Human Leucocyte Antigen-G (HLA-G) Polymorphism and Expression in Pneumonia & Human Immunodeficiency Virus (HIV) Patients

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DECLARATION

All the work presented in this thesis is my own and any other work is appropriately referenced. This thesis is the result of my own personal effort except where below.

Activity	**Individuals Responsible**
Submission and defence of ethics, study protocol	Ahmed
Clinical sampling	Assisted by Dr Georgios Pollakis Dr Bill Paxton Dr Daniel Wootton
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ABBREVIATIONS

CAP	Community-acquired pneumonia
CTL	Cytotoxic T lymphocytes
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HLA	Human leucocyte antigens
HLA-E	Human Leucocyte Antigen-E
HLA-F	Human Leucocyte Antigen-F
HLA-G	Human Leucocyte antigen-G
HRSN	High risk HIV seronegative
IDU	Intravenous drug users
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL	Interleukin
INF-γ	Interferon Gamma
KIR2DL4	Killer Immunoglobulin Like Receptor 2 Domain cytoplasmic tail 4 (KIR2DL4)
LIR-1	leukocyte Ig-like receptor 1
LIR-2	leukocyte Ig-like receptor 2
MHC	Major histocompatibility complex
MHC	Major Histocompatibility Complex
microRNA	Micro ribonucleic acid
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MSM	Men who have sex with men
NHS	National Health Service
NK	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline

PRRs	Pattern recognition receptors
sHLA-G	Soluble Human Leucocyte Antigen-G
SNPs	Single Nucleotide Polymorphisms
T reg	T regulatory cells
TCR	T cell receptor
TGF-β	Transforming Growth Factor-Beta
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-Alpha
UTR	Untranslated region

ABSTRACT.

Human Leucocyte Antigen-G (HLA-G) Polymorphism and Expression in Pneumonia & Human Immunodeficiency Virus (HIV) Patients

PhD Thesis by Ahmed Saleh Alyami

HLA-G is a non-classical class I HLA molecule involved in protection of the fetus against maternal immune responses and also suppression of immune responses in infectious diseases and tumours. The purpose of this study is to investigate HLA-G polymorphism and expression in a) pneumonia patients in relation to cytomegalovirus status and b) different groups of HIV patients, in relation to clinical outcomes.

Blood samples from 144 pneumonia patients were analysed for HLA-G genotype and serum soluble HLA-G in relation to healthy controls. The only significant difference between patients of distinct genotypes was that those homozygous for a 3'UTR allele of HLA-G linked with high levels of expression were associated with longer stays in hospital.

Previous work has shown that CMV can induce HLA-G expression, particularly in B cells, CD56+ T cells and monocytes. This was the rationale behind looking at the influence of CMV in pneumonia incidence and outcomes. When leucocytes from healthy subjects were stimulated in vitro with antigens from *Streptococcus pneumoniae* there were significant

increases in proportions of CD4+ and CD8+ T cells expressing HLA-G. Healthy donors revealed a significant increase in proportions of cells expressing KIR2DL4, a ligand for HLA-G, in CD56+ T cells, CD16+ CD56dim and CD16- CD56bright NK cells following IL-2 stimulation, which was unrelated to KIR2DL4 genotype.

DNA samples from 'The Amsterdam Cohort' of HIV+ patients, comprising men who have sex with men (MSM), intravenous drug users (IDU), together with high risk HIV seronegative (HRSN) subjects exposed to HIV but remaining HIV negative, were analysed for HLA-G genotype and soluble HLA-G. While genotypes of MSM and IDU patients were similar to healthy controls, HRSN subjects showed a significantly higher proportion of HLA-G genotypes associated with lower levels of HLA-G expression. When leucocytes from healthy subjects were stimulated *in vitro* with a vector expressing an HIV antigen, HLA-G was expressed on an increased proportion of CD4+ and CD8+ T cells. Overall levels of HLA-G mRNA were shown to be increased in HIV vector-stimulated leucocytes but not significantly.

These results would be consistent with HIV being capable of upregulating HLA-G as a mechanism of immune evasion, although in the case of HIV this would be less likely in individuals with low HLA-G-expressing haplotypes.

1 INTRODUCTION AND LITERATURE REVIEW

1.1 An overview of the immune system

The immune system is a complex network of cells and molecules each with specialized roles in defending the host from infectious microbes and any other foreign intruder or cancerous cell. According to the speed and specificity of the reaction, immunity is divided into the innate and the adaptive responses Parkin and Cohen (2001), (Chaplin, 2003, Wills et al., 2007).

1.1.1 Innate Immunity

Broadly defined, innate immunity is the first line of defence and consists of barriers such as skin, tears, saliva and mucus, as well as an inflammatory response. The innate response uses neutrophils, monocytes, and macrophages as phagocytic cells, cells producing inflammatory mediators and natural killer cells. It consists also of bioactive small molecules and cell receptors that bind molecular patterns expressed on the surfaces of invading microbes (Parkin and Cohen, 2001, Chinen et al., 2014). The innate immune response recognizes evolutionarily conserved structures on pathogens, called pathogen-associated molecular patterns (PAMPs) and relies on a limited number of germ line-encoded pattern recognition receptors (PRRs) which includes the family of Toll-like receptors (TLRs). Upon PAMP recognition, PRRs trigger proinflammatory and antimicrobial responses. PRR-induced

signal transduction pathways ultimately lead to the synthesis of a wide range of molecules, including cytokines, chemokines, cell adhesion molecules, immunoreceptors and activation of gene expression. This together orchestrates the early host response to infection and can be an important link to the adaptive immune response (Mogensen, 2009).

1.1.2 Adaptive immunity

Unlike the innate response, adaptive immunity involves a humoral response mediated by antibodies, and a cell mediated response by B lymphocytes and T lymphocytes. This response has memory, so a more vigorous and rapid response would be observed following re-exposure to a specific antigen. Whereas adaptive immunity takes several days or weeks to develop, it is more specific and leads to less harm to normal tissues compared to the broad innate response. The major histocompatibility complex (MHC) regulates the cell-mediated adaptive immune response. The key role of the MHC is to present pathogens in the form of small peptides at the cell surface where they can be recognized by T cell receptors. In humans, the human leucocyte antigens (HLAs) on the cell surface are encoded by the MHC genes. MHC molecules display both 'self' peptides derived from their own proteins, and foreign peptides derived from invading pathogens. Monitoring the amount and type of MHC-

presented antigens helps MHC molecules to target and destroy abnormal cells (Murphy et al., 2007).

1.2 Cells of the immune System

Numerous cells are involved in the immune response to pathogens with complex interactions and activities. The innate response is carried out by such cells as: phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils and natural killer cells (NK). The subsequent adaptive immune response may take days to develop and involves activation of cells such as T-cells and B-cells (Chaplin, 2003). The two major surface co-receptor molecules, CD4 and CD8, define two separate T cell lineages with different functions. Where CD4+ lymphocytes recognize Ag in the context of MHC class II molecules, antigenic peptides presented by MHC class I molecules activate CD8+ cells and form effector cytotoxic T lymphocytes (CTL). Based on production of signature cytokines, activated CD4+ T helper cells can be subdivided into Th1, Th2, Th17 and Treg subsets (Broere et al., 2011). Since the discovery of T cell subsets and specific cell surface markers, these have become a useful target in the design of drugs for selective manipulation of the immune response (Broere et al., 2011).

1.3 Mechanisms of Immunosuppression

A variety of mechanisms have been evolved by the immune system to achieve and maintain tolerance. Tolerance is achieved intra- and extra-thymically through mechanisms including: activation-induced cell death, anergy, and the induction of regulatory T cells (Tregs). Tregs can suppress the activation of conventional T cells in a cell-contact-dependent, IL-10-independent and TGF-ß-independent, manner. Tr1 cells also utilize various surface molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) (Buckner, 2004). Tr1 cells can re-direct an inappropriate immune response against allergens or auto-antigens using a broad range of suppressor mechanisms. HLA-G is able to induce regulatory T (Treg) cells and thus, it participates indirectly in inducing tolerogenic properties on immune cells to avoid activation of immune cells and inflammation (Taylor et al., 2006).

In some viruses, for instance Lymphocytic choriomeningitis virus (LCMV) in mice, human immunodeficiency virus (HIV), Cytomegalovirus (CMV) and hepatitis C virus (HCV) in humans, escape of killing by the host's immune system, either by avoidance or manipulation, can establish persistent infection in the host. The viruses may establish persistence in the host by (a) activation or induction of Tregs, (b) expression of inhibitory molecules such as PD-1 on activated T-cells, and (c) production

of suppressor cytokines such as TGF-ß or IL-10. Thus the goal of the immune system to completely clear the virus is curbed (Sarikonda and G von Herrath, 2011).

1.4 The Human Major Histocompatibility Complex

The Human Major Histocompatibility Complex was discovered by characterisation of a specific gene cluster located on chromosome 6p21.3 (Figure1-1). Initially the MHC was known for its role in transplantation through its relation to histocompatibility antigens. The 'human leucocyte antigens' (HLA) has since become the commonly used nomenclature. Intensive research has been conducted across several decades to define the remarkable genomic content of the MHC. Other specific research interests are investigating how genetic variation within this region plays a key role in susceptibility to autoimmune, infectious and other diseases.

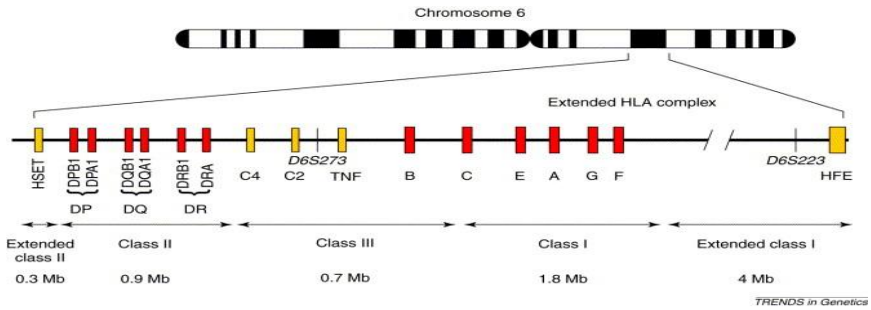

Figure1-1 Human MHC on chromosome 6.

The HLA complex and some of its genes. A total of 128 genes in the HLA complex (not including the extended HLA class I complex) are assumed to have an immune function. Red represents the genes encoding class I and II molecules, other genes in yellow. TNF is the gene encoding tumour necrosis factor, C2 and C4 are complement genes (Undlien et al., 2001).

1.4.1 The role of MHC molecules

There are three gene clusters representing the human MHC: class I, class II and class III. The main function of class I and class II molecules is to bind and present peptide fragments derived from pathogens for recognition by the appropriate T cells. The consequence is to eliminate or neutralize extracellular and intracellular pathogens such as bacteria or viruses through the activation of phagocytic macrophages, cytotoxic T cells and antibody producing B cells. Thus, there is strong selective pressure in favour of any pathogen that has mutated in such a way that it escapes presentation by an MHC molecule. MHC is also known for its role in transplantation and its ability to initiate allogeneic responses (Vandiedonck and Knight, 2009).

1.4.2 Structure of the MHC class I Molecule

Class I MHC and class II MHC are structurally closely related, although there are a few key differences. The classical HLA-A**,** HLA-B**,** and HLA-C genes of the class I region encode the heavy chains of class I molecules. The three domains of class I MHC formed by the α-chain and a single non-MHC encoded molecule named ß2-microglobulin. Both α1 and α2 domains serve to present the antigen to the T-cell receptor. The function of the α3 domain is to bind the CD8 co-receptor and the whole molecule to the cell membrane (Wieczorek, 2017).

The non-classical, or class Ib, MHC molecules, HLA-E, -F, and -G, show less polymorphic differences than MHC Ia and appear to possess distinct functions. The HLA-E molecule is known to present a peptide repertoire restricted to signal peptides cleaved from classical MHC class I molecules (Celik et al., 2016). It represents a significant self-recognition target for the natural killer (NK) cell inhibitory receptors NKG2A or NKG2C paired with CD94. There are currently four HLA-E alleles known. HLA-G is expressed selectively in extravillous trophoblasts the foetal cell population directly in contact with maternal tissues. It presents a wide array of peptides, is expressed in seven different alternatively spliced forms, and provides inhibitory signals to both NK cells and T cells, presumably in the role of maintaining maternofetal tolerance. The function of HLA-F remains largely unknown (Posch and Hurley, 2011, Choo, 2007).

1.4.3 Difference between classical and non-classical MHC class I molecules

Immune cells evolved to employ classical and non-classical major histocompatibility complex class I (MHC I) molecules as a major mechanism for the recognition of pathogens. Classical MHC class I (MHC-Ia) are highly polymorphic proteins which, together with ß2m, form a structure that presents antigenic peptide ligands on infected cells for

recognition by the classical αß T-cell receptor (TCR) on CD8+ T-cells. The key function for non-classical MHC I molecules is to provide inhibitory or activating stimuli to natural killer (NK) cells. They are are usually nonpolymorphic and tend to show a more restricted pattern of expression than their MHC-Ia counterparts (Halenius et al., 2015).

Human leucocyte antigen G (HLA-G) is an immune-suppressive non-classical major histocompatibility complex 1b molecule and which is involved in the escape of some tumour cells and viruses from elimination by Natural Killer Cell (NK) and CD8+ cytotoxic T-cells (Alegre et al., 2014). Further research on the neglected molecule HLA-G might lead to finding a new and more effective therapeutic exploration of existing current infections or at least in finding new targets (Halenius et al., 2015).

1.5 MHC class II molecules

MHC-II is very similar and is made up of an α and a ß chain, both of which are coded for by the MHC region in chromosome 6. Each chain has two domains; α1 and ß1 form the peptide binding cleft and both α2 and ß2 are membrane bound (Holling et al., 2004). The differences between class I MHC and class II MHC structures shown in (Figure 1-2) influence the types of immune responses that result. This mechanism acts to ensure that T cells are predominantly of the CD8 class responding when peptide antigens are presented by class I molecules, and similarly, that T cells

responding to class II pMHC complexes are predominantly CD4 (Vignali, 1994). However, the class III region contains genes for complement components (C2, C4, factor B), 21-hydroxylase, tumour necrosis factors (TNFs), and some others (Colten, 1984).

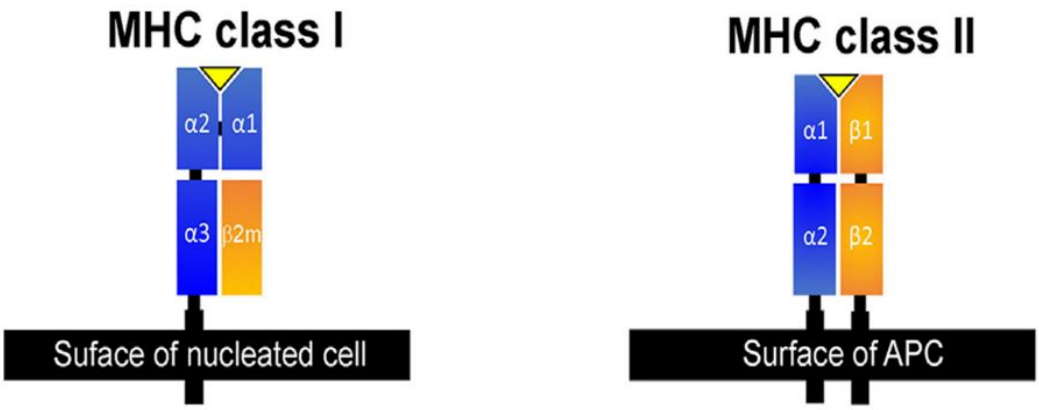

Figure 1-2 MHC class I & II domains.

The domain topology of Major histocompatibility complex class I and class II complex. MHC class I is composed of four domains originating from a single heavy α-chain (HC) and ß2-microglobulin , while in the case of MHC class II, the two chains are an α-chain and a ß-chain (Wieczorek et al., 2017).

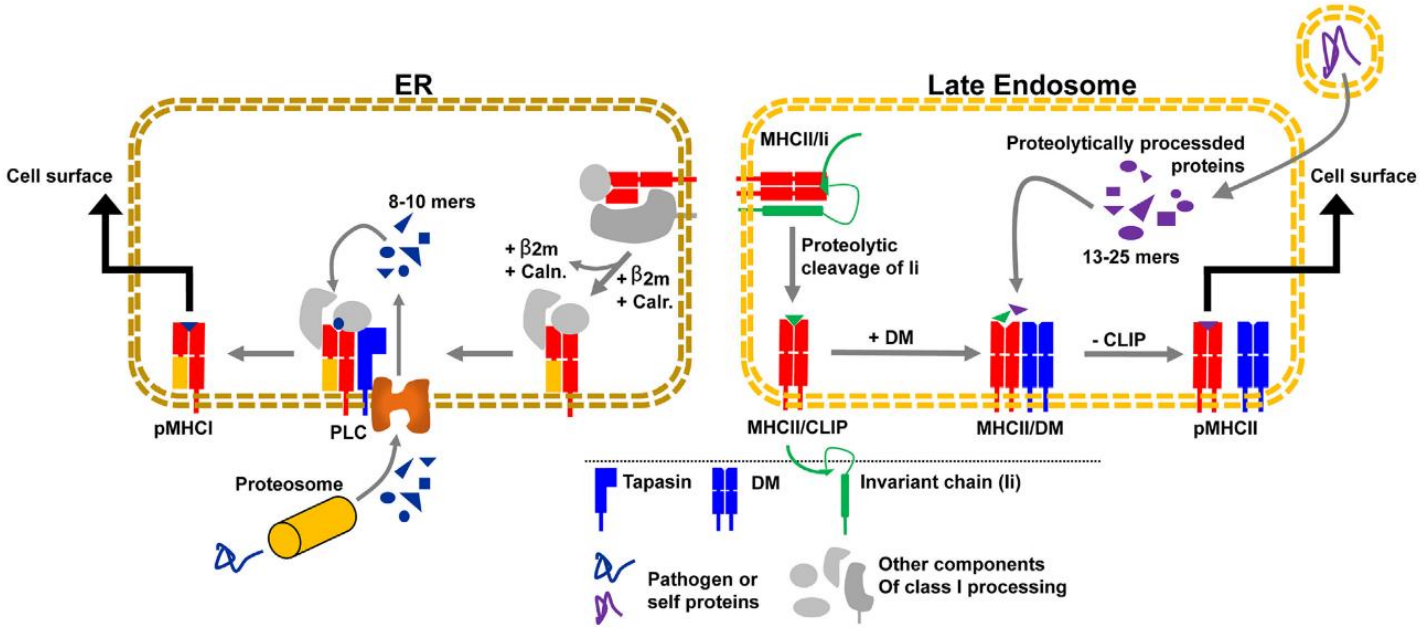

Figure 1-3 Processing peptide by MHC I & II.

Simplified illustration of MHC class I peptide-editing pathways (left). The proteasome primarily processes Cytosolic proteins. The processed peptides are then transported into the ER by TAP for subsequent assembly with MHC-I molecules. The MHC class II (right) processing pathway processes exogenous proteins. Several pathways are used to internalize antigens, including phagocytosis, macropinocytosis, and endocytosis, and eventually traffic to a mature or late endosomal compartment where they are processed and loaded onto MHC-II molecules. CLIP, class II-associated invariant chain peptide; Caln., calnexin; Calr., calreticulin; ER, endoplasmic reticulum; PLC, peptide loading complex (Wieczorek et al., 2017)

1.6 Human leucocyte antigen polymorphisms

Other commonly used nomenclature for MHC is the human leucocyte antigens (HLA). The antigen-presenting cells (APCs) and certain other cells usually express HLA class I and II complexes which present antigenic peptides to CD8 positive and CD4 positive T cells respectively. The extraordinary genetic polymorphism of HLA molecules guarantees the broadest diversity of recognized antigens and therefore protection against a wider range of pathogens. This mechanism involves an affinity between the HLA molecules and the antigenic epitopes which requires a degree of specificity for each HLA allele (Choo, 2007).

Most of the polymorphism is around the peptide binding groove and results in differences in the repertoire of peptides bound by MHC I or MHC II molecules. Beyond the antigen presentation function mediated by HLA molecules, MHC genes and individual polymorphisms were found to be involved in the regulation of the humoral immune responses and inflammatory reactions (Choo, 2007).

1.6.1 Genomic organization of the HLA system

To date, more than 20,088 HLA Alleles have been identified, which includes 14,800 different HLA Class I alleles and 5,288 different HLA Class II alleles (http://hla.alleles.org/nomenclature/index.html). There are three main MHC class I genes encoding HLA-A, HLA-B, and HLA-C.

Almost all cell surfaces express proteins from these genes. On the cell surface, the produced proteins are bound to protein fragments (peptides) that have been exported from within the cell (endogenous). MHC class I proteins present these peptides to the immune system as shown in (

Figure 1-3). If the cell is infected with a virus or bacterium, it presents peptides which are foreign (such as viral or bacterial peptides), CD8+ cytotoxic T cells of the immune system triggers the infected cell to self-destruct (apoptosis). Apoptosis is defined as a morphologically distinct form of cell death. The process occurs not only during development and aging as a homeostatic mechanism to maintain cell populations in tissues but also as a defence mechanism as part of the immune response against viruses or bacteria or when cells are damaged (Choo, 2007).

Humans have six main MHC class II genes, known as HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, and HLA-DRB1. The class II genes encode proteins that are present almost exclusively on the surface

of certain immune system cells such as on B lymphocytes, antigen-presenting cells (monocytes, macrophages, and dendritic cells), and activated T lymphocytes. Like MHC class I proteins, the peptides are displayed to the immune system but in this case peptides are exogenous in origin. An example of viral peptide binding class I and class II is shown in (Figure 1-4) in the case of HIV derived peptides.

The class III region contains genes for complement components (C2, C4, factor B), 21-hydroxylase, tumor necrosis factors (TNFs), and some others. However the class III region does not encode HLA molecules (Kochan et al., 2013).

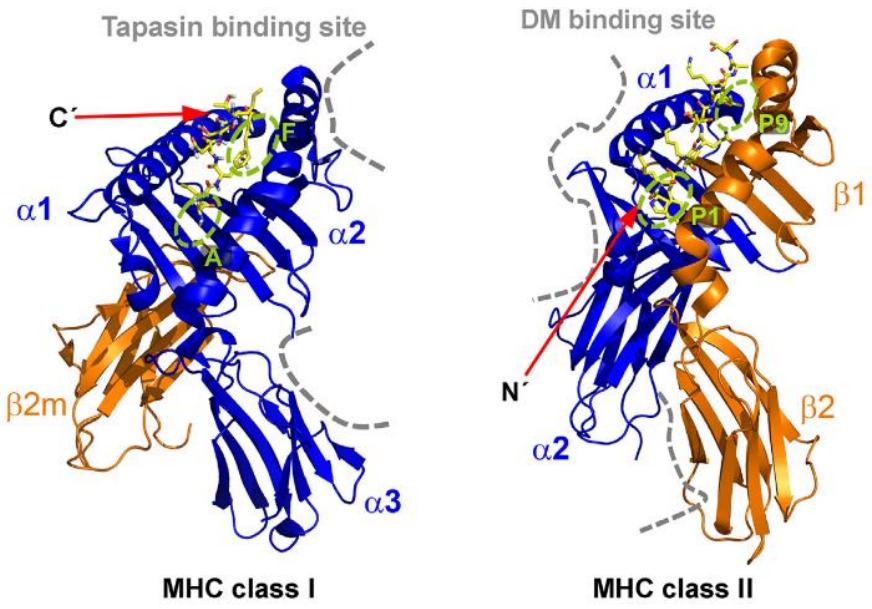

Figure 1-4 MHC in HIV infection.

Structure of HLA-A in complex with an HIV-derived peptide (PDB: 4HWZ, left) and HLA-DR1 in complex with a haemagglutinin-derived peptide (1DLH, right). Indicated are the supposed interaction sites of MHC class I with tapasin and of C class II with DM as dashed grey lines. The peptide is shown in yellow with its N and C-terminus marked and relevant pockets are labelled green (Wieczorek et al., 2017).

1.6.2 HLA haplotypes

The MHC region is polygenic in that it contains several different MHC class I and MHC class II genes. Every individual possesses a set of MHC molecules with different ranges of peptide-binding specificities. The MHC is highly polymorphic with multiple variants of each gene within the population as a whole. The polygeny and polymorphism may serve the immune system to respond to the multitude of different and rapidly evolving pathogens.

MHC is inherited as an HLA haplotype from each parent. Family HLA studies can assign the segregation of HLA haplotypes within a family. Two siblings have a 50% chance of being HLA haploidentical (sharing one particular haplotype), a 25% chance of being genotypically HLA identical, and a 25% chance that they share no HLA haplotypes. Certain HLA haplotypes are found more frequently in some populations than expected by chance, a phenomenon known as linkage disequilibrium (LD). LD is when non-random proportions of alleles at linked loci are inherited together more often than would be expected by chance. This may be due to the effect of some combinations of alleles at the two loci on the viability

of potential offspring. Or it could be due to actual genetic linkage that is the genes are closely located on the same chromosome. For example, with a frequency of 5% HLA-A1, B8, DR17 is the most common HLA haplotype among Caucasians (Choo, 2007). MHC alleles (variants) are inherited from both parents and equally expressed. Each person can express six different types of MHC-I. In the class-II locus, each person inherits six or eight functioning class-II alleles (Wieczorek, 2017).

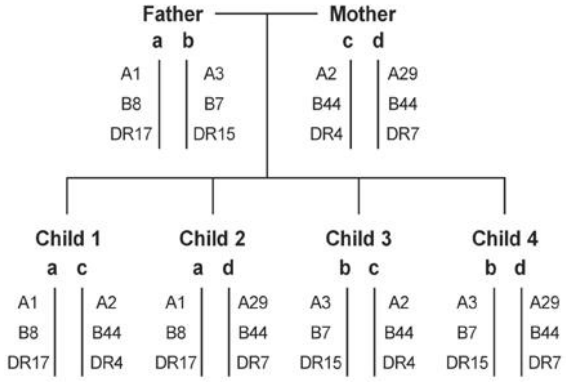

Figure 1-5 HLA haplotypes.

Mendelian inheritance of HLA haplotypes demonstrated in a family study. HLA haplotypes and genotypes can be inferred from phenotype data in an informative family study as illustrated. For example, the father's HLA phenotype is HLA-A1, 3; B7, 8; DR15,17 (Choo, 2007)

1.7 HLA-E

HLA-E is encoded by the HLA-E gene translated into five different proteins belonging to MHC-E. Out of five, two potential proteins (HLA-E*01:03 and HLA-E*01:01) have prominent immune effects. These

proteins usually have arginine and glycine at position 107 of the heavy chain alpha 2 domain (Joosten et al., 2016).

Under normal conditions, HLA-E can be expressed on a variety of human cells including T cells, B lymphocytes, NK cells, megakaryocytes as well as monocytes in addition to endothelial cells (Coupel et al., 2007).

NK cells express a specific receptor which is generated by C type lectin (NK group 2 and CD94) that acts as a ligand for HLA-E molecules (Lanier et al, 1998). The HLA-E molecule with heterodimeric structure consisting of non-polymorphic disulphide-linked polypeptide CD94, was also reported to be expressed on decidual NK cells (Verma et al, 1997) and can bind to the NK group A, B or C molecule (Cantoni et al., 1998). The CD94/NKG2 A, B molecules are known to have suppressor effects while the remaining CD94/NKG2 C and D molecules have stimulatory function, and those molecules are the ligands for MHC class E antigen (Borrego et al, 1998; Brooks et al., 1999). Binding of CD94/NKG2A to HLA-E leads to the suppression of NK, αß CD8+, γδ T lymphocyte function by means of disturbing the actin grid of the immune synapses (Masilamani et al, 2006). On the other hand, the ligation of HLA-E to the stimulatory CD94/NKG2C receptor results in no prominent effect (Garcia et al., 2002).

1.8 HLA-F

HLA-F is a non-classical MHC-I molecule that has limited polymorphism and a lower cell surface expression than its classical paralogues (Lima et al., 2016). These are 22 alleles that are translated as 4 protein variants (HLA-F*01:01, *01:02, * 01:03 and *01:04) (Pan et al, 2013). The domains of HLA-F consist of a heavy chain only that includes the standard HLA-I domain (alpha1, 2 and 3) with a binding groove in addition to the transmembrane portion and an intracellular tail (Goodridge et al, 2010). To date, the role of HLA-F is not fully described. Lima et al. has proposed that this molecule plays a similar role to HLA-G and HLA-E in terms of immune regulation and tolerance (Lima et al., 2016). It is also suggested that HLA-F has the ability to interact with inhibitory molecules of NK cells and monocytes, the immunoglobulin like toll (ILT) 2 and 4, which can exert regulatory effects on immunity (Lepin et al., 2000). Under physiological conditions, B lymphocytes, splenic cells, tonsils, and thymic cells express the HLA-F as well as *in vitro* in the HUT37 T cell line (Lee et al, 2010). While abundant expression of HLA-F in gastric cancer has been related to a metastatic form of the disease (Ishigami et al., 2015), its expression in such malignancies like lung carcinoma, oesophageal cancer, and breast carcinoma indicates poor prognosis (Lin et al., 2011; Zhang et al., 2013). Conversely, the expression of HLA-F is related to the activity

of systemic lupus erythematosus, where the higher the expression of this molecule, the lower severity of the disease (Jucaud et al., 2016).

1.9 Human leucocyte antigen G

1.9.1 Characterisation of HLA-G

Human leucocyte antigen G (HLA-G) is a non-classical human leucocyte antigen located within the major histocompatibility complex (MHC) at chromosome 6 in the p21.31 region. Unlike classical class I molecules, HLA-G differs in many biological features including: limited polymorphism, selective expression, restricted distribution, short cytoplasmic tail, structure and immune tolerance functions. Four membrane-bound isoforms (HLA-G1, -G2, -G3, and -G4) and three soluble forms (HLA-G5, -G6, and -G7) are commonly generated due to the alternative splicing of the primary transcript. HLA-G5, -G6, and -G7 isoforms are highly unusual because they are splice variants of the HLA-G mRNA and retain introns 4 and 2 (Figure 1-6). HLA-G structure variation appears to play a key role in the biological function of HLA-G and its regulation (Apps et al., 2008).

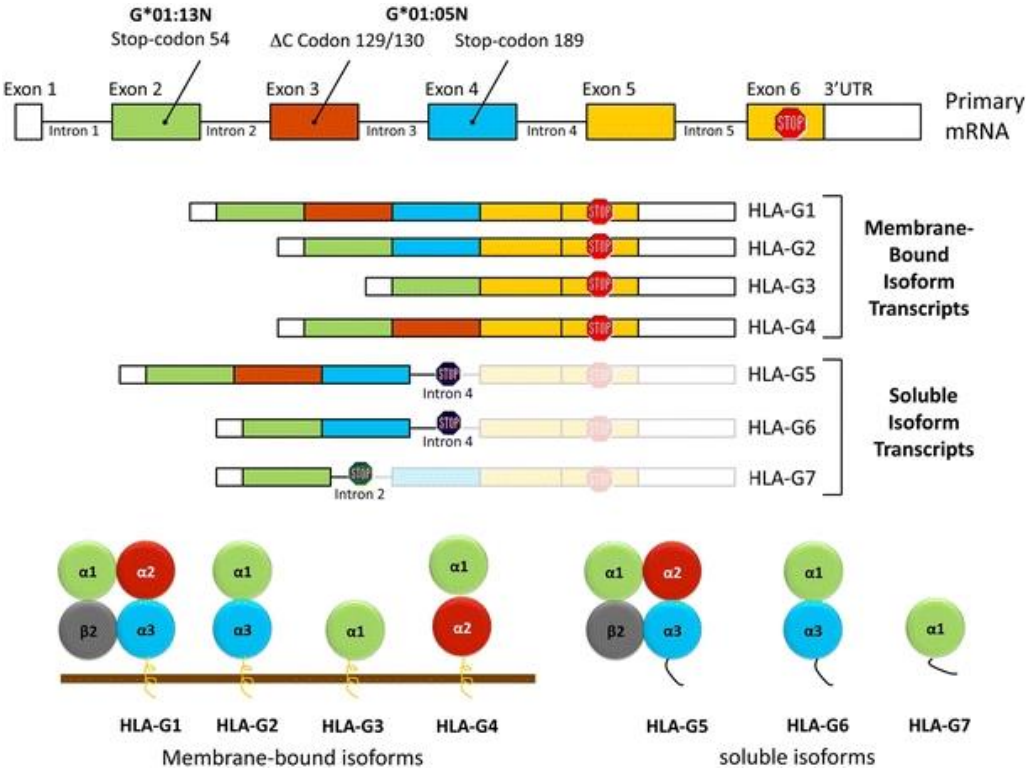

Figure 1-6 HLA-G gene structure.

The figure represents the HLA-G gene segments. These encode for mRNA and the figure depicts the alternative splicing which generate the isoforms of HLA-G proteins, HLA-G1 to −G7. The promoter region (5'-UTR) of the given gene regulates the functional mRNA level of a particular gene, as well as the rate of degradation, stability, localization and translatability of the specific mRNA. The HLA-G protein exhibits a heterodimer consisting of globular domains (α1, α2 and α3 domains, transmembrane and cytoplasmic domains) and a light chain (β2-microglobulin) called monomer (HLA-G7/5). HLA-G1 is the full-length HLA-G molecule, other mRNA transcripts lack either exon 3 or 4. HLA-G1 to −G4 are considered as membrane-bound molecules due to the presence of the transmembrane and cytoplasmic tail encoded by exons 5 and 6. HLA-G5 and -G6 are soluble forms due to the presence of intron 4. This intron contains a premature stop codon at exon 4 (blue stop signal), preventing the translation of the transmembrane and cytoplasmic tail. Due to the presence of intron 2, HLA-G7 is considered soluble. Intron 2 presents a premature stop codon (green stop signal). The G*01:13N allele is principally not expressed due to the presence of a premature stop codon at exon 2 (codon 54). A deletion of a cytosine (ΔC) at exon 3 of the G*01:05N allele leads to a stop codon at exon 4; (Donadi et al., 2011).

1.9.2 HLA-G function

Since HLA-G was first discovered in 1987, its relevance and role in immune response regulation in pathological conditions has been extensively targeted and explored in depth by the most recent research into how HLA-G affects particular mechanisms. HLA-G is known to be implicated in the immune escape by tumours and viral infections (Carosella et al., 2008, Rouas-Freiss et al., 2003). HLA-G has been shown to decrease the efficacy of the immune response including affecting all cell types involved in the immune response during infection (Amiot et al., 2014b). This occurs by inhibiting most mechanisms and steps of the immune response including: differentiation, proliferation, cytolysis, cytokine secretion, and immunoglobulin production. For example, HLA-G has been shown to alter antigen presentation to T lymphocytes and also affects cooperation with B lymphocytes (Amiot et al., 2015).

1.9.3 Inhibition of effector cells

The direct binding of both soluble and membrane-bound HLA-G to inhibitory receptors mediates the tolerogenic properties of HLA-G. T lymphocyte and NK cell function is inhibited by binding of their immunoglobulin like transcript ILT2 inhibitory molecules with HLA-G proteins. ILT2 is also expressed by a variety of immune cells such as B lymphocytes. In addition to DCs, monocyte and macrophages express

ILT4 and the binding of those proteins to HLA-G can modulate the activity of these cells or regulate the proliferative capacity in addition to enhancing the regulation of NK and T lymphocytes, respectively (Baffari et al., 2013) Colonna et al., 1997; Colonna et al., 1998). The inhibition involves changes in proliferation, cytotoxicity or the induction of regulatory T or NK cells. The expression of ILT2 was used to restore NK cell cytotoxicity to hepatocellular carcinoma (HCC) cells transfected with HLA-G1 following the addition of an inhibitory ILT2 HLA-G receptor (Manavalan et al., 2003). The receptor is expressed on B lymphocytes and may increase tolerance of liver allografts expressing HLA-G by inhibition of B-cell antibody secretion (LeMaoult et al., 2005).

1.9.4 Other HLA-G immune functions

The interaction of the CD8 receptor present on cytotoxic T cells and NK cells with sHLA-G triggers apoptosis. Elevated sHLA-G levels are observed in neoplastic diseases, as well as other soluble HLA class I antigens. This effect leads to inhibition of the immune response, particularly against liver cancers. NK cells and some CD8 T lymphocytes express the killer cell immunoglobulin-like receptor (KIR) 2DL4/p49 (CD158d) or KIR2DL4. This receptor may have an activating or inhibitory function. The immunoglobulin-like transcript 4 receptor, ILT-4 (CD85d; LILRB2) is expressed by dendritic cells (DCs), macrophages and

monocytes. The ILT-4/HLA-G interaction mediates the inhibition of the antigen-presenting function and DC maturation and allows tumors cells to escape from host immunity (Amiot et al., 2015).

1.9.5 HLA-G Expression

The restricted expression of HLA-G was initially described regarding the maternal-fetal interface, on extravillous cytotrophoblasts. Following this it was observed that in healthy subjects after birth the HLA-G protein is mainly expressed only on beta cells of the islets of Langerhans, endothelial precursors, the cornea, thymic medulla, mesenchymal stem cells and the nail matrix. When the HLA-G molecule is expressed, it appears to suppress the immune responses through exerting an inhibitory effect against immune cells and interacting with their expressed receptors such as LILRB1 (ILT2/CD85j), LILRB2 (ILT4/CD85d), and KIR2DL4 (CD158d), which are differentially expressed by immune cells (Dias et al., 2015). The interaction of HLA-G molecules with these inhibitory receptors induces apoptosis of activated cells, such as CD8+ T cells, and modulates the activity of NK cells and dendritic cells. Therefore, the immunomodulatory functions of HLA-G could inhibit host immune responses against infectious diseases and cancer (Sheu and Shih, 2007).

Aberrant HLA-G expression has been linked with several pathological conditions. These include: viral infections, tumours, transplantation,

autoimmune and inflammatory diseases with the effect of driving a reduction in the immune response. This tolerogenic property of HLA-G may have beneficial effects in circumstances such as pregnancy, transplantation, and inflammatory diseases. However, this immune evasion become deleterious when affecting the immune responses against tumours and viral infections by permitting the escape of tumour or virus-infected cells from the conventional anti-tumour or anti-viral responses (Amodio et al., 2015, Catamo et al., 2015, Polakova, 2003, Rajasekaran et al., 2014).

During viral infection, viruses can modulate the nonclassical HLA class I antigen HLA-G via diverse strategies to disrupt immune competent cell recognition. A previous study showed that cell surface HLA-G expression was significantly induced in H1N1 influenza virus–infected patients and seasonal H1N1–infected patients when compared to normal controls. The study suggests elevated cell surface HLA-G expression may favour the virus escaping from host immune responses (Chen et al., 2011). A significant difference in HLA-G expression on monocytes, plasma sHLA-G, IL-10 and IL-6 levels was associated with Enterovirus 71 (EV71) infection when compared to that in normal subjects (Zheng et al., 2014). Similarly, HLA-G expression has been reported also in the hepatocytes and biliary epithelial cells of the livers of patients with chronic hepatitis C. Plasma sHLA-G concentration levels were found to be higher in chronic

hepatitis C patients (n = 67) than healthy subjects (n = 129) (Amiot et al., 2014a). Taking these findings together, this suggests that viral infections can induce HLA-G expression, which could be an additional mechanism which helps viruses to subvert host defences.

1.9.6 HLA-G polymorphisms in the 3' untranslated region

There is a dimorphism of HLA-G dependent upon deletion or insertion of a 14bp sequence in the 3' untranslated region (UTR) which affects levels of HLA-G expression. Three 3'UTR polymorphic sites of HLA-G including the 14-base pair (14bp) Insertion/Deletion, +3142C-G and +3187A-G have been suggested to be associated with HLA-G mRNA regulation. In 187 French and 153 Brazilian healthy individuals, individuals presenting with the 14bp DEL/DEL showed higher plasma soluble HLA-G (sHLA-G, detected by ELISA) levels compared to 14bpINS/INS genotype. The differential expression of HLA-G seems to be either beneficial or harmful depending on the underlying medical condition. Therefore, understanding the association between individual genetic and differential expressions of HLA-G can help to define novel strategies of immune response toward the underlying medical causes (Martelli-Palomino et al., 2013). To date, 44 haplotypes of the HLA-G 3′ untranslated region have been identified which exhibit nine variable sites. UTR-1/2/3/4/5/6/7 are the most frequent 3'UTR haplotypes (Castelli et al, 2009a).

Haplotype	14bp	+3003	+3010	+3027	+3035	+3142	+3187	+3196	Freq
UTR-1	-	T	G	C	C	C	G	C	0.25
UTR-2	+	T	C	C	C	G	A	G	0.255
UTR-3	-	T	C	C	C	G	A	C	0.196
UTR-4	-	C	G	C	C	C	A	C	0.093
UTR-5	+	T	C	C	T	G	A	C	0.064
UTR-6	-	T	G	C	C	C	A	C	0.047
UTR-7	+	T	C	A	T	G	A	C	0.043

Table 1-1 Haplotypes of the HLA-G 3′UTR.

Green indicates alleles associated with increased HLA-G expression and red indicates alleles associated with decreased expression (Castelli et al, 2009a).

The most frequent haplotypes of the HLA-G 3′ untranslated region according to the 1000 Genomes data are shown in (Table 1.1). Haplotypes were named according to (Castelli et al., 2009a). Haplotype frequencies considering 21 worldwide populations are included with alleles associated with high expression highlighted in green (UTR-1). Alleles associated with low expression are highlighted in red (e.g. UTR-7). The sequences obtained were analyzed by observing the 14 bp INS/DEL (rs1704), +3003 C/T (rs1707), +3010 C/G (rs1710), +3027 A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142) and +3196 C/G (rs1610696) polymorphic sites and were individually annotated (Table 1-1). According to this figure, haplotype and genotype construction were assigned and named (Castelli et al., 2010).

Globally, these UTRs account for more than 96% of all the reported haplotypes. The predominant haplotypes are UTR-1 (~25%) and UTR-2 (~25%), which differ at five positions, followed by UTR-3. Some haplotypes have been associated with different levels of HLA-G expression and have been grouped in higher, lower and medium levels. UTR-1 was classified as the group that produced higher levels of sHLA-G. UTR-1 encodes all variants previously associated with high HLA-G expression (14 bp Del, +3142 C and +3187 G). UTR-5 and UTR-7 were previously associated with low HLA-G expression and with producing lower levels of sHLA-G and differ by a single position (+3027 C and A, respectively; rs17179101). One recent study has has reported an additional *HLA-G* alternative splicing pattern (Fujii, 1994). An mRNA isoform has been detected in some instances with the first 92 bp of exon 8 spliced out of the allelic form (including the 14-bp sequence). However, no further studies of this out-splicing of the 92-bp sequence have been reported. Interestingly, an association between the presence of the 14-bp sequence in homozygous third-trimester placenta biopsies and the lack or very low expression of the HLA-G3 mRNA isoform and possibly an overall low HLA-G mRNA expression has been reported. 14bp Ins may lead to alternate splicing of a 92bp sequence in exon 8 (Castelli, 2009). Interestingly, UTR-2 encodes the trio of variants found in UTR-5, and UTR-7 was classified as a medium producer of sHLA-G. Therefore, 3'UTR

variants have been suggested as potential prognostic biomarkers to determine susceptibility to infection (Castelli, 2010).

1.9.7 HLA-G polymorphism in the 5′ upstream regulatory region

The HLA-G gene is composed of eight exons and seven introns along with a stop codon at exon 6. In contrast to classi74cal class I loci, HLA-G has a low degree of polymorphism in the human genome compared with HLA class I and II antigens. The stop codon in exon 6 of HLA-G leads to a short cytoplasmic tail and exhibits a 5′ upstream regulatory (or promoter) region (5′ URR) extending at least 1.4 kb from the initial ATG (Berger et al., 2010) and has an extended 3′ untranslated region (3′ UTR). The 5′ upstream regulatory region (URR) of HLA-G, extending at least 1.4 kb, affects transcription regulation, and an extended 3′ untranslated region (3′UTR) influences mRNA stability (Dias et al., 2018). The 5′ URR and 3′ UTR regions polymorphisms of the HLA-G gene have been found to be associated with differences in HLA-G expression. This may affect HLA-G function and may play an important role in the regulation of expression (Hviid and Christiansen, 2005). The 14bp insertion–deletion located in exon 8 polymorphism lies in strong linkage disequilibrium with 5′ URR variants (Misra et al., 2013). It is also responsible for alternative splicing of the HLA-G transcript (Hiby et al., 1999). The alternative splicing process involves removal of the surrounding 92 bases from the mature transcript, which includes the pentameric initial AUUUG of the 14 bp sequence (Hviid et al., 2003). It is believed that the absence of such a motif

in the 92 base deleted transcripts may provide more stable mRNA (Rousseau et al., 2003).

In worldwide populations, the 5′URR shows 35 SNPs defining 68 haplotypes, of which 9–10 have been frequently observed (Gineau et al., 2015). With few studies analysing five 5′URR haplotypes, not much is known about the impact of 5′URR variations on the HLA-G activity. The in silico analyses reveals that 5′URR haplotypes are clustered into four groups, shaped by balancing selection. Due to putative differential transcription factor binding to regulatory elements, the clustering is likely to characterize different promoter region activity (Tan et al., 2005).

1.9.8 Influence of HLA-G polymorphisms on disease

The 14-bp insertion/deletion polymorphism at 3′ UTR attracts the attention of many scientific groups due to its role in HLA-G alternative splicing and regarding its role is RNA stability. A subgroup meta-analysis indicated the HLA-G 14 bp INS/DEL polymorphism was significantly associated with an increased risk of breast cancer (Li et al., 2015). Besides, the 14-bp INS/INS was associated with clinical symptoms suggestive of *H. pylori* infection. The odds of having the genotype INS/INS were 3.77 times greater among the HP+ patients compared to DEL/DEL than among controls (Genre et al., 2016b). Significant differences in allele and genotype frequencies of the HLA-G 14-bp polymorphism in HIV-exposed uninfected Brazilian children has been

reported, when compared to both seropositive children and healthy controls and the del allele has being associated with protection (Fabris et al., 2009). One study examined a C/G SNP at position +3142 in relation to microRNAs (miRNAs), the results of which indicates that +3142G might be associated with decreased expression of HLA-G. Yie et al. reported that a C/A SNP at position +3187 of exon 8 leads to reduced half-life of mRNA-transcripts (Yie et al., 2008).

Interestingly, there is disequilibrium linkage between the HLA-G 14 bp INS allele with both the +3142G and the +3187A SNPs. The 14 bp insertion has been repeatedly associated with lower levels of the HLA-G in different studies. The +3142G and +3187A alleles were previously associated with low mRNA availability and always accompanied by the 14 bp insertion. This indicates that the lower mRNA production is associated with the combined presence of these polymorphisms. To date, no correlation with other HLA-G polymorphisms (+3003C/T, +3010C/G, +3027A/C, +3035C/T and +3196C/G) and level of HLA-G expression has been observed.

The single nucleotide polymorphism C>G at position +3142 (rs1063320) within the 3′ UTR of the HLA-G gene is known to be within a putative binding site for microRNAs (miRNAs), which is suggested to influence the regulation of HLA-G gene expression. The +3142 G allele was reported to have a higher repression of translation and mRNA degradation through the targeting of three miRNAs (miR-148a, miR-148b and miR-152). It is

possible that this results in a higher repression of translation and mRNA degradation, consequently, low expression of HLA-G. In contrast, the C allele was thought to have a binding site with lower affinity for these miRNAs. Among patients with a history of respiratory tract infections, the +3142C allele was underrepresented. The +3142G allele was reported to increase the susceptibility to infections, in particular to HCV infection. This could suggest a possible interference of the +3142G allele of HLA-G molecule in the response to infections (Cordero et al., 2009).

1.9.9 Soluble HLA-G (sHLA-G)

sHLA-G is an anti-inflammatory protein that is synthesised primarily by the placenta. It contributes to the process of foetal tolerance during pregnancy (Beneventi et al, 2016). The tolerogenic effect is implicated in successful conception, and in order to accomplish this, several immune cells may fall under its influence such as cytotoxic T cells, NK cells and T reg. It was found that women who develop diabetes during pregnancy tend to have significantly increased levels of sHLA-G compared to healthy women. Undeniably, women who have repeated abortion, pre-eclampsia and embryo restricted maturation tend to have lower levels of sHLA-G (Beneventi et al., 2016).

In contrast, the shed isotype HLA-G1 is related to impaired capillary formation via blocking of FGF-2 induced angiogenesis (Fons et al., 2006).

During assisted fertilisation procedures (IVF), the detection of sHLA-G in the embryo media may give rise to high rates of implantation compared to certain conditions like frequent abortion and preeclampsia. (Rizzo et al, 2011). In patients with certain malignant tumours like breast and ovarian carcinoma, melanoma and glioma, sHLA-G has also been detected at high rates (Rebmann et al, 2003) and a similar observation was shown in HIV-1 infection (Huang et al., 2010).

1.9.10 Ligands for HLA-G: LIR-1 / 2 and KIR2DL4.

HLA-G is reported to bind the inhibitory receptors LIR-1 (leukocyte Ig-like receptor 1 or ILT-2) and LIR-2 (leukocyte Ig-like receptor 2 or ILT-4) that are expressed by a range of immune cell types including monocytes, NK cells, T cells, and macrophages (Figure 1-7). The family of KIR consists of two or three domain forms (KIR-2D or 3D) in addition to the cytoplasmic tail which can be either long (L) or short (S) and expressing inhibitory or activating properties respectively (Moradi et al., 2015). HLA-G also binds to the inhibitory receptor KIR2DL4 (killer Ig-like receptor 2DL4) expressed on NK cells. Recently, soluble HLA-G was found to be endocytosed into KIR2DL4-containing compartments in NK cells and cells transfected with KIR2DL4. The engagement of these molecules with HLA-G may directly reduce appropriate immune reactions against infections and tumours (Naji et al., 2007).

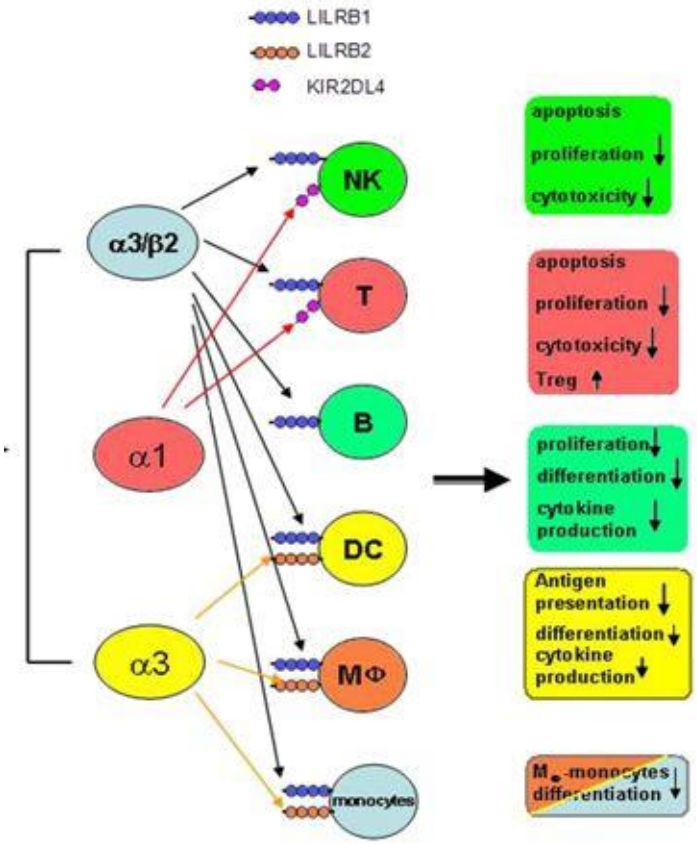

Figure 1-7 Immunoregulatory activities mediated by HLA-G, where the involved target cells and receptors are indicated.

The immune-inhibitory function of HLA-G is achived by interacting with leukocyte receptors including leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) and member 2 (LILRB2), and killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4). While LILRB1s are expressed on the surface of several leukocytes, such as NK and lymphomononuclear cells, LILRB2s are primarily expressed on the surface of a restricted set of cells, including monocytes and dendritic cells. The interaction between the immune tolerogenic molecule HLA-G and these inhibitory motifs receptors leads to the suppression of immune responses (Shiroishi et al., 2006; Gao et al., 2000; Dietrich et al., 2001).

NK cells have significant functionality in killing virally infected cells. They link between the innate and adaptive immune response and can induce cellular lysis via delivering toxic molecules like granzymes and perforin (Moretta et al, 2000). Likewise, in many inflammatory and infectious diseases NK cells were observed to generate cytokines like TNF-α and INF-γ (Biron et al, 1999) which potentially play a crucial role in the mobilisation of lymphocytes and proliferation to deter virally infected or malignant cells (Kikuchi-Maki et al, 2003).

The transmembrane portion of the KIR2DL4 gene region displays a dimorphism in which 9 or 10 adenines are expressed in exon 6 (Rajagopalan & Long, 2012). While the 9A form produces mRNA that encodes a truncated KIR2DL4 protein, the 10A form can be translated into full length KIR2DL4 protein. Surprisingly, it was observed that more 9A/10A genotypes of KIR2DL4 were present in the group of male partners from the miscarriage group in comparison to the men from the control group. Furthermore, no association was found between women's KIR2DL4 polymorphism with susceptibility to spontaneous abortion (Nowak et al., 2016). The KIR2DL4 protein does not bind with classical HLA-I molecules and retains the potency to ligate with HLA-G proteins (Ponte et al., 1999). This results in the production of IFN-γ and a low cytotoxic effect of NK cells at the fetomaternal interface to allow a proper environment for the developing embryo (Rajagopalan & Long, 1999).

1.10 Pneumonia

1.10.1 What is Pneumonia and Community Acquired Pneumonia?

Pneumonia is a syndromic, clinical diagnosis based upon a combination of signs, symptoms and where available, radiological findings. It describes a common infection of the lower respiratory tract in which there is inflammation of the alveolar air spaces of the lung and associated systemic features such as fever. It can be caused by viruses, fungi and parasites, but bacteria remain the most common aetiological agents. The typical classification of bacterial pneumonias is either as community-acquired (CAP) or hospital-acquired (Wootton and Feldman, 2014).

Unlike hospital acquired pneumonia, community acquired pneumonia (CAP) is described as a patient with pneumonia who has not had a recent hospital admission. CAP is thought to include those who have aspirated a large volume of oropharyngeal contents or vomitus. The elderly and those with neurological defects are thought to be more vulnerable to this phenomenon (Marik 2001). In routine clinical practice it is difficult to verify when pneumonia has developed as a consequence of aspiration which has meant that most studies of CAP exclude patients where aspiration pneumonia is strongly suspected. Defining aetiological agents that are associated with each pneumonia syndrome enables the clinician to empirically target the infection. (Wootton and Feldman, 2014).

1.10.2 Epidemiology of Hospitalized CAP in the UK

Each year, approximately 11 out of every 1,000 adults develop community acquired pneumonia (CAP) in the UK. In 1992/3, 16.3 million episodes of lower respiratory tract infection (LRTI) were treated by the National Health Service (NHS). A recorded 261,000 (1.6%) events were as a result of CAP, of which 32% were admitted to hospital. The age-standardized incidence admission to hospital with CAP has increased in the UK population (Trotter et al., 2008).

1.10.3 Seasonality

The UK peak of pneumonia infection is the cold winter months and typically around the weeks surrounding Christmas / New Year. This trend is related to climate and in particular the ambient air temperature has been demonstrated as a notable factor (Kumamaru et al., 2014).

1.10.4 Age

Pneumonia is common in children under the age of 5. Its incidence increases exponentially in adults for every decile above 55 years. In the UK, the greatest increase in adult hospitalisation (39%) has been in the most part related to the elderly (>85 years) (Rosychuk et al., 2010, Ewig et al., 2009).

1.10.5 Aetiology and antimicrobial treatment

Current evidence shows that the most frequent CAP in adults is caused by bacteria and as such patients are given antibiotics. However viral pneumonia can also occur at an incidence of around 30% with adenovirus and influenza being the most common cause.

Following the development of penicillin, CAP mortality rates have fallen dramatically. The most effective antibiotics need to be given as soon as possible to maximise the effect and therefore this does not give the time to determine which specific bacteria are causing the pneumonia (Lim et al., 2009). The process of determining which antibiotic the bacteria are susceptible to, using traditional diagnostic tests, takes 2-3 days. The process also requires culturing a clinical specimen and isolating the causative species. Therefore, the antibiotic of choice at the time of commencing treatment is a best guess based on the most likely causative organism. The detection rates are particularly low in CAP due to some bacterial strains being technically more difficult to culture (Howard et al., 2005). Also, if the patient has recently taken antibiotics, cultures of bacteria are likely to be impaired. Therefore, detection of a few bacterial species may require molecular testing. (Ingarfield et al., 2007). As a result, for >50% of adult patients the pathogen causing the pneumonia remains unidentified.

1.10.6 Outcome following CAP

There is a range of outcomes that can be measured following an episode of CAP against which we can prognosticate or assess the efficacy of interventions. The patient characteristics have been linked to a range of outcomes and each has its own inherent strengths, weaknesses and applicability. The validation of the traditional outcome measures used have shown a low rate of specificity. It is argued that the inclusion of patient based outcome measures in studies of CAP would be beneficial, particularly when matching outcome measures to the design of studies (Barlow et al., 2003).

1.10.7 Mortality

Each year 34,000 patients die from CAP, which makes its mortality rates in the UK amongst the highest in Europe. The auditing of CAP demonstrated an in-patient mortality of 21% and for CAP managed within UK hospitals these rates show over 50% of the 7% admitted to intensive care die. The same result confirmed that most patients hospitalised for CAP survive their initial infective insult to be discharged back to the community. Moreover, the mortality rate in the 70% of CAP patients treated in the community represents <1%.[6]. Therefore, mortality might be a useful outcome in certain populations where death rates are high (Lim et al., 2009).

1.10.8 Length of stay

Since a night in a hospital bed is so expensive, length of stay is thought to be an important measure for hospital managers and commissioners (Page et al., 2017). However, many factors that are not directly related to the severity or aetiology of the CAP or to the quality of care received may influence length of stay (Khosravizadeh et al., 2016, Wootton and Feldman, 2014). A hospital bed crisis happens within the NHS invariably in the winter just as most CAP occurs. Moreover, some hospitals will be more affected than others due to regional differences in demographics and socioeconomic factors. The lack of availability of residential and nursing home places can delay discharge and hospitals face significant pressure to discharge patients as soon as possible (McCormick et al., 1999).

1.10.9 Readmissions

Following an episode of pneumonia, a significant number of patients are re-admitted to hospital. In Spain a study showed that within 30 days, 2.5% of patients were re-admitted with pneumonia related problems and 4.6% were readmitted with non-pneumonia related problems (Carosella et al., 2008). A cohort study designed to capture medium and long-term morbidity associated with re-admission found 2% of patients were re-admitted with pneumonia within 30 days of hospital discharge and a further 9% by one year. In addition to these re-admissions, a study looked not only at re-admissions to hospital but contact with healthcare in

general following a CAP discharge shows a significant number of non-pneumonia re-admissions (Johnstone et al., 2008). Rates of re-admission are difficult to compare across countries due to different healthcare systems. The availability of out-of-hours primary care provision within regions of the UK is even influenced the variations. However these studies demonstrate that substantial ongoing morbidity is associated with CAP, despite being well enough to be discharged, for some patients (McCormick et al., 1999).

1.10.10 Immunology of Pneumonia

1.10.11 Innate immunity in pulmonary immunity

In the lung, innate immunity is mediated by several key cells (shown in Figure 1-8). The respiratory epithelium contains cells that vary in terms of morphology and gene expression as one moves down from the trachea to the respiratory bronchiole. The majority of cells are: the ciliated cell, the mucous secreting goblet cell, and the secretory Clara cell. In addition, there are submucosal glands that contribute to airway secretions in the upper airways. The sophisticated structure of the epithelium, however, can move inhaled or aspirated bacterial out of the lung through mucociliary transport. In addition to the epithelium, airway and alveolar macrophages represent the majority of resident myeloid derived cells that can mediate both opsonic and nonopsonic phagocytosis of inhaled or aspirated pathogens (Eddens and Kolls, 2012).

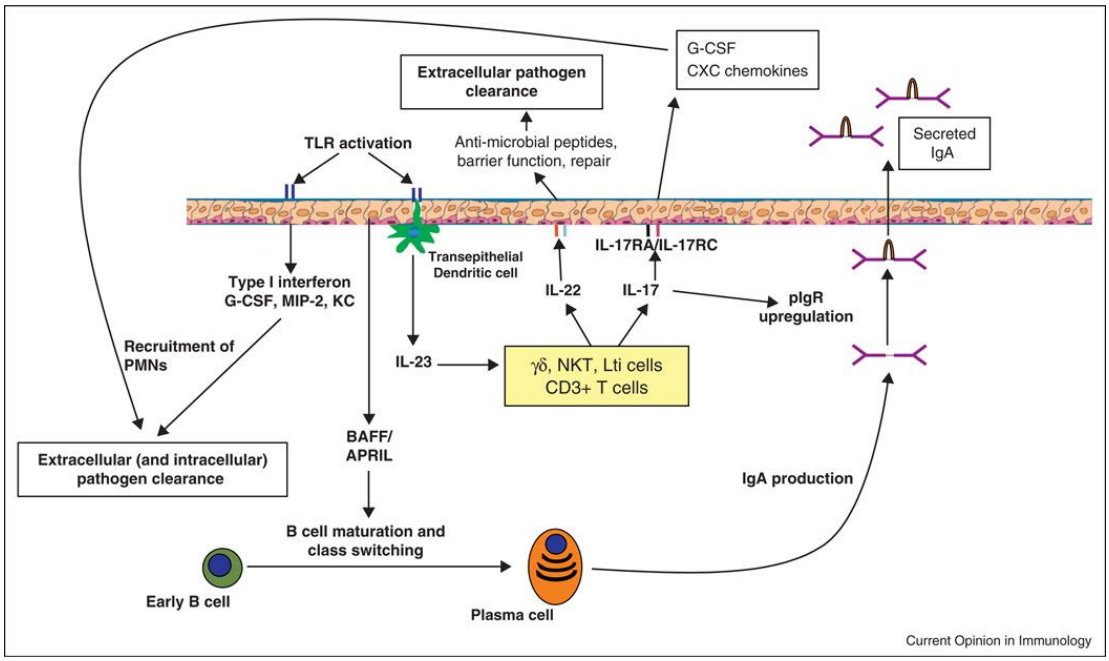

Figure 1-8 The emerging role of lung epithelia in immunity.

Lung epithelia and transepithelial dendritic cells respond to invading bacteria via TLRs. After activation, type I interferons, G-CSF, MIP-2, and KC are release by the lung epithelia which recruit polymorphonuclear cells, leading to enhanced pathogen clearance. Dendritic cells release IL-23, which stimulates innate producers of IL-17 and IL-22. Lung epithelia, which express IL-17RA, IL-17RC, and IL-22R, responds by augmenting the expression of antimicrobial peptides, increasing barrier function, and releasing G-CSF. In addition, BAFF and APRIL, proteins can be released by lung epithelial cells that promotes B cell maturation and class switching to IgA. Following IgA production, IL-17 can upregulate pIgR, leading to greater transcytosis of IgA into the lung lumen (Eddens, 2012).

1.10.12 Adaptive immunity in pulmonary immunity

The adaptive immune response plays a critical role in pulmonary immunity to pneumonia, specifically CD4+ T-cells (Eddens and Kolls, 2012). Differentiation of T-cells requires signaling events from class II expressing antigen presenting cells. After signaling, two CD4+ T- cells subsets initially develop: Th1 which mature under interferon-γ and Th2 cells which develop under IL-4 (Figure 1-9). One of the key effector

cytokines is interferon-γ which is produced by Th1 cells and can increase microbicidal activity of immune cells such as macrophages. However, Th2 cells express cytokines such as IL-4 and IL-13, the expression of which are thought to be critical for controlling pneumonia infection. Individuals with mutations in interferon or interferon receptor signaling are highly susceptible to infections such as pneumonia (Eddens and Kolls, 2012).

Adaptive immunity plays a key role in increasing the focus of vaccine induced immunity. In comparison to CD4+ T cell and humoral immunity, CD8+ T-cell immunity plays a limited role in most bacterial infections. The critical role of CD4+ T-cells and their subsets in pulmonary infection in orchestrating pulmonary immunity has been clearly illustrated (Figure 1-9).

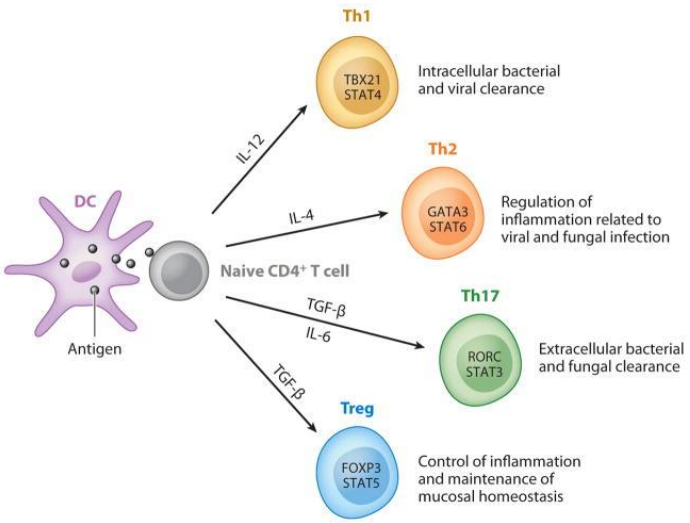

Figure 1-9 T cell subsets in pulmonary infection.

The figure shows CD4+ T cell subsets in pulmonary host defence after antigen encounter presented by DCs. Naive CD4+ T cells differentiate into Th1, Th2, Th17, and Treg cells. The process is controlled by certain cytokine environments (IL-12, IL-4, TGF-ß/IL-6, or TGF-ß, respectively. These major T cells play key roles in mediating pulmonary immunity (Chen and Kolls, 2013).

1.11 Human cytomegalovirus

1.11.1 Characteristics of Human cytomegalovirus

Human cytomegalovirus (HCMV) is a beta-herpesvirus which is widely prevalent in the population. It is the leading infectious cause of congenital infection and affects 0.2-2.4% of all infants in developing countries. Even though CMV infection is generally asymptomatic, it can cause fatal disease in immunocompromised individuals and neonates. The virus can also cause serious disease in organ transplant recipients, either after primary infection or due to reactivation of latent infection (Yinon et al., 2010). Despite efforts in controlling HCMV infection by early treatment with antivirals, challenges remain with regard to toxicity, the emergence of antiviral-resistant HCMV strains and the availability of antiviral drugs in developing countries. Knowledge of the underlying mechanisms controlling latency and reactivation of HCMV remains limited. A study shows that after allogeneic stimulation of HCMV latently infected monocytes, macrophages generated could produce HLA-G Ags during viral reactivation. This leads to partially down-regulated expression of classical MHC-I molecules (Onno et al., 2000a).

1.11.2 Immunology of CMV

CMV infection is not typically cleared from the host and immune surveillance mediated by the humoral and cellular immune systems can only suppress viral replication. The virus utilises a number of different

mechanisms that evade the human immune response including the ability to target the activation of the adaptive immune response (Reusch et al., 1999). During CMV infection, the classical and non-classical major histocompatibility complex class I (MHC I) molecules are major mechanisms for the recognition of CMV by immune cells (shown in Figure1-10). Whereas a key function for classical MHC I molecules is to present antigenic peptide ligands on infected cells to CD8+ T cells, non-classical MHC I molecules can mediate inhibitory or activating stimuli in natural killer (NK) cells (Halenius et al., 2015). A recent study shows that longstanding CMV infection leads to expansion of a large population of CMV-specific CD8+ and CD4+ T cells. This expansion leads to a reduction in the CD4/CD8 ratio, many of which are CD28- and hence non-functional (Wills et al., 2015b). CMV has recently been implicated in the process of immunosenescence, whereby in CMV+ subjects there is a progressive increase in proportions of T cells specific for CMV at the expense of T cells of other specificities. This may result in a decreased life expectancy in elderly subjects (Savva et al., 2013).

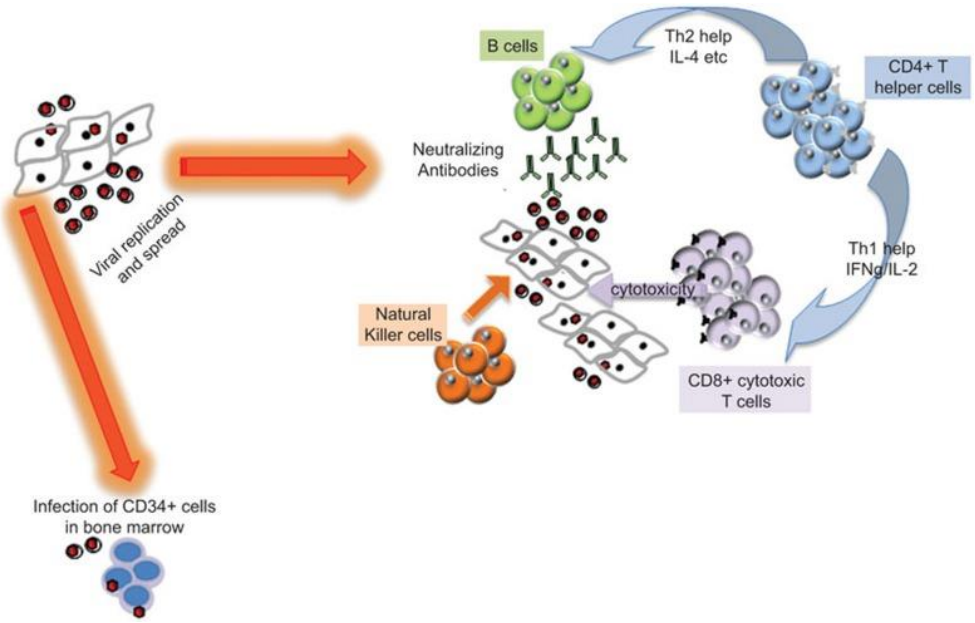

Figure1-10 Immune response Following HCMV infection.

Prior to the host generating an effective immune response, HCMV replicates and disseminates. Immune response includes natural killer cells, neutralizing antibodies, CD4+ and CD8+ T cells. Eventually viral replication is controlled and the CMV primary infection is resolved. (Wills et al., 2015a).

1.11.3 HLA-G in CMV infection

Some evidence shows that HCMV infection can induce HLA-G expression in monocytes and influence plasma soluble HLA-G and IL-10 levels (Onno et al., 2000a). Previous work from our group has shown that CMV can induce leucocyte sHLA-G production as well as expression of cell surface HLA-G, primarily on CD56+ T cells, B cells and monocytes (Albayati et al, 2017). HLA-G expression induced during HCMV infection could inhibit the functions of various immune cells involved in defence against HCMV infection. NK cells play a major role in limiting viral replication and

reducing viral load during early acute HCMV infection (Yan et al., 2009). As DCs constitute a key component in the immune response, the expression of HLA-G at transcriptional and protein levels during differentiation and maturation of the different DC subsets was assessed. HLA-G transcription was significantly induced during CD34+-derived DC differentiation and is associated with a cell-surface expression in half of the cases described and with a substantial secretion of soluble HLA-G in all cases (Friec et al., 2004).

1.12 Human Immunodeficiency Virus

1.12.1 Characterisation of Human Immunodeficiency Virus

Human Immunodeficiency Virus (HIV) is a lentivirus, which belongs to the Retroviridae family. The virus has a single stranded RNA genome contained within a capsid and a lipid envelope. There are two different viruses within the family of HIV: HIV-1 and HIV-2. While HIV-1 is the main strain of HIV and accounts for 95% of all infections worldwide, HIV-2 is seen in a few West African countries (Djomand et al., 2014).

HIV viral entry is required for infection and entails binding of the HIV protein envelope (Env) to its cellular receptor CD4 and then to a cellular co-receptor (Figure 1-11). HIV strains are classified based on their co-receptor usage. R5 HIV is named for viruses that use the chemokine receptor CCR5, where those that use CXCR4 are termed X4 HIV (Wilen et al., 2012). Although, both CXCR4 and CCR5 function as co-receptors

for HIV-1 entry into CD4+ cells, the majority of primary HIV-1 isolates in early disease use CCR5 as a co-receptor whereas during disease progression with the emergence of syncytium-inducing viruses, CXCR4 is also used (Bleul et al., 1997)

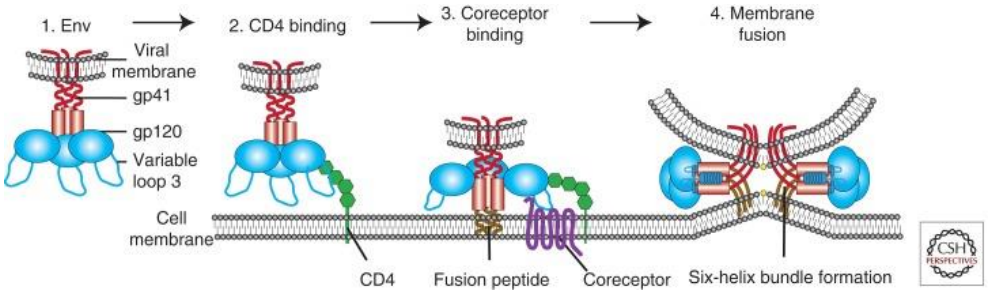

Figure 1-11 Cell entry of HIV-1 into host cells.

To to gain entry the cell, HIV Env, comprised of gp120 and gp41 subunits (1), first attaches to the host cell, binding CD4 (2). This leads to conformational changes in Env and allowing coreceptor binding,. It is mediated in part by the V3 loop of Env (3). The process initiates the membrane fusion process as the fusion peptide of gp41 inserts into the target membrane, followed by six-helix bundle formation and complete membrane fusion (4) (Wilen, 2012).

Globally, HIV-1 disease is a serious health issue with approximately 2 million new cases of HIV-1 annually. In 2014, an estimated 1.2 million people died from acquired immune deficiency syndrome (HIV/AIDS) AIDS-related illnesses (German Advisory Committee Blood, 2016, Fanales-Belasio et al., 2010). Both men who have sex with men and injecting drug users (MSM/IDU) have multiple risk factors for HIV acquisition and transmission and these groups continue to pose challenges for HIV reduction efforts. MSM has become one of the highest risk groups for HIV infection worldwide (Huang et al., 2016).

According to the replication characteristics and the pathogenic mechanisms of HIV-1 infection, diagnostic strategies are continuously revised (Fanales-Belasio et al., 2010). Upregulated HLA-G expression on monocytes and on some T lymphocytes in HIV positive patients with or without antiretroviral treatment was reported following infection with HIV-1 (Celsi et al., 2013). Therefore, understanding how HIV-1 establishes infection and causes AIDS with its association with HLA-G are important not only to identify and develop new effective drugs and vaccines but also to improve the current diagnostic strategies (Fanales-Belasio et al., 2010).

1.12.2 Immunology of HIV-1

The genetic variability of the virus, driven by viral evolution under pressure from the immune system or anti-retroviral therapy, is the most powerful weapon of HIV-1 and is attributed to the viruses' capacity to overcome host immunity. Variation in the clinical responses of individuals to viral infection is influenced by the properties of the infecting virus, the circumstances of infection and host genotype (Rouse and Sehrawat, 2010). One such example of this is "elite controllers" of HIV infection. Previously, the plausibility of a functional cure of HIV was guided by the demonstration that a certain rare group of HIV-positive individuals, these "elite controllers", can control viraemia in the absence of any treatment. However, a similar effect can be obtained with combination antiretroviral therapy (ART) (Lederman et al., 2016, Rouse

and Sehrawat, 2010). MSM who have remained seronegative despite high risk behaviour (HRSN) were compared to HIV-1 infected MSM to evaluate the susceptibility to HIV-1 infection. The study suggests that the 1650G allele of the cyclophilin A gene may be associated with a decreased susceptibility to HIV-1 infection in participants (Rits et al., 2008).

Both cellular and humoral (antibody) immune responses are important to prevent the ultimate progression of HIV-1 disease. Cellular responses have been noted to be more effective in controlling viraemia than humoral responses. These cellular responses, including CTLs (CD8+ cells) and helper T lymphocytes (CD4+ cells), can inhibit HIV-1 replication and produce soluble cytokine and chemokine antiviral factors. They include CD8 antiviral factor (CAF), TNF-α, IL-13, IFN-γ/α, macrophage-derived chemokine (MDC; CCL22) and others. Strong HIV-specific CD8+/CD4+ responses are associated with lower HIV-1 viral loads. HIV, as other human viral infections such as HCMV and HCV, is known to be become persistent and can cause severe chronic disease that is usually the consequence of an immune reaction to the infections. In HIV, the virus integrates into the genome of infected host cells and in addition to other multiple immune evasive properties, it is never removed from the body.

Additionally some memory CD4+ cells, which have undergone differentiation from activated infected CD4+ cells, may carry copies of the HIV-1 genome in a latent form within their own genetic code meaning

HIV-1 can persist long term within the host's own immune system (Chinen and Shearer, 2002).

1.13 Research background

To date, the HLA-G polymorphism and expression in pneumonia patients in relation to infection with CMV or other pathogens have rarely been investigated. The functional implications of HLA-G in pneumonia patients in relation to clinical outcomes and length of stay are yet to be empirically examined. Yet since HLA-G plays such a vital role in the immunomodulatory functions during bacterial and viral infections, it is possible that its expression could affect clinical outcomes. The INS/INS genotype of 14bp INS/DEL was found to confer a strong risk for many pathological conditions. The impact of the level of three functional polymorphisms, 14 bp INS/DEL, the +3142 G-C and the +3187 A-G and soluble HLA-G (sHLA-G), on the risk of specific pathogens has previously been evaluated in other diseases, but not in pneumonia patients.

However, in the case of relating HLA-G expressing to HIV-1 infection, transmission and susceptibility this knowledge would only be beneficial if there were some degree of HLA-G sequence variation between individuals. The HLA-G sequence variants would hypothetically influence the effects depending on changes in its function. Moreover, it would be of interest to attempt to correlate between protection provided against HIV-1 infection with the extent of HLA discordance. Comparisons of HLA-G polymorphisms, sHLA-G concentration, and

clinical outcomes of HIV and HCMV patients are required to assess the full spectrum of functional or clinical effects of HLA-G variation.

1.14 Rationale for the study

The Rationale of this study was to test whether increases in levels of HLA-G expression were related to clinical outcomes in pneumonia and HIV patients. In order to study how HLA-G polymorphism and expression may influence the clinical outcomes in pneumonia patients, it was important to consider three outcomes of interest: death, re-admittance and length of stay. Previous work has shown that CMV can induce HLA-G expression, particularly in B cells, CD56+ T cells and monocytes. This was the rationale behind looking at the influence of CMV in pneumonia incidence and outcomes.

1.15 Aim

The purpose of this study is to investigate HLA-G polymorphism and expression in pneumonia patients in relation to infection with CMV or, in other pathogens such as HIV, in relation to clinical outcomes. HLA-G alleles were determined by subjecting patient DNA extracted from stored blood samples to standard PCR and proportions of patients with the 14bp deletion/insertion compared with healthy controls. Patients will be subdivided into groups according to clinical outcomes and the proportions of HLA-G alleles in different groups will be compared. In HIV patients, the aim was to examine the impact of the level of three functional

polymorphisms, 14 bp INS/DEL, the +3142 G-C and the +3187 A-G on the risk of developing disease.

Previously, HLA-G expression was found to be significantly induced by CMV, particularly in B cells, CD56+ T cells and monocytes. The cytomegalovirus (CMV) serostatus of different groups of patients will be measured by means of an anti-CMV antibody ELISA. CMV status will be compared in different patient groups and also related to HLA-G genotype and levels of soluble protein. This is to investigate the influence of CMV in pneumonia incidence and outcomes.

Soluble HLA-G (sHLA-G) will be measured by enzyme-linked immunosorbent assay (ELISA) from serum specimens and differences in levels between different patient groups compared. The soluble HLA-G may mediate immune suppression in pneumonia patients in relation to infection with CMV or HIV, in relation to clinical outcomes.

Also, variations in the main ligand for HLA-G, KIR2DL4, will be studied. Relevant polymorphisms in KIR2DL4 will be tested in different patient groups by a combination of multiplex PCR and gene sequencing.

Therefore, we hope to understand the association between individual genetic and differential expressions of this ligand for HLA-G in relation to diseases in which HLA-G expression is upregulated. The study also aims to evaluate the proportions of cells from healthy subjects expressing KIR2DL4 in CD56+ T cells, and CD16+ CD56dim and CD56bright CD16-

NK cells following stimulation with IL-2, a nonspecific activator of T cells and NK cells.

2 MATERIALS AND METHODS

2.1 Patient and Healthy Control recruitment

2.1.1 Pneumonia patients

A total of 144 pneumonia patients were recruited from Aintree University Hospital NHS Trust (AUH) and the Royal Liverpool University Hospital (RLUH). The study was approved by Liverpool research ethics committees. (92%) of the cohort subjects had blood cultured in the NHS microbiology laboratories to investigate for a bacterial cause for their pneumonia.. Using these diagnostic modalities, 16/144 (11%) had potentially pathogenic bacteria reported from their samples. (Table 3-1)**Error! Reference source not found.** shows the general medical history of the pneumonia patients groups. The cohort was older, but did not differ from controls in ethnicity. 8 subjects were diagnosed and confirmed with Streptococcus pneumonia. Others include : Diptheroid bacilli, E.coli, Klebsiella pneumonia, Lactococcus lactus, Staphylococcus aureus and dysgalactiae equisimilis represent 0.055%. 128 out of 144 were diagnosed according to their symptoms and chest examination.

2.1.2 Healthy volunteers

47 healthy volunteers were obtained from the Institute of Infection and Global Health, University of Liverpool. All informed consent forms were collected prior collection.

2.1.3 HIV patients

The template DNA samples and more than 70 controls for HLA-G 3' UTR typing were provided from Dr N Kootstra, Dept of Experimental Immunology, University of Amsterdam, Netherlands. This is known as 'The Amsterdam Cohort' (Coutinho, 1998) and 275 serum samples were also obtained from some of the same groups of patients. Healthy donors were obtained from the Institute of Infection and Global Health, University of Liverpool. These were used for stimulating PBMC and measuring the expression of HLA-G on different cells.

2.1.4 Preparation of serum from pneumonia patients and healthy donors

Serum samples were obtained from several different sources. The whole blood was collected using commercially available tubes. After collection of the whole blood, we allowed the blood to clot by leaving it undisturbed at room temperature. The process usually takes 15–30 minutes. The clot was removed via centrifuging at 1,000–2,000 x g for 10 minutes. The samples were maintained at 2–8°C while handling, while the rest were apportioned into 0.5 ml aliquots, stored, and transported at –20°C or

lower. Samples which were hemolyzed, icteric or lipemic can invalidate certain tests and were excluded.

2.1.5 Anti-CMV IgG antibody ELISA

The anti-human-cytomegalovirus IgG ELISA (Product Number: 40-521-475073, GenWay Biotech, Inc. USA) was used following the manufacturer's protocol for the detection of IgG antibody to Cytomegalovirus (CMV) in healthy participants and pneumonia patients' serum. All reagents were allowed to reach room temperature (18-25ºC) before use.

Sample Dilution

Before assaying, all samples should be diluted 1+100 with IgG Sample Diluent. Dispense 10µl sample and 1ml IgG Sample Diluent into tubes to obtain a 1+100 dilution and thoroughly mix with a Vortex.

Procedure

1- Add 100 µl of negative control, positive control, calibrator, and samples (1:21 dilution) to coated strips after proper dilution.
2- The plate was incubated for 20-30 minutes at room temperature.
3- Wash wells with 300 µl of 1X wash buffer three times.
4- Dispense 100 µl of enzyme conjugate to each well and incubate the plate for 20 minutes at room temperature.
5- Repeat step 3 and remove liquid from all wells

6- Pipette 100 µl of TMB substrate into each well and incubate for 10 minutes at room temperature.
7- Add 100 µl of the stop solution.
8- Using a microwell reader, read the read O.D. at 450 nm within 15 minutes

Samples with measurements exceeding the OD of the cut off value of 0.613 were considered as positive for CMV, while those with an OD below the negative cut off value of 0.502 were considered as CMV negative, according to the manufacturer's instructions.

2.2 Measuring Levels of Soluble HLA-G (sHLA-G)

A BioVendor (Oxford Biosystems Ltd. UK) sHLA-G ELISA kit was used for the detection of soluble forms of human leucocyte antigen-G (sHLA-G) in serum samples from patients and healthy subjects. All preparations were made according to the manufacturer's instructions. A total of 47 samples from healthy donors, pneumonia and HIV patients were included to evaluate the soluble forms of s-HLA-G. Serum samples from healthy subject and patients were tested for sHLA-G content using an ELISA. According to the provider's protocol, the steps below were followed. The reagents were prepared fresh at the assay time and all reagents were brought to RT prior to use.

Reagent preparation

- Add 625µl dH_2O to the lyophilised powder vial of Master calibrator prior use, vortexed and left for 15 min at RT.
- Serial dilutions at: 125, 62.5, 31.25, 15.63 and 7.81 Unit/ml in 6 Eppendorf tubes were prepared.
- Dispense 1 volume of Conjugated solution 100X with 99 volumes of conjugate diluent buffer.
- Add 1 volume of wash Buffer 10X to 9 parts of dH_2O.

2.3 Sample preparation

Serum samples involved in the assay were diluted 8x with dilution buffer 2 provided in the kit for duplicates.

Procedure

1- Pipette 100µl of the controls, diluted serum and buffer (=blank) into the corresponding wells.
2- Without shaking, seal the plate incubate the plate at 2-8ºC for 16-20 hours,
3- Wash the plate 5 times with washing buffer using an ELISA washer (Dynex ELISA Washer, Dynex Technologies).
4- Following the last wash and until no residual fluid is observed in the wells, dry off the wells by tapping on tissue paper thoroughly.
5- Add 100µl prepared conjugate solution to all wells.

6- Leave the plate on an orbital shaker at 300 rpm for 60 minutes at RT.

7- Repeat step 3.

8- Pipette 100µl substrate solution (ready to use) into each well,

9- Incubate the plate in the dark for 25 min at RT.

10- Dispense 100µl of stop solution into all wells and read within 5 min using an ELISA reader (Thermo Electron Corporation) at 450nm and 630nm

11- Deduct the reading at 630nm from the reading at 450nm.

12- Subtract the blank value from the remaining measurements after calculating the average reading of all wells.

13- Create a standard curve using plotting the absorbance values against the standard concentrations on an Excel sheet and choose a polynomial curve or the best fit curve, an equation will be generated which facilitates the estimation of the concentration of unknown samples in Units/ml. The final values of the samples must be multiplied by the dilution factor as they were diluted in the sample preparation step.

2.4 Cellular work

Peripheral Blood Mononuclear cell isolation under careful and standard aseptic conditions, fresh blood (20-40ml) was drawn from each donor and aliquoted slowly into 50ml heparin sterile Falcon tubes (Fisher Scientific Inc. USA).

A biosafety cabinet II (MAT-2 CLASS II, Thermo Fisher Scientific Inc. USA) was used for preparation and processing of PBMCs as well as cell culturing. Blood was carefully layered over 1/3 volume of Ficoll Paque Plus solution (Sigma-Aldrich Ltd. UK) with minimal disturbance of the Ficoll. The tubes were centrifuged (Sorvall, Thermo Fisher Scientific Inc. USA) at 400×g (1800rpm) for 30–40 minutes at 20 °C without brake. The mononuclear cell layer was gently transferred to a new 50 mL conical tube before filling and mixing with buffer (phosphate-buffered saline (PBS), the conical tube was then centrifuged at 300×g for 10 minutes at 20 °C. The supernatant was completely removed. For the removal of platelets, the cell pellet was resuspended in 50 mL of buffer and centrifuged at 200×g for 10 minutes at 20 °C. The supernatant was completely removed. This step usually increases the purity of the mononuclear cells. The step was repeated to remove platelets which may remain in the supernatant upon centrifugation at 200×g. After that the cells of the pellet were counted and re-suspended in culture media with complete RPMI 1640 medium plus 2mM L-glutamine (with 10% FBS and 1% Penicillin-Streptomycin/ Sigma-Aldrich Inc. UK) and adjusted to 10^6 cells/ml concentration.

2.5 Cell counting and culture

A light microscope with haemocytometer were used to count the cells in 10µl of mononuclear cell suspension. RPMI medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10% FCS, 50 u/ml Penicillin (P) was

added to culture cells. The main incubator with 5% CO_2 atmosphere at 37ºC was used to incubate all cultured cells.

2.6 Stimulating PBMC with D39 antigen

The concentration of cells was adjusted to $1x10^6$ cells/ml in growth medium. The stimulation of PBMC cells was made using an extract of the *Streptococcus pneumonia* strain D39. The antigen was provided by Dr. Aimee Taylor (CIMI, IGH). Basically, the strain was produced by culturing nasopharyngeal swabs on blood agar (BA) plates. The density of strain load was measured semiquantitively following the methods described in (White et al., 2003). Cells were stimulated for 7 days with or without D39 antigen before analysing for HLA-G expression by flow cytometry.

2.7 Stimulating PBMC with HIV antigen

HIV antigen (CXCR4 tropic - LAI) was provided by Prof. Paxton's group. The LAI antigen originally is a chimeric construct in which two plasmids (p8.19 and pCSFLW) were used as backbone (L McKay, PhD student, University of Liverpool).The construct was used to infect cells and the supernatant containing the virus was taken. The supernatant finally were scaled harvested to different production volumes. The particles express the MERS-CoV spike protein and efficient knock-down of pseudotype virus entry using a polyclonal antibody directed against the spike glycoprotein.

PBMCs at a concentration of 1 x 10^6/ml were cultured with and without HIV construct fo 7 days in growth medium. The mean proportion of cells expressing HLA-G on PBMCs was observed during the stimulation period to measure the expression of HLA-G on NK cells, T cells, B cells and monocytes cells .

2.7.1 Phenotypic analysis

Antibody labelling

PBMCs were extracted at day 7 and following culture with Pneumonia or HIV antigens were phenotyped with a panel of monoclonal antibodies (mAbs) corresponding to the antigens expressed by defined groups of cells. The Abs (Table 2-2) were selected on the bases of optimal matching of four fluorochromes (FITC, PE, PerCPCy5.5 and APC) that can suit the BD Accuri C6 flow cytometer (BD Biosciences, Oxford); the main populations tested were NK cells, T cells, B cells and monocytes (Table 2-1).

The labelling protocol was applied according to the following technique:

1. Aliquot 400µl of cell suspension in 7 eppendorf Tubes 1.5 mL (Starlab, UK).
2. Dispense 4µl of anti-HLA-G Monoclonal Ab into tubes 1-5.
3. Dispense 4µl of anti-human CD3 FITC to tubes 1, 2 and 5.
4. Pipette 4µl of anti-human CD4 PE, anti-human-CD8 PE, anti-human CD19 PE, anti-human CD14 PE and anti-human CD56 PE into tubes 1, 2, 3, 4 and 5 respectively.

5. Add 4µl mouse IgG1 isotype APC into tube 6.
6. Leave tube 7 without staining as negative control.
7. Following incubation at 4° C for 30 min, wash the tubes with 2 ml PBS and centrifuge at 500g for 10 min.
8. Re-suspend the supernatant with 500µl PBS.
9. Use the Accuri C6 flow cytometer to acquire the cells.
10. Adjust the scales of the acquisition plot and gate the lymphocytes according to their scatter properties. For analysis of monocytes, the appropriate gate was used.

Table 2-1 Monoclonal Abs used for staining for HLA-G phenotyping

Tube	Cell type	Markers	Label
1	T helper	CD3+CD4+HLA-G	FITC+PE+PerCPCy5.5+APC
2	T cytotoxic	CD3+CD8+HLA-G	FITC+PE+PerCPCy5.5+APC
3	B cell	CD19+HLA-G	PE+APC
4	Monocyte	CD14+HLA-DR+HLA-G	PE+FITC+APC
5	NK cell	CD3+CD56+HLA-G	FITC+PE+APC
6	Isotype	Mouse IgG1 -	APC
7	Negative	-	-

Table 2-2 Monoclonal antibodies

Monoclonal antibody	Ab clone	Code #	Supplier
Anti-CD3-FITC	OKT3 IgG2a	317306	Biolegend, London, UK
Anti-CD4-PE	OKT4 IgG2b	317410	Biolegend, London, UK
Anti-CD8-PE	6F10 IgG1	9012-0087	eBioscience, Hatfield, UK
Anti-CD14-PE	63D3 IgG1	12-0149-42	eBioscience, Hatfield, UK
Anti-CD16-FITC	3G8 IgG1	11-0168-42	eBioscience, Hatfield, UK
Anti-CD19-PE	1D3/CD19 IgG1	12-0198-42	eBioscience, Hatfield, UK
Anti-HLA-G-APC*	MEM-G/9 IgG1	A15708	Life Technologies, Paisley,
Anti-CD56-PE	5.1H11 IgG1	12-0567-42	eBioscience, Hatfield, UK
Anti-CD69-PerCP/Cy5.5	FN50 IgG1	310926	Biolegend, London, UK
Anti-HLA-DR-FITC	L243 IgG2b	327006	Biolegend, London, UK
Anti-KIR2DL4-PE	mAb 33 IgG1	347006	Biolegend, London, UK
Isotype control-PE	27-35 IgG2b	ab91532	AbCam, Cambridge, UK
Isotype control-APC	27-35 IgG1	MG105	Life Technologies, Paisley,

Table 2-3 Monoclonal Ab staining for KIR2DL4

KIR2DL4 Staining			
Tube	**Cell type**	**Markers**	**Label**
1	NK Cell	CD3+CD16+CD56 +KIR2DL4	PerCPCy5.5+FITC+APC+PE
2	Isotype	Mouse IgG1 -	APC
3	Negative	-	-

2.8 Flow cytometry

Following the labelling steps, the Accuri C6 flow cytometer was used to assess cell labelling. The machine setting can determine the required cell number and type of population based on the forward and side scatter pattern that is yielded from cell acquisition. The gates were presented on the main plot and data were generated on another plot revealing the selected sets of cells according to the colours that have been allocated for each cell.

The machine allows data compensation according to colours used through real-time acquisition or later after acquisition was completed. The software provided by the company (Accuri C6 Plus software, BD Biosciences Inc. USA) was used to analyse the data generated from the machine. Both lymphocytes and monocytes were gated on the scatter graph. Those gates were then plotted to new graphs to be further refined on the scale of cell labelling that can define each group of cells. The isotype and the negative control values were used to determine and subtract from the test sample to give the final percentage of positive cells.

2.9 Flow cytometric staining of HLA-G

Cells were aliquoted in flow cytometry tubes. Up to four different antibodies conjugated with FITC, PE, PerCPCy5.5 or APC, respectively, were used. To evaluate the HLA-G expression on different cells, the cells were stained with 3µl of anti-human CD3, HLA-DR- FITC, anti-human CD4, 8, 14, 16, 19, 56- PE and HLA-G-APC respectively (Table 2-1).

Isotypes APC Mouse IgG2a, κ Isotype Ctrl (FC) Antibody was used. After adding the appropriate antibodies, all tubes were incubated in the dark at 4^{O} C for 30 minutes. The cells then were washed with PBS and centrifuged for 10 minutes at 400g at 4^{O} C. The cells were resuspended in 0.3-0.5 ml of PBS prior analysing them in a BD Accuri C6 Flow Cytometer. The cflow software was used to gate the lymphocyte and monocyte populations. The side scatter (SSC) and forward scatter (FSC) and fluorescence (FL1,2,3 and 4) were used for the quantification of specific cells subsets.

2.10 Molecular analysis

2.11 DNA extraction from healthy donors for standard PCR

A Nucleon kit for the extraction of genomic DNA from larger blood samples was used for 47 healthy volunteers. 3-10 ml of Sodium EDTA blood tubes were collected previously and stored at -20°C. 4 times the volume of Reagent A was added using an aseptic procedure. At room temperature and for 4 minutes the mixtures were rotated then centrifuged at 1300 g for 5 minutes. The supernatant was discarded. 2 ml of Reagent B was added to the pellet mixed briefly to resuspend the pellet. The suspension was transferred to a 15 ml screw capped polypropylene centrifuge tube. 500 µl of sodium perchlorate solution was then added and mixed by hand, through inverting the capped tube at least 7 times. 2 ml of chloroform was added, mixed by hand and the capped tube inverted at least 7 times. Without remixing the phases, 300 µl of Nucleon resin was

added then centrifuged at 1300 g for 3 minutes. Holding the tube vertically without disturbing the Nucleon resin layer (brown in colour), the upper phase (approximately 2.5 ml) was transferred to a clean tube of minimum volume 7.5 ml. 2 volumes of cold absolute ethanol was added and mixed by inversion until the precipitate appeared. The tubes were then centrifuged at top speed (minimum 4000 g) for 5 minutes to pellet the DNA. The supernatant was discarded and 2 ml cold 70% ethanol was added. The tubes were re-centrifuged and the supernatant was discarded. The pellet was air dried for 10 minutes and all the ethanol was removed. The final step involved re-dissolving the DNA in an appropriate volume of water or TE buffer (e.g.1.0–2.0 ml).

2.12 DNA extraction from stored blood samples for standard PCR

Frozen blood samples from all 144 pneumonia patients were submitted to DNA extraction using a QIAamp® DNA Mini Kit (Qiagen Inc. Germany).

Reagent preparation

As instructed in the leaflet provided, required amount of absolute Ethanol was added to Buffer AW1 and AW2 (Sigma Aldrich, Poole, Dorset) as indicated on each bottle. Before proceeding with the protocol steps, all the kit components were brought to RT.

Procedure

1. Add 20µl of Proteinase K provided into an Eppendorf tube.

2. Dispense 200µl of whole blood /200ml PBS.

3. Aliquot 200µl reagent AL, vortex thoroughly.

4. Incubate the tube at 56º C for 10min.

5. After that, load absolute ethanol 200µl to the Eppendorf tube and vortex again, centrifuge the tube to remove any residual attached.

6. Use a new QiaAmp spin column (provided) to load the content of the Eppendorf tube, and centrifuge at 8000 rpm for 1 minute.

7. Place the spin column into a new collection tube.

8. From the AW1 Buffer Add 500µl into the cartridge column, and centrifuge for 1 minute at 8000 rpm.

9. Repeat step 7.

10. Dispense 500µl AW2 reagent into the spin column, and centrifuge for 3 minutes at 14000 rpm.

11. Repeat step 7.

12. To dry the cartridge out of AW2 reagent, spin the column another 60 seconds.

13. Repeat step 7.

14. Add up to 200µl of reagent AE to the cartridge.

15. Leave the tube to sit for 1 min and centrifuge for 1 minute at 8000 rpm.

16. Quantify the harvested DNA using Nano Drop (Thermo Fisher Scientific) and store the sample at 4^{O} C for short term or at -20^{O} C for longer term.

2.13 HLA-G 3'UTR 14-bp Del/Ins gene polymorphism

The DNA samples produced from the extraction procedure for both healthy participants in addition to archived DNA samples were analysed to establish a link between the 14bp insertion/deletion dimorphism of HLA-G and pneumonia. The 14bp insertion/deletion dimorphism of HLA-G in exon 8 of those specimens were analysed. In short, the primers were designated via free commercial software (OligoPerfect™ Designer, Thermo Fisher Scientific Inc. Web Tools). Exon 8 was identified and the exact 14bp site was highlighted, after obtaining the standard nucleotide sequence of the HLA-G molecule (www.ensemble.org). Briefly, DNA was amplified with a set of two primers: 5′-GTG ATG GGC TGT TTA AAG TGT CAC C-3′, 5′-GGA AGG AAT GCA GTT CAG CAT GA-3′ respectively (Sigma Aldrich).

The PCR Reaction

The PCR reaction involved the following components; A 100 ng aliquot of DNA, 10mM forward and reverse primers, 8 µl MyTaq Red Mix (MyTaq™ Red Mix, Bioline Inc. UK) and 12 µl of dH_2O. A thermo cycler machine (Techne Thermal Cycler, Keison International Ltd. UK) was used for all tubes and the programme set as follows: Initial denaturation for at 94 for

5 min, followed by 30 cycles of denaturation at 94° C for 30 seconds, annealing at 64° C for 45 seconds, extension for 60 seconds at 72° C and final extension at 72° C for 10 min (Table 2-4/5).

Table 2-4 The master mix components for PCR amplification detection of the 14 bp HLA-G dimorphism.

PCR Components	Volume
DNA (100ng)	1µ
Forward Primer (10Mmol)	1µl
Reverse Primer (10Mmol)	1µl
Red Mix 2X	8µl
PCR water	12µl

Table 2-5 Thermo cycling condition for HLA-G amplification.

Program	Temperature	Time
Initial Denaturation	94° C	5 min
	30 cycles	
Denaturation	94° C	30 second
Annealing	64° C	45 second
Extension	72° C	60 second
Final Extension	72° C	10 min

2.14 Gel electrophoresis

DNA fragments were electrophoresed on 2% agarose gels containing SYBR® Safe. This was achieved by location of the bands using the UV transilluminator that is designed for the purpose. The insertion allele was visualized as a 224-bp band, while the deletion allele was seen as a 210-bp band (Eskandari-Nasab et al., 2013). 8 electrophoresed samples were sequenced for validation.

2.15 Polymorphisms in KIR2DL4

2.16 KIR2DL4 genotyping

The identification of 9 or 10 Adenines in exon 5 of the gene was made by genotyping the HLA-G ligand KIR2DL4. DNA samples from healthy individuals as well as pneumonia patients recruited in the study were genotyped. Using an online source (www.ensemble.org) the standard sequence of KIR2DL4 was obtained.

The two primers (5' TGCCTGGCAACCAAGAAATG '3) and (3' ACAATCAGGCAACGGTCTGT '5) were designed (OligoPerfect™ Designer, Thermo Fisher Scientific Inc. Web Tools) to target the 3' portion of intron 4 all the way down to the 5' end of intron 5. In 0.2ml PCR tubes, a total volume of 25µl PCR reaction was added with the following (100ng DNA, 20mM primers, 8µl 2X ready mix (MyTaq™ Red Mix, Bioline Inc. UK) and 12µl water (Table 2-6). The tubes were set in a thermocycler (Techne Thermal Cycler, Keison International Ltd. UK). The thermocycler was programmed as following; initial denaturation at 95° C for 5 min, then 30 cycles of denaturation at 95° C for 30 seconds, annealing for 40 seconds at 59° C, extension at 68° C for 1 min and a final extension at 68° C for 7 min (Table 2-7).

Table 2-6 The master mix components for KIR2DL4 amplification.

PCR Components	Volume
DNA (100ng)	1µl
Forward Primer (10Mmol)	1µl
Reverse Primer (10Mmol)	1µl
Red Mix 2X	8µl
PCR water	12µl

Table 2-7 Thermo cycling condition for KIR2DL4 amplification.

Program	Temperature	Time
Initial Denaturation	95° C	5 min
	30 cycles	
Denaturation	95° C	30 second
Annealing	59° C	40 second
Extension	68° C	60 second
Final Extension	68° C	7 min

2.17 PCR product purification

The amplified PCR product yields were purified with a PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific) and following the manufacturer's instructions.

Procedure

1. Use sterile PCR water to equilibrate the PCR product to 50µl.

2. Load 4:1 volume of B2 buffer to the PCR products and vortex the mixture well.

3. Add the sample into the collection tube.

4. Centrifuge the tube for 60 seconds at 10000g.

5. Discard the flow through.

6. Dispense 650µl W1 buffer into the column and centrifuge for 1 min at 10000g.

7. Discard the infiltrate and return to the same collection tube.

8. At full speed, centrifuge the column for 2-3 min and discard the collection tube.

9. Put the spin column in a new tube provided and add 50µl E1 buffer to the spin column.

10. Leave the tube to sit for 1 min and centrifuge again at full speed.

11. The recovered DNA can be then stored at 4° C short term or at -20° C for longer term storage.

2.18 Sequencing

All the cleaned purified PCR products were sent off for Sanger sequencing using a ABI Big Dye sequencer (Source Biosciences Inc. UK). The products were packed and coded with an online order. The reverse primer (3' ACACGTGTACTGTGGAAAGTT '5) was utilized for sequencing the DNA samples which was used for the amplification process mentioned earlier. The result was referred electronically and the data were accessed with commercial software to manipulate and organize the data.

SNPs Identification

The sequences obtained were analyzed by observing the 14 bp INS/DEL (rs1704), +3003 C/T (rs1707), +3010 C/G (rs1710), +3027 A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142) and +3196 C/G (rs1610696) polymorphic sites were individually annotated. According to a previous report, haplotype and genotype were identified and assigned. In addition to that, homozygosity was allocated to individuals carrying two copies of the same allele of a particular SNP, whereas heterozygosity was allocated to those carrying two different alleles.

2.19 Scoring the UTR alleles for high or low HLA-G expression

A scoring system was developed to quantify the potential influence of exon 8 SNPs on HLA-G expression. The 14bp DEL, +3142C and +3187G alleles associated with high HLA-G expression were allocated a score of 1 whereas a 0 score was allocated with low HLA-G expression: 14bp ins, +3142G and 3187A. Having two copies of the HLA-G gene permits each patient or healthy subject to be allocated a score of between 0 and 6, indicative of potential for low or high expression.

2.20 RNA extraction

The pellets yielded from stimulated and non-stimulated PBMC isolation were submitted to RNA extraction to assess the relative levels of HLA-G mRNA. The protocol was performed according to the manufacturer's

protocol (PureLink® RNA Mini Kit, Thermo Fisher Scientific) with standard aseptic conditions.

Reagent preparation

Wash reagent II prepared by adding 60 ml of absolute Ethanol and mixed by inverting up and down.

Dispense 10µl 2-Mercaptoethanol (Sigma Aldrich Inc. USA) to 1 ml lysis buffer, vortexed well.

Procedure

1. Centrifuge the cells at 2000g for 5 min and retain the pellet.

2. Add 300µl of lysis buffer with 2-Mercaptoethanol after discarding the supernatant.

3. Vortex thoroughly to lyse the pellets.

4. Aliquot the lysate to a new sterile Eppendorf tube and centrifuge for 120 seconds at 12000g.

5. Load 70% Ethanol (Sigma Aldrich Inc. USA) (one equal volume) to the suspension.

6. Vortex thoroughly.

7. Transfer up to 600µl of the lysate to the spin cartridge with collection tube provided.

8. Centrifuge the cartridge at 12000g for 30 seconds and discard the flow through and the collection tube.

9. Use a new collection tube to insert the column.

10. Add 500µl washing buffer II to the column.

11. Discard the flow through and the collection tube after spinning at 12000g for 30 seconds.

12. Insert the column in a new tube and repeat steps 10 and 11.

13. Transfer the column in a new recovery tube and add 30-100µl RNase free water to the column.

14. Leave the tube at RT for one minute.

15. Spin the column for 2 min to elute the RNA at maximum speed.

16. Store the RNA at either -20° C for short term or at -70° C for longer term use.

2.20.1 cDNA synthesis

The eluted RNA from stimulated and unstimulated PBMCs was quantified using Nano Drop (Thermo Fischer Scientific Inc. USA). The eluted RNA was used in the process of cDNA generation according to the supplier instructions (High Capacity cDNA Reverse Transcription Kits, Applied Biosystems Inc. USA). All steps were carried out on the bench (Table 2.8) with high standard aseptic conditions and minimal contamination risk. The kit components were ready to use.

Procedure

1. Thaw all the kit compartments on ice on ice or freezing rack (StarLab Internationals GmbH. Germany).
2. Prepare a master mix of the reaction in a final volume of 20µl.
3. Use new gloves, sterile 200µl Eppendorf tubes and filter tips throughout the whole steps.
4. Dispense 4µl of RNase free water into the master mix.
5. Load 2µl random primer.
6. Add 2µl RT buffer 10X.
7. Load 1µl 10X dNTP to the reaction.
8. Add 1µl reverse transcriptase.
9. Vortex well.
10. At 200ng/µl, transfer and mix 10µl of the master with 10µl total RNA.
11. Centrifuge thoroughly.
12. The mixtures were placed in a thermo cycler machine (Techne Thermal Cycler, Keison International Ltd. UK) programmed as one cycle at 37° C for 1 hour.
13. Store the tubes at -70 ° C for later qPCR step processing (Table 2.8). The master mix components for cDNA synthesis.

2.21 Real-Time PCR and HLA-G gene expression assay

The qPCR experiment was designed to evaluate HLA-G gene expression by stimulated and non-stimulated pBMCs induction at day 7 of the

experiment. The probes (Assay ID: Hs00365950_g1, Applied Biosystems Inc. USA) were designed to amplify exon 5 and 6 on chromosome 6 short arm of HLA-G region. The master mix (TaqMan® Universal Master Mix II, with UNG, Applied Biosystems) and the control positive housekeeping gene which was an appropriate control for blood samples (Assay ID: Hs01087168_m1, Applied Biosystems) were ordered from the same supplier.

The number of assays were set to cover a duplicate sample. The cDNA from the stimulated and non-stimulated, prepared master mix and the probes were added to a reaction plate in a final volume of 25µl per reaction. The experiment was carried out with a standard aseptic technique and spraying benches with bleach and changing gloves when possible.

Procedure

1. Dilute cDNA (from stimulated and non-stimulated PBMCs) 1:4 with RNase free water and vortex.
2. Add 11.25µl cDNA from stimulated PBMCs into wells 1-4.
3. Load 11.25µl cDNA from non-stimulated PBMCs into wells 5-8.
4. Use a sterile 200µl Eppendorf tube containing [1.25µl HLA-G probe and 12.5µl 2X TaqMan Universal Master Mix II] X4 to prepare a master mix of the qPCR reaction.
5. Prepare another master for the housekeeping gene probe following step 5.

6. Mix well.
7. In wells 1, 2, 5 and 6, pipette 13.75µl HLA-G master mix.
8. Dispense 13.75µl of Housekeeping gene mixture mix in wells 3, 4, 7 and 8.
9. Centrifuge briefly.
10. Load the plate into the programed qPCR machine (Applied Biosystems 7300 PCR system) with thermo cycling condition (the first stage at 50° C for 2 min, the second stage consists of 50 cycles at 95° C for 10 min and 95° C for 15 seconds followed by a third stage at 60° C for 2 min), label each well with the probe used and adjust the cycle number to 40-50.
11. The machine will automatically calculate the time and signal graphs for the amplification process in real time for each of the test wells.

2.22 Statistical analysis

The results of this thesis were analysed with Graph Pad Prism version 5 and IMB SPSS Statistics version 23, most of the comparison between groups were achieved with Paired and Unpaired t tests, one-way ANOVA, linear regression and logistic regression.

3 RESULTS : HLA-G IN PNEUMONIA PATIENTS

3.1 HLA-G in Pneumonia patients in relation to Infectious agents

The non-classical MHC class I molecule, HLA-G has been reported to be abundantly expressed in foetal trophoblast under physiological conditions (Kovats et al., 1990). The deletion/insertion (DEL/INS) dimorphism of 14 base pairs (14bp) polymorphism in the 3′-untranslated region of the HLA-G gene has been linked with the stability of HLA-G mRNA and HLA-G protein expression, with the DEL allele giving rise to higher levels of expression. The expression of HLA-G receptor was shown to be upregulated in various conditions including recurrent abortions, cancer, autoimmune and inflammatory diseases, and viral and parasitic infections (Genre et al., 2016a). Due to its tolerogenic properties the current study was designed to test whether the 14bp DEL/INS dimorphism is associated with susceptibility to pneumonia.

3.1.1 The Immune Risk Phenotype

The Immune Risk Phenotype (IRP) is a decline in cell-mediated immunity, particularly with respect to T cell function. The IRP predicts shorter survival in elderly patients with lung infections, but has not been evaluated as a possible risk factor in relation to CMV infection (Plonquet et al., 2011, Ndumbi et al., 2015). Our aim was to further explore the association of HLA-G polymorphism with susceptibility to pneumonia in relation to patient CMV serostatus.

3.2 Patient cohort description and assessment of CMV status

The anti-CMV immunoglobulin G (IgG) status in 144 patients compared to 47 controls was identified via one of the key immune methods used for CMV antibody detection: enzyme-linked immunosorbent assay (ELISA). The kit used to screen CMV IgG was GenWay Cytomegalovirus IgG ELISA Kit. This kit enables quantitative detection of anti-CMV IgG as readings exceeding the OD of the cut off value 0.6 were positive, while any OD below the negative cut off value 0.5 were negative. 3 samples with ambiguous results of the serological test, i.e., with IgG concentration 0.5–0.6 IU/ml were excluded from the analyses.

Table 3-1 Characteristics of pneumonia patients and healthy controls.

	Patients (n = 144)	**Controls (n = 47)**
Mean age	64.2	39.1
Gender M/F	75/69	28/19
CMV+/CMV-	105/39 (72.9% +ve)	29/18 (65.9% +ve)
***S pneumoniae*+**	8/144	
Viral pneumonia	0/144	
Diptheroid bacilli	1/144	
E.coli	2/144	
Klebsiella pneumoniae	2/144	
Lactococcus lactus	1/144	
Staphylococcus aureus	1/144	
Streptococcus dysgalactiae	1/144	
Undiagnosed	128/144	

3.2.1 CMV status in pneumonia patients compared to controls

A GenWay Cytomegalovirus (CMV) IgG ELISA Kit was used to distinguish CMV+ from CMV- patients and normal subjects (Table 3.1). Healthy subjects and pneumonia patients were screened with anti-CMV IgG ELISA. Readings exceeding the OD of the cut off value 0.6 were positive, while any OD below the negative cut off value 0.5 were negative. The data shows no significant difference between pneumonia patients and healthy controls as shown in (Table 3-2). It suggests no association of patient CMV serostatus with susceptibility to pneumonia (Figure 3-1).

Table 3-2 Frequency of anti-human-cytomegalovirus IgG in healthy subjects and *S pneumoniae*+ pneumonia patients.

	Subjects No.	CMV status	Frequency
Control	29	CMV+	0.61
	18	CMV-	0.38
S Patients	4	CMV+	0.73
	2	CMV-	0.26
Pneumonia patients	103	CMV+	0.72
	41	CMV-	0.28

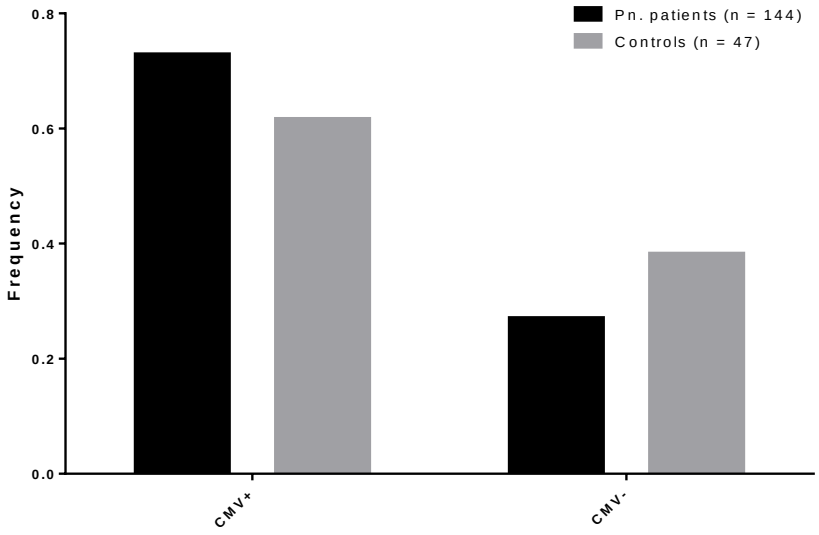

Figure 3-1 CMV status in pneumonia patients and controls.

The figure represents the frequency of anti- postive CMV immunoglobulin G (IgG) antibody status in 144 pneumonia patients and 47 control sera. There were no statistically significant differences between the groups.

3.2.2 CMV status in *S pneumoniae* patients compared with controls.

Six samples from patients with confirmed *S pneumoniae*+ pneumonia were selected to assess the presence of CMV by means of the standard ELISA. CMV IgG were detected in 4 of 6 (73%) samples and absence in 2 of 6 (26%) *S pneumoniae*+ samples as presented in (Figure 3-2).

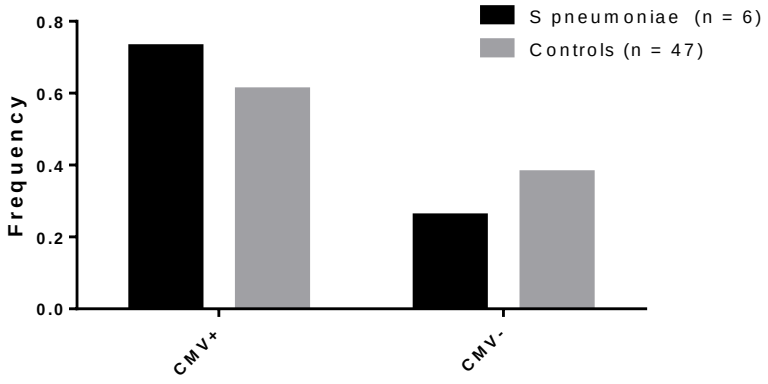

Figure 3-2 Comparison of CMV status in *S pneumoniae*+ pneumonia patients with controls.

The figure represents the frequency of anti-negative CMV immunoglobulin G (IgG) antibody status in *Streptococcus pneumoniae* patients and 47 control sera. There were no statistically significant differences between the groups.

Even though the difference was not significant, there was a higher incidence of CMV IgG in the *S pneumoniae* positive samples compared to control sera. Following that, the genotyping of HLA-G was tested for any correlation between the 14bp insertion/deletion dimorphism and susceptibility to pneumonia following assessing CMV IgG status.

3.2.3 The 14bp insertion/deletion polymorphism in exon 8 of HLA-G

The occurrence of the 14bp insertion/deletion polymorphism in exon 8 of HLA-G were evaluated to establish any correlation with pneumonia patients' outcome. Genomic DNA was extracted from peripheral blood mononuclear cells using a Nucleon kit (Figure 3-3).

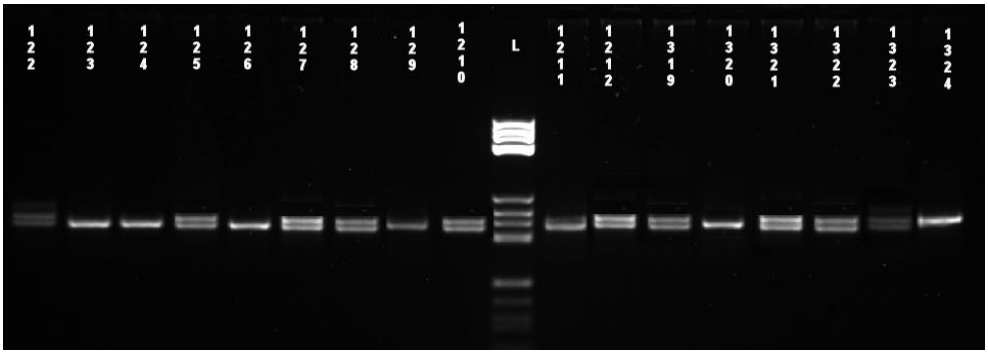

Figure 3-3 Shows the HLA-G 14-bp dimorphism of exon 8.

The L lane represents 50bp DNA ladder. The lanes 122,125,127,128,1210,1212,1319,1321, 1322 and 1323 represent (+/-) heterozygotes, the lanes 123,124,126,129,1211 and1320 represent (-/-) 14bp deletion homozygotes, while lane 1324 illustrates a (+/+) 14bp insertion homozygote.

The deletion/insertion (DEL/INS) HLA-G 14 bp polymorphism was genotyped by polymerase chain reaction. The frequency of (DEL/DEL) homozygotes was higher among the pneumonia patients than healthy controls, although there was no significant difference between pneumonia patients and healthy controls. The frequency of the DEL allele was 0.51 in controls (92) compared to 0.56 in pneumonia patients (288). Both controls and patients show similar INS frequencies with 0.48 and 0.43. No significant differences were noted when a paired t test was applied (two tails), P value >0.05; (Table 3-3/4). The data reveals the allele frequencies of HLA-G 14bp insertion/deletion polymorphisms is not correlated to the susceptibility to pneumonia following assessing CMV IgG status.

Table 3-3 Allele frequencies of HLA-G polymorphisms in pneumonia patients and controls

	Del	Ins
Pneumonia patients (144)	164/288 (0.57)	124/288 (0.43)
Controls (47)	48/94 (0.51)	46/94 (0.49)

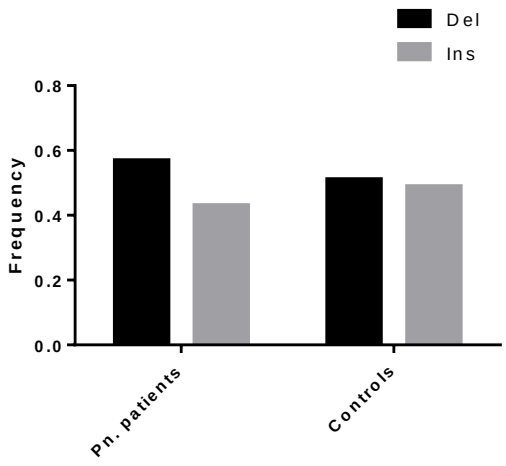

Figure 3-4 Allele frequencies of HLA-G polymorphisms in pneumonia patients and controls.

The figure represents the allele frequencies of 14-bp insertion/deletion polymorphism of HLA-G gene in pneumonia patients and controls, there were no statistically significant differences between groups.

3.2.4 Genotype frequencies of the 14 bp insertion/deletion polymorphism of HLA-G polymorphisms in pneumonia patients and controls

In this study, 144 pneumonia patients and 47 normal control were analyzed for 14 bp insertion/deletion polymorphism (**Error! Reference source not found.**). The amplified PCR products was either 224 or 210 bp or both in the case of heterozygotes depending on the 14-bp deletion homozygotes, 14-bp insertion homozygotes, or +14-bp /−14-bp

heterozygotes of exon-8. The results showed no significant differences for 14 bp allele and genotype frequencies between patients with pneumonia and controls (see,Table 3-4/5).

Table 3-4 Genotypes and alleles of 14 base pairs (14bp) polymorphism INS/DEL of controls, *Streptococcus pneumoniae* and pneumonia patients.

Genotype	Control (47)	*Streptococcus pneumoniae* (6)	pPneumonia patients (144)
Ins/ Ins	14 (0.30)	4 (0.67)	23 (0.15)
Del/ Del	15 (0.32)	0 (0.00)	43 (0.29)
INS/DEL	18 (0.38)	2 (0.33)	78 (0.54)
Alleles	Control (94)	*Streptococcus pneumoniae* (12)	Pneumonia patients (288)
Del	48 (0.51)	2 (0.17)	124 (0.43)
Ins	46 (0.49)	10 (0.83)	164 (0.57)

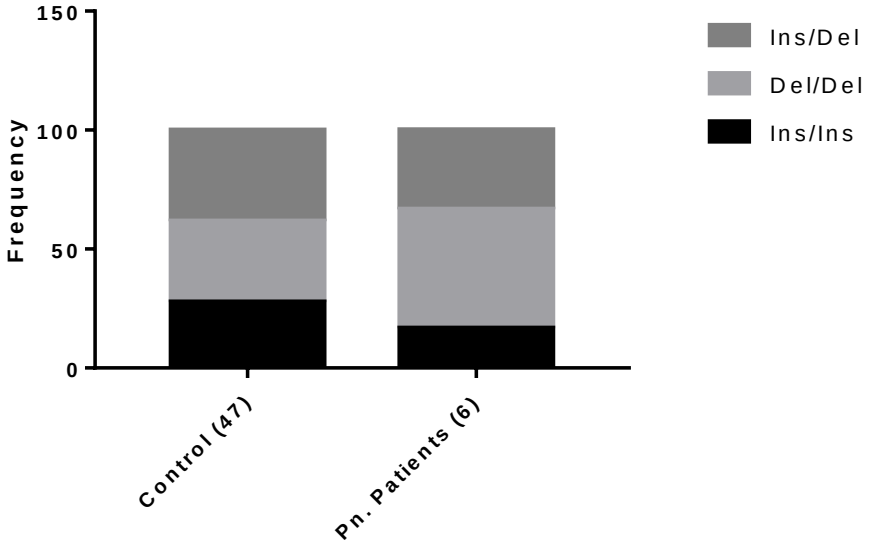

Figure 3-5 The HLA-G exon 8 14bp deletion/insertion (insertion (+) and deletion (-)) genotypes in pneumonia patients and controls.

The frequency of homozygous -/- genotype (50 %) was the highest among the control and pneumonia patients. There were clearly differences of homozygous -/- genotype between both healthy and patient's groups. The homozygous -/- genotype in healthy was higher than patients. But both are not significant using Chi-square test (P value >0.05).

There were more pneumonia patients who were homozygous -/- genotype than among the controls; however, the frequency of the +14-bp insertion allele was higher in control group as compared to the patients. When taken together, the data here suggested to us that there was no a detectable relationship between susceptibility to pneumonia and the presence of a +14-bp insertion allele in the HLA-G locus.

3.2.5 HLA-G 14bp INS/DEL polymorphism in pneumonia patients in relation to CMV status.

Differences in the HLA-G 14 bp genotype and allele frequencies between S pneumoniae and patients compared to controls were also tested. In this study, no significant difference in allele frequencies of the *HLA-G* 14 bp insertion/deletion polymorphism was observed between the two groups (

Figure 3-6/7/8). However, an increased frequency of HLA-G DEL/DEL 14 bp homozygotes was seen in hCMV-infected pneumoniae patients compared to control, p value < 0.05 as shown in (Figure 3-9). The DEL/DEL 14 bp genotype is associated with higher expression of HLA-G and may express higher levels of cell surface or soluble HLA-G, suppressing the immune response to S. pneumoniae.

Table 3-5 The occurrence of the 14bp HLA-G exon 8 insertion (+) and deletion (-) in healthy CMV+ and CMV- subjects. Differences were not statistically significant (p=0.49).

		Ins	Del	INS/DEL
Control (18)	CMV -	27.59	34.48	37.93
Patients(2)	CMV -	50	0	50

Control (29)	CMV+	22.22	38.89	38.89
Patients(4)	CMV+	0	75	25

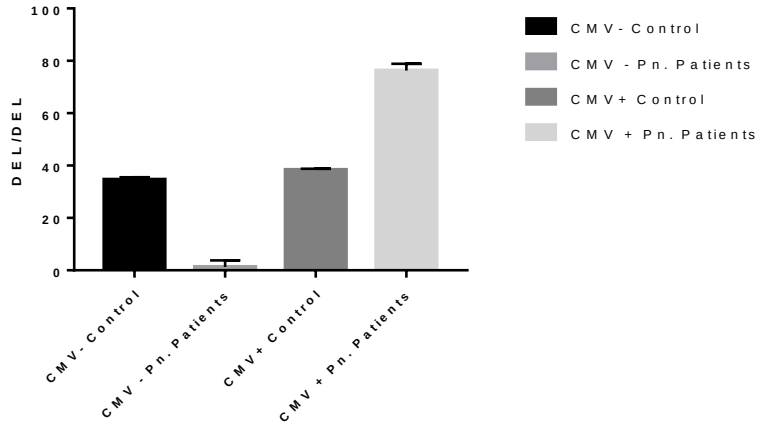

Figure 3-6 Frequencies of the 14 bp deletion /deletion polymorphism of HLA-G gene in hCMV-infected pneumoniae patients and normal controls.

Ddeletion (-) in patients and controls in relation to anti-human-cytomegalovirus IgG. The frequency of deletion// deletion 14 bp homozygotes genotype in controls shows no significant differences using the Chi-square (p value > 0.05).

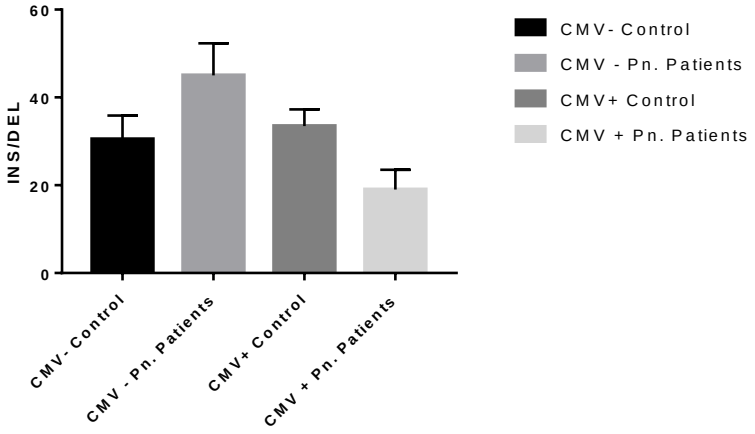

Figure 3-7 Frequencies of the 14 bp insertion/deletion polymorphism of HLA-G gene in hCMV-infected pneumoniae patients and normal controls.

Insertion (+) and deletion (-) in patients and controls in relation to anti-human-cytomegalovirus IgG. The frequency of HLA-G deletion/insertion genotype in controls shows no significant differences using the Chi-square (p value > 0.05).

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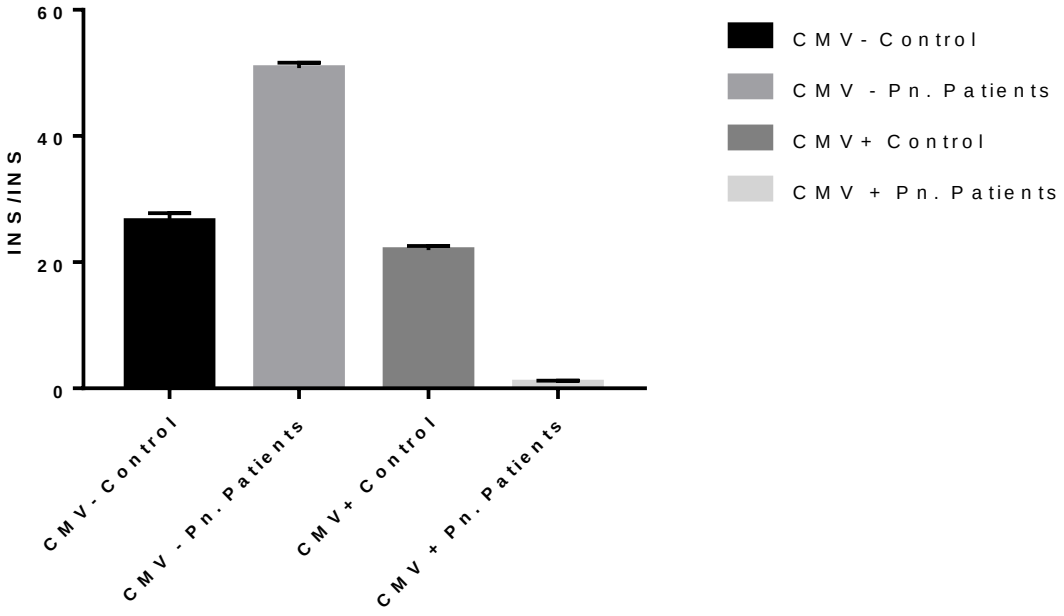

Figure 3-8 Frequencies of the 14 bp insertion/ insertion polymorphism of HLA-G gene in hCMV-infected pneumoniae patients and normal controls.

The frequency of Insertion/ Insertion 14 bp homozygotes genotype in controls shows no significant differences using the Chi-square (p value > 0.05).

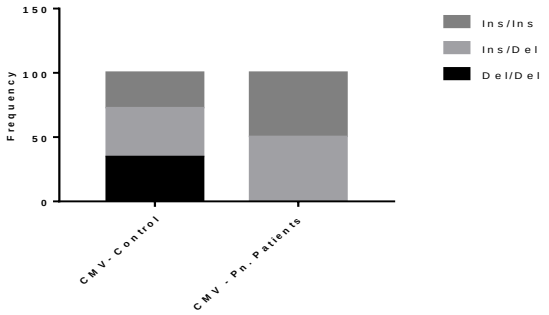

Figure 3-9 Frequencies of the 14 bp insertion/deletion polymorphism of HLA-G gene in CMV- ve S pneumoniae patients and normal controls.

While the homozygous INS/INS genotype was absent in CMV+ patients, No significant association was found between HLA-G DEL/DEL14 bp homozygotes in hCMV-infected pneumoniae+ patients compared to controls, Chi-square (p value > 0.05).

3.2.6 Allele frequencies of HLA-G polymorphisms in pneumonia patients and *Streptococcus pneumoniae*+ patients.

The 14 bp insertion/deletion polymorphism of the HLA-G gene in all pneumonia patients compared to *Streptococcus pneumoniae*+ patients were analysed. An increased frequency of the HLA-G Ins 14 bp allele was found in *Streptococcus pneumoniae*+ patients compared to total patients. The increased frequency of HLA-G DEL/DEL14 bp homozygous and Del/Ins heterozygous was also observed when compared pneumonia and *Streptococcus pneumoniae* patients (Figure 3-10).

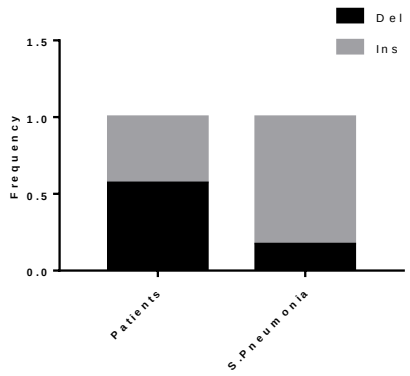

Figure 3-10 Allele frequencies of HLA-G polymorphisms in pneumonia patients and *Streptococcus pneumoniae*+ patients.

No significant association was found between of Allele frequencies of HLA-G Ins 14 bp in the two groups (p value > 0.05)..

However, when the data were analysed according to HLA-G INS/DEL haplotypes, there was no statistically significant difference between the groups (Figure 3-11). Thus, the data suggested to us, in part, that there was no a detectable relationship between susceptibility to pneumonia and the presence of the +14-bp insertion allele in the HLA-G locus.

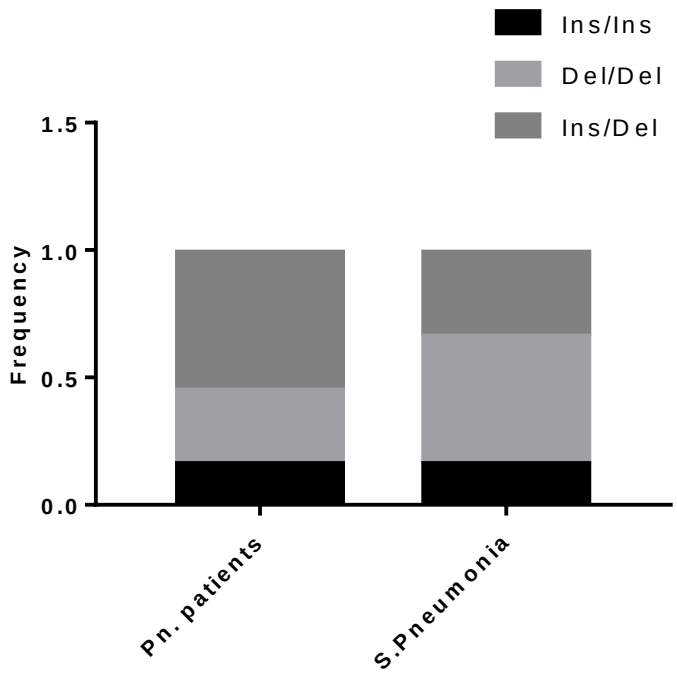

Figure 3-11 Frequencies of the 14 bp insertion/deletion polymorphism of HLA-G gene in pneumonia patients and *Streptococcus pneumoniae*+ patients.

Frequencies of HLA-G 14 bp Del/Ins genotypes in total and *Streptococcus pneumoniae*+ pneumonia patients. No significant association was found between HLA-G 14 bp genotype in the two groups.

3.2.7 Relationship of HLA-G genotype with clinical parameters

The patient data were subdivided into groups according to clinical outcomes and other parameters (age and gender). The comparison of

cytomegalovirus (CMV) serostatus of different groups of patients related to HLA-G genotype shows no statistically significant differences. Next, three outcomes of interest: death, re-admittance and length of hospital stay with the influence of age, sex, CMV status and HLA-G were assessed for any association. With the exception of length of hospital stay, none of these showed any significant association (Table 3-6 The correlation between clinical parameters in pneumonia patients.Table 3-6).

Table 3-6 The correlation between clinical parameters in pneumonia patients.

	Age	**CMV status**	**HLA-G**
Death	.035*	0.69	0.198
Re-admittance	0.469	0.89	0.05*
Length of hospital stay	.023*	.580	.048*

*. Correlation is significant at the P<0.05 level (2-tailed).

3.2.8 Length of stay in hospital with respect to HLA-G genotype

Overall hospitalization was measured from date of sampling to date of death (event) or last follow-up (censoring). The analysis was performed by the Kaplan-Meier method. Three outcomes of interest were assessed: death, re-admittance and length of stay. The influence of the following variables: age, sex, CMV status and HLA-G were considered to explore the length of stay in hospital.

The association of the chosen variables with length of stay was then considered. To use linear regression, a linearity assumption had to be tested with the outcome length of stay (x-axis). When a plot for age (the

only continuous variable) was produced a straight line fit through the points was found so the assumption is valid. A univariate analysis was the performed and showed that HLA-G genotype was significantly associated with length of stay. A multivariable model with all variables included was performed and again HLA-G genotype was found to be significantly associated with length of stay. Finally, stepwise selection was used to determine the best fitting (parsimonious) model. The model just included HLA-G genotype. The result was that HLA-G genotype was significantly associated with length of stay (p=0.025). In addition, the results can be interpreted as follows: patients with the DEL/DEL genotype showing a significantly longer mean stay in hospital (P = 0.025;Figure 3-12). Statistical tests were performed using GraphPad Prism 6.0 software (Graphpad Software, San Diego, CA, USA).

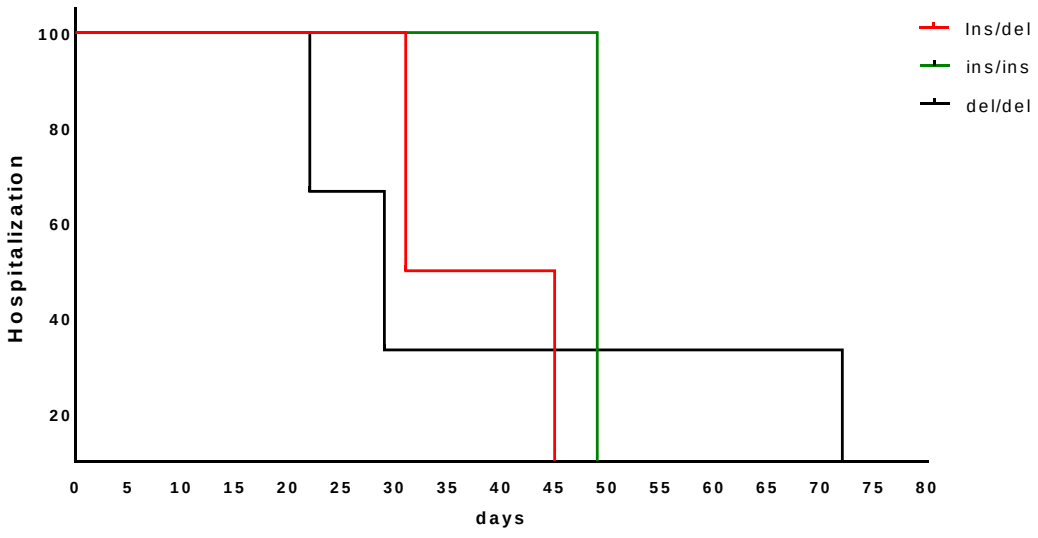

Figure 3-12 Length of stay in hospital with respect to HLA-G genotype.

HLA-G was significantly associated (p=0.025) with the length of stay in hospital. The predicted length of stay in hospital was 3 times higher in the DEL/DEL group than for those

in other groups. There is a slight increase with the frequency of DEL/DEL in pneumonia patients (0.56) but with no significant differences. The frequency of DEL/DEL is 0.51 in controls. The INS/INS frequency for both controls and patients was similar (0.48 and 0.43).

3.3 Stimulation of PBMCs with *Streptococcus pneumoniae* antigen D39

HLA-G expression has been reported to be upregulated in various conditions including virus infections (Lu et al., 2011), and tumours (Mociornita et al., 2013). The tolerogenic properties of this protein has led us to test whether HLA-G could be upregulated on PBMCs from healthy subjects following culture with or without pneumococcal culture supernatant (CCS) derived from wild type S. *pneumoniae* strain D39.

3.3.1 HLA-G expression in PBMC from healthy subjects stimulated with D39 pneumococcal antigens

The proportions of HLA-G+ cells in various PBMC populations stimulated for 7 days of culture with Streptococcus pneumoniae Ag D39 were compared to proportions of HLA-G+ cells in control unstimulated populations. Representative flow cytometry profiles are shown in (Figure 3-13). There were clearly differences between these data but not significant. CD3+CD4+ lymphocytes were revealed to have the highest difference, followed by CD3+CD8+ cells. The remaining subsets of PBMC showed no difference between Day 0 and Day 7. Live cells were gated according to forward and side scatter, having higher forward scatter and lower side scatter than dead cells.

3.3.2 Stimulation of PBMCs with Streptococcus pneumoniae Ag D39

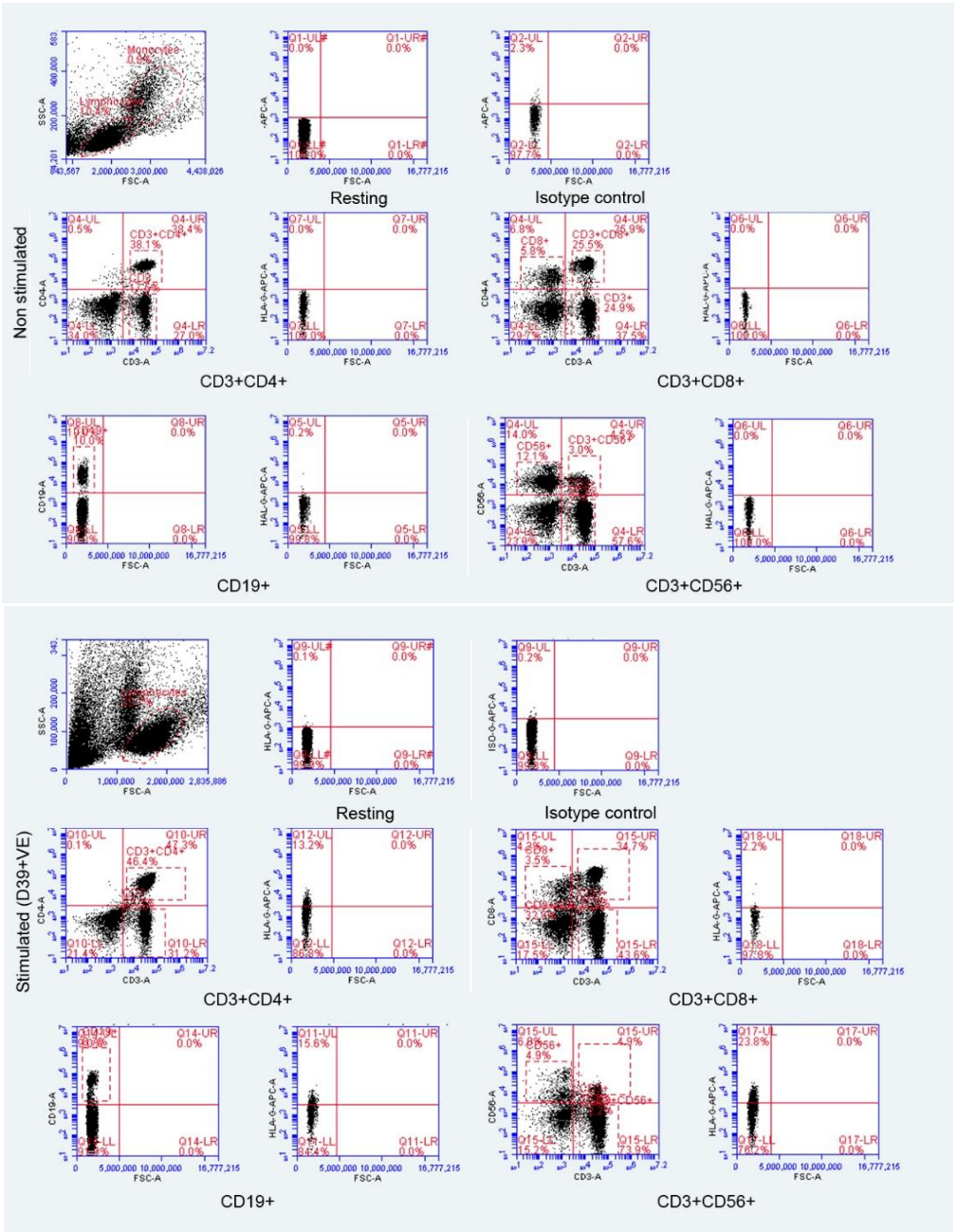

Figure 3-13 Representative dot plots of HLA-G expression on PBMC subsets stimulated with D39 antigen compared to unstimulated controls.

Representative dot plots of Accuri C6 flow cytometry showing staining for lymphocytes and monocytes with anti HLA-G after a week of culture with or without pneumococcal culture supernatant (CCS) derived from wild type strain D39. The cells were gated and analysed as CD3+CD4+, CD4+CD8+, CD19+ and CD3+CD56+ populations using specific antibodies respectively. There was an increase in the proportion of HLA-G expressing cells following the stimulation but not statistically significant using Chi-square test (P value >0.05).

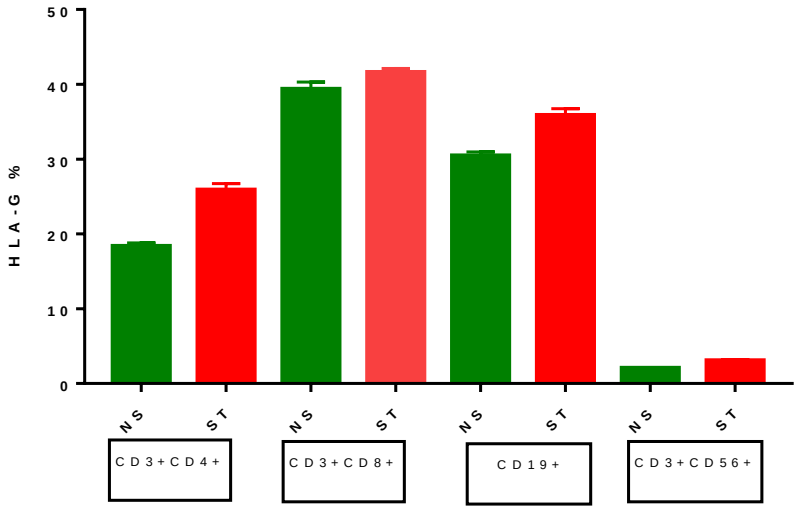

Figure 3-14 Comparison of proportions of HLA-G+ cells for various PBMC subsets non-Stimulated (NS) and stimulated (ST) with *S. pneumoniae* antigens.

PBMCs were treated for 7 days with (fred columns) or without (green columns) *S. pneumoniae* D39 antigens. HLA-G expression was assessed on the surface of a range of subsets of lymphocytes and monocytes separated from healthy donors after a week of culture with or without pneumococcal culture supernatant (CCS) derived from wild type strain D39. Results are expressed as mean percentage +/-sem. CD3+4+ and CD3+8+ cells showed differences but not significant using paired t-test, n = 11.

3.4 Serum levels of soluble HLA-G molecules

The soluble HLA-G (sHLA-G) levels have been reported to be significantly increased in patients with melanoma, neuroblastoma, lymphoproliferative disorders, breast, ovarian and colorectal carcinoma when compared to healthy controls or subjects with benign neoplasms (Cao et al., 2011). The aim of this experiment was to investigate whether or not the serum levels of sHLA-G in CMV+ and CMV- pneumonia patients and healthy controls are different. Serum of CMV+ and CMV- pneumonia patients and healthy controls were assessed with a sHLA-G ELISA kit.

The level of s-HLA-G from CMV+ and CMV- pneumonia patients displayed no significant difference when compared to healthy controls. However, the mean serum sHLA-G titres were generally higher in CMV- than CMV+ patients and normal subjects (Figure 3-15).

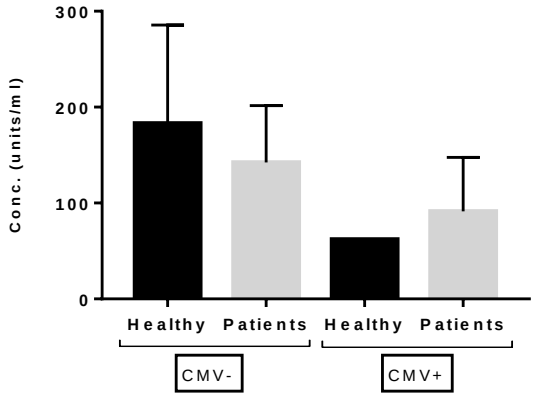

Figure 3-15 The levels of soluble HLA-G molecules (units/ml) in serum from pneumonia patients and healthy controls.

The mean serum sHLA-G average titre was lower in CMV- pneumonia patients compared to control (142.4/183.2) U/ml while in CMV+ individuals, the patients showed a slight increase versus control subjects (91.3/62.1) U/ml. No significant difference was found between the two groups.

The comparison of soluble HLA-G levels between pneumonia patients and healthy controls displayed no significant difference. An extension of the above work was to study variations in one of the ligands for HLA-G, KIR2DL4 and relevant polymorphisms within this ligand. The work aimed to to understand the association between individual genetic and differential expressions of this ligand for HLA-G in relation to diseases in which HLA-G expression is upregulated.

3.5 KIR2DL4 expression on resting lymphocytes in CMV+ subjects

KIR2DL4 is a primary ligand for HLA-G and is expressed mainly by NK cells and a subset of T cells (Rajagopalan et al., 2001). The receptor is known to have potent immunoregulatory behaviour of either inhibiting or stimulating other immune cells (Vilches and Parham, 2002). As a ligand for HLA-G, the role of this protein was evaluated on the basis of CMV status and according to the incidence of 9A and 10A alleles in the KIR2DL4 gene which has been reported to influence its expression in NK cells (Goodridge et al., 2003).

In this part, the expression of KIR2DL4 was assessed on IL-2 cultured NK cells and lymphocytes from PBMCs extracted from 12 healthy subjects of known CMV status. All subjects were labelled with mAbs to measure the level of cell surface KIR2DL4 expressed by resting NK cells and other lymphocytes and after IL-2 culture for 14 days. Sequencing was used to identify the occurrence of 9A and 10A genotype of the KIR2DL4 gene. Following the sequencing, those healthy individuals were classified to 9A and 10A to indicate any relevant influence on surface KIR2DL4 protein expression (Figure 3-16).

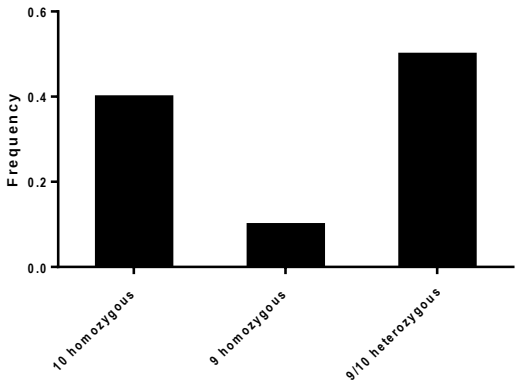

Figure 3-16 Polymorphic nucleotides in exon 6 and intron 6 of KIR2DL4 for 9A and 10A sequence.

This figure shows the occurrence of genotypes of 9A and 10A alleles in samples from 8 normal subjects, (9 homozygous: grey columns,, n = 1) and (9 homozygous: black columns, n =3) (9/10 heterozygous: dark grey columns, n = 4). Results are expressed as frequency, ($P > 0.8795$).

Variations in the main ligand for HLA-G, KIR2DL4, were tested. It was to investigate the association between individual genetic and differential expressions of this ligand for HLA-G in relation to diseases in which HLA-G expression is upregulated. No significant differences in KIR2DL4 genotype were noted in healthy subjects according to CMV status, although sample sizes were small (Table 3-7) .

Table 3-7 Summary of proportions of KIR2DL4 genotypes according to CMV status in healthy subjects.

KIR2DL4 Genotypes	CMV- (3)	CMV+ (5)	Total
10 homozygous	1	2	3
9 homozygous	0	1	1
9/10 heterozygous	2	2	4

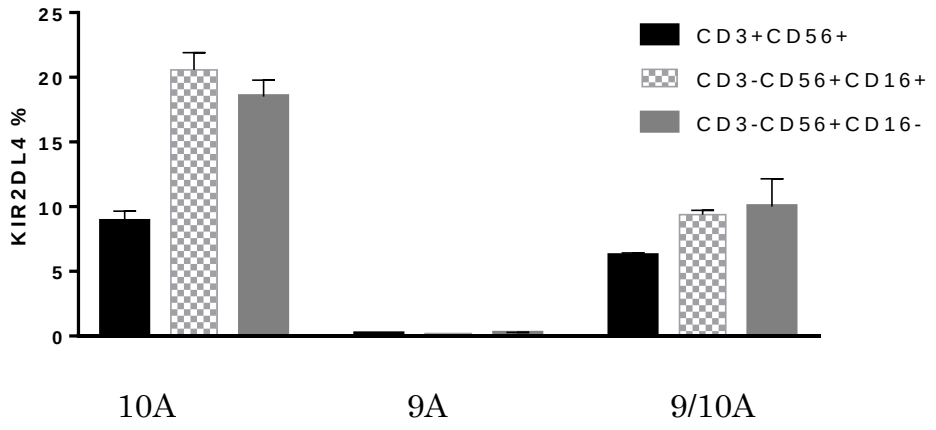

Figure 3-17 The expression of KIR2DL4 on NK cells.

Proportions of NK cells expressing KIR2DL4 according to the occurrence of 9A and 10A alleles in normal subjects samples; n:8. Results are expressed as mean +/- SEM, P>0.05;.

The result indicated no significant differences in proportions of KIR2DL4+ cells between CD16+ and CD16- NK cells and CD56+ T cells for 10A homozygotes and 9A/10A heterozygotes in healthy donors (Figure 3-17). As expected, 9A homozygotes showed almost no detectable cell surface expression, as previously reported (Goodridge et al, 2007). The expansion of the work was to test gene frequencies for the of 9A and 10A KIR2DL4 alleles in healthy donors either negative or positive for CMV.

Table 3-8 Polymorphic nucleotides in exon 6 and intron 6 of KIR2DL4 for 9A and 10A sequence and with different HLA-G 14bp Ins/Del genotypes CMV status. Results are expressed as %, P > 0.8795.

	I/I	D/D	I/D
10 homozygous CMV-	100	0	0
10 homozygous CMV+	25	25	50
9 homozygous CMV-	0	0	0
9 homozygous CMV+	100	0	0
9/10 heterozygous CMV-	0	33.33	66.67
9/10 heterozygous CMV+	0	50	50

There were no significant differences in gene frequencies for the 9A and 10A KIR2DL4 allele frequencies in healthy donors either negative or positive for CMV (Table 3-8). Next, we tested the proportions of cells from healthy subjects expressing KIR2DL4 in CD56+ T cells, and CD16+ CD56dim and CD56bright CD16- NK cells following stimulation with IL-2, a nonspecific activator of T cells and NK cells for 14 days.

3.5.1 Flow cytometric staining of lymphocytes with anti-KIRDL4

The cell surface KIR2DL4 expression was assessed using mAbs and Accuri C6 flow cytometry and the obtained data were analysed with the CFlow Plus software. Proportions of KIR2DL4+ cells were assessed on lymphocyte subsets extracted from 10 subjects. Fresh PBMCs were stained with anti-CD3, CD16, CD56 and KIR2DL4 monoclonal Abs. The CD56+CD3- population consists of both CD56bright and CD16+CD56dim populations. All lymphocyte subsets revealed significant increases in % KIR2DL4+ cells with CD3+CD56+ (P=0.0032) CD16+CD56dim (P=0.0097) and CD56 bright NK cells (CD56+CD16-) (P=0.0016) following IL-2 stimulation (Table 3-9).

Table 3-9 Proportions of lymphocytes with and without IL-2 stimulation staining with anti-KIRDL4 antibody in individual healthy subjects.

CD3+CD56+		CD3-CD56+CD16+		CD3-CD56+CD16-	
NS	ST	NS	ST	NS	ST
7	15.87	6.89	38.41	8.4	23.19
6.64	12.14	6.61	21.01	1.75	20.19
5.39	18.1	6.34	29.81	3.06	21.49
7.64	19.42	13.9	31.84	7.62	28.4
6.17	7.55	6.28	11.06	1.93	7.39
6.68	12.41	10.2	18.05	2.57	12.3
3.03	7.89	4.08	7.05	1.02	6.24
P value	**0.003**		**0.0097**		**0.0016**

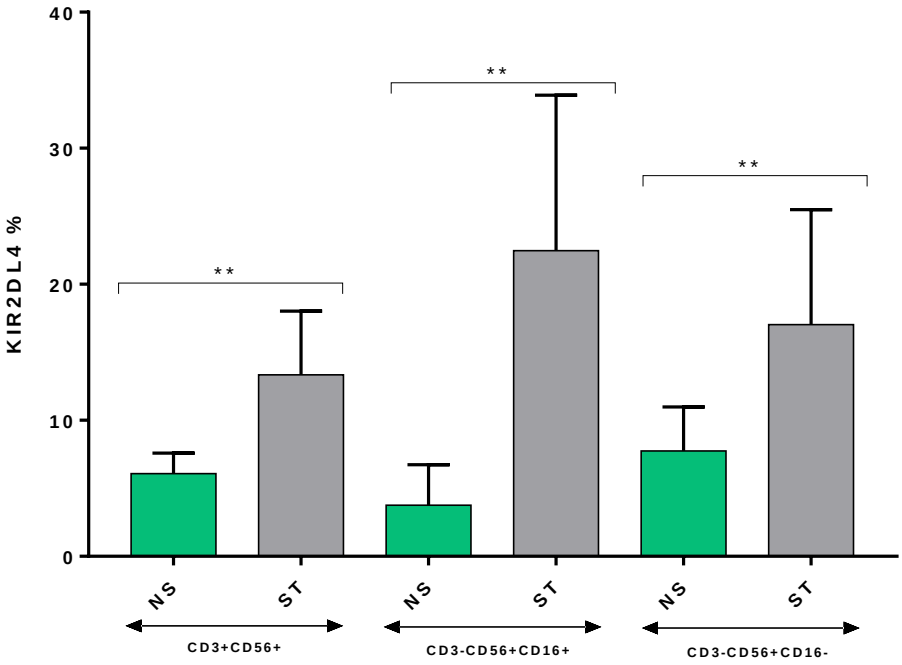

Figure 3-18 Expression of KIRDL4 on CD3+CD56+ and NK cells with (ST) or without (NS) IL-2 stimulation.

The highest differences were observed in regards to the NK cells (CD16+CD56dim). KIR2DL4 expressers of CD56bright NK cells (CD56+CD16-) were lower compared to CD16+CD56dim populations in day 14. All lymphocyte subsets revealed significant differences using a paired t-test with CD3+CD56+ (P=0.0032) CD16+CD56dim (P=0.0097) and CD56bright NK cells (CD56+CD16-) (P=0.0016), results expressed as mean +/-sem, paired t-test, n = 7.

The data have demonstrsated that there was a significant increase in the proportion of cells expressing KIR2DL4 in CD3+CD56+ lymphocytes and NK cell subsets following IL-2 induction (Figure 3-18). The current results have also demonstrated that the expression of KIR2DL4 was influenced by the KIR2DL4 9A and 10A genotype as the receptor was not found on the cell surface in 9A homozygotes (Figure 3-17).

4 RESULTS: HLA-G POLYMORPHISMS IN HIV PATIENTS

In the previous chapter, there were indications that HLA-G expression was increased in some lymphocyte subsets following stimulation with bacterial antigens. In the following chapter, this work was extended to a viral infection of international importance, namely HIV. This virus targets CD4+ T cells and antigen-presenting cells and if untreated leads to a progressive decline in immunocompetence. The virus fails to induce a protective immune response and there is the possibility that induction of HLA-G expression may be one means of achieving this.

The Amsterdam Cohort of patients is a well-studied population of individuals of known clinical features and outcomes. Both DNA and serum samples were obtained from groups of HIV+ men who have sex with men (MSM), intravenous drug users (IDU) and high risk seronegative patients exposed to HIV but without developing disease (HRSN), together with healthy controls. These were subjected to HLA-G genotypic analysis, in relation to well-studied 3'UTR dimorphisms previously associated with alterations in levels of HLA-G gene expression.

4.1 HLA-G 3′ UTR typing in 3 HIV groups.

To perform HLA-G 3′ UTR typing in 3 HIV patient groups, 275 individuals were evaluated from the Amsterdam Cohort: 133 Men who have sex with men (MSM), 50 intravenous drug users (IDU) and 32 high-risk HIV-seronegative (HRSN) and 85 control subjects who yielded data regarding

3'UTR alleles, genotype and haplotype. These included: the seven previously reported (Castelli et al., 2010), HLA-G 3'UTR polymorphic sites [14-bp INS/DEL (rs1704), +3003 C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142) and +3196 C/G (rs1610696)] were examined in the 3 HIV groups of individuals. No other polymorphic sites were examined in this region. The genotype frequencies of the eight HLA-G 3'UTR polymorphic sites observed in the groups are shown in (Table 4.1). Upon performing a Chi-Square test to assess the occurrence of these SNPs among the four groups, the statistical differences between the groups were significant for some of the SNPs. Histograms showing the data for each SNP are presented individually in (Figure 4-1/2/3).

Table 4-1 Genotype frequencies of HLA-G SNPs observed in the HIV patient groups and controls

Group	Cont 85		MSM 133		HRSN 32		IDU 50		P value
Genotype	N	Freq	N	Freq	N	Freq	N	Freq	
3003CC	2	0.01	4	0.03	0	0.00	2	0.04	
3003CT	23	0.27	78	0.59	4	0.13	12	0.24	
3003TT	60	0.71	51	0.38	28	0.88	36	0.72	*0.004
3010GG	26	0.29	20	0.15	3	0.09	15	0.3	**0.001
3010GC	43	0.51	100	0.75	16	0.50	21	0.42	
3010CC	16	0.19	13	0.10	13	0.41	14	0.28	
3027CC	74	0.87	128	0.96	26	0.81	48	0.96	
3027CA	11	0.11	5	0.04	6	0.19	2	0.04	
3027AA	0	0	0	0.00	0	0.00	0	0	**0.001
3035CC	65	0.76	121	0.91	19	0.59	47	0.94	
3035CT	18	0.21	12	0.09	12	0.38	3	0.06	
3035TT	2	0.01	0	0.00	1	0.03	0	0	
3142CC	22	0.26	26	0.20	4	0.13	14	0.28	**0.001
3142CG	46	0.54	100	0.75	16	0.50	23	0.46	**0.001
3142GG	17	0.19	7	0.05	12	0.38	13	0.26	**0.001
3187AA	35	0.41	55	0.41	17	0.53	23	0.46	*0.021
3187AG	42	0.49	71	0.53	13	0.41	23	0.46	**0.001
3187GG	8	0.08	7	0.05	2	0.06	4	0.08	
3196CC	40	0.47	36	0.27	13	0.41	21	0.42	
3196CG	36	0.42	91	0.68	14	0.44	23	0.46	
3196GG	9	0.09	6	0.05	5	0.16	6	0.12	*0.05
14bpII"	15	0.18	6	0.05	11	0.34	7	0.14	**0.001
14bpDD"	26	0.31	23	0.17	5	0.16	19	0.38	**0.001
14bpID"	44	0.51	104	0.78	16	0.50	24	0.48	**0.001

* p value <0.05; **P value <0.001. 14bpI/D=14bp insertion/deletion polymorphism. "II: insertion/ insertion, DD:deletion/ deletion, I/D deletion/ insertion.

Genotype frequencies of the 3' UTR polymorphic sites observed in 'The Amsterdam Cohort' of HIV+ patients, comprising men who have sex with men (MSM), intravenous drug users (IDU), together with high risk HIV seronegative (HRSN) subjects exposed to HIV but remaining HIV negative. 7 different HLA-G 3' UTR haplotypes were observed for the whole group of individuals, and were designated as previously described

(Table 1-1). The differences in genotype frequencies is shown as P value using Chi-square test in (Table 4-1).

Since the expression levels of the HLA-G gene has been reported to be associated with the 14-bp Ins/Del, +3142 C/G and +3187 A/G polymorphic sites, these polymorphic sites were analysed (Figure 4-1/2/3). The presence of variation in the sites associated with high production (14 bp Del/+3142C/+3187G) or with low production (14 bp Ins/ +3142G/ +3187A) in control and three HIV groups were compared. As UTR-5 and UTR-7 contained the 14 bp Ins, +3142G and +3187A alleles, these 3'UTR haplotypes are associated with lower production of sHLA-G. In contrast, other 3'UTRs exhibiting the 14 bp Del/+3142 C/+3187 G variation sites, like UTR-1, UTR-3, UTR-4, UTR-6, have been associated with high or intermediate expression of HLA-G.

To perform these analyses, 275 individuals from 'The Amsterdam Cohort' (Coutinho, 1998)) were analysed and and more than 70 controls regarding 3'UTR allele, genotype and haplotype. The analysis also included the eight previously reported HLA-G 3'UTR polymorphic sites [14-bp Ins/Del (rs1704), +3003 C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035 C/T (rs17179108), +3142 C/G (rs1063320), +3187 A/G (rs9380142) and +3196 C/G (rs1610696). No other polymorphic variation was observed in this region in any of the sequences obtained. The allele and genotype frequencies of the eight HLA-G 3'UTR polymorphic sites observed for both populations are shown in (Table 4-1/2).

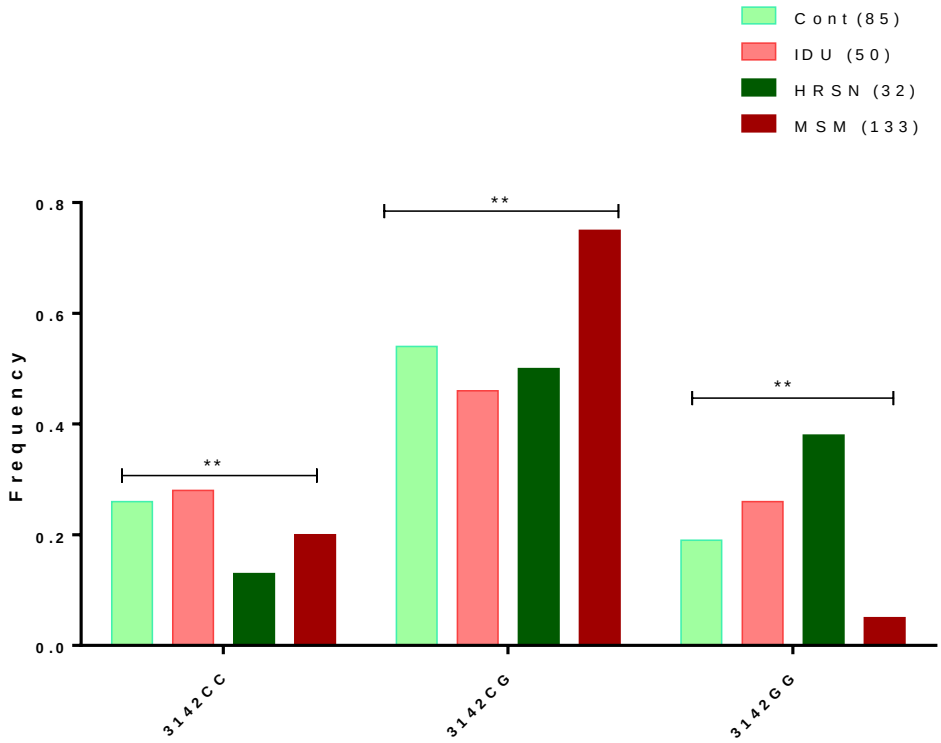

Figure 4-1 Haplotype frequencies observed at the HLA-G 3'UTR polymorphic site (3142C/G) in cntrols and three HIV patient groups.

The frequencies of the 3142C/G genotype observed in control and three HIV patient groups. Significant differences were observed when these frequencies were compared between the groups, Chi-square test ** P value <0.05.

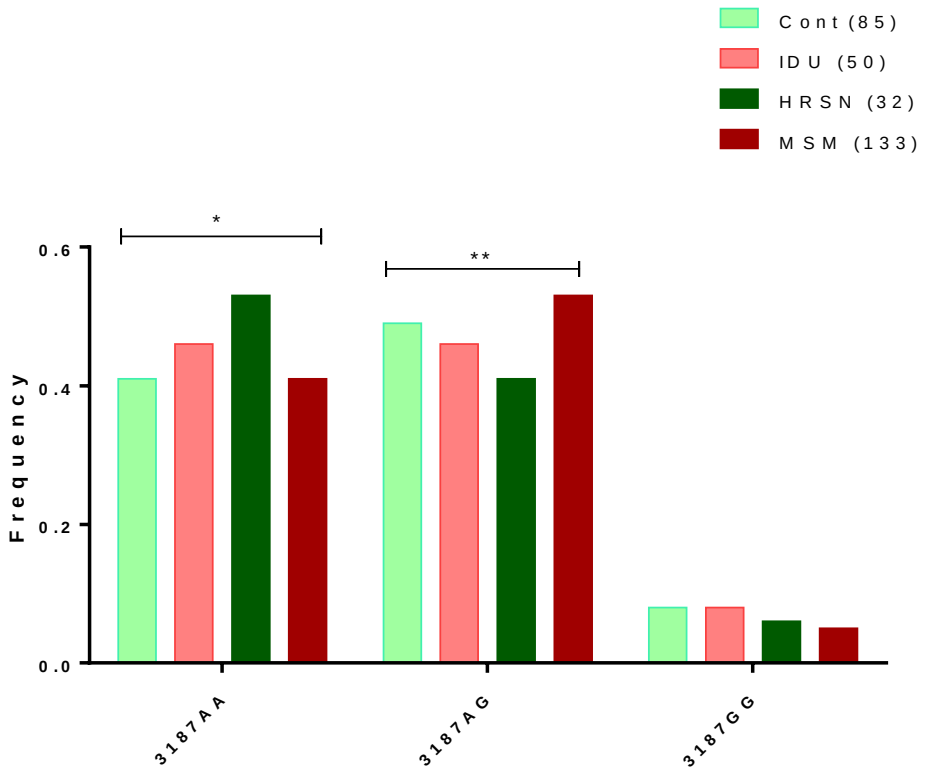

Figure 4-2 Haplotype frequencies observed at the 3187A/G polymorphic site in control and three HIV patient groups.

Significant differences were observed when these frequencies were compared between the three (3187A/G) genotype groups, using Chi-square test ,* p value <0.05; ***P value <0.001.

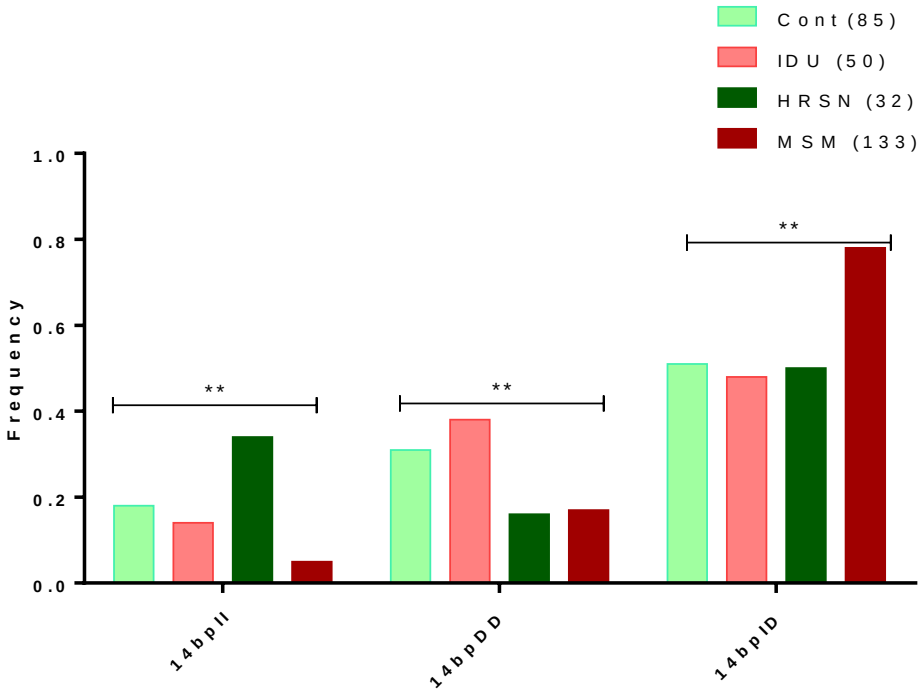

Figure 4-3 HLA-G 3'UTR 14bp INS/DEL genotype frequencies in three HIV patient groups and controls.

Significant differences were observed when these frequencies were compared between the three groups. Chi-square test ** P value <0.05.

Table 4-2 Allele frequencies of HLA-G SNPs and the 14bp INS/DEL dimorphism observed in the HIV patient groups and controls

Group	Control (170)		MSM (266)		HRSN (64)		IDU (100)		P value
Alleles	N	Freq	N	Freq	N	Freq	N	Freq	
3003C	27	0.15	86	0.32	**4**	**0.06**	16	0.16	<0.001**
3003T	143	0.84	180	0.68	60	0.94	84	0.84	
3010G	94	0.55	140	0.53	22	0.34	51	0.51	
3010C	76	0.44	126	0.47	**42**	**0.66**	49	0.49	<0.04*
3027C	158	0.92	261	0.98	58	0.91	98	0.98	
3027A	12	0.05	5	0.02	6	0.09	2	0.02	
3035C	149	0.87	254	0.95	50	0.78	97	0.97	
3035T	21	0.12	12	0.05	**14**	**0.22**	3	0.03	<0.046*
3142C	91	0.53	152	0.57	24	0.38	51	0.51	
3142G	79	0.46	114	0.43	40	0.63	49	0.49	
3187A	113	0.66	181	0.68	47	0.73	69	0.69	
3187G	57	0.33	85	0.32	17	0.27	31	0.31	
3196C	117	0.68	163	0.61	40	0.63	65	0.65	
3196G	53	0.31	103	0.39	24	0.38	35	0.35	
A4bp I"	74	0.43	116	0.44	38	0.59	38	0.38	
A4bp D"	96	0.56	150	0.56	26	0.41	62	0.62	

Chi-square test ; * P value <0.05, ** P value <0.001.

" I: Insertion, D:Deletion

Table 4-2 shows a significant difference between some allele frequencies of the HLA-G 3'UTR polymorphic sites, since the expression levels of the *HLA-G* gene has not been associated with these polymorphic sites, they were not included. The presence of variation sites associated with high production was further analysed (14 bp Del/+3142C/+3187G variation sites) or with low production (14 bp Ins/ +3142G/ +3187A) in control and

three HIV groups (Figure 4-4/5/6). However, no significant differences were observed when these frequencies were compared between the groups using using a Chi-square test.

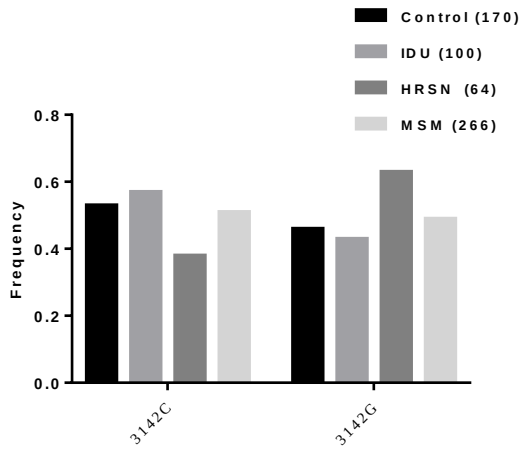

Figure 4-4, +3142G/C Allele frequencies of HLA-G observed in the HIV groups.

The +3142C allele has most lower frequencies among the HRSN group, and higher +3142G allele compared to groups compared. However, No significant differences were observed when these frequencies were compared between the groups using a Chi-square test.

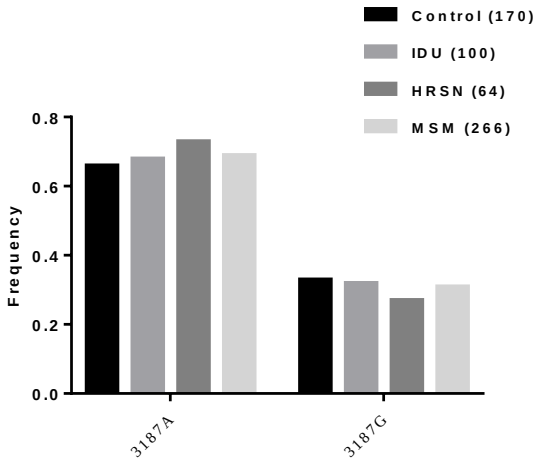

Figure 4-5, +3187 A/G Allele frequencies of HLA-G observed in the HIV groups.

No significant differences were observed when these frequencies were compared between the groups using Chia-square test.

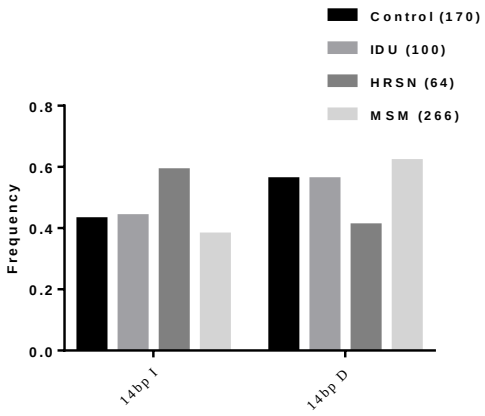

Figure 4-6, 14-bp INS/DEL allele frequencies of HLA-G observed in the HIV groups.

The HRSN group shows highest frequency among the groups when these +14-bp INS/INS allele were compared. The MSM group shows higher +14-bp Del frequency compared to other groups but not statistically significant.

4.2 Haplotypes of the HLA-G UTRs in MSM IDU and HRSN

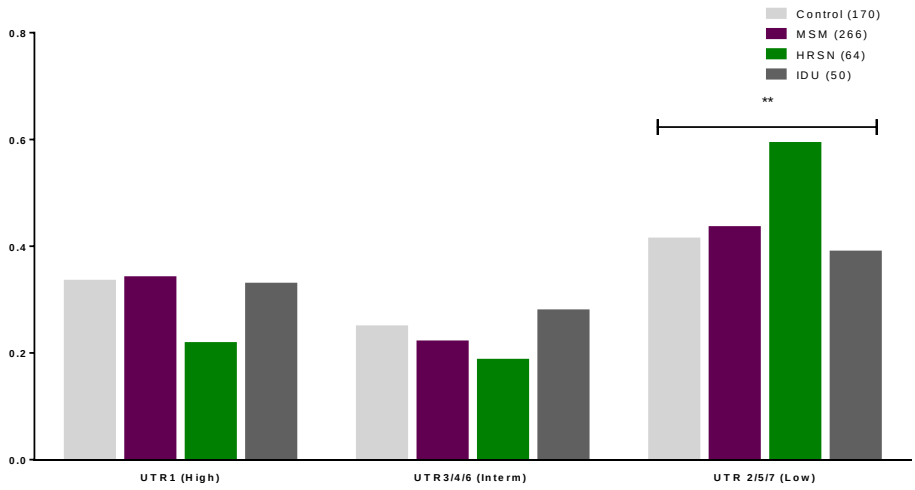

Figure 4-7 Haplotypes of the HLA-G UTRs in HIV groups: MSM, IDU and HRSN AND controls.

Seven different HLA-G 3' UTR haplotypes were observed in 3 groups. Men who have sex with men (MSM) HIV group, injection drug users (IDU) HIV group and high-risk HIV-seronegative (HRSN) group, and were designated as previously described (Castelli et al., 2010). Comparison of the haplotypes defined for the groups showed that HRSN exhibited a significantly higher proportion of low expressers (UTR2/5/7). The frequency of the low expresser (UTR2/5/7) differed significantly between the groups after performing a Chi-square test ; (**P=0.0217).

Following the PCR product sequencing, the indicative haplotype constitution was allocated (see Table 1-1). A database containing all SNPs identified at the 3 UTR of the HLA-G gene was designed for the analyses. The most frequent haplotypes of the HLA-G 3′ untranslated region UTR-1/2/3/4/5/6/7 were tested to check any variation sites associated with UTR-1 high or (UTR-2/5/7) low expressers. Haplotype allocation was achieved for all but a very small number of the total sequences, which may have included examples of the rarer haplotypes. The HRSN group showed a significantly higher proportion of low expressers (UTR2/5/7) than controls or the other patient groups as shown in (Figure 4-7). This suggests that low HLA-G expression alleles may play a role in protecting against HIV infection.

4.3 HIV group scoring

A scoring system was developed to quantify the potential influence of exon 8 polymorphisms on HLA-G expression. The 14bp del, +3142C and +3187G alleles associated with high HLA-G expression were allocated a score of 1 whereas a 0 score was allocated with low HLA-G expression: 14bp ins, +3142G and 3187A. Having both copies of the HLA-G gene permits each patient or healthy subject to be allocated a score of between 0 and 6, indicative of potential for low or high expression.

Upon comparing the scores in different groups of HIV+ and HIV– subjects, although there was a tendency for the HRSN group to have a lower mean score (1.93) compared to controls (2.71) and other patient

groups (2.70; 2.61), no statistical significance was found among the groups (P>0.05) when a two tailed Mann Whitney test was performed (Figure 4-8).

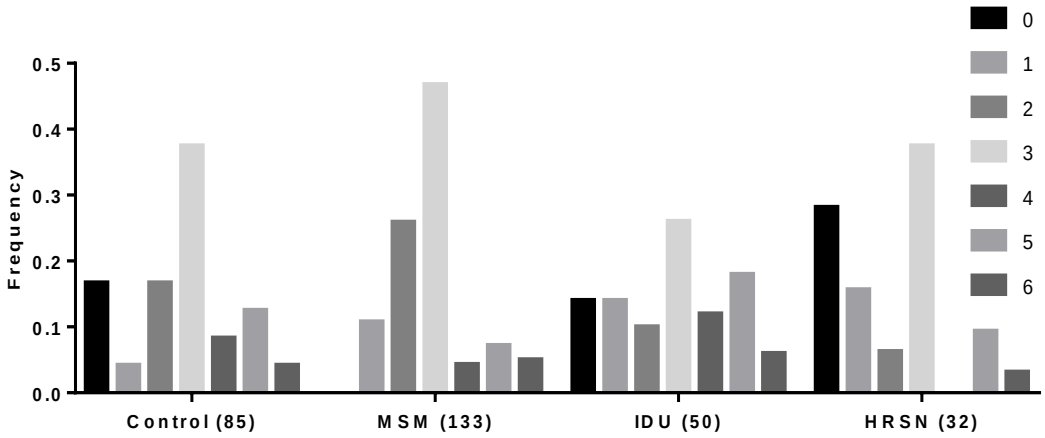

Figure 4-8 Scoring in MSM, IDU and HRSN groups scoring (High score reflecting potential high HLA-G expression)

Scoring the 14bp insertion/deletion polymorphisms (0=+/-, 1=+/+ and 2=-/-) with C/G +3142 (0=G/G, 1=C/G and 2=C/C) and A/G +3187 (0=A/A, 1=A/G and 2=G/G) in panels of HIV patients and control healthy subjects.

4.4 s-HLA-G in HIV patient sera

In healthy individuals, sHLA-G molecules are found in serum and the levels may increase in physiological and pathological conditions, such as pregnancy, acute rejection episodes after organ transplantation, autoimmune diseases, and some cancers. Interestingly, sHLA-G serum levels are increased elevated in viral infections, including hepatitis B and C, influenza, cytomegalovirus, and HIV-1 infections. Previously, the serum levels of sHLA-G were reported to be significantly elevated in HIV-infected subjects, which influences disease progression and development

of the acquired immune deficiency syndrome (AIDS) and virologic and immunologic response to antiretroviral therapy.

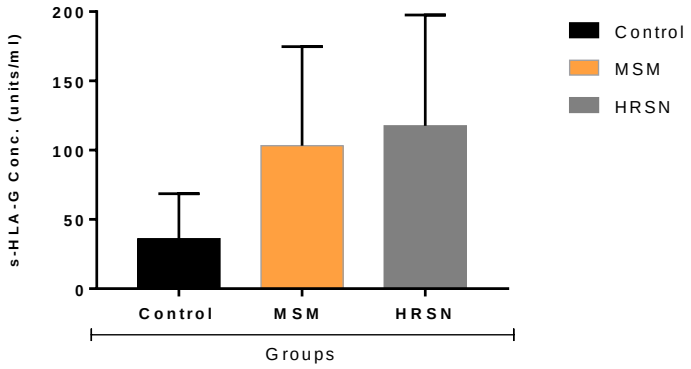

Figure 4-9 Mean soluble HLA-G (sHLA-G) levels (+/-SEM) in controls and HIV patient groups.

Mean and standard deviation of s-HLA-G level in Men who have sex with men (MSM) HIV group n:20, and high-risk HIV- seronegative (HRSN), n:20 and control group n:27. No significant differences were noted between the groups when using a Mann–Whitney U test (P>0.05).

The present experiment, therefore, assessed the sHLA-G serum levels in HIV patients from different groups. Comparing sHLA-G serum in Men who have sex with men (MSM) HIV group, and high-risk HIV-seronegative (HRSN) and control group shows no significant differences (P>0.05) (Figure 4-9).

4.5 Stimulating PBMC with an HIV viral vector and measuring the expression of HLA-G on different cells

4.5.1 Rationale for the experiments

The non-classical MHC class I molecule HLA-G was reported to be abundantly expressed in foetal trophoblast cells (Slukvin et al., 2000).

The molecule was found with low percentage on resting mononuclear and other cells (Lozano et al., 2002). HLA-G expression has been reported to be upregulated in various conditions including virus infections (Lu et al., 2011), and tumours (Mociornita et al., 2013). The tolerogenic properties of this protein has led us to test whether HLA-G could be upregulated on PBMCs from healthy subjects following culture with or without HIV-1 $_{LAI}$ strain provided by Prof. Paxton's group.

The aim was to explore whether HLA-G expression is upregulated on any lymphocyte populations following culture with an HIV-expressing vector, which would further support the association of HLA-G with HIV-1 infection. Therefore, we could have a better understanding as to how to elucidate the role played by this molecule in escaping immune surveillance.

4.5.2 Stimulation of PBMC with LAI antigen encoding vector

PBMC from a panel of 11 healthy subjects were stimulated for 7 days in vitro with HIV LAI antigen encoding vector. Unstimulated cells served as a control. Different leucocyte populations were then assessed for HLA-G expression by flow cytometry. Representative histograms are shown in (

Figure 4-10) and comparison of proportions of HLA-G+ cells for various PBMC subsets stimulated with HIV-1 antigens are summarised in (Figure 4-11). There were slight differences between HLA-G expression by different leucocyte subpopulations. CD3+CD4+ lymphocytes were revealed to have the highest increase in proportion of HLA-G expressing cells, followed by CD3+CD8+ cells, then CD19+ cells, although none of these increases were statistically significant. The remaining subsets of PBMC showed no difference between Day 0 and Day 7.

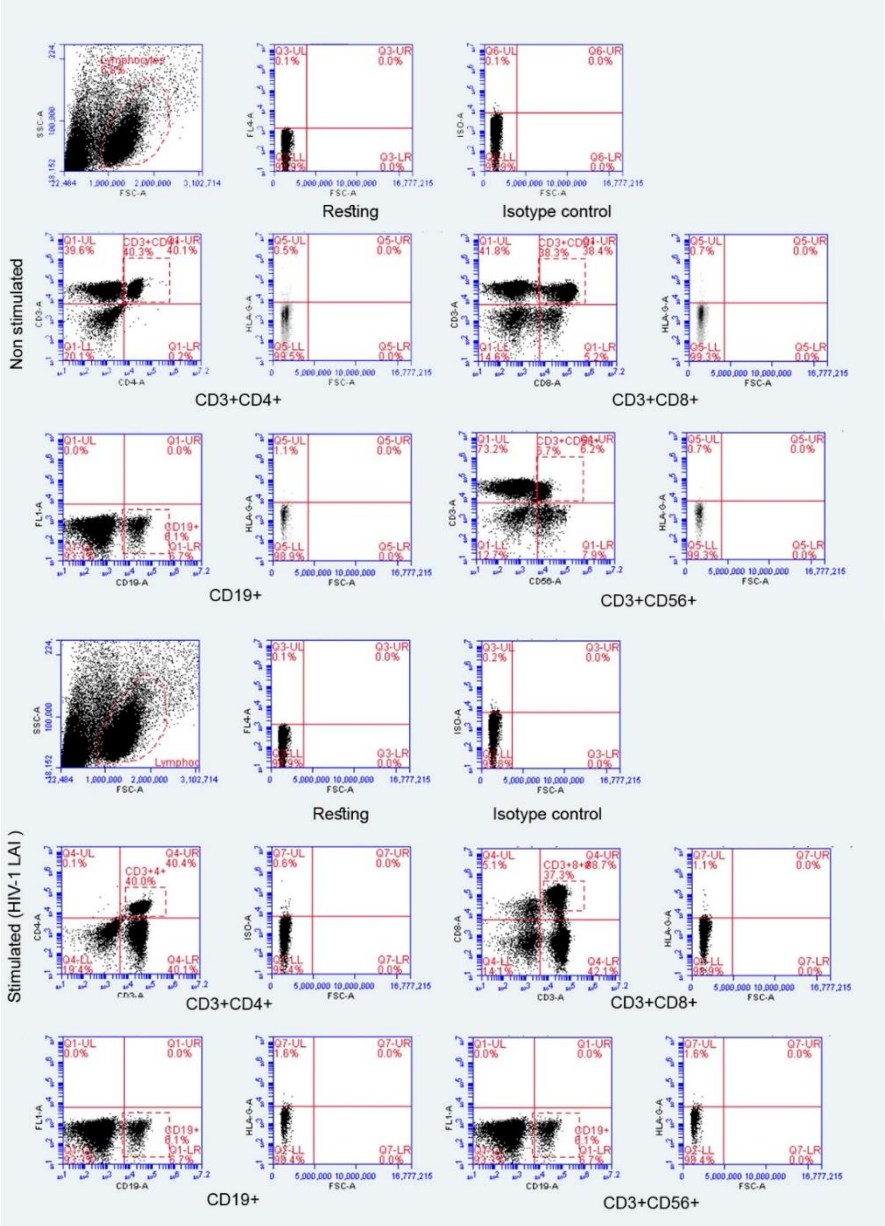

Figure 4-10 PBMC were stimulated with or without HIV-1 envelopes and co-receptors (CXCR4 = LAI) for a week.

Representative acquisition plots of Accuri C6 flow cytometry. Profile staining for lymphocytes and monocytes with anti HLA-G after a week of culture with or without LAI Ag. The figure shows flow cytometry profiles staining for lymphocytes and monocytes with Anti HLA-G. Isotype IgG2 APC control on lymphocytes and monocytes. Three colour staining of the T cell helper subset with CD3, CD4 and HLA-G. Cytotoxic T cells stained with CD3, CD8 and HLA-G. Two colours staining of B lymphocyte cells with CD19 and HLA-G. Three colour staining of CD56+ T cells with CD3, CD56 and HLA-G. All subsets showed differences but not statistically significant using Chi-square test (P value >0.05).

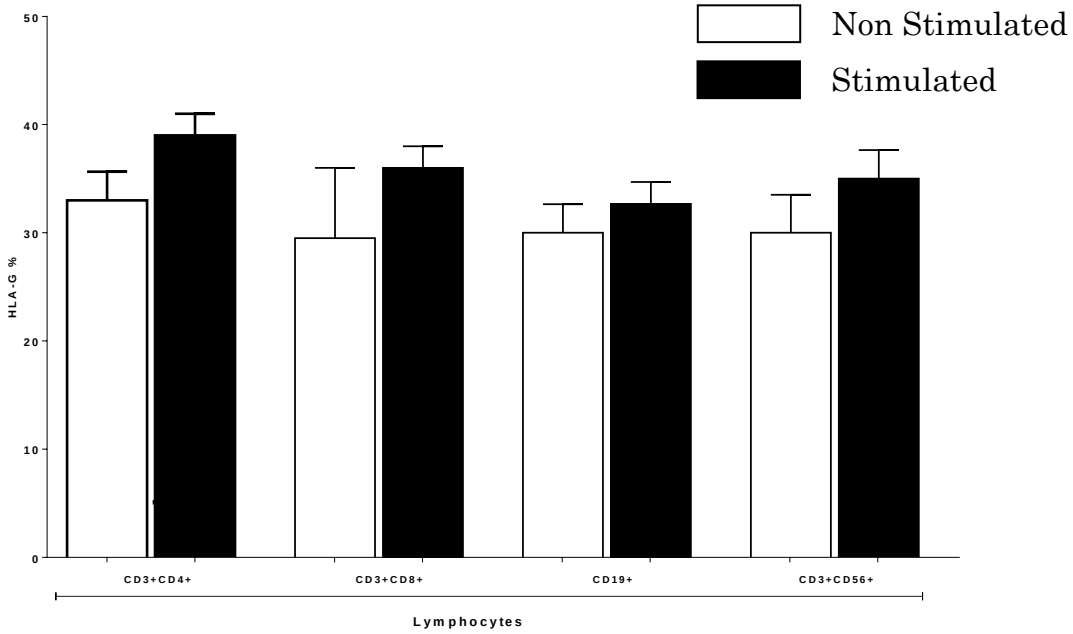

Figure 4-11 Comparison of proportions of HLA-G+ cells for various PBMC subsets stimulated with HIV-1 antigens.

PBMCs were treated for 7 days with (filled columns) or without (empty columns) HIV-1 antigens. HLA-G expression was assessed on the surface of a range of subsets of lymphocytes separated from healthy donors after a week of culture. Results are expressed as mean percentage. All subsets showed differences but not statistically significant; as mean +/- SEM, n = 11.

4.6 Changes in HLA-G gene expression following stimulation with HIV antigen-encoding vector

In order to test whether PBMCs may substantially increase fold expression of HLA-G following stimulation with HIV antigen, PBMCs from healthy donors were cultured with or without HIV antigen for 3/7 days. mRNA was extracted, reverse transcribed and analysed by real-time PCR.

The CT (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (ie exceeds background level). Based on the relative quantitation of gene expression and the calibration

of ΔCT by subtraction of the house keeping gene cycle number from the tested and the control cycles generated by the qPCR machine, the qPCR results were calculated. The measurements were then expressed as percentages and these values (ΔCT) were estimated via the equation (2ΔCT).

Although no statistical significance was observed upon comparing the mean gene expression folds of the non-stimulated HLA-G cells (19.02) with the stimulated lymphocytes (20.94), according to our analysis with Wilcoxon matched-pairs signed rank test stimulated PBMCs cells were more likely to increase fold in expression of HLA-G (Figure 4-12).

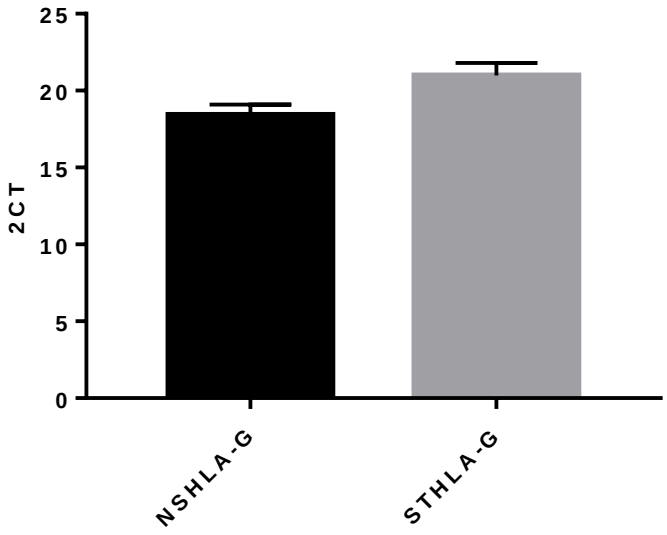

Figure 4-12 The relative HLA-G gene expression on whole PBMCs.

These from healthy subjects cultured with and without HIV protein, NSHLA-G = unstimulated cells; STHLA-G = stimulated cells, n =9. P>0.05, Wilcoxon matched pairs signed rank test.

5 DISCUSSION

5.1 HLA-G in pneumonia patients

Human leucocyte antigen G (HLA-G) has been linked with several pathological conditions. These include: viral infections, tumours, transplantation, autoimmune and inflammatory diseases with the effect driving a reduction in the immune response. Its tolerogenic property may have beneficial effects in circumstances such as pregnancy, transplantation, and inflammatory diseases. It however becomes deleterious when affecting the immune responses against tumours and viral infections by permitting the escape of tumour or virus-infected cells from the conventional anti-tumour or anti-viral responses (Amodio et al., 2015, Catamo et al., 2015, Polakova, 2003, Rajasekaran et al., 2014). This study was designed to investigate HLA-G polymorphism and expression in pneumonia patients in relation to infection with CMV or, in other pathogens such as HIV-1, in relation to clinical outcomes.

In the study of pneumonia patients, the HLA-G genotypes of patients, in relation to the 14bp deletion/insertion, were compared with healthy controls. There were no significant differences in proportions of patients with the 14bp del and ins alleles compared to healthy controls. This indicates that patients with higher potential expression of HLA-G are not significantly more susceptible to the development of pneumonia. However, as pneumonia can result from bacterial, viral and fungal infections, this heterogeneity in disease pathogenesis may mask any

genuine effect of HLA-G in pneumonia susceptibility. Although the mean age of the healthy controls was lower than that of pneumonia patients, this was not thought to have greatly affected the results.

Following that, patients were subdivided into groups according to clinical outcomes and other parameters. The cytomegalovirus (CMV) serostatus of different groups of patients were measured to be compared in different patient groups and also related to HLA-G genotype and levels of soluble protein. Proportions of pneumonia patients who were CMV+ were higher than in healthy controls, although this was not unexpected as patients were on average older than controls and the incidence of CMV positivity is declining in developed countries. Also, there were no statistically significant differences in HLA-G genotype between CMV+ and CMV- patients. CMV is capable of upregulating HLA-G on several leucocytes subsets in healthy subjects (Albayati et al, 2017). The results of the present study suggest that CMV does not have a substantial effect on disease incidence in patients of different HLA-G genotypes. However, it is possible that some of the CMV+ patients were more susceptible to pneumonia as a result of immunosenescence brought about by the immune risk phenotype (Pawelec, 2014).

Next, the genotyping of HLA-G was tested for any correlation between the 14bp insertion/deletion dimorphism and susceptibility to pneumonia following assessing CMV IgG status. The data were acquired using gel electrophoresis of amplified exon 8 that contains the 14bp region. By

assessing three outcomes of interest: death, re-admittance and length of stay with the influence of age, sex, CMV status and HLA-G, only HLA-G shows a significant association with the length of stay in hospital (P value <0.024). The results showed that patients with HLA_G DEL/DEL, have 3 times higher predicted length of stay in hospital than for those in the other group (INS/DEL + INS/INS). This may reduce the uncertainty in length of stay and permitting effective scheduling and manpower allocation. This is a genotype associated with higher expression of HLA-G and would be consistent with these patients being able to express higher levels of cell surface or soluble HLA-G, suppressing the immune response to *S. pneumoniae.*

Additionally, soluble HLA-G (sHLA-G) was measured to check for any differences in levels between different patient groups. However, no significant differences were seen between serum sHLA-G levels in pneumonia patients and healthy subjects, either in CMV+ or CMV- groups.

5.2 Ability of Streptococcus pneumoniae antigens to induce HLA-G expression

Streptococcus pneumoniae is the most frequent bacterial cause of pneumonia in adults, despite only a small number of patients in the present cohort being identified as positive. A natural extension of the above work, was to test whether HLA-G could be upregulated on PBMCs

from healthy subjects following culture with or without pneumococcal culture supernatant (CCS) derived from wild type strain D39.

The proportions of HLA-G+ cells in various PBMC populations harvested on day 7 of culture with Streptococcus pneumoniae Ag D39 were compared to non-stimulated cells. The findings of the present work have revealed that there were differences between these data but not statistically significant. CD3+CD4+ lymphocytes were revealed to have the highest increase, followed by CD3+CD8+ cells after applying a paired t-test. The remaining subsets of PBMC showed no difference between Day 0 and Day 7. Monocytes after 7 days show did not upregulate the protein following culture with D39 antigens. Onno et al. have shown HCMV induces the expression of nonclassical MHC-I HLA-G molecules in vitro. In the same study, alveolar macrophages collected from patients undergoing HCMV pneumonitis displayed HLA-G molecules in ex vivo (Onno et al., 2000b). In our previous work, CMV antigens were shown to be able to induce HLA-G expression mainly on B cells, CD56+ T cells and monocytes (Albayati et al, 2017). These are different leucocyte subsets from those found to upregulate HLA-G in response to *S. pneumoniae* antigens. Thus, D39 Ag used in the current study might be sufficient for HLA-G up-regulation but predominantly on classical T cell subsets. This may have implications for the lack of clearance of *S. pneumoniae* in some patients or healthy subjects if adaptive immune responses to the bacteria are inhibited by T cell expression of HLA-G. Several different immune

evasion mechanisms have been described for *S. pneumoniae* but there are no reports of any involvement of HLA-G (Weiser et al., 2018).

5.3 sHLA-G concentrations in pneumonia patients and healthy subjects

Soluble HLA-G levels were measured from serum specimens and differences in levels between different patient groups was compared. The soluble HLA-G (sHLA-G) levels have been reported to be significantly increased in a variety of pathological circumstances such as melanoma, neuroblastoma, lymphoproliferative disorders, breast, ovarian and colorectal carcinoma when compared to healthy controls or subjects with benign neoplasms (Cao et al., 2011). The level of s-HLA-G from CMV+ and CMV- pneumonia patients (91.3 / 142.4) U/ml displayed no significant difference when compared to healthy controls (62.1/ 146) U/ml. Previous studies reported increased secretion of sHLA-G in response to CMV induction and were thought to indicate the possibility of this protein to interact with receptors for HLA-G like KIR2DL4, ILT-2 and ILT-4 which mediate inhibitory behaviour of some immune cells in the presence of CMV infection (Albayati et al., 2017). Rizzo et al. showed a significant induction of sHLA-G in serum samples from primary CMV+ pregnant women versus CMV- and CMV carrier control (Rizzo et al., 2012). Similarly, sHLA-G levels in sera were elevated in Hepatitis B virus infection and chronic hepatitis C virus infection when compared to normal subjects (Shi et al., 2011, Weng et al., 2011). However, these data differ

from those in the current study, mainly due to the fact that those healthy subjects involved in the assessment of sHLA-G are unlikely to have had acute pneumonia infection or CMV at the time of blood sampling.

5.4 KIR2DL4 expression in healthy subjects

A part of this work was to study variations in one of the ligands for HLA-G, KIR2DL4 and relevant polymorphisms within this ligand. Therefore, we hope to understand the association between individual genetic and differential expressions of this ligand for HLA-G in relation to diseases in which HLA-G expression is upregulated. The data presented in this study reveals a significant increase in the proportions of cells from healthy subjects expressing KIR2DL4 in CD56+ T cells, and CD16+ CD56dim and CD56bright CD16- NK cells following stimulation with IL-2, a nonspecific activator of T cells and NK cells, for 14 days. CD56 bright (CD56+CD16-) and (CD16+CD56dim) NK cells revealed significant increases in comparison with untreated cells; CD16+CD56dim (P=0.0097) and CD56bright (P=0.0016). Following this, relevant polymorphisms in KIR2DL4 were tested in different patient groups by a combination of multiplex PCR and gene sequencing. Consequently, following the sequencing of healthy individuals they were classified to 9A and 10A alleles to indicate any relevant influence on surface KIR2DL4 protein expression.

There were no significant differences in gene frequencies for the of 9A and 10A KIR2DL4 alleles in healthy donors either negative or positive for

CMV. However, these data are not compatible with observations made by (Kikuchi-Maki et al., 2005) who showed the expression is limited to CD56bright cells in peripheral blood. T cells and NK cells expressing the KIR2DL4 receptor could bind with HLA-G and can result in the modification of the repertoire of cytokines released by NK cells (Rajagopalan et al., 2001). The process can decrease the cytotoxic function of NK cells and downregulate HLA-G expression on the cell surface (Kikuchi-Maki et al., 2005, Goodridge et al., 2003).

The current data shows the expression of KIR2DL4 may be not influenced by the KIR2DL4 9A and 10A genotype as the receptor was found on the cell surface in both 9A and 10A variants. It was reported (Goodridge et al., 2003) that the 10A allele of the KIR2DL4 gene encodes the full length protein, unlike the 9A variant which influences KIR2DL4 expression. Frame shifting after the 9A sequence results in generation of a stop codon in exon 7 which affects the cytoplasmic domain, consequently, the product of this gene is unlikely to be expressed at the cell surface (Witt et al., 2000). However, the presence of the 10A allele could influence the role of KIR2DL4 by the activating function of the protein being dominant (Goodridge et al., 2007).

5.5 Variation in HLA-G genotype in different groups of HIV patients

The HIV infection and virus escape process have been extensively studied. However, the ability of the virus to infect cells and deceive the immune

system remains a puzzle for researchers. Although an apparently appropriate immune response is initially induced by HIV, it later successfully evades the immune system (Tripathi and Agrawal, 2007). Several studies suggested that there is an increased expression of HLA-G after HIV infection and the immunomodulatory molecule HLA-G is capable of suppressing immune responses but the real involvement of this molecule in virus infection and immune responses to the virus remain elusive (Lajoie et al., 2009).

In the present study we investigated the association between the 14 bp INS/DEL and other HLA-G gene polymorphisms with HIV infection. The evaluations were performed in patients and ethnic-matched controls from the Amsterdam Cohort on HIV-1 infection and AIDS (ACS). We observed an increased frequency of the genotypes related to low HLA-G expression (UTR-2, 5 and 7) in high risk seronegative subjects (HRSN) as compared to men having sex with men (MSM), intravenous drug users (IDU) and uninfected individuals, suggesting that low HLA-G expression alleles may play a role in protecting against HIV infection.

Previous studies have associated the HLA-G genotypes and HIV vertical transmission risk in Brazilian children. The 14 bp deletion allele was observed to have a protective effect. Fabris et al hypothesized that the 14 bp deletion is associated to a higher HLA-G expression and could induce a local Th2 immune response thus reducing the production of pro-inflammatory cytokines, which can contribute to an increased risk of

mother-to-child HIV-1 transmission (Fabris et al., 2009, Segat et al., 2014). Our observations differ from this in that we found that low expressing haplotypes had a protective effect against HIV infection. Together, these data suggest an influence of 3′ UTR polymorphisms not only in vertical, but also in horizontal HIV transmission.

We acknowledge that our findings are restricted to patients of a particular ethnic origin and that results derived require some considerations. Different results were shown according to the population evaluated when studies evaluating the influence of the 14 bp polymorphism in HIV horizontal transmission (Fabris et al., 2009). Among African Americans a protective effect of the 14 bp DEL/DEL genotype was observed (Aghafar et al., 2012). However, in European-derived populations, no association was observed. The vast majority of the Amsterdam Cohort is of European origin and also we considered several of the other HLA-G polymorphisms associated with altered HLA-G expression, not just the 14bp INS/DEL dimorphism.

These studies support our findings and raise the importance of controlling for ethnicity in association studies. Some investigative works on the influence of other HLA-G alleles in HIV infection susceptibility in women from Italy, Zimbabwe and Kenya presented controversial results. This observation highlights a need of further investigations on the influence of HLA-G genetic variants in HIV susceptibility in populations with

different genetic backgrounds (da Silva et al., 2014) including Southern Brazil and in a confirmatory cohort of HIV+ patients from Italy.

We observed a decreased frequency of the genotypes related to low HLA-G expression in European-derived-HIV-infected as compared to uninfected individuals, suggesting that low HLA-G expression alleles may play a role in protection against HIV infection. In addition, we sought to determine the influence of HLA-G genotype on HLA-G expression in HIV patients by studying sHLA-G levels, however the current data showed no significant correlation between genotype and expression levels of HLA-G.

Some recent works have discovered that HLA-G 3′UTR mRNA has a number of binding sites for miRNAs. Castelli and co-authors in 2009 have made an extensive in silico analysis of the HLA-G 3′UTR region, seeking the putative miRNA binding site. They found that different miRNAs bind to this region and moreover the vast majority of these sequences encompass eight highly polymorphic sites (Castelli et al., 2009). One of them above all, the C/G polymorphism at position +3142, is the putative binding site for at least three miRNAs: hsa-mir-148a, hsa-mir-148b, and hsa-mir152, and has been subsequently confirmed to be the binding site for mir152 and it is noted that this binding reduces HLA-G expression (Zhu et al., 2010). However, the effect of this polymorphism in miRNA binding has been recently questioned by Manaster and colleagues. They confirmed previous results, finding that mir-152 and mir-148a bound to

HLA-G 3′UTR and downregulate its mRNA, but they also found that the C/G polymorphism at +3142 has no effect on miRNAs binding and efficacy (Manaster et al., 2012). Interestingly, they found in placental tissue low levels of these miRNA and they suggest that this could be a regulatory mechanism allowing HLA-G expression only where needed and not where the immunosuppressive activity of this molecule could be detrimental. In an interesting parallel, the hsa-mir-148a has been proposed to bind HLA-C 3′UTR and a polymorphism in the binding site for this miRNA, which increase the binding strength, has been associated with poor HIV-1 infection control. Thus, the connection between miRNA, HLA-G expression, and HIV-1 needs to be further explored because it can reveal novel information about HIV-1 control of the immune system. In the data of (Figure 4-7) there was a tendency for the HRSN group to have a higher proportion of patients with the allele associated with lower levels of HLA-G expression.

5.6 sHLA-G concentrations in HIV patients and healthy subjects

Previously, the serum levels of sHLA-G were reported to be significantly elevated in HIV-infected subjects, which influences disease progression and development of the acquired immune deficiency syndrome (AIDS), and virological and immunological response to antiretroviral therapy. sHLA-G has the ability suppress T lymphocyte all proliferative activity (Lila et al., 2002). sHLA-G could potentially interact with one of its ligands, KIR2DL4, found on NK cells and other PBMCs could moderate

inflammatory mediator synthesis ((Albright and Oppenheim, 1991, van Bergen and Trowsdale, 2012). In addition, the binding of sHLA-G with ILT-4 on DC can result in activating T reg which in turn participate in induction of tolerance favouring virus evasion (LeMaoult et al, 2004). The induced sHLA-G could be due to the influence of viruses and pathogens as a strategy by which they can escape the effects of immune system. The same was reported by Spaggiari et al. about the ability of sHLA-G to provoke self-killing in NK cells via the activation of the Fas/FasL pathway when CD8 antigen interacts with sHLA-G molecules (Spaggiari et al., 2002). The current study assessed the sHLA-G serum levels in HIV-1 patients from different groups, namely control, HRSN and MSM groups. No significant differences in sHLA-G concentration were noted in serum from the HIV patients groups and controls.

This could indicate that the sHLA-G protein is not the main mediator of immune suppression in HIV patients, but rather that cell plasma membrane-associated HLA-G is more important functionally. Levels of sHLA-G were highly variable between patients and may not be directly related to levels of cell-associated HLA-G. Levels and patterns of expression of its receptors like KIR2DL4, ILT-2 and ILT-4 that mediate inhibitory behaviour may also vary in the presence of HIV infection.

5.7 HLA-G expression in in response to HIV-1 envelope antigen

Changes in HLA-G expression were explored in response to HIV-1 envelope antigens and co-receptors (CXCR4 = LAI) in healthy subjects to

check if these antigens may exert any up or down regulation following in vitro culture with a viral vector for a week. The proportions of HLA-G+ cells in various PBMC populations harvested on Day 7 of culture with HIV Ag were compared to resting proportions of HLA-G+ cells. CD3+CD4+, CD3+CD8+ and CD3+CD56+ lymphocytes revealed slight elevation of HLA-G expression on Day 7 but not statistically significant.

Numerous studies were aimed at observing the expression of the molecule HLA-G in the early stage of infection by HIV and its progression. In 2004, Derrien and colleagues demonstrated that during HIV-1 infection the HLA-G1 isoform was downregulated (Derrien et al., 2004). The HLA-G1 isoform is known to be able to present of viral peptides to $CD8^+$ T lymphocytes (Lenfant et al., 2003); therefore, the recognition by $CD8^+$ T lymphocytes of HIV-1-infected cells could depend on the expression of HLA-G1 (Derrien et al., 2004).

In contrast, Lozano and colleagues have observed elevation of surface expression of HLA-G on monocytes and on some T lymphocytes in HIV-1 positive patients with or without antiretroviral treatment. They assumed that the high expression of HLA-G was indirectly induced by infection with HIV-1. The study was related to the pathogenesis of the infection, considering that HLA-G expression on the monocytes, which are unlikely to be infected (Lozano et al., 2002). It was also possible to explain this increase is caused indirectly as a consequence of high levels of cytokines such as IL-10 (Moreau et al., 1999).

In 2009, a longitudinal study evaluated sHLA-G plasma levels in association with progression of HIV-1 infection. Lajoie et al. observed elevated levels of sHLA-G in the early stages of HIV-1 infection compared to treated normal progressors. With respect to HIV-1-negative individuals, the high concentration of IL-10 in rapid progressor patients justify these data. Matte et al. in 2004 observed a significant association between the HLA-G*0105N allele (null allele) and protection from HIV-1 infection. The authors hypothesized that this allele could facilitate natural killer cell-mediated destruction of HIV-1-infected cells (Matte et al., 2004). Segat and colleagues in 2010 reported a significantly increased incidence of the HLA-G*0105N allele in HIV-1-positive women patients versus HIV-1-negative women controls (Fabris et al., 2009). This suggests that the different results reported by Matte et al. were imputable to the different ethnicity of the studied populations.

5.8 Final summary and Future work

In conclusion, the study was aimed to investigate HLA-G polymorphism and expression in pneumonia patients in relation to infection with CMV or, in other pathogens such as HIV, in relation to clinical outcomes. No significant differences in proportions of pneumonia patients with the 14bp del and ins alleles compared to healthy controls. This suggests that patients with higher potential expression of HLA-G are not significantly more susceptible to the development of pneumonia. However, incidence of pneumonia patients who were CMV+ were higher than in healthy

controls. It is likely because patients were on average older than controls and the incidence of CMV positivity is declining in developed countries.

It would be of greater relevance to repeat these experiments using controls of the same average age to avoid risk factors based on age-related differences. Following the stimulation with *Streptococcus pneumoniae* Ag D39, changes in the proportions of HLA-G+ cells in various PBMC were observed. It is unclear whether D39 Ag can induce proliferation of HLA-G+ cells or whether cells initially negative for this molecule have induced HLA-G expression. This could be assessed using CFSE- labelling of cells following stimulation with D39 Ag and measuring HLA-G expression on proliferated cells by flow cytometry.

In the current experiments, antigens from the D39 strain of *S. pneumoniae* were used. However, this is long-established laboratory strain that may have undergone genetic modifications. It would be more relevant to culture nasopharyngeal swabs to extract viable clinical *Streptococcus pneumoniae* isolates having a complete genome and conduct similar experiments with antigens derived from these to test for the ability of *S. pneumoniae* to influence HLA-G expression *in vitro*.

In HIV patients, the association between the 14 bp INS/DEL and other HLA-G gene polymorphisms with HIV infection were also investigated. Our finding is that an increased frequency of the genotypes related to low HLA-G expression (UTR-2, 5 and 7) were found in HRSN as compared to other patient groups and uninfected individuals. This suggests that low

HLA-G expression alleles may play a role in protecting against HIV infection. It would be of great interest to observe changes in HLA-G expression in HIV patients to ascertain any additional effect. An unclear issue is the effect of HLA-G genotype on HLA-G expression in response to HIV and it was not possible to confirm the previously reported genetic associations between SNPs and levels of expression and s-HLA-G. This could be tested in future work by using healthy donors of defined HLA-G genotype and comparing levels of HLA-G mRNA with and without a complete HIV genome antigen treatment using quantitative RT-PCR.

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