PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Quantitative analysis of the CD8$^+$ T–cell response to readily eliminated and persistent viruses

P. C. Doherty, J. M. Riberdy and G. T. Belz

Phil. Trans. R. Soc. Lond. B 2000 **355**, 1093-1101
doi: 10.1098/rstb.2000.0647

THE ROYAL SOCIETY

doi 10.1098/rstb.2000.0647

Quantitative analysis of the CD8$^+$ T-cell response to readily eliminated and persistent viruses

P. C. Doherty*, J. M. Riberdy and G. T. Belz

Department of Immunology, St Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38104, USA

The recent development of techniques for the direct staining of peptide-specific CD8$^+$ T cells has revolutionized the analysis of cell-mediated immunity (CMI) in virus infections. This approach has been used to quantify the acute and long-term consequences of infecting laboratory mice with the readily eliminated influenza A viruses (fluA) and a persistent γherpesvirus (γHV). It is now, for the first time, possible to work with real numbers in the analysis of CD8$^+$ T CMI, and to define various characteristics of the responding lymphocytes both by direct flow cytometric analysis and by sorting for further *in vitro* manipulation. Relatively little has yet been done from the latter aspect, though we are rapidly accumulating a mass of numerical data. The acute, antigen-driven phases of the fluA and γHV-specific response look rather similar, but CD8$^+$ T-cell numbers are maintained in the long term at a higher 'set point' in the persistent infection. Similarly, these 'memory' T cells continue to divide at a much greater rate in the γHV-infected mice. New insights have also been generated on the nature of the recall response following secondary challenge in both experimental systems, and the extent of protection conferred by large numbers of virus-specific CD8$^+$ T cells has been determined. However, there are still many parameters that have received little attention, partly because they are difficult to measure. These include the rate of antigen-specific CD8$^+$ T-cell loss, the extent of the lymphocyte 'diaspora' to other tissues, and the diversity of functional characteristics, turnover rates, clonal life spans and recirculation profiles. The basic question for immunologists remains how we reconcile the extraordinary plasticity of the immune system with the mechanisms that maintain a stable *milieu interieur*. This new capacity to quantify CD8$^+$ T-cell responses in readily manipulated mouse models has obvious potential for illuminating homeostatic control, particularly if the experimental approaches to the problem are designed in the context of appropriate predictive models.

Keywords: T cells; tetramers; influenza virus; γherpesvirus; lymph nodes; inflammation

1. INTRODUCTION

The capacity to stain antigen-specific CD8$^+$ T cells directly with tetrameric complexes (tetramers) of major histocompatibility complex (MHC) class I glycoprotein + peptide (Altman *et al.* 1996) has revolutionized the analysis of cell-mediated immunity (CMI) in virus infections. Accurate quantification of virus-specific CD8$^+$ T-cell populations is now an achievable reality, with the frequencies measured generally being comparable to those detected following short-term stimulation with peptide in the presence of Brefeldin A (Murali-Krishna *et al.* 1998; Flynn *et al.* 1998), followed by fixation and staining (Pepγassay) for cytoplasmic γ-interferon (IFN-γ). Otherwise, there is still relatively little understanding of the functional capacity and diversity of these virus-specific T-cell sets, though being able to select viable CD8$^+$ tetramer$^+$ populations in the flow cytometer obviously opens the way for such experiments. Most of what we have to say so far focuses on the numbers.

Having to deal with real numbers is a new and exciting experience for those who have been studying antigen-specific CD8$^+$ T-cell responses. The relatively few laboratories with access to the tetramers have been accumulating a mass of data very rapidly indeed. This raises two very basic questions. What does the information really mean? How do we design further experiments in ways that are most informative? If we are to access the underlying 'big' question, the nature of homeostatic control in the immune system, it becomes increasingly obvious that we must learn to think quantitatively, an unfamiliar experience for many experimental immunologists. We need to access the insights of mathematicians, and form linkages with them. In turn, it is important for theoretical biologists to have some understanding of the characteristics, limitations and uses of both the basic experimental systems and the experimentalists.

2. ACCESSING THE 'BLACK BOX'

What the T-cell dynamicists (Tanchot *et al.* 1997; Dutton *et al.* 1999; Sprent *et al.* 1999; Murali-Krishna *et al.* 1999; Swain *et al.* 1999; Doherty & Christensen 2000) are

*Author for correspondence (peter.doherty@stjude.org).

trying to understand at one level is the biology of the immune system, particularly the nature of homeostatic control (Freitas & Rocha 1993; Doherty *et al*. 1997*a*). At another level, we want to know how to exploit this knowledge to deal optimally with virus infections, cancer and autoimmune diseases in humans. It is essential that we recognize both the strengths and limitations of the available models. Mouse experiments provide insights, but mice are not humans. Contemporary viral immunology studies are generally done with C57BL/6J (B6) mice, because most of the genetically disrupted (−/−) 'knockouts' are developed with the H-2^b-congenic 129 strain. Just as all human beings are not HLA-A2$^+$ Caucasians, there is ample evidence that the B6 mouse is not 'every mouse', both with respect to T-cell numbers and cytokine response profiles. The B6 mouse has about $1–2\times10^8$ peripheral T cells with the CD4:CD8 ratio being approximately 1:1 (J. Sprent, personal communication). These lymphocytes are distributed in the blood, lymph, secondary lymphoid organs and the somatic tissue 'diaspora'. Mice are considered old by two years, but we have no real evidence that one mouse year is equivalent to 35 human years. The following is thus a 'B6 mouse world' view of viral immunity.

(a) *Anatomy of the host response*

Viruses are obligate intracellular parasites. Although viruses can grow in any tissue site where there are susceptible cellular targets expressing appropriate surface receptors, the 'naive' host response to a previously unencountered virus will always occur in organized lymphoid tissue, the lymph nodes, spleen and more specialized sites such as the Peyer's patches in the gut. The basic function of these lymphoid organs is to provide anatomically constrained micro-environments where the precursors of the CD8$^+$ 'killers', the CD4$^+$ 'helpers' and effectors of 'delayed type hypersensitivity', the antigen-presenting dendritic cells and the B lymphocytes that give rise to the antibody-producing plasma cells, can be brought into proximity. Apart from the various cellular elements, the key components are antigen (MHC I or MHC II glycoprotein + peptide for the T cells, protein for the B cells), various cytokines (IL-4, IL-2, IFN-γ, etc.) and chemokines, and a spectrum of adhesion molecules (e.g. CD40, B7, etc.).

Recruitment to the lymphoid tissue is the first step in any virus-specific immune response. This has probably been best worked out for localized respiratory infections (Doherty *et al*. 1992, 1997*b*). Dendritic cells that line the upper part of the respiratory tract become infected with the virus (McWilliam *et al*. 1997; Usherwood *et al*. 1999), then detach and transit via the afferent lymph to the regional mediastinal lymph nodes (MLN). Concurrently, various cytokines (IFN-α is known to be involved) induce the localization of naive (CD62L^{hi}) T cells and B cells to the regional nodes, an effect that can cause a transient lymphopenia (Hou *et al*. 1995). This is non-specific in the sense of antigen recognition, but selective from the aspect that the process occurs only in the lymph nodes draining a localized site of viral pathology. With a systemic infection, of course, this recruitment process would be expected to involve many more sites.

The consequence is selection of the few lymphocytes that can respond to the inducing pathogen, clonal expansion and differentiation to effector function. Although at least some CD4$^+$ helper T cells must produce cytokines in the lymph node, whether or not fully functional CD8$^+$ cytotoxic T lymphocytes (CTL) can be detected depends on the local concentration of antigen. Potent CTL populations are found in the lymphoid tissue of mice responding to lymphocytic choriomeningitis virus (LCMV), which grows in this site (Lynch *et al*. 1989), but both productive infection and CTL activity is largely limited to the respiratory tract following intranasal challenge with the negative-strand RNA viruses, such as the influenza A viruses and Sendai virus (Doherty *et al*. 1992, 1997*b*).

The whole story then becomes progressively more speculative, though immunologists do not necessarily present their case in this way. A proportion of the CD8$^+$ and CD4$^+$ T cells localize to the site of virus-induced pathology, differentiate to become fully functional effectors and control the infectious process (Doherty *et al*. 1997*b*). The bone marrow supports continued, long-term antibody production by terminally differentiated plasma cells (Hyland *et al*. 1994; Slifka *et al*. 1995; Sangster *et al*. 2000), but most effector T cells either return to a 'resting' state (large nucleus, little cytoplasm) or are eliminated. Many are thought to die very rapidly (Marrack *et al*. 1993), but the formal proof that this is indeed the case for the virus models is not all that compelling. Part of the problem is that there is as yet little information on the size of the lymphocyte 'diaspora' to other tissue sites. Some of the responding lymphocytes are clearly destroyed in the liver (Wack *et al*. 1997; Belz *et al*. 1998), while others remain to constitute the memory T- and B-cell pools (Doherty *et al*. 1996; Ahmed & Gray 1996).

Memory (CD44^{hi}CD62L^{lo}) and naive (CD44^{lo} CD62L^{hi}) CD8$^+$ and CD4$^+$ T cells distribute differently through the body. With respect to the lymphoid tissue, memory CD4$^+$ T cells are found almost exclusively in the spleen, while the CD44^{hi}CD8$^+$ set is detected at significant prevalence in the lymph nodes (Picker 1994). This is thought to reflect the fact that CD8$^+$ memory T cells exit the blood, migrate through solid tissues and return to the nodes via the afferent lymph, thus bypassing the CD62L-mediated interaction that allows lymphocytes to traverse the 'gate' constituted by the high endothelial venules. This 'gate' does not operate in the spleen, and is in some way subverted in Ig$^{-/-}$ μMT mice that lack both B cells and follicular dendritic cells (Kitamura *et al*. 1991; Topham *et al*. 1996). The likely possibility is that, although T-cell localization profiles are determined by the molecular anatomy of the various endothelial barriers, the 'cell-surface language' of the endothelium is in turn influenced by the physiological state of the lymphoid organ (Doherty 1995).

When it comes to the quantitative analysis of immunity, the availability of the tetramers means that we are generally in much better shape for CD8$^+$ than CD4$^+$ T cells. Apart from the greater difficulty of making the tetramers with MHC II molecules, we have generally failed (with considerable effort) to find the very prominent peptides that are detected routinely for the MHC I-restricted responses (Yewdell & Bennink 1999). With the acquisition of sufficient 'events' in the flow cytometer, it is possible to get an accurate estimate of tetramer$^+$CD8$^+$ T-cell numbers down to levels of about 0.1% of a CD8$^+$ population. Beyond that it is necessary to dilute the cells and use

the ELISPOT assay, which requires *in vitro* restimulation and culture (Butz & Bevan 1998). The correspondence between virus-specific $CD8^+$ T-cell numbers determined by ELISPOT (using peptide stimulation) and tetramer staining varies between about 1:1 and 1:3, depending on the experimental system and the laboratory. The ELISPOT is still the best technique available for the $CD4^+$ set (Topham & Doherty 1998*b*; Christensen & Doherty 1999), but most experiments with $CD4^+$ T cells have the further limitation that they depend on the use of virus-infected stimulators rather than the peptides that are available to probe the $CD8^+$ T-cell response. We have recently discussed the quantitation of $CD4^+$ T CMI elsewhere (Doherty & Christensen 2000), and focus for the remainder of this article on the $CD8^+$ set.

(b) *The two virus models*

Over the past two years we have used the tetramer-staining approach to analyse the acute and long-term consequences of exposing B6 mice to two very different types of viruses — one a negative-strand RNA virus (orthomyxovirus) with eight genes (Webster 1997), and the other a DNA virus (herpesvirus) with more than 90 genes (Efstathiou *et al.* 1990; Virgin *et al.* 1997). Respiratory challenge with both the influenza A viruses (fluA) and the murine γherpesvirus 68 (γHV-68) leads to replicative infection in the epithelial cells of the respiratory tract, which is controlled after eight to 12 days by the virus-specific host response (Doherty *et al.* 1992; Nash & Sunil-Chandra 1994; Virgin & Speck 1999). The fluA is then thought to be totally eliminated, while γHV-68 persists as a latent infection in (at least) B lymphocytes and macrophages. There is some indirect evidence that γHV-68 is regularly reactivating to the lytic phase.

The most important immune effector in both infections is the $CD8^+$ T cell (Doherty *et al.* 1992; Nash & Sunil-Chandra 1994; Virgin & Speck 1999), though the less virulent fluA viruses can be also be eliminated by $CD4^+$ T cells operating in the absence of the $CD8^+$ subset (Eichelberger *et al.* 1991). These fluA-specific $CD4^+$ T cells function principally via the provision of T help to B cells (Mozdzanowska *et al.* 1997; Topham & Doherty 1998*a*), but it is not clear how the consequent antibody response terminates the infectious process. Both $CD4^+$ and $CD8^+$ T-cell-mediated effector mechanisms are important for limiting the lytic phase of γHV-68 infection (Nash & Sunil-Chandra 1994; Virgin & Speck 1999; Christensen *et al.* 1999). As in the fluA system, the γHV-68-specific $CD8^+$ effectors are thought to operate via perforin or fas-mediated cytotoxicity (Doherty *et al.* 1997*b*; Topham *et al.* 1997), while the protective effect of the $CD4^+$ set is abrogated by treating the mice with a monoclonal antibody to IFN-γ (Christensen *et al.* 1999).

The immunodominant ASNENMETM peptide in the $CD8^+$ T-cell response to fluA (Townsend *et al.* 1986) is provided by the viral nucleoprotein ($NP_{366–374}$), while there are also other significant epitopes (Yewdell & Bennink 1999) including SSLENFRAYV (Belz *et al.* 2000*b*) derived from the polymerase 2 gene ($PA_{224–232}$). The responding T cells have been quantified by the Pepγassay, or by staining with the D^bNP_{366} or D^bPA_{224} tetramers (Flynn *et al.* 1998; Belz *et al.* 2000*b*). The most prominent targets in the γHV-68 response are p56 (AGPHNDMEI, D^bp56 tetramer) derived from a single-stranded DNA-binding protein (ORF6) and p79 (TSINFKVI, K^bp79 tetramer) from ORF61, which encodes the large ribonucleotide reductase subunit (Stevenson *et al.* 1999*a*). The response to p56 seems to be greater in the acute phase of the primary response while p79, which may be preferentially expressed following viral reactivation in persistently infected B cells, is more prominent in the long term.

Both NP_{366} and PA_{224} are present in the H1N1 (PR8) and H3N2 (HKx31) fluA viruses that we use in most of our experiments (Kilbourne 1969). The fact that PR8 and HKx31 have very different surface haemagglutinin (H) and neuraminidase (N) glycoproteins means that there is no cross-reactivity at the level of the neutralizing antibody, so intranasal challenge of an H1N1-immune mouse with the H3N2 virus leads to a secondary $CD8^+$ T-cell response in the context of a primary antibody response (Doherty *et al.* 1977; Flynn *et al.* 1998). Prime and boost experiments with γHV-68 (Stevenson *et al.* 1999*b*; Belz *et al.* 2000*b*) have used recombinants made with vaccinia virus (vacc-p56 and vacc-p79) or with the H1N1 WSN influenza A virus (WSN-p56). The general approach is that either naive or previously immunized (memory) mice are challenged intranasally with virus. The development of the specific host response is then monitored using tetramer staining (or the Pepγ) assay to characterize $CD8^+$ T cells from the regional MLN, the spleen and the inflammatory population recovered by bronchoalveolar lavage (BAL) of the virus-infected lung.

3. PRIMARY AND SECONDARY $CD8^+$ T-CELL RESPONSES

The overall impression is that naive and memory T cells are functionally different (Ahmed & Gray 1996; Doherty *et al.* 1996; Borrow *et al.* 1998; Sprent 1997). Memory T cells are much easier to stimulate, requiring smaller amounts of antigen and cytokine, and being less dependent on the presence of co-stimulatory molecules. The molecular basis of these differences is currently receiving some attention (Bachmann *et al.* 1999), although it was ignored to a remarkable extent for many years. When we talk about virus-specific $CD8^+$ T-cell numbers, however, it is important to recognize that there are no data on the magnitude of the naive population. The numbers are apparently too low to measure. Any insights we have concerning the naive $CD8^+$ set in normal mice come from analysing the total $CD44^{lo}CD62L^{hi}$ T-cell pool (without regard to specificity), or from studying alloreactivity where the enormous diversity of the response gives measurable frequencies prior to encountering the alloantigen. Otherwise, most of our information is derived from transgenic mice that express one or other T-cell receptor (TCR) αβ pair. How 'normal' are these TCR-transgenic lymphocytes?

(a) *Precursor T-cell numbers as a determinant of magnitude*

The difference between primary and secondary $CD8^+$ T-cell responses rests in the size and differentiation status of the 'resting' precursor populations. This review deals only with the quantitative aspects.

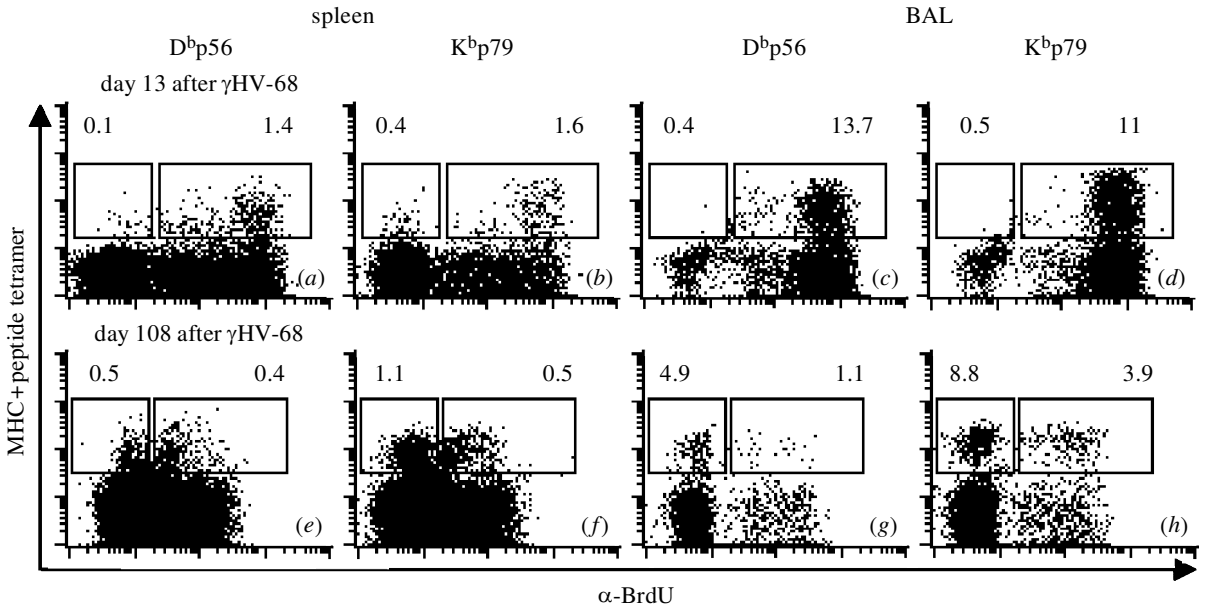

Figure 1. The B6 mice were infected intranasally with 600 plaque-forming units of γHV-68 and given 0.8 mg ml^{-1} BrdU in their drinking water for six days prior to sampling ((*a–d*) day 13 and (*e–h*) day 108) the spleen (*a*,*b*,*e*,*f*) and BAL (*c*,*d*,*g*,*h*) populations. The spleen cells were enriched for the CD8$^+$ set by incubating with mAbs to CD4 (rat) and MHC class II glycoprotein (mouse) followed by goat anti-mouse, and rat magnetic beads. The BAL cells were adhered for 1 h at 37 °C to remove macrophages. The cells were stained with the D^{b}p56 and K^{b}p79 tetramers and anti-CD8, then fixed and stained with anti-BrdU. Cumulating the data (gated on CD8$^+$ T cells) in the top quadrants of each figure segment gives the percentage tetramer$^+$ lymphocytes, while the top right quadrants show the percentage BrdU$^+$ tetramer$^+$. These data are from a more extensive set of experiments (G. Belz, P. Stevenson and P. Doherty) that is currently being prepared for publication.

(i) *The primary response*

Measurable numbers of naive CD8$^+$ T cells cannot be detected for either the major fluA (D^{b}NP$_{366}$ and D^{b}PA$_{224}$) or γHV-68 (D^{b}p56 and K^{b}p79) epitopes. What we can say is that there is massive clonal expansion during the primary response, with every CD8$^+$ tetramer$^+$ T cell showing evidence of proliferation in mice fed the thymidine analogue bromodeoxyuridine (BrdU) throughout that interval (Flynn *et al.* 1999). A relatively late time-point (day 13) in the γHV-68-specific response is shown in figure 1, which also illustrates the concentration of the proliferating virus-specific CD8$^+$ set in the site of inflammatory pathology (day 13 BAL, figure 1). Much of this cell division may, however, occur in the lymphoid tissue before the T cells exit into efferent lymph and travel via the blood to the virus-infected lung.

The development and resolution phases of the primary peptide-specific CD8$^+$ T-cell responses show the same general profiles for γHV-68 (day 0–25, figure 2) and for HKx31 (primary, figure 3). Comparison of these two sets of data also illustrates an important point with respect to the quantification of the T-cell response. Although the virus-specific CD8$^+$ set is detected at higher frequency in the BAL than in the spleen (day 13, figure 1; primary, figure 3), the relative sizes of these two cell populations are such that the total numbers of responding lymphocytes are not very different in the two sites. At least some of the decrease in fluA-specific CD8$^+$ T-cell numbers in both the BAL and spleen (primary, figure 3) reflects elimination in the liver (Belz *et al.* 1998), although we have not looked closely at the extent of dissemination to other lymphoid and non-lymphoid sites.

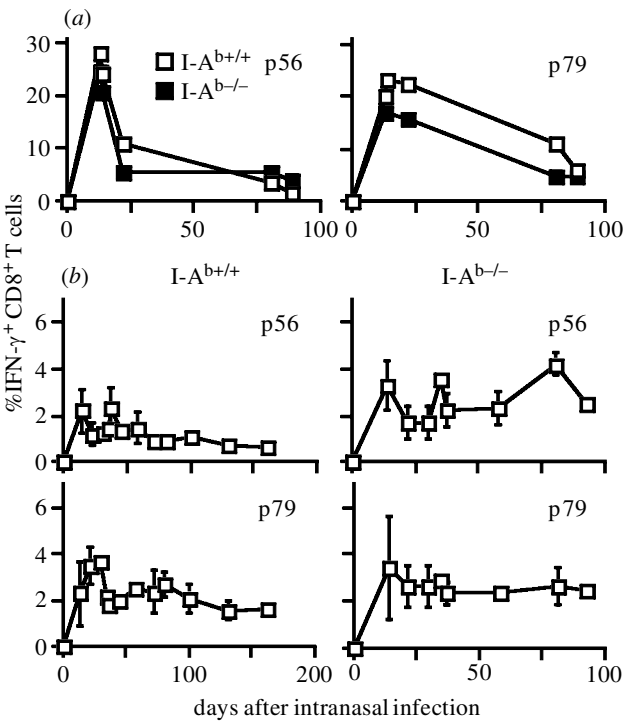

Figure 2. The prevalence of p56- and p79-specific CD8$^+$ T cells was determined for (*a*) BAL and (*b*) spleen populations from I-A$^{b+/+}$ (B6) and congenic I-A$^{b-/-}$ mice sampled at intervals after intranasal infection with 600 plaque-forming units of γHV-68. These data are cumulated from several different studies (Stevenson *et al.* 1998, 1999*b*; Belz *et al.* 2000*a*), and the values are generally comparable to those determined by tetramer staining.

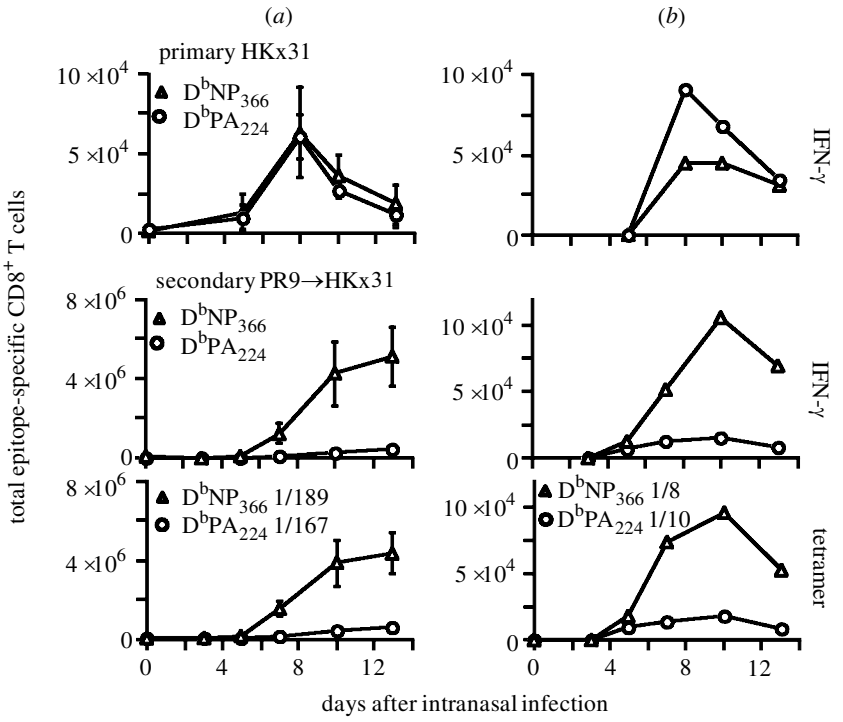

Figure 3. Naive or previously primed B6 mice were infected intranasally (in) with $10^{6.8}$ EID_{50} of the HKx31 (H1N1) fluA virus, then (*a*) spleen and (*b*) BAL populations were sampled at intervals and stained with the D^bNP_{366} and D^bPA_{224} tetramers and a mAb to CD8α. The immune mice had been infected intraperitoneally at least six weeks previously with $10^{8.5}$ EID_{50} of the PR8 (H1N1) virus. These data are reproduced from Belz *et al.* (2000*b*).

(ii) *The secondary response*

The prevalence of the D^bNP_{366}-specific cells in naive mice infected intranasally with the HKx31 (H3N2) virus (primary, figure 3) or intraperitoneally with the PR8 (HlN1) virus falls to < 1.0% of the $CD8^+$ population in spleen within three to four weeks (Flynn *et al.* 1998), then remains at levels that are close to the limits of detection by flow cytometry (primary, figure 4). Secondary challenge of PR8-primed mice (HKx31→PR8) also causes massive clonal expansion of the $CD8^+D^bNP_{366}{}^+$ set (secondary, figures 3 and 5), and again gives the impression that all the virus-specific $CD8^+$ Tcells divide more than once (Flynn *et al.* 1999). Increasing the $CD8^+$ T-cell precursory frequency from a level that is unmeasurable (primary) to about 0.5% thus causes an enormous boost in the magnitude of the fluA-specific response (figures 3–5). The effect is very obvious in both the virus-infected lung (figures 3 and 5) and in the spleen (figures 3–5), although evidence of replicative infection in mice challenged with the HKx31 virus is limited to the epithelial cells of the respiratory tract.

These results on the D^bNP_{366}-specific response also show very clearly that the great majority of the virus-specific $CD8^+$ T cells are found in the lymphoid tissue (compare BAL and spleen, secondary, figure 3). Most fluA-specific $CD8^+$ memory T cells may never enter the virus-infected lung and encounter a high dose of antigen, and would thus not be expected to become CTL effectors at any stage of their clonal history. The numbers fall off with time (Flynn *et al.* 1999), but this looks to be a gradual process (secondary, figure 5). Pulse–chase analysis of $BrdU^+CD8^+D^bNP_{366}{}^+$ T cells that proliferated during the initial, antigen-driven phase of the host response indicates that significant numbers of these fluA-specific $CD8^+$ memory T cells do not divide further, while others continue to cycle (Flynn *et al.* 1999). The overall picture from eight-day BrdU pulse experiments is that about 3–7% of the total $CD8^+D^bNP_{366}{}^+$ seems to be turning over through the course of long-term memory.

The protective efficacy of the fluA-specific secondary $CD8^+$ T-cell response is very limited, with virus clearance being enhanced (Flynn *et al.* 1998, 1999; Riberdy *et al.* 1999) by only about two days (figure 5). However, when we challenged the secondary HKx31→PR8-primed mice with a third, non-cross-reactive (H7N7) fluA virus, the duration and magnitude of this tertiary infection was much reduced (Christensen *et al.* 2000). Increasing memory $CD8^+$ T-cell frequencies from a level of < 0.5% to > 5.0% thus substantially enhancing the extent of protection.

The same effect was seen in mice primed with vacc-p56 and boosted with WSN-p56 (Stevenson *et al.* 1999*b*). The initial phase of replicative infection in the lung was almost totally prevented following intranasal challenge with γHV-68. Even so, there were equivalent numbers of persistently infected B lymphocytes in the spleens of the WSN-p56vacc-p56-primed and naive control mice within three weeks of exposure to γHV-68, and the infectious mononucleosis-like disease caused by γHV-68 (Tripp *et al.* 1997) was delayed in onset but not prevented. At least for the γHVs, early $CD8^+$ T-cell-mediated control directed at a lytic phase epitope may be of little consequence for the infectious process in the long term (Stevenson *et al.* 1999*b*; Liu *et al.* 1999).

4. PERTURBATION BY ANTIGEN

The magnitude of any virus-specific $CD8^+$ T-cell response can be thought of as a function of the numbers of virus-specific naive or memory precursors and the antigen load. This nexus is illustrated most clearly in figure 5 for the primary and secondary responses to the HKx31 fluA virus. Virus growth during the first three to four days after challenge was equivalent for the naive and

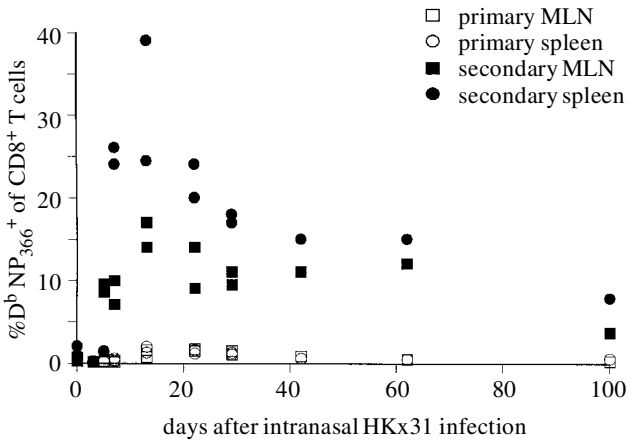

Figure 4. Reciprocal frequencies are shown for the splenic $CD8^+D^bNP_{366}^+$ set in B6 mice sampled at intervals after primary (HKx31) and secondary (HKx31→PR8) challenge with the same fluA virus (see figure 2 legend). in, intranasal. The data are abstracted from (Flynn *et al.* 1999).

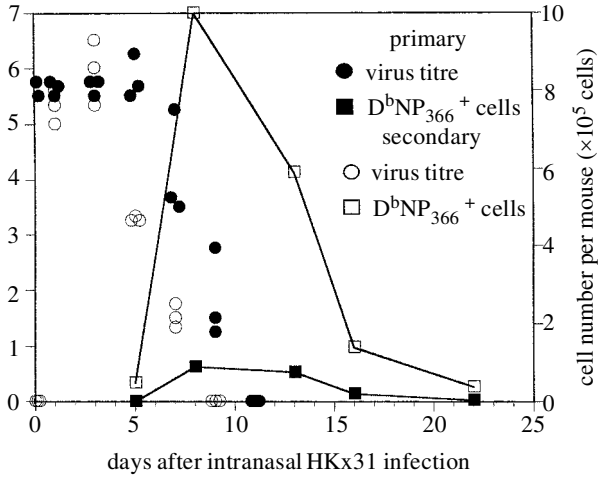

Figure 5. The control of HKx31 infection was analysed for the acute phase of the analysis described in figure 4 by titrating lung homogenates in embryonated hen's eggs. The numbers of $CD8^+D^bNP_{366}^+$ T cells in individual BAL populations were calculated from the total cell counts and the percentage staining with the tetramer. in, intranasal. The data are reproduced from (Flynn *et al.* 1999).

PR8-primed mice, but the magnitude of the secondary $CD8^+D^bNP_{366}^+$ response measured in the BAL (figure 5) and spleen (figures 3–5) was much greater for the 'memory' group (Flynn *et al.* 1998, 1999).

However, we have not yet looked closely at what happens when we vary the level of antigen challenge in the fluA-specific secondary $CD8^+$ T-cell response, so we do not know if the BAL results (secondary, figures 3–5) represent the peak values for a linear dose response. Also, the massive numbers of virus-specific $CD8^+$ T cells in the spleen, together with the fact that there is no evidence of replicative infection in this site, suggest that a relatively small amount of antigen may be able to drive a very substantial secondary response.

(a) *Antigen persistence*

The reason we started to work with the very complex γHV-68 model after years of focusing on readily eliminated, relatively simple viruses (fluA, Sendai) was that we wanted to see what happens to 'memory' T cells under conditions of antigen persistence. This is illustrated very clearly for normal $I\text{-}A^{b+/+}$ and CD4-T cell-deficient $I\text{-}A^{b-/-}$ mice in figure 6. Both groups of mice are persistently infected with γHV-68, but there is much greater evidence of reactivation to replicative infection (both p56 and p79 are lytic phase epitopes) in the $I\text{-}A^{b-/-}$ group (Cardin *et al.* 1996; Stevenson *et al.* 1998). While the $I\text{-}A^{b+/+}$ mice remain clinically normal, the $I\text{-}A^{b-/-}$ group develops symptoms of a chronic wasting disease 90–100 days after the initial exposure to virus and all eventually succumb (Cardin *et al.* 1996). Virus-specific $CD8^+$ T cells capable of producing IFN-γare still present at substantial frequency (> 2%) in both the BAL and spleen (figure 2) when the $I\text{-}A^{b-/-}$ mice are symptomatic (Stevenson *et al.* 1998); the primary immune defect seems to be the absence of an IFN-γ-producing $CD4^+$ effector T-cell population (Christensen *et al.* 1999).

Several tentative conclusions may be drawn from this study (figure 2). The first is that the numbers of virus-specific $CD8^+$ T cells in spleen (figure 2) remain higher for the persistent γHV-68 than for the readily eliminated HKx31 virus (primary, figure 5). Also, although the level of continued antigen stimulation is likely to be greater in the γHV-68-infected $I\text{-}A^{b-/-}$ mice, the actual numbers of D^bp56 and K^bp79-specific T cells do not appear to be much different from those in the $I\text{-}A^{b+/+}$ controls. Bear in mind that there are no $CD4^+$ T cells in the $I\text{-}A^{b-/-}$ mice, so the percentage of $CD8^+$ cells in the spleen will be increased accordingly (figure 2).

It does seem that the reactivation of γHV-68 to lytic phase may also be a feature of the well-controlled, persistent infection in the $I\text{-}A^{b+/+}$ mice. Feeding BrdU to $I\text{-}A^{b+/+}$ mice infected for longer three months with γHV-68 showed that at least 30% of the γHV-68-specific $CD8^+$ T cells cycled during a six-day interval (spleen, day 108, figure 1). The comparable value over eight days for the memory $CD8^+D^bNP_{366}^+$ set in HKx31-primed mice would be about 5%.

Persistent infection thus maintains virus-specific $CD8^+$ T-cell numbers at a higher level, partly as a consequence of continued proliferation (figures 1 and 2). However, the total size of these virus-$CD8^+$ sets seems still to be regulated, maintaining the numbers of responding lymphocytes at a fairly constant level that may not be too finely tuned by the extent of antigen stimulation. Again, we come back to the central mystery of immunology, the nature of homeostatic control.

(b) *Post-exposure vaccination of persistently infected mice*

Given the indications of continuing (or sporadic) reactivation to lytic phase (figures 1 and 2) in both $I\text{-}A^{b+/+}$ and $I\text{-}A^{b-/-}$ mice infected with γHV-68, we thought that further exposure to antigen would have relatively little effect. Much to our surprise, a single intraperitoneal challenge with vacc-p56 or vacc-p79 boosted the cognate response (Belz *et al.* 2000*a*) at least tenfold (figure 6). Furthermore, the numbers of D^bp56^+ and K^bp79^+ $CD8^+$ T cells remained high in the long term. The fact that these recombinant vaccinia viruses generally replicate rather poorly made this finding even more unexpected.

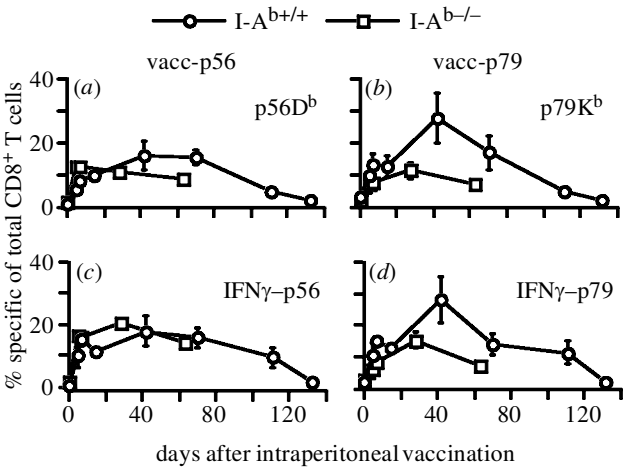

Figure 6. The I-A$^{b+/+}$ (B6) and congenic, CD4⁺ T-cell-deficient I-A$^{b-/-}$ mice were infected intranasally with 600 plaque-forming units of γHV-68, then challenged intraperitoneally with vacc-p56 or vacc-p79 one month later. The data (abstracted from Belz *et al.* (2000*a*)) show the percentage of CD8⁺ T cells staining with the appropriate tetramer (*a*,*b*), or detectable by the Pepγassay (*c*,*d*).

The net consequence for the I-A$^{b-/-}$ mice was a slight increase in survival time, but these mice again succumbed when the CD8⁺D^{b}p56⁺ and CD8⁺K^{b}p79⁺ T-cell counts fell to about the levels detected on day 100 in the unmanipulated I-A$^{b-/-}$ group (figure 2).

5. IMMUNODOMINANCE EFFECTS AND THE 'CLONAL BURST' HYPOTHESIS

Earlier studies completed before the tetramer and Pepγ approaches became available indicated that the magnitude of the virus-specific CD8⁺ memory T-cell pool reflects the size of the antigen-driven 'clonal burst' (Hou *et al.* 1994). This model still seems to be useful for most virus-specific CD8⁺ T-cell populations, but there is an exception. The primary CD8⁺ T-cell response to the recently identified D^{b}PA$_{224}$ epitope (Belz *et al.* 2000*b*) is initially as great as that to the immunodominant NP$_{366}$ peptide (primary, figure 3). Furthermore, the numbers of memory CD8⁺D^{b}PA$_{224}$⁺ and CD8⁺D^{b}NP$_{336}$⁺ T cells seem to be moderately equivalent when determined by tetramer staining (frequency values, (*a*) bottom panel, figure 3).

In the secondary response, however, there is massive expansion of the CD8⁺D^{b}NP$_{336}$⁺ but not the CD8⁺D^{b}PA$_{224}$⁺ set (secondary, figure 3). This is probably why the PA$_{224}$-specific population was not discovered earlier. We emphasize this point for several reasons. The first is that immunodominance hierarchies can greatly skew response profiles depending on the particular spectrum of MHC I glycoproteins that are present in a responding individual (Doherty *et al.* 1992; Yewdell & Bennink 1999). The second is that vaccine strategies need to take such effects into account. The third is that numbers are not everything.

6. CONCLUSIONS

The first experiments with tetramers in mouse model systems were published in 1998 (Murali-Krishna *et al.* 1998; Busch *et al.* 1998; Flynn *et al.* 1998), so the quantitative analysis of virus-specific CD8⁺ T-cell responses is still at a relatively early stage. Other papers in this issue discuss the studies that have been done in humans (Altman *et al.* 1996; Callan *et al.* 1998; Jeffery *et al.* 1999; Wodarz *et al.* 1999; Bieganowska *et al.* 1999). The large numbers of tetramer⁺ T cells that have been found in the circulation of people infected with the human immunodeficiency virus (HIV), human T-cell leukaemia virus type 1 (HTLV1) and Epstein–Barr virus (EBV) give the impression that all virus-specific CD8⁺ T-cell responses are massive, and dominate the CD8⁺ T-cell pool in the blood and lymphoid tissue. This view has been reinforced by the mouse experiments with LCMV (Murali-Krishna *et al.* 1998), a most unusual virus that is non-lytic, causes little pathology in T-cell-deficient mice and grows extensively in lymphoid tissue (Buchmeier *et al.* 1980). It is intriguing that the ongoing response to the persistent γHV-68 (figure 2) is at no higher level than that to LCMV strains that are thought to be readily eliminated by the acute response. Perhaps this implies that the primary LCMV response achieves a higher 'set point' during the initial phase of high antigen dose, a conclusion that is in accord with the 'clonal burst' hypothesis (Hou *et al.* 1994).

The mouse experiments with fluA are probably more typical of what happens in most virus infections (Flynn *et al.* 1998, 1999). The numbers of virus-specific CD8⁺ T cells generated following primary stimulation generally stabilize at a level below 0.5% of the CD8⁺ set in lymphoid tissue. Similar findings have been recorded for a coronavirus (Bergmann *et al.* 1999), an enterovirus (Johnson *et al.* 1999) and the bacterium *Listeria monocytogenes* (Busch *et al.* 1998). The fluA-specific CD8⁺ T-cell counts increase greatly following secondary infection. Repeat infection with related fluA viruses is a regular occurrence in human populations, and it will undoubtedly be informative to probe this further with the tetramer approach.

The experiments that have been done so far with the tetramers have focused largely on the overall nature of the virus-specific response without trying to dissect the effects that determine magnitude. Is it worthwhile, or even possible, to attempt careful dose-response curves in such a complex system? One set of experiments that clearly needs to be done is to make a better estimate of the size of the T-cell diaspora to other lymphoid and non-lymphoid sites. Numbers will also be determined by the rates of lymphocyte turnover and loss, and we are beginning to get some insights into these parameters. Are there useful theoretical models that can be probed with these experimental systems?

This research was supported by US Public Health Service grants (USPHS) AI 29579, AI 38359, CA 21765 and the American Syrian Lebanese Associated Charities. The mouse studies were all done in the ALAC-approved St Jude's Children's Research Hospital (SJCRH) Animal Resources Center, subsequent to approval by the appropriate institutional committee operating on guidelines determined by the USPHS. Other members of the laboratory, particularly Dr Philip Stevenson, Dr Kirsten Flynn, Dr Jan Christensen, Ms Kristen Branum and Ms Suzette Wingo were also involved in the acquisition of published data that is reproduced in this review article. The tetramers used in most of

these studies were produced in the laboratory of Dr J. Altman at Emory University, Atlanta, while the D^bPA_{224} tetramer was made by Dr Weidong Xie at SJCRH.

REFERENCES

Ahmed, R. & Gray, D. 1996 Immunological memory and protective immunity: understanding their relation. *Science* **272**, 54–60.

Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J. & Davis, M. M. 1996 Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94–96.

Bachmann, M. F., Gallimore, A., Linkert, S., Cerundolo, V., Lanzavecchia, A., Kopf, M. & Viola, A. 1999 Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells. *J. Exp. Med.* **189**, 1521–1530.

Belz, G. T., Altman, J. D. & Doherty, P. C. 1998 Characteristics of virus-specific $CD8^+$ T cells in the liver during the control and resolution phases of influenza pneumonia. *Proc. Natl Acad. Sci. USA* **95**, 13 812–13 817.

Belz, G. T., Stevenson, P. G., Castrucci, M. R., Altman, J. D. & Doherty, P. C. 2000*a* Post exposure vaccination massively increases the prevalence of γHV-specific $CD8^+$ T cells but confers minimal survival advantage on CD4-deficient mice. *Proc Natl Acad. Sci. USA* **97**, 2725–2730.

Belz, G. T., Xie, W., Altman, J. D. & Doherty, P. C. 2000*b* A previously unrecognized H-$2D^b$-restricted peptide prominent in the influenza A virus-specific $CD8^+$ T cell response is much less apparent following secondary challenge. *J. Virol.* **74**, 3486–3493.

Bergmann, C. C., Altman, J. D., Hinton, D. & Stohlman, S. A. 1999 Inverted immunodominance and impaired cytolytic function of $CD8^+$ T cells during viral persistence in the central nervous system. *J. Immunol.* **163**, 3379–3387.

Bieganowska, K. (and 13 others) 1999 Direct analysis of viral-specific $CD8^+$ T cells with soluble HLA- A2/Tax11-19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. *J. Immunol.* **162**, 1765–1771.

Borrow, P., Tough, D. F., Eto, D., Tishon, A., Grewal, I. S., Sprent, J., Flavell, R. A. & Oldstone, M. B. 1998 CD40 ligand-mediated interactions are involved in the generation of memory $CD8^+$ cytotoxic T lymphocytes (CTL) but are not required for the maintenance of CTL memory following virus infection. *J. Virol.* **72**, 7440–7449.

Buchmeier, M. J., Welsh, R. M., Dutko, F. J. & Oldstone, M. B. 1980 The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Adv. Immunol.* **30**, 275–331.

Busch, D. H., Pilip, I. M., Vijh, S. & Pamer, E. G. 1998. Coordinate regulation of complex T cell populations responding to bacterial infection. *Immunity* **8**, 353–362.

Butz, E. A. & Bevan, M. J. 1998 Massive expansion of antigen-specific $CD8^+$ T cells during an acute virus infection. *Immunity* **8**, 167–175.

Callan, M. F., Tan, L., Annels, N., Ogg, G. S., Wilson, J. D., O'Callaghan, C. A., Steven, N., McMichael, A. J. & Rickinson, A. B. 1998 Direct visualization of antigen-specific $CD8^+$ T cells during the primary immune response to Epstein–Barr virus *in vivo*. *J. Exp. Med.* **187**, 1395–1402.

Cardin, R. D., Brooks, J. W., Sarawar, S. R. & Doherty, P. C. 1996 Progressive loss of $CD8^+$ T cell-mediated control of a gamma-herpesvirus in the absence of $CD4^+$ T cells. *J. Exp. Med.* **184**, 863–871.

Christensen, J. P. & Doherty, P. C. 1999 Quantitative analysis of the acute and long-term $CD4^+$ T-cell response to a persistent gammaherpesvirus. *J. Virol.* **73**, 4279–4283.

Christensen, J. P., Cardin, R. D., Branum, K. C. & Doherty, P. C. 1999 $CD4^+$ T cell-mediated control of a gamma-herpesvirus in B cell-deficient mice is mediated by IFN-γ. *Proc. Natl Acad. Sci. USA* **96**, 5135–5140.

Christensen, J. P., Doherty, P. C., Branum, K. C. & Riberdy, J. M. 2000 Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of $CD8^+$ T cell memory. *J. Virol.* (Submitted.)

Doherty, P. C. 1995 Anatomical environment as a determinant in viral immunity. *J. Immunol.* **155**, 1023–1027.

Doherty, P. C. & Christensen, J. P. 2000 Accessing complexity: the dynamics of virus-specific T cell responses. *A. Rev. Immunol.* **18**, 561–592.

Doherty, P. C., Effros, R. B. & Bennink, J. 1977 Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. *Proc. Natl Acad. Sci. USA* **74**, 1209–1213.

Doherty, P. C., Allan, W., Eichelberger, M. & Carding, S. R. 1992 Roles of αβ and γδ T cell subsets in viral immunity. *A. Rev. Immunol.* **10**, 123–151.

Doherty, P. C., Topham, D. J. & Tripp, R. A. 1996 Establishment and persistence of virus-specific $CD4^+$ and $CD8^+$ T cell memory. *Immunol. Rev.* **150**, 23–44.

Doherty, P. C., Hamilton-Easton, A. M., Topham, D. J., Riberdy, J., Brooks, J. W. & Cardin, R. D. 1997*a* Consequences of viral infections for lymphocyte compartmentalization and homeostasis. *Semin. Immunol.* **9**, 365–373.

Doherty, P. C., Topham, D. J., Tripp, R. A., Cardin, R. D., Brooks, J. W. & Stevenson, P. G. 1997*b* Effector $CD4^+$ and $CD8^+$ T-cell mechanisms in the control of respiratory virus infections. *Immunol. Rev.* **159**, 105–117.

Dutton, R. W., Swain, S. L. & Bradley, L. M. 1999 The generation and maintenance of memory T and B cells. *Immunol. Today* **20**, 291–293.

Efstathiou, S., Ho, Y. M., Hall, S., Styles, C. J., Scott, S. D. & Gompels, U. A. 1990 Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein–Barr virus and herpesvirus saimiri. *J. Gen. Virol.* **71**, 1365–1372.

Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R. & Doherty, P. C. 1991 Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted $CD8^+$ T cells. *J. Exp. Med.* **174**, 875–880.

Flynn, K. J., Belz, G. T., Altman, J. D., Ahmed, R., Woodland, D. L. & Doherty, P. C. 1998 Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia. *Immunity* **8**, 683–691.

Flynn, K. J., Riberdy, J. M., Christensen, J. P., Altman, J. D. & Doherty, P. C. 1999 *In vivo* proliferation of naive and memory influenza-specific $CD8^+$ T cells. *Proc. Natl Acad. Sci. USA* **96**, 8597–8602.

Freitas, A. A. & Rocha, B. B. 1993 Lymphocyte lifespans: homeostasis, selection and competition. *Immunol. Today* **14**, 25–29.

Hou, S., Hyland, L., Ryan, K. W., Portner, A. & Doherty, P. C. 1994 Virus-specific $CD8^+$ T-cell memory determined by clonal burst size. *Nature* **369**, 652–654.

Hou, S., Hyland, L., Bradley, L. M., Watson, S. R. & Doherty, P. C. 1995 Subverting lymph node trafficking by treatment with the Mel-14 monoclonal antibody to L-selectin does not prevent an effective host response to Sendai virus. *J. Immunol.* **155**, 252–258.

Hyland, L., Sangster, M., Sealy, R. & Coleclough, C. 1994 Respiratory virus infection of mice provokes a permanent humoral immune response. *J. Virol.* **68**, 6083–6086.

Jeffery, K. J. (and 16 others) 1999 HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. *Proc. Natl Acad. Sci. USA* **96**, 3848–3853.

Johnson, A. J., Njenga, M. K., Hansen, M. J., Kuhns, S. T., Chen, L., Rodriguez, M. & Pease, L. R. 1999 Prevalent class I-restricted T-cell response to the Theiler's virus epitope D^b:VP2121-130 in the absence of endogenous CD4 help,

tumor necrosis factor alpha, gamma interferon, perforin, or costimulation through CD28. *J. Virol.* **73**, 3702–3708.

Kilbourne, E. D. 1969 Future influenza vaccines and the use of genetic recombinants. *Bull. WHO* **41**, 643–645.

Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K. 1991 A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature* **350**, 423–426.

Liu, L., Usherwood, E. J., Blackman, M. A. & Woodland, D. L. 1999 T-cell vaccination alters the course of murine herpesvirus 68 infection and the establishment of viral latency in mice. *J. Virol.* **73**, 9849–9857.

Lynch, F., Doherty, P. C. & Ceredig, R. 1989 Phenotypic and functional analysis of the cellular response in regional lymphoid tissue during an acute virus infection. *J. Immunol.* **142**, 3592–3598.

McWilliam, A. S., Marsh, A. M. & Holt, P. G. 1997 Inflammatory infiltration of the upper airway epithelium during Sendai virus infection: involvement of epithelial dendritic cells. *J. Virol.* **71**, 226–236.

Marrack, P., Hugo, P., McCormack, J. & Kappler, J. 1993 Death and T cells. *Immunol. Rev.* **133**, 119–129.

Mozdzanowska, K., Furchner, M., Maiese, K. & Gerhard, W. 1997 CD4⁺ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. *Virology* **239**, 217–225.

Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J., Zajac, A. J., Miller, J. D., Slansky, J. & Ahmed, R. 1998 Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* **8**, 177–187.

Murali-Krishna, K., Lau, L. L., Sambhara, S., Lemonnier, F., Altman, J. & Ahmed, R. 1999 Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377–1381.

Nash, A. A. & Sunil-Chandra, N. P. 1994 Interactions of the murine gammaherpesvirus with the immune system. *Curr. Opin. Immunol.* **6**, 560–563.

Picker, L. J. 1994 Control of lymphocyte homing. *Curr. Opin. Immunol.* **6**, 394–406.

Riberdy, J. M., Flynn, K. J., Stech, J., Webster, R. G., Altman, J. D. & Doherty, P. C. 1999 Protection against a lethal avian influenza A virus in a mammalian system. *J. Virol.* **73**, 1453–1459.

Sangster, M., Topham, D. J., D'Costa, S. D., Cardin, R. D., Marion, T. N., Myers, L. K. & Doherty, P. C. 2000 Analysis of the virus-specific and non-specific B cell response to a persistent γherpesvirus. *J. Immunol.* **164**, 1820–1828.

Slifka, M. K., Matloubian, M. & Ahmed, R. 1995 Bone marrow is a major site of long-term antibody production after acute viral infection. *J. Virol.* **69**, 1895–1902.

Sprent, J. 1997 Immunological memory. *Curr. Opin. Immunol.* **9**, 371–379.

Sprent, J., Zhang, X., Sun, S. & Tough, D. 1999 T-cell turnover *in vivo* and the role of cytokines. *Immunol. Lett.* **65**, 21–25.

Stevenson, P. G., Belz, G. T., Altman, J. D. & Doherty, P. C. 1998 Virus-specific CD8⁺ T cell numbers are maintained during gamma-herpesvirus reactivation in CD4-deficient mice. *Proc. Natl Acad. Sci. USA* **95**, 15 565–15 570.

Stevenson, P. G., Belz, G. T., Altman, J. D. & Doherty, P. C. 1999*a* Changing patterns of dominance in the CD8⁺ T cell response during acute and persistent murine gamma-herpesvirus infection. *Eur. J. Immunol.* **29**, 1059–1067.

Stevenson, P. G., Belz, G. T., Castrucci, M. R., Altman, J. D. & Doherty, P. C. 1999*b* A gamma-herpesvirus sneaks through a CD8⁺ T cell response primed to a lytic-phase epitope. *Proc. Natl Acad. Sci. USA* **96**, 9281–9286.

Swain, S. L., Hu, H. & Huston, G. 1999 Class II-independent generation of CD4 memory T cells from effectors. *Science* **286**, 1381–1383.

Tanchot, C., Lemonnier, F. A., Pèrarnau, B., Freitas, A. A. & Rocha, B. 1997 Differential requirements for survival and proliferation of CD8 naïve or memory T cells. *Science* **276**, 2057–2062.

Topham, D. J. & Doherty, P. C. 1998*a* Clearance of an influenza A virus by CD4⁺ T cells is inefficient in the absence of B cells. *J. Virol.* **72**, 882–885.

Topham, D. J. & Doherty, P. C. 1998*b* Longitudinal analysis of the acute Sendai virus-specific CD4⁺ T cell response and memory. *J. Immunol.* **161**, 4530–4535.

Topham, D. J., Tripp, R. A., Hamilton-Easton, A. M., Sarawar, S. R. & Doherty, P. C. 1996 Quantitative analysis of the influenza virus-specific CD4⁺ T cell memory in the absence of B cells and Ig. *J. Immunol.* **157**, 2947–2952.

Topham, D. J., Tripp, R. A. & Doherty, P. C. 1997 CD8⁺ T cells clear influenza virus by perforin or Fas-dependent processes. *J. Immunol.* **159**, 5197–5200.

Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. 1986 The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**, 959–968.

Tripp, R. A., Hamilton-Easton, A. M., Cardin, R. D., Nguyen, P., Behm, F. G., Woodland, D. L., Doherty, P. C. & Blackman, M. A. 1997 Pathogenesis of an infectious mononucleosis-like disease induced by a murine γ-herpesvirus: role for a viral superantigen? *J. Exp. Med.* **185**, 1641–1650.

Usherwood, E. J., Hogg, T. L. & Woodland, D. L. 1999 Enumeration of antigen-presenting cells in mice infected with Sendai virus. *J. Immunol.* **162**, 3350–3355.

Virgin, H. W. & Speck, S. H. 1999 Unraveling immunity to γ-herpesviruses: a new model for understanding the role of immunity in chronic virus infection. *Curr. Opin. Immunol.* **11**, 371–379.

Virgin, H. W., Latreille, P., Wamsley, P., Hallsworth, K., Weck, K. E., Dal, C. A. & Speck, S. H. 1997 Complete sequence and genomic analysis of murine gammaherpesvirus 68. *J. Virol.* **71**, 5894–5904.

Wack, A., Corbella, P., Harker, N., Crispe, I. N. & Kioussis, D. 1997 Multiple sites of post-activation CD8⁺ T cell disposal. *Eur. J. Immunol.* **27**, 577–583.

Webster, R. G. 1997 Influenza virus: transmission between species and relevance to emergence of the next human pandemic. *Arch. Virol.* **142**, 105–113.

Wodarz, D., Nowak, M. A. & Bangham, C. R. 1999 The dynamics of HTLV-I and the CTL response. *Immunol. Today* **20**, 220–227.

Yewdell, J. W. & Bennink, J. R. 1999 Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *A. Rev. Immunol.* **17**, 51–88.