Sizing up the key determinants of the CD8$^+$ T cell response

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Abstract | Naive CD8$^+$ T cells give rise to cytotoxic T lymphocytes (CTLs), which promote the effective eradication of viruses and tumours. Although the past decades have seen enormous advances in cellular immunology, a precise understanding of the key elements that determine the specificity and magnitude of primary CTL responses has been lacking. However, recent technological advances have allowed us to more accurately identify, characterize and quantitate key determinants that define the specificity and magnitude of CD8$^+$ T cell-mediated immunity. This Review discusses the technical and conceptual advances that have markedly changed our understanding of the determinants of primary CTL responses.

Peptide–MHC class I (pMHCI). A complex of peptide (often derived from virus in case of infection) and MHC class I molecule, which is expressed on the surface of cells and recognized by specific CD8$^+$ T cells through their T cell receptor.

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doi:10.1038/nri3905
Published online 9 October 2015

Effector CD8$^+$ cytotoxic T lymphocytes (CTLs) have long been considered to have a role in the eradication of viruses and tumours. However, the view that an optimally mobilized CTL response can be protective against virus challenge and play a key part in eliminating solid tumours is only now gaining substantial acceptance. For a range of cancers — including melanoma, and renal and lung cancers — immunotherapies that promote antitumour CTL responses by blocking inhibitory molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD1), are showing significant evidence of clinical success[1]. In the control of viruses and bacteria, sterilizing immunity conferred by pre-existing neutralizing antibodies has long been viewed as the ideal host response and is the primary goal of vaccine design. However, for a number of important human pathogens — such as HIV, malaria, *Mycobacterium tuberculosis* and many of the herpesviruses — vaccines that induce antibody responses are poorly effective due to constant antigenic changes or antibody inaccessibility. Meanwhile, evidence continues to emerge demonstrating the importance of CTL-mediated immunity in protection from diseases caused by these pathogens (BOX 1), supporting the view that T cell-based vaccines (or vaccines with a T cell component) will be important for effective protection[2,3].

The establishment of CTL immunity to any given virus is characterized by a limited range of polyclonal CD8$^+$ T cell responses targeting different virus-derived peptides presented by various MHC class I (MHCI) molecules. These peptide–MHC class I (pMHCI)-specific CTL subsets are typically arranged in reproducible immunodominance hierarchies, wherein a response may be defined as either immunodominant (large) or subdominant (small). Although immunodominant CTL responses are typically considered to be the most effective at driving antiviral immunity, naturally subdominant CTL responses have also been shown to provide protection from infection in various contexts (BOX 2). Importantly, however, protection mediated by typically subdominant populations is generally a consequence of these responses being heightened, either naturally following infection or deliberately through vaccination (BOX 2). Thus, although CTL functionality is essential for an effective CD8$^+$ T cell response, the magnitude of any given CTL response remains an independently critical aspect of CD8$^+$ T cell immunity and will be the focus of this Review.

The need for a complete understanding of the factors dictating the characteristics of the pMHCI targets and magnitudes of epitope-specific CTL responses is driven by findings suggesting that the identity[4,5] and breadth of epitopes targeted by CTLs[6,7], along with their relative magnitudes, are key determinants of viral control. An improved definition of the primary CTL response is essential if we are to develop a better understanding of both the acute effector and persistent memory phases of such immunity.

What elements influence the primary virus-specific CTL response? Obvious factors are: first, the number of cells in the pre-immune (naive) T cell repertoire; second, the affinity of T cell receptor (TCR) binding to pMHCI complexes; third, the phenotypic composition of the naive CD8$^+$ T cell repertoire; fourth, the possible

Box 1 | **The role of CTL immunity in protection from virus infection**

The importance of CD8+ T cell responses in providing protection from virus infection has been inferred both by the robustness with which CD8+ T cells are induced by virus infection and by their demonstrated role in animal models of disease. Solid evidence of the protective role of cytotoxic T lymphocytes (CTLs) against a number of important human viruses has only relatively recently begun to accumulate. In influenza A virus (IAV) infection, for example, a retrospective study on individuals during the 1957 IAV pandemic suggested that an accumulation of heterologous T cell immunity was responsible for improved protection from infection in adults compared with children[90]. Also in 2006, McElhaney and colleagues[91] demonstrated that the likelihood of elderly individuals developing symptoms of IAV infection following vaccination was inversely correlated with the functionality of their IAV-specific CD8+ T cells and unrelated to antibody titres. These findings confirmed those of McMichael and colleagues[92] in 1983, which were derived from experimentally infected individuals. In 2013, the novelty of the 2009 H1N1 IAV pandemic was exploited to show that, in the absence of cross-reactive neutralizing antibodies, the frequency of CD8+ T cells specific for conserved viral epitopes was significantly inversely correlated with disease severity[93], representing the most definitive evidence to date that CD8+ T cells have a key role in protection from IAV infection.

Evidence that the potent CD8+ T cell responses elicited by viruses such as cytomegalovirus, Epstein–Barr virus and HIV are also critical for control of viral replication continues to emerge. Selective diminution of T cell responses, as observed in immunosuppressed transplant recipients, is strongly associated with herpesvirus reactivation[94,95] and can be prevented or treated by adoptive transfer of virus-specific CD8+ T cells[96]. Immune pressure exerted by HIV-specific CTLs is associated with the emergence of escape mutants during acute and chronic infection[97,98]. Moreover, there is a strong link between disease non-progression and particular MHC class I alleles[99], and depletion of CD8+ T cells in rhesus macaques results in an inability to control simian immunodeficiency virus (SIV) replication[100,101] (reviewed in REF. 2). More recently, a fine dissection of the HIV-specific CTL responses — which involved tracking responses to founder and subsequently mutated T cell epitopes in HIV-infected individuals — demonstrated a previously underappreciated role for CD8+ T cells in the early and rapid control of acute viral titres, as well as in the long-term control of established viral set-points[102]. Collectively, evidence of the importance of CD8+ T cells in the control of many important human viruses is strong and continues to accumulate. What remains unclear are the kinetics and mechanisms by which CD8+ T cells exert their antiviral effects in various conditions (for example, in the presence versus the absence of neutralizing antibodies, or in acute versus chronic infections).

interactions between T cells of different specificities during priming; and finally, the nature and dose of viral antigens. In this Review, we examine each of these influences in turn, highlighting recent technical advances that have improved our understanding of CTL responses and discussing the questions that remain in the field.

Detection of CTL precursors

Assessing the pre-immune CTL precursor repertoire. Substantial indirect evidence points to the importance of naive CTL precursors (CTLPs) for determining the quantitative and functional outcome of CTL responses[8–10]. However, the inability to directly enumerate naive pMHCI-specific CTLPs has impeded our understanding of the true dynamics of antigen-driven responses and the determinants of CTL immunodominance hierarchies. Even when pMHCI tetramers became available (in 1996) for the direct identification and quantification of antigen-specific CD8+ T cells by flow cytometry, the conventional staining approach used was insufficiently sensitive to detect the exceedingly low numbers of MHCI-specific T cells (approximately 1–90 per million T cells) present in naive individuals[11]. Historically, a number of attempts were made to probe the naive CTLP repertoire and determine its impact on CTL response magnitudes. Several strategies have been used to deduce naive CTLP frequency, including *in vitro* expansion of T cell micro-clones by limiting dilution analysis[12,13], adoptive transfer of graded numbers of TCR-transgenic CD8+ T cells before antigen challenge followed by comparison to endogenous response magnitude[12,13], and inference from immune epitope-specific TCR complementarity-determining region 3β (CDR3β) characteristics[14,15]. In retrospect, such indirect strategies were either insufficiently sensitive to detect CTLPs, or they relied on assumptions (such as no loss of transferred cells, equivalent TCR–pMHCI avidities and contributions of all cells to the response, and uniform distribution of TCR diversity throughout the repertoire) that we now know to be overly simplistic. Consequently, these strategies yielded CTLP frequency predictions that tended to overestimate or provide excessively broad numerical ranges (TABLE 1). Importantly, when the same epitope-specific CTLP populations were determined using different indirect strategies[16] (TABLE 1), they lacked reproducibility and spanned two orders of magnitude, indicating that such protocols provided, at best, relative rather than absolute measures of CTLP frequency.

Direct detection of naive CTLPs. Detecting low numbers of antigen-specific CD8+ T cells in naive individuals, even when tetramers became available, was still beset by the linked problems of having to analyse a sample of sufficient size to ensure the identification of exceedingly small populations while avoiding false positives. Simply enriching naive T cells on the basis of CD8 expression before tetramer staining enabled the detection only of epitope-specific CTLPs found in unusually high numbers in healthy individuals (for example, precursors specific for a peptide from melanoma-associated antigen 1 ($MART1_{26–35}$) presented by HLA-A2) but was not sufficiently sensitive to detect other naive T cell populations present at more typical, lower frequencies[16]. Direct detection of naive CTLPs was finally achieved by adopting an approach pioneered by Moon *et al.*[17], in which naive epitope-specific CD4+ T cells were magnetically

CTL precursors
(CTLPs). Naive peptide–MHC class I-specific CD8+ T cells that have not encountered cognate antigen.

Complementarity-determining region 3β
(CDR3β). An amino acid (or nucleotide) sequence that is found within the most variable region of the T cell receptor-β chain and is used as an identifier of distinct T cell clones.

Box 2 | **Subdominant CD8⁺ T cell responses: small but mighty?**

Immunodominant cytotoxic T lymphocyte (CTL) responses have received much attention with respect to their role in protection from infection and their potential for therapeutic vaccine strategies. However, in both human virus infection and in animal models of infection, protection from disease has been associated with epitope specificities that need not be immunodominant[103–106]. Indeed, some recent studies have suggested that immunodominant epitopes may act as decoys to undermine effective viral control[107,108]. The importance of these subdominant responses can manifest in one of three ways: first, protection may be associated with one or more subdominant responses induced naturally following infection[104,106]. For example, consistently low viral loads in HLA-B*1503⁺ individuals infected with HIV clade B (versus clade C) infected have been associated with superior recognition of a number of subdominant epitopes, despite all individuals sharing the immunodominant response[106]. Second, protective capacity can be associated with the dominance of epitope-specific CTL responses that are typically subdominant or undetectable in a control group of more susceptible individuals. This is exemplified in a study showing that HIV-1-exposed, persistently seronegative individuals exhibit epitope reactivities that are significantly different from those in HIV-1-infected individuals and furthermore that a late conversion from HIV-1 resistance to HIV-1 infection was associated with a loss or subjugation of those protective responses[105]. Finally, some typically subdominant CTL responses are able to clear the virus if their response is boosted exogenously, either via vaccination or release from cytokine-mediated suppression[109,110]. Collectively, these studies indicate that the natural size of a viral epitope-specific CTL response is not necessarily predictive of its intrinsic antiviral capacity, and they suggest that the identification of typically subdominant responses, and targeting these using CTL-based vaccines, has the potential to elicit effective novel responses, as well as to increase the breadth of the antiviral CTL response.

enriched on the basis of pMHCII tetramer staining, a strategy previously exploited for the identification of antigen-expanded T cell populations[18,19]. Adapting this strategy for mouse[20,21] and human CTLPs[22] (reviewed in REF. 11) gave ~100-fold enrichments of epitope-specific cells[17,23] with a limit of detection of ~1 epitope-specific cell per million CD8⁺ T cells; which was a vast improvement on the sensitivity of conventional tetramer staining (which had a detection limit of ~1 in 10,000)[16,23,24]. Tetramer-based magnetic enrichment is now the protocol of choice for isolating and enumerating naive CTLPs, and it is transforming our understanding of the dynamics of primary CTL responses and the impact of naive antigen-specific CTLPs on these responses.

Determinants of CTL response magnitude

Naive CTLP frequencies. In mice and humans, the frequencies of naive CTLPs specific for a given pMHCI are highly reproducible between MHC-matched individuals but vary according to pMHCI specificity[20,22,25]. It is likely that, as was shown for CD4⁺ T cells, this frequency will be dictated for CTLs by negative selection of specificities that cross-react with self peptides and by the ability of specific peptide residues to provide a generally favourable landscape for TCR recognition[26]. In terms of their impact on the magnitude of responses, a strong correlation between naive CTLP frequencies and total CTL numbers following primary antigen challenge has been found for several pMHCI-specific T cell populations (reviewed in REF. 11). These data suggest that CTLP frequency is indeed the key determinant of response magnitude, and they imply that the complete recruitment and equivalent expansion of naive precursors during the immune response is the norm[27].

However, not all studies are in accordance with this model[20,28–30]. First, in influence A virus (IAV)-infected B6 mice, the relative numbers of CTLs specific for the immunodominant nucleoprotein-derived peptide ($NP_{366–374}$) and polymerase acidic-derived peptide ($PA_{224–233}$) presented in the context of H2-D^b (that is, H2-D^b–$NP_{366–374}$- and H2-D^b–$PA_{224–233}$-specific CTLs) correlated well with their precursor frequencies. However, this did not extend to CTLs specific for subdominant epitopes (namely, a peptide derived from an alternative open reading frame within the gene encoding the basic polymerase subunit 1 (PB1-$F2_{62–70}$) presented on H2-D^b (H2-D^b–PB1-$F2_{62–70}$), a peptide from nonstructural protein 2 presented on H2-K^b (H2-K^b–$NS2_{114–121}$) and H2-K^b-$PB1_{703–711}$); for these epitopes, the CTLP pools were substantially larger than those recognizing the immunodominant epitopes[20] (K. M. Quinn and N.L.L.G, unpublished observations). Second, Obar *et al.*[20] demonstrated that, despite a fourfold difference in naive CTLP frequency, epitopes derived from the murine cytomegalovirus M45 protein (H2-D^b–M45) or the vesicular stomatitis virus (VSV) nucleoprotein (H2-K^b–VSV-N) elicited comparable response magnitudes when mice were infected with recombinant VSV expressing these epitopes. Third, in humans, the immunodominance of an HLA-B27-restricted hepatitis C virus (HCV)-specific response could not be explained by a higher number of CTLPs relative to other specificities[29]. Fourth, following HIV infection in HLA-B27⁺ individuals, the CTL response specific for the HLA-B27-restricted Gag-derived epitope KK10 (residues 263–272) is almost universally dominant, despite the fact that the CTLP frequency for this population is equivalent to (or lower than) those found for other HIV-specific CD8⁺ T cell populations[30]. Finally, factors such as immunodomination[31] and peptide affinity for MHCI[21] have been found to act in concert with CTLP frequencies to shape the magnitude of responses.

In summary, although CTLP populations can be accurately quantitated and characterized in mice and humans, the extent to which they define immune response magnitudes seems to be variable and more nuanced than expected. Precisely what dictates the contribution of naive CTLP pools to immune response magnitude remains to be determined.

Recruitment of naive CTLPs. Why do CTLP frequencies predict the magnitudes of some but not other pMHCI-specific CTL responses? Beyond the factors noted above, the answer may lie in varying profiles of recruitment from the available CTLP pool. When Schumacher and colleagues adoptively transferred naive OTI TCR-transgenic CD8⁺ T cells marked by a DNA 'barcode' and then challenged the recipient mice with a variety of recombinant pathogens containing the target ovalbumin-derived peptide $OVA_{257–264}$, almost all of the transferred naive CTLPs were recruited into the primary response, irrespective of the vector or the antigen dose[32]. This was also true in an oligoclonal setting, in which T cells expressed a limited number of TCRs (comprising a fixed OTI-β chain and ~40 different TCRα chains)

Table 1 | **Naive CTL precursor frequencies calculated by direct versus indirect methods**

Specificity of TCR and source of peptide	Species	CTL precursor frequencies calculated (per 10^6 $CD8^+$ T cells)	
		By tetramer-based magnetic enrichment (direct method)	Using alternative indirect method (method indicated in parentheses)
H2-Db–$GP_{33–41}$	Mouse	• 26* (REF. 21) • 17* (REF. 20)	• 5‡ (adoptive transfer)[12] • ~100‡ (adoptive transfer)[13] • ~100§ (TCRβ sequencing)[15]
HLA-A2–$MART1_{26–35}$	Human	~100 (REF. 22)	• ~100 (limiting dilution)[16] • 0–10 (IFNγ ELISPOT)[16] • 1000–2000 (tetramer staining)[16]
H2-Db–$NP_{366–374}$	Mouse	• 1 (REF. 121) • 2 (REF. 25)	5–55 (TCRβ sequencing)[14]
H2-Db–$PA_{224–233}$	Mouse	• 4 (REF. 121) • 4 (REF. 25) • 7 (REF. 20)	5–55 (TCRβ sequencing)[14]

ELISPOT, enzyme-linked immunospot assay; GP, lymphocytic choriomeningitis virus glycoprotein; IFNγ, interferon-γ; MART1, melanoma-associated antigen 1; NP, influenza virus nucleoprotein; PA, influenza virus acid polymerase; TCR, T cell receptor. *Calculation based on assuming that there are 1.7×10^7 $CD8^+$ T cells in the mouse spleen and major lymph nodes. ‡Calculation based on assuming that there are a total of 3×10^7 $CD8^+$ T cells in a mouse. §Calculation based on assuming that there are 1×10^7 $CD8^+$ T cells per mouse spleen.

with differing affinities for the pMHCI complex. Similar findings came from a study by Zehn *et al.*[33] who also used the OTI TCR system and observed full recruitment of OTI T cells, even under conditions of suboptimal OTI T cell stimulation (achieved using altered peptide ligands).

Other studies analysing TCRβ-transgenic CTLPs (with limited diversity due to the presence of endogenously rearranged TCRα chains) have, however, identified clones in the naive antigen-specific CTLP repertoire that are not involved in the immune response[34,35]. Such incomplete CTLP recruitment was also observed after VSV infection of mice, based on a pMHCI-specific subpopulation that failed to upregulate CD69 (REF. 20). A possible explanation is that T cell priming and recruitment of low-avidity epitope-specific cells is normally suppressed by $CD4^+$ regulatory T (T_{Reg}) cells, as depletion of T_{Reg} cells from mice before immunization or infection with *Listeria monocytogenes* resulted in the elicitation of a novel, low-avidity $CD8^+$ T cell population[36]. Our own analysis in the IAV infection model demonstrated that, while recruitment of three subsets of epitope-specific CTLPs was almost complete (based on assessment of bromodeoxyuridine (BrdU) incorporation and CD44 upregulation), the subdominance of one pMHCI-specific population (namely, a population of cells specific for H2-D^b–PB1-$F2_{62–70}$) reflected a failure to recruit a substantial proportion (~20%) of the naive CTLP pool[25].

Collectively, the evidence suggests that in many (possibly most) cases, the vast majority of naive tetramer-binding $CD8^+$ T cells are recruited into the antigen-driven immune response. However, incomplete recruitment of naive CTLPs has been observed in a number of models of pathogen-induced, polyclonal $CD8^+$ T cell activation and, in such cases, it can markedly shape both CTL response hierarchies and profiles of TCR repertoire diversity. Notably, a study in humans found that negative selection of the $CD8^+$ T cell pool was inefficient and that tolerance is mediated by reduced intrinsic functionality[37]. This model predicts that the ability of CTLPs to acquire function might be as important as their frequency in determining response efficacy. It also offers a potential explanation for the poor recruitment of T cells specific for some foreign epitopes (mentioned previously), if those epitopes exhibit high homology to self peptides. However, this remains to be shown, and it is still possible that CTLP recruitment may be manipulated by altering the context, dose, or route of antigen delivery. In essence, the question is whether recruitment is limited by intrinsic or extrinsic mechanisms, or by a combination of both. This is a critical consideration, as it represents the difference between untapped potential within particular epitope-specific naive subsets, and naive CTLPs that are either refractory to recruitment and/or of limited use even when forcibly recruited into the response.

Expansion of naive CTLP populations. Although CTLP recruitment is a key factor, the extent of subsequent division must obviously be a major determinant of the ultimate magnitude of any given CTL response. Some evidence to date (but not all; see above[20,27]) suggests that while a broad range of CTLPs may undergo initial rounds of division, only clones with relatively high affinity for pMHCI continue to proliferate and become dominant in the primary response[33,35] (discussed further below). Similarly, IAV infection elicits CTL populations defined by varying kinetics and expansion rates for four different pMHCI specificities[38]. This fits with the view that CTL responses can be dominated by clones expressing 'optimal' TCR variable (V) regions, CDR3 motifs, or entire CDR3 regions (classified as type I – type III bias)[39,40]. This is seen clearly for HIV infection, in which particular TCRs selected following natural exposure to the virus are associated with better virus control[41,42]. It is perhaps most obvious in CTL responses to persistent

Altered peptide ligands
Peptides that have one or more residues mutated from the original cognate peptide and that are typically used to alter the strength of recognition through the T cell receptor.

Type I–type III bias
Types of bias observed in antigen-specific T cell repertoires: type I bias is the preferential usage of particular T cell receptor-α (TCRα) chain variable (TRAV) or TCRβ chain variable (TRBV) gene segments; type II bias is the preferential usage of particular TRAV or TRBV gene segments along with conserved amino acids at designated positions in the complementarity-determining region 3 (CDR3); and type III bias is repeated use of the same variable (V) region, joining (J) region and CDR3 amino acid sequence.

Epstein–Barr virus (EBV) and cytomegalovirus (CMV) infections (reviewed in REF. 39); in EBV infection, for example, the majority of the HLA-B8-restricted CTL response to the EBV-derived nuclear antigen 3 (EBNA3) epitope (FLRGRAYGL) is reproducibly comprised of a single clone[43].

What is less clear from these human studies is whether such biases in CTL responses pre-exist in the naive repertoire or whether they arise as a consequence of differential clonal expansion throughout the course of the response. An extreme (>80%) TCRβ variable 17 (TRBV17) bias is detected in the HLA-A*0201-restricted CTL response to the IAV matrix peptide ($M1_{58–66}$) in influenza virus-exposed adults[44], whereas naive CTLPs from cord blood that are specific for HLA-A*0201–$M1_{58–66}$ show a broad range of TCR Vβ usage[45]. Moreover, direct comparison of naive and primary immune IAV-specific CTL TCRs in mice shows a marked skewing in clonal distribution, from almost completely diverse in naive repertoires to highly skewed in immune populations, with this being the case for four distinct epitope-specific populations[25,46]. Differential expansion of individual $CD8^+$ T cell clones is perhaps most directly demonstrated by elegant studies in which individual pMHCI-specific T cells (distinguishable by congenic markers or DNA barcodes) were adoptively transferred into the same recipient and challenged with antigen in the context of virus or bacteria. Each of the single cell progenies was found to vary over three orders of magnitude, revealing that the bulk of infection-driven expansion is based on proliferation of a small proportion of precursors[47,48]. Collectively, these data suggest that many (if not most) immune CTL responses are a product of unequal clonal expansions, which may go some way to explaining the observed discrepancies between naive CTLP numbers and response magnitudes. Generally, tight correlations between pre-immune CTLP frequencies and response magnitudes will only apply for those subsets that are recruited and expanded to comparable extents.

TCR affinity for antigen. The notion of selective clonal recruitment and expansion leads to a discussion of the impact of TCR affinity as a determinant of primary CTL response magnitude. Analyses of both $CD4^+$ T helper cell and CTL responses have indicated that clonal prevalence within epitope-specific CTL responses may be reflective of TCR affinity for pMHC[49–52]. Affinity-mediated repertoire selection could be an indirect consequence of the fact that precursors with exceptionally low affinity for antigen are not recruited[35], or it may reflect the continued expansion of high-affinity clones in pathogen-specific responses[33]. In addition to determining proliferation, the characteristics of TCR binding to pMHC complexes may also regulate clonal apoptosis; a study by Wensveen *et al.*[53] found that T cells expressing TCRs with a subthreshold affinity for pMHCI preferentially undergo apoptosis early in the response via a process that is regulated by the BCL-2 family members BIM (also known as BCL2L11), NOXA (also known as PMAIP1) and MCL1. In addition, T_{Reg} cells may control clonal representation in an immune response by preferentially inhibiting the priming of low-affinity $CD8^+$ T cells. T_{Reg} cells have been found to suppress the expression of CC-chemokine ligand 3 (CCL3), CCL4 and CCL5, which are chemokines that function to stabilize the interaction between antigen-presenting cells (APCs) and low-affinity CTLPs[36].

Many of the above studies demonstrate that the selection for higher-affinity TCRs influences not only the quality of the responding T cells but also the magnitude of the primary response. For example, minor CTL responses elicited in the absence of the pro-apoptotic molecule NOXA[53] or T_{Reg} cells[36,54] are significantly augmented relative to the situation found for wild-type mice. In addition, the magnitudes of OTI CTL responses stimulated *in vivo* with a range of variant OVA pMHCI epitopes correlated with the affinity of the OTI TCR for the different pMHCI complexes[33]. It thus follows that the relative prevalence of high-affinity CTLPs within a pMHCI-specific population will influence the prominence of the epitope within that immunodominance hierarchy. Certainly, a distinct respiratory syncytial virus-specific CTL immunodominance profile, observed following infection of neonatal versus adult mice, was associated with epitope-specific alterations in $CD8^+$ T cell affinity and functionality in neonatal T cell populations[55].

Others who have investigated the discordance between CTLP frequency and response magnitude have proposed that the immunodominant $CD8^+$ T cell populations may arise from naive subsets containing a preponderance of high-affinity CTLPs, although this was not directly tested[28,30]. In our own studies, it was found that subdominance of the IAV-derived epitopes H2-D^b–PB1-$F2_{62–70}$ and H2-K^b–$NS2_{114–121}$ in the B6 model of IAV infection is associated with the relative paucity of high-avidity T cells in the pre-immune and immune repertoires, but not with CTLP numbers or antigen dose[25,38]. In fact, attempts to equalize epitope abundance using a variety of immunization strategies failed to align immune responses with relative CTLP frequency[38]. Together, these findings indicate that the intrinsic TCR–pMHCI avidity characteristics of responding CTLs have the potential to act as a dominant influence on primary CTL response magnitudes (FIG. 1).

Memory phenotype of 'naive' T cells. Naive $CD8^+$ T cells that have a memory phenotype can arise due to lymphopenia-induced expansion or elevated interleukin-4 levels during thymic development, and they comprise a small but significant (~10–25%) proportion of the endogenous, unprimed $CD8^+$ T cell repertoire in wild-type mice[25,56]. Termed virtual memory T cells, they arise independently of foreign pMHCI recognition and, compared with naive-phenotype cells, exhibit elevated proliferative capacity and effector functions when challenged with antigen[56]. Consequently, it has been suggested that the virtual memory T cell subset may have a proliferative advantage after pathogen encounter and that the relative frequency of virtual memory T cells within pMHCI-specific populations may contribute to CTL

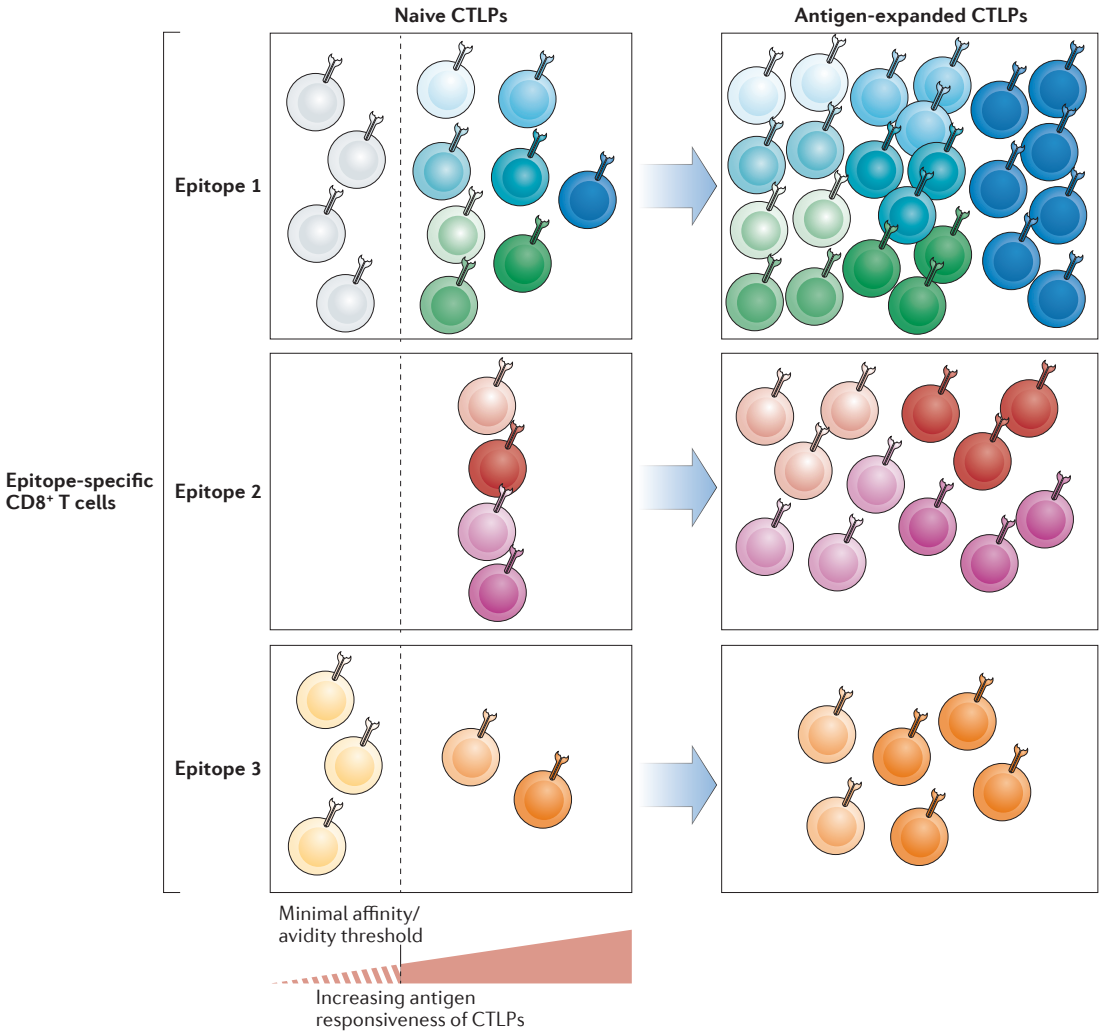


Figure 1 | **A model for the contribution of T cell receptor–peptide–MHCI affinity/avidity and CTL precursor frequency in determining CTL response magnitude and composition.** This model predicts that the peak magnitude of an antigen-driven immune response, for a given antigen dose, is determined not by the total number of epitope-specific cytotoxic T lymphocyte (CTL) precursors (CTLPs) as defined by tetramer staining, but by the number of epitope-specific CTLPs above a minimal affinity/avidity threshold (panels on the left; threshold indicated by dashed vertical line). CTLPs expressing T cell receptors (TCRs) with a subthreshold affinity for peptide–MHC class I (pMHCI) complexes are either not recruited into the response or show poor clonal expansion and contribute minimally to the effector CTL pool. Several studies have shown that within an epitope-specific CTL population, TCR affinity/avidity for pMHCI complexes (denoted by increasing colour intensity for each epitope) positively correlates with preferential clonal expansion and over-representation in the effector repertoire, compared with the naive, epitope-specific $CD8^+$ T cell repertoire. Distinct colours represent different CTL clones.

immunodominance[56]. Intriguingly, unlike virtual memory T cells in young adults, memory-phenotype cells that accumulate in aged mice seem to function poorly and express a transcriptional signature that is characteristic of exhaustion[57], reminiscent of $CD8^+$ T cells in elderly humans (reviewed in REF. 58).

In contrast to memory-like cells, the possibility that some CTLs might recognize cross-reactive epitopes from different organisms means that previous infections may create a population of memory-phenotype cells that participate in the response to a heterologous pathogen[59]. In this case, in which the T cells represent conventional memory populations, they exhibit numerical and proliferative advantages that, in turn, can substantially alter the hierarchy of the $CD8^+$ T cell response. It has been suggested that such heterologous immunity may be the cause of variability in CTL immunodominance hierarchies observed in response to viruses such as HIV or HCV[59]. Especially in humans, who encounter a multitude of pathogens over a prolonged lifespan, heterologous immunity might be predicted to significantly influence the magnitude of epitope-specific responses. Certainly, recent work has demonstrated a substantial population of memory-phenotype $CD4^+$ T cells in humans unexposed to the pathogens from which the designated epitopes were derived[60].

Collectively, the possibility that some CD8$^+$ T cells in a pre-immune repertoire may respond like memory T cells, and not like classical naive T cells, further confounds any capacity to predict response magnitude from precursor frequency alone. Moreover, it is possible that pre-immune CTLPs that show a memory phenotype can be selectively expanded to elicit effective T cell immunity.

Interactions between T cells of different specificities. Conceived as reflecting the complexities of interactions among polyclonal CTLPs and APCs during priming, the idea of immunodomination proposes that subdominant CTLPs are in some way inhibited, suppressed or overwhelmed by concurrent immunodominant responses. The effect is most convincingly demonstrated when subdominant responses are boosted by removing (or segregating) the immunodominant pMHCI complexes from the immune response. An intuitive form of immunodomination is the inhibition of naive T cell responses by pre-existing memory T cells[61,62]. However, here we will focus on immunodomination that occurs in primary, concurrent CTL responses to different pMHCI epitopes. There are various mechanisms by which immunodomination in this context may occur: competition for CTLP access to APCs; preferential MHCI-mediated presentation of particular epitopes at the expense of others (owing to differential peptide affinity for the same MHCI molecule); killing of APCs or production of cytokines to suppress APC activity by 'early responders' to inhibit the priming of subsequent specificities; or the more rapid elimination of virus by 'faster onset' CTLs resulting in incomplete priming of the subdominant subsets.

Immunodomination occurs more readily when CD8$^+$ T cells target the same antigen. Studies have clearly demonstrated that the adoptive transfer of high numbers of TCR-transgenic CD8$^+$ T cells specific for a particular epitope (either H2-K^b–OVA$_{257–264}$ or the lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived epitope H2-D^b–GP$_{33–41}$) before antigen challenge or infection severely limits the endogenous host-derived response to that epitope, although not typically to other epitopes[13,61,63]. Moreover, the weight of evidence from the analysis of endogenous antiviral responses suggests that inter-epitope immunodomination is relatively uncommon. Removal of the immunodominant peptides from IAV and *L. monocytogenes* in B6 and BALB/c models of infection, respectively, revealed no increase in CTL responses to other subdominant pMHCI complexes after infection[64,65]. In humans, although there are many examples of CTL immunodomination in response to minor histocompatibility antigens (MiHAs; reviewed in REF. 66), studies of human antiviral CTL responses demonstrate little evidence for competition between CTLs with different epitope specificities[66,67].

Minor histocompatibility antigens
(MiHAs). Non-MHC-encoded antigens that exhibit polymorphism between individuals and so can elicit immune responses when presented in complex with MHC class I molecules.

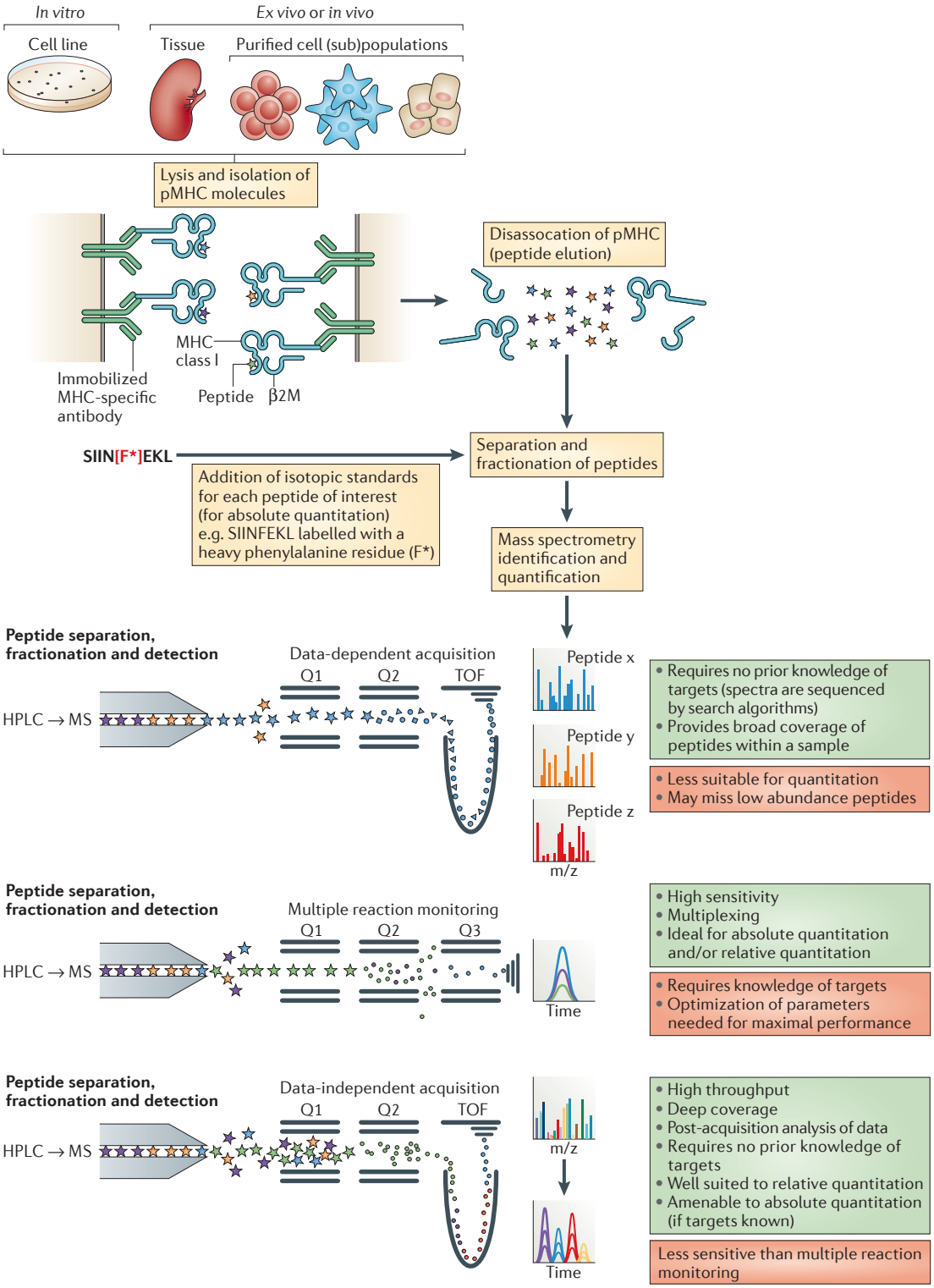
Despite this, inter-epitope immunodomination does occur, typically under conditions of limited resources that drive direct competition. For example, immunodomination is observed between CTL populations recognizing pMHCI complexes on the same, but not on different, APCs[61,68], with this effect being overcome by the transfer of excess numbers of antigen-bearing dendritic cells (DCs)[61]. Until recently, demonstrations of inter-epitope immunodomination in animal models were made exclusively in MiHA-specific CTL responses or in virus infection models following adoptive transfer of high numbers of TCR-transgenic cells. This prompted the suggestion that, in natural virus infections, the numbers of pMHCI-expressing APCs may be far in excess of what is required for the activation of low numbers of naive CTLPs and therefore not subject to immunodomination[62,66].

Recent studies in vaccinia virus- or IAV-infected mice have, however, clearly demonstrated that CTL immunodomination occurs in endogenous primary CTL responses[69,70]. In these experiments, viral antigen dose was controlled by altering the route of infection, an approach that in turn limited the capacity of the challenge virus to either grow productively (in the case of IAV infection)[70] or to establish systemic infection (in the case of vaccinia virus infection)[69]. Under these conditions of limited antigen availability, subdominant responses were selectively disadvantaged, whereas greater viral loads resulted in blunted (or abrogated) CTL immunodominance, supporting the notion that limiting antigen dose promotes immunodomination[63,69,70]. Lin and colleagues[69] also demonstrated that the overexpression of co-stimulatory molecules (namely, CD80 and CD86) could alleviate the observed immunodomination in the vaccinia virus system, again suggesting that limiting antigen is likely to restrict the number of APCs available to stimulate CTL responses, which in turn drives competition for all necessary APC-derived signals, including co-stimulation.

Antigen abundance. It seems obvious that the amount of antigen available to stimulate a given CTLP population will influence the final magnitude of its response, but because the analysis (at the level of the APC) is technically difficult, there are few unambiguous demonstrations of this principle in the literature. Perhaps the most elegant analysis focused on immune responses generated by mice immunized with a set of recombinant vaccinia viruses that expressed graded levels of IAV NP fused with the SIINFEKL epitope of OVA. This study showed a strong correlation between expression level and immunogenicity for lower antigen levels but found that immunogenicity reached a plateau, beyond which increasing the amount of antigen had no effect[71]. The recognition that there are such thresholds is crucial for understanding the role of antigen dose, as it indicates that a higher abundance of pMHCI complexes will not always result in an increased CTL response. Furthermore, it suggests that increasing the virus dose will not affect all CTL specificities equally. Indeed, this has recently been shown and modelled mathematically for IAV infection in mice, in which low and high doses of virus favoured H2-D^b–NP$_{366–374}$- and H2-D^b–PA$_{224–233}$-specific CTL responses, respectively[70].

Rapid advances in mass spectrometry instrumentation and methods are enabling both the relative and absolute quantification of pMHC complexes on *in vitro* cells, and are bringing the capacity to accurately quantify

multiple pMHCI complexes on cells infected *in vivo* within reach[72]. Whereas traditional data-dependent mass spectrometry methods provide ideal workflows for epitope discovery, the gold standard in quantification is the ability to target specific peptide sequences for detection through a process termed multiple reaction monitoring (MRM). Furthermore, the high-resolution capabilities of modern mass spectrometers can now be used to enable data collection that can be iteratively mined for simultaneous peptide discovery and quantification (FIG. 2). Our own study — which used a targeted liquid chromatography coupled to MRM mass spectrometry (LC-MRM MS) approach to directly quantify several different pMHCI complexes expressed



by vaccinia virus-infected cells — failed to find a correlation between antigen abundance and the sizes of responding CTL populations, despite the results and considerations presented above[73]. This lack of any obvious association was still observed when the differing presentation kinetics of the various pMHC complexes were taken into account. Perhaps the explanation is that pMHCI levels are a relatively weak determinant of CTL responses, but it is also possible that cell lines infected *in vitro* do not reproduce what is happening *in vivo* with the relevant APCs.

Beyond quantification, what other aspects of antigen presentation can be studied to shed light on the impact of peptide dose across antigens? In this regard, the quality of peptide binding to MHCI molecules, the manner in which antigen is acquired by DCs for presentation on MHCI molecules, and (to some degree) gene and protein expression levels are all factors that merit attention and are discussed below.

pMHCI affinity and stability. The capacity of any given peptide to bind an MHCI glycoprotein determines the half-life of these pMHCI complexes on the surface of cells and, as such, is a fundamental variable affecting antigen dose. Such binding is a function of the basic chemistry that should not vary according to cell type, presentation pathway or experimental system. In the original studies, affinity for MHCI was determined in competitive assays and compared with immunogenicity across a range of peptides, leading to the suggestion that peptides with IC_{50} values above 500 nM were unlikely to be immunogenic[74]. This value has largely stood the test of time, although it has been shown to be MHCI allomorph specific[75]. A different way to assess the strength of peptide binding to MHCI is to measure the stability of complexes over time[76]. Although they are clearly related, affinity and stability do not rank peptides identically, and there remains some argument in the literature as to which measure gives the better correlation with pMHCI immunogenicity[77]. Data from recent comprehensive papers with two viral systems (vaccinia virus and LCMV) show that as a single parameter, MHCI binding correlates to some extent with the relative immunogenicity of pMHCI complexes during infection[21,78]. Of note, the correlation between pMHCI affinity and the magnitude of CTL responses improves when mice are primed with synthetic peptides in adjuvant rather than when they are infected with virus[78]. This presumably reflects the role of other variables in antigen presentation during virus infection, such as variation in protein expression levels and perhaps competition for priming between CTLs specific for distinct pMHCI complexes (discussed above)[5].

Antigen presentation pathways. An early approach for manipulating the effective peptide dose was to change the context or attributes of the antigen. For example, compared with unmodified proteins, rapidly degraded antigens or minimal epitope constructs ('minigenes') expressed in recombinant vaccinia virus[79] constructs showed evidence of enhanced immunogenicity. The design of these experiments was guided by a molecular understanding of the endogenous processing pathway for MHCI-presented antigens and assumed that the CTLs were primed by exposure to infected APCs. This premise was later supported by observations that the same modifications increased the abundance of the relevant pMHCI complexes on cells infected with these recombinant vaccinia virus constructs[80]. Around the same time, however, it was becoming clear that cross-presentation, or the capture and processing of viral antigen by DCs that remain uninfected, is required for some viruses to prime CTLs[81]. Furthermore, the attributes of antigens that improve presentation by the endogenous (or direct) and cross-priming mechanisms are diametrically opposed. Unstable antigens and minigenes are favoured by the direct route, whereas more stable forms of protein are more efficiently cross-presented[82,83]. Depending on which process is of greater importance, characteristics such as protein stability will thus alter CTL responses in a virus-specific and maybe antigen-specific manner. For example, stable protein was more immunogenic than a minigene when expressed from an adenovirus vector, despite producing fewer pMHCI complexes on cells infected *in vitro*[84]. To date, there is no study comparing the range and abundance of pMHCI complexes that are endogenously presented versus cross-presented for any virus, so this remains an important unknown. Overall, the relative significance of these two pathways remains somewhat contentious and is not well dissected for many viruses (BOX 3).

◀ Figure 2 | **Mass spectrometry-based detection and quantification of MHC-bound peptides.** Peptide–MHC (pMHC) complexes can be isolated from a variety of sources (such as cell lines, tissues or purified cell populations) through mild lysis and (typically) immunoaffinity-based capture by immobilized MHC-specific antibodies. Peptides are dissociated from MHC molecules through acid elution. Precise quantities of isotopically-labelled internal standards for each epitope of interest can be incorporated at this step, such that downstream mass spectrometry (MS) can detect both the native (light) and isotopic (heavy) peptides and the ratio of their signal can be compared to enable absolute quantification of the peptide of interest. Peptides may be separated and fractionated by a variety of techniques (for example, reversed-phase high performance liquid chromatography (HPLC)) in order to reduce sample complexity and remove undesirable components (namely, MHC heavy chains and β2-microglobulin (β2M) in the case of MHC class I), followed by MS for peptide detection. For discovery-based workflows (in which quantification is not needed or is used as a primer for downstream quantitative MS), data-dependent acquisition uses high-resolution MS instruments (such as a quadrupole time-of-flight (Q-TOF), depicted here) to fragment peptides such that their spectra can be searched by algorithms against sequence databases. For the purposes of quantification, two MS modalities can be used: multiple reaction monitoring and data-independent acquisition. In the former, knowledge of the peptide sequences of interest is required in order to instruct a triple quadrupole MS to selectively filter precursor and product ions in the quadrupoles Q1 and Q3, respectively. Multiple reaction monitoring is ideally suited to absolute quantification and can also be multiplexed such that hundreds of target peptides can be detected simultaneously. Conversely, data-independent acquisition can be achieved using high-resolution MS instruments, whereby small sequential windows allow all peptides within that window through for fragmentation, theoretically fragmenting all ions within a sample. Data-independent acquisition experiments can be run with no prior knowledge of the analyte, with all analysis occurring post-acquisition. This modality is well suited to relative quantification of peptides across different samples or conditions, but it is also amenable to absolute quantification provided that isotopic standards are included for each epitope of interest.

Box 3 | **Endogenous presentation versus cross-presentation of antigen**

The contribution of direct presentation and cross-presentation to priming antiviral T cell responses has only rarely been directly examined. For example, the importance of direct presentation and cross-presentation has been shown for vaccinia virus and herpes simplex virus, albeit only for individual antigens[111–113]. For other viruses, the presentation pathway has been implied from knowledge that the relevant dendritic cell (DC) or process is associated with cross-presentation. A good example is the common interpretation of the finding that *Batf3*$^{-/-}$ mice, which lack cross-presenting DCs, prime antiviral CD8$^+$ T cells poorly[114]. However, this ignores the possibility that these same DC populations might be equally capable of direct presentation[115]. Similarly, paralyzing cross-presentation using systemic maturation of DCs has unanticipated effects on direct presentation, so this tool is not useful for dissecting these pathways[116]. Studies with mice deficient in proteins uniquely required for cross-presentation — for example, interferon-γ-inducible lysosomal thiol reductase (GILT; also known as IFI30) — should offer less complicated interpretations[117]. The identification of receptors that promote the cross-presentation of internalized antigen, such as DNGR1 (also known as CLEC9A), offers another possible way to dissect presentation pathways[118,119], but again the impact of knocking out such receptors on direct presentation needs to be rigorously established. Apart from direct visualization of priming by intravital microscopy[111], knowledge of the major substrates for direct presentation and cross-presentation may be the most reliable tool for dissecting antigen-presentation pathways[112,116]. Finally, mechanisms such as 'cross-dressing' that allow DCs to acquire pre-formed peptide–MHC complexes need to be considered, although it remains unclear if this is a major mechanism for priming antiviral responses[120].

Proteome
The entire set of proteins expressed by a cell or organism.

Protein abundance versus translation rate. As noted above, enhancing protein expression levels improves immunogenicity and, at least for cross-presentation, there is no reason to doubt that the more stable the protein, the better it will be at inducing an immune response. However, for endogenous presentation, it remains contentious whether the steady-state proteome or the extent and rate of translation is the best predictor of the range of presented peptides[85,86]. Supporting the predictive value of steady-state protein levels, a recent computational approach based on proteomics data and published sets of peptides eluted from MHCI molecules found that the abundance of the source protein predicted the likelihood that a peptide would be presented[87]. However, the use of metabolic labelling shows that many peptides are presented rapidly after translation and that protein turnover does not correlate well with pMHCI levels[88]. These studies suggest that presentation is more often linked to translation rates rather than to steady-state protein abundance. Most relevant to viral infection, a link between the onset of viral protein production and epitope presentation has been confirmed for several vaccinia virus epitopes[73]. Furthermore, there is evidence for EBV that mRNA structures that function to reduce the translation rate, but not the ultimate protein levels, reduce the efficiency of pMHCI presentation[89]. Notably, all of the above studies relate gene expression and pMHCI levels, although comprehensive comparisons of viral gene expression levels or proteomes and immunogenicity are so far lacking.

Conclusion

There has long been a perception that the relative numbers and TCR avidities of naive CTLP populations are, along with pMHCI abundance on APCs, likely to be the key determinants of primary CTL response magnitude and effector function. More recently, probing primary CTL responses using improved technologies for the detection, measurement and characterization of these parameters has provided new insights into the differential effects of these determinants on the clonal expansion of naive CTLP populations in response to infection or vaccination. Even so, the relative contributions of such key variables are not necessarily predictable and seem to be dependent on the context in which antigen is encountered. Thus, although our understanding of the factors that can, and do, influence primary CTL response magnitude has undoubtedly improved, the results to date suggest no absolute criteria that can be adopted for clinical application, particularly vaccine development. A global approach is thus required for future studies to elucidate how the context of antigen encounter drives the contribution of the various response determinants, thereby determining both epitope selection and the relative magnitudes of individual epitope-specific responses following natural infection and vaccination protocols.

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Acknowledgements
The authors wish to acknowledge the invaluable work that, owing to space limitations, could not be cited here. They thank F. Mercuri who assisted with editing of the manuscript. D.C.T. and N.P.C. are supported by a National Health and Medical Research Council (NHMRC) Project Grant (AIAPP1084283). D.C.T. is funded by an Australian Council of Research (ARC) Future Fellowship (FT110100310). N.L.G and P.C.D. are supported by an NHMRC Program Grant (5671222). N.L.G. is supported by an NHMRC Project Grant (AI1046333), and a Sylvia and Charles Viertel Senior Medical Research Fellowship.

Competing interests statement
The authors declare no competing interests.