Chapter 4

VIRUS SPECIFIC T-CELL RESPONSES

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Abstract: CD8+ and CD4+ T-cells play a key role in the maintenance of our immunity against viruses. Recent technological developments, such as the use of MHC-peptide tetrameric complexes, have permitted significant improvements in the study of these cells. It is now possible to assess precisely frequencies as well as phenotypic and functional features of virus specific T-cells from the onset of many viral infections onwards. Different virus specific T-cell populations exhibit distinct functional characteristics and can be positioned at different stages of a process of post-thymic development, which we are drawing near to understanding the significance. Still, further work is needed before consensus is reached as regards what defines and how to induce the optimal virus specific T-cell response which will confer long lasting immunological protection in humans.

Key words: Viral infection, CD8+ T-cell, CD4+ T-cell, tetramer, function, cytotoxic, differentiation, protective immunity.

1. INTRODUCTION

1.1 The importance of CD8+ T-cells in viral infections

The principal constituents of the adaptive cellular immunity arm are helper CD4+ T-cells, which participate in antigen recognition and in regulation functions; and cytotoxic CD8+ T-cells (CTL), which can kill virus infected cells and thus prevent viral spread. The importance of CD8+ T-cells in controlling viral infections was first illustrated in experiments of adoptive transfer with virus specific CTL, which conferred protection to mice exposed

D. Nagorsen and F.M. Marincola (eds.), Analyzing T Cell Responses, 83-101.
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to lethal doses of various viruses (e.g. influenza virus (1), respiratory syncytial virus (RSV) (2), herpes simplex virus (3), Sendai virus (4) and Moloney murine sarcoma virus (5). In simian immunodeficiency virus (SIV) primate models, depletion of CD8+ T-cells during both acute and chronic SIV infection in monkeys results the complete loss of viral control (6, 7). A conclusive role for CTL in the control of human viral infections has been more elusive. Nevertheless, influenza specific CD8+ T-cell levels were reported to correlate with protection after challenge with life virus (8), and adoptive transfer of Epstein-Barr virus (EBV) or cytomegalovirus (CMV) specific CTL were shown to represent a benefit in the treatment of EBV and CMV associated complications respectively (9, 10). Moreover the majority of persistent viruses have developed strategies to escape CD8+ T-cell recognition, for instance through specific mutations in the epitopes recognized by cytotoxic T lymphocytes or by blocking the process of antigen presentation, which also argues in favor of a strong role for CTL (11). Overall, it is clear that T-cell mediated immunity serves as the one of the principle defense mechanisms against virus infections and is essential for human survival. Its study is central to medical research.

Since the discoveries by Rolf Zinkernagel and Peter Doherty that CD8+ T-cells were able to recognize lymphocytic choriomeningitis virus-(LCMV) infected cells in an MHC-dependent manner (12, 13), and by Alan Townsend and Andrew McMichael that influenza specific CTL recognize target cells presenting virus derived peptides (14), our understanding of the mechanisms involved in cellular immunity has expanded considerably. Considerable knowledge about the dynamics of CTL responses in viral infections has been obtained from studies in mouse models, however I will concentrate in this chapter on the characterization of virus specific T-cells in humans, which is principally related to the focus of this book.

1.2 Methodological development in the study of CD8+ T-cells in humans

1.2.1 Measuring CD8+ T-cell activity

For obvious questions of practicality, detail analyses have been more difficult to perform in humans than in mice. Progress in the study of human CTL responses and of their involvement in the control of virus infections has been largely dependent on the development of several methods. The early established methods are based on the ability of CTL to lyse appropriate target cells *in vitro*. This can be performed under limiting dilution conditions (limiting dilution assay, LDA) to provide a quantitative

measurement of the antigen-specific CTL precursor cells which are able to grow and divide *in vitro* (15). The use of other recently-developed techniques including the measurement of cytokine production by CD8+ T-cells (by intracellular cytokine staining- ICS) (16) and the Interferon-gamma (IFN-γ) ELISpot assay (17), has shown that the LDA may significantly underestimate the true frequency of circulating CTL with specificity for viral antigens. However, all these techniques represent methodological limitations. They address different aspects of antigen-specific CD8+ T-cell activity. Limiting dilution analysis measures the lytic ability of specific CTL, but is also dependent on their ability to grow and divide in tissue culture conditions. ELISpot and ICS assays examine the ability of antigen-specific CD8+ cells to secrete a single cytokine, usually IFN-γ, on contact with their cognate antigen, but do not address their lytic function; it is also clear that not all antigen-specific CD8+ cells are able to produce IFN-γ upon specific stimulation or secrete other soluble factors. Moreover, since *in vitro* manipulation may significantly alter the composition and functional properties of the populations of interest, a detailed analysis of virus specific CD8+ T-cells is difficult to perform.

1.2.2 The tetramer era

In 1996, after more than 20 years of abortive efforts to measure the CD8+ T-cell response with precision, immunologists at last had at their disposal a tool which enabled a direct visualization of antigen specific CD8+ T-cells: the introduction of peptide-MHC class I tetrameric complex (tetramers) technology initiated a profound revolution in the field of cellular immunology (18). The use of tetramers to stain antigen-specific CD8+ T-cells is based on the ability of their T-cell receptor to interact specifically with a complex of the appropriate HLA molecule assembled with a relevant peptide with sufficient avidity to allow read-out by flow cytometry. The analysis of tetramer positive CD8+ T-cells has provided a method that reliably quantitates the number of specific CD8+ T-cells present in peripheral blood and secondary lymphoid organs (19, 20). The benefit of such a technology is not only limited to the precise quantification of these cells. The next step in the use of tetramers is the detailed analysis of the characteristics of the antigen-specific T-cell population. Together with the technological progress of multicolor flow cytometry (21), the reliable identification of virus-specific CD8+ T-cells by tetramers offer the possibility of obtaining additional information about their phenotype through co-staining for cell surface markers, including markers of activation (22), T-cell receptor Vβ usage (23), and other differentiation and homing markers (e.g. CD family and chemokine receptors) (24). Moreover, the combination

of intracellular labeling techniques with the use of tetramers enables us to characterize in detail the function of virus-specific CD8+ T-cells by assessing practically all aspects of the life of the cell (25). Caveats compelled with tetramer studies in humans still remain, and include generally the analysis of a limited number of epitopes per virus, and its restriction to the peripheral blood compartment. Nevertheless, the tetramer technology and its combination with cellular functional analysis provides for the first time the mean to uncover specific T-cell attributes associated with anti-viral protection.

2. STUDYING VIRUS SPECIFIC CD8+ T-CELLS

2.1 Frequency

The number of tetramers available for the study of CD8+ T-cell populations specific for various viruses is increasing [e.g. influenza (26), HIV (19), EBV (27), CMV (28), hepatitis B virus (HBV) (29), hepatitis C virus HCV (30), human papillomavirus (HPV) (31), human T-cell lymphotrophic virus type 1 (HTLV-1) (32), RSV (33) and dengue virus (34)], covering a large range of HLA types. Together with these technological advances, a better identification of individuals in early stages of viral infections have facilitated more detailed studies of virus specific CD8+ T-cells found in human peripheral blood from primary infection to the establishment of viral latency. During primary infection, most viruses induce a substantial antigen-driven activation and expansion of CD8+ T-cells, referred to as the "effector phase" (35). The use of tetramers has revealed that T-cell frequencies in diverse viral infection are much higher than estimated previously. The expansion during the primary infection is such that a population specific for one single viral epitope (from an EBV lytic protein) was shown to reach up to 44% of the total CD8+ T-cells at that moment [27]. Responses specific for more than one viral epitope are usually elicited. Subsequently as the viral burden is lowered or cleared, the majority of the CTLs undergo apoptosis, giving place during the "memory phase" to a long lasting pool of resting T-cells, which provide a faster and more effective response to reinfection or virus rebound, upon reactivation and expansion of these cells. The frequency of virus specific CD8+ T-cells detected following primary infection vary according to the virus considered. For instance HIV, EBV and notably CMV seem to induce particularly strong responses; during chronic infection, it is not unusual to detect 1% to 10% CD8+ T-cells specific for these viruses. This phenomenon is most certainly

related to continuous antigenic stimulation in the light of persistence of these viruses. In HIV infection, suppression of viral replication using drugs results in a decline of HIV specific T-cell numbers (36). Moreover, T-cell frequencies for viruses that cause limited infection of the host (e.g. influenza) are usually low (<0.1%) after resolution of primary infection (26). There is no consensus as regards a correlation between frequencies of virus specific CD8+ T-cells and viral control. It is certain though that the presence of virus specific CD8+ T-cells *per se* does not prevent viral spread and the development of virus associated complications in some patients. This has led to hypothesizes of dysfunctional CTL enable to suppress viral replication, for instance due to poor effector functions, anergy or exhaustion, and to direct studies on the functional and phenotypic characteristics of the CD8+ T-cells to verify this hypothesis.

2.2 Activation, proliferation and apoptosis

In vivo, infection by pathogens leads to a marked activation of T-cells whose eventual consequences can be as diverse as proliferation or apoptosis of the cells (35). CD8+ T-cells can also apoptose following lysis of their targets (37). Assessing these features is essential to understand the dynamics of the T-cell response. The activation status of antigen-specific CD8+ T-cells *ex vivo* can easily be monitored by staining for cell surface activation markers (e.g. CD38, HLA class II molecules) (22, 38). The nuclear antigen Ki67 is a commonly used marker to assess cell proliferation *ex vivo*; its expression coincides with activation markers. Following permeabilisation, tetramer positive T lymphocytes can easily be stained for Ki67, thereby providing a measurement of the proliferation of virus-specific CD8+ T-cells, for example during acute viral infection or viral reactivation (39, 40). Apoptosis is conducted through two main pathways: the first involves the engagement of death receptors such as Fas (or CD95) through interactions with their ligands (e.g. Fas ligand) (41) and the second pathway is governed by the Bcl-2 family which includes both pro-apoptotic (e.g. Bax, Bik) and anti-apoptotic (e.g. Bcl-2, Bcl-xL) members, promoting or preventing death signals from diverse cytotoxic stimuli (such as cytokine deprivation, DNA or mitochondrial damage) (41, 42). The measurement of the expression of cell surface death receptors such as CD95 *ex vivo*, as well as various intracellular mediators of the apoptotic pathway such as members of the Bcl-2 family provides information as regards the susceptibility to apoptose of the cells. Their expression is also closely linked to the level of T-cell activation. Overall, the expression of these molecules serve as *ex vivo* indicators of the reactivity of virus specific CD8+ T-cells to a viral challenge. As a consequence of active viral replication during primary infection and

secondary infection or viral rebound, virus specific CD8+ T-cell populations exhibit a similar phenotype of activated antigen experienced cells, expressing CD38, HLA-DR, Ki67 and low Bcl-2 levels (38, 43) (Figure 1).

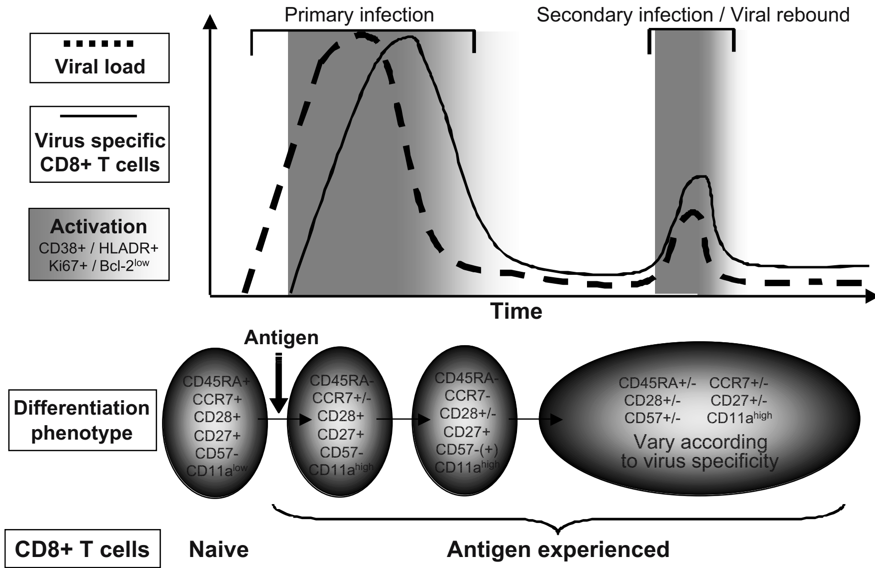

Figure 1. Common pattern of CD8+ T-cell response in human viral infections.

An important technical advance has been the possibility of monitoring cell division, for up to 8 discrete cycles, by following the serial halving of the dye CFSE (carboxyfluorescein diacetate succinimidyl ester) (44). CFSE can easily be loaded into lymphocytes, which retain their normal cell function throughout the assay. Following diffusion into the intracellular environment, the molecule loses its permeant ability and becomes highly fluorescent. CFSE is then partitioned with remarkable fidelity between daughter cells: proliferation results in progressive dilution and ultimately extinction of the dye, observed by flow cytometry. Using CFSE labeling in combination with tetramer staining *in vitro*, it was shown in the context of HIV-1 infection that non progression towards the disease is associated with the presence of HIV specific CD8+ T-cells capable of proliferating rapidly upon antigenic stimulation (45). This is in keeping with mouse studies suggesting that protective immunity is principally conferred by CD8+ T-cells displaying a strong proliferative capacity (46). The limitation of this technique is that this dye cannot be used *in vivo* in humans, restricting its use to *in vitro* assays. Nevertheless, it may be only a matter of time before innovative techniques, such as deuterated glucose and water labeling (47),

which enable the study of proliferation and turn over *in vivo* in humans, are combined with the tetramer technology, and make possible the study of virus specific T-cell populations.

2.3 Effector function

A major issue in the understanding of the relationship between T-cell response and anti viral control resides in the assessment of the CD8+ T-cell effector characteristics. Virus-specific CD8+ T-cells can mobilize two main effector mechanisms: cytolysis of infected cells and production of cytokines, chemokines and microbicidal molecules. The exploration of the capacity to execute these functions is particularly important in pathologies in which failure of immune surveillance is postulated to be significant (e.g. HIV infection).

2.3.1 Production of anti-viral soluble factors

CD8+ T-cells produce various soluble factors, including cytokines and chemokines, which play an active role in the immune response. For instance, IFN-γ plays an important role in the induction of cellular antiviral proteins (48) and through its ability to activate macrophages (49); IL-2 is a strong inducer of T-cell proliferation; and TNF-α is able to inhibit viral gene expression and replication (48). The CC-chemokines RANTES, MIP-1α and MIP-1β coordinate chemoattraction of lymphocytes and macrophages to the infection site, and have direct inhibitory effects against HIV infection (50). CD8+ T-cells produce cytokines rapidly upon stimulation (51), and reliable methods have been developed to measure intracellular cytokines by flow cytometry (52) following *in vitro* antigenic stimulation. Intracellular cytokine staining (ICS) for IFN-γ, TNF-α, IL-2, and MIP-1β can be successfully combined with tetramer staining. Staining with tetramers prior to activation can help significantly in preserving a reasonable level of tetramer staining, which is usually reduced due to activation triggered downregulation of the T-cell receptor (53). While few cells seem able to secrete IL-2 *ex vivo*, a majority (>50%) of CMV, HIV and EBV specific CD8+ T-cells can producing IFN-γ, TNF-α and MIP-1β during chronic infection, suggesting that these cells are functional, at least on the basis of cytokine production (54-56). This capacity to produce IFN-γ seems to be altered with the time in HIV infection; its reduction has been reported with late stages of the infection (57). HCV specific CD8+ T-cells have been shown to produce little IFN-γ, thus designated as stunned cells (30). A difficulty with the study of cytokine production relates to the interpretation of the results: although we know for certain that these cytokines have a role

in anti viral immunity, the importance of their production by CD8+ T-cells is not completely solved. For instance, what is the suitability of measuring IFN-γ secretion as a marker of effector function? What are the most relevant cytokines secreted by CD8+ T-cells to suppress viral replication? Relevance may even vary from one viral infection to another.

2.3.2 Cytotoxic potential

CD8+ T-cells mediate cytotoxicity through two main pathways: the perforin-dependent cytotoxic pathway and the Fas/FasL pathway (58). FasL (Fas ligand) present on the CD8+ T-cell surface binds to target cell-surface death receptor Fas (or CD95), which induces apoptosis of the target cell. Unfortunately the study of FasL expression on virus specific CD8+ T-cells remains difficult, due principally to a lack of sensitivity of the method or low levels of expression in the cells. Perforin is contained in lytic granules, and is thought to form pores in target cell membranes to enable the entry of granzymes which activate an apoptotic cascade resulting in cell death (59, 60). These molecules can be stained intracellularly. It is also possible to monitor the presence of intracellular lytic granules using monoclonal antibodies specific for TIA-1 (or GMP-17), a protein found in the granule membrane (61, 62), or the ability to degranulate by surface staining for CD107 (or LAMP) (63), also present in the membrane of cytotoxic granules, but which is transiently expressed onto the cell surface upon antigenic stimulation and degranulation. These tools provide an indication as regards the cytotoxic potential of T-cells *ex vivo*. Increasing evidence suggest that the majority of virus specific CD8+ T-cells exhibit such cytotoxic potential (with lytic granules containing perforin and granzymes) (62-65), even though this one may vary in intensity, as various levels of perforin and granzymes are found in different virus specific populations. The significance of these differences in cytotoxic potential for viral control remains to be understood. Moreover, staining for these molecules is only indicative of a potential, but is not a direct proof of cytotoxic efficiency *in vivo*. *Ex vivo* lysis assays (using chromium loaded target cells) with fresh PBMCs may be thought to provide a better view of cytotoxicity; however a recent study in the mouse actually suggests that these assays are not a true reflection of *in vivo* cytotoxic capacities (66), and so these measurements may be misleading as to what constitutes a protective “effector cell”.

2.4 CD8+ T-cell differentiation in viral infections

2.4.1 Post thymic development

With the large number of monoclonal antibodies against a variety of molecules (e.g. co-stimulatory receptors, chemokine receptors, adhesion molecules) available for flow cytometry, analysis of CD8+ T-cell subsets has been very extensive in human studies, resulting in the characterization of many phenotypically distinct subpopulations. The phenotype of a naïve cell is agreed by all and is characterized by expression of: CD45RA, the lymph node homing receptors CCR7 and CD62L, and the co-stimulatory receptors CD28 and CD27, low expression of the integrin CD11a, and lack of expression of markers such as CD57, granzymes and perforin (67). However, beyond the phenotype of naïve cells, complexity begins, with the description of a multitude of antigen experienced subsets characterized by distinct pattern of expression for the above cited markers (62, 68, 69). Nonetheless, there seems to be consensus that the expression of many of these molecules follows a common pattern, thus marking a linear pathway of CD8+ T-cell differentiation, as depicted simply in Figure 2. Such model is supported by longitudinal *ex vivo* analysis of virus specific CD8+ T-cells in human viral infections (Figure 1) (38, 43), as well as telomere length (see later) measurement in the different subsets and *in vitro* data following naïve T-cell priming (70). The *in vivo* significance and role of these distinct subpopulations has become a matter of intense debate, for instance with the use by different investigators of terms as varied as effector, senescent or suppressor cells to designate a same subset. In order to avoid confusion with the use of any particular terminology, the pathway of differentiation described above may be seen simply as the post-thymic development of T-cells, along which T-cell subsets are more or less differentiated; the attribution of particular functions or protective properties to these subsets remains unclear.

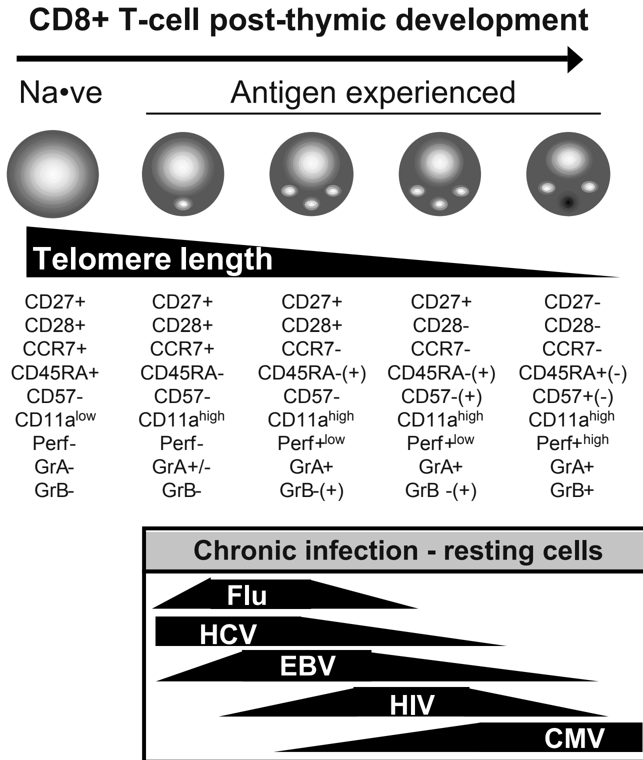

Figure 2. Distribution of virus specific CD8+ T-cells along T-cell differentiation during chronic virus infections. Partially reproduced from Appay V, Rowland-Jones SL. Lessons from the study of T-cell differentiation in persistent human virus infection. Seminars in Immunology. 2004 Jun;16(3):205-12 -Eelsevier Publishers.

2.4.2 Virus specific CD8+ T-cell differentiation

During the primary stage of most viral infections, virus specific CD8+ T-cells are present at early stages of differentiation with expression of CD27, as well as CD28, and no expression of CCR7 and CD45RA (43, 62, 71-73). During chronic infection, however, the picture is less simple: considerable heterogeneity is observed in the extent of differentiation of the CD8+ T-cell populations, and interestingly, this appears to be related to the viral specificity of the cells (62, 74, 75). For instance, the pattern of expression of differentiation markers varies between HCV-, EBV-, HIV- and CMV-specific CD8+ T-cells suggesting that these populations are at distinct stages of post thymic development (Figure 2). Resting Influenza A specific CD8+ T-cells usually show an early differentiated phenotype. Additional

differences reflecting particular antigen specificity have also become apparent. CD8+ T-cells specific for EBV latent proteins present a less differentiated phenotype that those specific for EBV lytic proteins (72). Cells recognizing the HLA-B8 restricted HIV nef epitope (FLKEKGGL) have been reported to be further differentiated compared to other HIV specific CD8+ T-cells (57). Nonetheless, this diversity is not as striking as those associated with different viral specificities. The basis for these differences and their significance in viral control remain to be determined. One hypothesis would be that the different differentiation states observed in peripheral blood for particular viral infections may relate to compartmentalization of some virus specific CD8+ T-cells in secondary lymphoid organs or at sites of viral persistence. This may be particularly relevant in the context of chronic HCV and HBV infection, where a significant number of virus-specific cells may be located in the liver.

Alternatively, the differentiation state of anti-viral T-cells with different viral specificities may reflect distinct conditions associated with particular viral infections, such as variations in antigen load, persistence, anatomical location and antigen presentation. The level of stimulation may be an important factor in this process (70). Elevated CD8+ T-cell activation seems to drive more extensive differentiation, as seen in both *in vitro* experiments using priming of naïve CD8+ T-cells and *ex vivo* analysis of virus specific CD8+ T-cells. In this context, antigen specific CD8+ T-cells may be subjected, from priming onwards, to variable intensity of activation or co-stimulation which differs in different infections, thereby dictating their development (i.e. expansion and differentiation). In this scenario, CMV would constitute a particularly potent stimulus for CD8+ T-cells, promoting extensive differentiation of CMV-specific cells. The regulation of T-cell differentiation is likely to be multi-factorial, and may also depend on the cytokine environment, the presence of T regulatory cells and the involvement of viral immune evasion strategies.

3. PERSPECTIVES

3.1 The importance of virus specific CD4+ T-cells

Giving focus only on virus specific CD8+ T-cells is only half a story told. CD4+ T-cells play also a major role in the establishment of effective anti viral immune responses, performing helper or co-ordination functions. The presence of virus specific CD4+ T-cells has been associated with long term non progression in HIV infection (76, 77), with control of CMV

replication after transplantation (43), and with the resolution of HCV viremia (78). The characterization of CD4+ T-cell populations specific for viral antigens represents a major challenge in human immunology. There is a substantial gap in our knowledge between CD8+ T-cells and CD4+ T-cells. This is in part due to the apparent complexity relative to the nature of these cells. On the basis of the pattern of cytokines produced, CD4+ T-cells have been divided into distinct functional subpopulations: T helper-0 (Th-0) cells , Th-1 cells and Th-2 cells; in addition, there are subsets of CD4+ T-cells that have regulatory – suppressing functions, or also cytotoxic potential. A further difficulty relates to the lack of tools adapted to their detailed study. Current approaches to study virus specific CD4+ T-cells rely on particular functions of the cells, such as proliferation and cytokine (often IFN-γ) production. This way, it is possible to detect CD4+ T-cell activity specific for most human viruses; although antigen specific CD4+ T-cells are usually present at significantly lower frequencies compared to their CD8 counterparts, which may be related to a slower division rate of the CD4+ T-cells, and represents an additional obstacle for their study. Moreover, these techniques do not permit *ex vivo* characterization of T-cell populations with a defined peptide specificity; the development of MHC class II /peptide tetrameric complex technology, adapted to CD4+ T-cell population analysis, has been more difficult than for CD8+ T-cells (79). At the cost of intensive efforts, the generation and use of reliable class II tetramers have recently begun (80); it should not be long before in depth characterization of virus specific CD4+ T-cells is performed. Furthermore, in order to overcome the problem of low frequency and to distinguish populations of interest from background, investigators have combined tetramer staining with magnetic bead capture techniques, thus enriching the tetramer positive cells to obtain satisfactory data (78).

In the recent years, the study of CD4+ T-cell subsets has also become more intensive and reveals a differentiation process, resembling CD8+ T-cell differentiation. Similarities involve phenotypic as well as functional changes that occur along the post-thymic development pathway: for instance, loss of expression of molecules such as CD27, CD28 and CCR7, gain in expression of CD57 as well as perforin, re-expression of CD45RA, and reduced capacity to proliferate (81, 82). Eventually, highly differentiated CD4+ T-cells, which have become cytotoxic, show striking resemblances to late differentiated CD8+ T-cells. Strong CD4+ T-cell differentiation seems particularly likely to occur under similar conditions of strong or chronic activation, such as in HIV-1 infection (83). Interestingly, virus specific CD4+ T-cells also exhibit distinct differentiation phenotypes in different infections: HCV, HIV and EBV specific CD4+ T-cells are less differentiated than CMV specific CD4+ T-cells (78, 84, 85), in parallel with the

observations made in CD8+ T-cells. Although these observations remain to be understood, this suggests that similar mechanisms are involved in the differentiation of these two lineages. Much work is needed to detail the exact implication of virus specific CD4+ T-cells in conferring protective immunity, as well as the mechanisms involved.

3.2 Understanding senescence and the maintenance of proliferative capacity

Since recent studies both in humans and in mice suggest an association between the capacity of virus specific T-cells to proliferate and the establishment of protective immunity (45, 46), it is most important to further our understanding of the loss and maintenance of such capacity by T-cells. Continuous activation can drive clonal exhaustion (86) as well as aging of lymphocytes, which eventually reach a state of replicative senescence (87); this process seems to be associated with T-cell differentiation, with highly differentiated drawing near senescence (70). Various mechanisms, under investigations, are involved in the onset of replicative senescence (88). One possible way to address the aging of T-cells resides in the measure of telomere length (89). Cellular expansion, involving extensive numbers of divisions, results in shortening of hexameric DNA sequences found at the end of chromosomes referred to as telomeres. With each division, 30 to 60 base pairs of telomeric DNA are lost, due the inability of DNA polymerase to replicate fully the extreme ends of chromosomes. Telomeres are responsible for maintaining chromosomal stability and integrity, and their shortening may eventually lead to cell cycle arrest and replicative senescence (90). The measurement of telomere length by flow cytometry is possible by flow-FISH (fluorescence in situ hybridization by flow cytometry) (91, 92), a technique recently associated with staining of virus specific CD8+ T-cells by tetramers (93). During primary infection and despite intensive T-cell proliferation, telomeres are not shortened, due to the expression of the telomerase (the enzymatic complex necessary for the maintenance of telomere length) (94). However, the induction of human telomerase expression appears to decrease in T-cells thereafter, when cells are stimulated again (95), resulting in telomere shortening. Interestingly, constitutive expression of telomerase by transduction in an HIV-1-specific CTL clone resulted in enhanced proliferative capacity and cytolytic capabilities in *in vitro* experiments (96).

3.3 Concluding remark

Understanding protective immunity is the gateway to vaccine development. Long term immunological protection is likely to depend on both the quantity and the quality of the antigen specific CD4+ and CD8+ T-cells that are generated. The level of antigen provided and the manner of presentation do certainly impact on both these parameters. However what defines exactly these parameters and how to govern them are not known yet. Although we are becoming better at inducing antigen specific T-cell responses *in vivo* using various vaccine strategies (e.g. antigen with adjuvants, antigen presenting dendritic cells, recombinants viral vectors, DNA), interrogation and controversy still remain as regards which are the most relevant T-cell phenotypic subpopulations or functional characteristics (i.e. effector functions as well as T-cell receptor usage and affinity - avidity) to generate. The path is still long before we reach a complete understanding of all T-cell mediated immunity mysteries; the study of virus specific T-cells is necessary to shed light on these points.

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