Aspects to T-cell Phenotype During Infection With HIV, CMV And Hepatitis C Virus.

"The LORD is my light"

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Dr John W Northfield

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Abstract:

This work concerns itself with understanding the organisation of cellular immune responses to three major human pathogens - HIV, CMV and Hepatitis C (HCV). Each was studied to form three projects, each undertaken with a different approach - arrived at independently - and largely owing their origins to opportunity and circumstance as much as design. Each project led to exploration of a particular aspect of T-cell phenotype (that is the expression of particular molecular markers on T-cells) and its' broader biological significance. I found that T-cell phenotype was strongly linked to the magnitude of T-cell responses (CMV) and the ability of T-cells to control infection (HIV). Finally I explored the significance of expression of a molecule known as CD161 on the surface of HCV-specific CD8+ T-cells, indicating a phenotype of T-cell that may not follow the 'normal rules' applicable to T-cells in general.

Chapter 1 – Introduction.

The human immune system operates to detect and eliminate pathogens that breach epidermal surfaces through a complex and sophisticated set of molecular and cellular interactions. On the other hand, 'successful' pathogens (best defined as those that are able to set up persistent and transmissible infections) have an array of methods to circumvent these immune defences. An overview of this dynamic balance is useful in order to place the work of this thesis in the correct context.

1:1 An overview of the immune system:

The immune system is comprised of innate and adaptive immune systems. The basic distinctions are that innate immunity operates with only crude recognition of the foreign materials that compose pathogens and operates only on a per-cell basis. However it is rapidly triggered the moment natural barriers to infection are breached. On the other hand the adaptive immune system is highly specific for foreign pathogens, and can be amplified from a per-cell basis to ultimately involve billions of cells. Its' specificity, however, means that it can operate with precision with a potent armament to prevent infection whilst also limiting damage to the host, or auto-immunity. However it takes time for the adaptive immune response to reach full strength after infection, a problem that is curtailed to some extent by the ability to form 'immunological memory'. This enables a much brisker and more powerful response with the same specificity if a second

infection with the same pathogen occurs, usually conferring nearly complete protection against re-infection.

1:1:1 The innate immune system.

The body is in continuous contact with a high burden and wide variety of microbes. Many microbes however are non-pathogenic and under normal conditions don't breach epidermal surfaces to cause invasive disease. The innate immune system is the first point of contact with the host immune system for microbes, and it is important in rendering many microbes non-pathogenic, and limiting the capacity of pathogens to cause severe disease and death.

Macrophages are a key cell of innate immunity. They are able to recognise pathogen associated molecular patterns (PAMPs). PAMPs are characteristic molecular structures of pathogens that are not found in host tissues, and are recognised by an array of different cell surface receptors known as pathogen recognition receptors (PRRs). PAMPs are therefore also known as 'danger signals' – giving an early warning of a breach of surface integrity and the characteristic 'molecular signature' of the presence of a pathogen. Typical of PRRs, and perhaps the most important, are the Toll-like Receptors, of which there are nine (TLR 1-9). TLR 3, TLR 7 & TLR 9 are probably the most important PRRs for combating viral infections (Kawai and Akira 2006). These TLRs are expressed on macrophages and plasmacytoid dendritic cells (pDCs) as well as by other cells, and recognise fragments of viral RNA and other nucleic acid sequences characteristic of viruses. TLR triggering results in Type I interferon secretion (such as IFN-α species and

IFN-β). Type I interferons work to inhibit viral replication through impairment of virus construction and assembly (eg induction of RNAse L (Liang, Quirk et al. 2006)). They also promote secretion of chemokines that attract macrophages and lymphocytes to the site of infection. Type I interferons also impact on the generation of adaptive immune responses through other mechanisms (see below) and thus form part of the bridge that integrates innate and adaptive immunity.

Another component of innate immunity is the activity of natural killer cells (NK cells). NK cells are able to recognise cells that have down regulated MHC class I as a consequence of infection. According to the 'missing self' hypothesis, the absence of triggering of NK receptors (rather than direct ligation) reduces inhibitory signals to the NK cell. This, in concert with positive signalling from ligation of activatory receptors and binding to cellular targets upregulated with cell stress, such as MIC-A, results in activation of effector mechanisms leading to death of the infected cells. These effector mechanisms include cytotoxcity and IFN-γ secretion. Thus NK cells are able to destroy the infected cells thus limiting viral replication through a variety of mechanisms (Bryceson and Long 2008). Other mechanisms of innate immunity exist (eg complement activation) but a more detailed discussion is beyond the chosen scope of this thesis.

1:1:2 The adaptive immune system.

The adaptive immune system is designed to generate a highly focussed, specific and potent response with the subsequent formation of immunological 'memory'. It is based on the activity of CD4+ and CD8+ T-cells, as well as B-cells. These complex cell

populations have the ability to recognise and respond with high specificity to precise protein sequences – or peptides - of an invading pathogen. In the case of T-cells this ability is based on their ability to express a massive array of specific receptors for the huge diversity of peptide sequences that compose the protein components of pathogens. The system is set up before the host is ever exposed to a pathogen – as it develops *in utero*. The diversity itself is possible through re-arrangement of the 'V', 'D' and 'J' segments of the alpha and beta T-cell receptor gene and additional modification of the 'joining' segments during T-cell development. One T-cell expresses just one rearranged beta chain (although potentially more than one alpha chain) for the T-cell receptor sequence, and the ability to transcribe other sequences is lost. This process is so refined and effective that a repertoire of 'naïve cells' each with one of up to 10^{16} different T-cell receptors can be generated by the developing immune system. T-cell receptors are exposed to 'self' antigens in the thymus, and if they react strongly they are largely deleted through negative selection to avoid auto-immunity (Kyewski and Klein 2006). Those showing minimal reactivity to self antigens are allowed to egress the thymus ('positive selection'). In addition to this 'central tolerance', mechanisms of 'peripheral tolerance' are also required.

The wide array of T-cell receptors results in the immune system being able to develop immune responses to a wide array of pathogen-derived peptides. However for the system to work it is necessary for a large proportion of the T-cell receptor repertoire (or 'peptide library') to 'see' a given peptide sequences until it is ultimately recognised and a T-cell response can be mounted. Antigen presenting cells (such as dendritic cells) have a

specific capacity to sample peptide sequences (antigens) from the environment. They thus act as highly efficient librarians, quickly identifying the T-cell(s) with the correct reference, or peptide sequence. The T-cell 'library' is stored in lymph nodes. Thus APC's migrate to lymph nodes to 'present' antigen to T-cells that bear a wide array of T-cell receptors. This can be visualised by video microscopy when dendritic cells can be seen to rapidly scan large numbers of T-cell in lymph-nodes at a rate of around 500 T-cells per hour each (Bousso and Robey 2003). However, antigen-specific T-cells can be seen to form stable contacts and 'cluster' around dendritic cells for several hours with up to ten T-cells per dendritic cell. Priming or activation of dendritic cells requires the presence of 'danger signals' (eg IFN-α) produced on activation of innate immune pathways. These danger signals lead to changes such as maturation of dendritic cells (reflected by expression of maturation markers such as CD40, CD80 & CD86) (Rescigno, Winzler et al. 1997). Maturation of dendritic cells encourages their migration through lymphatic vessels and thus enables them to liaise effectively with T-cells. The need for 'danger signals' provides a neat mechanism to ensure that adaptive immune responses are 'licensed' in a proportionate manner to combat any nascent infection.

The requirement for actual 'presentation' of pathogen peptide fragments on the surface of dendritic cells to T-cells adds another layer of complexity to the immune system, and is performed as follows: pathogens, or fragments of them, are endocytosed by dendritic cells in infected tissue, to form an intracellular endosome. After fusion with a lysosome (to form an endolysosome), digestion of proteins occurs through the action of proteases to form short peptide sequences. Meanwhile MHC class II molecules are synthesised in the

endoplasmic reticulum and golgi apparatus. Eventually an exosome containing MHC class II molecules fuses with the peptide-containing endo-lysosome. Peptide fragments are then loaded into the 'groove' of the MHC class II molecules after displacement of a molecule known as 'li'. (li is then cleaved to form CLIP). The now peptide-laden MHC II molecule can now be transported to the cell surface. Dendritic cells use class II molecules to present antigen to CD4+ cells. For antigens that are new to the immune system presentation occurs to naïve cells in a process known as T-cell priming. Cytokines such as IL-12 and Il-18 facilitate priming (Kapsenberg 2003). Presentation can also be to antigen-specific memory cells, enabling a 'recall' or 'memory response' to be mounted. In addition to interactions between TCR's and peptide laden MHC complexes, additional co-stimulatory signals are required such as those between CD28 and B71/B72 molecules (Croft 2003). Dendritic cells and other professional antigen presenting cells (ie B-cells) express MHC class II molecules constitutively. Many other cell types may express class II in response to IFN-γ. These latter cell types include fibroblasts, endothelial cells and epithelial cells. Expression of class II is under the control of the class II transcription activator (CIITA) (LeibundGut-Landmann, Waldburger et al. 2004).

Responding 'helper' CD4+ cells, as their name suggests, have several important roles in supporting the immune response:

1) They can stimulate B-cells to secrete antibody. Like T cells B-cells are antigen specific and recognise antigen through an immunoglobulin receptor.

2) CD4+ T-cells also contribute to the immune response through activating macrophages and contribute to DC maturation through CD40L/CD40 interactions (van Kooten and Banchereau 1997).
3) CD4+ T-cells support the CD8+ T-cell response through 'licensing' the formation of CD8+ memory responses and secretion of IL-2.
4) CD8+ T-cells generated in the absence of CD4+ T-cells show reduced potential to respond to re-infection with a pathogen as they show reduced clonal expansion and reduced secretion of IFN-γ (Sun and Bevan 2003).
5) CD4+ T-cells secrete anti-viral factors such as IFN-γ.

Many pathogens however (especially viruses such as HIV, CMV and Hepatitis C) are sequestered within a variety of cell types where they can replicate, and thus avoid the extra cellular apparatus (such as antibody and macrophages) that would otherwise eliminate them. CD4+ cells, in general, do not have a role in targeting cells harbouring pathogens as they lack the capacity, on their own at least, to exert sufficient anti-viral activity against these important intracellular pathogens. This is because, on the whole they do not contain the cytolytic apparatus that CD8+ T-cells possess (see later). Although anti-viral immunoglobulin and other mechanisms can limit free transport of virus through the body, and thus limit the number of infected cells (at least to some extent), persistent infection (perhaps with progressive damage to infected host tissues through ineffectual immune responses and infection of host cells) will be all but inevitable unless infected cells can be identified with precision throughout the body and eliminated with a minimum of 'collateral damage' to healthy host tissues.

Although (as indicated earlier) NK cells are able to recognise and destroy infected cells (as downregulation of MHC class I may occur, for example, thus triggering NK cell activity), recognition and destruction of virally infected cells also falls within the remit of adaptive immunity. This role is necessary otherwise viruses could operate by stealth and be harboured in cells during their life and replication cycle This role falls in large part to CD8+ T-cells. CD8+ cells, like NK cells, elaborate molecules needed to destroy infected cells. These molecules include perforin and granzyme B. Perforin is a complex of proteins that can form channels spanning the membrane of infected cells. Granzymes are enzymes that can enter the cell through these pores and induce apoptosis. However an entirely different pathway for antigen processing exists for CD8+ T-cells to recognise infected cells. This is probably in large part due to the fact that recognition in the class II pathway requires endocytosed material. Intracellular pathogens, on the other hand, reside in the cytosolic domain (including the endoplasmic reticulum and golgi apparatus). Viral proteins have thus been constructed within the normal (albeit hijacked) cellular synthetic pathways, and are therefore not endocytosed material. Thus an alternative pathway for antigen presentation is required. This occurs as follows: even in healthy cells a portion of endogenous protein is broken down by a complex of cytosolic enzymes, called the proteasome. The peptide sequences thus generated are transported into the endoplasmic reticulum via a transporter protein known as TAP. The peptides can then be loaded onto MHC class I molecules and transported to the cell surface. MHC class I molecules are similar to class II molecules, but unlike class II molecules (which consist of a heterodimer of α and β chains) they consist of an alpha chain in conjunction with a

molecule known as β_2-microglobulin. Virtually all cell types express MHC class I on their surface, thus all cells have the capacity to present endogenous viral peptide sequences for recognition by CD8+ - or 'cytotoxic' T-cells. It is believed that naïve CD8+ T-cells, like CD4+ T-cells, can also be primed by dendritic cells in a process known as 'cross presentation' or 'cross priming'. This probably depends on the 'trafficking' of endocytosed material through the class I pathway, as direct infection of dendritic cells by pathogens does occur but only to a limited extent. (For a helpful disussion see (Bevan 2006)). This pathway is potentially of great importance as otherwise CD8+ T-cells would not benefit from the unique positioning and ability of dendritic cells to sample huge amounts of antigen crossing mucosal and epidermal surfaces and their ability to then migrate to lymph nodes to present antigen to a vast array of T-cells.

Recent evidence has suggested a 'feedback loop' which allows dendritic cells to be eliminated once sufficient numbers of effector cells are generated: Effector cells do not usually enter 'resting' lymphnodes' as they lack the '3-digit code' of receptor-ligand pairings that allow entry. Naïve and memory cells do contain this code which consists of: 1) L-selectin, (which binds to peripheral node addressin (PNAd)), 2) the chemokine receptor CCR7 (which binds to CCL21) and, 3) the integrin LFA-1 (which binds to ICAM-1). This 'code' excludes effector cells that might destroy potentially infected or MHC class I bearing dendritic cells before an adequate immune response is triggered. However, pro-inflammatory signals (for example TNF-α) are generated in lymphnodes containing licensed dendritic cells causing the lymphnodes to become 'reactive'. This results in upregulation of a marker known as CXCL9 on the venules draining lymph

nodes. This allows effector cells (that contain the apparatus capable of destroying cells bearing viral antigens) to enter as they express the chemokine CXCR3 (which binds CXCL9) and may curtail the activity of dendritic cells (and hence further immune activation) by destroying them. Previously it had been considered that the dichotomy of 'central' and 'effector' memory cells allowed effector cells to be segregated from the site where the immune response is orchestrated. Now it seems that effector cells may have a vital role in curtailing the immune response by access to lymph nodes that is normally reserved for memory and naïve T-cells (De Boer, Oprea et al. 2001; Guarda, Hons et al. 2007), thus preventing deleterious ongoing or secondary reactivation of the immune system.

1:2 Introduction to T-cell Homeostasis

Once a naïve CD8+ T-cell recognises antigen it is triggered to activate with multiple cycles of proliferation enabling an exponential increase in T-cells recognising the specific antigen. Experiments assessing this have indicated that up to fifteen divisions occur after stimulation – theoretically producing 2^{15} daughter cells from each naïve cell. (The number of divisions occurring on stimulation of CD4+ is far less than this – 2^9 (Seder and Ahmed 2003). This probably reflects the role of CD4+ T-cells in orchestrating the immune response, rather like 'officers' organising a huge army of 'infantry' CD8+ T-cells. In acute LCMV infection in mice, around eight such CD8+ T-cell responses are formed, ultimately accounting for between 80-95% of all CD8+ T-cells in acute infection (Masopust, Murali-Krishna et al. 2007). The response is therefore designed to combat the

pathogen (at a time when it may be poised to overwhelm the host having evaded innate immune responses) and thus further progression of infection is halted.

In the case of either CD4+ or CD8+ T-cell responses, as infection is surmounted, contraction of the antigen-specific T-cell population occurs with formation of a residual T-cell population, which forms the 'memory' population. Often the memory cell pool is still at a relatively low frequency (although considerably greater than that of the naïve cell precursors). Thus a rapid 'contraction' of the responding population is required, typically described as a loss of greater than 90% of the T cells (De Boer, Oprea et al. 2001). Contraction is required to stop huge and increasing expansion of T-lymphocyte pools with repeated cycles of infection and immune activation with increasing age. It is believed that 'contraction' of the response occurs through sequestration within the gut – which some suggest contains more lymphocytes than lymphnodes, liver and spleen put together (Rocha and Tanchot 2006). However, recent work indicates that the gut may contain fewer T-cells than previously thought (perhaps less than twenty percent of the total found elsewhere) (Ganusov and De Boer 2007). In addition a large number of lymphocytes undergo apoptosis and are destroyed in a process termed Activation Induced Cell Death (AICD) (Badovinac, Porter et al. 2002).

Despite returning to a low precursor frequency, (perhaps through a combination of apoptosis and sequestration), memory cells have, paradoxically, improved survival capacity compared to naïve cells. It is estimated that virus specific memory cell populations (even following vaccination, rather than actual infection) can survive 75

years or more (Hammarlund, Lewis et al. 2003). Memory cells are able to respond far more rapidly once an infection (or more specifically a peptide sequence from within it) is encountered again. This is accomplished in several ways (Rocha and Tanchot 2006):

1) Memory cells 'rest' at the late G1 phase of division whereas naïve cells are not in the cell cycle (ie they are at G0), and can therefore divide about 1-2 days earlier after stimulation than naïve cells. Memory cells contain lower levels of the inhibitor of DNA replication p27kip and high levels of cyclin D3/CDK6 complexes are retained in the cell cytoplasm, ready to translocate the neuclear membrane to promote DNA transcription.
2) Memory cells also survive the demands of rapid and fast mitosis better than naïve cells. The huge expansion of T-cells after activation takes a huge toll of cell death, because of breaks in DNA during synthesis that there is insufficient time to repair. This is limited to some extent in memory cells, although the mechanisms for this 'protection' are unclear.
3) A greater proportion of activated memory cells develop effector functions such as TNF-α and IFN-γ production. Overall this may increase up to a hundred fold, with 90% of cells producing IFN-γ mRNA (as opposed to 10% of naïve cells) and 100% producing TNF-α mRNA (as opposed to <1% of naïve cells). This is attained by epigenetic modifications that improve the accessibility of cytokine and presumably other genetic loci. For example, in memory cells demethylation of the IFN-γ locus is found. In addition key intermediaries of the signal-transduction pathway of the T-cell receptor (such as CD3ε and ZAP-70) are pre-phosphorylated. In addition, memory T-

cells have larger lipid rafts, (aggregates of cell membrane proteins) which might enable more rapid and better organised responses to activation.

Cytokines, particularly the Common Cytokine Receptor γ-Chain Cytokines, or CD132 cytokines (which include Interleukin 2 (IL-2), IL-4, IL-7, IL-15 & IL-21) are likely to have an important role in shaping the course of T-cell responses through effects on the initiation, clonal expansion, contraction and memory-generation phases (Schluns and Lefrancois 2003; Waldmann 2004). For example, IL-2 produced by central memory T-cells is able to promote positive autocrine effects on T-cell proliferation. In addition, and paradoxically, IL-2 augments AICD and is therefore proapototic. Thus it may also be important in modulating the contraction phase. IL-15 also promotes T-cell proliferation and acquisition of effector functions. Mice deficient in IL-15 or IL-15 Receptor alpha chain (IL-15Rα) have reduced numbers of memory cells, suggesting the IL-15 pathway might counter the pro-apoptotic effects of IL-2 in the contraction phase of responses (Waldmann 2004). What is perhaps surprising is that IL-15 can cause proliferation of naïve T-cells, loss of their distinctive phenotype, induction of cytotoxic activity and production of pro-inflammatory cytokines, in the absence of T-cell receptor triggering (Alves, Hooibrink et al. 2003). Thus it is likely that T-cell activation and distribution into their functional subsets and development of effector functions can be autonomous, or at least semi-autonomous, of triggering of the T-cell receptor by cognate antigen and is promoted by a microenvironment containing pro-inflammatory signals (such as IL-15).

I have, in this thesis, used the term T-cell homeostasis broadly to mean T-cell regulation before, during and after infection, and not to mean specifically the 'housekeeping' of T-cell pools that must presumably occur in the absence of an infection. 'Housekeeping' of T-cell pools clearly occurs, as, after T-cells are depleted artificially, proliferation of T-cells occurs, allowing reconstitution of T-cell pools with naïve cells. IL-7 may mediate this effect (Fry and Mackall 2005), whereas IL-15 promotes differentiation of non-naïve T-cell pools to 'effector' status. Interestingly, the effects of IL-7 and IL-15 were not seen in MHC class I deficient mice. This indicates that T-cell pools may be maintained by exposure to self-antigens, or at least by (weak) non-antigen specific TCR-MHC interactions, or perhaps Killer Inhibitory Receptor (KIR)-MHC interactions (discussed in due course) with class I molecules in general (Cho, Boyman et al. 2007). It certainly is unclear what the relative contributions each of these different classes of interaction make to the T-cell pool.

1:2:1 Selection of T-cells into memory pool

Although activated effector cells that form memory cell populations develop features that protect them against cell death (see earlier), most activated cells are far more susceptible to cell death (or possibly sequestration (Johansson-Lindbom and Agace 2007)) compared to naïve cells. Thus mechanisms presumably exist to polarise effector cells in to two groups: (1) a relatively small number that will become memory cells, and (2), the majority that will die (or be sequestrated). The factors involved in the process of 'polarisation' are vital to the healthy functioning and maintenance of the immune

response, but are not understood well. Some clues to the possible underlying molecular mechanisms (or broad strategies) employed by the immune system exist. These include:

1) Patterns of expression of Killer Immunoglobulin-like inhibitory receptors (KIRs) These inhibitory natural killer cell receptors enable healthy cells to inhibit NK cells as they bind to MHC class I (thus protecting themselves from NK-cell mediated cytotoxcity). It has been suggested that these may have an important role in modulating the function of T-cells as particular host KIR genotypes have been related to progression of HIV. This may be through allowing synergy between class I bearing T-cells, and particular KIR's (on NK cells) or because of the presence of KIR on CD8+ T-cells (rather than on NK cells). These possibilities are suggested by the finding of linkage disequilibrium of KIR alleles with MHC class I alleles, and, through further linkage analysis, that their presence may explain the association between specific class I alleles and HIV progression (Altfeld and Goulder 2007), at least in some cases. Specifically, in the context of T-cell homeostasis, it has been proposed that KIR+ CD8+ T-cells may be resistant to AICD, are relatively few in number and are therefore ideally placed to become 'memory cells' (Young 2001; Young and Uhrberg 2002).

2) Expression of CD8+ alpha homodimers on CD8+ T-cells, rather than the normal CD8alpha/beta heterodimers can occur resulting in the generation of CD8α/α T-cells (Madakamutil, Christen et al. 2004). This might promote memory cell development,

possibly through binding of the CD8α/α homodimer to an MHC class I like molecule known as 'TL' (Romero, Cerottini et al. 2005).

3) Apoptosis induced by binding of Fas Ligand (FasL) to Fas (CD95) may require distribution of this molecule into lipid rafts (Muppidi and Siegel 2004). Generally lipid rafts enable conglomerations of multiple receptors of the same or different varieties, enabling their activity to be coordinated and synergised effectively. Thus cells that do not generate CD95+ lipid rafts may be relatively protected from AICD, and this may enable their survival as memory cells.

4) CD8+ T-cells produce molecules capable of inducing cell death in target cells. These include Fas, and in addition the Granzymes (A and B) and perforin. Perforin can form a channel through the target cell membrane allowing entry of Granzyme molecules that can induce apoptosis. Interestingly, although specific CD8+ T-cell subsets contain significant levels of perforin and granzymes when stained directly *ex vivo* (and are contained in intracellular cytosolic 'granules'). It is, however believed that preformed molecules may not be critical in mediating target cell death (Isaaz, Baetz et al. 1995). Fas binds to FasL on infected cells, inducing apoptosis. Interestingly as indicated above, although Fas is a T-cell 'effector molecule', Fas can 'turn on' the T-cell that synthesised it: 'Fas' deficiency, in a disease known as auto-immune lymphoproliferative syndrome (AILS), results in over-proliferation of T-cell subsets. This suggests that the process of AICD may be impaired. The mechanism for diseases in AILS may be as follows: FasL is expressed on the cell surface after degranulation

(Bossi and Griffiths 1999), and in healthy persons may lead to death of T-cells through exposure to Fas. If Fas is deficient (as in AILS) T-cells will continue to be active and proliferate. T-cells do however undergo some cell death in these patients, indicating that a compensatory mechanism exists. There is now evidence that this compensatory mechanism is dependent on Granzymes and Perforin, perhaps preformed in granules (Mateo, Menager et al. 2007). These molecules (perforin, granzymeB and Fas-FasL) may therefore have a role in regulating T-cell responses in healthy persons. Thus the process of delivering a large number of potent effector cells is dependent on them operating 'Kamikaze' style, and committing suicide as they deliver their death sentence, or perhaps even before they fail to identify a target cell within a pre-defined time window. This would neatly explain why only T_{CM} subsets (which contain little, if any, perforin or granzymes) appear to be left behind after immediate immune responses.

5) Expression of the IL-7 receptor component IL-7Rα may occur in a small proportion of activated CD8+ T-cells and select them for future memory functions (Huster, Busch et al. 2004). Interestingly, epithelial cells in the intestine secrete large amounts of IL-7, and this may have considerable effects on T-cells that may be sequestered into the intestine. This may favour long term survival of memory T-cells in the intestine (Yang, Sun et al. 2007).

However, it is notable that in each example cited (as well as not being mutually exclusive) the factors that determine which cells are 'marked out' for death or, on the

other hand, survival as memory cells (through expression of CD8α/α, Kir's, IL-7Rα or CD95 distribution into lipid rafts, for example) are unclear. The process may be stochastic. However, models proposing the basic pathways and strategic mechanisms have been proposed:

1) The strength of signal received may determine which cell populations become memory cells - the model of Sallusto et al. This is a non-linear model (Gett, Sallusto et al. 2003).
2) There may be a linear pathway (T_{NAIVE} → $T_{EFFECTOR}$ → T_{MEMORY}), where memory cells develop from the last generation of daughter cells (Model of Ahmed et al (Opferman, Ober et al. 1999)). Thus three cycles of division allow acquisition of effector markers, whereas five may be needed for acquisition of memory status (as shown by inoculation of these cell populations into naïve hosts). However this model would predict large numbers of memory cells as the exponential population expansion means that the latter cycles of replication would result in largest numbers of daughter/ memory cells (Unless only small proportion of effector cells are 'selected' for the final cycles of replication to memory status).
3) It is possible that effector cell and memory populations develop independently of each other after priming of responses. This is the model of Pannetier et al. This model is supported by the observation that only around five percent of TCR sequences are shared between central and effector memory cells in healthy persons, indicating that they contain T-cells with different TCR's and hence that are of different specificities (Baron, Bouneaud et al. 2003). This is consistent with the signal-strength model of

Sallusto et al, but other factors may also determine the fate of T-cell populations. Some studies suggest that the local balance in concentration of IL-2 and IL-15 may be important. Thus in the presence of large amounts IL-2, generation of large numbers of 'effector' CCR7-ve cells is favoured. However if IL-2 concentrations are low, or if there is additional IL-15, then formation of CCR7+ memory cells is favoured (Sallusto and Lanzavecchia 2001). It is possible also the specific tissues from which activated T-cells derive determines their phenotype when they traffic back into blood. Thus intra-epithelial lymphocytes in the gut assume a CD62L low phenotype predominantly (ie effector memory phenotype), whereas those that migrate to the spleen do express CD62L (ie central memory phenotype) (Masopust, Vezys et al. 2006). This study showed also that the status of T-cells was mutable and could be modified by transfer to a different lymphoid organ.

Recent evidence indicates that T_{EM} cells can develop from T_{CM} cells but not vice versa, as adoptive transfer of antigen specific T_{CM} cell clones into immune-naïve macaques can construct a long-lived memory pool, but transferred T_{EM} clones were unable to mediate this effect. This indicates that T_{EM} cells are short lived and probably do not confer protective immunity (Berger, Jensen et al. 2007). However this is consistent with a unidirectional linear model of T-cell differentiation, as opposed to a bidirectional model of T-cell differentiation.

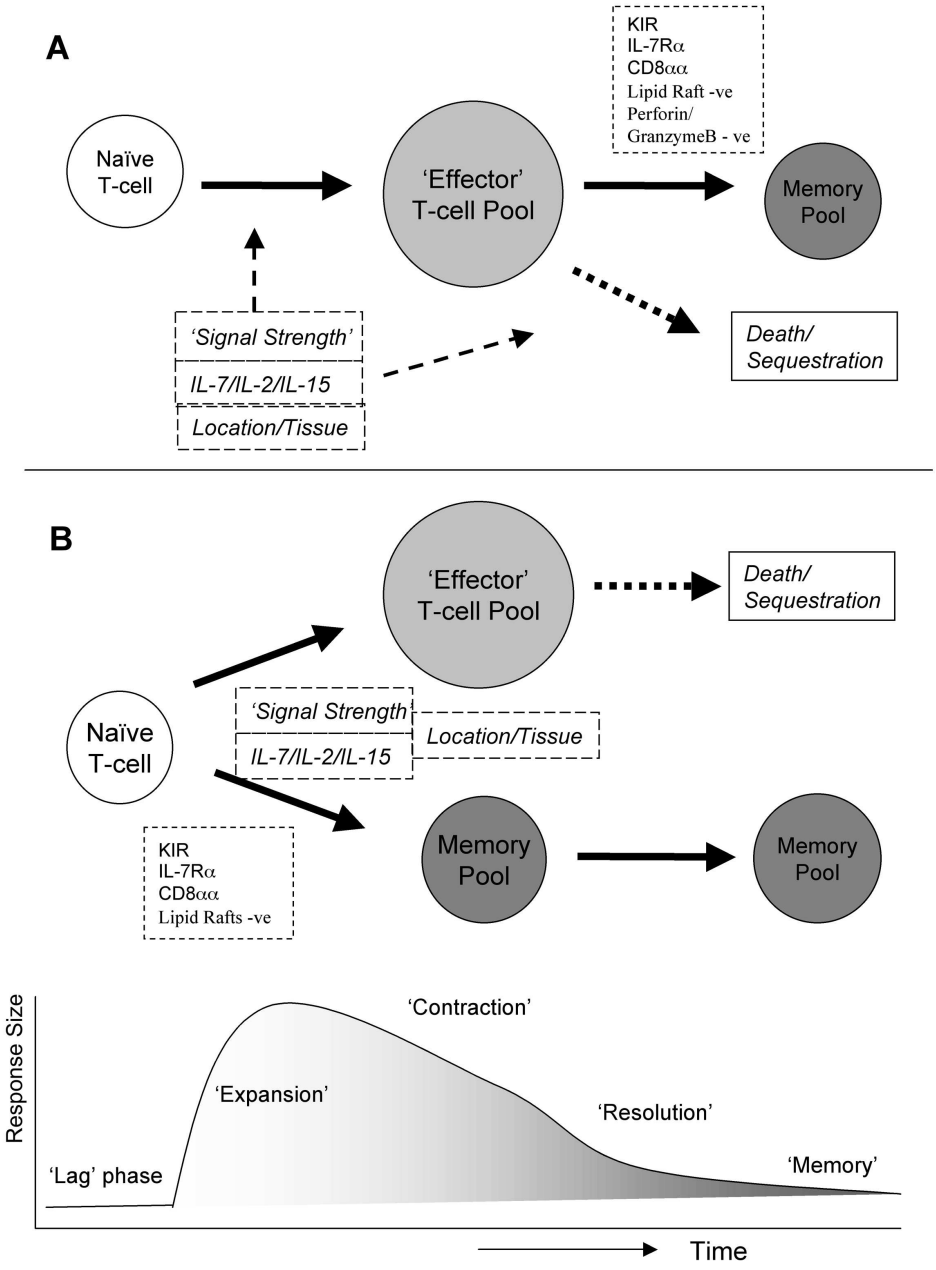

Figure 1:1: Diagram outlining modes of T-cell differentiation. There are two main potential modes of T-cell differentiation: A (Top) indicates a 'linear' model of T-cell differentiation. B (middle) indicates a 'branched' model of T-cell differentiation. Within both models there is scope for an influence of 'signal strength' of T-cell stimulation by APC's, cytokines and the site of T-cell stimulation/ differentiation to modulate T-cell differentiation. This may affect the size of the response and that of the residual memory population (as well as its other functional and phenotypic characteristics). Expression of KIR, IL7Rα and CD8αα and absence of perforin/granzyme B and lipid rafts may serve to 'predetermine' the fate of particular cells as future memory populations, by enabling them to avoid death (apoptosis/AICD) and/or sequestration. Below a typical time course of an immune response is superimposed.

1:2:2 Diversity of anti-viral T-cell functions:

The mechanisms regulating overall control of the various phases of the immune response have not been fully worked out. However a key (although not entirely validated) principle that underlies organisation of the T-cell response, including the memory response, is the functional diversity of different T-cell populations. Central to this is the proposed dichotomy of 'central memory' (T_{CM}) and 'effector memory' (T_{EM}) cells. Initially these were defined and characterised by Sallusto et al (Sallusto, Lenig et al. 1999) amongst CD4+ and CD8+ cells using florescence activated cell sorting (FACS) and surface staining with monoclonal antibodies. Later combined staining with two markers enabled identification and characterisation of four populations. The two markers used were the lymphnode homing marker CCR7, and a specific splicing variant of CD45, the leucocyte-common antigen CD45RA (Tchilian and Beverley 2006). The four populations have thus been characterised as follows:

1) Firstly, CD4+ and CD8+ naïve cells were identified and sorted for expression of CD45RA and CCR7 (CCR7+CD45RA+). They showed prompt and extensive proliferation and secretion of interleukin-2 (IL-2) (in response to polyclonal, non-antigen specific stimulation). Because naive cells express CCR7 and L-selectin (CD62L), (receptors that enables them to migrate to lymphnodes), they are ideally placed to liase with dendritic cells for priming during the early phases of the immune response.
2) CCR7+CD45RA- cells (for both CD4+ and CD8+ T-cells) shared the functional properties of naïve cells (ie high proliferation, IL-2 secretion and lymphnode

homing capacity). Like naïve cells they thus express high levels of lymph node homing markers, presumably enabling them to circulate through lymphoid tissues. Unlike naïve cells however, they are antigen experienced, and are known as 'central memory' or T_{CM} cells. As memory cells they respond quicker and more effectively to antigen re-exposure (as outlined earlier)

3) CCR7-CD45RA- cells, showed reduced production of IL-2 but high production of IFN-γ. In addition, for CD8+ cells, only CCR7- cells express intracellular perforin. These are 'effector memory', or T_{EM} cells. T_{EM} (and T_{CM} cells) do express an alternative splicing variant of CD45, known as CD45RO (but not CD45RA itself).
4) CCR7-CD45RA+ cells are found, in the main, amongst CD8+ T-cells. It is believed that they are T_{EM} cells that have re-expressed the CD45 splicing variant found on naïve cells. It is unclear what the functional consequence of CD45RA 'reversion' is. However Sallusto et al found that they had abundant perforin – more so than other CCR7- T_{EM} cells. They are known as T_{EMRA} cells.

The different functions of 'central' and 'effector' memory cells *in vitro* may enable them to perform complementary roles in recall responses. T_{EM} cells may be available to respond immediately to kill infected cells and halt viral infection in its tracks, whereas T_{CM} may be able to generate large numbers of T_{EM} cells (whose existence could not be supported in the long term in health) to reinforce the initial response and perhaps generate further memory cells ready for future episodes of infection. However, T_{EM} may not show

long term survival in the absence of ongoing generation of this sub-population via stimulation of T_{CM} cells with persistent antigen.

Recent work from murine models (Hikono, Kohlmeier et al. 2007) suggests that the relationship between 'memory status' and immunological function, at least in its' simplest expressions, may be simplistic. Thus CCR7 and CD62L simply define the capacity of T-cells to re-circulate through lymphoid tissues. Other markers, for instance CD43 and CD27 may also define populations of cells that have differential capacity to respond to re-challenge (Hikono, Kohlmeier et al. 2007). In fact in this study, two years after infection with the self-limiting murine respiratory pathogen known as Sendai virus, virtually all of the memory response (greater than ninety percent - compared to around thirty percent one month after infection), is $CD43^{lo}/CD27^{hi}$, a cell type that seems to be consistently associated with greater capacity to respond to re-challenge. Finally CD127 (or IL-7Rα) and KLRG1 are a further pair of markers that can be used to define T-cells in humans. Thus memory CD8+ T-cells to resolved infections (such as influenza, which does not cause persistent infection) are $CD127^{hi}/KLRG1^{lo}$ (indicating that they are memory cells), whereas those to persistent infections (such as HIV) are $CD127^{lo}/KLRG1^{hi}$ (Ibegbu, Xu et al. 2005; Bengsch, Spangenberg et al. 2007).

In fact there is a huge array of cell surface markers that can be readily studied, with hugely divergent functions. These can be reciprocally or exclusively expressed meaning that unravelling those markers with direct importance in T-cell functions, and hence classification, is very difficult. A lot of preliminary work on T-cell classification was

driven when there was an inability to study multiple markers simultaneously, leading to inevitably simplistic conclusions, thus most classifications use only a pair of phenotypic markers. Polychromatic flow cytometry that enables far larger numbers of markers to be studied simultaneously is now available but unravelling the interrelationships between overlapping T-cell subsets and their link to function *in vivo* across the entire time period of infection is extremely difficult (De Rosa, Herzenberg et al. 2001). These concerns are echoed in chapter five of this thesis, where we describe a marker (CD161) that is restricted in its expression to Hepatitis C specific T-cells, or at least responses to specifically hepatic infections. Nevertheless this thesis argues overwhelmingly in favour of T-cell phenotype being a powerful concept to explore T-cell biology, when it is rightly understood. In chapter three I show that phenotype is linked to the magnitude of responses and the composition of lymphocyte pools in blood, and in Chapter four I link future control (or lack thereof) of HIV, with CD8+ T-cell phenotype in early infection. It seems likely that different T-cell populations exist with different functions, but current analysis is akin to a blindfolded city dweller trying to analyse the functions of animals and equipment in a country farm using only touch, and only one touch for every scientific paper! However, a pragmatic and critical approach to the concept of T-cell phenotype may ultimately unlock the doors to the many of the many mysteries of T-cell biology. It also remains possible that receptor-ligand pairings, when evaluated appropriately, may allow a far greater resolution and discrimination of functions for different T-cell populations than currently recognised.

1:3 T-cells and Chronic Viral Infections

1:3:1 The Host Response:

The outline above refers, in the main, to immune responses following acute and resolving infection. The three viruses, Cytomegalovirus (CMV), Hepatitis C (HCV) and Human Immunodeficiency Virus (HIV-1), or rather their counterpart virus specific immune responses, that are the focus of this thesis are characterised by their ability to cause persistent infection. In the case of CMV this takes the form of latent infection when, although circulating virus is not detectable in blood, reactivation of infection can occur if immunosuppression supervenes. This suggests that the immune system is actively and continuously involved in detecting and suppressing infection. In the case of HIV and HCV, high levels of viral replication continue after acute infection, with the immune system perhaps limiting, but by no means containing infection. Thus the classical sequence of events following acute infection, (lag-phase, expansion, plateau, contraction and memory phase), are unlikely to be applicable in terms of understanding both the generation of immune responses and immune system homeostasis in health and disease in both chronic viraemia and latent infection.

What is clear, however, is that immune responses in chronic infection with HIV and HCV do not reach the scales seen in acute infection, with the capacity for ongoing exponential growth. From a general perspective it is unclear whether the responses seen in chronic phases of infection are designed specifically - arguing teleologically - to meet the circumstance of chronic infection, perhaps effectively reaching a '*coup detente'* with the

invading pathogen (Wherry and Ahmed 2004). This could be beneficial to the host as it may limit the physiological demands of maintaining an immune response at peak capacity, and also limit 'collateral' host damage and immunopathology. Otherwise the 'immunological solution' may be more harmful to the host than the pathogen itself. Alternatively immune responses in chronic infection, especially for HIV (and perhaps HCV) may represent to a large extent a 'hangover' from failed acute immune responses, with the patterns of responses and observations that can be made on the immune system in general simply reflecting an immune system 'struggling to pick up the pieces' following a successful challenge by the pathogen. In any case there is evidence that although having clearly failed to completely repel the invading pathogen, immune responses continue to limit viral replication and may therefore limit the resulting disease severity.

Several observations have been made to document differences in response between those in acute and chronic infection (Wherry and Ahmed 2004). Thus responses with different peptide specificity come to the fore in chronic infection. Secondly the tissue distribution of T-cells may vary with a greater tissue density of T-cells in chronic infection. Thirdly CD8+ T-cells may become 'exhausted', with progressive loss of effector functions from cytotoxic activity, to IFN-γ secretion, and finally loss of proliferative responses.

Recently the cellular and molecular mechanisms underlying T-cell 'exhaustion' have been elucidated. Thus chronic immune activation is associated with the activity of regulatory T-cells. These are specialised CD4+ T-cells that are high in expression of

CD25 ($CD25^{HI}$) and contain an intracellular transcription factor 'foxP3'. Their presence is associated with poor proliferative capacity to antigen *in vitro* (Rushbrook, Ward et al. 2005). A role in HIV (Aandahl, Michaelsson et al. 2004), and HCV infection (Rushbrook, Ward et al. 2005) has been proposed. In addition a molecule known as programmed death -1 (PD-1) has been found on CD8+ T-cells in chronic infections. Blockade of this molecule with antibody appears to restore T-cell functions in a striking manner (Sharpe, Wherry et al. 2007).

1:3:2 General Strategies for Immune Evasion By Persistent Viral Infections:

Generally viruses seem to fall into two main groups termed 'small' and 'large', as their physical size appears to directly relate to their capacity to adopt various escape mechanisms (Lucas, Karrer et al. 2001). 'Small' viruses, such as HIV, HCV and, in the mouse, lymphocytic choriomeningitis virus (LCMV) can, by virtue of their size, replicate extremely rapidly, enabling them to rapidly obtain a foothold in the host. Thus they can escape host defences because of 'speed'. In addition they usually have a high propensity to escape T-cell responses, through mutation, or 'shape change' which enables them to change the antigenic determinants recognised. On the other hand 'large' viruses such as CMV and others do not replicate so rapidly and thus do not generate such a large number of escape mutations. However their size is, in part, due to a large amount of molecular apparatus that enables them to hide from immune responses ('camouflage') and also subvert immune responses ('sabotage').

1:3:3 Human Cytomegalovirus Infection:

Cytomegalovirus (CMV) is a herpes virus that has established an extensive biological niche in humans. Acute infection is usually asymptomatic and occurs in up to fifty percent of individuals, as evidenced by high rates of seropositivity in healthy persons. CMV lies dormant after acute infection without any clinical manifestations unless immunosuppresion supervenes (i.e. in persons with AIDS, or those who receive immunosuppressant drugs). Thus CMV has the ability to survive host immune responses and remain 'latent' for many decades. As a 'large' virus CMV has the ability to avoid host responses in the long term. Multiple molecular pathways enable the virus to survive (Michelson 1999). For example CMV can hide from immune responses through expression of UL18. This binds to the inhibitory receptor LIR-1 on NK cells and CD8+ T-cells and thus inhibits NK-mediated cytotoxicity of infected cells (an example of 'sabotage'). In addition UL83 blocks processing and presentation of the CMV protein 'IE', thus avoiding T-cell recognition (camouflage).

1:3:4 Human Immunodeficiency Virus:

HIV is a retrovirus that can replicate extremely rapidly and reaches over 10^6 copies per millilitre of blood three weeks after infection. It has an error-prone reverse transcriptase (that provides a DNA template for protein synthesis from viral RNA). This enables novel mutants to be generated with ease – each with the potential to enable the new mutant to escape immune recognition, at least in part. By targeting CD4+ T-cells for destruction, especially in the gastro-intestinal tract (Guadalupe, Reay et al. 2003), it can also 'sabotage' the immune system.

1:3:5 Hepatitis C virus:

Hepatitis C is a RNA virus, meaning that not only does it have no need to store its genetic information as a hard copy in the form of DNA, it also does not need to generate a DNA template after infection. Thus HCV replication is done in a 'slap dash' but extremely rapid manner, with up to a 10^9 virus particles formed per day, even in chronic infection, with a high propensity of these to be novel mutants (Neumann, Lam et al. 1998). The virus actually exists in the infected host as a swarm of different strains, or 'quasispecies' (Liu, Netski et al. 2004), and each quasi species can mutate extremely rapidly. This obviously poses severe difficulties to the host immune response, as even the rapid immune responses generated as described previously seem like a slow hammer liable to constantly miss a large number of ever moving nuts!

1:4 Outline of Thesis

In the face of the emerging picture of hugely dynamic and complex host immune defences locking 'horns' with highly aggressive and adaptable pathogens, it would perhaps be surprising if measurements derived from the immune system were to correlate with important biological parameters. Such relationships are important to evaluate however as they may yield insights with scientific and ultimately clinical relevance.

1) In chapter 3 of this thesis I evaluated the hypothesis that T-cell phenotype (a measure of T-cell differentiation) was linked to the overall size of CD8+ T-cell responses to 'latent' CMV. (Published as (Northfield, Lucas et al. 2005)).

2) In chapter 4 I tested the hypothesis that CD8+ T-cell phenotype in early infection would predict future control of infection (determined by the level of viral replication in established chronic infection – the set point). (Published as (Northfield, Loo et al. 2007))
3) In Chapter 5 I describe expression of a novel marker on HCV-specific T-cells called CD161, and argue that its' expression may relate to the presence of a distinctive form of response. The response be functionally limited and this may therefore explain, in part, why it is unable to curtail HCV replication in most cases. (Published as (Northfield, Kasprowicz et al. 2008).

Chapter 2: Methods.

In this chapter I will outline in detail the basic methods that underpinned this thesis. These are extended in each chapter in line with their particular emphases. The thesis, broadly speaking, utilised methods in cellular immunology and mainly used fluorescence activated cell sorter (FACS) based techniques, such as phenotypic analysis itself, tetramer staining, intracellular staining and functional assays. In addition cell sorting using magnetic beads was utilised. The experiments were generally performed in a category III glass cabinet in category III laboratories to avoid any risk of infection, and also to ensure sterility of the samples (which is especially important for cell culture).

2:1 Cell Separation:

Isolation of peripheral blood mono-neuclear cells (PBMC's) was performed on fresh blood or buffy coats. Initially 15mls of lymphoprep (Invitrogen) was placed in labelled 50ml tubes. Blood was diluted 1:1 or 2:1 with RPMI (Sigma-Aldrich) and then 20-25mls of blood was layered onto the lymphoprep gently using a battery-powered 25ml pipette. The 50ml tubes were then placed in a centrifuge and spun at 2200rpm for 22mins. The tubes were then collected and the PBMC layer was carefully aspirated using a 5ml pastette. The PBMC's were washed twice in 25mls of R10 (RPMI 10% supplemented with foetal calf serum (FCS), L-Glutamine, Penicillin and Streptomycin). The cells were then counted using trypan blue and a cell counting chamber or slide. If the cells were not used fresh they were frozen. For cell freezing the cell pellet (after washing and

centrifugation) was mixed with FCS with 10% DMSO) at a concentration of 10-20^6 cells per ml and placed in cryovials (1ml of cell suspension per vial). The cells were then transferred to a -80°C Freezer, where they were stored for a 2-3 days before transferring to the liquid nitrogen freezer (-150°C). Cells were thawed immediately prior to use by removal from liquid nitrogen and thawed rapidly in a 37°C water bath. The vials were immediately opened carefully and 1ml of R10 was placed in the tube and gently mixed using a 1ml pipette. The solution was then aspirated and placed in 10mls of R10, and washed twice, before counting and assessment of viability.

2:2 Tetramers and Tetramer staining of T-cells:

Tetramers allow quantification and phenotypic analysis of antigen specific T-cells directly *ex vivo*. The basic principle is to provide fluorescently labelled antigen-class I complex specific for the TCR of the T-cell population under study. Monomers of class I do not stain samples cells reliably. Thus tetramers of class I are used in order to induce stable contacts with the T-cell membrane and possibly internalisation as well (Klenerman, Cerundolo et al. 2002). Their strength as a tool is to allow precise quantification and *ex vivo* analysis of specific T-cell populations. Their limitation is that without multiple tetramers it is impossible to characterise the breadth of response to a specific pathogen. In addition, if the response does not recognise the consensus sequence of the pathogen (for example it may recognise an 'escape' sequence) then the T-cell response will not be identified.

The tetramers were either purchased from Beckman Coulter, Pro-immune (as pentamers), or synthesised 'in house'. For this I acknowledge with great appreciation Andrew Loughry and Allison Turner who prepared tetramers for use. The basic protocol uses MHC class I heavy chain synthesised in recombinant *E. Coli*. The –COOH terminus is engineered to allow biotinylation. The heavy chain is then refolded with peptide and β2-microglobulin. The monomers thus formed are then tetramerised after biotynilation and addition of streptavidin. A flourochrome is then added, such as Phycoerythrin or Allophyocyanin.

Tetramer staining was performed by transferring cells to a 5ml FACS tube or a 96 well plate, with 0.5^6 – 2^6 cells in 50 - 100μL of Phosphate Buffered Saline (PBS). Pre-titrated amounts of tetramer (or pentamer) were added and the cell suspension gently mixed. The cells were then transferred to a 37°C incubator for twenty minutes. The cells were then washed in PBS. They were then ready for phenotype staining. Wash steps were performed by centrifugation at 1500rpm for 5 minutes.

2:3 Antibody Staining:

For antibody staining cells were transferred to a 5ml FACS tube or a 96 well plate, with 0.5^6 – 2^6 cells in 50 - 100μL of Phosphate Buffered Saline (PBS). Antibody was then added in amounts determined after prior titration. Cells were then placed at 4°C for twenty minutes. Antibody was then washed off using PBS. (Two washes were needed if staining was performed in a 96 well plate). The cells were then fixed with 1% paraformaldehyde (PAF) and then stained.

2:4 Intracellular staining:

Intracellular staining was performed frequently to allow for staining of cells for Ki67 (a marker of cell proliferation), Perforin and Granzyme B, and intracellular cytokines (after stimulation of cells). I used eBiosciences permeabilisation buffer. Permeabilisation buffers rely on the presence of Saponin which increases permeability of the cell membrane, allowing the antibody to enter. The buffer is toxic so the cells are first fixed in 4% PAF with incubation at room temperature (RT) for twenty minutes. Permeabilisation buffer is then added with antibody in pre-titrated amounts and the cells incubated for a fifteen minutes at room temperature. The cells are then washed in 1% PAF to remove excess antibody and permeabilisation buffer.

2:5 Whole blood staining:

Chapter 3 used whole blood for FACS experiments. This relies on a buffer that lyses red cells but is able to leave PBMC's intact. I used a commercial buffer from BD. The lysis step was performed after tetramer and antibody staining. Washed cells are spun down and excess supernatant carefully aspirated. 250µL of dilute cell lysis buffer is added and the cells suspended within it. The samples were then left at RT in the dark for ten minutes. The plates were then centrifuged and the lysis buffer aspirated. Residual lysis buffer was then washed off with PBS in a further two wash steps, and the samples fixed in 1% PAF

2:6 Intracellular Cytokine Staining (ICS):

This usually used non-antigen specific stimulation using phorbol 12-myristate 13-acetate PMA (1µg/ml) and Ionomycin (1µg/ml), except in chapter 4 where HIV peptides were

used (and Staphylococcal enterotoxin B (SEB) as a positive control), and chapter 5 when a FLU peptide was used. Staining was performed in 96 well plates. Cells were placed in 250μL of R10 in the wells, and PMA/I added. The cells were then incubated for five hours at 37°C. After 1 hour 'golgistop' (BD) was added. This contains monensin, a chemical that stops protein transport within T-cells. Cytokines are therefore retained within the golgi apparatus (rather than secreted) and are thus amenable to intracellular staining. At the end of incubation cells were washed and intracellular staining was then performed.

2:7 FACS analysis:

Usually a FACScalibur machine was used (BD). (In chapter 4, however a LSR II cytometer was used for polychromatic flow cytometry - PFC). A FACS machine works by streaming of cells so that they passage individually through laser beams. Initial scattering of light can either be 'forward' (forward scatter – FSC) or 'sideways' (side scatter - SSC). The extent of FSC correlates with the cell size and SSC correlates with the granularity of cells contents. Readings of FSC and SSC for each cell are placed on a dot plot which allows resolution of specific PBMC populations. Thus granulocytes, monocytes and lymphocytes can be examined separately for other characteristics. Use of different fluorochromes allows staining with up to four different antibodies (or a tetramer and three antibodies) simultaneously for each cell. The flourochromes used were usually fluorescein isothiocyanate (FITC), Phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and Allophycocyanin (APC). The FACScalibur machine uses two lasers of different light wave length to identify fluorescence for all these flourochromes. The

fluorescence caused by the laser is detected with four different channels (designated fl-1, fl-2, fl-3 and fl-4). These channels can be adjusted in order to resolve the wavelength of emitted light specific for each flourochrome into its' specific channel. Adjustment is referred to as compensation, and is important as it is vital for independent resolution of the four flourochromes studied and the signal intensity to be measured specifically within each channel, without leaking into neighbouring channels. Compensation is performed either with beads or cells labelled for each flourochrome on its own in turn, to check that the other channels do not 'pick up' the flourochrome. Generation of sample data is termed 'sample acquisition'. PFC follows basically the same principals but the LSR II has four lasers to allow for multiple channels, and requires additional flourochromes. Flourochromes can be joined to form 'tandem conjugates' to generate a larger number of flourochromes. The compensation process is so complicated that it is performed by software once the samples are acquired, and it can adjust for imperfections in compensation for the different channels. (FACSdiva (BD) software is used initially during acquisition, and Flowjo (Tree-star) software once the data has been acquired). The LSR II also requires preparation of fluorescence minus one (FMOs). FMO's are samples stained with all the flourochromes except one. Thus 1 FMO is formed for each study flourochrome). Flowjo performs compensation provided that the correct FMO's and single stains have been performed. Cellquest can be used for the FACScalibur acquisition and file analysis, but not for the LSR II, whereas Flowjo can be used for both.

2:8 Cytometric Bead Array (CBA):

This was used in Chapter 5. It is a FACS based technique that allows quantification of multiple cytokines simultaneously in relatively small aliquots of serum or culture medium. It utilises beads coated with antibody to the cytokine studied ('capture' beads). Usually six bead types are mixed allowing six cytokines to be studied. They can be visualised differentially on the cytometer as they have variable intrinsic fluorescence conveyed by a flourochrome. Addition of a mixture of antibody to the cytokines studied (conjugated to Phycoerythrin – PE) allows the amount of cytokine 'captured' on the beads to be inferred. The kit is calibrated using samples with known dilutions of cytokines (the standards). A simple software program models the flourescense – [cytokine] relationship and enables the raw data to be transformed into data on [cytokine].

Briefly, standard cytokine dilutions were prepared using recombinant cytokine provided with the kit. Beads were also mixed, and 25µL of beads was then transferred to the wells (1 well for each test sample and standard dilution). 25µL of a PE detection reagent was also added, and then 25µL of each sample and standard dilution was added. The samples were then incubated for 3 hours at RT. The samples were then washed to remove excess reagent and sample. Special beads allowed the cytometer to be set up with careful compensation for fl-1 to fl-3. This allowed precise resolution of each bead population, according to the software requirements.

2:9 CMV Enzyme Linked Immunosorbant Assay (ELISA):

I used an ELISA from IBL Hamburg to determine CMV serostatus in chapter 3. Samples were diluted 1:100 with sample diluent. These were then added to CMV-antigen coated wells in the ELISA plate, along with substrate controls. The plate was then coated with foil and incubated for 1 hour at 37°C. The wells were then aspirated and washed three times with 300µL washing solution. Excess washing solution was removed by tapping the plate flat on to tissue paper. 100µL of CMV anti-IgG Conjugate was then added except for the 'blank' well and the plate was then incubated for 30minutes at 37°C. The plate was then washed as before. 100 µL tetramethylbenzidine/hydrogen peroxide (TMB solution) was then added and the plate incubated for 30minutes at 37°C. 100µL of sulphuric acid was added to the wells to 'stop' the TMB solution. The plate was then read immediately in a BioRad 550 plate reader. The plate reader was set to zero using the substrate blank, and absorbance read at 450nm. Determination of seropositivity was then used using the cut off controls provided with the kit.

2:10 Cell Sorting:

Miltneyi-Biotec magnetic beads were used for isolation of CD161+ and CD161- cells in Chapter 5. This method takes advantage of magnetic microbeads that can adhere to PBMC's by virtue of attached monoclonal antibodies for the marker(s) in question – magnetism assisted cell sorting (MACS). The PBMC's can then be passed through a column containing beads which is attached to a magnet. The microbeads with the targeted cells are retained in the magnet, and the remaining PBMC's are then gathered underneath

the column – 'negative selection'. Removing the column from the magnet and a flush of fluid through it allows the selected cells with the attached beads to be gathered – 'positive selection'.

I used the CD8+ T-cell isolation kit II for isolation of pure CD8+ T-cell populations. This kit uses 'negative selection' of CD8+ T-cells. The kit contains a cocktail of antibodies to non-CD8+ T-cell PBMC's. Thus it contains biotinylated antibodies for CD4, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (glycophorin A). The kit thus removes NK-cells, Monocytes, CD4+ T-cells and γ/δ-T-cells by 'positive selection'. It has the advantage that the CD8+ cells are relatively untouched, as antibodies and beads do not bind to them. Briefly cells were suspended at a cell concentration of 10^7 cells in 40μL of MACS buffer (This is sterile, chilled PBS with 1% Foetal Calf serum and 5mM EDTA). 10μL of biotinylated anti-body cocktail was added to every 40μL of cell suspension. The sample was then mixed and then placed at 4°C for ten minutes. After this an additional 30μL of buffer was added, and then 20μL of anti-biotin antibody conjugated microbeads – to form 100μL of suspension. After mixing the sample was then placed at 4°C for a further fifteen minutes. The excess beads and antibody was then washed off by adding 2ml of buffer and centrifugation of samples at 300g for 10minutes. Supernatant was then carefully aspirated from above the cell pellet. Cells were then resuspended in 500μL (as long as the total cell number did not exceed 10^8).

A range of columns are available for cell sorting, which slot in to an appropriately sized magnet. Larger columns provide purer populations of cells by 'negative selection'

whereas smaller columns give a purer 'positive selection'. Larger columns reduce the efficiency of the separation and only a small number of 'positively selected' cells may be attained. In both cases both positive and negative selected populations can be purified further by passing through a fresh column. I used LS columns, recommended for the CD8+ T-cell kit. The columns are prepared by passing 3ml of buffer through them. The cells are then aspirated from the sample tube and carefully pipitted on to the top of the column. Once the fluid has drained through, 3 lots of 3ml of buffer are placed through the column in turn. The fluid is then collected as the 'negative sort' population – in this case containing CD8+ T-cells. After passing a further 3ml of buffer whilst the column is still in the magnetic field, the column is then removed from the magnet and 3ml of buffer is placed on the top, and by using a plunger provided, the 'positive sort' population is eluted. This is then spun down and suspended (after counting of cells) into the required amount of R10 for the experiments in question. The purity of the sort was tested in each case by FACS after staining with anti- CD3 and anti- CD8 antibodies. The CD8+ cells could then be sorted into CD161+CD3+CD8+ and CD161-CD3+CD8+ T-cells as described below. This provided pure cell populations that could be used in functional assays. Supernatants could be collected for CBA analysis (without concerns about cytokine secreted by contaminating cell populations).

For cell culture that utilised intracellular cytokine staining (ICS) or staining for Ki67 it was not necessary to pre-sort into pure CD8+ populations as cells were studied on an intra-cellular basis. Thus CD161+ and CD161- cell fractions were sorted in bulk. This utilised the same basic techniques but use of anti -CD161 antibody conjugated to PE was

used. Miltenyi-biotec provide anti-PE anti-body conjugated microbeads which can then be used after washing off CD161-PE antibody. Thus anti-PE microbeads enable sorting to be performed using any antibody conjugated to PE. In these intra-cellular assays that used prolonged culture (ie assessment of proliferation as opposed to intracellular cytokine staining) it was considered important to correct for any difference in the numbers of monocytes, CD4+ and other cells that occurred in the CD161+ and CD161- fractions. Thus I used positive selection of CD8+ T-cells from PBMC's using DYNA-Beads (Invitrogen, Ltd), to provide CD8-depleted PBMC's. This provided a cellular substrate for functional assays that did not interfere with studies of CD8+ cells. For this technique cells were diluted 10^7 cells per ml of MACS buffer. 50μL of mixed CD8 Dynabead suspension was added for each ml of cell suspension. The sample was then placed at 4°C for twenty minutes on a rotating device, to stop the cells and beads from clumping. The sample was then placed in a magnetic field, which propelled the beads with attached CD8+ T-cells to the internal surface of the sample tube. After two minutes the remaining supernatant was carefully aspirated, avoiding dislodging the smear of cell/bead mixture from the side of the tube. The sample was then washed and resuspended in R10 ready for use.

2:11 Antibodies:

Marker	Flourochrome	Company & Notes
Anti-mouse IgG Fab	FITC	R&D (Used for 2° Stains)
Anti-mouse IgG Fab	APC	R&D (Used for 2° Stains)
CCR7	FITC	R&D systems
CCR7	PE-Cy7	BD (Tandem-Conjugate)
CCR9	FITC	R&D
CD103	FITC	BD
CD161 (DX12)	FITC	BD
CD161 (DX12)	PE	BD
CD161 (191B8)	PE	Beckman-Coulter
CD3	Unconjugated	Beckman-Coulter
CD3	ECD	Beckman-Coulter
CD4	APC-Cy7	BD (Tandem-Conjugate)
CD4	PE	BD
CD45RA	APC	BD
CD45RA	Biotin	BD
CD57	FITC	BD
CD8	PerCP	BD
CD8	PE-Cy5.5	BD (Tandem-Conjugate)
CD85j(ILT-2/LIR-1)	FITC	BD
CD85j(ILT-2/LIR-1)	PE	Beckman-Coulter
CXCR6	PE	R&D
GranzymeB	APC	Caltag
HLA-A2	FITC	Abcam
IFN-γ	FITC	BD
IFN-γ	APC	BD
Ki67	FITC	BD
Perforin	FITC	BD
Steptavidin	Pacific-Blue	Beckman-Coulter
Viaprobe	Fl-3	BD
β-7 integrin	PE	BD

2:12 Manufacturers (Names, and Addresses):

Company	Address
Abcam	332 Cambridge Science Park, Cambridge, CB4 0FW, UK
Beckman Coulter	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, Buckinghamshire HP11 1JU
Becton-Dickenson (BD)	The Danby Building, Edmund Halley Road, Oxford Science Park Oxford, OX4 4DQ, United Kingdom
BIORAD	Bio-Rad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX
Caltag/Invitrogen	As for Invitrogen
Chiron	Chiron Healthcare SAS, 10 Rue Chevreul, 92150 Suresnes, France
eBiosciences	eBioscience, Inc., 6042 Cornerstone Court West, San Diego, CA 92121, USA
IBL	IBL GESELLSCHAFT FÜR IMMUNCHEMIE UND, IMMUNBIOLOGIE MBH, FLUGHAFENSTRASSE 52a, D-22335, HAMBURG, GERMANY
Invitrogen	3 Fountain Drive, Inchinnan Business Park, Paisley. UK, PA4 9RF
Miltenyi-Biotec	Almac House, Church Lane Bisley GU24 9DR Surrey
Pro-immune	The Magdalen Centre, Oxford Science Park, Oxford, OX4 4GA, UK
R&D systems	19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, UK
Sigma-Aldrich	The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK

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Chapter 3: A Relationship Between the Size of T-Cell Populations and Their Phenotype.

3:1 Abstract:

The phenotype of T-cells is believed to reflect basic T-cell properties (from naïve T-cells to 'central' and 'effector' memory pools'). This chapter looks at the basic 'phenotype' of CD4+ and CD8+ T-cells in persons who have not been infected with HIV, HCV or CMV. I look at the relationship between age and T-cell phenotype, using a cross-sectional approach. I then go on to compare these same observations with those who are 'latently' infected with CMV. Finally, I looked at CMV specific T-cells and relate their phenotype to the magnitude of responses. Multivariate regression analysis and a high throughput approach (using multiple samples) were used to evaluate and clarify these relationships. This new approach to evaluating T-cell 'macro' biology resulted in several interesting findings. We found that: 1) Predictable changes in the phenotype of T-cell pools occur with age, 2) CMV has marked effects on T-cell phenotype and its association with age and, finally 3) We find that the 'size' of memory responses to CMV (and possibly in general for all CD8+ T-cells) is related to their phenotype. The implications of these findings are discussed.

3:2 Introduction

This chapter utilises CD8+ T-cell responses to CMV as a model to explore general relationships between T-cell responses to viruses and their phenotypic characteristics. However, it is important to review the biology and clinical aspects of CMV infection.

CMV is a β-herpes virus. These are large DNA viruses and in the case of CMV there are 213 genomic open reading frames (ORF's), each encoding a specific protein product (Fields, Knipe et al. 2007). CMV virions consist of three parts: an inner neucleocapsid (containing DNA) which is embedded in a middle tegument layer and finally, an outer covering of envelope. CMV is a near-ubiquitous virus, with very high rates of seropositivity in healthy persons (reflecting previous infection). These range from 52% (France) to 99% (Turkey). Infection in the vast majority occurs before the age of six (or younger in developing countries) and continues to increase until the fifth or sixth decade. It seems to be more common in immigrant populations, developing countries, in lower socio-economic groups and institutional settings. Seasonal variation in infection does not seem to occur (Fields, Knipe et al. 2007).

Primary infection in non-pregnant, immunocompetent persons is usually (ninety percent) asymptomatic and of no consequence. Ten percent of persons may develop an 'infectious mononeuclosis' like syndrome (like that caused by Epstein-Barr virus (EBV)). ('Infectious mononeuclosis' refers to the presence of atypical lymphocytes on blood films). Flu-like synptoms, rash, lymphadenopathy and hepatosplenomegaly may occur. Severe disease in normal hosts occurs rarely, if at all (Fields, Knipe et al. 2007). Infection

is however, a concern in pregnant woman, particularly in the first trimester, when neurological damage to the unborn child can occur, ranging from severe problems to mild hearing impairment (Fields, Knipe et al. 2007). Severe complications for the newborn may also occur with peri-natal infection. Infection is also a concern for persons who are immunosuppressed. Immunosuppresion may arise from either congenital immunodeficiency, acquired immunodeficiency syndrome (AIDS) arising as a result of HIV infection, or as a result of immunosuppressive medical regimes (for treatment of autoimmune disease or to allow bone marrow or solid organ transplantation). Clinical problems include encephalitis, retinitis, pneumonitis, hepatitis, colitis and leucopenia. Failure and rejection of transplanted grafts is also associated with infection. It has not been possible to identify strains of CMV with especially high virulence, but this may be due to the large size of the virus, the genomic complexity and near-infinite possible number of recombinations of genomic contents (Britt and Boppana 2004; Fields, Knipe et al. 2007).

Serological tests are particularly useful at diagnosing past infection by looking at IgG levels. Appearance of maternal IgG after a prior negative result suggests seroconversion and that the unborn child may be at risk. Acute infection is more difficult to diagnose serologically as IgM may be expressed in reactivated infection and is often non-specific. However it is useful for diagnosis of infection in pregnant women. Antibodies to particular virus components appear at different times after infection and the pattern of antibody responses may be used to infer a time after infection.

Clinical samples can be inoculated onto cell culture material and the cytopathic effect of infectious virus detected. These assays have been refined by using antibodies to identify expression of 'immediate early' CMV antigens within 24 – 48 hours post inoculation. For many years the most reliable diagnostic test was to look for evidence of CMV antigens in the peripheral blood mononeuclear cell fraction of peripheral blood using mono-clonal antibodies. Such tests have been used to pre-empt clinical disease in transplant cohorts for example. Detection of viral neucleic acid is also now available by polymerase chain reaction (PCR). However, although this is often very sensitive, it may detect viral replication at low levels and correlation of a positive test with clinical disease prospectively is often not possible. This may be because significant viraemia may occur in apparently healthy individuals. Thus viraemia is seen months after symptoms resolve following primary infection. Viral shedding into urine (determined by culture or by PCR) may occur at even more remote time points after infection (Fields, Knipe et al. 2007). Virus is also evident in saliva, breast milk and genital secretions and thus provide mediums for transmission to uninfected persons (Fields, Knipe et al. 2007).

There are several antiviral agents active against CMV. Ganciclovir is phosphorylated intracellularly to become a synthetic analogue of deoxyguanosine triphosphate (dGTP). It competitively competes with dGTP for incorporation into DNA by viral DNA polymerase. Its activity results in inhibition of DNA replication. It is poorly absorbed and requires intravenous administration. A prodrug of Ganciclovir, Valganciclovir is better absorbed and can therefore be administered by the oral route. Cidofovir is an acyclic nucleoside phosphonate that does not require phosphorylation *in vivo*. It too inhibits viral

DNA synthesis. Foscarnet is a non-competitive inhibitor of DNA polymerase. It works as a pyrophosphate 'mimic', acting on viral but not human polymerase.

Anti-CMV immunoglobulin has also been used to prevent or pre-empt clinical disease with CMV but it does not appear to have any benefit superior to the above anti-viral agents (Hodson, Jones et al. 2007).

CMV infects a wide variety of cell types and organs including monocyte derived macrophages, T-lymphocytes, granulocytes, endothelial cells, epithelial cells, fibroblasts, stromal cells, neuronal cells, smooth muscle cells and hepatocytes (Jarvis and Nelson 2002). The emergence of CMV in previously healthy persons who are immunosuppressed implies that CMV results in a state of chronic infection characterised by the absence of significant viraemia or clinical disease. This state is termed 'latent infection'. Monocytes are the predominant infected cell in peripheral blood, and macrophages are the predominant infected cell type in tissues in healthy persons with latent infection. In contrast, in persons with clinical disease with CMV such as liver and renal transplant recipients for example, CMV-infected leucocytes are seen in biopsies. Thus latent infection may be harboured in monocytes and MDM, and in reactivated disease these are able to transmit productive infection to leucocytes. IFN-γ and IL-2 from CD8+ T-cells may have a key role in activating monocytes that are latently infected to allow for infection productive of CMV (Jarvis and Nelson 2002). Other interactions between different cell populations may occur. Thus infection of endothelial cells leads to upregulation of ICAM-1 to which monocytes adhere. This may allow transmission of

infection. CMV antigens such as IE-1 and IE-2 can stimulate upregulation of ICAM-1, thus CMV actively plays an active role in transmission of infection. Infected monocytes may in turn be able to infect previously uninfected endothelial cells (Jarvis and Nelson 2002). Endothelial cells may be a key cell type for infection in acute and latent CMV infection. Interestingly, infection of monocyte progenitors does not appear to play a role in generating fresh populations of infected monocytes. Monocytes do not replicate and are short lived (Jarvis and Nelson 2002). Thus recurrent cycles of sub-clinical infection may be required for latent infection to be maintained. Clinical reactivation of infection may thus reflect a breakdown in a complex set of mechanisms that permit subclinical cycles of paracellular infection which avoids full blown activation of the immune system which, in turn, could result in more widespread active viral replication in monocytes and increased transmission to other cell populations resulting in clinical disease.

Multiple immune processes are affected by CMV proteins. These include: 1) Modulation of intracellular events, such as transcription, apoptosis and Type I IFN pathways, 2) Disruption of innate immunity pathways controlled by IFN, chemokines, cytokines and NK cells 3) Deflection of T-cell mediated immunity (Fields, Knipe et al. 2007).

T-cell responses to CMV have often been used as a model to explore the biology of immune responses. This is because large, immunodominant T-cell populations can be readily studied in healthy persons (Champagne, Ogg et al. 2001). Recently, in a huge project, the full spectrum of CD4+ and CD8+ T-cell responses to CMV was mapped in 43 persons aged from 19-45 (33 CMV seropositive) (Sylwester, Mitchell et al. 2005).

This study used intracellular cytokine staining and required 13,687 peptides mixed in 232 groups. These corresponded approximately to the 'open reading frames' of the virus and thus to the full range of CMV proteins. On average eight CD8+ T-cell responses were seen, each averaging 1.1% of all memory CD8+ T-cells. Thus in healthy seropositive persons, approximately 8.8 percent of all memory CD8+ T-cells recognise CMV peptides – an astonishing figure. CD8+ T-cell responses to 107 of 213 CMV viral proteins were seen in at least one person. The occurrence of responses was correlated with the abundance of each protein as measured by proteomic analysis of CMV particles, and was also related to viral variability as determined by comparison of six full length sequences of specific viral strains. Recognition was also according to timing of expression according to the viral life cycle. CMV proteins are produced in order according for their need at particular times in the construction of virus. Thus immediate early (IE), early (E), early-late (EL) and late (L) proteins exist. Immediate early late antigens were recognised at proportionately greater frequency compared to other antigens. A broad range of MHC class I genotypes were selected for the cohort. Despite the huge number of peptides studied, none were recognised in CMV seronegative persons.

It is inferred (as discussed in the introduction) from the array of complex molecular and cellular machinery, and from the high stakes involved in the potential to develop massive uncontrolled immune responses on one hand and, on the other hand, ineffectual responses and subsequent overwhelming infection on the other, that the immune responses operates within narrowly defined and regulated limits. This activity ranges from relatively short periods of explosive activity to long periods of quiescence with a state of readiness. Thus

there is a need for homeostatic maintenance of naïve and memory T-cell pools over a human life span. However, little published work concerns itself with phenotypic determinants of the size of T-cell responses (and the size of T-cell subpopulations in general), or inter-relationships between T-cell pools.

In this chapter I describe a novel approach to addressing these issues using human samples and flow cytometry. We obtained data on CD4+ and CD8+ T-cell phenotype in a large number of persons and related this to CMV serostatus. In addition I was able to use MHC class I tetramers to study an extremely common CD8+ T-cell response to CMV (an epitope from the CMV pp65 protein) in a large number of persons and relate this to expression of markers commonly used to define subtypes of memory T-cell. Pp65 (derived from open reading frame UL83) is a tegument protein. It is expressed in the DE (delayed early) phase of viral replication but may only be required at later stages (Fields, Knipe et al. 2007). pp65 is not critical for viral replication, however (Britt and Boppana 2004). It is also able to block the activity of type I interferons (Browne and Shenk 2003), and therefore helps the virus disarm anti-viral defence mechanisms. The peptide NLVPMVATV was the pp65 epitope studied.

Previously the lab has shown in a cross-sectional analysis that CMV-specific responses to the pp65 epitope increase with age (Komatsu, Sierro et al. 2003). This has been termed ‘memory inflation’, and had been inferred previously by the finding of very large responses in apparently healthy elderly individuals. Occasionally up to 24% of CD8+ T-cells in peripheral blood may respond to this single CMV epitope (Khan, Shariff et al.

2002; Ouyang 2003). Such responses are very impressive, given that in theory, on average, another seven such responses will be present (Sylwester, Mitchell et al. 2005). Thus virtually all CD8+ T-cells in some healthy seropositive elderly subjects may be CMV-specific, assuming that non-pp65 specific responses follow the same dynamics. It has been shown that there is a linear increase in the size of the pp65 response with increasing age, thus indicating that this T-cell expansion at least developed systematically from early youth to old age and is therefore not idiosyncratic of a few rare individuals. Interestingly, longitudinal studies in the lab have also demonstrated the same phenomenon in (murine) CMV in mice (Karrer U 2003). However, in murine CMV, only specific epitopes show memory inflation (Karrer U 2003). Interestingly this occurs for the IE-1 peptide pp89 but not specific antigens expressed at other time points (which led the authors to propose that these 'early' responses prevent full expression of viral antigens by halting reactivation in its tracks). In contrast, in humans 'inflation' to a DE antigen (pp65) occurs. In spite of these important caveats memory inflation to the pp65 epitope provides a readily available method to explore the associations between phenotype, age and response magnitude.

3:3 Methods:

As stated, the work was based on protocols that had been developed in the lab that allowed large numbers of individuals spanning the full age spectrum to be screened for CMV-specific CD8+ T-cell responses by flow cytometry after staining with MHC class I tetramers for an HLA-A2 restricted immunodominant response to the pp65 protein of

CMV (Komatsu, Sierro et al. 2003). Conveniently, responses to this peptide are extremely common, and are seen in twenty-five percent of unselected persons, or fifty percent of HLA-A2+ persons (fifty percent of all persons are HLA-A2+ve, approximately). I was granted local ethics committee permission to utilise routine EDTA containing samples sent to the haematology department of the John Radcliffe Hospital. This permission is granted on the basis that samples are anonymised: the patient's age is recorded but no other data. Use of ninety-six well plates and multi-channel pipettes enables samples to be processed quickly and conveniently. Thus data on huge numbers of CMV-specific responses and accompanying CD4+ and CD8+ T-cell pools can be generated with relative ease.

I expanded this approach to include staining for T-cell phenotype markers. Briefly, samples are initially screened for HLA-A2, and these HLA A2+ve persons are then stained with a tetramer of an HLA-A2-restricted immunodominant epitope from the CMV pp65 tegument protein (NLVPMVATV), conjugated to APC (Allophycocyanin). After washing off the tetramer antibody to anti-CD8 is added along with antibodies for up to two additional markers.

Individuals aged 13 – 94 years were studied. CMV-specific population sizes are given as a percentage of all CD8+ T-cells. Previously tetramer staining in A2+ individuals has been taken to correspond approximately with seropositivity to CMV. In this project information on both was available in a large number of individuals enabling this relationship to be evaluated. Serology for CMV was done using a commercial ELISA

(IBL-Hamburg) set up in house according to the manufacturer's instructions (as described in chapter 2). In some individuals serology was done in parallel at the NHS virology lab at the JRH.

I chose the following phenotypic markers: 1) CCR7-FITC 2) CD57-FITC (BD), 3) CD85j (ILT-2/LIR-1)-PE & 4) A combination of CCR7 and CD45RA. CCR7+/CD45RA- cells are 'central memory cells' (T_{CM}), CCR7-CD45RA- cells are 'effector memory cells' (T_{EM}), and CCR7-CD45RA+ cells are 'CD4RA revertant effector memory cells' (T_{EMRA}). Thus these markers enabled T_{CM} status &T_{EM} status to be evaluated, along with CD57 and a novel marker that was not known to fit into the previously described memory paradigms (LIR-1). CD57 indicates a terminally differentiated group of 'senescent' T-cells (Brenchley, Karandikar et al. 2003).

Samples were acquired and analysed using FACScalibur (Becton Dickenson), CellQuest software and Prism Graph Pad. Regression analysis was performed using Stata (Stata corporation). Wilcoxon's test was used to compare paired observations, and the Mann-Whitney U-test was used to compare unpaired observations. Correlation was assessed using Spearman's correlation co-efficient.

3:4 Results:

3:4:1 an association between the size of CMV-specific T-cell populations and age:

As before (Komatsu, Sierro et al. 2003), it was possible to correlate the size of CMV-specific populations with age in a cross sectional demonstration of memory inflation (R=0.3291, p=0.0058, n=69).

3:4:2 CMV Specific CD8+ T-cells are of an 'Effector Memory' Phenotype.

There was no significant difference in the age of CMV-positive and negative persons (64.6 years vs 59.68 years, respectively, p=0.249). In order to assess the viability of cells collected and stained in the manner described I stained 31 samples collected according to the usual protocol and stained with viaprobe – a marker of cell viability. A median of 10.2% of CD8+ cells were viaprobe positive (SE=2.4), indicating, on the whole, reasonable levels of cell viability (89.8%).

In common with other work (Appay 2002; Lucas, Vargas-Cuero et al. 2004), I found that CMV specific CD8+ T-cells had an effector memory phenotype. Figure 3:1 shows the proportion of tetramer positive (TET+) that were T_{CM}, T_{EM} and T_{EMRA}. As can be seen, compared to non-TET+ cells from persons who had tetramer staining, relatively few cells had a T_{CM} phenotype (median=2.94%), whereas the majority of them had a T_{EMRA} phenotype (median=52.13%). The advantage of performing this analysis with analysis of both CCR7 and CD45RA is that we were able to exclude naïve cells from this analysis

(which might otherwise skew the analysis in favour of tetramer positive cells). CD57 was highly expressed on CMV-specific CD8+ T-cells, (median=35.27% +/-3.67%), and CCR7 showed only weak expression (5.69 +/ 10.5).

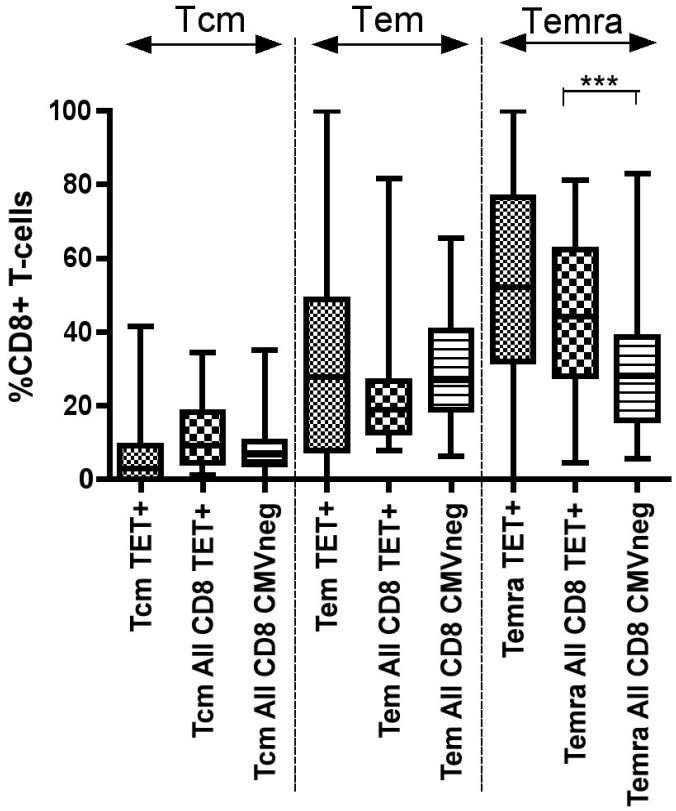

Figure 3.1: This graph compares the phenotype of Tetramer positive, that is CMV-specific (dotted squares) with non-tetramer positive cells in 39 persons with tetramer staining (checkered squares) and CD8+ cells in 59 persons who were CMV seronegative (horizontal lined). From left to right T_{CM}, T_{EM} and T_{EMRA} cells are shown, as indicated. CMV-specific cells were predominantly T_{EMRA} cells (median=52.3%). Error bars show the 95% confidence interval. In addition I compared non-tetramer positive CD8+ T-cells from persons with tetramer staining with those from CMV seronegative persons. As can be seen tetramer staining was associated with a 'pervasive effect' on the remaining CD8+ T-cells with a marked increase in the number of T_{EMRA} cells ($p<0.005$- Mann-Whitney U-test), compared to CMV-seronegative persons. (*** indicates $p<0.005$).

3:4:3 Designating 'memory status' to a novel phenotypic marker: CD85j (ILT-2/LIR-1) May be Regarded as a Maturation Marker, Similar to CD57.

We also evaluated staining of LIR-1(CD85j-ILT-2) on CMV-specific cells. This is a marker found on NK-cells in addition to CD8+ T-cells, and binds class I. It may deliver an inhibitory signal to T-cells when they bind class I molecules using their T-cell receptor. This may enable tolerance to self-epitopes, failure of T-cell activation if the MHC class I molecule does not contain the correct epitope, or prevent over activation of the T-cell if the right T-cell receptor and MHC-class I/epitope complex pairing is made (Young 2001; Young 2002). I found this marker to be highly expressed on CMV-specific (tetramer positive) cells, even when compared to non-tetramer positive CD8+ T-cells from the same person (median=69.37 +/- 4.13, compared to 59.74 +/- 21.9 on tetramer negative cells ($p=0.029$ – Wilcoxon's test).

I suspected that LIR-1 is an established marker of antigen experienced T-cells with effector memory function. In order to explore this further, in 21 individuals, (both CMV positive and negative), co-staining of CD8+ T-cells with CD57 and CD85j (ILT-2/LIR-1) was performed. In line with data from CD3+ T-cells in previous work, I show that co-expression of CD57 and LIR-1 is common (see figure 3.2). Furthermore, there was a strong and positive correlation between the numbers of CD8+ T-cells staining for CD85j (ILT-2/LIR-1) and CD57 ($R=0.848$, $p<0.0001$). This suggests that CD85j (ILT-2/LIR-1) can generally be regarded, like CD57, as an effector memory marker.

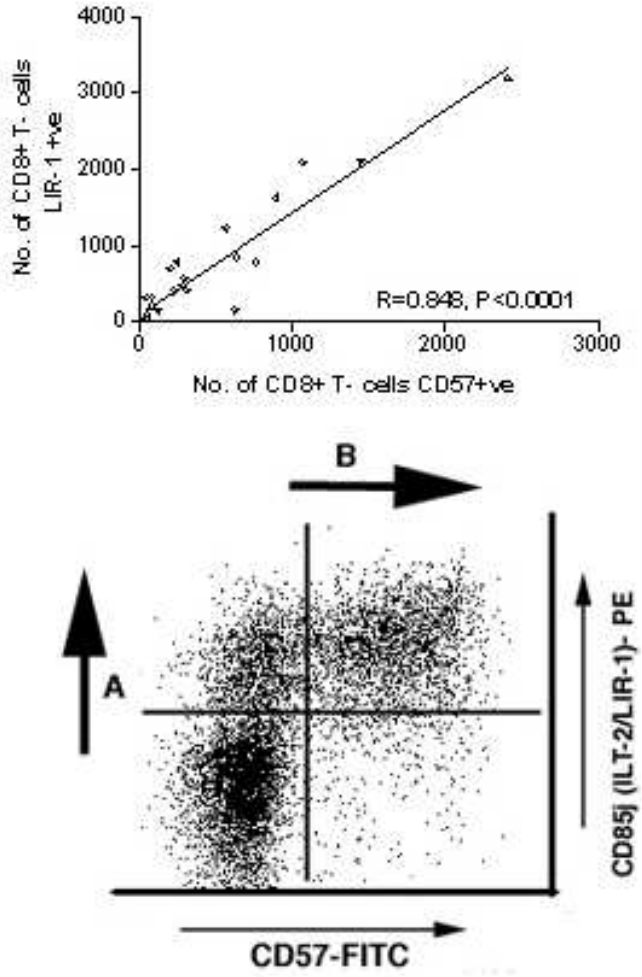

Figure 3.2: Top: CD8+CD57+ 'events' and CD8+LIR-1+ 'events' on flow cytometry were highly correlated indicating that CD57 and LIR-1 are closely related in their expression. Bottom: We suspect that CD57 is expressed subsequent to LIR-1 (assuming a linear model of T-cell differentiation) as LIR-1+CD57- events (top left quadrant) were far more common that LIR-1-CD57+ events (bottom right quadrant). A significant number of CD57-LIR-1+ve events, however indicates that CD57 and LIR-1 are not completely interchangeable as memory markers.

3:4:4 Relationship between CMV sero-positivity and tetramer positivity:

The relationship between antibody status and 'CMV-specific T-cell status' (as indicated by tetramer staining) in immune responses to CMV has not received much attention. In A2 positive persons it was possible to relate how closely antibody responses were usually associated with tetramer staining and vice versa. Out of 39 persons who were A2+ and had tetramer staining assessed, 6 had negative CMV serology. I did not perform duplicate wells for each serum samples for each well (although this is recommended by the manufacturer), which may have led to impaired sensitivity and specificity of the ELISA. I evaluated this by asking for these samples to be tested at the John Radcliffe Hospital Virology Department. Out of the 6 with positive tetramer staining, only 1 of these persons had negative serology confirmed at the John Radcliffe Hospital Virology Department (this was performed on an aliquot of serum from my anonymised samples). This person had tetramer staining for 2.4% of CD8+ T-cells. A further person's serology was 'equivocal' at JRH despite a large tetramer response - 10.23% CD8+ T-cells being CMV positive. These two persons could have had clinical disorders of anti-body production (for example multiple myeloma) or may have had acute infection (with IgM rather than IgG production). It is also possible that antibodies are lost in rare cases of prolonged latency. It seems unlikely that the pp65 cross reacts with epitopes for other infections as this was not seen in previous studies (Sylwester, Mitchell et al. 2005). Unfortunately my anonymised system made it impossible to track these patients to follow this up. A further four randomly selected persons (CMV seropositive and CMV sero negative) who had no tetramer staining, had serology checked at JRH and there was

concordance with my in house ELISA. There were no gross differences in the phenotype or size of the responses in the 6 tetramer staining positive persons with negative serology (data not shown).

In 54 A2+ve CMV seropositive persons tetramer staining was performed. 42.6% of these had tetramer staining to the pp65 peptide. Interestingly these persons (CMV-seropositive, tetramer-positive) did have differences in the overall phenotype of their (non-CMV specific) CD8+ T-cells compared to CMV-seropositive persons in whom no tetramer staining was found. The biggest difference was in the number of T_{EMRA} cells (Tetramer +ve: 48% +/- SE 4.0% vs Tetramer –ve: 27.8% +/- SE 3.2%, p=0.0038 – see Figure 3:3). This percentage (in tetramer negative persons) approximated that seen in CMV sero-negative persons. Although there may be an argument about the validity of the ELISA (see above), there may be a rationale for there to be a - CMV seropositive - tetramer –ve – 'normal' CD8+ T-cell phenotype – population. It is possible that in these individuals lacking responses to pp65, in a manner analogous to that proposed in the murine model (Karrer U 2003), responses to IE-1 may come into play, and the dynamics revert to that found in this model. Thus responses to IE-1 may be present, whereas response to later antigens such as pp65 may no longer be triggered in an ongoing fashion in latent infection. This would be a highly interesting hypothesis to explore in future studies.

In summary, there appears to be minor discordance between CMV status as determined by tetramer staining of CD8+ T-cells and that determined by serology. It is possible that small numbers of persons have large tetramer positive populations but lacked positive

serology. An interesting distinction was found for persons who were CMV seropostive (and HLA-A2+ve) but lacked tetramer staining and those who had tetramer staining.

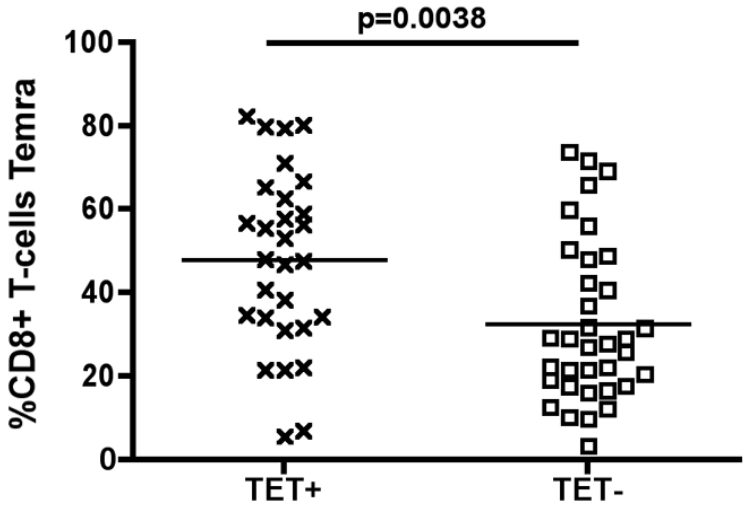

Figure 3.3: A large number of HLA-A2+ve, CMV-seropositive persons did not have detectable staining with the tetramer used in this project. These persons (squares) had fewer T_{EMRA} cells amongst their bulk CD8+ T-cell populations compared to persons who did have tetramer staining (crosses).

3:4:5 CMV-specific T-cell frequency shows an exponential relationship with changes in T- cell phenotype:

It was of interest to address if antigen-specific CD8+ T-cell population size (CMV-specific) followed a normal distribution. This could be assessed using a statistical test known as a symmetry plot. Using this test I showed that population size did not follow a normal distribution, but was significantly 'skewed' in favour of smaller population size. This could be corrected in part by logarithmic transformation of the values. This is illustrated by figure 3.4. The top graph shows a distance above/below median plot for untransformed data. Significant deviation from the straight line predicted by a normal distribution is shown. On the other hand the values do follow the line after logarithmic transformation (bottom graph).

I found that logarithmic transformation of population size values enabled meaningful relationships to be determined on regression analysis with other parameters. The finding that log-transformed values followed a normal distribution (on the whole), but non log-transformed values didn't, helped validate this approach.

I predicted that T-cell populations of different sizes might vary in their predominance of different T-cell memory subtypes. Crude analysis does indicate such a link. In figure 3:5 FACS 'contour' plots for 'small' (left) and 'large' (right) T-cell populations are placed side by side and the proportion staining for LIR-1, CD57 and co-staining with CCR7 and CD45RA is shown. (CCR7+/CD45RA+ cells are 'naïve'. CCR7+/CD45RA- are 'central memory' (T_{CM}), CCR7-CD45RA- are 'effector memory' (T_{EM}), and CCR7-CD45RA+

cells are 'CD4RA revertant effector memory cells' (T_{EMRA})). Larger populations had evidence of greater maturity with increased proportions of CD57 positive populations and T_{EMRA} cells. Larger populations also expressed more LIR-1.

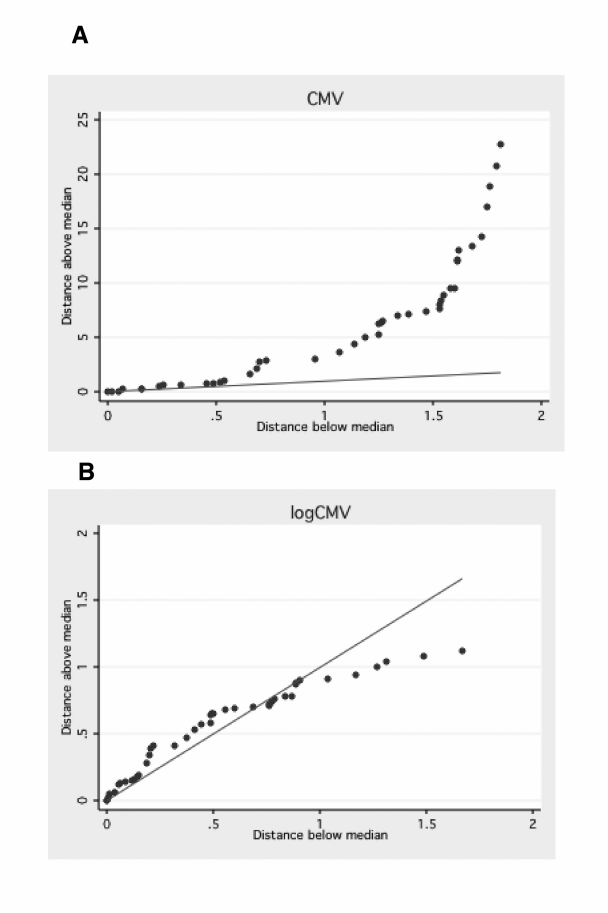

Figure 3.4: 'Symmetry plots' of CMV-specific population size were performed before (top) and after logarithmic transformation (bottom). The curved fall of the data (top) indicates that population size is skewed significantly and does not follow a normal distribution, whereas logarithmic transformation fits much more closely with a normal distribution of data. Thus logarithmic transformation of population size was used to link this with T-cell phenotype.

I went on to characterise the relationship between 'maturity' of 'memory status' of virus T-cells. Statistically significant correlations were seen. There was a negative correlation between population size and CCR7 expression and positive correlations between expression of LIR-1, CD57 and T_{EMRA} status (see table 3.1). Regression analysis indicated that exponential relationships between T-cell phenotype and population size provided the best model of fit. Figure 3.6 shows an overlay of the fitted regression lines for each subset (CD57+, LIR-1+, CCR7 and T_{EMRA}). Individual data points are not shown in order to keep the broad relationships visible. The R^2 values and p-values are shown in table 3.1. In addition a factor 'X' was calculated by exponentiating the regression co-efficients of the regression analysis and multiplying by 100. This figure predicts the proportional percentage increase (or decrease) in population size that occurs with every ten percent increase in expression of each given marker (or pairs of markers).

The relationship between phenotype and population size was independent of age. Multivariate regression analysis (controlled for age) showed a relationship between LIR-1 and population size: n=42, $R^2 = 0.52$, coefficient = 0.018, (SE = 0.003), $p<0.001$. There was no independent relationship between age and the phenotype of CMV-specific cells ($p=0.79$).

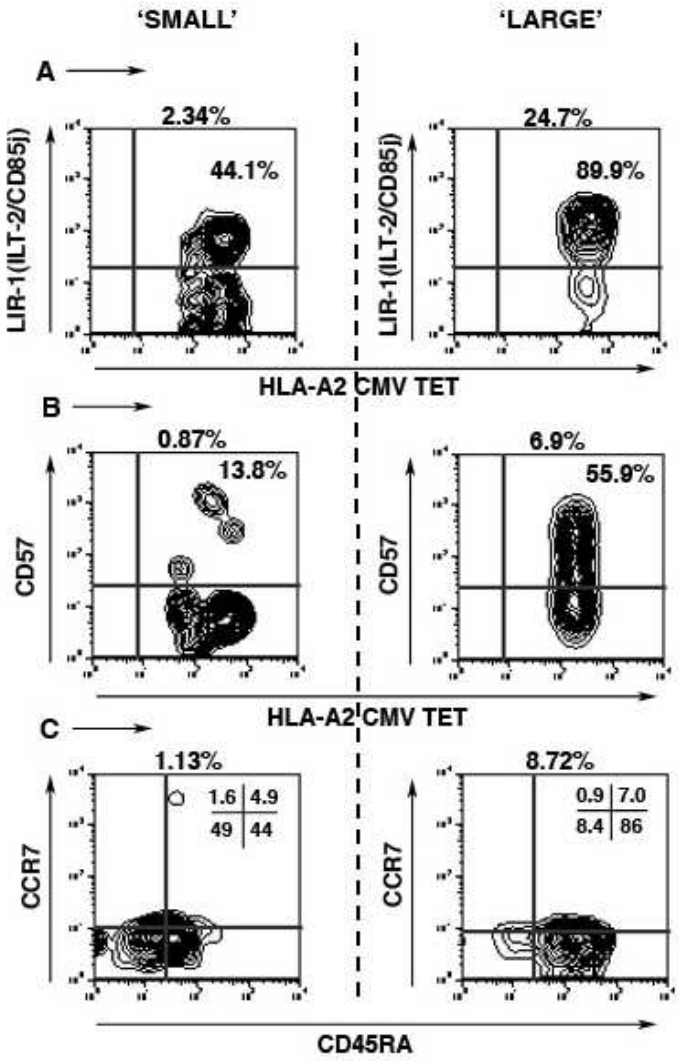

Figure 3.5: Staining of three pairs of CMV-specific CD8+ T-cell populations (gated on tetramer positive cells) is shown by contour plot demonstrating the link between 'size' of response and phenotype. On the left hand side, small responses are shown. On the right hand side large responses are shown. The size of response is indicated (as a percent of CD8+ T-cells) on the top of each FACS plot. As can be seen larger responses show high expression of LIR-1(CD85j/ILTR-2) (top), high expression of CD57 (middle), and are low in expression of CCR7, but have high numbers of T_{EMRA} cells.

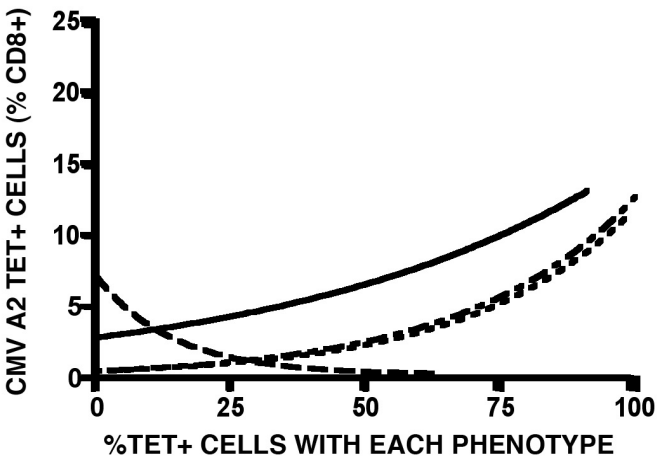

Figure 3.6: The relationship between CD8+ T-cell phenotype and size of CMV-specific populations is shown for four markers: CCR7 (long dashes), LIR-1 (solid line), CD57 (alternate short and long dashes) and T_{EMRA} (short dashes). Individual date points and confidence intervals are not indicated to keep the graph clear. 95% confidence intervals for regression co-effcients are, however indicated in table 3.1

	N	'R'	'P' (2)	R^2	*'X'*	'P' (1)
CCR7	69	-0.469	0.0026	0.18	67 (51 – 89)	0.007
T_{EMRA}	39	0.447	0.0043	0.27	128 (112 – 146)	0.001
CD57	37	0.466	0.0037	0.27	132 (113 – 155)	0.001
LIR-1	47	0.68	<0.0001	0.52	152 (135 – 172)	0.000

Table 3.1: This table summarizes the statistical analysis for the link between T-cell phenotype and population size for each phenotypic marker. In the first and second columns the correlation co-efficient ('R') value for Spearman's correlation and the 'p' value – 'P'(2) are shown. In the third column the R^2 value of the exponential regression model is indicated. In the fourth column exponentiated regression co-efficients multiplied by 100 (with 95% confidence intervals) are shown giving 'factor X'. CMV specific population size increases by 'X' percent for every ten percent increase in the corresponding marker. LIR-1 gives the best and most consistent relationship with population size, and can be taken to relate to 52% of the variability in population size (the R^2 value). 'P' (1) is the 'p' value of the regression analysis.

3:4:6 Relationship between phenotype of CD4+ and CD8+ T-cells:

Because CD4+ T-cell help assists in the generation of CD8+ T-cell responses (see introduction), I hypothesized that there may be a link between the phenotype of CD4+ and CD8+ T-cells. The data set compiled in this project gave me the perfect opportunity to test this hypothesis. I found close links between the proportion of T_{EM}, T_{EMRA}, T_{CM} and Naïve CD4+ and CD8+ (See Figure 3.7).

I compared these relationships in CMV seropositive (n=59), CMV seronegative persons (n=64), and also for 21 HCV positive persons (CMV status not determined) - See figure 3.7. Generally the strength of the correlation, and the 'slope' of the relationship (on linear regression analysis) was surprisingly similar in each case, given the apparent 'pervasive effect' of CMV described here, and that of HCV described in (Lucas, Vargas-Cuero et al. 2004), on bulk CD8+ T-cells. The T_{EMRA} subpopulation was the exception to this rule, and interestingly only in CMV positive (but not CMV seronegative or HCV positive persons) was the proportion of T_{EMRA} related in CD4+ and CD8+ compartments.

3:4:7 Relationship between CMV status and Phenotype of CD4+ and CD8+ T-cells.

We compared the phenotype of CD4+ and CD8+ T-cells between persons who were CMV seropositive and seronegative. T-cell phenotype was defined by expression of CCR7 and CD45RA. Interestingly the phenotype of CD4+ cells was nearly identical in CMV positive and negative persons with respect to expression of these markers. However, there were marked differences in the phenotype of CD8+ cells, with a relative

reduction in the number of naïve CD8+ cells and a relative increase in the number of T_{EMRA} cells. See Figure 3.8.

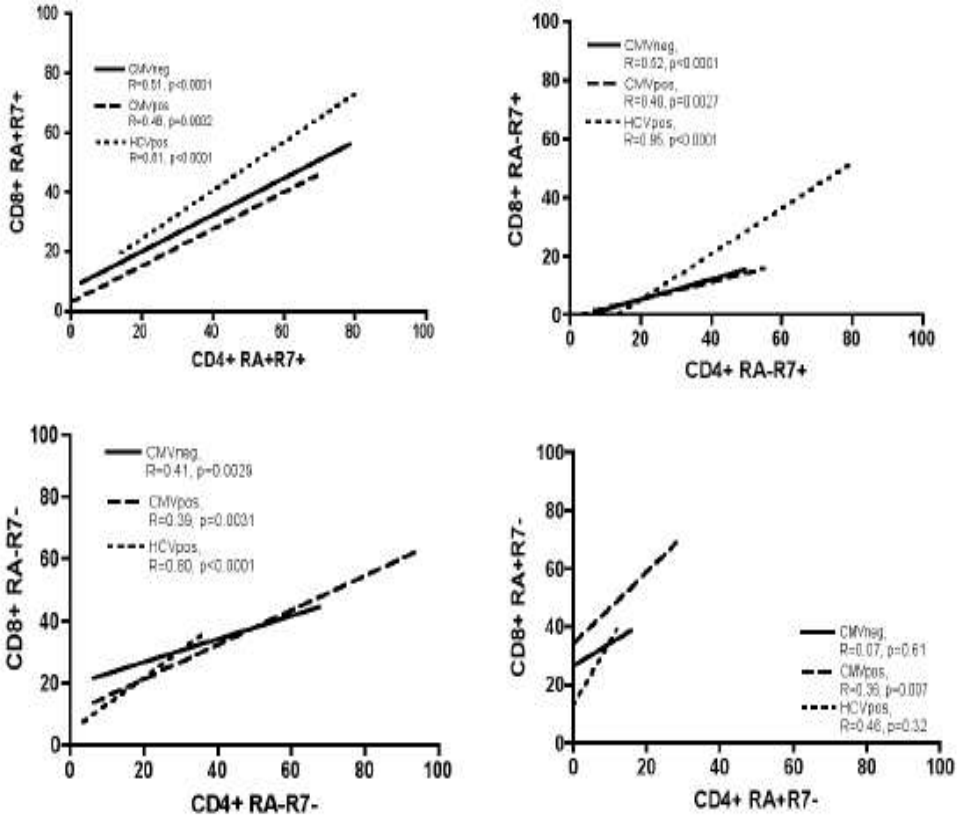

Figure 3.7 shows the relationship between the phenotype of CD4+ and CD8+ in CMV-seropositive, HCV-seropositive (CMV-status not determined) and CMV-seronegative persons for naïve, T_{CM}, T_{EM} and T_{EMRA} cells. Individual data points are omitted for the sake of clarity. In each graph the summary of the correlation analysis is indicated. Usually a strong correlation is seen between CD4+ and CD8+ T-cells . However this was not seen for T_{EMRA} cells - except in CMV-seropositive persons.

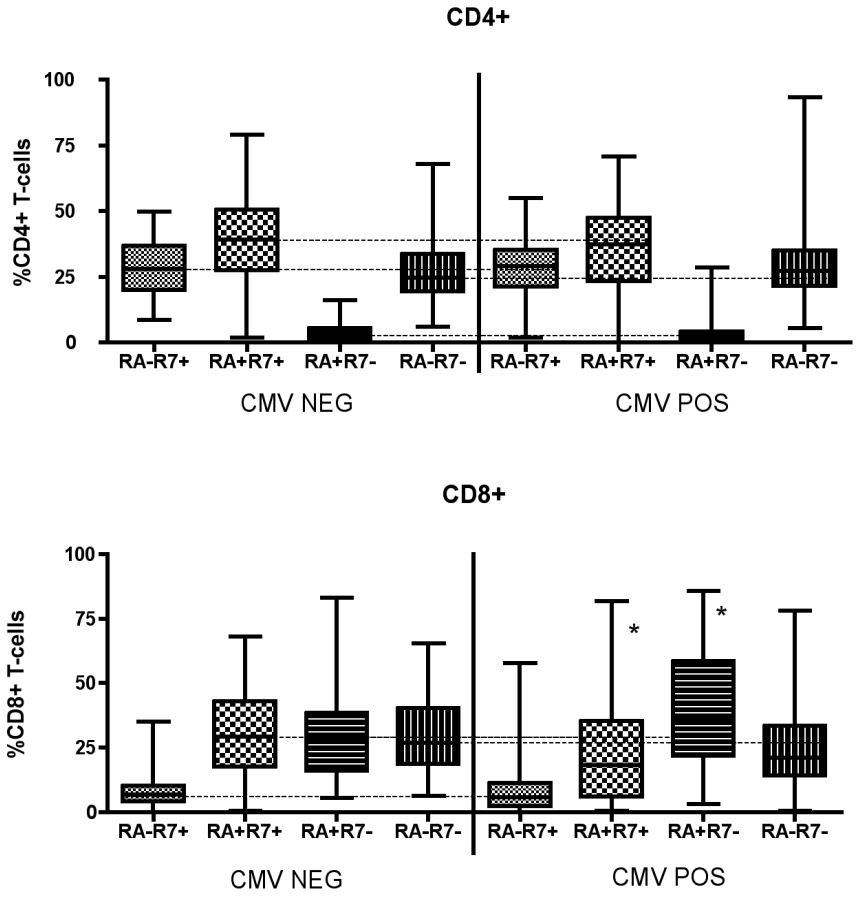

Figure 3.8: Multiple samples were obtained and CMV serostatus determined by ELISA. In parallel, whole blood was stained to examine the phenotype of both CD4+ and CD8+ T-cells with respect to expression of CCR7 and CD45RA by flow cytometry. The phenotype of CD4+ was similar regardless of CMV status, whereas in CMV positive persons there was marked relative loss of naïve cells and an increase in T_{EMRA} cells – see text. *$p<0.05$.

In this project we did not look at absolute cell numbers, thus we were unable to tell if the changes seen in CD8+ T-cell pools were caused either by reduction in naïve T-cells or an increase in T_{EMRA} cells or both. Both would be plausible. As we shall see later these changes occurred in a linear fashion with increasing age and were related to a progressive increase in the relative numbers of CD8+ cells (CD4/CD8 ratio). Overall this data suggests that probably the number of CD8+ T_{EMRA} cells is increasing – although numbers of naïve cells may be falling in addition.

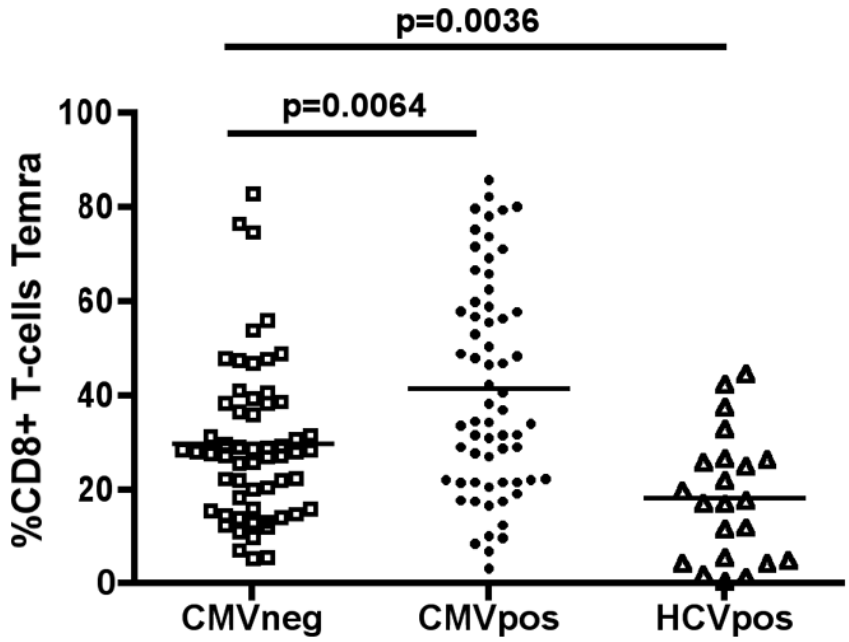

Figure 3.9: The phenotype of bulk CD8+ T-cells was compared with respect to the T_{EMRA} subscript in CMV seropositive (filled circles) and negative (squares). Date from a cohort of persons with chronic Hepatitis C was included for comparison, showing that HCV is associated with a reduction in T_{EMRA} frequency.

Some data was also available from persons with chronic hepatitis C. These individuals were from Oxford or Italian patients under study in Oxford. HCV was associated with a reduction in numbers of T_{EMRA} cells. Lack of maturity of non-HCV-specific (CMV-specific) cells has been described in Hepatitis C previously by the group (Lucas 2004). See figure 2.9.

3:4:8 CMV and macro-expansion of T-cell pools:

So far I have shown the following: 1) CMV specific CD8+ T-cell populations are often extremely large; 2) CMV seropositivity is associated with marked differences in the phenotype of CD8+ T-cell pools (but not CD4+ T-cells), and 3) Changes in T-cell phenotype can be associated mathematically with expansion of CMV populations. In this section I developed an analysis to see if the changes in overall T-cell phenotype could be linked in a general fashion to an overall expansion in CD8+ T-cell pools. It has been observed that CMV is linked to an expansion in numbers of CD8+ T-cells, and, only to a lesser extent, numbers of CD4+ T-cells (Khan, Shariff et al. 2002; Moss and Khan 2004). The notion that CMV had bigger effects on CD8+ than CD4+ T-cell pools is also supported by the finding that the composition of CD4+ T-cell pools was identical in CMV sero-positive and sero-negative persons (See Figure 3.8). Thus we were able to evaluate the evolution of CD8+ T-cell pools (in terms of bulk and phenotype) by comparing it with CD4+ pools. (In marked contrast to CD4+ T-cells, CD8+ T-cells, in CMV seropositive persons show a relative but marked increase in CCR7-CD45RA (T_{EMRA}) and a loss of CCR7+CD45RA+ (Naïve) CD8+ T-cells). The CD8/CD4 ratio (calculated by dividing the number of CD8+ 'events' by the number of CD4+ 'events' on

the cytometer) enabled us to assess the 'outgrowth' of CD8+ T-cells (relative to CD4+ T-cell pools) and its relationship to age, phenotype and CMV status. Thus although we had no data on the absolute numbers of CD4+ and CD8+ cells per μL of blood, we could nevertheless accurately assess the relative abundance of the two T-cell populations, and relate this to relative changes in CD8+ T-cell phenotype (compared to CD4+ T-cell phenotype).

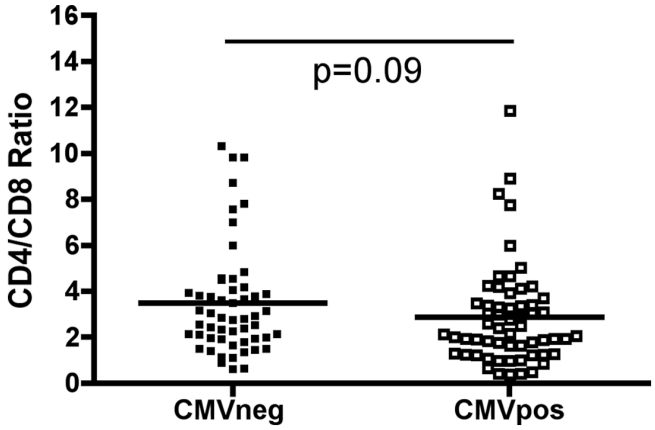

Figure 3.10: The CD4/CD8 ratio was calculated by dividing gated CD4+ events by gated CD8+ events. The CD4/CD8 ratio was compared in CMV seropositive and seronegative persons. There was no clear cut difference.

As an important aside in this analysis, there was no significant difference in the CD4/CD8 ratio between CMV seropositive and negative individuals. However there was a trend for it to be higher in CMV seronegative (n=54) and seropositive (n=59) individuals (median = 2.84 (SE = 0.32) vs 2.17 (SE = 0.28), p=0.09) (See Figure 3.10). Interestingly there was no overall correlation between the CD4/CD8 ratio and age seen (R=0.20, p=0.07, n=79), or on separate analysis for CMV seropositive or seronegative

persons (R=0.18, p=0.28, n=39 & R=0.26, p=0.11, n=40, respectively. However, other variables may have been confusing the picture.

A crude analysis to relate the 'outgrowth' of CD8+ T-cells to the overall phenotype of CD8+ T-cells was possible by dividing the proportion of CD8+ that were T_{EMRA} by the proportion of CD4+ T-cells that were T_{EMRA} to give a figure that I termed the 'T_{EMRA} ratio'. I attempted to correlate this with the CD8+/CD4+ ratio. Interestingly a strong positive correlation was seen in CMV seropositive persons (R=0.57, p<0.0001), but only a weak one in CMV seronegative persons (R=0.28, p=0.03) – See figure 3:11. It is possible that CMV enables a link between overall T-cell phenotype and magnitude of response to be exaggerated, because of the large outgrowth of (CMV-specific) CD8+ T-cells seen and the big 'shifts' in T-cell phenotype. Alternatively, because of the presence of antigen driving CD8+ T-cell pools, the effect of homeostatic turnover of T-cells (which may follow different dynamics) may be negligible, whereas in CMV-sero negative individuals it has a dominant influence on T-cell pools.

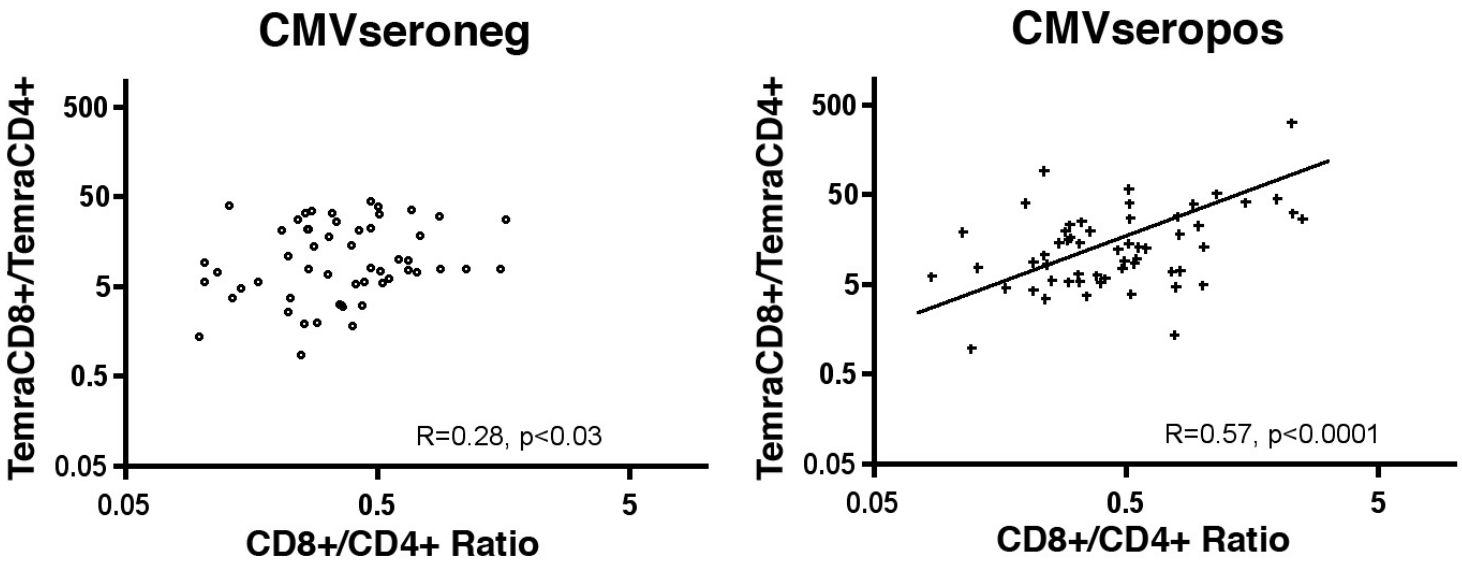

Figure 3.11: In order to explore the hypothesis that CD8+ T-cell expansion is linked to phenotype we analysed the linked the relative abundance of T_{EMRA} cells amongst CD8+ T-cells and relative abundance of CD8+ T-cells (the CD8+/CD4+ ratio) – see text. In CMV seropositive persons (right) this link was especially strong. Please note logarithmic transformation of scales.

3:4:9 Age, CMV and the Phenotype of CD4+ and CD8+ T-cells:

There was marked loss of CCR7 expression on bulk T-cell populations with age, and this appeared to be due to relative loss of naïve cells, as the number of T_{CM} cells was unchanged. In addition although there was a relative increase in 'effector memory' cells with age, as indicated by increased staining for CD57 and LIR-1(CD85j/ILT-2), this was largely accounted for by an increase in the T_{EMRA} subset of 'effector memory' cells, as the T_{EM} subset (CCR7-CD45RA-) *per se* did not change significantly with time (data not shown). The relationship between age and T-cell phenotype varied with CMV status. For CD57, the link between phenotype and age was lost in CMV seropostive persons, whereas for T_{EMRA} cells, a relationship between age was seen for CMV seropositive persons, but not seronegative persons. For CCR7 and LIR-1 however, a relationship was seen in both CMV seropositive and seronegative persons, and the 'slope' of the regression line was similar. However, at any given age, CMV was associated with a similar difference in CCR7 and LIR-1 expression.

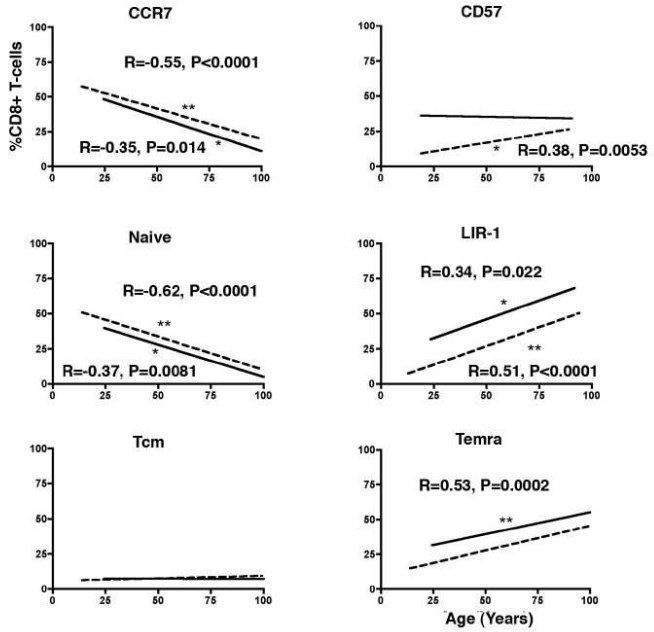

Figure 3.12: Graph showing relationship between age and phenotype in CMV seropositive persons (solid line) and CMV seronegative persons (dashed lines). The phenotype studied is indicated on the title for each graph.* indicates that the 'p' value for Spearman's correlation coefficient was <0.05, ** indicates that it was <0.005. Where there is no asterisk adjacent to the fitted regression line the correlation co-efficient was >0.05. The 'R' value and exact 'p' value is indicated (where p<0.05 or <0.005) immediately above or below regression lines.

Using regression analysis I calculated the difference in intercept, the average co-efficient and the likelihood that the co-efficients were equal for bulk CD8+ cells in CMV seronegative and seropositive persons and CMV specific cells where correlation co-efficient models were significant. This analysis is indicated below (table 3.2):

	LIR-1	CD57	T_{EMRA}	CCR7
CMV TET+	0.24 (+/-0.099) R^2=0.14, p=0.016	0.13 (+/-0.17) R^2=0.02, p=0.44	-0.03 (+/-0.12) R^2=0.004, p=0.76	-0.48 (+/-0.25) R^2=0.07, p=0.62
Bulk CD8+ CMVpos	0.38 (+/-0.12) R^2=0.20, p<0.001	-0.12 (+/-0.14) R^2=0.02, p=0.402	0.18 (+/-0.10) R^2=0.04, p=0.074	-0.32 (+/-0.09) R^2=0.13, p=0.001
Bulk CD8+ CMVneg	0.47 (+/-0.089) R^2=0.25, p<0.002	0.56 (+/-0.20) R^2=0.14, p=0.007	0.711 (+/-0.20) R^2=0.26, p=0.001	-0.65 (+/-0.1) R^2=0.34, p<0.001
Comparison	0.53 (+/-0.17) R^2=0.39, p=0.99 Intercept=+18.7	-	-	-0.52 (+/-0.09) R^2=0.28, P=0.341 Intercept=-16.1
AGE (CMV-S)	9.6 (7.28-14.2)	-	-	-
AGE (CMV)	35 (26.7-51.7)	-	-	30.1 (26.4-37.4)

Table 3.2: This table outlines the regression analysis linking phenotype and age for CMV-specific cells (tetramer positive) top line, bulk CD8+ cells (CMV seropositive) second line and bulk CD8+ cells (CMV seronegative) persons (third line). Where correlation co-efficients were significant (see figure 3.8) and a linear regression model was significant ($p<0.05$), regression analysis was used to compare the models. This could be done for all three groups for LIR-1, and for bulk CD8+ cells for CMV seropositive and seronegative persons for CCR7. Age is linked with phenotype changes as was CMV seropositivity, thus CMV could be deemed to have an 'ageing effect'. By dividing the difference in intercept by the co-efficient, an 'ageing effect' could be calculated using CCR7 and LIR-1. Both figures fell within similar ranges (35 years for LIR-1(CD85j/ILT-2), and 30.1 years for CCR7) with overlapping 95% confidence intervals (in brackets), demonstrating that an 'ageing effect' could be estimated as being similar for LIR-1 and CCR7 (despite these markers being very different both at the molecular and cellular level). CMV therefore has an 'ageing effect' of approximately thirty years. Because for LIR-1(CD85j/ILT-2) all three groups of CD8+ T-cells' phenotype significantly changed with age, the relative age of CMV-tetramer positive cells could be calculated. This was 9.6 years. In other words tetramer positive cells had the characteristics seen in cells from a person nearly ten years older than those in the actual host. For LIR-1(CD85j/ILT-2) the co-efficients were surprisingly uniform for the three T-cell groups ($p=0.99$) whereas for CCR7 on bulk CD8+ T-cells in CMVpos and CMVneg there was a higher likelihood of a significant difference ($p=0.34$).

By dividing the difference in intercept (between CMV positive and CMV negative persons) by the regression co-efficient it was possible to estimate an 'ageing effect' for CMV. This was 35 years (95% confidence interval 26.7 – 51.7) for LIR-1(CD85j/ILT-2) and 30.1 years (26.4-37.4) for CCR7. These two sets of values were surprisingly harmonious considering the wildly different biology and relationship to T-cell functions of LIR-1(CD85j/ILT-2) and CCR7.

3.5 Discussion:

3:5:1 The link between the size of CMV-specific T-cell Responses and their Phenotype

This is the first study to characterise the relationship between the maturation of anti-viral memory T-cell populations and their frequency across such a wide range of ages. I found that an increase in three separate phenotypic subsets of T-cells (T_{EMRA}, CD57, LIR-1) were associated with the size of CMV specific responses, and an increase in CCR7 was related to smaller sizes of T-cell responses. A loss of CD27 has also been related to increasing sizes of CMV-specific populations in a mixed cohort including both healthy volunteers and renal transplant recipients with evidence of CMV reactivation. (Loss of CD27 is indicative of increased maturation to an effector memory phenotype (Gamadia, van Leeuwen et al. 2004)). This work corroborates the findings of this study and also suggests that similar relationships between T-cell phenotype and frequency may be seen in both latent CMV and reactivated CMV. (Acute, primary CMV infection, an uncommon clinical presentation, requires further study).

Virus specific populations differ in their size. HCV specific populations are scanty, HIV-specific populations have intermediate size whereas CMV specific populations are often extremely large (as discussed). If our findings for CMV specific populations (increasing population size with greater expression of effector memory markers) are transferable to other virus specific populations we would expect greater expression of markers of effector memory cells on CMV specific cells, less on HIV-specific cells and the least expression of these markers on HCV specific cells. This was found to be the case when different virus specific T-cell populations were evaluated (Appay 2002). However, the relationships between phenotype and frequency of anti-viral T-cells in HCV and HIV infection require exploration to confirm a generalised link between the size of responses and phenotype. Although re-activation of CMV-specific T-cells can lead to transient re-expression of CCR7 on T_{EM} cells (van Leeuwen, van Buul et al. 2005), it is unlikely that this affected our findings, as CCR7+ CMV-specific cells, were on the whole, extremely rare. In fact I assumed these were T_{CM} cells. This assumption was supported by the negative correlation between CCR7 expression and population size, as loss of CCR7 seemed to be associated with a drive towards larger virus specific populations. It would be of interest to study this in acute infection. The analysis linking the CD8+/CD4+ ratio with changes in T-cell phenotype also suggests T-cell phenotype is generally linked with expansion, although this wasn't seen in CMV seronegative persons.

Gamadia et al linked their findings with the molecular biology of CD27. However, we find that expression several markers is related to expansion of T-cell pools, thus it is not

clear which markers *'drive'* T-cell expansion and which are simply *'passenger'* interactions that may have other functions for T-cell biology. Several factors may account for increased number of T-cells in peripheral blood. These include increased proliferation, increased exposure to antigen, increased sensitivity and or exposure to growth factors, reduced trafficking to tissues and lymphoid tissue and increased survival. The breadth of these possibilities indicates scope for a larger number of molecular and cellular pathways to be involved.

Given the correlation with CD57 as demonstrated here, CD8+ LIR-1 expression may also define a subset of T-cells with considerable effector potential -but poor proliferative potential. However, in contrast to CD57, LIR-1 may be more likely to be a *'driver'* rather than a passenger marker: LIR-1 has inhibitory functions on T-cells through binding to class I molecules (Saverino 2001; Saverino 2002) and may, potentially, contribute to the development of large T-cell populations, as its expression may limit the ability of T-cells to suppress CMV (or other virus) replication.

CMV may be able to exploit expression of LIR-1 on T-cells as it contains DNA for a MHC-class I homolog known as UL18 which has exquisitely high affinity for LIR-1 (Chapman 1999). Theoretically, UL18 expression in tissues may greatly augment the inhibitory functions of LIR-1, compared to MHC class I molecules (its physiological ligand), when T-cells engage with CMV infected cells. This would neatly explain the persistence of latent infection in the face of high, and indeed expanding T-cell frequencies, and fit with the suggestion that CMV specific CD8+ T-cells may be

dysfunctional (Ozdemir, St John et al. 2002), as the resultant persistence of antigen would drive further accumulation of CMV-specific T-cells. UL18 appears to be expressed in tissues – at least during clinical CMV disease (Saverino 2004). Furthermore, Young et al have observed that in vitro expansion of CMV specific T-cells with CMV antigen is associated with increasing expression of LIR-1 – (personal communication – data not shown). Although, this is probably an over-simplistic model of the situation *in vi*vo, it is consistent with the hypothesis that persistent, low-level antigen exposure drives the accumulation LIR-1+ CD8+ T-cells. We did not however perform *ex vivo* functional assays in an attempt to evaluate true 'effector status' in this study. In theory, IL-15 may have a role here, as it has been implicated in CD45RA-reversion and can drive T-cell proliferation and maturation in a non-antigen specific manner (Alves, Hooibrink et al. 2003; Dunne, Belaramani et al. 2005) Thus some *'drivers'* of T-cell expansion may not be established T-cell phenotypic markers. Indeed the IL-15 Receptor (IL-15R) showed low expression on virus specific T-cells, but was expressed on a small proportion of T-cells after activation (data not shown), indicating that expression of the IL-15R is probably a poor surrogate for total IL-15 activity over time. We considered measuring IL-15 concentrations in the serum samples but felt that the storage at room temperature prior to collection would probably lead to degradation of IL-15.

It is likely that the dynamics of the pp65 response and those to other CMV-specific peptides such as IE-1 are different. Indeed the responses may interact significantly, as proposed in section 3:3:4.

3:5:2 The relationship between the phenotype of CD4+ and CD8+ Bulk T-cell Pools:

Recent work has elucidated pathways that might explain the phenotypic coupling of CD4+ and CD8+ T-cells observed in this study. It has been postulated that antigen presenting cells bearing the relevant epitope may transfer some membrane components to CD4+ T-cells when immunological synapses are formed. This could occur as an intrinsic part of T-cell priming or recall responses. This has been termed trogocytosis (from the Greek, *trogo* or 'gnaw'). The CD4+ T-cells may therefore bear APC co-stimulatory molecules, and, most importantly, MHC class I – peptide sequences. These CD4+ T-cells may therefore act as potent stimulators of CD8+ T-cells. The capacity of CD4+ T-cells to act as potent stimulators of CD8+ T-cells has been established, at least in principle (Adamopoulou, Diekmann et al. 2007). This process would neatly explain the observations of CD4+ and CD8+ coupling made in this study. The discordance between the phenotype of the T_{EMRA} subset in (except in CMV seropositive persons) indicates that this subset may have the CD4+ CD8+ link 'uncoupled'.

A second mechanism to explain CD4+ - CD8+ coupling is through CD4+ T-cell secretion of IL-21. This γ-chain cytokine seems to be specific in its secretion to activated CD4+ T-cells. Recently it has been found that IL-21 can support differentiation of naïve CD8+ T-cells with development of effector memory functions such as cytotoxicity (Casey and Mescher 2007). An interesting aside is that the CD8+ T-cells generated in such a manner are low in programmed death molecule-1 (PD-1). PD-1 is a novel marker, the discovery of which has promised new avenues with which to modulate anti-viral immunity. It is

highly expressed on HIV-specific CD8+ T-cells (Day, Kaufmann et al. 2006), and seems to render the cells refractory to stimulation, a state termed 'exhaustion', Anti-PD-1 antibody has potent immunostimulatory effects on CD8+ T-cells, suggesting a way to exploit latent reserves of potential CD8+ T-cell activity, with obvious potential benefits to HIV control. It is possible that IL-21 deficiency as a consequence of CD4+ T-cell depletion (which occurs to a huge extent in the gastro-intestinal tract in early HIV-1 infection (Guadalupe, Reay et al. 2003)), results in reduced production of IL-21, and subsequently impaired CD8+ T-cell responses, which are in a PD-1 high, exhausted state.

3:5:3 CMV responses and the phenotype of bulk CD8+ T-cell pools:

It is clear that CMV has marked effects on the overall CD8+ T-cell compartment. T_{EMRA} cells are found at far higher levels in CMV positive persons. The overall effect makes the CD8+ T-cell compartment resemble that from more elderly person. Regression analysis of our data enabled us to more precisely define this as representing an 'ageing' of approximately 30 – 35 years in response to CMV infection. Similar estimates were found using LIR-1 (an effector memory marker) and CCR7 (a central memory marker) suggesting that the analysis was robust and consistent for functionally disparate markers. The relative loss of naïve cells would appear to partly explain this 'ageing effect' as CCR7+ T_{CM} cells were similar across all ages and seemed independent of CMV-status. The link between CMV and the overall phenotype of T-cells was restricted to the CD8+ compartment as CMV seemed to have little, if any, effect on CD4+ T-cell phenotype. This finding is contradicted by other recent work, where CMV was associated with significant differences in expression of several markers on bulk CD4+ T-cell pools

(Pourgheysari, Khan et al. 2007). However, the analysis in that study was restricted to persons over 65 years old, whereas a far wider age range was studied here. This suggests that on average, CMV does not significantly affect the phenotype of bulk CD4+ T-cell pools.

It is possible that the effect of CMV on bulk CD8+ T-cell pools occurs through the progressive expansion of multiple, CMV specific, clonal CD8+ T-cell populations. At the molecular levels these populations could accrue through interactions such as that postulated between CD85j (LIR-1/ILT-2) and UL18. Consistent with this notion is the finding from previous work that CMV is associated with absolute increases in numbers of CD4 and CD8+ T-cells (Moss and Khan 2004), as well as affecting the overall phenotype of the CD8+ T-cell pool. It also now seems feasible for a large proportion (perhaps the majority, if naïve cells are excluded) of CD8+ T-cells to be committed at least in some cases to CMV – especially in the elderly - given the breadth of the anti-CMV response (Sylwester, Mitchell et al. 2005).

Although I did not prove in this study that latent infection with CMV can drive maturation and proliferation of non-CMV-specific T-cell pools, the group was able to establish that hepatitis C infection has such an effect: in the presence of HCV infection, CMV specific T-cells showed greater expression of central memory markers, including CCR7 (Lucas, Vargas-Cuero et al. 2004). It is unclear at this stage whether this effect of HCV is due to impaired maturation, or premature loss of T-cells. CMV may have a similar pervasive effect on CD8+ T-cells, albeit – in contrast to hepatitis C - one that may

result in increased maturity of T-cell pools. It would be interesting to evaluate the phenotype and frequency of EBV or Flu specific T-cells in persons who are CMV seropositive to see if such a 'pervasive' effect does occur.

CD57 expression is associated with shortened telomeres, indicative of a history of many replicative cycles and limited potential to proliferate further (Brenchley, Karandikar et al. 2003). On the other hand, T-cell proliferation may be restricted to CCR7+ subpopulations (Sallusto, Lenig et al. 1999). In simple terms, therefore, younger individuals may generally have small CMV populations of 'low replicative history' but 'high replicative potential'. In contrast, elderly subjects show T-cells of 'high replicative history', but 'low replicative potential'.

Generally the CD8+ T-cell compartment as a whole shows reduced expression of CCR7, but increased expression of CD57 with age. CMV specific cells show reduced expression of CCR7 and increased expression of CD57 compared to the CD8+ T-cell pool as a whole and can therefore be conceptualised as showing accelerated maturation, as if on a 'fast track', with more rapid loss of CCR7 and more rapid acquisition of CD85j (LIR-1/ILT-2) and CD57. (Loss of naïve cells probably cannot explain the entire ageing effect of CMV, as only Naïve and T_{EMRA} cells and not T_{EM} or T_{CM} cells changed significantly with age). Presumably accumulation of T_{EMRA} cells is driven by continuous, low level, antigen exposure. Radiolabelling of T-cells with deuterated water (which is incorporated into DNA during cellular mitosis) enables their kinetics to be evaluated. Most CD4+ and CD8+ T-cell subsets show rapid loss of this marker over a period of five days indicating

that even in healthy persons T-cell turnover is relatively rapid. However the CD8+ T_{EMRA} subset retains cells containing this marker for far longer, indicating that they persist in the circulation . Although it is unclear if this is a feature of this subset in elderly persons specifically, it seems likely that latent CMV is linked to long lived CD8+ T_{EMRA} cells especially in elderly subjects. It is unclear why CD8+ T_{EMRA} cells are long lived, since they are laden with perforin and granzyme – containing granules and these markers are linked to AICD (– see earlier). Possibly CMV (perhaps via immunoevasins such as UL18) is able to block degranulation and therefore prevents activation of the pro-apoptotic pathway that leads to AICD (for example by blocking the appearance of FasL on T-cells). Alternatively the changes seen in elderly subjects may be partly set in motion by conditions related to acute infection with CMV. For example UL18, and other CMV immuno-evasins, could have enduring effects if they are highly expressed in tissues during acute infection.

It seems that CMV may be unique among common pathogens in the duration and magnitude of the effect it has on the immune system after acute infection in otherwise healthy individuals. These findings have implications for the healthy functioning of the immune system. In elderly persons the effect of CMV may reach a climax with development of a 'high-risk immune phenotype' (Ouyang 2003). Our data provides a detailed analysis as to how this develops systematically in the years spanning youth to old age. In addition to this CMV may also have important effects in specific circumstances in younger individuals. For example, CMV infection has a deleterious effect on the progression of HIV infection to AIDS, even in the era of highly active anti-retroviral

therapy (Deayton JR 2004). This finding may be related to the relative expansion of CD8+ CD57+ T-cells in CMV seropositive, HIV positive persons compared to CMV seronegative HIV positive persons (Looney RJ 1999) - an effect that is independent of HIV viral load and absolute CD4+ T-cell counts. CMV may therefore be a dominant influence on the phenotype, and perhaps size of the CD8+ T-cell pool - in the context of HIV infection as well as healthy persons. It is likely that CD8+ T-cells have a role in preventing CMV reactivation in HIV-positive persons as persons who have reactivated CMV have lower CD8+ T-cell responses to CMV (Garber, Silvestri et al. 2004).

It is surprising that there is no clear evidence from clinical studies for an effect of latent CMV on functioning of the immune system, except maybe at the extremity of life and during HIV infection, given the magnitude of the systematic effect on the immune system described here.

Chapter 4: 'A link between phenotype of HIV-specific T-cells and control of HIV'

4:1 Abstract

CD8+ T cells are believed to play an important role in control of HIV-1 infection. However, despite intensive efforts, generally it has not been possible to link the overall magnitude of the CD8+ T cell response with control of HIV-1. In this study I investigated whether qualitative analysis of HIV-1-specific CD8+ T cell phenotype would be a better predictor of viral control, compared to quantitative analyses alone, in a cohort of ten persons with early infection. My results demonstrate that both a larger proportion and absolute number of CD8+ T_{EMRA} (CCR7-CD45RA+) cells were associated with a lower future viral load set point. Conversely, a larger absolute number of HIV-1-specific CD8+ T_{EM} cells (CCR7-CD45RA-) cells was not related to the viral load set point. Overall the findings suggest that T_{EMRA} cells have superior anti-viral activity, and indicate that both qualitative and quantitative aspects of the CD8+ T cell response need to be considered when defining the characteristics of protective immunity to HIV.

4:2 Acknowledgements:

For this chapter I acknowledge with special gratitude the help of Professor Douglas Nixon, Christopher Loo and Dr Jakob Michaelsson in San Franscico. I was given a lot of help in obtaining samples, designing the experimental protocol and executing the

experiments (not least in performing experiments on the LSR II!). I also acknowledge their help in assisting in analysis of the data.

4:3 Introduction

The spread of Human immuno-deficiency virus (HIV) infection is now best described as a global pandemic which continues to exert a huge toll of death and morbidity in communities worldwide (Garber, Silvestri et al. 2004). Efforts to combat its spread by design of an effective vaccine have so far been frustrated, and long-term therapy with anti-viral drugs remains sub-optimal because of drug toxicity, viral drug resistance and high cost.

HIV was first identified in the 1980's. Twenty five million persons are now infected in Africa, and epidemics are also still developing in South East Asia and China. HIV is a retrovirus. Retroviruses contain RNA and a Reverse Transcriptase that transcribes RNA into pro-viral DNA. Pro-viral DNA can thus be used as a template for mRNA synthesis and for integration into the host genome. HIV resembles Lentiviruses. These are viruses that infect a wide number of animals and are characterized by a chronic course of infection that affects haematopoietic tissues. HIV consists of 9 ORF's. In common with other retrovruses'gag', 'pol' and env' genes are present. The 'gag' gene encodes matrix (MA), capsid (CA), neucleocapsid (NC) and p6 proteins. The 'pol' gene contains the code for key enzymes: Reverse transcriptase (RT), Protease (P) and Integrase (IN). The 'env' gene encodes for surface glycoproteins gp120 and gp41. In addition to these

proteins HIV contains the following 'accessory' proteins: Vif, Vpr, Nef, Tat and Rev. These proteins have various functions that assist at multiple aspects to HIV biology, such as: 1) Facilitation of transcription of pro-viral DNA into mRNA for protein synthesis (Tat); 2) Release of virus particles (VpU), 3) Evasion of innate immune pathways (Vif inhibits a cellular enzyme that degrades viral RNA) and 4) Evasion of cellular immunity via downregulation of MHC class I, (for example Nef) (Frankel and Young 1998; Fields, Knipe et al. 2007).

Two main subspecies of HIV exist: HIV-1 and HIV-2. Both viruses probably originate from Simian (monkey) viruses: SIV_{CPZ} (CPZ = chimpanzee) and SIV_{SM} (SM = Sooty Mangabeys), respectively. Unlike other lentiviruses there is a huge amount of variability in HIV sequences. This is encouraged by the error prone tendency of the RT enzyme, recombination between different HIV sequences in the same cell, the high levels of virus replication (10^9 copies per day, or 150-300 replication cycles per year) and the large number of infected persons. Even within the same individual different quasispecies can be found. Three groups of HIV clades have been defined. These are 'M' (Main) = 95% of cases, 'O' (Outlier) and 'N (non-M and non-O). 9 'clades are described in the 'M' group (A,B,C,D,F,G,H,J,K). These clades are genetically equidistant. In addition 15 circulating recombinants CRF clades are present. Clade C is responsible fifty percent of infections worldwide as it is the predominant clade in Africa. Clade B is the predominant clade in the United States and in the EU clades A,B,C and G are found. Interestingly HIV-1 and HIV-2 strains do not recombine. Infection is either through sexual transmission or by entry of infected blood into uninfected persons. Co-infection with other sexually

transmitted diseases, or infections such as TB and malaria, may facilitate spread. On the other hand circumcision may lead to a 60% decrease in risk of infection (Fields, Knipe et al. 2007).

HIV is able to enter cells expressing CD4. Thus CD4+ Helper T-cells are targeted. In addition CCR5 and CXCR4 are important co-receptors. Thus macrophages and dendritic cells can become infected. DC-SIGN is a lectin that can enable dendritic cells to transmit HIV to CD4+ T-cells without the virus needing to enter the dendritic cell (Stevenson 2003).

Despite the high levels of viral replication, like CMV, HIV is able to latently infected cells. These may be CD4+ T-cells or macrophages/ dendritic cells. Latently infected cells need to be in a resting state and out of cell cycle (Go). However, the G_1 stage of cell cycle may be more permissive to HIV integration into the genome as access to sites of integration into cellular DNA need to be available (Stevenson 2003). Latent infection is important as it limits the capacity of anti-viral drug regimes and/or immune responses to sterilize the host. Activation of a latently infected cell will result in replication of HIV along with other cellular proteins (Stevenson 2003).

HIV probably causes AIDS though direct cytopathogenic effects on the CD4+ T-cell compartment, through pro-apoptotic effects on uninfected cells and through stimulating the immune system into a state of chronic activation (Stevenson 2003). HIV may infect 30-60 percent of gut T-cells within the first 4 days of infection. The prognosis for the

newly infected persons varies widely, and can naturally range from 6 months to 25 years (Fields, Knipe et al. 2007). Peak viral load in acute infection can be $10^6 – 10^7$ copies per ml, and this drops to a mean of 3^4 in chronic infection. The viral load 'set point' is the viral load after acute infection and predicts the prognosis (Mellors, Rinaldo et al. 1996). As well as the viral load set point the CD4+ count predicts prognosis (Fields, Knipe et al. 2007).

Acute infection may have non-specific symptoms with flu like symptoms. A rash may be found in 70% of cases. Other features are lymphadenopathy and, in some cases, meningoencephalitis. Leucopenia and mild hepatitis may occur. The manifestations of chronic HIV depend on the degree of immunosuppresion and range from non-specific findings such as seborrhoeic dermatitis and unusual malignancies such as Kaposi's sarcoma.

The diagnsosis of HIV infection can be made by looking for anti-HIV antibody. This is usually present by three months post infection, but may take up to six months. Western blots to HIV proteins can be used to confirm a positive test. Assays for HIV antigens (p24 antigen) in serum can be used to detect HIV in the 'window' period prior to seroconversion. In addition PCR can be used to detect and measure virus neucleic acid (Fields, Knipe et al. 2007).

HIV can be treated by anti-retroviral therapy. The mortality may be reduced by 80% by regimes of multiple drugs (Highly active anti-retroviral therapy – HAART). Reverse

transcriptase inhibitors can work competitively to block neucloside/neucleotide binding (Neucleoside/neucleotide reverse transcriptase inhibitors – NRTI) and non-competitive non- neucleoside/neucleotide reverse transcriptase inhibitors –NNRTI). Protease inhibitors block processing of the 'gag' and 'pol' genes. Enfuviritide can be used to block virus fusion with the cell membrane. Combinations of drugs are used to avoid resistance to the drugs from developing. Full suppression of virus is linked to a better immunological and clinical recovery. Ongoing treatment is necessary because of the stubborn persistence of a small number of latently infected cells. It is estimated that there may be around 10^6 of these, and they may have a half life of over 40 months and would require up to 60 years of full HAART for their elimination to be complete. Immunostimulation (with IL-15, for example), which by activating T-cells may 'flush out' virus, so far has not shown significant benefit (Williams and Greene 2005). Control of virus pharmacologically has the potential to undermine the immune responses as levels of exposure to antigens may be less. However strategic structured interruptions of therapy (STI) have not shown any clinical benefit and potentially my expose the host to further cycles of viral replication (Pai, Lawrence et al. 2006).

It is clear that the immune system can control HIV-1 replication to a certain extent, albeit with large inter-individual variation. The CD8+ T cell response is assumed to play an important role, and is regarded as being sufficiently robust to exert some control of viral replication (Gandhi and Walker 2002; Klenerman, Wu et al. 2002) following acute infection, and probably helps to establish the 'viral load set point' – a key long term prognostic indicator (Mellors, Rinaldo et al. 1996). However, progression to AIDS in

untreated HIV-1-infected individuals is, in general, inevitable, although some patients progress only slowly to AIDS, if at all. These patients, known as long-term non-progressors (LTNP's), may have superior immune responses, suggesting that in the majority of infected persons, the CD8+ T-cell response does not attain its full potential. Therefore manipulation of the CD8+ T-cell response (through prior administration of a vaccine, for example) may pave the way to partial and perhaps even permanent control of HIV infection.

However despite strong evidence for a role for CD8+ T cells in control of HIV-1 infection (Musey, Hughes et al. 1997; Gea-Banacloche, Migueles et al. 2000; Betts, Ambrozak et al. 2001; Edwards, Bansal et al. 2002; Addo, Yu et al. 2003; Masemola, Mashishi et al. 2004; Kiepiela, Ngumbela et al. 2007), attempts to statistically link the magnitude of the CD8+ T cell response with control of HIV-1 replication in vivo have yielded contradictory results, although this may reflect variations in methodology (Gea-Banacloche, Migueles et al. 2000; Addo, Yu et al. 2003; Masemola, Mashishi et al. 2004; Betts, Nason et al. 2006). Indeed fifty percent of studies have not confirmed an association of the magnitude of the CD8+ T cell response with control of HIV-1 viral replication, and one study actually showed that a large HIV-1-specific CD8+ T cell response was linked to higher HIV-1 viral loads (Masemola, Mashishi et al. 2004) – See Table 4:1. In the light of these studies, use of the magnitude of HIV-1-specific CD8+ T cell responses alone as a predictor of clinical outcome may not be appropriate. However, studies combining quantitative and qualitative examination of the CD8+ T cell responses may enable its input in control of HIV-1 infection to be determined. For example, a

recent study using multi-color flow cytometry showed that long-term non-progressors (LTNPs) have more 'multi-functional' HIV-1-specific CD8+ T cells compared to persons with progressive disease (Betts, Nason et al. 2006).

In addition to examining the role of 'specialist' multi-functional cells, or selecting the responses according to what part of the virus is targeted, it is plausible that the general phenotype of the HIV-1-specific CD8+ T cell response is linked to its anti-viral capacity. Different 'memory' subsets, defined according to phenotype, have different functional capabilities and roles (Sallusto, Lenig et al. 1999; Champagne, Ogg et al. 2001; Lanzavecchia and Sallusto 2005), and their relative contribution to the overall size of the response may relate to its overall efficacy. For example, 'effector memory' (T_{EM}) cells, which are CCR7-, have powerful and direct anti-viral capacity, whilst on the other hand, 'central-memory' (T_{CM}), which are CCR7+, have little direct anti-viral activity but probably serve to replenish and sustain CD8+ T_{EM} populations through secretion of IL-2 and proliferation. In addition some T_{EM} cells lose CD45RO and re-express CD45RA (CD45RA reversion) although the significance of this is uncertain currently. This part of the thesis sets out to test the hypothesis that HIV-specific T-cell phenotype might relate to the overall potency of the response and therefore control of HIV viral replication. The basic approach was to use intracellular cytokine staining to detect and perform phenotypic evaluation of HIV-specific CD8+ T-cells responding to HIV-'gag' peptides. This was facilitated by use of polychromatic flow cytometry (PFC) that permits multiple aspects of T-cell biology to be evaluated simultaneously (De Rosa, Herzenberg et al. 2001). We studied T-cells in early HIV during early HIV infection, anticipating that any

link between T-cells and control of infection may be more demonstrable at this point. We also linked T-cell parameters in early infection with the 'viral load set point', with the aim of linking T-cells with subsequent enduring control of HIV.

AUTHOR & JOURNAL	YEAR	NUMBER OF PATIENTS	DISEASE STAGE	ASSAY	LINK WITH VIRAL LOAD?
MUSEY (NEJM)	1997	33	EARLY INFECTION	51Chr Release	Negative
OGG (SCIENCE)	1998	14	VARIABLE	Class I Tetramer	Negative
GEA-BANACLOCHE (J. IMMUNOLOGY)	2000	7 LTNP'S & 8 UNTREATED	CHRONIC	ICS	No link
EDWARDS (J. VIROL0GY)	2002	27	CHRONIC	ELISpot	Negative
ADDO (J. VIROLOGY)	2003	23	CHRONIC	ELISpot	No link
MASEMOLA (J.VIROLOGY)	2003	44	12 MONTHS	ELISpot	Positive
BETTS (BLOOD)	2006	79 & 9 LTNP's	CHRONIC	PFC & ICS	No Link
KIEPEILA (NATURE MEDICINE)	2007	578	Chronic	ELISpot	+ve with anti-'env' T-cells, -ve with anti-'gag' T-cells

Table 4:1: Summary of studies attempting to link the frequency of HIV-specific T-cells with control of infection. Studies vary according to size of cohort, stage of disease, assay for enumerating the HIV-specific response, the specificity of the T-cells for various parts of HIV and finally , result.

4:4 Methods:

4:4:1 Study samples

Peripheral blood samples from HIV-1-infected patients were obtained by venipuncture from 10 adult patients recruited after informed consent from a pre-existing UCSF cohort recruited for a study known as 'Options'. This cohort includes a number of persons who have been identified as having early infection – see table 3:1. The date of HIV-1 infection was estimated based on one of the three following approaches: (1) prior data on median time from exposure to acute retroviral syndrome (ARS) or an indeterminate HIV-1 antibody test, as described by (Lindback, Thorstensson et al. 2000) (2) the mid-point between last negative and first positive HIV-1 antibody tests, or (3) the level of a less sensitive HIV-1 antibody test if the optical density was between 0.5 and 1.0, a range in which there has been shown to be a linear relationship between the less sensitive antibody results and the days since seroconversion (Kothe, Byers et al. 2003). The UCSF Committee for Human Research approved this study and patients gave signed consent. All patients were treatment naïve at the time of sampling, and remained untreated for more than 16 weeks after sampling had finished (mean ± 1SD: 29 ± 7.6 weeks, range 16-40 weeks). The viral load setpoint was calculated by averaging all viral load measurements between six to twelve months after the estimated date of infection. The average length of time used to calculate the viral load setpoint was 4.6 months. PBMC were isolated from heparinized whole blood by Ficoll-Paque and density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden) and frozen in fetal bovine serum with 10% DMSO. Before utilisation, frozen cells were thawed, washed twice in

R15 medium (RPMI 1640 (MediaTech, Herndon, VA) supplemented with 15% FCS (Gemini BioProducts, Woodland, CA), L-glutamine, penicillin and streptomycin) and used directly in functional assays.

Patient	Viral Load[1]	CD4 count[2]	ETI[4]	Age	Gender
1	265	594	12	36	Male
2	68000	588	7	34	Male
3	70300	432	29	49	Male
4	61800	616	12	42	Male
5	28400	495	8	34	Female
6	1380	558	14	35	Male
7	20293	384	14	36	Male
8	69800	608	20	40	Male
9	534	720	14	33	Male
10	7670	640	26	23	Male

Table 4:2: Outline of patient's characteristics recruited from the 'Options' cohort – see text. 1 = Viral load at study entry. 2 = CD4 count per μL of blood. 3 = estimated date of infection (weeks).

4:4:2 Cytokine flow cytometry and phenotyping

Thawed PBMC were cultured with 1μg/ml Staphyloccoal enterotoxin B (SEB) (Sigma-Aldrich, St. Louis, MO), 1μg/ml HIV Gag p55 peptide mix (BD BioSciences), or with R15 medium alone for 15 hours. Brefeldin A (Sigma-Aldrich, St. Louis, MO) was added at a final concentration of 5 μg/mL after 1 hour of culture. After culture, cells were washed in FACS buffer (PBS with 2 mM EDTA and 1% bovine serum albumin), stained with APC-Cy7-conjugated anti-CD4, PE-Cy5.5-conjugated anti-CD8, biotinylated anti-CD45RA, PE-Cy7-conjugated anti-CCR7, FITC-conjugated anti-CD57, PE-conjugated anti-CD27 (all from BD BioSciences San Jose, CA) for 30 min at 4°C. The cells were subsequently washed, fixed in 1% paraformaldehyde for 15 min and permeabilized in FACS Permeabilizing Solution II for 20 min prior to being stained with Energy coupled dye (ECD)-conjugated anti-CD3, Pacific Blue-conjugated streptavidin, and allophycocyanin-conjugated anti-IFN-γ. Finally, the cells were washed twice in FACS buffer before being acquired on a LSR II flow cytometer (BD) modified from the standard configuration by the addition of a 150 mW green (532 nm) diode laser, and the upgrade of the blue and red lasers to 100 mW and 25 mW respectively. The green diode was used for the excitation of all the PE tandem conjugates. All flow cytometry data was analyzed using FlowJo software (Tree Star, San Carlos, CA). The gating strategy used is depicted in figure 4:1

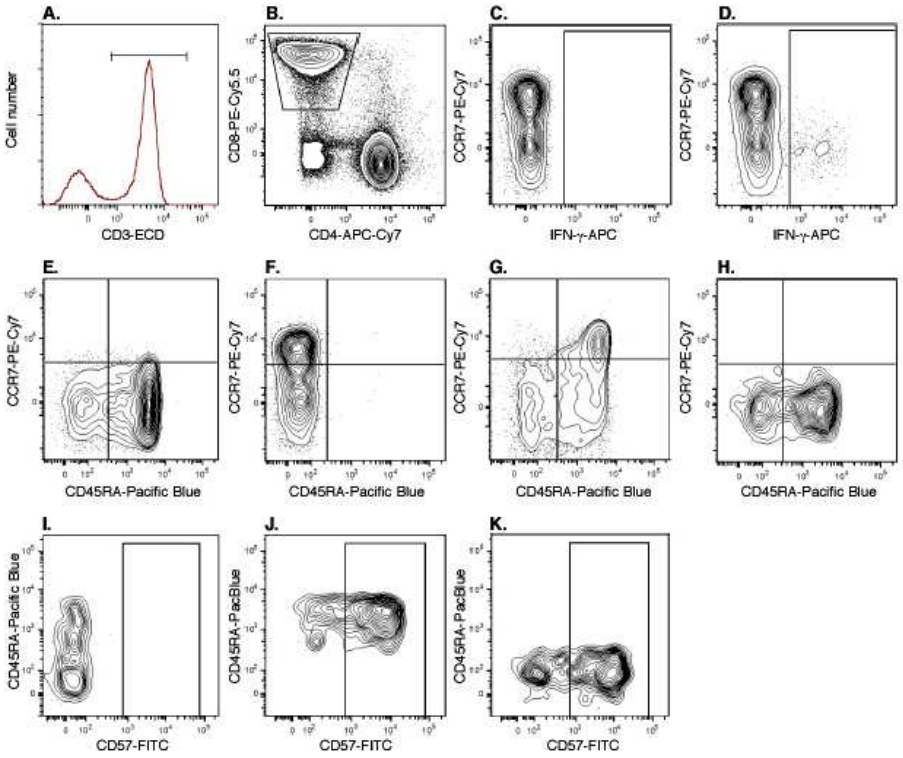

Figure 4:1 Gating strategy for phenotyping of HIV-1-specific CD8+ T cells. (A) T cells were selected based on CD3 expression. (B) CD8+CD4- T cell were selected in a CD8 versus CD4 plot. (C) The APC FMO sample was used to set the gate for (D) IFN-γ+ CD8+ T cells. (E) The PE-Cy7 FMO sample and (F) Pacific Blue FMO sample were used to set the gates for T_{EM} (CD45RA-CCR7-) and T_{EMRA} (CD45RA+CCR7-) CD8+ T cells. The expression of CCR7 and CD45RA was analyzed on (G) IFN-γ- CD8+ T cells and (H) IFN-γ+ CD8+ T cells. (I) The FITC FMO sample was used to set the gate for CD57+ cells. The expression of CD57 was analyzed on (J) HIV-1-specific CD8+ T_{EMRA} cells and (K) HIV-1-specific CD8+ T_{EM} cells. FMO is an abbreviation for "fluorescence minus one" - a sample stained with all antibodies except the one indicated.

A wide forward scatter (FSC) versus side scatter (SSC) plot was used to define lymphocytes, followed by exclusion of cell conjugates by using a FSC-Area versus FSC-Height gate. T cells were selected by gating on CD3+ cells, followed by selection of CD8+ T cells by gating on CD8+CD4- cells. IFN-γ+ cells were defined using an allophycocyanin-"fluorescence minus one" (FMO) sample. IFN-γ^+ and IFN-γ^- cells were further analyzed for expression of T cell memory markers in a CD45RA versus CCR7 plot. Quadrant gates were set using the PE-Cy7 FMO and Pacific Blue-FMO. T_{EM} (CCR7-CD45RA-) and T_{EMRA} (CCR7-CD45RA+) populations were further analyzed for expression of CD57 and CD27. The gates for CD57 and CD27 were set using the FITC FMO and PE FMO, respectively.

4:4:3 Statistical analysis

Regression analysis was performed using intercooled stata version 9.0 for Macintosh (StataCorp, College Station, TX). Linear regression was performed using a robust model. Other statistical analysis was performed using non-parametric tests, such as Wilcoxon's test for paired samples and Spearman's Correlation co-efficient using GraphPad Prism 4 for Macintosh (GraphPad Software, San Diego, CA).

4:5 Results.

4:5:1 The overall magnitude of CD8+ T cell responses to HIV-1 GAG early in infection is not a strong predictor of future viral load set point.

In this study I aimed to determine whether HIV-1-specific CD8+ T cell responses at early phases of infection were associated with future control of HIV-1, as assessed by the viral load set point. I made use of 8-color flow cytometry to simultaneously determine the frequency and memory phenotype of CD8+ T cells responding by IFN-γ production to a pool of HIV-1 GAG-derived peptides. Ten treatment naïve subjects with early HIV-1-infection were included in the study (Table 4:2). We evaluated the influence of HIV-1-specific CD8+ T cell responses on subsequent control of infection, by measuring the viral load set point (average of viral loads from six to twelve months post infection). In addition to the overall magnitude of the HIV-1-specific CD8+ T cell responses, we simultaneously determined the expression of CCR7, CD45RA, CD27 and CD57 on the HIV-1-specific CD8+ T cells. The gating strategy used to identify HIV-1-specific CD8+ T cells responding with IFN-γ production and their phenotype is depicted in figure 4:1.

The frequency of IFN-γ+ CD8+ T cells in samples cultured in medium alone was below 0.1%, and this was subtracted from the response to HIV-1 GAG. The HIV-1-specific responses thus calculated ranged from 0.1 to 1.8% of the CD8+ T cells, with an average of 0.44%, with responses being found in nine of ten subjects.

There was no correlation between either the estimated times post infection (R=0.067, p=0.86) or the CD4 count (R= - 0.167, p=0.68) and the viral load set point. Thus these parameters were unlikely to confound the findings of the study and were not used in the analysis from here onwards.

The frequency of responding HIV-1-specific CD8+ T cells was only weakly associated with the future viral load set point when analyzed with Spearman's correlation and linear regression analysis (R=-0.60, p=0.07), (Table 3:3 and Figure 4:2:A). In addition we calculated the absolute number of HIV-'gag' specific CD8+ T-cells (using the absolute CD8+ T-cell count derived from the clinical data), and detected no significant relationship between the absolute number of responding HIV-1-specific CD8+ T cells (per μL of blood) and viral load set point (R=-0.57, p=0.12), (Table 4:3 and Figure 4:2:B). A previous study did relate the size of response with control of viral replication in early infection (Musey, Hughes et al. 1997). This study did use a larger cohort of patients (thirty three), which might explain this discrepancy. However, there is still a possibility that other measurable aspects of the HIV-1-specific CD8+ T cell response, including the contribution of key CD8+ memory T cell subsets, could be linked to control of HIV-1.

4:5:2 HIV-1-specific $CD8^+$ T cells with a T_{EMRA} phenotype are linked to lower viral load set points.

The phenotype of HIV-1-specific $CD8^+$ T cells has been compared to those of $CD8^+$ T cells specific for other viruses, e.g., cytomegalovirus (CMV). The HIV-1-specific $CD8^+$ T-cell response appears to be skewed towards a T_{EM} phenotype, with lower frequencies of

T_{EMRA} cells (which are thought to represent more mature effector cells) than are present with CMV-specific $CD8^+$ T-cell responses (Champagne, Ogg et al. 2001). It has therefore been proposed that HIV-1-specific $CD8^+$ T cells are deficient in their maturation, and this may be one reason why control of HIV-1 infection is poor. If this is the case, it is anticipated that control of HIV-1 infection would be better if there were a greater number of T_{EMRA} cells. In line with this hypothesis, we found that early HIV-1-specific $CD8^+$ T-cell responses with a higher proportion of T_{EMRA} cells were strongly and negatively linked with a lower subsequent viral load set point ($r = –0.73$, $P = 0.03$) (Table 4:3 and Figure 4:2:C.). This finding supports and extends recently published data (Hess, Altfeld et al. 2004), where the fraction of HLA/HIV peptide tetramer-positive $CD8^+$ T cells displaying a T_{EMRA} phenotype was associated with better viral control in patients undergoing structured treatment interruption. Furthermore, responses were enriched for this subset in LTNPs (Hess, Altfeld et al. 2004). Overall, the data suggest that $CD8^+$ T_{EMRA} cells may have an enhanced capacity to enable sustained control of HIV-1 viral replication.

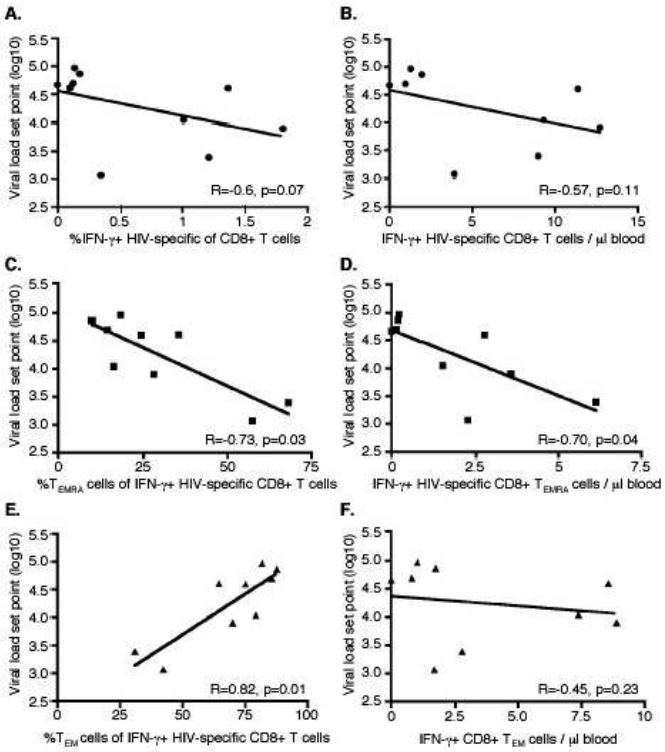

Figure 4:2: Occurrence of HIV-1-specific CD8+ T_{EMRA} cells is linked to future viral control. A) The percentage of CD8+ T cells responding with IFN-γ production in PBMC stimulated with HIV-1 GAG peptides versus future viral load set point. B) Absolute number of CD8+ T cells per µl of blood responding with IFN-γ production in PBMC stimulated with HIV-1 GAG peptides versus future viral load set point. C) The percentage of T_{EMRA} (CCR7-CD45RA+) cells among HIV-1-specific CD8+ T cells versus future viral load set point. D) The absolute number of HIV-1-specific CD8+ T_{EMRA} cells per µl of blood versus future viral load set point. E) The percentage of T_{EM} (CCR7-CD45RA-) cells among HIV-1-specific CD8+ T cells versus future viral load set point. F) The absolute number of HIV-1-specific CD8+ T_{EM} cells per µl of blood versus future viral load set point. The correlations between viral load set point and the HIV-specific CD8+ T cell responses were tested by Spearman's test.

Table 4:3 Analysis of Phenotype Linking CD8+ TEMRA Cells with Control of HIV Infection

	Univariate analysis (percentage terms)[a]			Univariate Analysis (Absolute – per μL blood)[b]			Multivariate Analysis (percentage terms)[c]		Multivariate Analysis (Absolute – per μL blood)[d]	
	'R' Spearman's Test	'p' value*	Co-efficient (SE)	'R' Spearman's Test	'p' value*	Co-efficient (SE)	Co-efficient (SE)	'p' – value*	Co-efficient (SE)	'p' – value*
Overall response	- 0.600	0.073	- 0.438 (0.229) p=0.092	- 0.57	0.121	-0.060 (0.033) p=0.114	0.496 (0.262)	0.131	-	-
T_{EMRA}	- 0.733	**0.031**	- 0.027 (0.006) p=0.002*	- 0.70	**0.043**	- 0.236 (0.039) p<0.001	- 0.053 (0.015)	**0.025**	- 0.263 (0.104)	**0.042**
T_{EM}	+ 0.817	**0.011**	+ 0.029 (0.006) p=0.002	- 0.45	0.230	- 0.035 (0.049) p=0.505	-	-	0.034 (0.059)	0.275
CD57+	- 0.317	0.410	- 0.015 (0.008) p=0.091	- 0.78	**0.017**	-0.218 (0.051) p=0.004	0.013 (0.013)	0.385	-	-
CD27+	- 0.233	0.552	- 0.004 (0.010) 0.627	- 0.633	0.076	- 0.065 (0.068) p=0.376	- 0.027 (0.010)	0.055	-	-

(a) Spearman's correlations coefficient and univariate linear regression analysis were used to relate the overall HIV-1-specific CD8+ T cell response (as a percentage of all CD8+ T cells), as well as the indicated phenotypes of HIV-1-specific CD8+ T cells (as a percentage of all HIV-1-specific CD8+ T cells), with viral load set point. (b) Univariate analysis of the relationship between the absolute number of HIV-1-specific CD8+ T cells with the indicated phenotypes, and viral load set point. (c) Multivariate regression analysis of the terms in (a) with the viral load set point (excluding the T_{EM} cells due to marked co-linearity of T_{EM} and T_{EMRA} subsets). (d) Multivariate regression analysis of the terms in (b) with the viral load set point.

However, several important caveats need to be addressed before this conclusion can be reached. Firstly, it is known that the overall magnitude of the CMV-specific CD8+ T cell response correlates with its phenotype (Gamadia, van Leeuwen et al. 2004; Hess, Altfeld et al. 2004; Northfield, Lucas et al. 2005). Therefore a greater number of T_{EMRA} cells may be linked with larger responses, rather than directly with control of HIV infection. In the current study, however, the fraction of HIV-1-specific CD8+ T cells displaying a T_{EMRA} phenotype was linked with viral load set point independently of the magnitude of the overall HIV-1- specific CD8+ T cell response, as tested by multi-variate regression analysis (Co-efficient = -0.053 SE +/- 0.015, p=0.025) (Table 4:3 and Figure 4:2:C).

Secondly, it remained to be determined whether better viral control was associated with relative enrichment of the T_{EMRA} subset, or relative deficiency of the T_{EM} cell subset: Nearly all of the responding HIV-1-specific CD8+ T cells displayed either a T_{EMRA} or a T_{EM} phenotype, and there was, predictably, a close and inverse correlation between the two ($R=-0.98$, $p<0.0001$). Not surprisingly therefore, a higher proportion of the response with a T_{EM} phenotype was linked strongly and positively with higher viral load set point ($R=0.82$ $p = 0.01$), (Table 4:3 and Figure 4:2:E).

4:5:3 The Absolute Number of HIV-1-specific T_{EMRA} cell is Inversely Related to the Viral Load Set Point, and this is Independent of the Number of HIV-1-specific CD8+ T_{EM} cells.

In an attempt to determine whether more HIV-1-specific CD8+ T_{EMRA} cells, rather than fewer HIV-1-specific CD8+ T_{EM} cells were associated with viral control, we compared

the viral load set point with the absolute number of HIV-1-specific CD8+ T_{EMRA} and T_{EM} cells. We found that a higher absolute number of HIV-1-specific CD8+ T_{EMRA} cells was strongly associated with a lower viral load set point (R= - 0.70, p = 0.04) (Table 4:3 and Figure 4:2:D). In contrast, the absolute numbers of HIV-1-specific CD8+ T_{EM} cells was not related to viral load set point (R=-0.45, p = 0.23) (Table 4:3 and Figure 4:3:F). Furthermore, the relation between the absolute number of HIV-1-specific CD8+ T_{EMRA} cells and viral load set point was independent of the absolute number of HIV-1-specific CD8+ T_{EM} cells, as tested by multivariate regression analysis (- 0.263 lower log10 HIV-1 RNA per each additional CD8+ T_{EMRA} cell measured (per μL blood), p=0.04) (Table 4:3). These results suggested that it is likely that T_{EMRA} cells are directly beneficial to viral control.

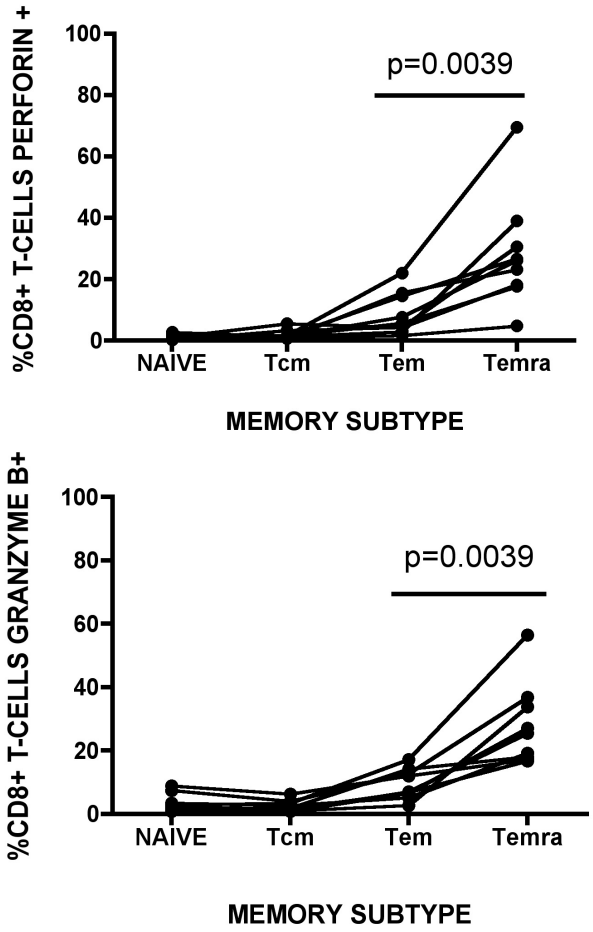

Figure 4:3. In order to explore the basis for the apparent link between the CD8+ T_{EMRA} subset and control of HIV viraemia we compared expression of the lytic granule molecules perforin and Granzyme B on bulk naïve, T_{CM}, T_{EM} and T_{EMRA} cells from nine healthy persons. In common with other studies (see text) we found that T_{EMRA} cells contained the highest levels of these molecules suggesting a mechanism for improved anti-viral function.

In order to explore the mechanism linking the T_{EMRA} subset with control of infection, we evaluated intracellular Granzyme B and Perforin in bulk CD8+ T-cells in nine healthy individuals within the four cell subsets – Naïve, T_{CM}, T_{EM} and T_{EMRA}. Granzyme B and Perforin are released on activation of CD8+ T-cells and can induce cell death (Isaaz, Baetz et al. 1995). Their presence within T-cells may therefore reflect cytolytic function (see introduction). Analysis of bulk CD8+ T cells from healthy individuals revealed that a 4 to 5-fold larger fraction of the CD8+ T_{EMRA} cell subset contained perforin and granzyme compared to CD8+ T_{EM} cells (see Figure 3:3), a finding consistent with previous studies suggesting enhanced cytotoxic function for this subset (Wills, Carmichael et al. 1999; Champagne, Ogg et al. 2001; Dunne, Belaramani et al. 2005). Together with the independent and apparently sustained effect of HIV-1-specific CD8+ T_{EMRA} cells on viral load, these results further strengthened the proposed link between HIV-1-specific CD8+ T_{EMRA} cells and better viral control, and the hypothesis that these cells have stronger anti-viral activity *in vivo.*

4:5:4 CD57 is expressed by a larger fraction of HIV-1-specific CD8+ T_{EMRA} cells compared to T_{EM} cells.

The maturation of CD8+ T cells has been described using several models, of which 'senescence', as measured by CD57 expression (Brenchley, Karandikar et al. 2003), and 'terminal differentiation' – as indicated by T_{EMRA} status (Champagne, Ogg et al. 2001), are commonly used. A larger fraction of the HIV-1-specific CD8+ T_{EMRA} cells expressed CD57 compared to HIV-1-specific CD8+ T_{EM} cells ($p=0.004$ by Wilcoxon matched pairs

test (Fig. 4A). On average 69% of the HIV-1-specific CD8+ T_{EMRA} expressed CD57, compared to 43% of the T_{EM} cells - although a large amount of overlap in CD57-expression is apparent.

The fraction of HIV-1-specific CD8+ T cells expressing CD57 was not a strong predictor of viral load set point (R= - 0.32, p=0.4), (Table 4:3). However, the absolute number of CD57+, HIV-1-specific CD8+ T cells was associated with a lower viral load set point (R=-0.78, p = 0.02), (Table 4:3). We found that the absolute number of HIV-1-specific CD8+ T cells expressing CD57 was closely correlated with the absolute number of HIV-1-specific CD8+ T cells with a T_{EMRA} phenotype (R=0.983, p<0.0001; Fig. 4C). It is possible therefore that the linkage between absolute numbers of HIV-1-specific CD57+ cells and viral load set point was dependent on the T_{EMRA} subset. However, the marked co-linearity between HIV-1-specific CD57+CD8+ T cells and T_{EMRA} cells prevented us from further dissecting the contribution of these two subsets to viral control, in terms of absolute cell numbers. However, when controlling for the fraction of CD57 expressing cells among HIV-1-specific CD8+ T cells with multi-variate regression analysis, the link between viral load set point and the fraction of HIV-1-specific CD8+ T cells displaying a T_{EMRA} phenotype remained significant (Table 4:3). This suggests that the T_{EMRA} subset (rather than the CD57+ subset) was the subset more independently and consistently linked with control of HIV infection.

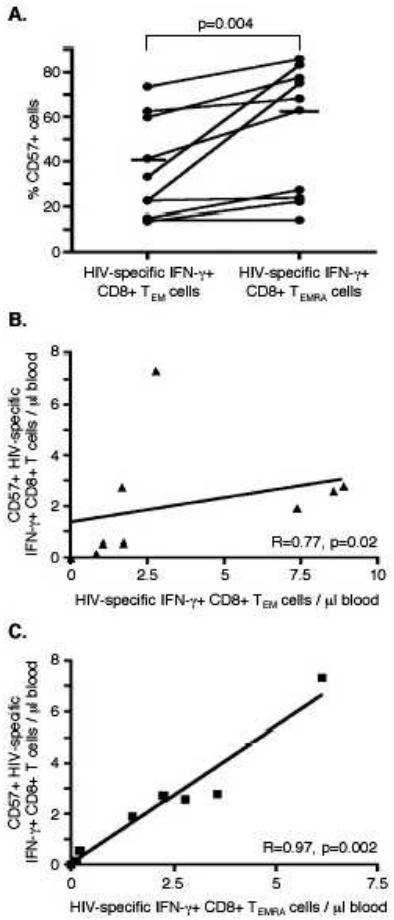

Figure 4:4: CD57 is expressed by a larger fraction of HIV-1-specific $CD8^+$ T_{EMRA} cells than T_{EM} cells. (A) Percentages of HIV-1-specific $CD8^+$ T_{EM} and T_{EMRA} cells that express CD57. Statistical significance was tested by the Wilcoxon matched-pairs test. (B) The absolute number of HIV-1-specific $CD8^+$ T_{EM} cells correlates with the absolute number of HIV-1-specific $CD57^+$ $CD8^+$ cells (r = 0.77, P = 0.02), as tested by Spearman's test. (C) The absolute number of HIV-1-specific $CD8^+$ T_{EMRA} cells correlates with the absolute number of HIV-1-specific $CD57^+$ $CD8^+$ cells (r = 0.98, P < 0.0001), as tested by Spearman's test.

4:6 Discussion

One of the major goals of HIV-research is to develop a highly immunogenic (and safe) vaccine for use on a global scale. Necessarily, the need to better define correlates of immune protection in naturally infected individuals has to be a major driver in the overall research effort. The evidence presented here, indicating that both quantitative and qualitative aspects of the CD8+ T cell response need to be considered when evaluating its overall efficacy in early infection, is therefore timely and important. Specifically, by showing that higher HIV-1-specific CD8+ T_{EMRA} cell numbers, either in absolute or relative terms, are independently associated with a lower future viral load set point, we are able to present a cogent argument that this subset has superior anti-viral activity in vivo. Furthermore, the argument for dichotomy of efficacy (eg that of T_{EM} and T_{EMRA} subsets) advanced here probably explains why, in general, it has not been possible to link the CD8+ T-cell response with actual control of infection in many previous studies. On the other hand, the evidence for dichotomy of function *in vivo*, partly validates the findings from *in vitro* studies of phenotype

Most studies addressing control of HIV have not addressed links between immune responses in early infection and the viral load set point. I was fortunate to gain access to the Options cohort, as access to samples from untreated persons with early infection is unusual. However, it is possible that studies that have, perhaps necessarily, studied chronic infection, may have limited capacity to unravel the overall interplay between T-cells and viral control. At best immune responses of chronic infection may be related in

terms of their character and magnitude with responses in acute infection, or may reflect a minor role in ongoing control of the virus. At worst, studies of chronic infection may be seriously misleading as the assumption of a link between *ex vivo* studies of immune function with a role *in vivo* at a specific point in time may be flawed – especially in chronic infection. This is especially apparent when the apparent validity of the viral load set point in projecting control of infection is considered (Mellors, Rinaldo et al. 1996). On the other hand, given that it maybe that peak CD8+ T-cell responses reach a peak at around eight to twelve weeks post infection (Altfeld, Rosenberg et al. 2001; Karlsson, Malleret et al. 2007), and we studied responses at a mean of 15.4 weeks post infection (range 6.7 to 29 weeks), it maybe that closer links between response size (and phenotype) would be obtained at more closely clustered, earlier time points post infection. The capacity of immune response to suppress infection depends not only on response size (or phenotype) but presumably on the density of target infected cells in tissues (rather than at the peak of their presence in peripheral blood). Furthermore responses could feasibly develop independently of ongoing viral replication, and be determined by factors governing the initial priming of responses. Thus if phenotype is determined by initial 'signal strength' then it may be a surrogate marker for responses with higher avidity. However, a link between phenotype and control of CMV (a virus with relatively conserved sequence) has been documented when only two responses were studied. These were the CMV pp65-derived peptide NLVPMVATV (used in Chapter 3) and an HLA-B7 restricted peptide TPRVTGGGAM. Thus even when the studied responses are rigidly controlled, phenotype may again predict viral control (Gamadia, Remmerswaal et al. 2003)

Two basic questions are raised by this study. Firstly, why may response magnitude be unrelated to control of virus (at least in some studies)? Secondly why may phenotype be related to control of virus replication? In answer to the first question, as discussed already, the timing of sampling and the number of samples may be critical. In addition it may be important to study the correct response. Thus anti –'gag' responses may be related to control of virus as 'gag' peptides will be in the cell cytoplasm after virus entry whereas anti – 'env' responses may have no effect until virus assembly is near complete. Secondly envelope proteins, unlike 'gag' proteins, are able to accommodate sequence variation without any fitness cost. Thus progressive increases in variability driven by immune selection pressure may essentially lead to impotence of responses. This may explain why anti-'gag' responses (but not anti-'env' responses) are linked to improved viral control) whereas – anti-'env' responses seem to increase as viral load increases (Kiepiela, Ngumbela et al. 2007). Except for the most potent responses, virus may drive T-cell responses, rather than T-cell responses drive virus control, as T-cell responses diminish during structured treatment interruptions (STI) (Altfeld, van Lunzen et al. 2002). Certainly the link between 'gag' responses seems statistically to be related to a trade off between viral fitness and an ability to escape immune responses. Thus the link between anti-'gag' responses and lower viral load is related to the number of responses and thus the HLA type of the host. Multivariate regression analysis (controlling for co-existence of other responses perhaps positively related to viral control) estimates a -0.21-$\log_{10}$ fall in viral load for each additional anti-'gag' response. Thus so-called 'passenger' epitopes (Zafiropoulos, Barnes et al. 2004) seem to undermine the benefits of protective

responses although the reasons for this are unclear. These studies have not taken into account the cascade of response that may account on viral escape as responses to intra-individual sequences has not been studied. This study also emphasised the importance of the number of responses, as opposed to the magnitude of responses (Kiepiela, Ngumbela et al. 2007).

Thus although response magnitude is often used to imply immune efficacy this is dependent on the total number of responses and their actual efficacy (which is dependent on the region of the virus targeted). Phenotype may actually, assuming, a 'signal strength' model of differentiation, reflect those more 'mature' responses that have not selected for escape variants and are still actively 'seeing' and potentially suppressing infection. In fact studies of T-cell phenotype in HIV may feasibly 'cut the Gordon knot' of complex tensions between immune pressure and viral evolution. It would be interesting to compare the phenotype of anti-'gag' and anti-'env' responses and relate this to passenger/driver status of epitopes targeted. It is logical to suggest that phenotype may relate to viral control as different subsets may have specialist anti-viral functions (although these remain to be better defined). In any case, there is little reason to assume that response magnitude is any better indication of the benefit of specific responses than phenotype, although the latter has not been so frequently studied. Studies in larger numbers of people should be performed to explore these relationships in more detail. Further functional studies of T-cells are needed.

The statistical approaches used in this study are novel, suggesting new ways in which data sets exploring the links between T-cell immunity and control of HIV (and other viruses) can be evaluated. For example not all studies calculate the total number of particular T-cells (defined either by their specificity or by their phenotype) per unit volume of blood. In this study we estimated that one 'extra' T_{EMRA} per μL of blood is associated with - 0.263 lower log10 HIV-1 RNA load. This is the first such estimation of actual anti-viral capacity of T-cells to be considered. It is problematic as the efficacy of this cell type seems to be surprisingly strong. However, the link between this calculation, and tissue density of T_{EMRA} cells and their turnover is not known. One 'extra' T_{EMRA} cell may equate to far greater tissue densities of these cells, and a huge number over a given period of time – especially in early infection when 'contraction' of the immune response is underway and needs to be taken into consideration. It is apparent that cell turnover is augmented in HIV, with survival perhaps being shortened by more than two thirds (Hellerstein, Hanley et al. 1999).

Multi-regression analysis also enabled us to dissect out the contribution of different cell types, which is important when the link between phenotype and magnitude of responses (Gamadia, van Leeuwen et al. 2004; Northfield, Lucas et al. 2005), (and, importantly, vice versa) is considered. I attempted to tease out the role of specific cell subsets, despite the co-linearity seen between particular cell subsets as a proportion of the response.

I suggest that the capacity of T_{EMRA} cells to secrete cytolytic molecules is the 'key' to their efficacy. However other effector molecules may also have a role, such as anti-viral

chemokines. Furthermore, it is unclear what the significance of intracellular granule contents is, given that preformed perforin and granzyme B are not required for cytotoxicity (Isaaz, Baetz et al. 1995), and may, in fact, have a role in mediating activation induced cell death, as discussed in the introduction. HIV-specific CD8+ T-cells do express perforin after they have proliferated *in vitro* – at least in LTNPs (Migueles, Laborico et al. 2002). Thus the potential for development into T_{EMRA} cells presumably exists, but may be inhibited through lack of CD4+ T-cells or through the activity of regulatory T-cells (T_{regs}). It might also be questioned whether analyzing healthy volunteers to assess cytolytic potential in this manner is appropriate. However, the transcription profiles on micro-array analysis of CD57+ CD8+ T-cells (a subset related to T_{EMRA} cells) in HIV+ve and HIV-ve persons is indistinguishable (Le Priol, Puthier et al. 2006).

Both the preceding chapter and this one have explored CD45RA reversion on virus specific cells and its link with response size and control of viral replication. Thus latent CMV infection seems to be characterised by extensive outgrowth of this subset, whereas in HIV infection, a predominance of this subtype may be linked to an ability to control viral infection better. In chapter 3 I advanced the hypothesis that outgrowth of T_{EMRA} may be aberrant, and driven by a partially futile attempt by CD8+ T-cells to eliminate latent CMV that is 'hidden' by immunoevasins such as UL-18. Thus CMV may not be a valid comparator to understand the role of T_{EMRA} cells in HIV infection although it has been proposed that HIV-specific cells display aberrant differentiation (compared to CMV-specific responses) (Champagne, Ogg et al. 2001). T_{EMRA} cells do seem to be related to

anti-viral control as they are found in abundance in LTNP's (Hess, Altfeld et al. 2004), in HIV exposed but uninfected persons (Schenal, Lo Caputo et al. 2005), and in those persons destined for improved viral control (as proposed here). T_{EMRA} cells may have improved anti-viral activity (as discussed earlier). Furthermore their apparent prolonged survival *in vivo* (Wallace, Zhang et al. 2004), and presumably highly clonal nature, reflecting (perhaps) especially high avidity, (Khan, Shariff et al. 2002) may add to their potential beneficial properties. It may be that this subset can be augmented in HIV infected persons (perhaps by treatment with cytokines such as IL-15), perhaps leading to better viral control. However exogenous IL-15 may have other effects on the immune system as well. In terms of vaccine development it remains to be seen what adjuvants or immunogens may favour outgrowth this subset. A key stumbling block is also their long term survival in persons who are vaccinated but uninfected. Thus their capacity to act as true memory cells (in the absence of latent infection) is uncertain. So far, transfer experiments suggest only T_{CM} may be able to confer long term protection (Berger, Jensen et al. 2007). T_{EMRA} cells may have a niche role in patrolling for isolated 'bursts' of viral replication in viral infections with the capacity for latency (such as HIV and CMV), and the potential for this role to be exploited therapeutically may be limited. It would be interesting to study this subset during HAART, to see if it is relatively preserved in a situation when other subsets may reduce in size (Altfeld, van Lunzen et al. 2002).

Chapter 5: 'A Novel Pathway of T-cell Differentiation'

5:1 Abstract:

Hepatitis C virus (HCV) causes chronic infection accompanied by a high risk of liver failure and hepatocellular carcinoma. CD8+ T-cell responses are important in control of viraemia. However, the T-cell response is weak both in absolute numbers and in the range of epitopes targeted. In order to explore the biology of this response further we looked at expression of a panel of natural killer cell markers (NK-cell markers) on HCV compared to other virus specific T-cell populations as defined by MHC class I tetramers. It was found that CD161 was significantly expressed on HCV–specific cells (median 16.8%) but not on HIV (3.3%), CMV (3.4%) or Influenza (3.4%) specific CD8+ T-cells. Expression was seen in acute, chronic and resolved disease, and was greatest on intra-hepatic HCV-specific T-cells (median 57.6%, $p<0.05$). Expression of CD161 was also found on HBV-specific CD8+ T cells. Generally CD161+ CD8+ T-cells were found to be CCR7-ve 'effector memory' T-cells, that could produce pro-inflammatory cytokines (IFN-γ and TNF-α), but contained scanty amounts of cytolytic molecules (granzyme B and perforin) and proliferated poorly *in vitro*. Expression of CD161 on CD8+ T cells was tightly linked to that of CXCR6, a chemokine with a major role in liver homing. **In conclusion,** I propose that expression of CD161 expression indicates a unique pattern of T-cell differentiation that might be relevant to understanding mechanisms of HCV immunity and pathogenesis.

5:2 Acknoweldgements:

For this chapter I need to specially acknowledge help from several groups with whom I collaborated. Firstly I would like to acknowledge the help of Professor Bruce Walker's group in Boston: Dr. Victoria Kasprowicz carried out the experiments on samples from persons with acute Hepatitis C infection and also carried out work on some liver-derived cells. Drs Georg Lauer and Arthur Kim also contributed from this lab with helpful discussion and the opportunity to perform preliminary work to refine and develop the ideas generated. Secondly I would like to acknowledge the help of Professor Robert Thimme's group in Freiberg. Dr Bertram Bengsch performed work on samples from persons with acute Hepatitis B and Nadine Kersting performed studies on liver-derived cells. I also owe a special debt of gratitude to Dr Michaela Lucas, who originally documented the expression of CD161 on HCV-specific T-cells and allowed me to capitalise on it! Finally I would like to thank Professor Paul Klenerman and Andrew Loughry for performing the experiments establishing the link between CD161 and CXCR6.

5:3 Introduction:

In the previous chapters I examined the association between T cell phenotype and response magnitude (Chapter 3), and T cell function (chapter 4). Two prototypical viruses were studied: CMV, which is normally controlled well by the immune system, and HIV,

which is typically controlled poorly. In this chapter I study HCV, a virus which may be controlled well (ie without persistence) or poorly (with persistent viremia) and focus on a specific aspects of T cell phenotype which might be relevant.

HCV is an RNA virus belonging to the flavivirus group. IT replicates using an RNA replicase which generates huge amounts of diversity – greater than that seen for HIV. There are six main genotypes which differ by around 33% of base pairs from each other. It is transmitted between persons by sharing of needles by intravenous drug users and in resource-poor medical settings. It can also be transmitted sexually, especially between male homosexual partners, and transplacentally. It has been estimated that 130 x 10^6 may be infected worldwide, and ranged in incidence from <1% (UK) to 15-20% (Egypt) (Fields, Knipe et al. 2007) .

Subtype 1a is the most common subtype worldwide. Subtype 1b is widely distributed in Europe and South America and is typically found in older persons who may have been infected by transfusions. Genotype 2 is found predominantly in older persons in the Mediterranean regions and in Asia. Genotype 3 is also found in Europe amongst intravenous drug users. Genotype 4 is found in the Middle East, Genotype 5 is found in South Africa and Genotype 6 is found in South-East Asia.

HCV consists of a single stranded positive-sense RNA genome approximately 9.6 kilobytes in length. It consists of a single open reading frame that encodes a polyprotein of approximately 3000 residues. It has 3 main regions; the NH_2- terminal region (coding

for core, E1 and E2 proteins), a central region (coding for P7 and NS2) and finally the COOH- terminal region (coding for the non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B). The virus itself is approximately 55 to 65 nm in diameter. In blood it may associate with lipoproteins LDL/VLDL.

Hepatitis C mainly infects the liver with 10^8 to 10^{11} copies per gram of tissue and from 5 to 50% of hepatocytes are infected. Other cell types (e.g. lymphoid cells) may be infected but the extent of replication is uncertain. An important co-receptor for cell entry is CD81 (Fields, Knipe et al. 2007).

Acute infection is often completely asymptomatic although mild jaundice may occur. Complete resolution of infection occurs in approximately 20% of cases but persistent infection is the outcome in the remainder. Genotype 3 virus seems to be more readily cleared than genotype 1. Persistent infection frequently leads to cirrhosis (after 20 years of infection), and hepatocellular carcinoma (mean 28 years). Alcohol consumption and co-infection with HIV act as co-factors, markedly accelerating disease progression (Fields, Knipe et al. 2007). HCV is estimated to cause around 280,000 deaths per year. Hepatitis C is now the major indication for liver transplant in Europe and the United States (Poynard, Yuen et al. 2003).

Diagnosis can be made by detecting antibodies to Hepatitis C. This can be in the form of a enzyme immunoassay (EIA). Usually a confirmatory recombinant immunoblot assay

(RIBA) is required. Confirmation of active viral replication (as opposed to resolved infection) can be provided by PCR for viral neucleic acids (Fields, Knipe et al. 2007).

The main stay of therapy consists of recombinant interferon-α and ribaviran. Pegylation of interferon improves its' pharmokinetics and is associated with a better therapeutic response. Genotype1 infection can be cured in 42 to 46% if cases whereas in genotype 2 and 3 response rates between 76 to 82% are possible. Novel therapies such as NS3/4A protease inhibitors and NS5B RNA polymerase inhibitors are in development but viral resistance is emerging as an important problem (Fields, Knipe et al. 2007).

CD8+ T-cell responses may play a role in determining the overall success of the immune response in acute infection (Lechner, Wong et al. 2000). Not surprisingly, considerable effort has been applied to define the characteristics of successful CD8+ T-cell immune responses (Bowen and Walker 2005). Successful responses (in acute infection) may be of higher magnitude, or broader, targeting a greater number of epitopes, or target specific epitopes (Lauer, Lucas et al. 2005). However the outcome of infection also includes important contributions from innate, CD4+ T cell and humoral responses.

In chronic infection the CD8+ T-cell response is narrow in both breadth and magnitude despite high levels of replication. Evidence suggests that there is progressive attrition of responses as chronic infection ensues (Cox, Mosbruger et al. 2005). Thus a failure to maintain responses may be the characteristic of chronic infection (as opposed to an inability to generate responses initially).

As discussed previously, the function of T-cells is reflected to some extent by the combination of molecular markers expressed on their surface – known as the T-cell phenotype (Sallusto, Lenig et al. 1999). HCV-specific CD8+ T-cells during chronic infection have a phenotype that is described as 'immature' or of a 'central' memory phenotype (Appay, Dunbar et al. 2002; Lucas, Vargas-Cuero et al. 2004). This contrasts sharply with the phenotype of HIV and CMV -specific T cells, which are consistently larger and more 'mature' with 'effector' potential (Appay, Dunbar et al. 2002). The low frequency of CD8+ T-cells with their immature phenotype and narrow repertoire are the most salient features of HCV immunology, and to date, are unexplained (Northfield, Harcourt et al. 2005).

It is unclear whether these features are merely positioned on a continuous spectrum of maturity and efficacy, or whether the HCV-specific response develops along a distinctive paradigm of T-cell differentiation and homeostasis compared to that to HIV and CMV. This may be because the site of infection is the liver, where the environment for antigen presentation and the surrounding cell types is unique. The liver is well known as an immuno-tolerant site. The liver can be transplanted across MHC-barriers. In the mouse, hepatocytes can present antigen but T-cells stimulated in such a manner appear to undergo apoptosis (Willberg, Barnes et al. 2003). In an attempt to evaluate the hypothesis that a distinctive type of response may develop to Hepatitis C, we compared expression of a panel of NK-receptors on different virus-specific CD8+ T-cells as defined by MHC

class I tetramer complexes specific for HCV, HIV, FLU, CMV and finally HBV, to see if discriminating features could be identified.

I found that the C-type Lectin CD161 is expressed on a significant subset of HCV-specific T-cells, but not on CMV, HIV or Influenza specific responses. It was, however, found on HBV-specific CD8+ T cells. Exploration of the properties of CD161+ CD8+ T-cells in uninfected persons showed that these are 'effector' cells that produce pro-inflammatory cytokines, such as IFN-γ and TNF-α, but express low levels of granzyme B and perforin, perhaps indicating reduced ability to lyse virus-infected cells. CD161 was linked strongly to CXCR6 - a chemokine receptor strongly implicated in liver homing (Kim, Kunkel et al. 2001; Wang, Holmes et al. 2004; Heydtmann, Lalor et al. 2005; Sato, Thorlacius et al. 2005). These findings suggest another dimension to T-cell biology beyond the 'central' and 'effector' memory dichotomy (Sallusto, Lenig et al. 1999) with implications for mechanisms of virus persistence, and more broadly for models of T-cell differentiation and homeostasis.

5:4 Methods:

5:4:1 Patients:

Studies on healthy laboratory volunteers used fresh buffy coats from the National Blood Transfusion Service, from healthy laboratory volunteers and from routine clinical haematology samples as described in Chapter 3. HIV+ responses were obtained from 16 HIV positive patients with HLA-A2 restricted responses to SLYNTVATL undergoing study in Oxford. HCV- specific responses were obtained by screening patients at the John Radcliffe Hospital (JRH) Oxford; and from Massachusetts General Hospital (MGH) Boston. Hepatitis B positive patients, and studies of intrahepatic HCV-specific T-cell responses were enrolled as in (Boettler, Panther et al. 2006).

5:4:2 Tetramers

Tetramers were either obtained commercially, or synthesised as described. Staining was performed on whole blood or on fresh or thawed, previously frozen PBMC as in the introduction. After preparation, cells were incubated with tetramer at 37°C for twenty minutes, before staining for surface antibodies.

5:3:3 Antibodies & Phenotyping:

The following antibodies were used: CD161 antibody (from Beckman-Coulter (clone 191B8 – PE) or BD biosciences (DX12 clone conjugated to FITC or PE), IFN-γ-FITC,

CCR7-FITC, anti- CXCR6/Bonzo-PE and anti-mouse IgG-Fab APC, IgG-Fab-FITC. CD103-FITC, β7-integrin-PE, Ki67-FITC, Perforin-FITC, IFN-γ-FITC, CD3-FITC, CD3 and CD8-(peridinin-chlorophyll protein complex-(PerCP), CD8-APC, CD45RA-Allophycocyanin (APC), Unconjugated anti-CD3 (Beckman Coulter) and Anti-Granzyme B-APC antibody.

Cells were stained with antibody, incubated for twenty minutes at 4°C, washed and then fixed in 1% Paraformaldehyde solution (Sigma-Aldrich). Phenotyping of bulk CD8+ T-cells was performed using whole blood from HIV/HCV uninfected persons as described in the introduction.

Liver-infiltrating lymphocytes were isolated from 0.5–1 cm of hepatic needle biopsy. The tissue was homogenised in 2–3 ml of Dulbecco's Phosphate-Buffered Saline by using a Dounce tissue grinder. Cell suspensions were incubated with magnetic beads coupled to anti-CD8 (Dynabeads) as below. The purity of each T cell subset was confirmed by FACS analysis and was always_95%. The isolated intrahepatic CD8+ T cells were then plated into separate wells in 24-well plates in 1 ml of 10% human AB+ serum, 100 units/ml IL-2), 0.04 μg/ml anti-human CD3 monoclonal antibody (Immunotech, Marseilles, France), and 2 x 10^6 irradiated autologous PBMC as feeder cells. Twice a week, 1 ml of media was exchanged and 100 units/ml IL-2 added. After 2 wk, the expanded T cells were tested for HCV-specific responses with tetramer.

5:3:4 Cell Sorting:

Miltenyi-Biotec microbead cell sorting was utilised. (CD8+ T-cell Isolation kit II, anti-PE microbeads and LS columns), using freshly isolated PBMC's from buffy coats or laboratory volunteers, as outlined in the methods chapter..

PMA (1µg/ml)/Ionomycin (1µg/ml) was used to stimulate cytokine secretion. I used a CBA (BD) assay on supernatants of purified CD161+ or CD161- CD3+ CD8+ T-cells. Median purity (percent of all events acquired) was 76.9% (SE = 9.9), and 81% (SE = 8.4), respectively. Cells were plated in a ninety-six well round bottomed plate at 0.1 – 0.5 x 10^6 cells in R10 (RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, penicillin and streptomycin). Cells were stimulated overnight at 37°C.

For intracellular cytokine staining, unsorted PBMC's were sorted on CD161 as above, (purity (gated on CD3+CD8+ cells) consistently >95%), and placed in a 96 well plate as above. Cells were incubated for 5 hours. Golgiplug (BD) was added after an hour.

5:3:5 Cell Proliferation:

Cells sorted on CD161 were placed with CD8-depleted PBMC's (<1% CD8+ T-cells - CD8-DYNA beads (Invitrogen) in a ratio of 1:2 (PBMC's sorted on CD161: CD8-depleted PBMC's).. (Addition of CD8-depleted cells provided accessory cells). IL-2 (Chiron) was added at 200U/ml. Cells were then incubated at 37°C for three days before staining for intracellular Ki67 (a marker of cell proliferation).

5:3:6 Effect of Cytokines on Expression of CD161:

An *ex-vivo* re-stimulation assay for a FLU response was performed in a person known to have a response to the FLU peptide GILGFVFTL. 2 x 10^6 cells/ ml were placed in a 48-well plate and 10μg/ml of FLU-peptide was added, along with 100 IU/ml of (IL-2) Either recombinant IL-12 (2ng/ml) or TGF-β (10ng/ml) (R&D systems) were added as indicated at the time of initial culture inception. Cells were left for 10 days with fresh R10 and IL-2 every three to four days. Collected cells were stained as above.

5:3:7 Intracellular Cytokine and Ki67 Staining:

Cells were stained with surface antibodies as above and re-suspended in 4% Paraformaldehyde (PAF – Sigma-Aldrich) in PBS and left in the dark at room temperature for thirty minutes. Cells were then washed and then re-suspended in permeabilisation buffer, and intracellular antibody to CD3 and either perforin or IFN-γ or Ki67 was added. Cells were then incubated with antibody and permeabilisation buffer at room temperature for fifteen minutes, washed and then fixed with 1% PAF solution. (Intracellular staining for Granzyme B and Perforin was performed on sorted cells as this was found to allow more precise determination of staining of CD161+ cells, due to the occasional low background frequency of this population).

5:3:8 Data Acquisition and Statistical Analysis:

Samples were acquired using a FACScalibur FACS machine (BD) using Cell quest software, or using a LSR II cytometer. Files were analysed using Flowjo software. Data

and statistical analysis was performed using GraphPad Prism Version 4. Wilcoxon's matched pairs test was used for analysing paired data, Mann Witney – U – test was used for comparing unpaired data.

5:4 Results:

5:4:1 Expression of NK receptors on HCV, FLU, HIV and CMV specific T-cells

CD161 and LIR-1 (CD85j/ILT-2) showed significant expression on HCV-specific T-cells median =16.85% (SE = 4.9) and median = 10.53% (SE = 2.9), respectively (Figure 5:1A). KIRs were not expressed on HCV-specific cells (median = 0.2%, SE = 0.64). Unlike LIR-1 (CD85j/ILT-2) (which is known to be expressed on CMV-specific T-cells for example (Northfield, Lucas et al. 2005), amongst HCV, CMV, FLU and HIV-specific cells, only HCV-specific cells expressed significant levels of CD161 (range 0-74.9%) (Figure 5:1B & C), indicating that HCV-specific responses frequently contain a significant CD161+ subset.

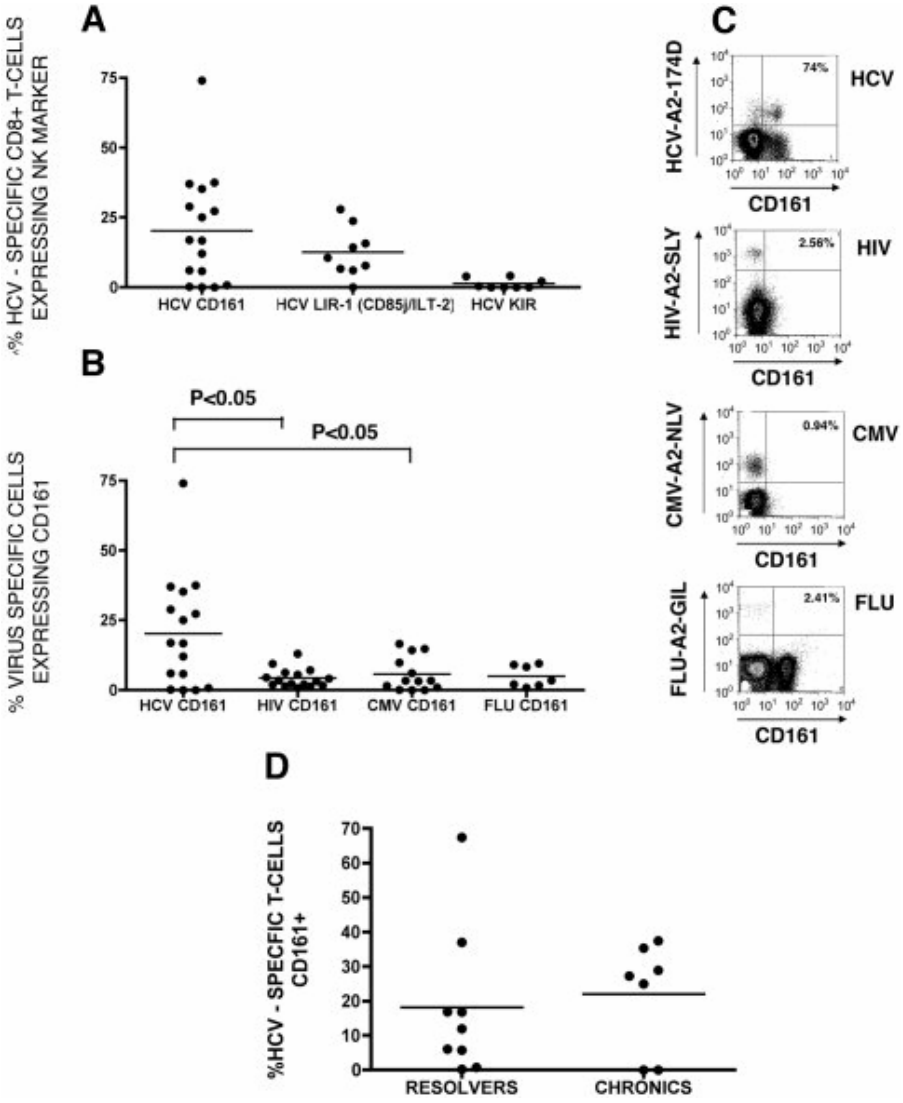

Figure 5:1 Staining of virus-specific CD8+ T-cells for CD161 and other NK markers: A) PBMCs from a range of HCV+ donors were stained with HLA Class I peptide tetramers and NK markers as described in Methods. The % of HCV tetramer+ cells which stained +ve for a given marker is indicated. B) The expression of CD161 was compared on tetramer+ cell populations specific for peptides derived from cytomegalovirus (CMV), HIV and influenza (FLU). The expression of CD161 is significantly elevated on HCV-specific cells compared to populations with other specificities. C) Representative staining of different virus specific populations is shown (top to bottom HCV, HIV, CMV, FLU). In each case, the population illustrated is live gated CD8+ T cells and the binding of tetramer and CD161 expression are shown. D) The data for HCV specific T cells was analysed with respect to the clinical status of the donor (HCV PCR+ or PCR-). There was no significant difference in expression of CD161 in responses from persons with chronic and resolved HCV.

No significant difference in CD161 expression was seen in those with chronic or resolved HCV (Figure 5:1D), or in three persons with acute Hepatitis C. (Figure 5:1E and Figure 5:2). Expression was sustained at similar levels until at least four months post infection (see Figure 5:2).

5:4:2 Analysis of CD161 expression on HBV-specific T cells

Since CD161 expression appeared to be selectively elevated on HCV specific T cells, we next addressed whether CD161 expression was a feature of T-cells targeting another Hepatitis virus – Hepatitis B (Figure 5:3A). With this aim I looked for CD161 expression in five persons with acute Hepatitis B virus (HBV) infection (in those with persistent infection tetramer staining is typically below the level of detection ex vivo from blood) (Maini, Boni et al. 2000). Expression varied from 3.3 to 27.30% of antigen-specific T cells, indicating significant CD161 expression in some cases on HBV-specific cells. These expression levels were significantly higher than those on CMV- and HIV-specific T cells ($p<0.05$), and not significantly different from those found on HCV-specific CD8+ T cells in acute disease (Figure 5:3B). Thus CD161 expression may reflect a more general feature of T cells specific for liver associated infections.

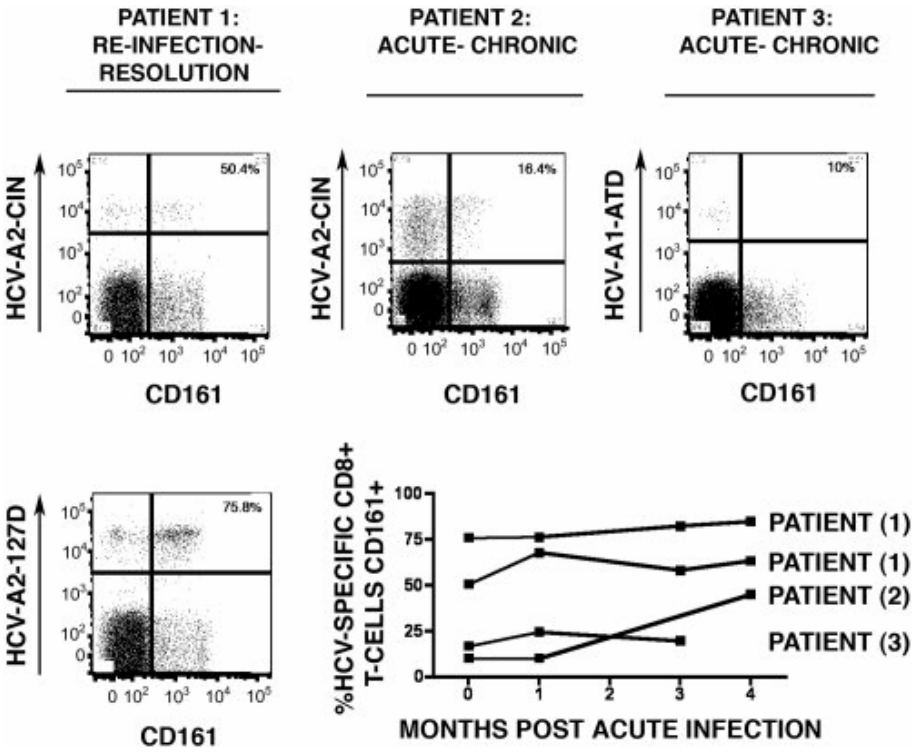

Figure 5:2 Staining of HCV-specific CD8+ T-cells in acute Hepatitis C infection: Three persons with acute HCV were used to determine CD161 expression on CD8+ T-cells specific for HCV in acute disease. One individual (first column – two responses) went to on resolve his/her infection, whereas two further individuals (top middle and top right developed chronic HCV infection. The graph (bottom right) shows CD161 staining on these four responses over time (up to 4 months post infection). CD161 was expressed on virus-specific CD8+ T cells during responses to acute HCV infection and this was maintained into chronic and resolved disease.

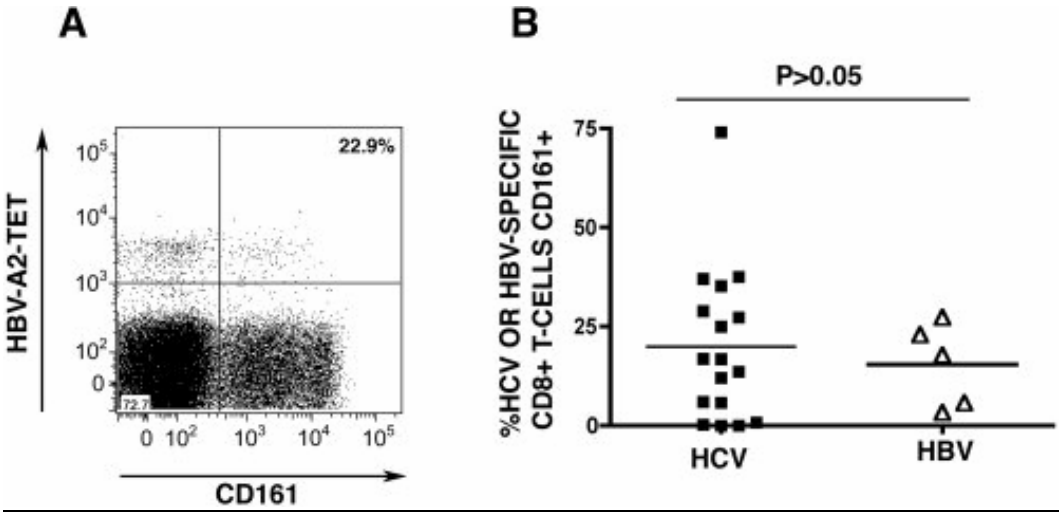

Figure 5:3 Expression of CD161 on HBV-specific CD8+ T cells Five donors with acute HBV infection where substantial HBV tetramer+ populations were identified were analysed for CD161 expression. As illustrated in panel A, expression of CD161 on HBV specific cells was also identified. The plot is gated on live CD8+ T lymphocytes. B) Staining for CD161 was similar on HCV-specific cells derived from donors with chronic/ resolved HCV (nonacute) and acute HBV p=n.s. As discussed in the text, the levels of CD161 expression on HBV specific T cells were significantly higher than those on CD8+ T cells specific for other viral infections

5:4:3 Study of Expression of CD161, CCR9 and CD103 on Intra-hepatic T-cells.

I next determined expression of CD161 on HCV specific T-cells in the liver from four persons (see Figure 5:4A & B). The median expression was 57.6% (SE = 14.39) (Total of 6 responses), compared to 16.85 (SE = 5%) in peripheral blood (p=0.03). Thus, a 'step up' in CD161 expression between peripheral blood and the liver was seen. Two individuals also had intra-hepatic FLU-specific responses that showed low levels of CD161 expression (5.2% and 2.7%) (Figure 5:4B).

CD161 has been linked with the gut and expression of gut homing markers (Iiai, Watanabe et al. 2002; O'Keeffe, Doherty et al. 2004; Rodriguez, Paquet et al. 2004). Thus HCV-specific T-cells might express other markers related to T-cell homing to the liver and intestine. I therefore firstly looked at expression of CCR9 which may be linked to gut (Hosoe, Miura et al. 2004) , and possibly liver homing (Eksteen, Grant et al. 2004). I found extremely low levels of CCR9 expression on HCV-specific cells in the liver and peripheral blood. CD103 has also been linked to CD8+ T-cell homing to the liver in HCV (Shimizu, Minemura et al. 2003). Although CD103 showed limited expression on HCV-specific T-cells in peripheral blood (median = 4.0%, SE= 3.1, n=5), intra-hepatic HCV-specific CD8+ T-cells showed relatively high expression (16.8 – 43%), consistent with previous work (Shimizu, Minemura et al. 2003).

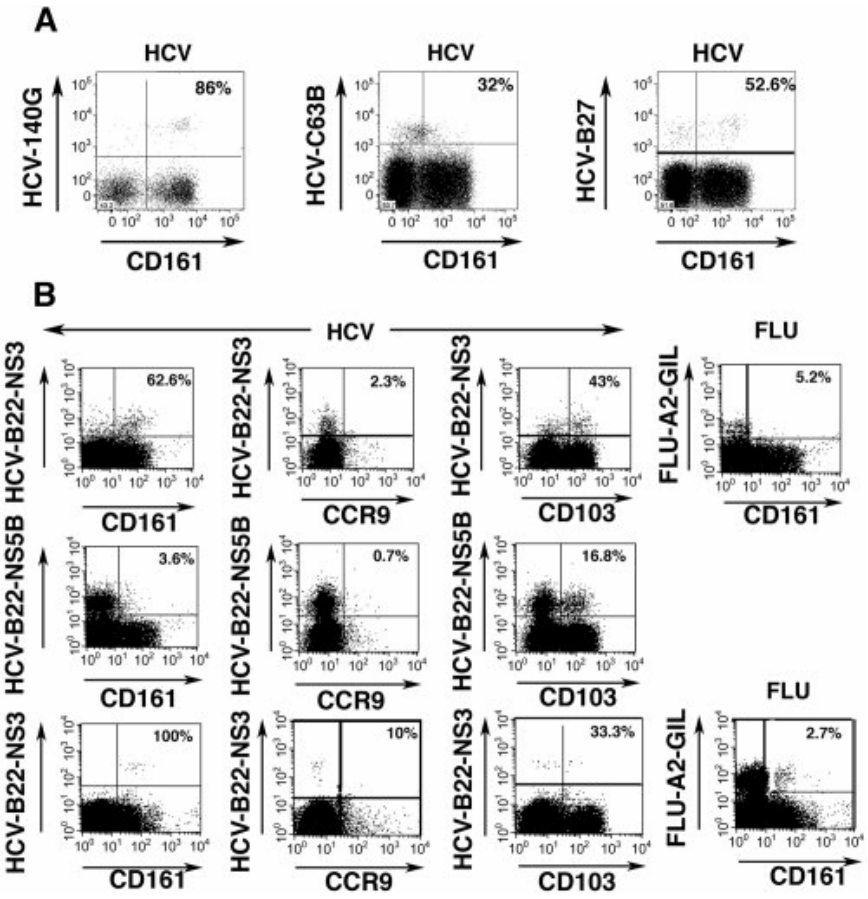

Figure 5:4 Expression of CD161 and other markers on intra-hepatic virus specific T-cells: A) Expression of CD161 on intra-hepatic antigen-specific T-cells is illustrated in two HCV+ patients. In one patient (right hand two panels) two responses were studied. B) Expression of CCR9 and CD103 was studied alongside that of CD161 on intra-hepatic HCV specific T-cells in an additional two persons. (Two responses were studied from one HCV+ patient (middle two rows)). In both these patients intra-hepatic FLU-specific responses were also found and assessed for CD161 expression as illustrated.

TGF-β upregulates CD103 (Shimizu, Minemura et al. 2003), and may be produced by regulatory T-cells in the liver. We found that CD161+ CD8+ T-cells (bulk populations from peripheral blood of normal donors) expressed more CD103 than CD161- cells – median = 12.2 (SE = 2.7) and 3.1 (SE = 0.63), respectively ($p<0.005$). This suggests that peripheral blood CD161+ CD8+ T-cells are more likely to have recently been exposed to TGF-β, and /or trafficked through gastro-intestinal tissues. CD161+ cells were also relatively high in expression of the beta-7 chain of the integrin heterodimer α4/β7 ($p=0.065$; data not shown).

5:4:4 Linkage between CD161 expression and CXCR6 expression on CD8+ T cells

The strongest link with CD161 and liver homing was found by analysis of CXCR6 expression. CXCR6 and its ligand CXCL16 have been strongly implicated in liver homing (Kim, Kunkel et al. 2001; Wang, Holmes et al. 2004; Heydtmann, Lalor et al. 2005; Sato, Thorlacius et al. 2005). CXCR6 is found on CD8 and CD4 T lymphocytes, NK cells, and NK-T cells. On the other hand CXCL16 mRNA is found in normal and in inflamed liver but the exact pattern of cellular expression is not clear (Heydtmann, Lalor et al. 2005). There was a striking association between the presence of CD161 and the presence of CXCR6 in a large series of HCV-ve donors (n=30; examples in Figure 5:5A and 5:5B. Similar findings were seen in peripheral blood of 7 HCV+ donors and a single donor with acute hepatitis B ($p=0.02$; examples in figure 5:5A)

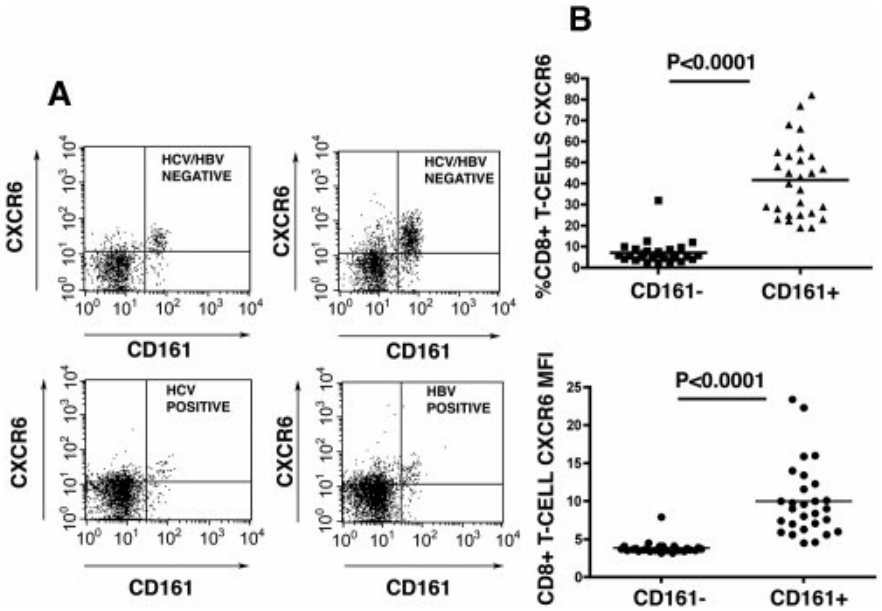

Figure 5:5 Co-expression of CD161 and CXCR6 on CD8+ T cells CD3+CD8+ T cells were analysed for co-expression of CD161 and CXCR6. Panel A shows examples from such staining. The upper FACS plots are gated on live CD8+ T cells from HCV- donors and the lower FACS plots from HCV+ (Chronic infection) and HBV+ (acute infection) donors respectively. Panel B shows composite data from a panel of 30 HCV- donors. The upper graph shows the distribution of CXCR6+ cells amongst CD161+ and CD161- CD8+ T cell subsets. The lower graph shows the mean fluorescence intensity (mfi) for the expression of CXCR6 in the CD161+ and CD161- CD8 T cell subsets. Analysis of CD3- and CD3+CD8- subsets did not reveal a consistent co-expression pattern of this nature

5:4:5 Phenotype of CD161+ CD8+ T-cells

FACS analysis with co-staining for CCR7 and CD45RA (Champagne, Ogg et al. 2001) in 11 uninfected individuals enabled us to determine what 'memory' subtype CD161+ CD8+ T-cells are (ie bulk populations in peripheral blood of normal individuals). I found that CD161 expression was very low on naïve T-cells (median 0.89%) and was only weakly expressed on T_{CM} cells (8.41%). However, significant expression was seen on both 'effector memory' subtypes (T_{EMRA} and T_{EM}) (25.4% and 23.7%, respectively) see Figure 5:6A. Thus CD161+ CD8+ T-cells are, in the main, a sub-population of 'effector memory' cells. This data is consistent with the linkage between CD161 and CXCR6 expression, since CXCR6 is known to be expressed on effector memory (CCR7-) populations (Kim, Kunkel et al. 2001).

5:4:6 CD161 expression is not upregulated by Interleukin 12 or TGF-β.

In order to explore further what the determinants of CD161 expression might be I explored if exposure to specific cytokines might induce CD161 expression. PBMCs from a donor already defined as positive for a FLU-specific tetramer+ (CD161-) population were re-stimulated with peptide in vitro as described in methods (Fig 5:6:B-D). Interleukin-12 (IL-12) has been reported to upregulate CD161 on NK cells (Poggi, Costa et al. 1998), and TGF-β may be secreted by regulatory T-cells that might modulate HCV-specific T-cell function (Rushbrook, Ward et al. 2005). However none of these cytokines were able to modulate CD161 expression. Thus I found no evidence that exposure to

these specific cytokines could be responsible for CD161 expression, at least in the short term, in vitro.

5:4:7 CD161 Expression is not modulated by T-cell Activation:

In order to address if CD161 might be modulated by T-cell activation I looked at CD161 expression on sorted CD161+ or CD161- T-cells (mixed with CD8- PBMC's as above) for three days with CD2/CD3/CD28 coated beads (Fig 5:6E). No alteration in CD161 expression was seen for CD161- cells (remained 99% CD161-) or CD161+ cells (remained 97.6% CD161+). In addition, the lack of CD161 expression after re-stimulation of FLU-specific cells strongly suggested that CD161 is not simply an activation marker.

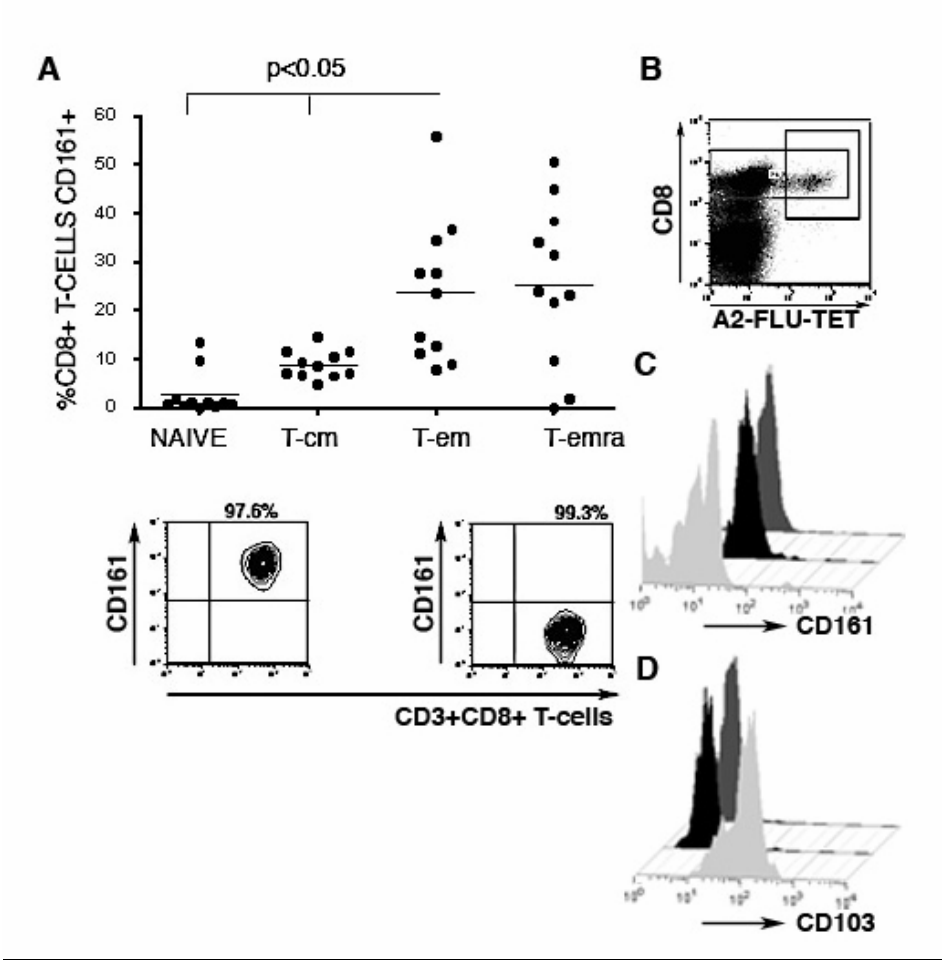

Figure 5:6 CD161 expression on different T-cell populations and is seen mainly on 'effector memory' T-cells in vivo: A) CD161 expression on different 'memory' CD8+ T cell memory subtypes was determined in 10 HCV uninfected persons. This shows that CD161 was greatest on the two 'effector memory' subpopulations (TEM and TEMRA). B) PBMC from a healthy HCV-ve donor with a previously defined CD8+ T cell response specific for influenza matrix peptide (FLU) were restimulated in vitro as described in the methods. After 10 days restimulation in vitro the cultured cells were stained with the relevant tetramer stain and co-stained with CD161. This panel shows the expanded CD8+ tetramer+ population. C) CD161 expression was determined on FLU-specific cells from the restimulated culture identified in panel B; these are illustrated after culture without additional cytokine (dark grey histogram), with 2ng/ml IL-12 (black histogram) and 10ng/ml TGF-β (light grey histogram). No effect on CD161 expression was seen. In comparison (D) TGF clearly caused upregulation of CD103 on the same FLU-specific population. E) Activation of sorted CD161+ (left) and CD161- (right) CD8+ T cells from a healthy donor was studied in vitro as described in the methods. Activation did not influence expression of CD161 on CD8+ T cells when co-cultured with CD8- depleted 'accessory' cells.

5:4:8 Function of CD161+ CD8+ T-cells

I next explored the cytokine secreting potential of CD161+ T-cells (Fig 5:7). Supernatants of CD161+ and CD161- CD3+CD8+ T-cells from five persons (sorted bulk populations from the peripheral blood of uninfected individuals) showed that they could elaborate IFN-γ, TNF-α and Interleukin 2 (IL-2), but not IL-10 or IL-4 (Figure 5:7A). Intracellular cytokine staining gave similar findings (see Figure 5:7B). Like CXCR6 (Kim, Kunkel et al. 2001), therefore, CD161 expression is linked to a Tc1 phenotype. In contrast to the clear capacity of CD161+ T-cells to produce pro-inflammatory cytokines, CD161+ T-cells were low in expression of lytic granule components perforin ($P<0.05$) and granzyme B ($p<0.05$) compared to CD161- cells, suggesting that they may have diminished capacity to kill virally infected cells through this pathway, or require further activation in order to do so (Figure 5:7C and 5:7D). Sorted CD161+ CD3+ CD8+ T-cells also showed reduced capacity to proliferate compared to sorted CD161- cells (as indicated by staining for Ki67) in response to non-specific stimuli even when accessory cells and additional IL-2 were provided (Figure 5:7E).

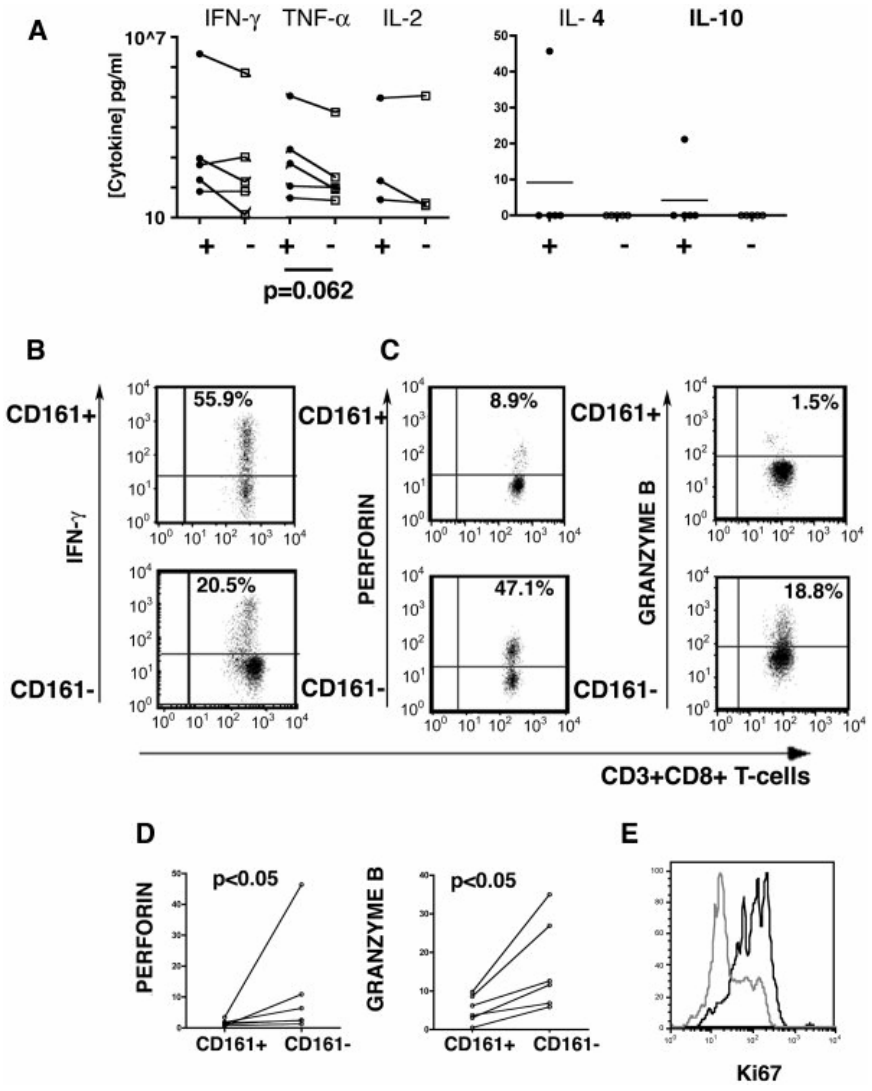

Figure 5:7 Functional Characteristics of CD161+ CD8+ T-cells: A) Sorted CD161+ and CD161- CD3+CD8+ T-cells produce copious amounts of cytokines. Graphs compare cytokine secretions from 5-paired assays (+ = CD161+, - = CD161-). CD161+ cells produce little IL-4 and IL-10. B) Sorted CD161+ cells also produce IFN-γ when assessed by intracellular cytokine staining. C) Ex vivo staining of sorted cells showed little expression of perforin and granzyme B by CD161+ cells. Comparison from 5 pairs of samples is shown in figure D. E) CD161+ cells (grey histogram) do not proliferate, as indicated by Ki67 expression three days after stimulation of sorted cells compared to CD161- cells (black histogram).

5:5 Discussion:

CD161 (KLRB1/NKRP1A) is a C-type lectin that shows high expression on NK and NK T-cells (Exley, Porcelli et al. 1998; Poggi, Costa et al. 1998). The cell surface ligand for CD161 has been recently identified as Lectin-Like Transcipt 1 (LLT-1) (Aldemir, Prod'homme et al. 2005; Pozo, Vales-Gomez et al. 2006). Unlike other NK receptors therefore, CD161 does not interact with class 1 molecules. Interactions between CD161 and LLT-1 may have marked effects on multiple cell processes through effects on acid sphingomyelinase activity (Pozo, Vales-Gomez et al. 2006), including NK and T-cell functions (Exley, Porcelli et al. 1998; Poggi, Costa et al. 1998).

This is the first study to examine CD161 expression on virus specific T-cells. The findings have been illuminating, as CD161 expressing cells account for a significant subset of Hepatitis C specific cells, whereas virtually no expression is seen on other prototype anti-viral T-cell responses. Trogocytosis (acquisition of CD161 from other cells in the liver (Caumartin, Favier et al. 2007) (Bengsch, Spangenberg et al. 2007)) seems an unlikely explanation as other NK markers (notably KLRGI) show low expression in HCV (Bengsch, Spangenberg et al. 2007). In addition an HCV specific cell line also showed expression of CD161 (data not shown), whereas trogocytosis would predict dilution and loss of expression after proliferation in culture.

My study indicated reduced levels of granzyme B and perforin in CD161+ T-cells. HCV specific T cells also show low expression (although maturation can occur upon in vitro

culture) (Appay, Dunbar et al. 2002; Lucas, Vargas-Cuero et al. 2004; Wang, Holmes et al. 2004). It is possible that CD161+ T-cells, although 'effector memory' cells, may have relatively defective killing independent of HCV status. The mechanisms involved in killing of HCV infected cells in the liver have not been fully elucidated, but this may be relevant to the intrahepatic environment. A number of studies have indicated the importance of IFN-γ secretion over perforin as a mediator of control over intrahepatic pathogens (Guidotti 2003). The group has recently shown using in vitro models that hepatocytes are relatively resistant to non-Fas mediated killing after exposure to inflammatory stimuli (Willberg, Ward et al. 2007). Further work is required to define the cytolytic function of these cells in vivo, especially after further activation and/or ligation of the CD161 receptor.

I observed using in vitro assays that CD161+ T-cells do not proliferate as well as their CD161- counterparts. This is consistent with previous work (Takahashi, Dejbakhsh-Jones et al. 2006). I did carefully control for numbers of CD8- accessory cells and supplemented IL-2, although, as a caveat, CD161- cells are enriched for central memory cells, which have high proliferative capacity compared to "effector memory cells" (Champagne, Ogg et al. 2001). The issue of proliferative capacity is important as 'amplification' of priming and recall T-cell responses to HCV may potentially be defective. Some studies have suggested limited proliferative capacity of HCV-specific CD8+ T cells (Wedemeyer, He et al. 2002) and this, together with our findings could go some way to explaining the low frequency of HCV specific T-cells in chronic infection.

Consistent with the phenotypic data showing an "effector memory" phenotype (CCR7lo), CD161+ T cells did retain other effector functions. They were able to produce pro-inflammatory cytokines (notably IFN-γ and also TNF-α). They did not produce any type II cytokines (such as IL-10 and IL-4), however, suggesting that they are not involved in anti-inflammatory processes. IL-10 secretion from HCV specific T cells has been observed in liver (Abel, Sene et al. 2006), so further analysis of CD161+ cell function, especially amongst antigen-specific cells, in the liver will be of interest to establish if this phenotype/function link is maintained. The expression of IFN-γ, as described above, could be very relevant to a potential role in clearing hepatotropic viruses (Gruner, Gerlach et al. 2000).

I found CD161 expression in acute disease, in chronic infection and on memory responses once HCV infection had resolved. We were also unable to show that CD161 expression could be modulated by T-cell activation, or by TGF-β and IL-12. This may indicate that CD161 is 'hardwired' into the basic properties of T-cells, and expression (or lack of expression) is relatively consistent over time for different T-cell populations. This stability is in contrast to many other markers which may be either modulated by short-term (e.g CD38) or long term (CD127, CD27, CD28, CD85j) antigenic exposure. Clearly, specific signalling pathways must contribute to the upregulation of CD161 early in T cell maturation. Further studies to establish the signalling pathways active in CD161+ cells and their link to CXCR6 expression are underway.

Expression of CD161 may relate to the site of HCV infection (the liver) as enrichment of CD161+ cells is seen here and elsewhere (Iiai, Watanabe et al. 2002; O'Keeffe, Doherty et al. 2004). In addition there is evidence of some CD161 expression on HBV specific cells indicating its expression is not restricted to HCV specific cells. Although the numbers of acute HBV+ patients studied remains limited, the CD161 level was nevertheless significantly higher than in the larger CMV and HIV groups studied ($p<0.05$). Finally the strong link of CD161 to CXCR6 observed here, and to a lesser extent with CD103 and β-7 integrin, suggests a link with liver-associated T-cell pools as these markers, particularly CXCR6, have been linked to liver and gut homing. It will be important to assess whether signalling through CXCR6 impacts on CD161 expression and vice versa or whether expression of both is linked to a common signalling history.

In summary, I propose that CD161 expression is the 'tip of an iceberg' of biological properties that discriminate HCV-specific responses from those to other commonly studied pathogens. Micro-array analysis of HCV-specific T-cells, compared to other virus specific T-cells – or more feasibly – between CD161+ and CD161- cells in healthy persons may help address these questions. It will also be important to see if antigen presenting cells derived from the liver promote CD161 expression during T-cell priming. Prospective analysis of CD161 expression in large cohorts of acute infection may enable it to be related to outcome of infection. For the first time however, outcome of a specific infection and its immunopathogenesis has been linked with a qualitatively distinct pathway of T-cell differentiation. Important features of CD161 include: 1) the relationship to hepatic T-cell pools, 2) the linkage to CXCR6, 3) the potential for T-cell

modulation through CD161 by interaction with LLT-1 (which awaits exploration with functional studies with mono-clonal antibody to CD161 or LLT-1 transfectants), and finally, 4) the low expression of cytolytic markers (such as perforin and granzyme B) by CD161+ T-cells. These features clearly have implications for the pathogenesis and persistence of HCV infection and its treatment. Thus CD161 - LLT-1 interactions have the potential to be an important pharmacological target. In addition any vaccine candidate for Hepatitis C infection will have to circumvent the apparently dampened nature of CD161+ HCV specific immune responses whilst ensuring adequate homing of T-cells to the liver. The findings thus provide a tangible handle with which to explore the enigmatic nature of T-cell responses to chronic HCV infection.

Chapter 6: Discussion

This body of work has made important observations of relevance to an understanding T-cell biology. The key findings are summarised in figure 6:1. Some findings seem to be generalisable to T-cell populations in general. Thus the linear increase in representation of the T-cell subset T_{EMRA} in the bulk CD8+ T-cell pool with age is an important finding as it strongly suggests both a linear and signal strength model of T-cell maturation. The 'independent' model of Pannetier et al does not seem to be supported. However the differences in TCR Vβ-repertoire of T_{CM} and T_{EMRA} is potentially explained by selection of Vβ chains with highest avidity over time as observed by (Karrer U 2003). Thus good evidence now supports a 'linear, signal strength' model of T-cell differentiation, with repeated exposure to antigen, high levels of antigen or stimulation via TCR of especially high avidity all driving T-cell differentiation forward in a progressive manner. An important refinement would also be the acknowledgement of potential pervasive effects of HCV (Lucas, Vargas-Cuero et al. 2004), and also, possibly also CMV on T-cells of other specificities. It will be important to document whether CMV has such a pervasive effect, by studying the phenotype of Flu specific responses, for example, in CMV positive persons. Pervasive effects of co-infection may be driven by the concentrations of specific cytokines such as IL-15 (Alves, Hooibrink et al. 2003). It is also possible that reactions with heterologous antigens may also drive this onwards differentiation of the T-cell pool. Heterologous immunity, that is cross-reactivity of TCR's with pathogens which they did not originally target does appear to occur (Selin, Varga et al. 1998). Homeostatic

peripheral expansion (HPE) induced by IL-7 also occurs in lymphopenic hosts, and may also be associated with T-cell phenotypic alterations (Fry and Mackall 2005). HPE may be dependent on multiple weak MHC-TCR contacts as it doesn't occur in MHC deficient hosts.

A key finding for CMV-specific responses (which may be relevant to T-cell populations in general) is the link between T-cell phenotype and the size of CMV-specific populations. In figure 6:2 I outline a model which links general strategies of immune evasion with T-cell phenotype and outcome of infection. Thus 'camouflage' and 'sabotage' are linked with latent infection and the T_{EMRA} phenotype whereas 'speed' and 'shape change' are linked a T_{CM} phenotype. It may be that the dynamics of relationship between phenotype and response size are contingent on the immune escape strategy of the virus. It will be important to link HCV response size with phenotype. It may be that immunoevasins such as UL-18 do affect killing by CMV-specific T-cells and this awaits careful functional assays. Although HCV is generally regarded as not existing in a latent state after resolution of acute infection, it seems that low levels of HCV may persist in these patients (Carreno, Pardo et al. 2006), thus the model proposed in figure 6:2 may not be quite so clear cut. However the existence of latency in an immuno-privileged site such as the liver may explain the lack of anti-HCV specific T_{EMRA} cells, especially if the viral burden in latency is low.

The relationship between phenotype and viral load suggested for HIV infection is also important. This also seems to be applicable in re-activated CMV (Gamadia,

Remmerswaal et al. 2003). It will be important to reproduce these findings in HIV infection in a larger cohort of patients. In figure 6:2 I propose that viral escape may lead to a return of the T-cell population to a T_{CM} phenotype. It would be highly interesting to compare the phenotype of anti-'gag' and anti-'env' responses given their discordant associations with viral load (Kiepiela, Ngumbela et al. 2007). The concept of 'original antigenic sin' would, however, suggest ongoing stimulation of the original responses by escape variants (Klenerman and Zinkernagel 1998), which might drive a positive relationship between anti-'env' responses and viral load – as well as ongoing differentiation of T-cell phenotype (Klenerman and Zinkernagel 1998). It will also be important to carry out careful functional assays on T_{EMRA} cells to establish a mechanism for such a phenotype-viral load link. It will also be important to see if the putative T_{GUT} phenotype, that is CD161+, will be related to viral load, although it is noteworthy that similar levels of CD161 expression are seen in acute, chronic and resolved HCV. Prospective studies of acute HCV may ultimately be able to relate CD161 with disease outcome and viral load, however.

Recently it has been established that T-cell priming by intestinal dendritic cells results in expression of adhesion molecules that favour recirculation of T-cells back to the intestine. The opposite is true of peripheral lymph node dendritic cells (Mora, Cheng et al. 2005). It is unclear what the functional consequences for T-cells of imprinting homing characteristics are. However potentially functional characteristics appropriate for access to immunoprivileged sites is conveyed. Potentially CD161 expression may be conveyed in such a way. The gut and liver do have dissimilar homing requirements, although in

disease (such as in ulcerative colitis and primary sclerosing cholangits) cross over may occur in homing requirements (Eksteen, Grant et al. 2004). This is interesting as HCV may replicate in the gut (Beld, Sentjens et al. 2000; Deforges, Evlashev et al. 2004). It will be important to identify the determinants of CD161 expression by priming experiments using liver and gut-derived dendritic cells. Micro-arrays to compare CD161+ and CD161- cells may also provide useful information. Analysis of the patterns of expression of LLT-1 and the effects of CD161 binding to LLT-1 on CD8+ T-cell function may be highly important.

In summary this work has provided important insights into the utility of T-cell phenotype and has brought these and multiple disparate insights from the wider literature together. T-cell phenotype may have a role as an important clinical parameter. It can also be used as bridge to link an understanding of virus escape mechanisms with a broad understanding of T-cell biology.

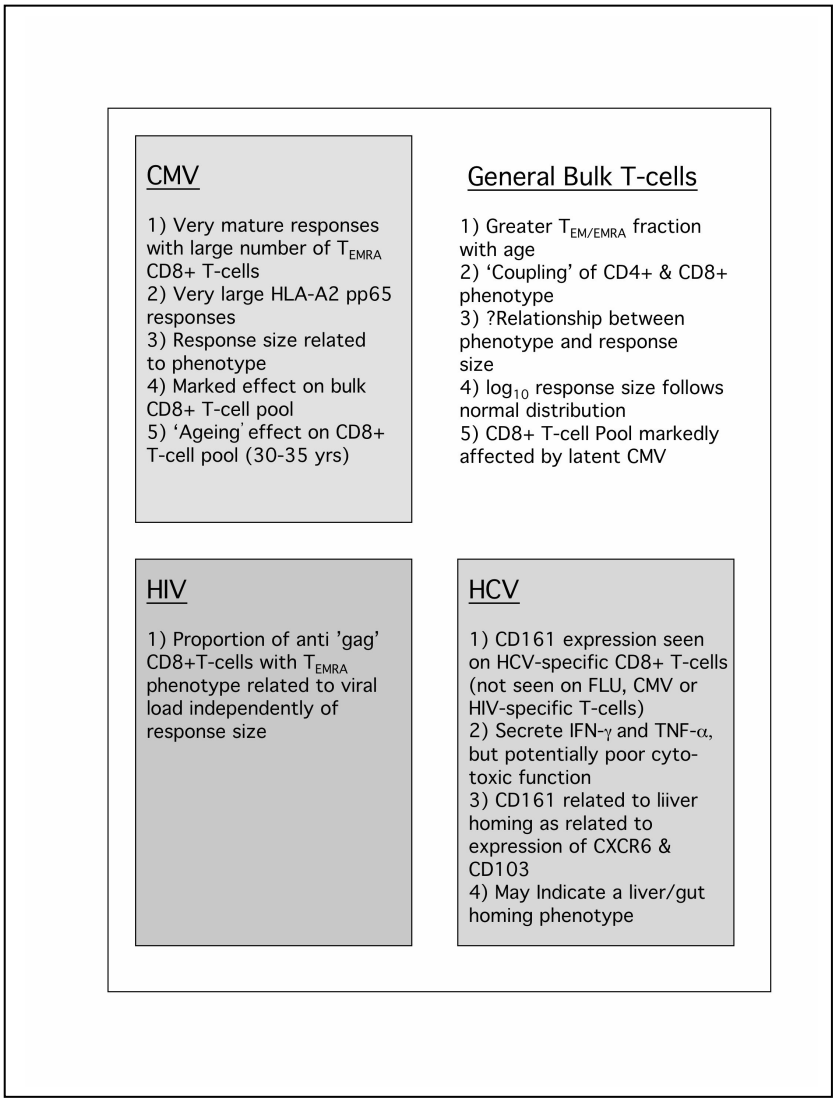

Figure 6:1. Summary of Key findings. The large rectangle represents the entire CD8+ T-cell pool, and within it the findings of general relevance (or potential relevance) to the biology of CD8+ T-cells are listed. The findings derived from specific virus-specific responses are also listed separately, each represented by a separate rectangle. See text for discussion.

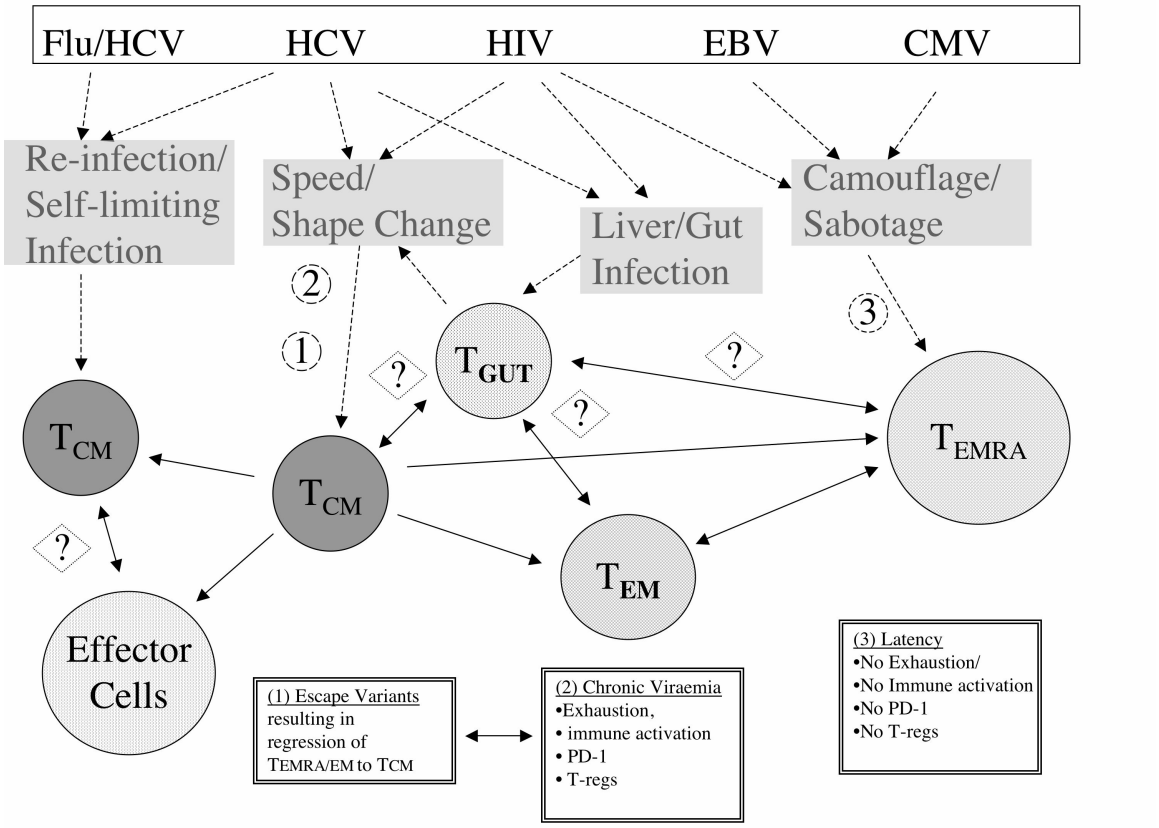

Figure 6:2 A model of T-cell differentiation that draws together the findings of this study with established work. The top long horizontal rectangle lists the protagonist viruses and their counterpart immune responses. The grey borderless rectangles link the main strategies utilized by each virus as discussed in (Lucas, Karrer et al. 2001). Viral infections which resolve spontaneously are identified (these range from influenza infection to resolving HCV infection). The potential strategy of infection of immuno privileged sights such as the liver and gut is also included. The potential outcome of infection is listed in the double-bordered rectangles at the bottom. It is proposed that in general, viruses that use 'camouflage' and 'sabotage' as their escape method (such as CMV) result in latent infection. On the other hand viruses using the strategies of 'speed' and 'shape change' to evade immune control result in chronic viraemia (which results in exhaustion – mediated by PD-1 expression and T-regs - and chronic immune activation). Viral escape mutations also enable viruses to slip out of immune control, and exacerbate the exhaustion and immune activation, and so on. These outcomes to infection are correlated with T-cell phenotype. Thus HCV is characterized by a T_{CM} of responses as escape variants may no longer trigger responses primed by the initial sequence, and thus these cells return to a 'central memory' phenotype (as if tricked into thinking the infection had resolved). Immune exhaustion and immuno privilege may also tend to result in a response with this phenotype. On the other hand CMV (and to a lesser extent HIV) exist in latent form and immune exhaustion is less likely to occur and the T_{EMRA} phenotype, perhaps specialised for such a scenario, predominates. HIV exists between the two extremes as it shows less diversity than HCV, but more readily establishes a state of latency. This model would seem to reconcile T-cell phenotype with pathogenic and virus escape mechanisms neatly. The question marks in small diamonds indicate pathways of T-cell differentiation that have not been clearly established. Thus it is not clear how the putative phenotype T_{GUT} (CD161+) arises and its relationship to T-cells with classical markers

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