JCI insight

New epitopes in ovalbumin provide insights for cancer neoepitopes

Sukrut Hemant Karandikar, … , Alan Jerry Korman, Pramod Kumar Srivastava

JCI Insight. 2019. https://doi.org/10.1172/jci.insight.127882.

Research In-Press Preview **Immunology**

MHC I-restricted epitopes of chicken ovalbumin (OVA) were originally identified using CD8 T cells as probes. Here, using bioinformatics tools, we identify four additional epitopes in OVA in addition to a cryptic epitope. Each new epitope is presented in vivo, as deduced from the lack of CD8 response to it in OVA-transgenic mice. In addition, CD8 responses to the known and novel epitopes are examined in C57BL/6 mice exposed to the OVA-expressing tumor E.G7 in several ways. No responses to any epitope including SIINFEKL are detected in mice with growing E.G7 or mice immunized with the tumor. Only in E.G7-bearing mice treated with an anti-CTLA4 antibody which depletes tumor-infiltrating regulatory T cells, CD8 responses to SIINFEKL and the novel epitope EKYNLTSVL are detected. Finally, all epitopes fails to treat mice with pre-existing tumors. These observations force an important re-consideration of the common assumptions about the therapeutic value of neoepitopes detected by CD8 responses in tumor-bearing hosts.

New epitopes in ovalbumin provide insights for cancer neoepitopes

Sukrut Hemant Karandikar[1], John Sidney[2], Alessandro Sette[2], Mark Joseph Selby[3], Alan Jerry Korman[3] and Pramod Kumar Srivastava[1,4]

1 Department of Immunology, University of Connecticut School of Medicine, Farmington, CT 06030
2 Division of Vaccine Discovery, La Jolla Institute of Allergy and Immunology, La Jolla, CA 92037
3 Bristol-Myers Squibb Company, Redwood City, CA 94063
4 Carole and Ray Neag Comprehensive Cancer Center, University of Connecticut School of Medicine, Farmington, CT 06030

Correspondence: Pramod K. Srivastava: Srivastava@uchc.edu

CONFLICT OF INTEREST STATEMENT

SHK has declared that no conflict of interest exists.

ABSTRACT

MHC I-restricted epitopes of chicken ovalbumin (OVA) were originally identified using CD8 T cells as probes. Here, using bioinformatics tools, we identify four additional epitopes in OVA in addition to a cryptic epitope. Each new epitope is presented in vivo, as deduced from the lack of CD8 response to it in OVA-transgenic mice. In addition, CD8 responses to the known and novel epitopes are examined in C57BL/6 mice exposed to the OVA-expressing tumor E.G7 in several ways. No responses to any epitope including SIINFEKL are detected in mice with growing E.G7 or mice immunized with the tumor. Only in E.G7-bearing mice treated with an anti-CTLA4 antibody which depletes tumor-infiltrating regulatory T cells, CD8 responses to SIINFEKL and the novel epitope EKYNLTSVL are detected. Finally, all epitopes fails to *treat* mice with pre-existing tumors. These observations force an important re-consideration of the common assumptions about the therapeutic value of neoepitopes detected by CD8 responses in tumor-bearing hosts.

INTRODUCTION

The antigen processing machinery enables surveillance of the intracellular protein milieu by $CD8^+$ T cells. The intracellular proteins undergo partial proteolysis. The peptides, mostly eight to 11 amino acids in length, generated by proteolysis, are loaded onto MHC class I molecules and presented on the cell surface (1). Recognition of the MHC-presented peptides by $CD8^+$ T cells endows them with the status of an epitope. The characteristic of a peptide to serve as an epitope was termed by Sercarz as epitypicity (2, 3), and we use this term in that same meaning. Epitypicity is the same as immunogenicity with the nuance that it is not whole antigens but fragments of antigens that are recognized by the immune system.

Tumors result from driver events deregulating cell division. With progression, tumors acquire additional driver or passenger mutations. The antigen presenting machinery presents normal as well as mutated peptides (neoepitopes) on MHC I molecules to cognate $CD8^+$ T cells (4–6). Current efforts at designing personalized cancer vaccines seek to exploit the inherent immunogenicity of tumors by identifying tumor-specific neoepitopes using high throughput genomics and bioinformatics, and by directing $CD8^+$ T cell responses in the patients against the identified neoepitopes. The success of these efforts depends upon accurate definition of the epitopes that make tumors immunogenic, and immune response to the neoepitopes which can protect a host against tumor growth.

Here, we have used the model antigen ovalbumin (OVA) as a tumor-specific antigen. Since tumor-specific antigens are overwhelmingly mutated self-antigens, which are indeed non-self or foreign with respect to the germline, the choice of chicken OVA (which is also a foreign or non-self-antigen for mice) as a tumor-neoantigen is reasonable. We have used the very

same bioinformatics tools that are now used to predict potential cancer neoepitopes, to identify the immunogenic epitopes of OVA; thus far, this has been accomplished purely through the use of anti-OVA CD8 T cells. We have analyzed the immunogenicity of the two known and four newly identified epitopes of OVA by immunizing mice with individual peptides or whole OVA and testing the CD8 response on peptide-pulsed targets. We have then analyzed the CD8 T cell response to each individual epitope of OVA in mice with progressively growing as well as regressing OVA-expressing tumor E.G7 (7). Our studies reveal significant lessons for our current efforts to identify neoepitopes that may be used to immunize cancer patients with a view to elicit immunological protection from tumor growth.

RESULTS

Novel putative immunogenic peptides of OVA

The primary sequence of OVA was analyzed using NetMHC (3.0). (NetMHC 4.0 identifies four additional peptides which were not tested.) Any eight, nine or 10 amino acid-long sequence with a potential to bind K^b or D^b (i.e. Profile Weight Matrix or PWM score > the default threshold score 7.61 for K^b and 8.08 for D^b, *or* a predicted IC50 < the default threshold value 500 nM for either allele) was considered a putative epitope. Nineteen sequences fit the PWM, or IC50 or both criteria. All three previously reported epitopes (8–10) are included in this group of 19 and are predicted to have the potential to bind K^b, but not D^b (Table 1). Thus, we identified 16 novel potential epitopes for K^b or D^b. Of these, 10 putative epitopes were predicted to bind K^b alone, four to D^b alone, and two to K^b as well as D^b (Fig. 1A).

Immunogenicity of novel peptides of OVA

Ability of each of the 19 peptides within OVA (Table 1 and Fig 1A) to elicit $CD8^+$ T cell responses in C57BL/6 mice was tested. In order to determine the appropriate dose of peptide for effective immunization, SIINFEKL (257-264) was used as a guide. Naïve C57BL/6 mice were immunized with doses of peptide 257-264 (emulsified in an adjuvant) varying from 1 μg to 100 μg per mouse. All doses of immunization elicited clear CD8 responses (data not shown). Following immunizations were performed at a dose of 10 μg peptide per immunization, emulsified with TiterMax, injected in the footpad of naïve C57BL/6 mice. Seven days later, the draining lymph nodes (dLN) were harvested, and the single cell suspensions generated were stimulated in vitro for 12 h with the immunizing peptide or not stimulated. All 19 peptides were tested (Fig. 1B). As expected, peptides 55-62 (9) and

SIINFEKL were immunogenic (Fig. 1B). Four out of the 16 novel, predicted peptides of OVA (peptides 27-35, 97-105, 208-216 and 256-264), which have not been reported to be immunogenic before, were noted to elicit significant IFNγ secreting, $CD44^{hi}$, $CD8^{+}$ T cells (Fig 1B).

As typical examples of these experiments, expression of IFNγ by the $CD44^{hi}$ $CD8^{+}$ T from the immunized mice in response to peptide re-stimulation was tested using peptides 176-183 (reported earlier), 208-216 (a novel epitope) and the well-known SIINFEKL (Fig 1C). SIINFEKL was clearly immunogenic, but the peptide 176-183 which has been reported to be immunogenic by CD8 cytotoxicity assays previously (el-Shami et al., 1999; Lipford et al., 1993), was not observed to be immunogenic by the IFNγ assay. The novel putative epitopes, peptide 208-216, was significantly immunogenic (Fig. 1C).

Among all the predicted K^{b}-binding peptides, 214-222 has the strongest predicted affinity (Table 1) but this peptide was not observed to be immunogenic. SIINFEKL and peptide 208-216 are the peptides with the next strongest predicted affinities, and they are both immunogenic. The other known epitopes as well as the novel peptides shown in Fig. 1B have moderate predicted for K^{b} (170-393 nM IC_{50}). The remaining 12 peptides had a range of affinities for K^{b} (17-13639 nM IC_{50}), but were not immunogenic. None of the four novel predicted peptides have a significant affinity for D^{b}.

Of the four new antigenic peptides, one (256-257) is a single n-terminus amino acid extension of the peptide 257-264. In order to test whether 256-264 and 257-264 are immunologically distinct, mice were immunized with 256-264 or 257-264. $CD8^{+}$ T cells from

mice immunized with any one peptide recognized both the peptides indicating 256-264 and 257-264 are cross-reactive and not immunologically distinct (data not shown).

Epitypicity of novel peptides in context of immunization with whole OVA

The epitypicity of all predicted epitopes was now tested in the context of immunization with whole OVA protein, in contrast to the context of immunization with individual peptides as tested in Fig. 1. Naïve mice were immunized with an emulsion containing an adjuvant and 450 μg of OVA, the approximate molar equivalent of 10 μg peptide. Control mice were immunized with an emulsion lacking the antigen. The cells from the harvested dLNs of immunized mice were stimulated with each of the 19 individual peptides (Table 1) or not stimulated; the expression of IFNγ by $CD44^{hi}$ $CD8^+$ T cells in response to stimulation in vitro was tested (Fig. 2A). As expected, peptides 55-62 and SIINFEKL successfully stimulated the $CD8^+$ T cells from OVA-immunized mice (Fig. 2A). Interestingly, peptide 176-183 (previously reported to be immunogenic by el-Shami et al., 1999; Lipford et al., 1993) which was non-immunogenic upon peptide-immunization (Fig. 1B) was observed to be non-functional as an epitope upon OVA-immunization as well. Among the novel peptides, peptides 97-105, 99-107, 250-258, 256-264 and 289-297 stimulated OVA-primed $CD8^+$ T cells. Mice immunized with the emulsion lacking the adjuvant (not shown) had no $CD8^+$ responses to OVA. Peptide 256-264 was excluded from consideration in the following experiments due to its immunological similarity to SIINFEKL.

Table 2 summarizes the relationship of all six novel sequences (and the two previously known epitopes) with their immunogenicity. Of the four novel peptides observed to be epitypic in the context of immunization with the whole OVA, one (97-105) had also been

observed to be weakly immunogenic in the context of immunization with individual peptides (Fig. 1B). The remaining three were not immunogenic by peptide-immunization but are immunogenic by protein-immunization (i.e. epitypic). Conversely, of the three peptides observed to be immunogenic by peptide-immunization, two (27-35 and 208-216) are observed to be non-epitypic in the context of whole OVA-immunization (Fig. 2A). Since peptides 27-35 and 208-216 are immunogenic as peptides but not recognized by the natural immune response to OVA, they are likely to be cryptic by definition (11).

Peptides must demonstrate sufficient binding affinity for the expressed MHC I alleles in order to be recognized by the $CD8^+$ T cells. MHC I-peptide binding with IC50 value of 500 nM or less is considered strong and peptides capable of such interactions are more likely to be immunogenic as per the current consensus. Most of the nineteen peptides tested here that are recognized by $CD8^+$ T cells: 27-35, 55-62, 97-105, 208-216, 257-264 and 289-297 have strong affinity for K^b as measured using competitive binding assays (Table 1 and Fig. 1B and 2A). Peptide 99-107 binds D^b with an IC50 of 572 nM which is very close to the threshold of 500 nM. However, peptide 250-258 does not adhere to the current paradigm and shows weak affinity for both K^b and D^b (IC50 of 5110 nM and 17967 respectively) despite being recognized by $CD8^+$ T cells (Table 1 and Fig. 2A).

CD8 responses to 55-62, 97-105, 99-107, 257-264 and 289-297 were also tested in mice immunized with irradiated E.G7-OVA (E.G7) cells. E.G7 is a murine thymoma line derived from EL4 and constitutively expresses whole OVA. Control mice were immunized with the protein. Surprisingly, responses to EL4 cells, E.G7 cells or any of the tested epitopes of OVA were not detected in mice inoculated with irradiated E.G7 cells (Figure 2B; blue bars). As

expected, $CD8^+$ T cells from the mice immunized with OVA protein recognized E.G7 cells but not EL4 cells. Control mice also showed responses to OVA as observed previously (Fig. 2B; red bars).

Antigenicity of extended precursors of non-immunogenic precise epitopes

Peptides 99-107, 250-258 and 289-297 were noted to be epitypic (Fig. 2A) but not immunogenic (Fig. 1B). The possibility that longer precursors of peptides 99-107 and 250-258 may be antigenic, was tested. C57BL/6 mice were immunized with long peptides in which the precise peptide was flanked by 10 (10-90-10 or 10-250-10) or 20 (20-90-20 or 20-250-20) naturally flanking amino acids on both termini. Control mice were immunized with the precise peptide or whole OVA. $CD8^+$ T cell response was tested by stimulating the dLN cells with the corresponding precise peptide. It was observed that peptide 250-258, which was not immunogenic by itself as also seen in Fig. 1B, was not immunogenic here as well, but its 10-amino acid and 20-amino acid extended variants were (Fig. 2C). Interestingly, extended versions of peptide 99-108 did not elicit $CD8^+$ T cells, and its even longer variants were not tested. Longer variants of peptide 289-297 were not tested.

Epitopes of OVA present within endogenous antigens induce immunological tolerance.

Peptides 36-43 and 214-222 (Table 1) were observed to be non-immunogenic despite having strong affinities for K^b with IC50 values of 20 nM and 39 nM respectively. The possibility of their homology with sequences in the mouse proteome was considered. Peptides 36-43 and 214-222 were aligned with all the mouse (taxid:10090) non-redundant protein sequences using BLAST. Sequences identical to OVA peptide 36-43 but not peptide 214-222 were

observed to be present in two mouse proteins, leukocyte elastase inhibitor c and serpin B8, both being members of the Serpin superfamily (Fig 3A). Indeed, OVA is also known to be a member of the Serpin family (12). This observation suggests that the lack of immunogenicity of peptide 36-43 may result from negative selection during thymic maturation. Lack of immunogenicity of peptide 214-222 may be due to a possible hole in the repertoire that does not result from negative selection.

We argued that expression of OVA by mice as an endogenous self-antigen should also induce tolerance towards all MHC I-restricted epitopes of OVA. Act-mOVA mice express a membrane bound form of OVA ubiquitously (13). To test whether Act-mOVA mice are tolerized towards OVA, immunizations were performed with complete OVA. C57BL/6 mice were also immunized with complete OVA as positive controls. $CD8^+$ T cell responses were tested in OVA- immunized mice against epitopes 55-62, 97-105, 99-107, 257-264 and 289-297. As hypothesized, $CD8^+$ T cell responses were not observed against any of the tested epitopes of OVA in Act-mOVA (Fig. 3B; blue bars). As observed previously, immunizations with OVA elicited $CD8^+$ T cell responses against each of the tested peptides in C57BL/6 mice (Fig 3B; red bars). Presumably, the lack of response to any of the OVA epitopes in Act-mOVA mice derives from deletion of the T cells recognizing those "self-epitopes", thus indicating that all the epitopes including the epitopes newly identified by us here, are physiologically presented.

Cryptic epitopes, by definition, undergo intra-epitope proteolysis during the processing of the antigen. As a result, cryptic epitopes are not presented on MHC I and consequentially, cryptic epitopes should neither elicit CD8 responses upon immunization with exogenous

antigens harboring the cryptic epitopes nor induce immunological tolerance when present within “self-antigens”. However, synthetic peptides mimicking cryptic epitopes, which do not require intracellular processing, elicit CD8 responses upon immunization. Immunization with OVA, which is an exogenous antigen to mice, does not elicit CD8 responses against peptides 27-35 and 208-216 (Fig 2A) but the synthetic peptides are immunogenic (Fig 1B). We hypothesized peptides 27-35 and 208-216 to be cryptic. Act-mOVA mice, which are tolerized towards all epitopes of OVA (Fig 3B), including epitopes 55-62 and 257-264, were immunized with peptides 27-35, 55-62, 208-216 and 257-264 emulsified in TiterMax; C57BL/6 mice were immunized as controls. All peptides elicited strong responses in C57BL/6 mice as observed previously. In Act-mOVA mice, CD8 responses to peptides 55-62 and 257-264 were not observed as expected (Fig 3C). As hypothesized, peptide 208-216 elicited significant responses in Act-mOVA mice, which is in accordance with the nature of cryptic epitopes.

Surprisingly, immunized Act-mOVA mice did not show CD8 responses against peptide 27-35. Two non-mutually exclusive possibilities, not tested here, might explain this observation. The epitope is obviously presented directly, but it may be unable to be cross-presented. It may be a sub-dominant epitope as classically defined; the reasons for sub-dominance are of course unclear.

Epitypicity of novel peptides in contexts of an OVA-expressing tumor

$CD8^+$ responses to five epitopes of OVA: 55-62, 97-105, 99-107, 257-264 and 289-297 were tested in mice with progressively growing E.G7 tumors. $CD8^+$ T cells from the inguinal nodes and spleens of the tumor bearing and naïve mice were isolated and stimulated with epitopes

of OVA or E.G7 cells. Surprisingly, $CD8^+$ T cells from E.G7 -bearing mice also failed to detect E.G7 or the splenocytes pulsed with individual epitopes of OVA (Fig 4A). The $CD8^+$ T cells, however, expressed IFNγ and upregulated CD44 when stimulated non-specifically with PMA and ionomycin (Fig 4A).

Response to $CD8^+$ T cell epitopes was tested in the context of depletion of tumor-infiltrating T regulatory cells (Tregs). E.G7 tumor-bearing C57BL/6 mice were treated with 9D9 (IgG2a), an anti-CTLA-4 monoclonal antibody, which inhibits CTLA-4:B7 interaction but also depletes Tregs from the tumor micro-environment (14, 15). The tumors underwent complete regression in mice treated with this antibody (Fig. 4B). Upon co-culturing $CD8^+$ T cells isolated from mice rejecting E.G7 tumors with splenocytes presenting individual epitopes of OVA, the $CD8^+$ T cells were found to respond to peptides 257-264 (SIINFEKL) and 289-297 but not 55-62, 97-105 or 99-107 (Fig 4C, right panel). $CD8^+$ T cells from non-tumor bearing mice treated with antibody 9D9 did not recognize any epitopes of OVA (Fig 4C, left panel).

In addition to OVA, E.G7 tumor cells must express any neoepitopes that are present in the parental line EL4. CD8 responses against such neoepitopes of E.G7 cells were tested by co-incubating $CD8^+$ T cells from E.G7-rejecting-mice with EL4 cells. EL4 cells were recognized by $CD8^+$ T cells indicating that responses against the neoepitopes of E.G7 were generated in 9D9-IgG2a treated, E.G7 bearing mice.

Since $CD8^+$ responses to peptides SIINFEKL and 289-297 are always concordant, the possibility that they are cross-reactive, was tested. Splenocytes pulsed with SIINFEKL but

not peptide 289-297 were able to stimulate the B3Z hybridoma that specifically recognized SIINFEKL-K^b (Fig 4E). Ability of SIINFEKL and peptide 289-297 to protect naïve C57BL/6 mice from an E.G7 challenge was tested. Mice immunized twice with either peptide, or whole OVA, or un-immunized mice were challenged with E.G7 cells. Mice immunized with SIINFEKL and those immunized with whole OVA (data not shown) were completely protected, but mice immunized with peptide 289-297 showed no inhibition of tumor growth (Fig 4F). Parenthetically, we tested peptide 289-297 for anti-tumor activity even though we had observed previously (Fig.1) that it does not elicit CD8+ response; we did this in order to consider the possibility that the CD8 response may be below the level of detection, and yet may be effective in slowing the tumor growth.

SIINFEKL as a therapeutic vaccine against E.G7 tumors

The objective of identifying cancer neoepitopes is to treat cancer-bearing hosts by immunizing with such neoepitopes and obtaining a favorable change in the course of disease. Cancer neoepitopes are universally recognized on basis of their ability to elicit immunity in mice immunized before tumor challenge, or by their ability to elicit good CD8 responses. SIINFEKL meets both criteria. The efficacy of immunization with SIINFEKL in tumor-bearing mice was tested. Mice challenged with E.G7 cells were immunized with SIINFEKL admixed with an adjuvant on the day of (i.e. concurrent with) or three- or five-days post-tumor challenge. Immunization with SIINFEKL on the day of the tumor challenge conferred mice with weak immunity against E.G7 tumors and resulted in the delayed onset of tumor growth in some mice (Fig S1; left panel). However, the tumor burden in mice immunized three or five days post-tumor challenge increased unabated (Fig S1; middle and right panels).

DISCUSSION

Our studies utilize bioinformatics and immunological approaches to reveal six novel peptides of immunological relevance in the well-studied model antigen OVA. The six peptides fall into three categories. (i) One of the six peptides is immunogenic by itself and is also recognized as an epitope upon immunization with OVA (one additional such peptide, 256-264, is a single amino acid extension of SIINFEKL, and is not considered a novel peptide). (ii) An additional three peptides are not immunogenic, i.e. do not elicit $CD8^+$ T cell response upon immunization with peptides, but are perfectly epitypic, in that they are recognized by $CD8^+$ T cells elicited upon immunization with whole OVA. The discordance between immunogenicity and epitypicity here must arise from instability of the immunizing peptides during immunization and processing within the dendritic cells. For one of these three peptides, we show that an extended version of the peptide can immunize effectively. (iii) One peptide (27-35) is immunogenic, but is not seen as an epitope upon immunization with whole OVA, suggesting that this epitope is subdominant in the context of immunization. One additional peptide (208-216) cannot be naturally generated in vivo, and is thus cryptic, by the original definition of the term (3).

Viewed in the context of tumor antigenicity, the four novel epitopes (i.e. the six peptides identified minus the two cryptic epitopes and also excluding the single amino acid extension of SIINFEKL) behave in un-expected ways. In mice immunized with irradiated E.G7 cells, no $CD8^+$ response to any of the OVA epitopes (including SIINFEKL) was detected. Mice with growing E.G7 cells too showed no response against any novel epitope nor against SIINFEKL. Only in mice with regressing tumors (as a result of CTLA-4 blockade and $Fc\gamma R$ mediated depletion of T regulatory cells), was a response detected, and it was confined to the known

epitope SIINFEKL and a new epitope reported here, peptide 289-297. No $CD8^+$ T cell response to the previously reported epitope 55-62 was detected. These data indicate epitopes of OVA besides SIINFEKL and 289-297 are subdominant to mutation-encoded neoepitopes of E.G7/EL4. Interestingly, while immunization with SIINFEKL can protect mice against a subsequent challenge with E.G7 tumor cells, immunization with 289-297 has no anti-tumor activity. The latter observation is consistent with our observation that 289-297 is not immunogenic in and of itself even as it is perfectly epitypic. It is likely that immunization with an extended version of 289-297 may indeed elicit anti-tumor immunity; however, such extended peptides could not be synthesized due to technical difficulties. Parenthetically, immunization with 55-62 or 97-105 (both immunogenic and epitypic) also does not have anti-tumor activity. Table 3 summarizes these data. Of note, the experiments with SIINFEKL and those that revealed the novel MHC I-restricted epitopes of OVA were performed in non-tumor bearing mice. Therapeutic immunization with SIINFEKL does not control tumor burden because of the possibility that responses elicited by SIINFEKL in tumor-bearing mice could be less in magnitude than the responses observed in tumor-free mice. Additionally, the pre-established tumors could exclude SIINFEKL-specific T cells. Further, whether immunization with OVA elicits equally broad responses in mice with pre-existing tumors is unclear and needs to be tested.

These results have a cautionary lesson for our current aspirations at neoepitope-based immunotherapy of human cancers. Neoepitopes that elicit $CD8^+$ T cell responses in cancer patients are considered to be particularly useful indicators of a successful anti-tumor immune response, and also as potential antigens as cancer vaccines. If the results with OVA are broadly applicable, mice with growing E.G7 tumor show no $CD8^+$ response to the potent

antigen SIINFEKL which can indeed mediate tumor rejection. Conversely, in mice with regressing tumors, equally vigorous $CD8^+$ responses to SIINFEKL and 289-297 are seen, but only SIINFEKL can elicit anti-tumor immunity. There is thus the risk of finding a false negative as well as a false positive! And lest we dismiss OVA because it is OVA, it is useful to remember that everything that has been discovered with OVA, immunologically, has thus far found to be true broadly.

MATERIALS AND METHODS

Mice

Naïve C57BL/6J (00064) and Act-mOVA (005145) female mice were purchased from The Jackson Laboratory. Mice were maintained in the animal facility at UConn Health. Mice aged 6-10 weeks were used for all the experiments.

Cell lines and reagents

B3Z hybridoma was a gift from Dr. Nilabh Shastri (Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, University of California, Berkeley, CA). EL4 (Cat. no. ATCC TIB-38) and E.G7 (Cat. no. ATCC CRL-2113) cell lines were purchased from American Type Cell Culture. Phosphate-buffered saline (Cat. no. 10010023), Hank's balanced salt solution (Cat. no. 14025092), RPMI 1640 (Cat. no. 11875085), sodium pyruvate (Cat. no. 11360070), non-essential amino acids (Cat. no. 11140050), penicillin, streptomycin, glutamine (Cat. no. 10378016) and 2-mercaptoethanol (Cat. no. 21985023) were purchased form ThermoFisher Scientific. Fetal bovine serum (Cat. no. F8067-500 ml), OVA (Cat. no. A5503) and dimethyl sulfoxide (Cat. no. D2650) were purchased from Sigma Aldrich. All cells were cultured in RPMI containing 5% fetal bovine serum and the supplements mentioned above.

All fluorescently labelled antibodies: anti-CD8-PerCP/Cy5.5 (100734), anti-CD3-Alexa Flour 488 (100210), anti-CD44-APC (103012), anti-IFNγ-PE (505808) were purchased from Biolegend. Fluorescent fixable viability dye (65-0865-14) was purchased from eBioscience.

The 9D9-IgG2a antibody against CTLA-4 was generated as described earlier (15)

Synthetic peptides were purchased from different vendors: Genemed Synthesis Inc., Advanced peptides, JPT Peptide Technologies and Genscript.

Putative epitope prediction

The primary structure of OVA was obtained from uniprot.org (entry number P01012). Primary structure of OVA was analyzed with NetMHC (3.0) available at http://www.cbs.dtu.dk/services/NetMHC-3.0/ to predict all eight, nine, 10 and 11 amino acids long sequences with the potential to bind H-2K^b or H-2D^b. Both artificial neural network (ANN) and profile weight matrix (PWM) algorithms were utilized.

Testing peptide-MHC binding affinities

The binding capacities of synthetic peptides were measured in classical competition assays using high affinity radiolabeled ligands and purified MHC molecules, as detailed elsewhere (16). Six different concentrations of peptides were typically tested in at least three independent experiments. The IC_{50} value determined by this method approximately represents the dissociation constant value (17, 18).

Testing immunogenicity of peptides

Synthetic peptides were dissolved in DMSO to prepare a master stock. Working stocks of peptides were prepared by diluting the master stocks in PBS. Required quantities of peptides from the working stocks were mixed with TiterMax, an adjuvant, in 1:1 ratio and emulsified. Mice were anesthetized and immunized in the rear footpads with 10 μg of

individual emulsified peptides. To test $CD8^+$ T cell responses, mice were euthanized seven days after immunizations and the draining popliteal nodes were harvested. A single cell suspension was generated from the lymph nodes (LN) by crushing the nodes with butts of syringe plungers and then passing them through 100 μm cell strainer (Fisher scientific: 08-771-19). The lymph node cells were then re-stimulated with 10 μm immunizing peptide or PBS for 12 hours in the presence of Brefeldin A. Cells were stained for CD8, CD3, CD44 and viability at the end of the incubation and fixed and permeabilized using BD Cytofix/Cytoperm (BD: 554714). Cells were finally stained for IFNγ and analyzed by performing flow cytometry on a MACSQuant Analyzer. The data generated through flow cytometry was analyzed using FlowJo.

Testing MHC class I restricted antigenicity of OVA

OVA was dissolved in PBS and then emulsified with TiterMax. Mice were immunized with 450 μg (or as indicated) of emulsified OVA in rear footpads. Seven days later, the single cell suspension generated from the draining lymph nodes was re-stimulated with individual peptide or not in the presence of Brefeldin A for 12 hours. Expression of IFNγ by the re-stimulated cells was tested using intracellular cytokine staining assay as mentioned above.

Testing cross-reactivity of SIINFEKL and 289-297 with B3Z

Splenocytes from naïve C57BL/6 mice were pulsed with 10 μM of indicated peptide. Forty-thousand splenocytes were co-cultured with 100,000 B3Z for 18 hours. Expression of b-galactosidase by the cultured B3Z cells was tested by adding a lysis buffer containing

NP-40 and CPRG was added to the cultures. Absorbance at 595 nm was measured 12 hours after addition of CPRG.

Testing homology between sequences within OVA and proteins endogenous to mouse.

Peptides 36-43 and 214-222 were aligned with all the mouse (taxid:10090) non-redundant protein sequences using the Basic Local Alignment Search Tool (pBLAST) available of National Center for Biotechnology Information website.

Testing immunogenicity of E.G7

E.G7 cells were subjected to 3,400 rad of γ radiations. Cells were washed three times with HBSS to completely remove fetal bovine serum. Mice were subcutaneously inoculated with $2*10^7$ irradiated cells twice, seven days apart. Ten days after the second inoculation, the draining inguinal lymph nodes and spleens were harvested, and $CD8^+$ T cells were enriched using a magnetic cell enrichment kit (STEMCELL Technologies: 19853). Enriched $CD8^+$ T cells were co-cultured with naïve splenocytes pulsed with 10 μM indicated peptide or with E.G7 or EL4 cells for 12 hours in the presence of BFA. Intracellular cytokine staining and flow cytometry was performed to test the expression of IFNγ by the cultured $CD8^+$ T cells.

Tumor challenges

E.G7 cells were harvested from the cultures and washed three times with HBSS and maintained in HBSS after the final wash. Mice were challenged subcutaneously with $5*10^5$ cells on the right flank. Growth of tumors was recorded as the measured average length

of two perpendicular diameters. Mice carrying tumors with the average diameter of 20 mm were euthanized.

Antibody mediated immunomodulation

An anti-CTLA-4 monoclonal antibody 9D9 (isotype IgG2a) was used to apply checkpoint blockade in mice. E.G7 tumor bearing C57BL/6 mice were administered 100 μg of the antibody admixed with PBS intraperitoneally on days five and 10 post-tumor challenge. Non-tumor bearing mice were subjected to identical regimen as a control.

Testing $CD8^+$ T cells responses in tumor bearing mice

Mice were challenged with $5*10^5$ E.G7 cells and then either treated with antibody 9D9 or not. The mice treated with 9D9 were euthanized after tumors were completely rejected, usually on day 20 post-challenge. The untreated tumor-bearing mice were euthanized nine days after the challenge. The draining inguinal lymph nodes and spleens were harvested and $CD8^+$ T cells enriched from them. Responses against the epitopes of OVA or E.G7 or EL4 cells were tested by co-culturing the isolated $CD8^+$ T cells with appropriate target cells.

Statistical analysis

Biological replicates were tested in each group in every experiment to enable statistical analysis. The statistical differences between experimental groups were determined by performing unpaired t test (two-tailed) or t test (two-tailed) with Welch's correction. A P value less than 0.05 was considered significant. Prism software (by GraphPad Software) was used to perform the analysis.

Study approval

All experimental protocols involving use of laboratory animals were approved by Institutional Animal Care and Use Committee at UConn Health (Farmington, CT).

AUTHOR CONTRIBUTIONS

Author contributions: SHK and PKS designed and performed experiments and wrote the manuscript. JS and AS measured MHC-peptide binding affinities and reviewed the manuscript. MJS and AJK generated anti-CTLA-4 9D9-IgG2a antibody, discussed its application to the study, and reviewed the manuscript.

ACKNOWLEDGEMENTS

We acknowledge Sara Pan (UConn School of Medicine) for preliminary experiments, and Drs. Sreyashi Basu (University of Texas) and Ion Mandoiu (UConn) for valuable discussions**.**

REFERENCES

1. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. *Annu. Rev. Immunol.* 2013;31:443–473.

2. Jerne NK. Immunological speculations. *Annu. Rev. Microbiol.* 1960;14:341–358.

3. Sercarz EE et al. Dominance and crypticity of T cell antigenic determinants. *Annu. Rev. Immunol.* 1993;11:729–766.

4. Duan F et al. Genomic and bioinformatic profiling of mutational neoepitopes reveals new rules to predict anticancer immunogenicity. *J. Exp. Med.* 2014;211(11):2231–2248.

5. Gubin MM et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature* 2014;515(7528):577–581.

6. Yadav M et al. Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. *Nature* 2014;515(7528):572–576.

7. Moore MW, Carbone FR, Bevan MJ. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 1988;54(6):777–785.

8. el-Shami K et al. MHC class I-restricted epitope spreading in the context of tumor rejection following vaccination with a single immunodominant CTL epitope. *Eur. J. Immunol.* 1999;29(10):3295–3301.

9. Lipford GB, Hoffman M, Wagner H, Heeg K. Primary in vivo responses to ovalbumin. Probing the predictive value of the Kb binding motif. *J. Immunol.* 1993;150(4):1212–1222.

10. Rötzschke O et al. Exact prediction of a natural T cell epitope. *Eur. J. Immunol.* 1991;21(11):2891–2894.

11. Assarsson E et al. A quantitative analysis of the variables affecting the repertoire of T cell specificities recognized after vaccinia virus infection. *J. Immunol.* 2007;178(12):7890–7901.

12. Benarafa C, Remold-O'Donnell E. The ovalbumin serpins revisited: perspective from the chicken genome of clade B serpin evolution in vertebrates. *Proc. Natl. Acad. Sci. U.S.A.* 2005;102(32):11367–11372.

13. Ehst BD, Ingulli E, Jenkins MK. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am. J. Transplant.* 2003;3(11):1355–1362.

14. Waight JD et al. Selective FcγR Co-engagement on APCs Modulates the Activity of Therapeutic Antibodies Targeting T Cell Antigens. *Cancer Cell* 2018;33(6):1033-1047.e5.

15. Selby MJ et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol Res* 2013;1(1):32–42.

16. Sidney J et al. Measurement of MHC/peptide interactions by gel filtration or monoclonal antibody capture. *Curr Protoc Immunol* 2013;Chapter 18:Unit 18.3.

17. Gulukota K, Sidney J, Sette A, DeLisi C. Two complementary methods for predicting peptides binding major histocompatibility complex molecules. *J. Mol. Biol.* 1997;267(5):1258–1267.

18. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 1973;22(23):3099–3108.

Table 1. Putative epitopes of OVA and their binding affinities for K^b and D^b alleles*.

Putative epitopes		PWM Score[a]		Predicted IC50[b] (nM)		Measured IC50[c] (nM)	
Position	**Sequence**	**H-2K^b**	**H-2D^b**	**H-2K^b**	**H-2D^b**	**H-2K^b**	**H-2D^b**
23-32	ANENIFYCPI	**10**	0.037	2756	33404	5914	11029
27-35	IFYCPIAIM	-3.3	-3.7	**272**	27578	**29**	15250
32-40	IAIMSALAM	0.5	3.2	**243**	**98**	3256	40972
35-43	MSALAMVYL	-0.5	2.4	**456**	2091	1070	13631
36-43	SALAMVYL	3.1	7.6	1773	**65**	**20**	46508
55-62	KVVRFDKL	4.8	1.4	**248**	13050	**25**	-
75-83	TSVNVHSSL	4.7	1.5	**134**	31877	1300	-
97-105	YSFSLASRL	2.7	2.8	**170**	15707	**248**	10830
99-107	FSLASRLYA	-2.7	4.2	13639	**441**	13219	572
164-172	SSVDSQTAM	5.9	**9.1**	501	**432**	3383	3933
176-183	NAIVFKGL	6.1	2.9	**269**	19457	**16**	77300
208-216	VQMMYQIGL	1.8	-5.7	**59**	14703	**1.5**	52427
214-222	IGLFRVASM	4.8	-0.2	**17**	18352	**39**	-
250-258	SGLEQLESI	5.8	7.1	**350**	8595	5110	17967
256-264	ESIINFEKL	6.9	7.3	**393**	760	**67**	741
257-264	SIINFEKL	7.6	2.3	**20**	15463	**10**	3795
289-297	EKYNLTSVL	5.1	-0.8	**470**	37427	**113**	-
307-315	SSSANLSGI	6.8	**8.7**	3668	10411	928	16669
313-321	SGISSAESL	4.8	**9.8**	**139**	11844	13162	5511

* All eight, nine and 10 amino acids sequences with potential to bind Kb or Db are listed. No 11-mer or 12-mer putative epitopes were identified. Values better than the selection threshold are in bold. Previously reported sequences are in red. PWM scores and predicted IC50 values were generated by analyzing the primary sequence of OVA with NetMHC 3.0.

[a] PWM: Position weight matrix, one of the two methods employed by NetMHC. Threshold scores for Kb and Db are 7.61 and 8.08 respectively
IC50: Concentration of the test peptide required to inhibit binding of the standard peptide to MHC I by half.

[b] Predicted IC50: Predicted IC50 of peptide-MHC binding.

[c] Measured IC50: Experimentally determined IC50 value of peptide-MHC binding. IC50 was measured by incubating varying concentrations of the test peptide with purified MHC I and a radiolabeled standard peptide. See Methods.

Table 2. Immunogenicity and epitypicity of OVA-derived peptides

Peptide sequence	27-35	55-62[a]	97-105	99-107	208-216	250-258	257-264 [a]	289-297
Immunization with the peptide [b]	+[d]	+	+	-	+	-	+	-
Immunization with whole OVA[c]	-[d]	+	+	+	-	+	+	+

[a] Previously defined epitopes
[b] Mice were immunized with synthetic peptides and CD8 response was tested against the immunizing peptide in vitro.
[c] Mice were immunized with whole OVA protein and CD8 response was tested against the indicated peptide in vitro.
[d] Symbols + and – indicate a CD8 response or lack of it, respectively.

Table 3. Context dependent epitypicity of OVA

	Epitypicity (Immunogenicity) in context of					
	Immunization by					
Peptide sequence	**Peptide** [b]	**Whole OVA Protein** [c]	**Irradiated E.G7 Cells**	**Growing E.G7 tumors**	**Regressing E.G7 tumors**[d]	**Ability to elicit rejection of E.G7 cells**[e]
27-35	+[f]	-[f]	-	-	-	-
55-62[a]	+	+	-	-	-	-
97-105	+	+	-	-	-	-
99-107	-	+	-	-	-	-
208-216	+	-	-	-	-	-
250-258	-	+	-	-	-	-
257-264 [a]	+	+	-	-	+	+
289-297	-	+	-	-	+	-

[a] Previously defined epitopes
[b] Mice were immunized with synthetic peptides and CD8 response was tested against the immunizing peptide in vitro.
[c] Mice were immunized with whole OVA protein and CD8 response was tested against the indicated peptide in vitro.
[d] Mice with growing E.G7 tumors were treated with an anti-CTLA4 antibody (9D9 IgG2a) with selectivity for intra-tumoral T regs (Selby et al. 2013).
[e] Mice were immunized with synthetic peptides and were challenged with live E.G7 cells.
[f]Symbols + and – indicate a CD8 response or lack of it, respectively.

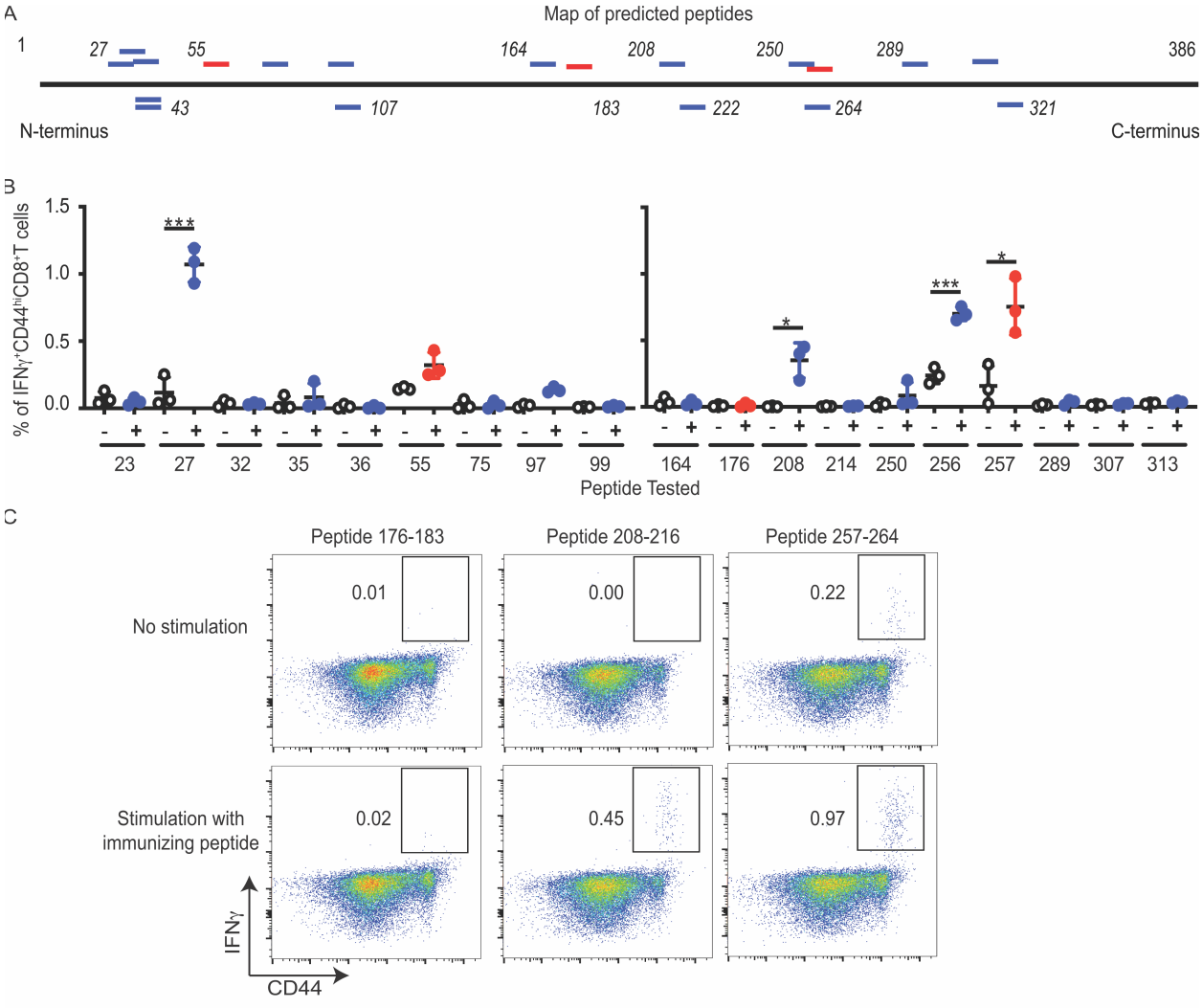

Figure 1. Antigenicity of novel putative and known epitypic peptides of OVA. (A) Positions of novel putative (blue) and the previously reported (red) epitopes of OVA, as detailed in Table 1. Horizontal black line represents the primary structure of OVA. Italicized numbers above and below OVA mark various positions. (B) Immunogenicity of each putative epitypic (blue) and the previously known (red) peptides of OVA shown in Table 1. "-" indicates no stimulation and "+" indicates stimulation of LN cells *in vitro* with the immunizing peptide for 12 h. Tested peptides are identified on x-axis with the position of their n-terminal residue (n=3; each peptide tested in nine different mice over four independent experiments; Welch's t test). (C) Representative assay of the ability of SIINFEKL (257-264), 176-183 (also previously known) and 208-216 (novel peptide) to elicit $CD8^+$ T cells upon immunization of C57BL/6 mice, as described in Methods (each peptide tested in nine different mice in several independent experiments). Flow cytometry plots of viable $CD3^+$ $CD8^+$ cells from LNs of immunized mice are shown. * means $p \leq 0.05$; ** means $p \leq 0.01$ and *** means $p \leq 0.001$.

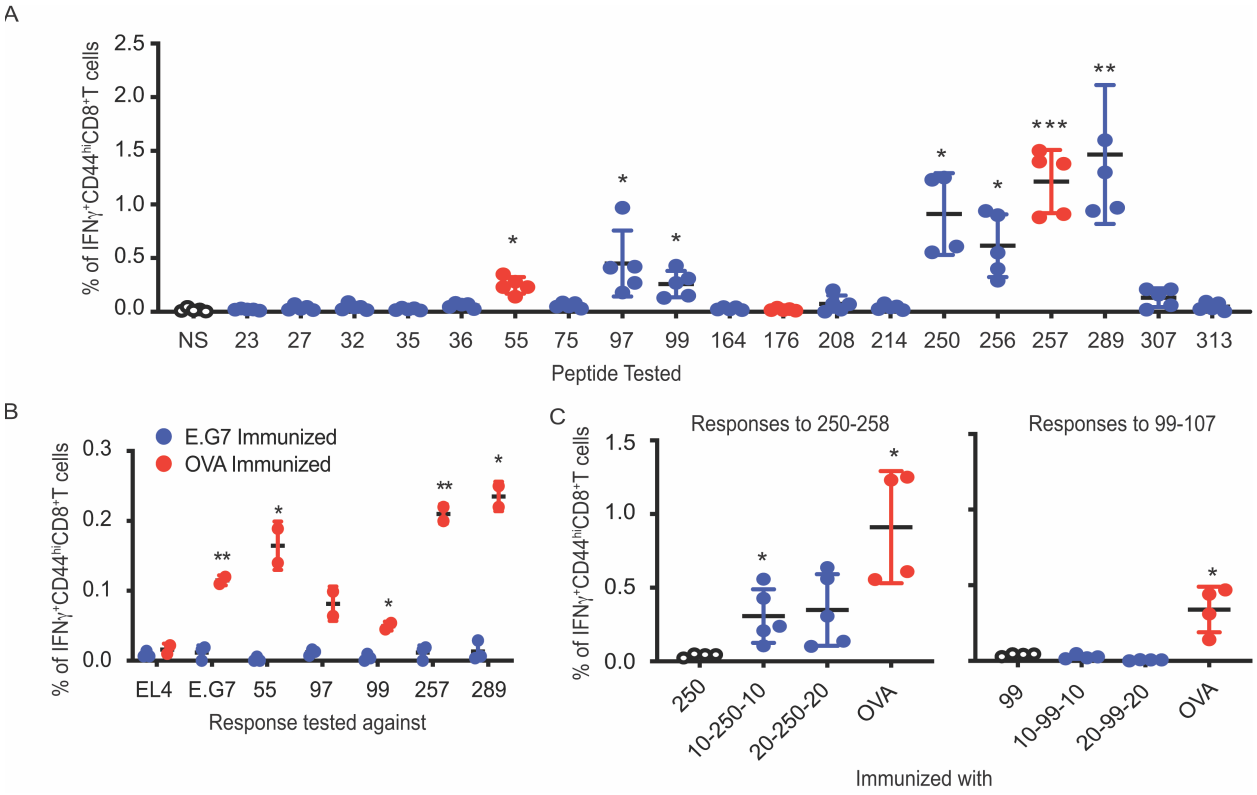

Figure 2. MHC I-restricted epitypicity of OVA. (A) CD8+ T cell responses to OVA elicited in C57BL/6 mice immunized with OVA emulsified with TiterMax. Responses against each of the novel putative (blue) and the previously known (red) epitopes of OVA (as listed in Table 1) were tested as in Fig.1; NS indicates no re-stimulation. p value reflects the significance of difference in % of IFNγ expressing $CD44^{hi}$ CD8+ T cells between non-stimulated (NS) and peptide-stimulated cultures (n=5; experiment performed three times; Welch's t test). (B) Inability of irradiated E.G7 cells to elicit CD8+ T cell responses against itself or OVA. CD8+ T cells from LN of immunized mice were stimulated with splenocytes pulsed with indicated peptide or EL4 or E.G7 cells for 12 h. p values indicate significance of difference between cultures stimulated with the indicated peptide or cells (n=3 for E.G7-immunized mice, n=2 for OVA immunized mice; experiment performed four times; unpaired t test) (C) Extended variants of peptide 250-258 are immunogenic. Mice were immunized with precise peptide (labeled 250 or 99), extended peptides with 10 (10-250-10 or 10-99-10) or 20 (20-250-20 or 20-250-20) flanking amino acids on either termini or whole OVA. Response to 250-258 and 99-107 were tested as in Fig. 1 (n=4 for 250 and OVA, n=5 for 10-250-10 and 20-250-20 in left panel; n=4 for all sets in the right panel; experiment performed two times; Welch's t test). * means $p \leq 0.05$; ** means $p \leq 0.01$ and *** means $p \leq 0.001$.

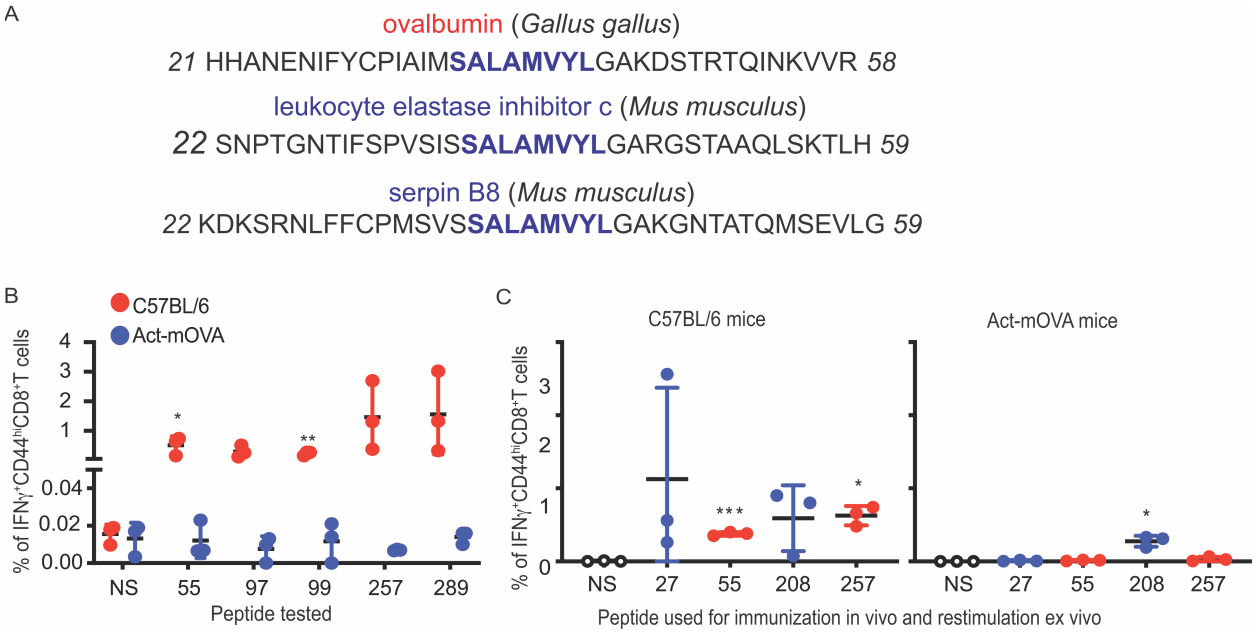

Figure 3 Epitopes of OVA induce tolerance when expressed endogenously. (A) Peptide 36-43 (SALAMVYL), is present in two proteins expressed by mouse. Peptide 36-43 was aligned against all non-redundant mouse (taxid:10090) protein sequences. Thirty-nine amino acids regions of OVA and two mouse proteins containing peptide 36-43 are shown. Numbers flanking the sequences indicate the position (from the N-terminus) of the first and the last depicted amino acid. (B) Act-mOVA mice are tolerized towards all epitopes of OVA. C57BL/6 mice (red) or Act-mOVA (blue) were immunized with OVA and $CD8^+$ T cell responses against the indicated epitopes were tested (n=3; experiment performed two times; unpaired t test). (C) Act-mOVA mice are not tolerized towards peptide 208-216. Wildtype C57BL/6 (left panel) or Act-mOVA (right panel) mice were immunized with peptides 27-35, 55-62, 208-216 and 257-264. CD8 responses were tested seven days after immunizations (n=3 for both panels; experiment performed two times; unpaired t test). * means $p \leq 0.05$; ** means $p \leq 0.01$ and *** means $p \leq 0.001$.

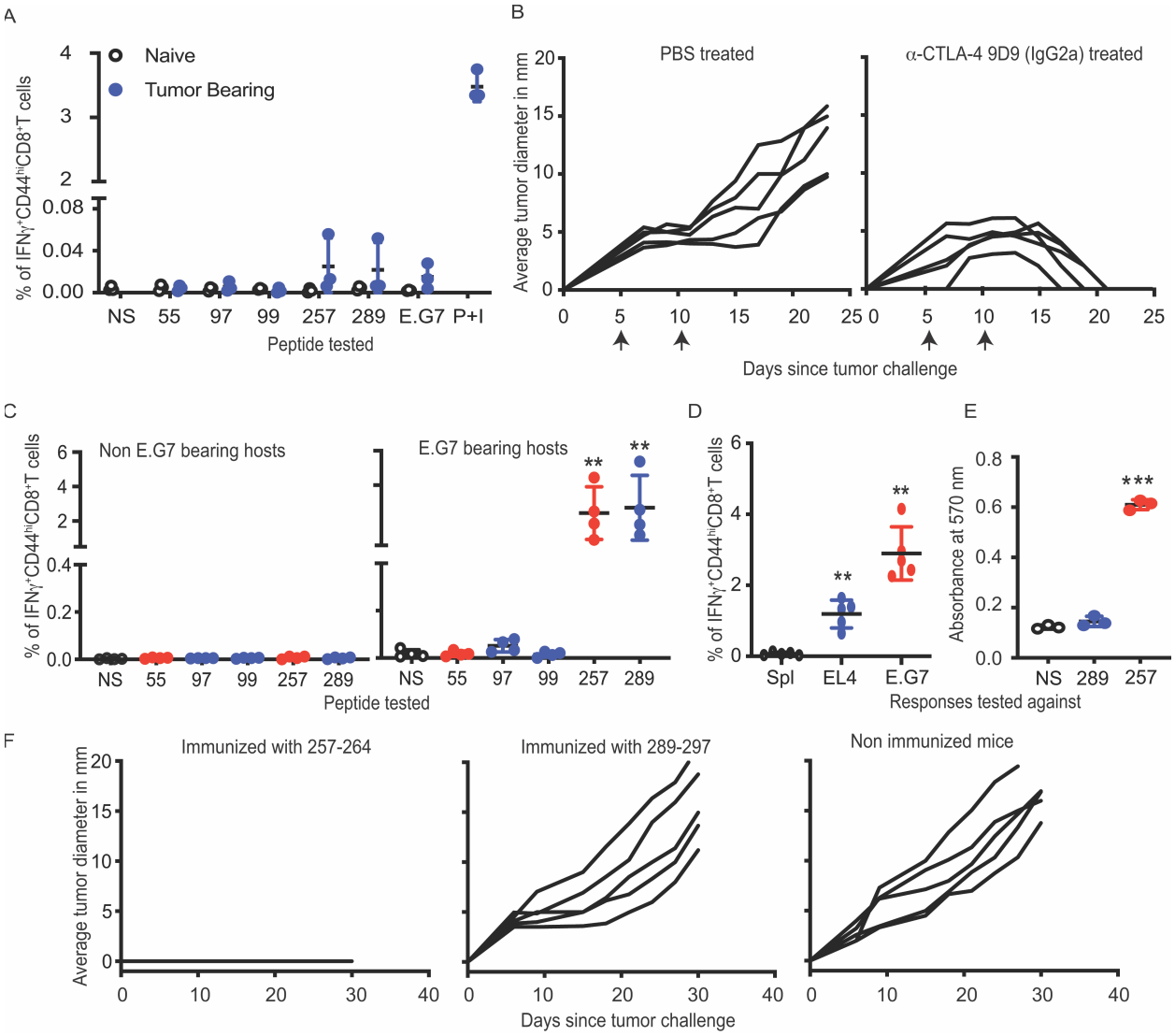

Figure 4. CD8⁺ T cell responses in E.G7 bearing mice. (A) Lack of CD8⁺ responses against E.G7 or OVA in E.G7-bearing mice. CD8⁺ cells from tumor bearing mice were stimulated with peptide-pulsed-splenocytes, unpulsed splenocytes (NS), E.G7 cells or PMA and ionomycin (P+I) (n=3; experiment performed three times). (B) Treatment with antibody 9D9 IgG2a (α-CTLA-4) causes complete regression of E.G7. E.G7-bearing mice were treated with 9D9 or PBS on the days indicated with arrows (n=5; experiment performed three times). (C) CD8⁺ responses to 257-264 and 289-297 are primed in mice rejecting E.G7. E.G7-bearing or naïve mice were treated with 9D9. CD8⁺ cells were enriched after tumors regressed and stimulated with peptide-pulsed-splenocytes or un-pulsed splenocytes (NS; n=4 for both panels; experiment performed three times; Welch's t test) (D) CD8⁺ cells from mice rejecting E.G7 were stimulated with splenocytes (Spl), EL4 or E.G7 cells (n=5; experiment performed two times, Welch's t test). (E) Splenocytes pulsed with 289-297 or SIINFEKL were co-cultured with B3Z (n=3; experiment performed one time; Welch's t test). (F) 289-297 does not elicit protective immunity while SIINFEKL does. Mice were immunized with SIINFEKL or 289-297 and challenged with E.G7 seven days later (n=5 for each panel; experiment performed two times). ** means $p \leq 0.01$ and *** means $p \leq 0.001$.