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Host factors influencing viral persistence

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With the aim of characterizing the antiviral immune response to a non-cytocidal virus, we studied the outcome of lymphocytic choriomeningitis virus infection in a number of gene knockout mouse strains. Two virus strains differing markedly in their capacity to spread and replicate inside the murine host were used. Our results reveal that very different outcomes may be observed depending on virus strain and immunocompetence of the host. Thus while $CD4^+$ cells are not critical during the initial phase of virus control, infectious virus reappear in mice lacking $CD4^+$ cells, B cells or CD40 ligand. Reappearance of virus is associated with impaired long-term $CD8^+$ T-cell mediated immune surveillance, and the time to virus resurgence is inversely correlated to the replication rate of the virus. Our studies also reveal that interferon-γ is a central cytokine, and depending on the rate of virus replication, mice lacking the ability to produce interferon-γ may develop either a severe, mostly fatal, T-cell mediated wasting syndrome or a chronic infection characterized by long-term coexistence of antiviral cytotoxic T lymphocytes and infectious virus. Mathematical modelling indicates that these different outcomes may be explained in relatively simple mathematical terms. This suggests that modelling may be used as a means to predict critical host and virus parameters. Therefore, combining mathematical modelling with precise, quantitative, *in vivo* analyses looks to be a promising approach in addressing central quantitative issues in immunobiology.

Keywords: antiviral immunity; $CD8^+$ Tcells; T-cell memory

1. INTRODUCTION

The outcome of any infection is decided by a race between the capacity of the infecting agent to invade and replicate inside the host and the ability of the host to respond efficiently, applying a plethora of potential effector systems. This statement is self-evident for infecting agents with the capacity to seriously disturb host organ functions, but even in the case of, for example, non-cytocidal viruses, this may be very important. Thus, if too many host cells in critical organ sites have become infected before an efficient immune attack is established, the host may succumb from its own attempt to clear the infection (see, for example, Marker *et al*. 1976; Thomsen *et al*. 1979; Leist *et al*. 1989; Ehl *et al*. 1998). In this context, it should be remembered that non-cytocidal viruses may replicate to high titres without seriously affecting the host—thus allowing the stage to be set for the above scenario. For the same reason, virus persistence may be established following infection with this category of viruses. In this situation, the virus load becomes the result of a constant struggle between antiviral effector systems and the capacity of the virus to avoid elimination (Nowak & Bangham 1996). Hence, analysing virus levels under such conditions becomes a sensitive and biologically relevant parameter to evaluate the capacity of the host to maintain effective immune surveillance on a long-term basis. However, a side-effect of this ongoing battle is chronic tissue damage. Therefore also under these conditions, immunopathology becomes the critical factor determining for how long the host will survive without a breakdown of vital organ functions. Under certain conditions the host may therefore be better off aborting the immune response than maintaining a response that is insufficient for complete control. At the present stage it suffices to conclude that although probably not the most common of events in nature, studying host interactions with non-cytocidal viruses may provide valuable information about vital host defence mechanisms and the factors involved in regulating their magnitude and stability; information that may not be obtainable through analysis of more virulent infections. Hence analysis of infections with non-cytocidal viruses has served and continues to serve as a useful tool to address central immunological issues such as memory, anergy and immune exhaustion (see, for example, Zinkernagel *et al*. 1993; Kundig *et al*. 1996)

2. THE VIRAL MODEL

Our group has for many years been using the murine lymphocytic choriomeningitis virus (LCMV) infection as a model to study various aspects of the interplay between viruses and immune system (Volkert *et al*. 1975; Bro-Jorgensen 1978; Marker & Thomsen 1987; Thomsen & Pfau 1993; Thomsen *et al*. 1998*a*). This virus is a natural

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murine pathogen that, in the absence of a potent immune response, causes little or no damage to its primary host. This is evident from the fact that transplacentally and neonatally infected mice may survive for the normal lifetime of a mouse as persistent virus carriers, and such early acquired infections probably constitute the most common type of LCMV infection under natural conditions. However, a chronic virus carrier state may also be produced by infection of adult T-cell-deficient mice (Cole *et al*. 1972; Christoffersen *et al*. 1976; Moskophidis *et al*. 1987).

This observation is central for two reasons. First, it demonstrates that the T-cell component of the immune response is pivotal to the outcome of this infection, both as regards immunopathology and virus control. (Note: we have deliberately chosen to use the word control rather than elimination based on the strong evidence suggesting that even in immunocompetent mice this infection may never be completely cleared just reduced to a level below the point of detection (Volkert & Lundstedt 1968; Ciurea *et al*. 1999).) Second, as a consequence, this finding also functions as a starting point for subsequent studies by showing that infection of adult mice with induced defects in their immune response may be used to study critical T-cell effector functions and their regulation. Thus, a similar outcome to that obtained in completely T-cell-deficient mice is to be found in mice deficient of $CD8^+$ T cells (Moskophidis *et al*. 1987; Lehmann-Grube *et al*. 1993; Christensen *et al*. 1994). This fact, together with the much older finding that virus control correlates with cytotoxic T-lymphocyte (CTL) activity *ex vivo* (Marker & Volkert 1973), for many years focused the attention entirely on virus-specific CTLs as the crucial effector subset. Although still correct in its essence—and indeed further confirmed by the inability of perforin-deficient mice to control this infection (Walsh *et al*. 1994; Kagi *et al*. 1994)—recent studies by several groups, including our own, have revealed a much more complex picture of the virus–host interplay.

In this report we have focused on two critical issues relevant to the general understanding of T-cell-mediated antiviral immunity. First, how is antiviral $CD8^+$ T-cell activity maintained, thus preventing the virus from regaining a positive growth rate subsequent to the initial phase of virus control. Second, what is the importance of different molecular effector systems in the normal functioning of virus-specific CTLs *in vivo*.

Essential to our analysis of the above issues has been the use of virus strains that differ in their capacity to replicate and spread in the host. Various strains of LCMV may be classified according to their ability to rapidly attain high titres in the viscera following intravenous or intraperitoneal infection, and previous studies have demonstrated a clear correlation between this ability and the capacity (at high doses of infection) to establish chronic infection in adult mice (Moskophidis *et al*. 1994*a*, 1995; King *et al*. 1990; Pfau *et al*. 1982). The chronic infection is generally associated with at least some degree of immune exhaustion, i.e. anergy or actual depletion of relevant T-cell populations obtained through extended exhaustive stimulation with antigen (Moskophidis *et al*. 1993, 1994, 1995; Ahmed & Oldstone 1988; Thomsen & Marker 1989; Zajac *et al*. 1998).

In our own studies we have been using two LCMV strains representing both ends of this spectrum. The LCMV Armstong strain represents a non-viscerotropic strain with limited capacity to persist even following inoculation with extreme doses of virus (Moskophidis *et al*. 1994*a*, 1995). The Traub strain on the other hand spreads rapidly to a number of organ sites and replicates to high titres (Moskophidis *et al*. 1994*b*; Thomsen & Marker 1989; Thomsen *et al*. 1998*b*). Moreover, chronic infection is easily induced as evidenced by the finding that a 100-fold increase in inoculum may change the course of infection with this strain from an acute type to a chronic pattern (Marker *et al*. 1985; Marker & Thomsen 1987).

3. THE IMPORTANCE OF $CD4^+$ HELP IN MAINTAINING EFFICIENT $CD8^+$ T-CELL SURVEILLANCE IN PERSISTENT VIRAL INFECTION

While $CD8^+$ T cells are central effectors in the control of LCMV infection, initial studies failed to reveal an essential role for $CD4^+$ T helper cells in determining the outcome of this infection (Moskophidis *et al*. 1987; Ahmed *et al*. 1988; Christensen *et al*. 1994). Thus, both acute (treatment with monoclonal antibodies) and chronic (major histocompatibility complex (MHC) class II deficiency) depletion of $CD4^+$ T cells failed to influence the magnitude of the CTL response and the efficiency of virus control *in vivo*. However, the original conclusions were based exclusively on analysis of the acute phase of the infection, i.e. up to around day 10 post-infection (p.i.). Furthermore, experiments were initially conducted with slowly invasive virus strains only.

Subsequent analysis by several groups revealed that in $CD4^+$-deficient mice, infections with viscerotropic strains of LCMV resulted in failure to control the infection and to establish efficient long-term CTL memory, thus revealing a critical role for $CD4^+$ T cells under conditions of chronic T-cell stimulation (Battegay *et al*. 1994; Matloubian *et al*. 1994). While these results were generally obtained using relatively high virus doses for inoculation, our own studies employing a relatively low dose of the viscerotropic Traub strain allowed a more precise definition of the role played by $CD4^+$ T cells in the anti-LCMV response. Thus by studying MHC class II-deficient mice infected with *ca*. 200 plaque-forming units (pfu) of LCMV Traub, a biphasic response pattern was disclosed (Christensen *et al*. 1994; Thomsen *et al*. 1996).

Under these conditions, the primary CTL response was unimpaired—except for a slightly premature decline of activity in some mice—and transient virus control was observed. However, after about two months CTL memory could not be demonstrated, and at about the same time high titres of virus could be detected in the blood as well as in several organs. Hence, it appeared that $CD4^+$ T cells were somehow essential to the long-term maintenance of virus-specific $CD8^+$ T-cell activity and immune surveillance in mice infected with rapidly replicating virus strains, whereas the initial $CD8^+$ T-cell expansion and differentiation did not critically depend on $CD4^+$ T-cell help. This conclusion was further supported by the finding that transient $CD4^+$ T-cell depletion during the acute response had little impact on the final

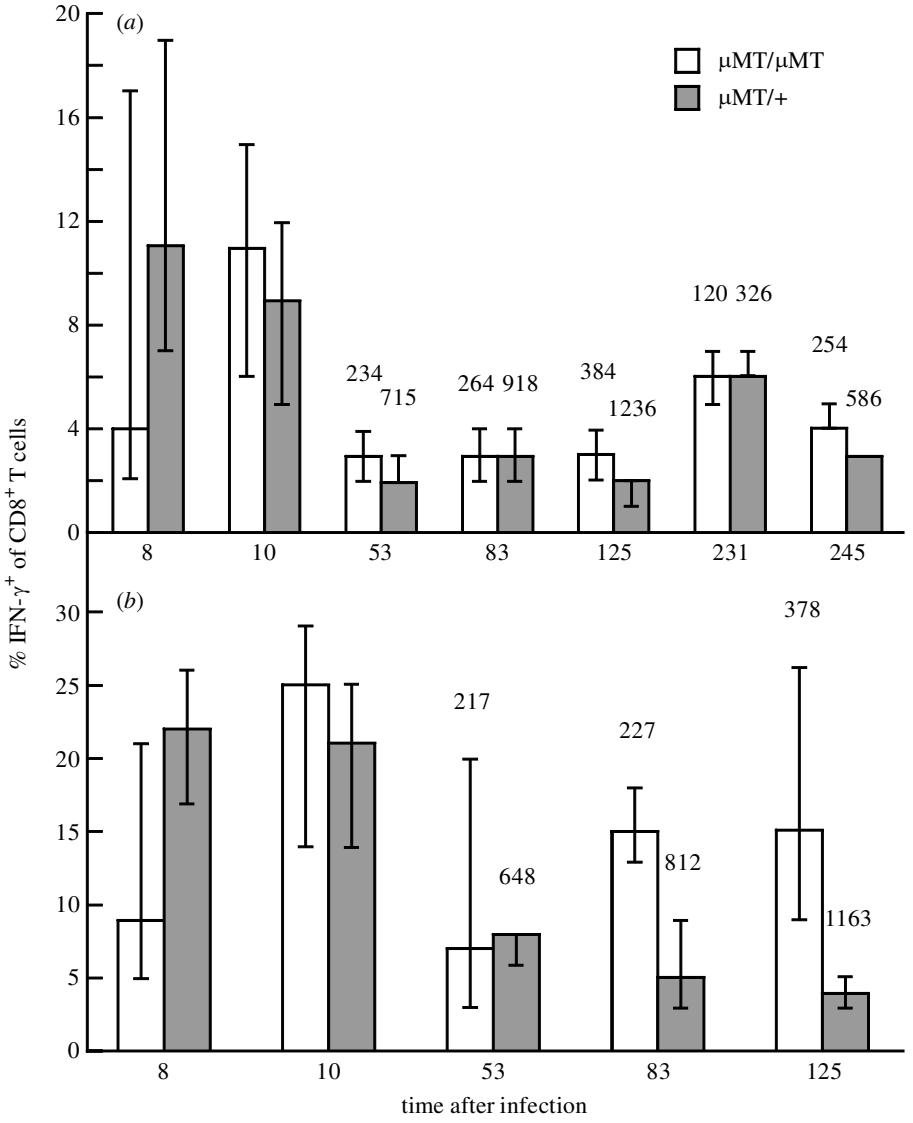

Figure 1. Frequency of LCMV-specific CD8+ T cells in LCMV Armstrong-infected B-cell deficient (μMT/μMT) and wild-type (μMT/+) mice as a function of time. Mice were infected intravenously with 4800 plaque-forming units of LCMV Armstrong, and on the indicated days (*a*) GP33-41- and (*b*) NP396-404-specific CD8+ T cells were visualized through detection of IFN-γ intracellularly (for details see Murali-Krishna *et al.* 1998 and legend to figure 2). Columns show the frequency of peptide specific CD8+ T cells per total CD8+ T cells (median and ranges of two to 13 mice). Figures above columns refer to mean fluorescence intensity of cells with regard to intracellular staining for IFN-γ; mean fluorescence was essentially similar for the two strains during the acute response (see figure 2 for representative FACS plots).

outcome of infection (Thomsen *et al.* 1996), except when very high doses of viscerotropic virus were inoculated (Matloubian *et al.* 1994).

4. THE ANTIVIRAL CD8+ T-CELL RESPONSE IN B-CELL-DEFICIENT MICE

One important function of CD4+ T cells is to help in B-cell activation, and since neutralizing antibodies might play a role in reducing the viral load that might otherwise exhaust the CD8+ T-cell population through chronic stimulation (Thomsen & Marker 1988; Battegay *et al.* 1993), we next analysed the response to LCMV infection in B-cell-deficient mice. Using infection with *ca.* 200 pfu of LCMV Traub, we observed a pattern not much dissimilar to that observed in MHC class II-deficient mice: the initial CTL response was only slightly impaired and transient virus control was observed in most of the infected B-cell-deficient mice (Thomsen *et al.* 1996). However, also in these mice we eventually noted a breakdown of virus control and failure to detect CTL memory. In contrast, B-cell-deficient mice infected with an even higher dose of the slowly replicating LCMV Armstrong strain were able to almost eliminate the virus (Asano & Ahmed 1996; Thomsen *et al.* 1996), and only low levels could be detected primarily in the lungs after four to five months (Thomsen *et al.* 1998*b*). Also with regard to maintaining CTL surveillance, the latter mice differed from Traub-infected mice. Thus, CTL memory was reduced only about fourfold, as demonstrated under bulk conditions (A. R. Thomsen, unpublished observation) and analysis of cytotoxic T-lymphocyte precursors (CTLp) frequencies yielded essentially similar results (Asano & Ahmed 1996).

Based on these findings, we concluded that the main function of CD4+ T cells was to help B cells, and that the

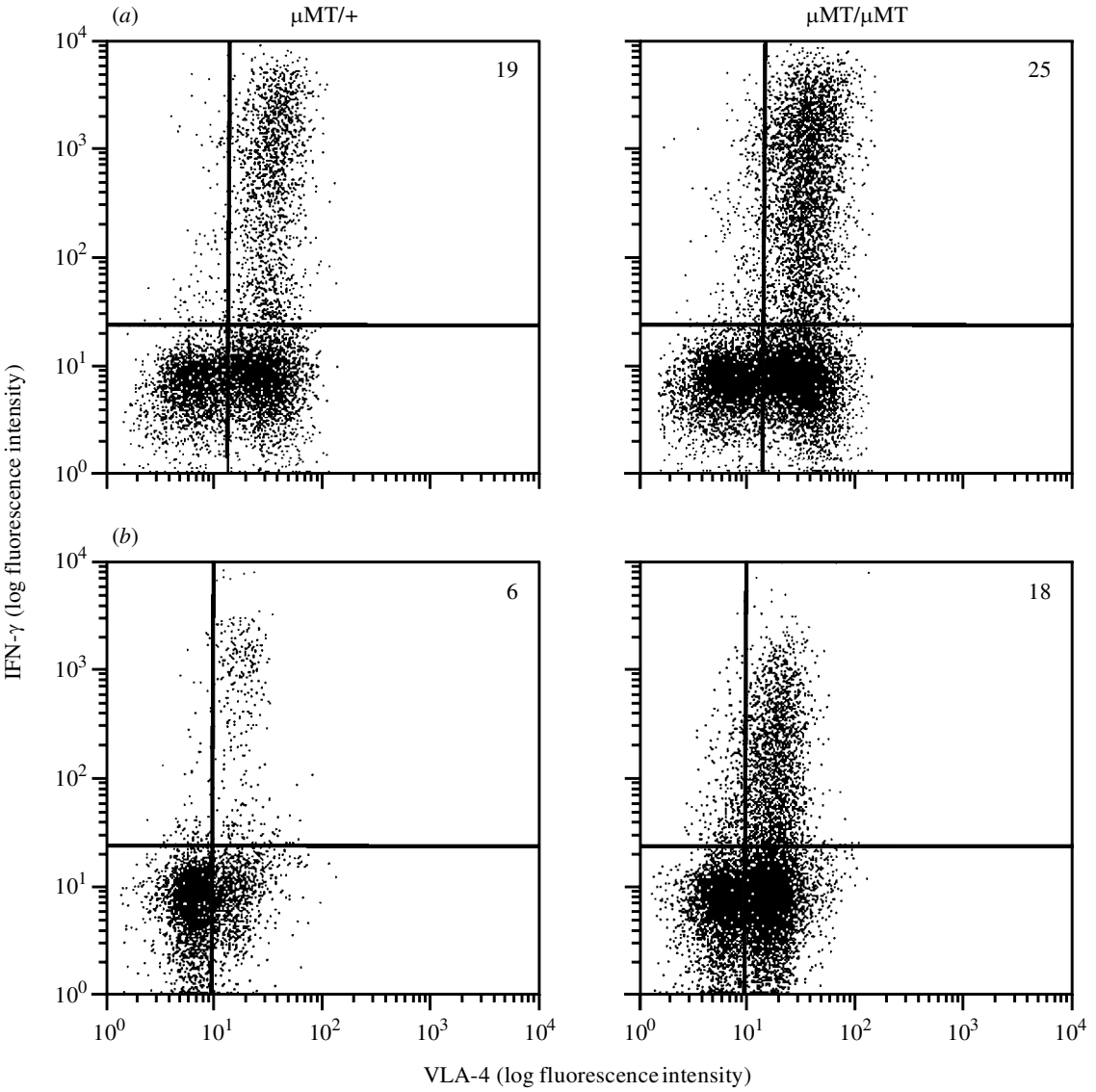

Figure 2. Impaired functional capacity of LCMV-specific $CD8^+$ memory T cells in B-cell-deficient mice. B-cell-deficient (μMT/μMT) and wild-type (μMT/+) mice were infected with 4800 plaque-forming units intravenously, and on day (*a*) 10 and (*b*) 125 post-infection splenocytes were harvested and stimulated with GP33-41 and NP396-404 for 5 h in the presence of monensin. Following *in vitro* incubation, cells were surface labelled with anti-CD8 and anti-VLA-4 (activation marker), permeabilized and stained with anti-IFN-γ. Gates have been set for $CD8^+$ T cells, representative results of three to five mice are depicted. Only results for NP396-404-specific cells are shown, but similar results have been obtained for GP33-41-specific cells.

lack of antibodies leads to an increased virus load, eventually resulting in exhaustion of the $CD8^+$ subset (Thomsen *et al.* 1996). However, more recent studies have pointed towards a possible role of B cells as critical modifiers of $CD4^+$ and $CD8^+$ activity perhaps in the role as antigen-presenting cells. Thus, Homann et al. (1998) have presented evidence indicating that the $CD4^+$ and $CD8^+$ T cells primed and present in LCMV-infected B-cell-deficient mice are qualitatively inferior to those primed and maintained in the presence of B cells.

To study this in greater detail, we analysed the kinetics of the virus-specific $CD8^+$ T-cell response in B-cell-deficient mice infected with 4800 pfu of LCMV Armstrong, visualizing antigen-specific cells through detection of intracellular interferon-γ (IFN-γ). By this approach we found that while the initial response tends to be slightly delayed, at least the same frequency of LCMV-specific $CD8^+$ T cells are generated in B-cell-deficient mice (figure 1), although the absolute number is lower due to the reduced size of secondary lymphoid organs in B-cell-deficient mice. However, as time passes, the LCMV-specific $CD8^+$ T cells in B-cell-deficient mice become qualitatively inferior to those in infected wild-type mice, as evidenced by lower mean fluorescence following staining for IFN-γ (figures 1 and 2). In other studies we have found that this is a relevant parameter for the capacity of the T cells to secrete IFN-γ.

In continuation of this finding, we analysed $CD8^+$ T-cell turnover and phenotype in both groups of mice, and found that more memory ($CD44^{high}$) $CD8^+$ T cells in B-cell-deficient mice were actively cycling for months after infection, as revealed by increased BrdU incorporation over a seven-day pulse period. Furthermore, a significantly higher fraction of the $CD8^+$ T cells in B-cell-deficient mice maintained an L-selectin^{low} phenotype, and this subset comprised the majority of cycling $CD8^+$ T cells (figure 3);

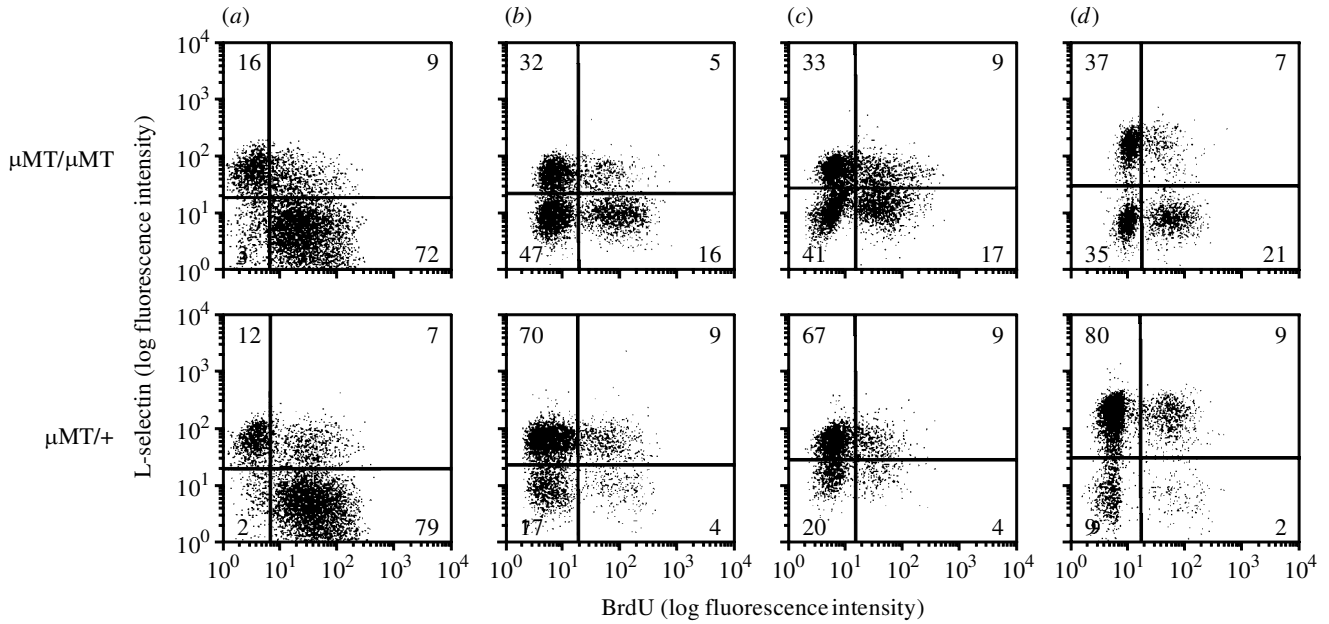

Figure 3. Permanently increased CD8$^+$ T cell turnover in LCMV Armstong-infected B-cell-deficient mice. B-cell-deficient and wild-type mice were infected with 4800 plaque-forming units of LCMV Armstrong intravenously, and for a period of either three (acute) or seven (memory) days prior to analysis, mice were given BrdU in their drinking water to label *in vivo* dividing cells. On the indicated days, splenocytes were harvested and cells were surface labelled with anti-CD8 and anti-L-selectin, permeabilized and stained with anti-BrdU. Gates have been set for CD8$^+$ T cells, and representative results of three to five mice are depicted; (*a*) day 8, (*b*) day 80, (*c*) day 125 and (*d*) approximately day 240 post-infection.

in wild-type mice a similar pattern is observed only during the acute response. Consequently this finding points to ongoing CD8$^+$ T-cell activation in B-cell-deficient mice.

Overall, these findings confirm and extend the conclusion that the CD8$^+$ subset behaves very differently in B-cell-deficient mice compared with similarly infected wild-types. Notably, the lower per cell capacity to produce IFN-γ suggests that the LCMV-specific CD8$^+$ T cells present in the former mice are partially anergized (Zajac *et al.* 1998). This, together with the evidence for chronic T-cell activation observed in B-cell-deficient mice, suggests that in the absence of B cells, the CD8$^+$ cell subset in LCMV-infected mice is subject to chronic stimulation leading to partial anergy. Since the virus load in LCMV Armstrong-infected B-cell-deficient mice remains limited for most of the observation period (Thomsen *et al.* 1998*b*), an indirect effect reflecting the lack of an antibody-mediated reduction in the viral load constitutes an unlikely mechanism. The presence of a normal B-cell population must therefore in itself provide an environment essential to the normal functioning of CD8$^+$ T cells in virus-infected mice. Therefore, conclusions based on results obtained in B-cell-deficient mice need to be interpreted with great care. Indeed, similar indirect mechanisms may explain recent findings in several autoimmune models implicating B cells in the pathogenesis, despite lack of any other evidence pointing to antibody-mediated effector mechanisms (Serreze *et al.* 1996; Wolf *et al.* 1996; Chan *et al.* 1999).

5. ROLE OF CO-STIMULATION IN THE MAINTENANCE OF CD8$^+$ T-CELL MEDIATED IMMUNITY

Another approach to study the mechanisms underlying the maintenance of CD8$^+$ effector capacity in persistently infected mice is to search for critical molecular interactions. In this context, a number of studies have pointed to CD40–CD40 ligand as a set of molecules central in the control of T-cell activation (for a review see Grewal & Flavell 1998; Laman *et al.* 1996).

Under normal conditions, naive T cells require two signals for activation (Davidson 1977; Lafferty & Cunningham 1975). The first is delivered through T-cell receptor binding to relevant peptide–MHC complexes on the surface of antigen-presenting cells (APCs). The second signal is provided through interaction with various molecules presented on the surface of professional APCs. A pre-condition for efficient delivery of the second signal, commonly known as co-stimulation, is previous activation of the APC (Liu & Janeway Jr 1991; Banchereau & Steinman 1998).

Based mostly on studies of autoreactive T cells, interaction between CD40 ligand expressed by the T cell and CD40 expressed by the APC has been found to be critical to this process (Grewal *et al.* 1996; Grewal & Flavell 1996, 1998). Initial studies in virus-infected animals quickly revealed that viral infection might by-pass the need for CD40–CD40 ligand in the activation of the CD8$^+$ T-cell response (Borrow *et al.* 1996; Whitmire *et al.* 1996). Complementing these findings it has been shown *in vitro* that viral infection of APCs may have the same effect on these cells as ligation of CD40 (Wu & Liu 1994). However, as it was the case in CD4$^+$ T-cell-deficient mice, subsequent extended analysis revealed a more complex picture. Thus, while CD40 ligand expression was dispensable during the acute response to slowly replicating LCMV Armstrong (Whitmire *et al.* 1996, 1999; Thomsen *et al.* 1998*b*; Borrow *et al.* 1996), the primary response collapsed rapidly in LCMV Traub-infected CD40 ligand-deficient mice (Thomsen *et al.* 1998*b*). In the latter mice,

initial CD8$^+$ T-cell proliferation and differentiation was normal, but already around day 10 p.i., the fraction of activated CD8$^+$ T cells was rapidly shrinking, and after two months of infection no LCMV-specific memory cells could be detected. Subsequent studies involving other viscerotropic strains have confirmed this picture (A. R. Thomsen, unpublished observation; Whitmire *et al.* 1999).

With regard to virus control, a matching pattern was observed: some mice were able to transiently control the infection, but after two months all were viraemic and harboured high titres of virus in their organs. In LCMV Armstrong-infected mice, on the other hand, the infection was initially controlled very efficiently, i.e. below the level of detection, but after about four to five months virus reappeared in the blood and substantial amounts were generally detected in the organs (Thomsen *et al.* 1998*b*). Extended analysis of the LCMV-specific CD8$^+$ T cells in LCMV Armstrong-infected CD40 ligand-deficient mice (Andreasen *et al.* 2000) revealed that although initially capable of responding normally, the per-cell capacity to produce IFN-γ was significantly reduced with time. Thus at two and four months p.i., mean fluorescence was markedly reduced, and peptide-stimulated release of IFN-γ was reduced by a factor of ten in CD40 ligand-deficient mice compared to matched wild-type mice. Thus, even in LCMV Armstrong-infected mice in which the virus load is virtually undetectable for a period of at least two months, virus-specific CD8$^+$ T cells appear to be driven towards a state of anergy in the absence of CD40 ligand expression. The result of this is reappearance of detectable levels of virus.

In conclusion, these findings reveal that although CD40–CD40 ligand interaction is dispensable during the acute response to LCMV, the maintenance of an efficient CD8$^+$ T-cell population somehow requires this molecular interaction even under conditions where the virus load is limited. However, following infection with rapidly replicating variants, the consequences of this suboptimal stimulation are aggravated, and the result is a total collapse of the CD8$^+$ T-cell response.

At the present time it is not completely clear whether CD40 ligand needs to be expressed by the CD8$^+$ T cells themselves or by the CD4$^+$ helper cells. However, a recent model for CD4 help states that CD4$^+$ T cells provide help primarily through 'conditioning' of the APCs, and that this requires expression of CD40 ligand (Bennett *et al.* 1998; Ridge *et al.* 1998; Schoenberger *et al.* 1998). A likely scenario is therefore, that while virally induced activation of the APCs suffices during the early phase of infection (Andreasen *et al.* 2000)—driving the initial CD8$^+$ T-cell expansion and differentiation—this signal wanes with time, and CD40 ligand-mediated activation therefore becomes a limiting factor in maintaining optimally stimulating APCs required for the maintenance of effector activity in the CD8$^+$ population. This interpretation is also consistent with the findings previously made in CD4$^+$ T-cell-deficient mice (Thomsen *et al.* 1996), and thus constitutes a simple consensus model for maintenance of CD8$^+$ T-cell function in persistently infected animals.

Given that a requirement for ongoing interaction with APCs is intrinsic to the above model, how can this be reconciled with recent findings indicating that memory CD8$^+$ T cells are maintained and may even proliferate in the absence of antigenic stimulation and cellular interactions (Di Rosa & Matzinger 1996; Hou *et al.* 1994; Lau *et al.* 1994; Murali-Krishna *et al.* 1999)? We believe that the answer to this question lies in the experimental conditions under which the latter findings have been obtained. Thus, we find it pertinent that the evidence supporting presentation-independent maintenance of memory CD8$^+$ T cells mostly derives from experiments involving adoptive transfer of primed CD8$^+$ T cells into a MHC class I-free environment. This constitutes a highly contrived situation in which the memory CD8$^+$ T cells exist in an immunological vacuum removed from interactions with the surrounding environment as well as from competition from expanding clones of other specificities. If indeed these results were applied without modification to the situation in a normal host, the consequence would be that once generated, a memory subset would persist forever. Given that there is a natural upper limit to the total number of T cells (and probably to the number of memory cells *per se*; Tanchot & Rocha 1998), this would—in the perspective of a lifetime—leave very little room for dynamic adaptation of the T-cell repertoire in the face of a changing environment. Consequently, although the available evidence indicates that, under certain conditions, a memory CD8$^+$ T-cell population may be maintained *in vivo* in the absence of T-cell receptor stimulation, we would tend to believe that this is not generally the situation. And particularly in the case of a persistent infection that requires long-standing T-cell surveillance, different rules are likely to apply. In this context, it might be relevant to bear in mind that memory probably constitutes an evolutionary design for the control of persistent infections rather than a mechanism for protection against exogenous rechallenge (Wodarz *et al.* 2000). Moreover, some of the findings presented below provide further evidence for a dynamic interaction continuing into the 'memory' phase of this persistent infection.

6. EFFECTOR MOLECULES INVOLVED IN THE CONTROL OF INFECTION WITH A NON-CYTOCIDAL VIRUS

Although CD8$^+$ T cells are often called by their eponym CTLs, and are assumed by many to serve predominantly as effectors in cell contact-dependent killing, they are also potent producers of several cytokines and may, like CD4$^+$ cells, be divided into at least two major functional subsets based on their cytokine profiles (Carter & Dutton 1996). Tc1 cells produce essentially the same cytokines as Th1 cells, in particular IFN-γ, the detection of which may be used as a means to quantitate CD8$^+$ responses (see § 4). Tc2 cells have the same cytokine profile as Th2 cells and may be detected by demonstration of IL-5 intracellularly. In general, viral infections are associated with Tc1 responses, whereas Tc2 cells are only found rarely (Seder & Le Gros 1995; Christensen *et al.* 1996).

In itself, the generality of this pattern could be taken to suggest that the production of type 1 cytokines would serve some functional purpose during the resolution of most viral infections. However, in the case of non-cytocidal viruses it has been suggested that production of

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IFN-γ is virtually redundant, whereas cell-dependent killing, primarily via the perforin pathway, is essential to virus elimination (Kagi & Hengartner 1996). However, this suggestion is based mostly on the study of LCMV Armstrong-infected mice analysed during the acute phase of infection, and as the following will reveal, this represents a far from complete picture as regards the involvement of IFN-γ in control of the LCMV infection.

The first indication that IFN-γ might play a role in controlling this infection came from findings in virus carrier mice adoptively immunized with primed donor cells. In this situation, capacity of the donor cells to produce IFN-γ was found to be of critical importance (Tishon *et al.* 1995; Planz *et al.* 1997). This finding led us to pursue the role of IFN-γ in the course of a normal LCMV infection in adult mice. Based on the assumption that cytokines were likely to be most important during infection with a rapidly replicating virus strain, IFN-γ-deficient mice and wild-type controls were infected with 200 pfu of LCMV Traub. Using these conditions (Nansen *et al.* 1999) we found that organ virus titres in IFN-γ-deficient mice on day 10 p.i. were as high as in mice having no T cells at all. With time, virus levels became somewhat reduced, but the majority of infected IFN-γ-deficient mice succumbed in the process (*ca.* 85% mortality). This outcome of infection was due to $CD8^+$ T-cell-dependent immunopathology, since depletion of this subset protected the mice. With the use of newly generated perforin–IFN-γ double knockout mice, we could further demonstrate that perforin-mediated cell killing was a central, though not the only, effector mechanism. Although most double knockout mice survived (*ca.* 90%), transient $CD8^+$ T-cell-mediated wasting was noted. Very limited disease was observed in similarly infected wild-type mice. Thus, the inability to produce IFN-γ resulted in augmented virus levels in the viscera, and under these conditions CTL killing of infected cells led to progressive tissue destruction and death.

In contrast, IFN-γ played a relatively minor role in LCMV Armstrong-infected mice, and all survived the acute phase. Thus, as opposed to perforin, which was essential for virus control irrespective of replication rate, the importance of IFN-γ varied with this viral parameter (Nansen *et al.* 1999). However, a small influence was noted, and extended analysis of virus levels in LCMV Armstrong-infected IFN-γ-deficient mice revealed that the infection was never completely controlled and significant levels of virus could be demonstrated in spleen and lungs for months after infection (Bartholdy *et al.* 2000). Notably, no impairment of either effector or memory T-cell generation was observed in these mice as evidenced by supranormal *ex vivo* CTL activity and increased numbers of CTL precursors as determined by limiting dilution. In fact, *ex vivo* CTL activity remained substantial for many months in chronically infected IFN-γ-deficient mice, whereas this response gradually disappeared in wild-type mice. Also in this situation, analysis of cell-cycle and phenotypic markers provided additional support for chronic ongoing $CD8^+$ T-cell activation in persistently infected mice. Thus, it appears that a new equilibrium is established in LCMV Armstrong-infected, IFN-γ-deficient mice with long-term coexistence of cytolytically active $CD8^+$ effector cells and target cells in several organs supporting active replication of the virus.

In conclusion, our results demonstrate that IFN-γ is by no means redundant to the control of LCMV infection. Indeed, it is evident that failure to produce this cytokine markedly affects the outcome of infection with this non-cytocidal virus. Thus lack of IFN-γ may transform an essentially asymptomatic infection with a rapidly replicating LCMV strain into a fatal disease, and infection with a slowly replicating strain results in chronic low-grade infection; in the latter case, the lack of cytokine production is partially compensated by permanently increased CTL activity. An important feature of the IFN-γ–LCMV Armstrong model is that although chronic T-cell stimulation is observed, we find no evidence pointing towards a collapse of immune surveillance: viral titres remain stable for months. This observation therefore highlights the fact that while chronic stimulation is clearly a precondition for T-cell exhaustion, the conditions surrounding this stimulation are essential in determining the final outcome. Thus, interestingly, preliminary results suggest that $CD4^+$ T-cell depletion in this situation tips the scale in favour of the virus, resulting in increased virus levels and immunopathology.

In a broader perspective, the finding of a chronically elevated level of $CD8^+$ T-cell surveillance in IFN-γ-deficient mice infected with LCMV Armstrong supports our assumption that persisting antigen may play an important role in maintaining effector T-cell activity into what is normally perceived as the memory phase. Although it may be argued that this represents a contrived situation, additional observations strongly suggest that the conclusion is valid also in the normal host. Thus, not unlike the pattern in LCMV Armstrong-infected, IFN-γ-deficient mice we find a somewhat protracted CTL response in wild-type mice infected with the more persistent LCMV Traub strain (figure 4). Moreover, preliminary data on virus-specific $CD8^+$ T cells enumerated through detection of intracellular IFN-γ also reveal a more protracted response in LCMV Traub relative to LCMV Armstrong-infected wild-type mice (A. R. Thomsen, unpublished observation).

7. THE USE OF MATHEMATICAL MODELLING AS A TOOL TO UNDERSTAND T-CELL DYNAMICS IN VIRAL INFECTIONS

It is a classical observation that the capacity of an LCMV strain to induce T-cell exhaustion when inoculated into normal adult mice at high doses critically depends on the ability of the virus strain to rapidly replicate in the host. Although still primarily an empirical observation, some new insight into this phenomenon and the underlying mechanisms has recently been gained by application of simple mathematical simulation (Wodarz *et al.* 1998). In the context of the studies mentioned above, we consistently noted that the choice of viral strain was critical to the results obtained, and that generally any effect observed would tend to become more exaggerated if mice were infected with a rapidly invasive strain. Although this may intuitively be felt as an obvious finding, it is nevertheless noteworthy that the rate of viral replication may still continue to play a decisive role even

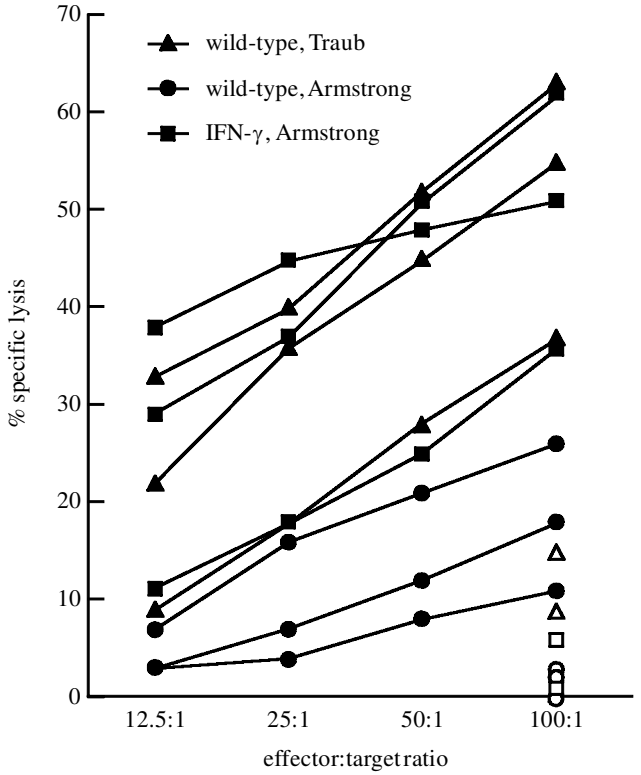

Figure 4. Prolonged CTL activity in LCMV Armstong-infected IFN-γ-deficient mice. IFN-γ-deficient and wild-type were infected with 4800 plaque-forming units of LCMV Armstrong, for comparison an additional group of wild-type mice were infected with 200 plaque-forming units of LCMV Traub. Four weeks after infection, splenocytes were harvested and tested in a ^{51}Cr-release assay against GP33-41 pulsed RMA-S tumour cells (closed symbols); unpulsed RMA-S cells served as control targets (open symbols). Note that while peptide-specific cytotoxicity in LCMV Armstrong-infected wild-type mice has declined to a low level, substantial cytotoxic activity is still observed in similarly infected IFN-γ-deficient mice as well as in wild-type mice infected with a lower dose of the more persistent LCMV Traub.

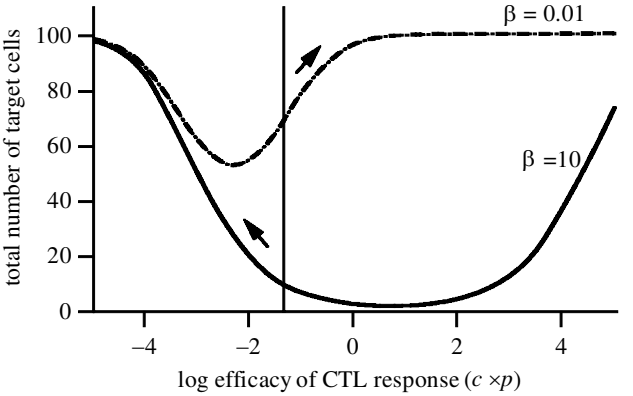

Figure 5. Basic properties of CTL-induced pathology, as predicted by a simple mathematical model describing the basic dynamics between a virus population, its target cells and a lytic CTL response (Nowak & Bangham 1996). We plot the total number of target cells (uninfected + infected) at equilibrium, in dependence of the efficacy of cell-mediated immunity and the replication rate of the virus. We assume that the virus is non-cytocidal. We define immunopathology by a reduction of the total number of target cells in the presence of CTL, compared to the absence of CTL. CTL-induced pathology is most likely to occur at a low or intermediate efficacy of the CTL response. In addition, the replication rate of the virus plays an important role. The faster the replication kinetics of the virus, the more severe the degree of pathology observed. If the virus replicates at a fast rate, a significant reduction in the total number of target cells will be observed even in the presence of a relatively strong CTL response. If the virus replicates slowly, any degree of immunopathology is observed only in the presence of inefficient CTL. Thus, for slowly replicating viruses, an increase in the CTL responsiveness is likely to benefit the host, while for faster replicating strains, the opposite applies (see vertical dashed line and arrows). β = replication rate.

at a relatively late stage in a more or less chronic infection. A good example of this may be found in the observation that intravenous infection with a very low dose of the viscerotropic Traub strain (2 pfu) results in a more rapid and marked breakdown of virus control in CD40 ligand-deficient mice than does infection of the same mice with a 1000-fold higher dose of slowly replicating LCMV Armstrong (Thomsen *et al.* 1998*b*). To understand this better we modified the original mathematical model describing the basic interactions between virus replication and CTL response (Nowak & Bangham 1996) so as to accommodate the above interpretation regarding the role of CD4$^+$ T cells–APCs in regulating the dynamics of the CTL response (D. Wodarz and A. R. Thomsen, unpublished observation). Without going into the details of our modifications—this analysis still requires some additional evaluation—it may be stated that the predictions so far fit the experimental observations well. For example, simply by putting an upper limit to the number of cell divisions CTLs can undergo in the absence of CD4$^+$ help, a biphasic infection course is predicted similar to that actually observed in MHC class II-deficient mice (Thomsen *et al.* 1996). Under these conditions, it is also predicted that the time-period until virus resurgence is inversely correlated to the replication rate of the virus. Thus mathematical simulation helps in identifying replication rate as a central viral parameter that significantly affects the outcome of a viral infection even under conditions where primary T-cell control may be obtained.

Similar to the analysis of T-cell dynamics under various conditions, the modelling approach may also be used to predict parameters critically affecting the degree of immunopathology induced by the generated effector T cells. This is very important since with non-cytocidal viruses this is the factor that determines the life or death of the infected host. An apparent paradox in this context is the observation that a partial immune defect may sometimes be associated with augmented immunopathology (see Marker *et al.* 1976; Leist *et al.* 1989). The reason for this becomes obvious when considering that a weakening of the T-cell response not only reduces the capacity to induce tissue damage, but also impairs antiviral effector capacity. As a consequence of the latter, the infection is less efficaciously contained, allowing more host cells to become infected and thus potential targets for the immune attack.

When this is analysed in mathematical terms (figure 5), it becomes evident that immunopathology is to be

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expected at intermediate levels of T-cell responsiveness and that the rate of viral replication plays an important role in determining the extent as well as the precise conditions under which immunopathology may be observed. This fits reasonably well with our observations in wild-type mice versus IFN-γ-deficient mice versus mice deficient in both IFN-γ and perforin (Nansen et al. 1999; A. R. Thomsen, unpublished observation). Thus, if LCMV Armstrong is used for infection, no immunopathology is associated with infection of wild-type mice. Eliminating IFN-γ has little effect, whereas lack of both IFN-γ and perforin is associated with high ($CD8^+$ T-cell-mediated) mortality (*ca.* 90%). In contrast, following infection with LCMV Traub, transient immunopathology is induced even in wild-type mice and elimination of IFN-γ results in high mortality (*ca.* 85%). However, additional elimination of perforin markedly reduces immunopathology (< 15%). Thus with the exception of very low mortality in Traub-infected double knockout mice, the experimental observations fit the predictions well. The reason for the latter discrepancy is probably that with a rapidly replicating virus, very reduced virus control leads to overwhelming infection, which not only increases the number of target cells (i.e. risk of immunopathology), but also rapidly exhausts the host response potential (i.e. further reduces T-cell effector capacity). We are presently testing whether this explanation holds true *in vivo*.

To further evaluate the use of mathematical modelling as a tool to predict the outcome of viral infection, we included into the basic model the assumptions that (i) generation of CTLs is independent of the presence of IFN-γ, and (ii) cytokine production contributes to inhibition of viral replication. Under these assumptions (Bartholdy *et al.* 2000), the model predicts that the outcome of infection in IFN-γ-deficient mice is critically dependent on the replication rate of the virus and that persistent infection in the absence of substantial immunopathology is only possible for slowly replicating strains. Moreover, in the latter situation the balance between virus load and CTL activity will settle at a new equilibrium characterized by a compensatory increase in CTL activity, which is precisely what we observe in IFN-γ-deficient mice infected with LCMV Armstrong.

Overall, the apparent success of mathematical modelling in predicting experimental outcomes suggests that simulation may be used as a tool to reveal critical parameters of both host and virus, and therefore may help in the designing of better-focused and more informative experiments.

8. CONCLUDING REMARKS

For about a decade the study of immunobiology has been dominated by a reductionistic view, analysing immune responses primarily at the single-cell level. This has clearly provided very important qualitative information about cell subtypes and molecular mechanisms. However, our understanding of important quantitative issues is still incomplete, and since many features of a normal immune response are of a quantitative nature, insight into the cell dynamics involved is a prerequisite for the understanding of the normal physiology of the immune system. Until recently it has been difficult to analyse immune responses in precise quantitative terms as there was no methodology to reveal real numbers of antigen-specific T cells. However, with the advent of recent technology making possible direct visualization of antigen-specific cells either through staining with MHC–tetramers (Altman *et al.* 1996; Murali-Krishna *et al.* 1998) or detection of cytokine production at the single-cell level (Butz & Bevan 1998; Murali-Krishna *et al.* 1998), things are changing fast. Through the use of this methodology, precise analysis of normal polyclonal T-cell responses in the context of several infections has recently been published (e.g. Murali-Krishna *et al.* 1998; Belz *et al.* 1998; Busch *et al.* 1998; Flynn *et al.* 1998; Hoshino *et al.* 1999; Stevenson *et al.* 1998). Although these studies in themselves provide crucial information, no doubt much more information on cell dynamics can be extracted at this stage through a collaborative effort of experimental immunologists and theoretical biologists. Thus combining precise quantitative analysis with mathematical modelling should lead to a rapid advancement in our perception of important issues such as regulation of lymphocyte homeostasis, memory and immune exhaustion.

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