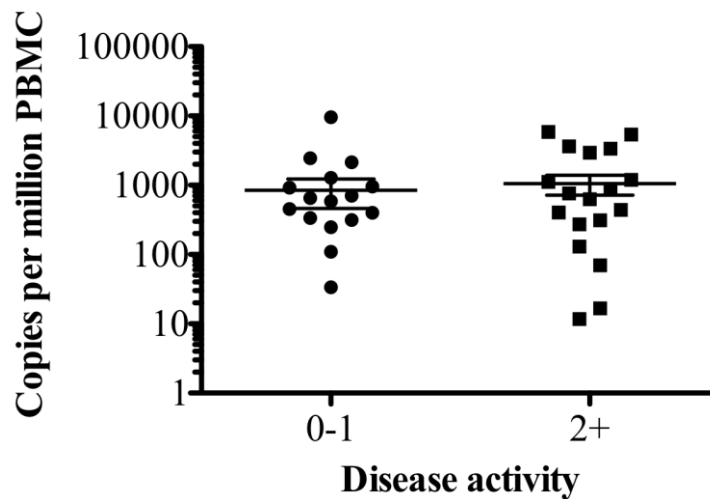
	No damage	Damage		No damage	Damage
CMV-	17	17	CMV-	17	17
CMV+	20	20	CMV+	20	20

**Figure 19: Association between seropositivity and clinical data. A. SLE patients were grouped based on HCMV (negative n=35, positive n=36) and EBV (negative n=4, positive n=58) seropositivity. B. Patients were grouped based on HCMV/EBV seropositivity and the presence of any damage. Bars represent mean  $\pm$  SEM.**

There is no association between HCMV and EBV seropositivity, detection of EBV load, or presence of T-cell responses, and any of the clinical parameters considered here (Figure 19). Furthermore, there is no association between magnitude of response to these viruses, by any method used, and any of the clinical parameters examined.

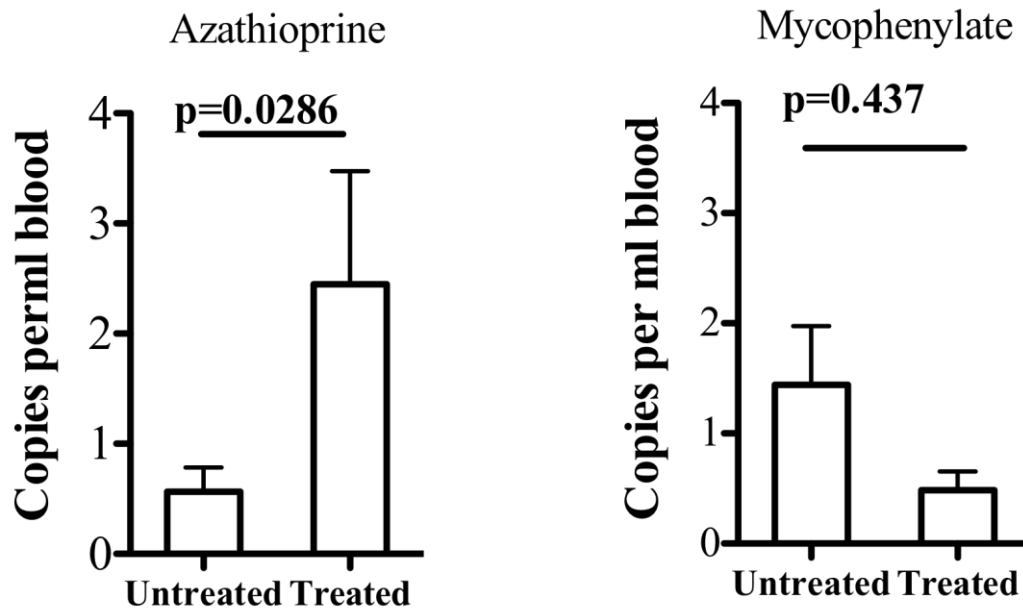
There were very few incidences of activity flares in the population studied during the period samples were taken. Median disease activity was one, indicating minimal activity on one system. Only four of 88 patients had disease activity greater than 10, and 17 had disease activity greater than five. The maximum disease activity recorded was 13. 14 patients had no activity at all. Median CRP was 3mg/L, and the maximum recorded was 44mg/L. Median C3 and C4 levels were 1.05 and 0.19 respectively. Median dsDNA titre was 30U, maximum recorded was 900U. There was no difference between any of the clinical parameters considered in patients younger or older than the median (data not shown).

In this data set, C3, C4, CRP and dsDNA did not correlate with current disease activity.



**Figure 20: Disease activity and EBV load.** Patients were categorised based on having current disease activity of 0 or 1 (minimal/no activity,  $n=25$ ), or disease activity of 2 ( $n=26$ ) or more (some disease activity). Disease activity and EBV load are from the same timepoint, only the first donation is shown for each patient. Bars represent mean  $\pm$  SEM. Points represent individual patients.

EBV load is not associated with disease activity (Figure 20), when any cut off of disease activity is considered. There is also no association between EBV load per ml of blood and disease activity. Neither is there any correlation between disease activity and EBV load. The magnitude of anti-HCMV and anti-EBV T-cell responses, both by IFN $\gamma$  production and by tetramer binding, are not associated with disease activity, or any of the other clinical parameters considered. Humoral responses to HCMV and EBV are also not associated with disease activity, or other disease parameters. EBV load is also not associated with any of the other recorded measures of disease activity.

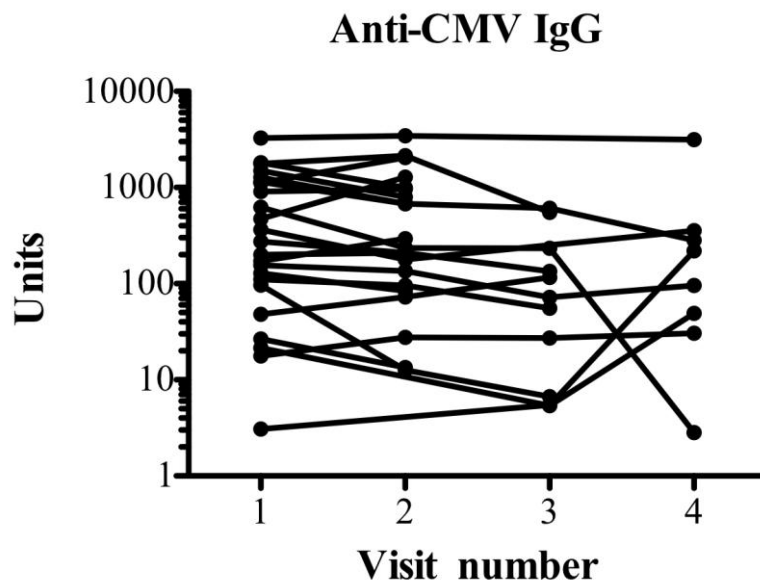
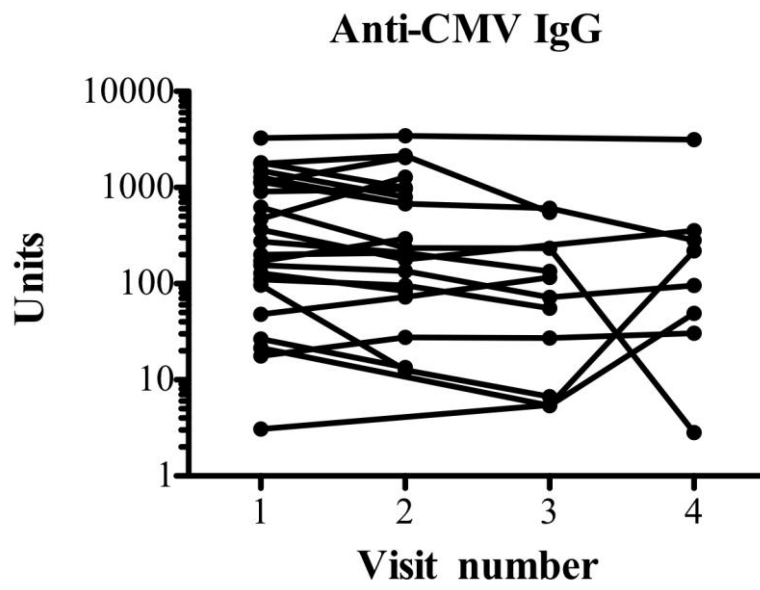


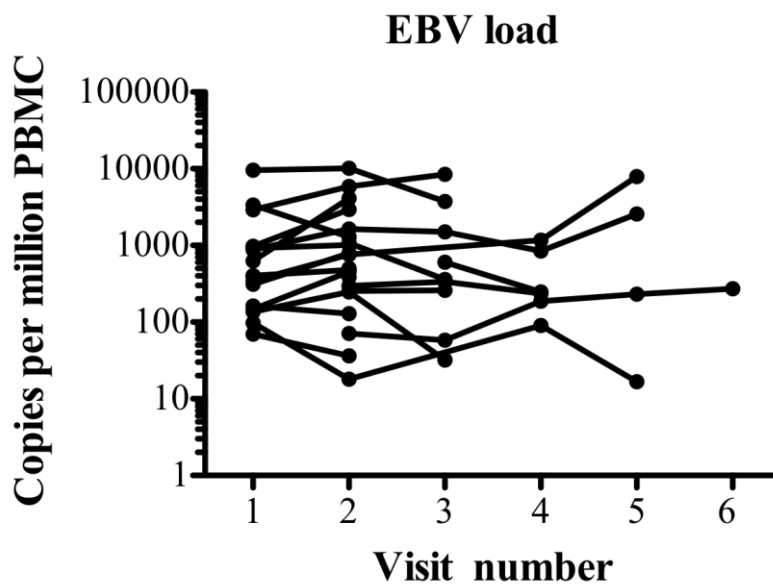
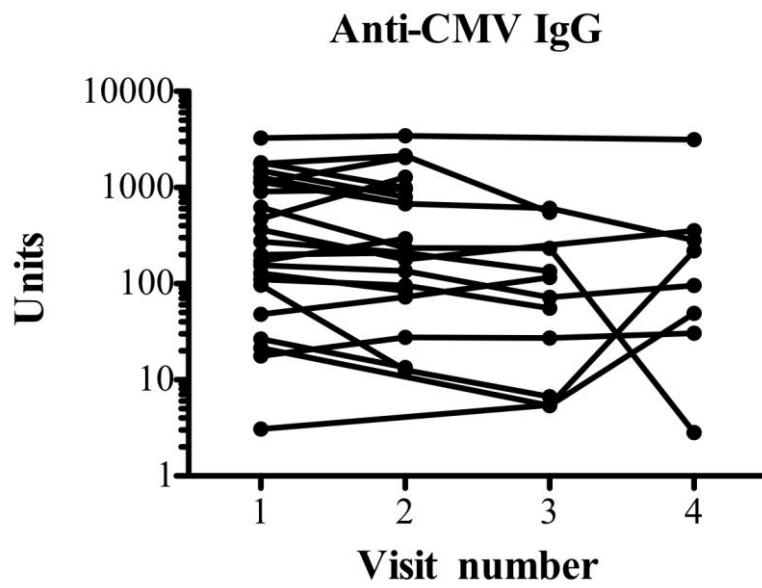
**Figure 21:** *Azathioprine (untreated n=40, treated n=13) and Mycophenolate mofetil (untreated n=30, treated n=23) treatment and EBV load.*

Treatment of SLE patients was heterogenous, but generally consistent for each individual. Therefore, only treatment at first blood donation has been considered. Most patients were receiving a number of different drugs. Most patients were treated with prednisiole (92.7%), and hydroxycholoquinne (76.8%). Smaller percentages were treated using azathioprine (28.0%) and mycophenolate mofetil (36.6%). A small minority were treated with methotrexate (9.8%), cylosporin (2.4%), rituxibab (1.22%) and tacrolimus (1.22%).

EBV load, both per PBMC and per ml blood, is elevated in patients treated with azathioprine (Figure 21,  $p=0.0286$ ). No other drug is associated with an increase or reduction in EBV load. No treatment had any association with the presence or magnitude of T-cell responses

against HCMV or EBV by tetramer or IFN $\gamma$  production. Humoral responses against HCMV, EBV and tetanus were unaffected by the presence of any one drug.





**Figure 22: EBV load and anti-HCMV IgG titre on consecutive donations. Samples were taken a minimum of three months apart, and a maximum of one year apart. Only patients HCMV positive at at least one donation were included.**

EBV load is fairly consistent over time (Figure 22). It was not possible to associate any changes in load with changes in disease activity or treatment. Healthy controls only provided one sample, making it impossible to compare the longitudinal variation of EBV load in SLE

patients, with that of healthy controls. Anti-HCMV IgG titre shows more variability over time, but it was not possible to associate any changes in response with changes in disease activity or treatment. Other methods of measuring anti-HCMV and EBV responses were not measured frequently enough to examine longitudinal changes.

## **Discussion**

### **Age of diagnosis**

HCMV positive patients develop disease 7 years later than seronegatives. A delay of this length is unlikely to be due to delayed diagnosis. It is not known when patients acquired the virus, therefore it is impossible to know whether HCMV seroconversion occurred before SLE diagnosis. It is possible that those predisposed to develop disease later, are more likely to acquire the virus, or that clinically relevant SLE makes it less likely to acquire virus. This hypothesis would fit with the finding that older SLE patients are less likely to be seropositive than age matched controls, and the finding that those diagnosed younger are less likely to have the virus. Clinically relevant disease may make it harder to acquire the virus, so those with disease had fewer years in which they were likely to acquire it. It seems implausible that a fully functioning immune system is necessary for infection, as HCMV infection is generally only pathological in those with impaired immune function i.e. neonates and those with HIV or immunosuppressive medication. However, HCMV associated pathogenesis in the immunosuppressed may be due to reactivation, from latency in the host or from donated tissue, rather than primary infection. It has previously been shown in a cohort of Mexican SLE patients that those HCMV IgG positive, IgM positive developed disease younger than IgG positive IgM negative. This suggests frequent virus reactivation is associated with earlier disease development. However, CMV negative individuals were excluded from the study, as they only made up 3% of the population [129].

### **Virus does not affect clinical outcome**

HCMV and EBV seropositivity have no discernable effect on disease activity or clinical parameters, therefore can not be involved in the exacerbation of disease. Elevated EBV load, suggestive of impaired T-cell control also shows no association with exacerbation of disease, therefore virus reactivation cannot be the causative agent of SLE exacerbation. It is not known whether CMV may have this effect. Conversely, given the lack of association, it is unlikely that disease activity is responsible for reactivation of EBV, or any of the alterations in anti-viral responses.

However, none of the recognised markers of disease activity recorded here correlate with disease activity as would be predicted. This suggests the data set is not large enough to show such correlations. It is also possible that the associations are not recognisable due to the relatively few episodes of elevated disease activity. The majority of patients have either no activity at all (17%) or very minimal activity, with BILAG scores of between 1 and 4 (63%), indicating only mild symptoms.

### **Treatment associations**

Azathioprine treatment is linked to elevated virus load, both per PBMC and per ml of blood, mycophenolate mofetil is not, and neither are any other drugs tested. As no form of treatment is associated with changes in T-cell or antibody responses, the alteration in load associated with Azathioprine may be due to alterations in antigen processing. Azathioprine is a purine analogue, it functions by inhibiting cell proliferation via interference with DNA replication. Doesch et al and Shubert et al showed in 2008 that EBV load post-heart transplant was elevated in patients treated with azathioprine.

In CMV seronegative recipients of CMV positive kidney transplants, HCMV seroconversion

was just as likely in those treated with mycophenolate mofetil, as those without such treatment. However, incidence of HCMV related disease (ie fever, leukocytopenia, thrombocytopenia) was more common among those receiving the immunosuppressive medication, but did not affect the severity of symptoms[138].

Elevated EBV load may also be due to historical impairment of T-cell responses or associated EBV control.

No individual medication used here, affected the ability of CD4+ or CD8+ T-cells to produce IFN $\gamma$ . This finding is similar to a recent study of the use of two immunosuppressive drugs used in SLE, in solid organ transplant patients. Treatment with cyclosporin A and tacrolimus in solid organ transplant patients significantly affects CD8+ T-cell's ability to produce IFN $\gamma$  and TNF $\alpha$ , but not their ability to reduce IFN $\gamma$  alone [129]. Polyfunctionality of T-cells -the ability to produce multiple inflammatory cytokines -is more closely associated with their anti-viral capacity, than is the production of IFN $\gamma$  alone (pantaleo correlates of). This means that T-cells in the SLE patients studied here, may well be impaired in their functionality, but not have impaired IFN $\gamma$  responses.

No individual medication affected anti-HCMV or anti-EBV responses. Immunosuppressive medications are frequently associated with the reactivation of one or both viruses following solid organ transplant [129].

Cytomegalovirus is frequently reactivated in patients with ulcerative colitis, however infection disappears without antiviral agents[129]. All immunosuppressive drugs used in this study have been previously associated with the reactivation of CMV or EBV.

### **EBV load and anti-HCMV IgG titres are fairly stable**

EBV load and HCMV IgG titre are fairly stable over time, as are disease activity levels. It was not possible to examine longitudinal changes in T-cell responses in SLE patients. It is likely that changes anti-HCMV IgG would correlate with magnitude of T-cell response longitudinally, as they correlate in cross section of the population. The alterations in load or anti-HCMV titre identified, were no associated with changes in disease activity or treatment. Unfortunately it was not possible to examine the stability of any responses in healthy controls as only one sample was obtained for each.

## **DISCUSSION AND CONCLUSIONS**

### **EBV is not necessary for SLE development and neither virus is associated with clinical outcomes**

The identification of SLE patients without the virus also shows that EBV is not necessary for disease development. Two patients, of 86, had no detectable EBV load, no anti-EBV antibodies and no T-cells producing IFN $\gamma$  in response to EBV peptide stimulation. Furthermore, no association has been found between the carriage of either virus, or the response to either virus, and disease activity. It is therefore unlikely that these two viruses are contributing to the etiology or exacerbation of disease.

### **HCMV delays the development of SLE**

It has always been assumed that the relationship between herpesviruses and autoimmune diseases is likely to be a negative one. However, in MS patients, HCMV seropositivity has been associated with better clinical outcome [129]. They also found that HCMV seropositivity is associated with later age of diagnosis, a finding that mirrors those of this study. The mechanism for both may be similar, HCMV may have a protective role in autoimmune disease. Unlike SLE, MS is a disease in which severity and degree of disability increase with age. A later onset of disease therefore may lead to better clinical outcome in MS. No association with age and clinical outcome was found in SLE patients, it was therefore less likely that increased age of diagnosis would improve outcome. It is unclear why HCMV is associated with later onset of disease. It may be that HCMV is more difficult to acquire in the autoimmune environment. This would result in those who develop disease younger having lower seropositivity. Those who develop disease later, have had more time with a normally functioning immune system, therefore, more time to acquire the virus, and have higher seropositivity.

**CMV protects SLE patients from developing SLE early by ‘exhausting’ the supply of IFN $\alpha$ .**

IFN $\alpha$  is found at lower concentration in plasma of HCMV positive SLE patients. It is possible that this reduction is responsible for the delay in disease development associated with HCMV seropositivity.

PDCs in SLE patients can become unresponsive to stimulation after excessive stimulation via TLR9, and stop producing IFN $\alpha$ , as their intracellular supplies of it become exhausted. HCMV infection causes the production of IFN $\alpha$ , via ligation of TLR9, so it is possible that repeated stimulation of pDCs by HCMV has lead to their anergy, and impaired IFN $\alpha$  production. This could explain the reduced levels of IFN $\alpha$  in HCMV positive SLE patients. SLE patients with elevated IFN $\alpha$  levels also have worse disease activity. This could also explain why HCMV+ SLE patients develop disease later, as their reduced plasma IFN $\gamma$  levels are insufficient to allow disease initiation.

### **Anti-CMV titre and T-cell response accumulate with age**

Anti-HCMV responses are strongly associated with age. This is either due to an accumulation of virus load as a result of multiple sub-clinical reactivations, or simply an accumulation of antibodies and T-cells specific for the virus. Either way, it is possible that anti-HCMV response could be used as a marker of duration of infection. EBV load also accumulates with age, but anti-EBV immune responses do not. EBV load may also be reflective of time since infection. It is probable that the balance between virus and host is different for each virus considered. SLE patients do not undergo the same alterations in anti-virus responses, EBV load, and phenotypic changes associated with aging, as controls. SLE patients' load and responses may be prematurely elevated, as a result of excessive reactivation, and not conducive to any further accumulation.

### **SLE patients have elevated EBV loads due to immunosuppressive treatment and impaired T-cell responses, not due to disease.**

Azathioprine treatment in SLE patients is associated with a five fold increase in EBV load per cell. An elevated load was also associated with reduced frequency of CD8<sup>+</sup> T-cells producing IFN $\gamma$ . It is therefore possible that azathioprine treatment is impairing CD8 T-cell responses and leading to an elevation in load. However, no association was found between treatment and IFN $\gamma$  responses. EBV load was only negatively correlated with responses to lytic cycle peptides, not latency peptides, suggesting impaired control of reactivation is responsible for elevated load. Anti-EBV IgG titre against the latency peptide EBNA-1 was

also not correlated with EBV load. Immunosuppressive treatment may be affecting the time taken to generate a protective response in reaction to reactivation. Raised titres of antibody against EBNA-1 in SLE patients may reflect increased historical reactivation, which would result in a larger pool of EBV specific plasma cells.

No associations were found between any either medication and any immunological parameters, however, many patients are on multiple medications, making it difficult to identify any direct links. Furthermore, as all patients are on some type of immunosuppressive agent, it is difficult to determine the effect of one drug over another. It would be useful to compare EBV load and anti-herpesvirus responses in SLE patients not receiving any medication. It would also be of interest to examine the effects of these drugs on herpes virus responses in other conditions receiving equivalent immunosuppression. Patients receiving immunosuppression post-transplant are generally on much higher doses of immunosuppression than SLE patients, so are not directly comparable. A more suitable comparison would be other conditions requiring immunosuppression such as rheumatoid arthritis or ulcerative colitis.

## **Limitations**

The lack of HCMV load information is probably the greatest drawback of this work. It is impossible to know whether the same relationship between T-cell response and load occurs for CMV as for EBV. Another significant weakness is the lack of enough samples to show whether correlation exists between EBV load and anti-EBV T-cell responses in controls. Without this information it is not possible to know whether the inverse correlation between these parameters is as a result of SLE disease and treatment, or the normal relationship between pathogen and host.

It is unfortunate that full blood counts were not available for controls, as it would have been interesting to compare the numbers of T-cells specific for each virus, rather than just frequencies. It would also have been useful, as in almost all studies, to have a greater number of controls, to allow better comparisons within the population. To determine the longitudinal changes in healthy controls, it would have been useful to have multiple samples, to examine whether the changes in anti-HCMV IgG and EBV over time in SLE patients reflect changes in disease activity, or whether they are just normal variations within each individual.

Historical information on patient HCMV status would have been useful, in order to discover whether patients were HCMV positive before disease developed. This would clarify whether HCMV delays disease onset, or whether SLE patients are less likely to acquire virus. Also, for all patients and controls it would be interesting to know how long ago infection occurred, to see whether anti-CMV IgG titre reflects time since infection.

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# Appendix

**Appendix 1. SLE patient disease activity and treatment at recruitment.**

Patient ID	Age of diagnosis	Sex	Age	Disease activity									Treatment									Clinical measurements					
				1.	2.	3.	4.	5.	6.	7.	8.	Total score	Hydroxychloroquine	Prednisolone	Azathioprine	Cyclophosphamide	Methotrexate	Cyclosporine	Mycophenolate	Rituximab	Tacrolimus	Damage score	CRP	C3	C4	dsDNA	Lymphocyte count x10^9/l
1	14	f	42	d	d	d	d	d	d	d	c	1	y	y	y							5	4	0.79	0.21	29	1
2	27	f	47	d	d	d	d	d	d	d	c	1	y	y	y							0	3	1.09	0.14	33	0.6
3	25	f	45	d	d	d	d	d	d	c	c	2	y	y				y			1	3	1.04	0.21	29	0.3	
4	25	m	57	d	d	d	d	d	d	d	c	1	y	y							3	5	1.03	0.14	24	0.6	
5	32	f	53	d	d	d	d	e	d	c	c	2		y					y		6	3	1.14	0.23	41	1.7	
6	26	m	63	d	d	d	d	d	d	d	c	1	y	y					y		3	3	0.82	0.16	27	1	
7	58	f	58	d	d	d	d	d	d	d	b	5		y	y						1	3	1.22	0.4	48	2.1	
8	29	f	49	d	d	d	d	d	d	d	d	0	y	y					y		1	3	1.29	0.18	0	1.9	
9		f		d	c	d	d	d	d	d	c	2	y	y					y		3	6	1.19	0.21	118	1	
10	42	f	62	d	d	d	d	d	d	e	d	0	y	y							8	3	1.28	0.28	21	1.5	
11	32	f	54	d	d	d	c	d	d	c	d	2		y							2	13	1.87	0.4	0	2	
12	33	f	51	d	d	d	c	e	d	d	d	1	y	y							2	17	0.97	0.1	244	1.9	
13	18	f	37	d	d	d	d	d	d	c	c	2	y	y	y						3	3	0.69	0.18	114	0.8	
14	25	f	44	d	d	d	b	d	d	c	c	7	y	y				y	y		1	3	1.02	0.24	0	0.6	
15	23	f	40	d	b	e	d	d	d	d	c	6	y	y					y		0	3	1.07	0.26	23	0.9	

16	20	f	40	d	d	d	d	e	d	e	d	0	y	y		y		0	3	0.89	0.15	0	
17	28	f	52	d	d	d	d	e	d	d	c	1	y	y	y			2	3	1.41	0.25	0	1.1
18	27	m	61	d	d	d	c	d	d	d	c	2	y	y	y			1	5	1.45	0.29	0	0.9
19	12	f	37	d	c	d	d	d	d	c	c	3	y	y	y			0	13	1.4	0.2	0	2.1
20	20	f	44	d	d	d	d	d	d	c	c	2		y		y		3	3	0.87	0.11	28	1.1
21	47	f	62	d	d	d	d	d	d	e	c	1		y	y			1	3	0.9	0.15	63	0.8
22	24	f	41	d	d	e	d	d	d	e	d	0	y	y				0	3	1.22	0.2	0	1.9
23	34	f	51	d	d	d	d	d	d	d	d	0	y			y		3	19	1.88	0.43	0	1.8
24	22	f	55	d	e	e	d	d	d	d	c	1		y				1	3	0.94	0.04	46	1.1
25	30	f	45	d	d	d	d	e	d	d	c	1		y				1	3	1.1	0.24	20	0.8
26	32	f	47	c	d	d	c	d	d	c	d	3	y	y	y			0	3	1.02	0.13	0	1.5
27	61	m	75	d	d	e	d	d	e	d	c	1	y	y				3	4	0.9	0.16	130	0.6
28	33	f	47	d	d	d	d	d	d	c	c	2	y	y		y		1	3	1.01	0.02	33	0.8
29	31	f	42	d	d	d	d	d	d	d	c	1	y	y		y		0	3	0.85	0.13	0	0.7
30	19	f	34	d	c	d	c	d	d	c	c	4	y	y			y	0	3	0.98	0.14	33	1.4
31	23	m	37	d	d	e	d	d	d	b	c	6	y	y			y	4		0.85	0.21	39	1
32		f	39	d	d	e	d	d	d	e	c	1			y			1	3	1.01	0.07	0	0.9
33	19	f	32	d	d	d	d	d	e	d	d	0		y		y		0	3	1.4	0.17	40	3.6
34	27	f	40	d	d	d	d	d	d	c	c	2	y	y	y			0	9	1.73	0.24	0	1.1
35	23	f	34	c	c	e	c	e	b	d	c	9	y	y				0	3	0.67	0.11	372	0.3
36	27	f	43	d	d	d	d	d	d	e	c	1	y		y			0	3	1.02	0.12	0	0.8
37	35	f	46	d	d	e	c	e	e	e	c	2	y	y				1	10	1.22	0.21	62	1.1
38	41	f	52	d	d	e	c	d	d	d	d	1	y	y		y		0	3	1.11	0.27	0	1.2
39	18	m	37	d	d	d	d	d	e	e	c	1	y	y				4	12	1.21	0.22	0	1.7
40	49	f	60	d	d	d	d	e	d	d	d	0	y	y		y		0	3	1.14	0.16	76	0.7
41		f	53	d	d	e	d	e	e	d	d	0			y			1	3	1.41	1.32	/	1.3
42		f	x	d	d	d	e	e	d	e	c	1	y	y				0	3	0.92	0.16	122	1.1
43	41	f	51	d	d	d	d	d	d	d	c	1	y	y				4	24	1.25	0.37	0	0.7
44	20	f	28	d	d	e	d	d	d	c	d	1		y	y		y	0	4	1.06	0.19	38	1.7
45	19	f	30	d	d	e	d	d	d	c	c	2	y	y	y			0	3	0.64	0.08	197	1.1
46	36	f	45	c	c	d	b	d	d	d	d	7	y	y			y	1	16	1.33	0.28	0	1.6
47	38	f	49	c	d	d	c	e	d	c	d	3		y		y		0	7	0.83	0.13	103	1.5

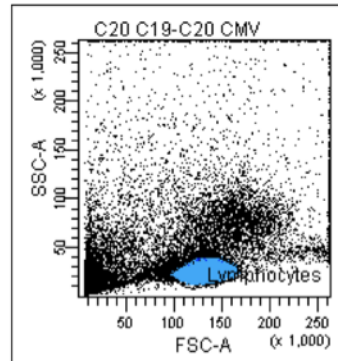
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49	14	f	25	d	c	e	d	d	d	b	b	11	y	y		y		1	4	1.11	0.12	41	0.7
50	34	f	40	d	d	e	d	e	e	d	c	1	y	y	y			0	26	0.91	0.16	26	0.7
51	20	f	29	d	d	e	d	e	e	e	d	0	y					1	3	0.86	0.19	0	2.7
52	25	m	36	d	d	e	d	d	e	c	c	2	y	y		y		2	5	1.02	0.25	179	1
53	41	f	47	d	b	c	d	d	d	e	c	7	y	y	y			3	3	1.59	0.33	0	0.9
54	26	f	34	d	d	d	b	e	e	d	d	5	y	y		y		0	7	1.37	0.29	211	1.6
55	36	f	43	d	e	d	b	d	d	c	d	6	y	y		y		1	4	1.12	0.22	24	2.8
56	39	f	46	d	e	d	d	e	e	c	c	2		y	y			0	3	1.15	0.11	62	0.5
57	40	f	46	e	e	d	d	e	d	d	b	5	y	y		y		2	3	1.1	0.35	0	0.6
58	21	f	26	d	c	e	d	e	d	c	c	3	y	y	y			0	25	1.83	0.25	33	2.4
59	29	f	34	d	d	e	d	e	e	d	c	1	y	y		y		1	3	1.17	0.39	42	1.1
60	21	f	30	e	d	e	d	d	d	d	c	1	y	y		y		0	17	0.62	0.04	900	0.8
61	20	f	24	d	d	e	d	d	d	d	e	0	y	y				0	3	1.15	0.22	0	
62	28	f	32	d	d	e	d	d	e	e	c	1	y	y				0	3	1.17	0.29	30	1.1
63	25	f	28	d	e	d	d	d	e	c	b	6		y		y		1	5	0.53	0.04	154	0.7
64	23	f	26	e	d	e	d	e	d	d	c	1	y	y		y		0	3	0.72	0.15	62	0.6
65	50	f	55	d	d	e	d	e	d	c	b	6	y	y		y		0	8	1.37	0.34	55	1.1
66	33	f	39	d	b	e	d	e	d	c	b	11		y		y		2	3	0.39	0.1	46	1.5
67	23	f	29	d	d	e	d	d	d	d	c	1	y	y				0	3	0.94	0.16	0	1
68	26	f	29	d	d	e	d	e	e	c	d	1	y	y	y			0	3	1.04	0.25	0	1.6
69	39	f	42	d	d	e	c	d	e	d	d	1	y	y		y		0	11	1.7	0.27	30	3.6
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71	23	f	24	c	c	e	b	d	b	c	d	13	y	y		y		0	3	1.24	0.15	0	1.9
72	24	f	26	d	c	d	d	e	d	d	c	2	y	y		y		0	3	0.89	0.16	0	0.7
73	25	f	26	e	d	e	d	e	e	d	b	5			y			0	3	1.02	0.17	188	0.8
74	57	f	57	e	d	e	d	e	e	c	c	2	y	y				0	3	0.79	0.05	129	1.4
75	27	f	27	d	d	e	d	d	e	c	d	1		y		y		0	3	0.89	0.24	69	2.2
76	18	f	18	d	d	e	d	e	e	d	d	0	y	y		y		0	3	0.99	0.09	0	1.5
77	25	f	27	e	d	e	d	d	d	c	c	2	y	y		y		0	3	1.07	0.2	100	1.2
78	36	f	37	e	a	e	d	e	e	d	d	12	y	y		y		0	4	1.15	0.28	0	1.6
79		f	21	d	d	d	d	d	d	c	d	1	y	y				0	3	1.16	0.15	34	

80	f	27	d	c	d	d	d	d	d	c	2	y	y	y			3	0.69	0.12	576	
81	f	22	d	d	d	d	d	d	d	d	0		y		y		0	3	0.89	0.12	44
82	f	43	d	d	d	d	d	d	d	d	0		y		y		1	3	1.01	0.26	0
83	f																				
84	m																				

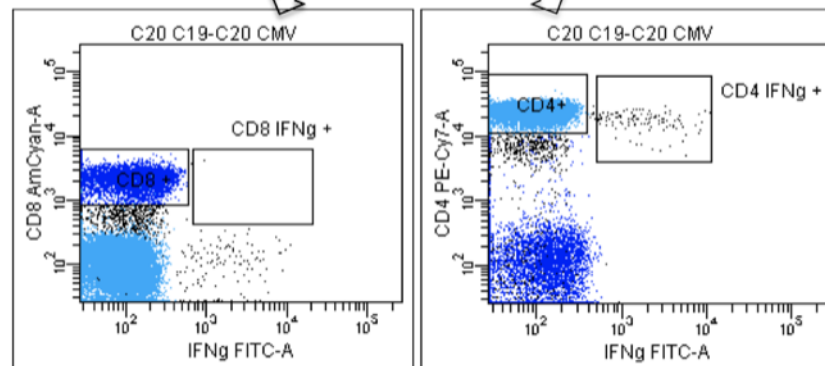
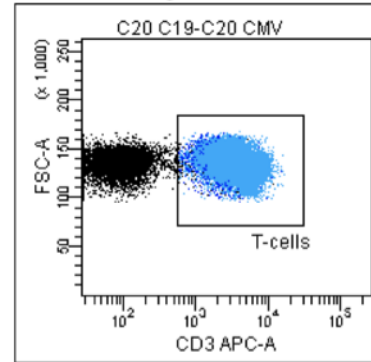
Appendix 1: SLE patients' disease and treatment. Y represents the patient is receiving the medication, dose was not considered. Disease activity is scored using the BILAG system, where a represents most severe disease activity, d and e represent no current activity and never had activity respectively.

## Appendix 2. Gating strategy for intracellular staining.

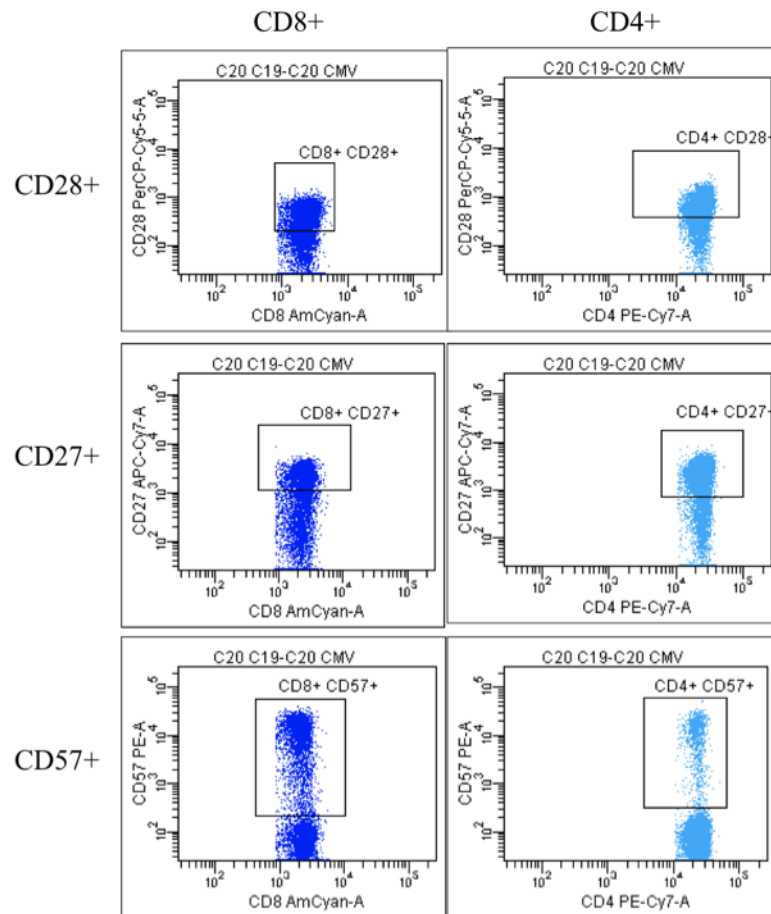
### 1. Identification of lymphocytes



### 2. Gating of CD3+ cells



### 3. Identification of CD8+ and CD4+ T-cells, and cells producing IFN $\gamma$



4. CD4+ T-cells and CD8+ T-cells, were gated on the presence or absence of differentiation markers