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Affinity Thresholds for Naive CD8+ CTL Activation by Peptides and Engineered Influenza A Viruses

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High-avidity interactions between TCRs and peptide plus class I MHC (pMHC) epitopes drive CTL activation and expansion. Intriguing questions remain concerning the constraints determining optimal TCR/pMHC binding. The present analysis uses the TCR transgenic OT-I model to assess how varying profiles of TCR/pMHC avidity influence naive CTL proliferation and the acquisition of effector function following exposure to the cognate H-2Kb/OVA257–264 (SIINFEKL) epitope and to mutants provided as peptide or in engineered influenza A viruses. Stimulating naive OT-I CD8+ T cells in vitro with SIINFEKL induced full CTL proliferation and differentiation that was largely independent of any need for costimulation. By contrast, in vitro activation with the low-affinity EINFEKL or SIIGFEKL ligands depended on the provision of IL-2 and other costimulatory signals. Importantly, although they did generate potent endogenous responses, infection of mice with influenza A viruses expressing these same OVA257 Variants failed to induce the activation of adoptively transferred naive OT-I CTLs, an effect that was only partially overcome by priming with a lipopeptide vaccine. Subsequent structural and biophysical analysis of H2-KbOVA257, H2-KbE1, and H2-KbG4 established that these variations introduce small changes at the pMHC interface and decrease epitope stability in ways that would likely impact cell surface presentation and recognition. Overall, it seems that there is an activation threshold for naive CTLs, that minimal alterations in peptide sequence can have profound effects, and that the antigenic requirements for the in vitro and in vivo induction of CTL proliferation and effector function differ substantially. The Journal of Immunology, 2011, 187: 5733–5744.

Virust-specific CD8+ CTLs recognize non–self-peptides complexed with self-MHC class (pMHC) I glycoproteins. This requirement for pMHC recognition focuses CTL effectors onto virus-infected cells and is mediated via clonally expressed TCR αβ heterodimers. Naive CTL clonal expansion and differentiation depends on the integration of signals subse-

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The coordinates presented in this article have been submitted to the Protein Data Bank (http://www.pdb.org/pdb/home/home.do) under accession numbers 3PAB and 3PDM.

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The online version of this article contains supplemental material.

Abbreviations used in this article: APL, altered peptide ligand; B6, C57BL/6; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; E1, EIINFEKL peptide; G4, SIIGFEKL peptide; ICS, intracellular cytokine staining; i.n., intranasal; MLN, mediastinal lymph node; N, SIINFEKL peptide; NA, influenza A neuraminidase gene segment; OT-I, Ly5.1+ TCR transgenic mouse with T cells specific for SIINFEKL, pMHC; PR8, influenza A/Puerto Rico/8/34 virus; Q4, SIQFEKL peptide; Rag−/−, Rag1/2-deficient; Tm, thermal denaturation; wt, wild-type; x31, influenza A/Hong Kong/x31 virus.

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a better understanding of how low-affinity interactions can impact pathogen-specific T cell responses. The present analysis uses mutant peptides to determine how altering the K\(^+\) OVA\(_{257-264}\) (N) epitope modifies the avidity of cognate TCR/pMHCI interactions. The normal N, EIIIFKEL (E1), and SIIGFEKL (G4) variants were used in vitro as free peptide to stimulate cultured, transgenic OT-I T cells, with or without added costimulation. Peptide-pulsed, bone marrow-derived dendritic cells (BMDCs), lipopeptide vaccine preparations (17), and influenza A viruses engineered to express E1, G4, or influenza A neuraminidase gene segment (NA) stalk (18) were then used to prime B6 mice that had been given naive, congenic OT-I T cells. The findings following in vitro and in vivo stimulation were very different and are considered in the context of TCR/pMHCI avidity/affinity measurements and pMHCI structural constraints. Overall, this analysis supports the idea that CD8\(^+\) T cell activation depends on TCR/pMHCI interactions reaching a minimal avidity threshold and that changes in pMHCI stability as a result of small alterations in peptide sequence represent a potential mechanism for viral escape. The experiments also indicate that, whereas in vitro studies of antigenicity provide important insights, they give only a partial reflection of what happens in an immune response.

### Materials and Methods

#### Mice

C57BL/6J (B6, H-2\(^b\)), and Ly5.2\(^+\), congenic Ly5.1\(^+\)OT-I (OT-I), and RAG1-deficient (Rag\(^-/-\)) mice were bred and housed under specific pathogen-free conditions at the Department of Microbiology and Immunology, University of Melbourne. All experiments followed guidelines stipulated by the University of Melbourne Animal Ethics Experimentation Committee.

#### In vitro stimulation

Pooled lymph nodes from naive OT-I mice were disrupted through a 70-mm sieve, and an aliquot was stained for CD8\(^{+}\) expression to determine the percentage of OT-I CTL precursors. Unlabeled or CFSE-labeled OT-I cells (2 \times 10\(^5\)) were stimulated with 1 \(\mu\)M peptide (E1, G4, or Q4 peptide (Auspep) with or without 5 \(\mu\)M anti-CD28 (clone 37.51; BioLegend) and 10 U/ml IL-2 (Apot) or ex vivo-purified dendritic cells (DCs) activated with LPS (19) and pulsed with 1 \(\mu\)M E1, G4, or peptide. Stimulation was carried out in round-bottom 96-well plates for 5 h to 4 d in 200 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mM nonessential amino acids, 5 mM HEPES buffer, 55 mM 2-ME, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10% FCS. For analysis of cytokine production at 1–4 d, OT-I cells were restimulated with 1 ng/ml IL-2 (Apollo) or ex vivo-purified dendritic cells (DCs) activated with LPS (19) and pulsed with 1 \(\mu\)M E1, G4, or peptide. Stimulation was carried out in round-bottom 96-well plates for 5 h to 4 d in 200 ml RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mM nonessential amino acids, 5 mM HEPES buffer, 55 mM 2-ME, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10% FCS. For analysis of cytokine production at 1–4 d, OT-I cells were restimulated with 1 \(\mu\)M peptide.

#### Infection and vaccination

B6 mice were anesthetized by inhalation of vaporized methoxyfluorane and infected with 10\(^3\) PFU influenza A/Hong Kong/x31 virus (x31; H3N2) or vaccinated with 25 nmol lipopeptide intranasally (i.n.). For analysis of recall responses, mice were primed i.p. with 1.5 \times 10\(^7\) PFU A/Puerto Rico/8/34 virus (PR8; H1N1) 6 wk prior to challenge with 10\(^4\) PFU x31 virus. Lipopeptide constructs (20) contained the CD4\(^+\) Th OT-II epitope (ISQA V-100 mM nonessential amino acids, 5 mM HEPES buffer, 55 mM 2-ME, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10% FCS). For analysis of cytokine production at 1–4 d, OT-I cells were restimulated with 1 \(\mu\)M peptide. Pooled lymph nodes from OT-I mice (10\(^7\) cells/ml in PBS/0.1% BSA) were labeled with 5 mM CFSE (Sigma-Aldrich) for 10 min at 37°C and washed in media containing 10% FCS. CFSE-labeled cells were then adjusted to the desired concentration for in vitro stimulation or adoptive transfer to B6 recipients via tail vein inoculation. B6 mice that had received OT-I cells were infected 1 d later with x31-N, x31-E1, x31-G4, or x31-Q4.

#### Depletion of tetramer\(^{+}\)CD8\(^+\) T cells

Naive K\(^+\)G4-specific T cells were depleted from B6 spleen and lymph nodes using the method developed by Moon et al. (25) and adapted by La Gruta et al. (7) for identifying naive, epitope-specific, CD8\(^+\) T cells. B6 mice were anesthetized by inhalation of vaporized methoxyfluorane and infected with 10\(^3\) PFU influenza A/Hong Kong/x31 virus (x31; H3N2) or vaccinated with 25 nmol lipopeptide intranasally (i.n.). For analysis of recall responses, mice were primed i.p. with 1.5 \times 10\(^7\) PFU A/Puerto Rico/8/34 virus (PR8; H1N1) 6 wk prior to challenge with 10\(^4\) PFU x31 virus. Lipopeptide constructs (20) contained the CD4\(^+\) Th OT-II epitope (ISQA V-100 mM nonessential amino acids, 5 mM HEPES buffer, 55 mM 2-ME, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10% FCS). For analysis of cytokine production at 1–4 d, OT-I cells were restimulated with 1 \(\mu\)M peptide.

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were cultured with graded concentrations of OVA APLs for 1 h and then transferred to 37°C for 2 h. H2-K\(^b\) cell surface expression was detected using the K\(^b\)-specific mAb, Y3, conjugated to FITC. Cells were analyzed using a FACSCanto II (BD Biosciences), and the mean fluorescence intensity was determined using FlowJo software (Tree Star). The RMA-S cells were washed three times in PBS to remove excess peptide and used to stimulate day 10 effector OT-I CTL, generated after adoptive transfer and subsequent infection as described above. After 5 h, samples were stained with anti-CD8a and anti-CD45.1 (for detection of OT-I cells), fixed, and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-IFN-\(\gamma\) (clone XMG1.2; BD Pharmingen) to detect intracellular cytokine production. Samples were analyzed by flow cytometry as described above.

**Statistical analysis**

Statistical analyses were conducted using the unpaired two-tailed Student \(t\) test, with significance denoted as *\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\).

**Protein expression, crystallization, and structure determination**

The H-2K\(^b\) and \(\beta_2\)-microglobulin molecules were expressed in *Escherichia coli* as inclusion bodies, refolded with each of the N, E1, and G4 peptides and then purified (27). The H-2KN complex crystals were obtained using the Kb-specific mAb, Y3, conjugated to FITC. Cells were analyzed (Kd values: 20–22 \(\mu\)M) in a solution of 10 mM Tris (pH 8) and 150 mM NaCl.

The H-2Kb and \(\beta_2\)-microglobulin molecules were expressed in *Escherichia coli* as inclusion bodies, refolded with each of the N, E1, and G4 peptides and then purified (27). The H-2KbN complex crystals were obtained as inclusion bodies, refolded with each of the N, E1, and G4 peptides (H-2KbN, 5 and 2.5 mM; H-2KbG4, 10 and 5 mM; and H-2KbE1, 6 and 3 mM) in a solution of 10 mM Tris (pH 8) and 150 mM NaCl. The Jasco Spectra Manager software was used to view and measure the CD profile when used in vitro to activate OT-I cells. This provides a model for assessing how hierarchies of TCR/pMHC1 affinity translate to diverse functional outcomes following different modes of T cell activation. For example, although K\(^b\)-E1 has been described as a weak agonist (16, 35) capable of triggering limited lytic activity in OT-I T cells (16, 38), K\(^b\)G4 induces poor activation and survival (11, 36).

The consequences of costimulation for the phenotypic and functional changes induced by in vitro culture of OT-I T cells with 1 \(\mu\)M of the N, E1, or G4 peptide ligands were analyzed over a 4-d interval following the addition of exogenous IL-2 and/or anti-CD28 Ab. As expected, activation of naive OT-I T cells with cognate N peptide resulted in extensive proliferation (Fig. 1A, top panel) together with the rapid upregulation of CD44 (Fig. 1B, top panel) and loss of CD62L (Fig. 1C, top panel). Interestingly, stimulation of OT-I T cells with concurrent CD28 cross-linking appeared to limit the extent of expansion (Fig. 1A, Supplemental Fig. 1). In response to G4 stimulation, almost all cells became CD44\(^+\) within 48 h, regardless of the secondary signals (Fig. 1B, middle panel), although significant accumulation was observed only if IL-2 was present in the culture (Fig. 1A, middle panel).

Irrespective of the signals provided by IL-2 and CD28 ligation, stimulation with the low-affinity E1 peptide failed to induce significant OT-I proliferation, with delayed CD44 upregulation and little change in the CD62L phenotype (Fig. 1B, 1C, bottom panels). Therefore, qualitative changes in cell surface phenotype are associated with the quality of TCR/costimulatory signals received during initial T cell activation.

**Varied cytokine profiles after in vitro activation with mutant peptides**

We next analyzed how these variant peptides influence the acquisition of cytokine production. Naive OT-I T cells were stimulated for 5 h in vitro in the presence of brefeldin A, and then, profiles of IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 production were determined by the flow cytometric intracellular cytokine staining (ICS) assay (Fig. 2A–C).

Much to our surprise, we found that naive OT-I T cells stained strongly for cytoplasmic TNF-\(\alpha\) protein within 5 h of stimulation with the cognate N peptide (Fig. 2A, top panel). This suggests that, at least from the aspect of TNF-\(\alpha\) production, naive T lymphocytes are “poised” for immediate effector function prior to division (39). Furthermore, this very rapid cytokine induction depends on a high-affinity TCR/pMHC1 interaction, as <4% of cells showed evidence of 5-h TNF-\(\alpha\) production after stimulation with the G4 peptide (Fig. 2A, middle panel), whereas E1 induced no detectable early response (Fig. 2A, bottom panel).

How persistent is this profile of very early TNF-\(\alpha\) production? From the 24-h time point in Fig. 2, naive OT-I cells that had been activated in vitro as described above were restimulated with PMA plus ionomycin for 5 h prior to reading the cytokine staining profile. This additional step bypassed any effect of TCR down-regulation following the initial peptide stimulation. Strikingly, there was a rapid continuing drop in TNF-\(\alpha\) production from 48 h in the N-stimulated OT-I cultures (Fig. 2A, top panel). The down-regulation effect was not a consequence of selective cell death as loss of TNF-\(\alpha\) production correlated with increased proliferation (Fig. 1A, top panel). Interestingly, sustained TNF-\(\alpha\) production was observed in the presence of costimulation (Fig. 2A, top panel), and there was evidence of IFN-\(\gamma\) synthesis within 24 h of exposure to the N peptide, with a subsequent, gradual increase over the culture period that was dependent on CD28 signaling (Fig. 2B, top panel). Conversely, IFN-\(\gamma\) expression in the G4- and E1-stimulated cultures was only observed in the presence of IL-2 (Fig. 2B, middle and bottom panels, respectively) with there being little dependence on CD28 when it came either IFN-\(\gamma\)
production or maintaining T cell numbers (Fig. 1A, middle and lower panels). Overall, these results suggest that acquisition of effector phenotype is differentially regulated depending on the varying quality of the TCR/pMHC interaction that, in turn, establishes differential requirements for costimulatory signals.

**Coexpression of cytokines during in vitro differentiation**

To examine the impact of TCR/pMHCI avidity on the coexpression of IFN-γ and TNF-α upon activation, naive OT-I cells were stimulated with either the N or G4 peptides in the presence of anti-CD28, and the extent of coexpression of IFN-γ and TNF-α was determined by ICS (Fig. 2C, 2D). As expected (from Fig. 1A), the naive OT-I CTLs were only making TNF-α at 5 h after N or G4 stimulation (Fig. 2C, 2D), whereas IFN-γ production was observed by 24 h in a subset of the TNF-α producers for both the N and G4 cultures (Fig. 2C, 2D). This acquisition of IFN-γ was, however, sustained only in the N-stimulated population and soon fell off for those activated by the G4 peptide (Fig. 2C, 2D). The apparent emergence of TNF-α–only producers at days 2–4 within the G4-stimulated cultures likely reflects the survival of undivided...
naive OT-I (Supplemental Fig. 1, middle panel). It thus seems that the acquisition and maintenance of cytokine polyfunctionality requires an optimal, high-avidity TCR/pMHCI interaction.

**Influenza A viruses expressing N variants induce potent endogenous responses**

The in vitro analysis shown in Figs. 1 and 2 established that low-affinity pMHCI ligands induce the activation of OT-I cells, although less optimally than the wt peptide. Are, however, these mutant epitopes immunogenic in normal mice? As described for N (18), recombinant influenza A viruses were engineered to express either the E1 or the G4 variant within the viral NA. Naive B6 mice were then infected separately with 10^4 PFU of the x31-N, x31-E1, or x31-G4 viruses, and lung virus titers were measured (days 3, 6, and 9) by plaque assay (Fig. 3A). Both x31-E1 and x31-G4 had grown to a higher titer than x31-N by day 3 (p < 0.01). Within an additional 3 d, however, the lung titers for x31-N and x31-E1 were comparable and greater than those for x31-G4 (p < 0.01). All viruses were cleared by day 9 (Fig. 3A).

The K^N-, K^E1-, and K^G4-specific CD8^+ CTL populations were measured by tetramer staining on day 10 postinfection. All viruses induced tetramer^+ CD8^+ CTL populations that were readily detected in the spleen (Fig. 3B), demonstrating that there is indeed a TCR repertoire specific for each of these variant epitopes. Furthermore, K^G4 tetramer^+ CTL expansion induced by infection with x31-G4 was significantly greater in magnitude than the endogenous K^N-specific response (Fig. 3C; p < 0.05), possibly contributing to the faster rate of virus clearance (Fig. 3A).

Despite these quantitative differences, the K^N-, K^E1-, and K^G4-specific CTL sets were qualitatively similar in terms of multiple cytokine production profiles (Fig. 3D, 3E). In addition, all three CD8^+ CTL populations showed a similar capacity to produce IFN-γ in response to decreasing doses of the homologous peptide (Fig. 3F), suggesting that the spectrum of “functional avidity” was broadly comparable for these different TCR/pMHCI interactions.

**Minimal in vivo activation of OT-I CTLs by the x31-E1 and x31G4 viruses**

Graded numbers of OT-I cells were adoptively transferred into naive B6 recipients, which were then infected separately with 10^4 PFU of the x31-N, x31-E1, or x31-G4 viruses. The various OT-I populations were then analyzed on day 10 to determine the extent of in vivo expansion and functional activation (Fig. 4). As expected, x31-N induced substantial cell dose-related proliferation of the OT-I sets, but somewhat surprisingly after the findings from the in vitro experiments (Figs. 1, 2) and from the endogenous response profiles (Fig. 3), exposure to x31-E1 or x31-G4 did not cause any significant increase in OT-I T cell numbers (Fig. 4A). This was further confirmed from the CFSE dilution profiles assessed for labeled OT-I CTLs at day 3 or 10 postinfection (Fig. 4B). Furthermore, the lack of obvious x31-E1 or x31-G4 virus-induced OT-I expansion correlated with the maintenance of C6D2L expression (Fig. 4C) and the absence of any increase in IFN-γ production following ex vivo restimulation with the wt N peptide (Fig. 4D). Thus, we see completely different in vitro (peptide stimulation) and in vivo (virus infection) proliferation and functional activation profiles for OT-I T cells exposed to these mutant epitopes, although the control responses to the wt N peptide were equivalent for both situations (Figs. 1, 2, 4).

One possibility for the absence of any immediate recall of IFN-γ production (Fig. 4D) for the OT-I sets recovered from the x31-E1– and x31-G4–primed mice is that the T cells may have been
rendered anergic as a consequence of suboptimal activation with the variant peptides and were thus refractory to secondary stimulation (40). To test this in vivo, B6 mice that had been given OT-I T cells were primed i.p. with the PR8-N, PR8-E1, or PR8-G4 virus, then challenged i.n. 6 wk later with the serologically different (H3N2 versus H1N1) x31-N virus (Supplemental Fig. 2) or, following PR8-N, with the x31-E1 or x31-G4 variants (Supplemental Fig. 3). Respiratory exposure to x31-N induced a secondary OT-I response in the PR8-N–immune mice and also triggered what looked to be a primary OT-I response (Fig. 3) in those that were first exposed to PR8-E1 and PR8-E4 (Supplemental Fig. 2). Reversing the prime/challenge protocol indicated that x31-G4 induced some activation of PR8-N–primed memory T cells, although the effect was minimal (Supplemental Fig. 3). In summary, although the mutant peptides can stimulate an OT-I response in vitro (Figs. 1, 2), there are no indications that in vivo exposure induces either anergy or promotes an effective cross-reactive recall response.

Lack of in vivo OT-I activation by G4 is not due to CTL immunodomination

Considering that the nonresponsiveness to G4 in vivo is not due to the nature of infection, we next wondered whether the sizeable endogenous response to KβG4 impedes OT-I cells responding to G4, because CD8+ T cell responses to the same epitope can impede one another, a phenomenon known as immunodomination (41, 42). To examine this possibility, we reconstituted RAG–/– mice that lack mature T cells, with either whole B6 lymphocytes preparations or with lymphocyte preparations from which the Kβ G4-specific cells had been depleted (6, 25). Mice were reconstituted for 4 wk prior to adoptive transfer of OT-I cells and subsequent infection with x31-N or -G4 i.n. (Fig. 5A). Following x31-G4 infection, a robust KβG4-specific response was generated in mice reconstituted with whole lymphocytes, whereas mice that received depleted lymphocytes showed a significantly diminished KβG4-specific response (Fig. 5B). Depletion of KβG4-specific CD8+ T cells did not result in activation of naive OT-I cells in response to x31-G4, because OT-I cells did not accumulate (Fig. 5C) and did not undergo greater division than naive controls (Fig. 5D). Thus, the endogenous KβG4-specific CD8+ T cell response does not impact upon the OT-I response to G4, and the inability of OT-I cells to respond to G4 is likely to be T cell intrinsic.

Probing alternative vaccination strategies with the N variants

The difference between the findings for in vitro (Figs. 1, 2) and in vivo (Figs. 3, 4, Supplemental Figs. 2, 3) activation suggests that the levels of pMHC epitope encountered by T cells exposed to peptide-pulsed versus in vivo-infected APCs may be very different. Naive B6 mice were thus primed with peptide-pulsed, LPS-activated BMDCs (Fig. 6A, 6B), and the KβN- and KβG4-specific responses were measured by tetramer staining. Although vaccination with G4-peptide–pulsed BMDCs induced an endogenous Kβ G4-specific response (Fig. 6A), it failed to trigger naive OT-I cells (Fig. 6B, 6C). This is in contrast the OT-I response in mice that received activated BMDCs pulsed with the wt N peptide (Fig. 6B, 6C). This is not due to an inability of OT-I cells to respond to
FIGURE 6. Alternative approaches for low affinity ligand presentation do not modify the in vivo activation profiles for OT-I T cells. A–D, Naive B6 mice were given $10^6$ naive OT-I cells and LPS-activated N-, G4-, or unpulsed BMDCs. The endogenous responses to the N and G4 peptides were measured by tetramer staining (A) of the recipient Ly5.1+CD8α+ T cells 8 d after the BMDC transfer, when the numbers (B) and CD62L profiles (C) were also measured for splenic OT-I T cells (D) OT-I cells ($2 \times 10^3$) were stimulated in vitro at a 5:1 ratio with N-, E1-, or G4-pulsed DCs, and the extent of proliferation was determined 3 d later by CFSE dilution. Data shown are mean + SEM ($n = 4$). E–H, Naive B6 mice were given $10^6$ CFSE-labeled OT-I cells 1 d prior to vaccination with 25 nmol lipopeptide-N, -E1, or -G4, or i.n. infection with x31-N virus. On day 9, the extent of CFSE dilution was determined for OT-I T cells 8 d after the BMDC transfer, with 25 nmol lipopeptide-N, -E1, or -G4, or i.n. infection with x31-N virus. Data shown are mean + sem ($n = 2–3$) and are representative of two independent experiments.

G4 presented by DCs, because N- and G4-pulsed LPS-activated ex vivo isolated DCs were able to induce proliferation by naive OT-I cells in vitro (Fig. 6D).

Priming naive mice with peptide bound (20) to a Pam2Cys lipid moiety (lipopeptide) activates DCs to induce potent wt CTL response (17, 43, 44) via TLR-2 ligation (17). Naive B6 mice were given OT-I cells, then vaccinated with then N-, E1-, or G4-lipopeptide constructs (Fig. 6E–H). As expected, the N-lipopeptide induced strong proliferation of the OT-I CTL (Fig. 6E). Interestingly, administration of both the E1- and G4-lipopeptides was also associated with limited division of the OT-I CTL sets within the draining lymph node (Fig. 6F, 6G) and caused some decrease (at least for E1) in cell surface CD62L expression (Fig. 6H), although this level of activation and proliferation did not lead to increased localization to the lung (Fig. 6G). Still, the lipopeptide formulations were more effective than the peptide-pulsed BMDCs at replicating the in vitro peptide cross-stimulation profile (Figs. 1, 2) in the in vivo situation.

TCR/pMHCI affinity differences and the level T cell activation in vivo

Infection with recombinant L. monocytogenes expressing the SIHQEKLL (Q4) mutant activates naive OT-I T cells in vivo (9) to levels greater than those seen for the E1 and G4 peptides used in this study (Figs. 3–6), although to a lesser extent than that observed for the wt N. Would we see the same thing if the Q4 response driven via infection with a modified influenza A virus? Mice that received adoptively transferred, naive OT-I CD8+ T cells (Fig 3) were thus infected i.n. with the engineered x31-Q4 virus. As expected from the L. monocytogenes study, x31-Q4 infection does activate OT-I cells, with the extent of early (to day 3) CTL division in the MLN being remarkably similar for mice exposed to x31-N or x31-Q4 (Fig. 7A). However, as observed previously by Zhen and colleagues following bacterial challenge (13), the extent of clonal expansion in the longer term was clearly diminished, with the magnitude of the OT-I response in the spleen on day 7 being significantly lower for the mice given x31-Q4 rather than x31-N (Fig. 7B; $p < 0.01$). Also, although no difference was observed for the cytokine production profile following high-dose N peptide restimulation in vitro (Fig. 7C), significantly more of the x31-Q4–primed OT-I cells remained CD62Lhi (Fig 7D; $p < 0.01$). A single experiment (data not shown) confirmed the observation made with the Listeria model that the OT-I T cells exit the lymph node more rapidly in the x31-Q4–primed mice, leading do an overall decrease in the extent of clonal expansion.

Do these response profiles (Figs. 3–7) correlate with the spectrum of “functional avidity” measured by IFN-γ production for in vivo-primed (day 10 x31-N) OT-I cells following in vitro stimulation with graded doses of the N, G4, or Q4 peptides (Fig. 7E)? Such effector OT-I CTLs showed 50% maximal IFN-γ responses at the $10^{-10}$, $10^{-9}$, and $10^{-8}$ M peptide concentration for the N, Q4, and G4 peptides, respectively (Fig. 7E). Stimulation of OT-I CTL with G4 had not yet reached saturation (Fig. 7E). Thus, the 50% maximal IFN-γ response at $10^{-7}$ M for the G4 peptide is likely underestimated. Furthermore, the spectrum of in vitro persistence for OT-I cells stimulated with graded doses of the N, Q4, and G4 peptides shows an equivalent hierarchy (Fig. 7F). Naive OT-I T cells failed to survive for 3 d following exposure to $10^{-7}$ M G4, whereas comparable Q4- and N-peptide–stimulated CTLs remained viable after stimulation with much lower doses ($10^{-8}$ and $10^{-9}$ M, respectively). This in vitro evidence that the avidity of the TCR/pMHCI interaction for G4 falls
MHC, with the side-chain hydroxyl group making two hydrogen bonds with p63-Glu in the MHC (Supplemental Fig. 4C, 4D). Interestingly, there was loss of two direct bonds with the Glu63 in the MHC in the KαE1 complex (Fig. 8B). Furthermore, the E1 side chain extended out of the MHC groove, becoming more solvent exposed in a manner that would likely impact OT-I TCR binding. Overall, the structural analysis indicates that substitution of p1 S to E within the N peptide may result in decreased peptide stability as a result of the loss of contacts around the secondary anchor residue, which could in turn impact TCR recognition and lead to lower avidity interactions.

The KαG4 complex also showed a highly similar structure to the KαN, with a root mean square deviation of 0.21Å within the MHC α1–α2 domains (Fig. 8B). Although most hydrogen bonds with the peptide main chain were conserved, there was loss of one direct hydrogen bond to the Arg155 (Fig. 8B). This MHCα2 residue has been described as a key MHC contact residue for all TCR/pMHC ternary complexes solved to date (45–47). Again, mutation of the p4N to G results in loss of an essential solvent exposed TCR contact. Taken together, this substitution may impact on TCR recognition via direct and indirect conformational changes.

An implication of the above results is that E1 and G4 may destabilize the peptide within the MHC groove because of the loss of specific hydrogen bonds between the variant peptides and the MHC (Fig. 8). To determine whether this was indeed the case, the thermostability of each complex was determined. The KαN complex shows high thermostability with a Tm point of 57.9°C, whereas the KαE1 and KαG4 complexes showed lower Tms of 50.2 and 50.3°C, respectively (Table II), indicative of less stable pMHC complexes. To determine whether this impacted cell surface stability of pMHC complexes, APL stabilization of Kα was determined by RMA-S assay using graded doses of peptide (Fig. 9). The Q4 and G4 peptides were able to stabilize Kα cell surface expression to a similar degree as the WT N peptide (Fig. 9A). Thus, for G4, the thermodynamic stability does not reflect ability to stabilize cell surface pMHC expression. In contrast, a greater concentration of the E1 peptide was required to obtain 50% maximal Kα stability when compared with the WT N peptide (Fig. 9A). Despite demonstrating a similar Kα stability profile to WT N peptide, the G4 peptide demonstrated a diminished capacity to stimulate effector OT-I CTL (Fig. 9B). Moreover, although the E1 peptide could stabilize pMHC cell surface expression, albeit less efficiently than WT N peptide, there was a lack of OT-I activation (Fig. 9B). Thus, combined with structural analysis, these data suggest that the inability of these variant peptides to activate naive OT-I CTLs in vivo likely reflects abrogation of optimal TCR recognition, rather than decreased pMHC stability postinfection.

**Discussion**

Stimulating naive OT-I CTLs in vitro demonstrated that, although low-affinity pMHC/TCR interactions can trigger clonal expansion, both survival and the acquisition of effector function are highly dependent on the quality of the inductive signals. Exposure to the agonist N peptide induced robust effector proliferation with rapid and sustained acquisition of polyfunctional cytokine production. The integration of multiple signals is clearly central to this induction of full effector CTL differentiation, because the absence of costimulation gave rise to OT-I cells that, despite robust proliferation and survival in culture, were functionally limited. Previous studies using the mutant G4 peptide as a stimulus have demonstrated that, whereas OT-I cells may be partially activated by KαG4, they do not persist (11).

Furthermore, although stimulation with the mutant G4 and E1 peptides induced CD44 expression, the level of surface CD62L...
staining remained high after exposure to the low-affinity E1 peptide. Also, in contrast to the wt N peptide, exposure to E1 in the presence of concurrent CD28 costimulation failed to rescue either sustained OT-I proliferation or to initiate the sustained acquisition of multiple cytokine expression. The findings from this in vitro analysis are thus in accord with the notion that only high-avidity TCR/pMHCI interactions are capable of triggering CTLs in a way that is both optimal and sustainable.

Our data support earlier observations (39) that strong TCR signals can induce the production of TNF-α within 5 h of exposing naive CD8⁺ T cells to a potent inductive stimulus, in this case, the cognate N peptide. This same functional capacity is not triggered by suboptimal (G4 or E1) OT-I TCR/pMHCI interactions, perhaps reflecting decreased proliferation as a consequence of diminished autocrine cytokine production (48, 49). Interestingly, our data suggest that added exogenous IL-2 is relatively dispensable for the acquisition and maintenance of cytokine synthesis by CD8⁺ T cells that are induced in culture via a high (N), although not a low (G4), avidity TCR/pMHCI interaction. This agrees with a recent publication reporting that greater exposure to IL-2 during the course of CD8⁺ T cell activation did not increase cytokine production. However, this same study also demonstrated that the expression of perforin and granzyme B was greatly enhanced by IL-2 (50). The obvious implication is that the various CTL effector functions may be regulated via different signaling pathways.

A striking finding was that although CD28 ligation was key to robust, sustained effector function following stimulation with the cognate N peptide, we also observed more limited initial proliferation and maintenance of a CD62L⁺ phenotype when this pathway was engaged. The maintenance of cell surface CD62L expression may be important to ensure that fully activated CTLs that produce multiple cytokines do not leave the lymph nodes and traffic to the site of infection too early. This limitation of OT-I cycling with CD28 engagement is, however, at odds with previous reports suggesting that costimulation promotes the proliferative capacity of CD8⁺ T cells (51). It is also possible, of course, that the effect observed in this study reflects the high concentrations of peptide and anti-CD28 mAb used in this in vitro system, leading to a situation that may never be seen under the physiological conditions that apply in the responding lymph node.

The OV A257–264 APLs Q4, G4, and E1 exhibit a spectrum of ligand potency for the OT-I TCR that correlates with different outcomes for immature OT-I T cell development. Although both the agonist OVA257–264 and weak agonist Q4 result in negative selection (15), the low-affinity E1 and G4 support positive selection of OT-I TCR transgenic T cells (15, 16). Interestingly, despite being able to support positive selection, infection of mice with recombinant influenza A viruses expressing either the G4 or E1 variant peptides failed to induce the activation of adoptively transferred naive OT-I cells. Our data indicate that OT-I recognition of the G4 and E1 peptides falls below a threshold required for inducing efficient T cell activation (52). The analysis of Zehn et al. (9) found that other p4 variants can activate OT-I T cells, and in particular, V4 was shown to induce a very small OT-I response with the expected consequences for diminished CTL expansion and effector function. Although V4 is slightly more immunogenic for OT-I T cells, the in vitro situation (13) was generally similar to that found for G4 in our study. Given these peptides represent the lower spectrum of APL potency for the OT-I TCR (15), these two ligands likely fall the minimal threshold for TCR-mediated OT-I CD8⁺ T cell activation under in vivo conditions. Importantly, a potential implication of these data are that self-ligands that support positive selection of a mature T cell repertoire are in themselves unlikely to be able to initiate activation of these selected T cells in the periphery as they fall below the affinity threshold for mature T cell activation.

The lack of OT-I responsiveness was not due to diminished antigenicity, because these viruses induced robust, endogenous CTL responses. Furthermore, competition for Ag was not obviously a factor, because the removal of K⁺G4-specific naive

### Table I. Data collection and refinement statistics

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<td>100 K</td>
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1Rmerge = Σ |hkl - < hkl > | / Σ |hkl |
2Rmerge = Σ | Fobs, | - | Fcalc, | / Σ | Fobs, |

Values in parentheses are for highest resolution shell.

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precursors did not restore the OT-I response. Also, the OT-I cells rendered anergic by exposure to the low-avidity KbG4 epitope, because they could be efficiently recalled when the mice were challenged 6 wk later with the x31-N virus. In addition, memory OT-I cells were not triggered by infection with x31-G4, suggesting that the heightened sensitivity characteristic of CD8+ T cell memory does not overcome the OT-I TCR/pMHCI signaling threshold required for effective CTL activation. This lack of recall was not a consequence of some immunosuppressive effect resulting from influenza A virus infection, because the x31-Q4 virus triggers robust OT-I responses. In fact, even in the context of peptide-pulsed BMDCs, KbG4 could not induce an OT-I response, suggesting that, whereas the OT-I TCR can to some extent be engaged by high concentrations of G4 peptide encountered in vitro, these conditions are not replicated in the in vivo situation.

An interesting observation was that administration of the E1 and G4 as lipopeptides did induce some OT-I proliferation, although not resulting in full effector CTL differentiation. An implication is that targeting of TLR2 (44), either on the DC or on the T cell, somehow lowers the affinity threshold enabling activation of OT-I CTL. A recent report has suggested that TLR2 signaling can enhance CTL activation and effector function in response to peptide activation (53). It is tempting to speculate that in our model, TLR2 targeting on OT-I lowers the threshold of activation and promotes activation in response to low-affinity E1 and G1 APLs. In support of this, we have previously reported (43) that lipopeptide vaccination resulted in a broader responding T cell repertoire due to recruitment of CTL with suboptimal TCR characteristics. Our interpretation of these data is that TLR2 signaling enabled activation and recruitment of low-affinity T cell clones, although this still remains to be tested.

What may influence this threshold is the level of pMHCI stability (54, 55). Earlier experiments have suggested that the reduced activation of naive CD8+ T cells by altered peptide ligands reflects that formed pMHCI complexes are less robustly presented at the cell surface (56). The decreased thermodynamic stability of both the KbE1 and KbG4 complexes (when compared with KbN) may reflect the loss of some hydrogen bonds that potentially impact the density and persistence of epitope presentation postinfection. This may be a factor in the inability of KbE1 and KbG4 to activate naive OT-I CTLs following in vivo priming. However, in agreement with others (38), we found the extent of N, G4, and E1 stabilization of Kb on RMA-S cells to be largely equivalent. As such, diminished thermostability does not necessarily translate to loss of stability at the cell surface. More importantly, we demonstrated that despite an ability to stabilize Kb cell surface expression but demonstrate diminished capacity to stimulate effector OT-I, RMA-S cells were incubated at 27°C overnight and then pulsed with graded concentrations of the N, E1, G4, or Q4 peptides for 1 h. The cells were then transferred to 37°C for 2 h, and Kb expression was detected by staining with the Kb-specific mAb (clone Y3) by flow cytometry. A. Shown is the mean fluorescence intensity (MFI) of Kb-specific staining on peptide-pulsed RMA-S cells. B. The RMA-S cells were then used as APCs to stimulate IFN-γ production by day 10 effector OT-I CTL. IFN-γ was detected by intracellular cytokine staining after 5 h of stimulation, and the percentage of IFN-γ+ effector OT-I of input cells was determined by flow cytometry.

Table II. Thermostability of different Kb complexes

<table>
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<th>Complex</th>
<th>Tm (°C)</th>
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<tr>
<td>KbN</td>
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<tr>
<td>KbE1</td>
<td>50.2 ± 1.9</td>
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<tr>
<td>KbG4</td>
<td>50.3 ± 1.6</td>
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E1 and K\(^B\)G4 epitopes, demonstrates that the major factor in the failure of these pMHC complexes to activate OT-I CTL appears to be at the level of the OT-I TCR/pMHC interaction.

In fact, the peptide substitutions analyzed in this study are associated with relatively minor alterations in pMHC topology, reflected in the addition or loss of prominent side-chain residues for the E1 and G4 peptides, respectively. In particular, P4-Asn has been previously described as a TCR contact residue (57), so substitution with a Gly at this location may be expected to result in compromised OT-I TCR ligation and subsequent poor activation. Interestingly, the structural analysis indicates that the Asn to Gly change at position 4 resulted in a small but significant modification of the side-chain contacts made to Arg\(^{155}\) within the MHC\(\alpha_2\) helix. Position 155 is invariably contacted in all the TCR/pMHC ternary structures solved to date and has been proposed as a “gate keeper” residue that is important for efficient TCR docking (45, 46, 58). Thus, it is tempting to speculate that any alteration in Arg\(^{155}\) conformation is sufficient to compromise efficient OT-I TCR binding onto the pMHC complex. In the case of the E1 peptide, the introduction of a prominent Glu side chain that extends out from the pMHC clef likely interferes with OT-I TCR ligation, leading to a low-avidity K\(^B\)E1/OT-I TCR interaction (34, 35) and consequent diminished CTL activation.

The present analysis thus establishes that small changes in peptide sequence that cause limited structural alteration can lead to low-avidity TCR/pMHC ligation and inefficient T cell activation. The substitution of key contact TCR contact residues and consequent modification in atomic contacts are sufficient to render an low-avidity TCR/pMHCI ligation and inefficient T cell activation.

Peptide substitutions analyzed in this study are as-

References


Acknowledgments

We thank Dr. Nicole La Gruta and Prof. Lorena Brown (University of Melbourne) for reagents and helpful discussion. We thank the staff at the Australian Synchrotron for assistance with data collection.

Disclosures

The authors have no financial conflicts of interest.


Supplementary Figure 1. OT-I cells divide in response to N variants when IL-2 is present. CFSE-labeled OT-I T cells (2x10^6) were incubated with 1μM N, G4 or E1 peptide with either 5μg/ml anti-CD28 (red) or 10U/mL IL-2 (grey) in vitro. At two, three and four days after stimulation, the division of OT-I cells was determined by CFSE dilution. Data shown are are representative of three independent experiments.
Supplementary Figure 2. Naïve OT-I are not rendered anergic by E1 and G4 in vivo. OT-I cells (10⁶) were adoptively transferred into congenic B6 hosts one day prior to priming with 1.5x10⁷ PFU PR8-N, -E1 or -G4 virus. Six weeks later mice were challenged with 10⁴ PFU x31-N and the recall of OT-I cells was assessed 8d later. The number of OT-I cells in the spleen (A) and BAL (B), CD62L phenotype of splenic OT-I cells (C), and proportion of IFNγ+ cells also producing TNFα was determined following ex vivo re-stimulation of splenic OT-I with 1μM N peptide (D) were determined. Data shown are mean±sem (n=3-4). * p<0.05, ** p<0.01
Supplementary Figure 3. Memory OT-I cells can be partially recalled by x31-G4 in vivo. OT-I cells (10^7) were adoptively transferred into congenic B6 hosts one day prior to priming with 1.5x10^7 PFU PR8-N virus. Six weeks later mice were challenged with 10^4 PFU wt x31 or x31-N, E1, or -G4 and the recall of memory OT-I cells was assessed 8d later. OT-I cells in the spleen (A) and BAL (B) were enumerated, and the CD62L phenotype of OT-I cells from the spleen (C) and BAL (D), and proportion of IFNγ+ cells producing TNFα was determined following ex vivo re-stimulation of splenic OT-I with N peptide (E) were determined. Data shown are mean±sem (n=3) and are representative of two independent experiments. *p<0.05.
Supplementary Figure 4. Conformation of variant peptides bound within H-2K^b. The conformation of the peptide is shown from the side (A, C, E) and above (B, D, F) the electron density map at 1 σ. N shown in green and H-2K^b shown in pale green (A, B), E1 shown in blue and H-2K^b shown in pale blue (C, D) and and G4 shown in pink with the H-2K^b shown in pale pink (E, F). A2 helix has been removed from all images, and mutated residues are shown in yellow (C-F).