Systemic TLR ligation and selective killing of DC subsets fail to dissect priming pathways for anti-vaccinia virus CD8$^+$ T cells

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Running title: Dissecting anti-viral CD8$^+$ T cell priming pathways

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Abstract

$CD8^+$ T cell responses can be generated by direct or cross priming mechanisms and several mouse models have been used to reveal which of these is the most important pathway for various viruses. Amongst these models is systemic treatment of mice with a CpG-containing oligodeoxynucleotide (CpG) to mature all dendritic cells (DCs) rendering them incapable of cross presentation. A second is the use of cytochrome c (cytc) as a selective poison of the subsets of DCs able to cross-present antigen. In this study using two vaccinia virus (VACV) strains, namely WR and MVA, we found that the CpG and cytc methods gave conflicting data. Moreover we show for both strains of VACV, that treatment of mice with CpG and cytc inhibited $CD8^+$ T cell responses to antigens designed to prime exclusively by direct presentation. Further investigation of the CpG method found that the extent to which priming is inhibited depends on the antigen examined, immunization route, replication ability of the virus and crucially immunization dose. We suggest greater caution is required when interpreting data using these methods and that priming pathways for anti-viral $CD8^+$ T cells are not simply separated according to DC subsets or their maturation state.

Introduction

Antigens for presentation to $CD8^+$ T cells can be acquired and processed by direct and cross presentation. In direct presentation, the peptides presented on major histocompatibility complex class I (MHC-I) are processed from antigens expressed within a cell, for example after infection by a virus (1). Conversely, in cross presentation the sources of peptides are exogenous antigens that are taken up by the cell, processed and presented (2). Direct presentation occurs in any cell that expresses MHC-I, however cross presentation is a property of very few cells and is largely restricted to a subset of dendritic cells (DCs) (3). Cross presentation by DCs allows them to prime anti-viral $CD8^+$ T cells when viruses either do not infected these cells or encode functions that inhibit antigen processing and presentation in infected cells (4, 5). The extent to which these pathways contribute to the priming of anti-viral $CD8^+$ T cells is challenging to investigate, especially when the virus is known to infect DCs (6).

Vaccinia virus (VACV) is an excellent model for viral immunology and has relevance as a vector for vaccines. Priming pathway is of practical interest in the context of VACV vectored vaccines because the requisites for antigen differ for direct and cross presentation (7, 8). Several lines of evidence suggest that VACV strain Western Reserve (WR), a virulent laboratory strain, primes $CD8^+$ T cells largely via direct presentation: (a) rapidly-degraded and minimal peptide constructs that improve direct presentation, but compromise cross presentation, have enhanced immunogenicity (7, 9, 10); (b) interactions between naive $CD8^+$ T cells and VACV-infected dendritic cells (DC) leading to T cell activation have been seen by *intra vital* microscopy (7, 11); (c) mice with MHC-I knockout bone marrow in wild type recipients mount strong $CD8^+$ T cell responses when infected by a VACV WR that expresses an MHC-I gene to complement the deficiency only in infected cells (10); (d) inhibition of cross priming by the systemic administration of a synthetic CpG-containing oligonucleotide (CpG) did not reduce $CD8^+$ T cell responses to

VACV WR (10, 12). However WR might not be representative of all VACV strains and data for the attenuated vaccine strain Modified Vaccinia Ankara (MVA) is conflicting. Gasteiger et al (13) found that MVAs expressing minimal epitopes and rapidly-degraded antigens elicited poor $CD8^+$ T cell responses and inhibition of cross priming using systemic CpG treatment abolished $CD8^+$ T cell responses to MVA. However, more recent in vivo imaging results found evidence for direct, but not cross priming by MVA (14). Recombinant vaccines are based on attenuated strains of VACV such as MVA, so understanding priming by such strains is arguably of most practical relevance. The lack of consistency in the literature across the strains adds to the interest in using VACV to study priming pathways for anti-viral $CD8^+$ T cells.

Two methods that that have been used to dissect $CD8^+$ T cell priming in wild type mice are: 1) Pre-treatment of mice with the TLR9 agonist CpG as noted above (10, 13). Such treatment causes systemic maturation of DCs resulting in their inability to cross present antigens, but direct presentation is apparently not affected (12). 2) Administration of horse cytochrome c (cytc) that acts as a selective poison of cross-presenting $CD8\alpha^+$ DCs owing to their unique ability to translocate extracellular proteins into their cytoplasm (15). These two methods have been very well published in studies of $CD8^+$ T cell priming pathways (10, 12, 13, 15-20). While the CpG method has been applied in separate studies to VACV WR and MVA, there has not been a direct comparison of cross and direct priming with these strains. Further, no single study has used both the CpG and cytc methods to see whether these two methods give similar results for any model system, viral or otherwise.

Here we report that rather than clarifying antigen presentation mechanisms for VACV WR and MVA, our data cast doubt on the utility of pre-treatment of mice with CpG and cytc for dissecting priming pathways. We found that the extent to which CpG treatment inhibited priming of anti-

VACV $CD8^+$ T cells was dependent on antigen, route and most crucially, dose. Dose is important because it is related to the replicative ability of viruses, a property that can vary widely between strains. Further, results obtained with CpG and cytc treatments were not consistent. The implications of these findings for VACV strains and anti-viral immunity in general are discussed.

Materials and Methods

Cells and viruses

Cell lines were grown at 37°C in 5% CO_2 in DMEM with 10% FBS. VACV was grown and titered according to standard methods. The unmodified viruses used were Strain WR and MVA, from B. Moss, NIH. Recombinants of strain WR (WR-SIIN and WR-OVA) were from J. Yewdell and J. Bennink, NIH, and an engineered MVA (MVA-SIIN) was made for this study. WR-SIIN and WR-OVA express an MSIINFEKL 'minigene' (SIIN) or the whole of ovalbumin (OVA), respectively under the VACV p7.5 promoter from the VACV thymidine kinase (TK) locus (9). MVA-SIIN was made to match WR-SIIN and expresses the MSIINFEKL minigene under p7.5 from TK of MVA. This virus was made for this study using a method based on transient dominant selection driven by a GFP-blasticidin selection marker (21). The TK targeting vector (pSC11GB) used had the pSS GFP-BSD cassette from pSSGB (21) inserted into the *Hind*III site of pSC11-SBAKN plasmid (22). MVA-SIIN was verified by sequencing the insertion site.

Mice

Female C57Bl/6 mice were obtained from the Animal Resources Centre (Perth, Australia) or the Australian Phenomics Facility (Canberra, Australia) and used at >8 weeks of age. Mice were housed and experiments were done according to the relevant ethics protocols.

Synthetic peptides

Synthetic peptides were purchased from Genscript (Piscataway) or Mimotopes (Clayton, Australia), 10mg/ml stocks in DMSO were diluted as required in DMEM (Table 1).

CpG and cytc treatments

Mice were injected intravenously (i.v.) with 20 nmol synthetic phosphorothioated CpG1668 oligonucleotide (TCCATGACGTTCCTGATGCT; Sigma) in 200µl PBS one day before immunization. For cytc treatment, mice were injected i.v. with 5 mg horse cytc (Sigma) in 100 µl PBS for five consecutive days starting one day before immunization. Control mice received PBS.

UV/psoralen treatment and infection of cells with VACV for immunizations

To make UV/psoralen-inactivated virus, VACV at $2x10^8$ PFU/ml was resuspended in 100 µl of PBS with 1 ug/ml 4,5,8-Trimethylpsoralen (psoralen; Sigma) and irradiated 10 mm below a 365 nm UV-A lamp (Vilber Lourmat, France) for 3 min. This treatment eliminates all PFU from the virus but allows early virus gene expression. To infect cells to use as a source of virus antigen for cross priming, 293A cells were infected with WR-OVA at 5 PFU/cell in DMEM at 37°C for 60 min with shaking followed by further culture for five hours. After three washes with PBS the cells were incubated at 60°C for one hour to kill residual virus (verified by plaque assay).

Immunizations

Virus: Mice were injected intraperitoneally (i.p.) with VACV in 200 µl PBS or were anesthetized by isofluorane inhalation and injected intradermally (i.d.) with VACV in 10 µl PBS into ear pinnae with doses indicated (23). UV/psoralen-inactivated virus: mice were immunized i.p. with the equivalent of $2x10^6$ PFU UV/psoralen treated virus in 200µl. For in vivo cross presentation assay, mice were injected i.p. with $1x10^6$ or $2.5x10^7$ WR-OVA-infected heat killed 293A cells in 200 µl PBS.

Detection of peptide-specific $CD8^+$ T cells

Mice were euthanized seven days after immunization and $CD8^+$ T cell responses were determined by an intracellular cytokine staining (ICS) protocol (24). Briefly, splenocytes were incubated with a

panel of VACV peptides (Table 1) for four hours in the presence of brefeldin A, followed by surface staining with anti-CD8α-PE (clone 53-6.7) and intracellular staining with anti-IFN-γ-APC (clone XMG1.2). Data were acquired with an LSR II cytometer and analysis was with Flowjo Software (Tree Star). Backgrounds as determined using no peptide in stimulations were typically below 0.2% of $CD8^+$ events and were subtracted from values reported.

VACV titration from isolated organs

Organs were taken from mice one or three days after immunization and stored at -80°C before use. Samples were thawed, homogenized with 1ml tissue grinders, frozen and thawed three times and sonicated. VACV titers were determined by plaque assay on BSC-1 cells.

Statistics

Data are presented as means ± Standard error of the means. Statistical comparisons were done using two-tailed, unpaired student's T test with GraphPad Prism software, $p<0.05$ is considered statistically significant and is indicated by asterisk (*).

Results

CpG inhibits cross presentation of a wide range of VACV antigens

To establish that CpG inhibits cross priming of a broad range of VACV antigens, mice were treated with CpG or PBS and were immunized i.p. with 1×10^6 WR-OVA-infected, heat killed 293A cells one day later. In addition to the full length OVA expressed from this recombinant virus, which is known to be cross-presented from VACV-infected cells (7), $CD8^+$ T cell responses to 14 native VACV antigens were measured (Fig 1A and B). Responses were detected for OVA and seven of the VACV peptides in control mice, but all were reduced >80% in CpG-treated mice (Fig. 1C). These data confirm and extend the range of antigens for which CpG treatment inhibits the cross priming of VACV antigens (10).

CpG inhibits direct presentation following dermal immunization with VACV

Having established the CpG treatment method we applied it to dissect the presentation of VACV antigens after immunization with virus. We chose to start with an i.d. route to model vaccination and examined responses to a set of 14 VACV epitopes, all of which are conserved between WR and MVA with the exception of C4-125 and B2-54 . CpG or PBS treated mice were immunized by i.d. injection of 2×10^6 PFU of VACV WR or MVA in the ear (25) and $CD8^+$ T cell responses were measured after seven days (Fig. 2). Contrary to published data for i.p. and subcutaneous immunizations (10), priming of $CD8^+$ T cell responses to all epitopes of the virulent WR were strongly inhibited by CpG treatment. Inhibition by CpG-treatment was >60% for all epitopes and in some cases >80% (Fig. 2C). Likewise for MVA, all $CD8^+$ T cell responses seen in controls were significantly inhibited by CpG treatment, but this time our obseravtion was in agreement with the literature (13). At face value, these data suggest that cross priming is the dominant pathway both for MVA and WR after i.d. immunization. This conclusion for WR after i.d. immunization has support from one paper, but more recent intravital observations using this route and data for another

peripheral route (subcutaneous) favor direct presentation for WR (11, 26, 27). For this reason we were concerned that the interpretation of our experiment might be more complicated and explored CpG treatment further.

To test directly whether CpG treatment genuinely dissects direct and cross priming after i.d. immunization we took advantage of a VACV recombinant referred to here as WR-SIIN. This virus expresses a 'minigene' version of the dominant epitope in OVA (MSIINFEKL) that primes OVA-specific $CD8^+$ T cells exclusively by direct presentation (7, 28). Further, we confirmed the above reports finding no OVA-specific responses after immunizing mice with 1×10^6 and upto 1×10^7 WR-SIIN-infected cells by i.d. and i.p. routes, respectively (not shown). In mice immunized with 2×10^6 PFU of WR-SIIN, nearly 2% of $CD8^+$ T cells were OVA-specific on day 7 (Fig. 3A), which is similar to responses induced by this virus after i.p. immunization (7; and see below). This suggests that direct priming is efficient after i.d. immunization. Moreover, in mice pre-treated with CpG this directly-primed OVA-response was inhibited by 80%, similar to the inhibition seen for the dominant VACV B8-20 epitope (Fig. 3A left). To extend the experiments we made MVA-SIIN, which was designed to match WR-SIIN using the same promoter and insertion site for the MSIINFEKL minigene (p7.5 and TK, respectively). Just as for WR-SIIN, when mice were immunized with MVA-SIIN by i.d. injection, responses to OVA were robust at around 1.5% of $CD8^+$ T cells, suggesting efficient direct priming by this virus. However, the directly-primed OVA-specific response was again inhibited by >80% when mice were treated with CpG (Fig. 3B). Taken together with our data using wild type VACVs, CpG treatment reduces $CD8^+$ T cell responses against all VACV antigens both from WR and MVA after i.d. immunization, even when they are targeted for direct presentation and cannot be cross primed.

200 *The effect of CpG treatment on CD8$^+$ T cell priming depends on antigen, immunization route and*
201 *virus replication*
202 To determine whether our results were specific to i.d. immunization in the ear and to recapitulate
203 the main body of previously published work more faithfully, we repeated our experiments with
204 WR-SIIN and MVA-SIIN using i.p. immunization. Unlike our i.d. experiments and consistent with
205 published data (10), CpG treatment did not reduce OVA- and B8-20-specific responses after i.p.
206 immunization with WR-SIIN (Fig. 4A, D). Surprisingly however, CpG significantly lowered the
207 responses against most of the other VACV antigens, suggesting that the effect of this treatment was
208 antigen-specific. After i.p. immunization with MVA-SIIN, CpG treatment significantly inhibited
209 responses to all epitopes examined, including B8-20 and the directly-presented OVA minigene (Fig.
210 4B, D). These show that we are able to repeat published data for WR (10) and MVA (13) when the
211 i.p. route and same antigens are used. However, our extension of the system to new epitopes (for
212 WR) and the inclusion of directly-primed SIIN (for MVA) demonstrates again that experiments
213 using CpG treatment are not reliably interpreted in terms of antigen presentation pathways.
214
215 It was of interest to know why the effect of CpG treatment on CD8$^+$ T cell priming was different for
216 WR and MVA after i.p. immunizations. While there are many genetic differences between WR and
217 MVA (29) the most obvious phenotypic disparity is the inability of MVA to replicate in vivo, even
218 highly immunocompromised animals (30, 31). To examine virus replication and the effect of CpG
219 treatment directly, we used WR-SIIN that was inactivated by UV/psoralen treatment (32). The
220 amounts of psoralen and UV exposure were carefully titrated to inhibit replication, but allow early
221 virus gene expression (33; YCW, unpublished). When mice were immunized i.p. with
222 UV/psoralen-inactivated WR-SIIN, responses to OVA, B8-20 and all VACV epitopes were reduced
223 by around 80% in CpG-treated mice (Fig. 4C, D). These results closely resembled those found
224 using MVA-SIIN. We conclude that differences in virus replication, rather than genetics explain

why CpG-treatment inhibits $CD8^+$ T cell priming by MVA and not WR after i.p. immunization. However we reiterate, this inhibition for non-replicating viruses is irrespective of priming pathway.

CpG treatment reduces virus loads, but this does not explain its impact on $CD8^+$ T cell priming

It remained puzzling why CpG should affect priming of $CD8^+$ T cells after WR infection by i.d. but not i.p. immunization. Given the sensitivity of the CpG treatment to virus replication after i.p. immunization we wondered if there might be important differences in amounts of WR available for priming in i.d. versus i.p. routes. Previous studies have shown that WR replication and spread is relatively limited after i.d. injection of ears compared with systemic routes (25, 34) and CpG treatment has been shown to reduce VACV spread due to induction of robust innate immune responses (35, 36). Perhaps the combination of these two factors reduces WR antigen amounts to levels that compromise $CD8^+$ T cell responses after i.d. ear immunization of CpG-treated mice. To address this we examined infectious WR levels in various organs including known priming sites after i.d. and i.p. immunization (34) of CpG-treated and control mice (Fig. 5). For i.d. immunization, there was detectable, but significantly less virus (around 1.5 $\log_{10}$) in the cervical lymph nodes (LN), but not ears of CpG-treated mice compared with controls at 1 and 3 days after infection. After i.p. immunization, although mean virus titers were always lower in CpG treated mice, the apparent difference was only statistically significant in the spleen on day 3. Overall, there was strong evidence that CpG treatment compromised antigen loads in priming sites after i.d. but less so for i.p. immunization. To explore whether reduction of virus alone might compromise responses after ear immunization, mice were immunized with 2×10^3 PFU of WR, a 3 $\log_{10}$ reduction on the usual dose. At day 1, <2 PFU of virus could be isolated from the cervical LNs of low dose-immunized mice (Fig. 5C). This was much less virus than was detected on day 1 in CpG-treated mice (Fig 5A), but virus levels in low dose immunized mice were more typical by day 3. Despite the reduced virus levels in draining LN on the first day, low dose-immunized mice had

strong $CD8^+$ T cell responses to all VACV epitopes, similar to those injected with 1000-fold more virus. (Fig. 5D, compare Fig. 2A). This experiment is not ideal because virus was available in the LN at day 3 after low dose immunization, but the first 24 hours after immunization is the most important time for priming $CD8^+$ T cells by VACV (26). Furthermore by day 3, CpG treatment significantly reduced virus in the spleen of i.p. immunized mice, but this did not affect OVA- or B8-20-specific $CD8^+$ T cell responses. These arguments suggest that differences in virus levels at day 3 are probably not as relevant here as those earlier. In conclusion, CpG treatment does reduce virus loads and this may have some impact after i.d. immunization with WR, but it seems unlikely that this complication completely explains the strong inhibition of anti-WR $CD8^+$ T cell responses seen for this route.

Increased antigen dose partially overcomes the inhibition of $CD8^+$ T cell priming by CpG

The various links between virus replication and CpG-induced inhibition of $CD8^+$ T cell responses explored above suggested that antigen dose was an important factor in the outcome of experiments. Therefore we wondered if increasing antigen dose alone could reverse the inhibition of priming by CpG treatment. First, we used MVA-SIIN and immunized mice twice, two days apart with 2×10^8 PFU to double the dosing and increase the inoculum by 100-fold (Fig. 6A). B8-20 responses were significantly lowered by CpG-treatment after this high-dose immunization, but the extent of the reduction was only 40-50%, compared with nearly 90% with the usual dose (Fig. 6A left compared with Fig 4D middle). On the other hand, OVA-specific responses were no longer significantly lower in CpG-treated mice and the apparent reduction in mean response was around 40%, compared with 90% for the usual dose.

Next we tested if a higher dose might also overcome CpG treatment-induced inhibition of bona fide cross priming. To do this CpG treated and control mice were immunized with $2.5x10^7$ heat-

inactivated WR-OVA-infected cells, which is 25-fold more than was used in the original experiments shown in Fig. 1. Similar to results for high-dose MVA, a significantly lower response was observed for B8-20- but not OVA-specific $CD8^+$ T cells in CpG-treated mice when the high dose of infected cells was used (Fig. 6B). Furthermore reductions in mean responses were now less severe for both epitopes compared with experiments using a more typical dose of 1×10^6 cells (Fig. 6B left and Fig 1C). Taken together, these data demonstrate that the extent to which CpG treatment inhibits $CD8^+$ T cell responses is sensitive to the antigen dose administered.

Cytc treatment fails to differentiate between direct and cross priming

Having found that the effect of CpG treatment more reliably reflects antigen dose than $CD8^+$ T cell priming pathway, we wanted to try a different strategy. Cytc treatment has also been published as a method that selectively inhibits cross presentation (15), but has not been previously applied to dissect priming pathways for VACV-specific $CD8^+$ T cells. First we showed that cytc-treatment reduced $CD8^+$ T cell responses primed by VACV-infected cells, but noted that the inhibition was less than we observed for CpG-treatment (Figure 7A). When cytc-treated mice were immunized with WR-SIIN by the i.p. route, we were very surprised to find enhanced $CD8^+$ T cell responses against OVA minigene and B8-20, while other responses were not affected (Fig. 7B). For MVA-SIIN, cytc-treatment caused a reduction of $CD8^+$ T cell responses against most antigens, including the directly-presented OVA minigene, but A47-171 was a notable exception to this trend (Fig. 7C). To determine again whether it is solely differences in replication that determine the disparate outcomes of cytc treatment for WR and MVA, cytc and control mice were immunized with UV/psoralen inactivated WR-SIIN (Fig. 7D). As was the case for CpG treatment, priming of inactivated WR-SIIN was affected in a manner most similar to MVA and not WR. A curious difference in this experiment was that for inactivated WR-SIIN, A47-171 did not escape the inhibition of priming. Therefore for this form of VACV alone, cytc and CpG treatments were

consistent in their effect on priming for all epitopes, though the latter was more efficient. However, more broadly across all the experiments and antigens cytc and CpG treatments did not give consistent results. Further, cytc like CpG can impair direct presentation. We conclude that cytc treatment fails to provide insight into the pathways involved in priming anti-VACV $CD8^+$ T cells.

Discussion

Our data show that use of CpG and cytc treatments to dissect priming mechanisms for anti-viral $CD8^+$ T cells in mice is far more complicated than has been appreciated. In the case of CpG treatment, this method inhibited direct priming by VACV where antigen was limited. Further, its ability to completely abrogate cross priming from virus-infected cells was partly overcome by increasing antigen dose. These findings highlight an important difficulty in comparison of virus systems used to examine direct and cross priming. When infectious virus is used, as is required to allow direct priming, replication occurs and antigen dose is usually very high, especially if infection is disseminated. By contrast, controls that restrict priming to cross presentation, such as infected cells, are always associated with low antigen levels. Non-replicating viruses such as MVA are also characterized by relatively little antigen. If these systems are combined with methods such as CpG treatment, which we show is sensitive to antigen levels, it might be that antigen dose differences, rather than priming pathways drive the outcome of experiments. We believe that this explains the results of experiments using CpG that at first glance suggest priming by MVA by any route and WR by i.d. injection in the ear requires cross priming. In both cases direct priming is possible, as shown by experiments with WR-SIIN and MVA-SIIN, but antigen dose is limited, either due to the extreme attenuation of MVA or because of the nature of the immunization site (34).

Our data do not clearly uncover what effect of CpG treatment effects anti-VACV $CD8^+$ T cell priming. However, the complex effects of systemic TLR ligation, which include splenomegaly, altered proportions of lymphocytes and changes to the organization of secondary lymphoid organs may all play a role (37). Additionally, CpG treatment increases indoleamine 2,3-dioxygenase expression and activation of regulatory T cells to suppress $CD8^+$ T cell immunity (38, 39). Finally, there may be a role for reduction of antigen dose for virulent viruses at some sites (as for WR after ear pinna immunization) because CpG treatment can reduce virus loads. While these effects

complicate experiments, it is less obvious how such broad changes might lead to differential suppression of responses across different VACV antigens (as in Fig. 3A). However, we note that $CD8^+$ T cell epitope-specific effects have been observed for co-administration of TLR-ligands with lymphocytic choriomeningitis virus, suggesting our results are unlikely to be VACV-specific (40). Alternatively, if the most relevant consequence of CpG treatment is the maturation of DCs, it is reasonable to suggest that this alters the outcome of VACV infection and/or the ability of DCs to process and directly present virus antigen. In this case, the high abundance of peptide-MHC complexes made by the immunodominant B8-20 and OVA minigene may have been what allowed responses to these to escape the reduction of priming caused by CpG treatment (41, 42). Perhaps also the relatively high frequency of $CD8^+$ T cells in the naïve repertoire with these specificities plays a role (43). In their original paper, Wilson et al (12) showed that direct priming of $CD8^+$ T cells against OVA expressed endogenously in DCs was not affected by CpG in CD11c-OVA transgenic mice. In that case one might expect that antigen was limited as for our experiments with MVA and infected cells, challenging our interpretation above. However in their model, antigen expression is established in the direct-presenting DCs prior to the CpG treatment and maturation might actually stabilize presentation (44). This is unlike the situation of immunization of mice after CpG treatent, where a virus must be able to infect and have its antigens processed and presented by DCs that have been matured many hours previously. We expect that our findings have implications for the use of other TLR ligands, such as synthetic dsRNA polyinosinic:polycytidylic acid (polyI:C) and lipopolysaccharide (LPS), that have been suggested to inhibit cross presentation *in vivo* (12, 45). Finally, there is no obvious reason why our data obtained with VACV and VACV-infected cells would not apply equally to other viruses.

CpG and cytc gave contradictory results and given the discussion above, it could perhaps be hoped that where the former fails, the latter provides more solid data. Two observations suggest this is not

the case. Firstly, the substantial enhancement of responses to B8-20 and especially OVA minigene when expressed from WR are impossible to explain if the only activity of cytc is to remove cross presenting DCs. Perhaps the killing of DCs by cytc disturbs the structure of secondary lymph organs, or creates an unexpected signal due to debris that somehow modulates $CD8^+$ T cell responses. Secondly and less ambiguously, cytc treatment inhibited priming by the OVA minigene when expressed from MVA-SIIN, demonstrating that this strategy impairs direct presentation. Why A47-171 was immune to the inhibition of priming for MVA, but not UV/psoralen inactivated WR is a mystery, but perhaps virus genetics plays a role. Mechanistically, treatment with cytc results in depletion of $CD8\alpha^+$ DCs (15) and this subset has been implicated in the direct priming of $CD8^+$ T cells by WR (46). If MVA is also presented by $CD8\alpha^+$ DCs, but relatively few are infected because MVA cannot replicate, a further reduction of these cells by cytc treatment might result in poor $CD8^+$ T cell priming.

While the main point of this paper is to discuss the failure of CpG and cytc to dissect priming pathways, our data have unavoidable implications for the presentation mechanisms that can be used by VACV strains WR and MVA. The use of WR-SIIN and MVA-SIIN by two routes, each of which prime similarly strong OVA-specific responses demonstrates that both strains of VACV are capable of robust direct priming. This confirms reports for WR, but contradicts one regarding MVA (10, 13). The reasons for the difference seen for MVA are not clear, but we note that the insertion site of the recombinant antigen was different (TK versus Del III, though the same promoter was used) (13). In support of a broader role for direct priming by VACV, we have similar results for other MVA and WR recombinants where MSIINFEKL (or another minigene) was expressed from regions other than TK (YCW and DCT, unpublished). This means the data here are not simply due to the use of TK^- VACVs. However, in view of the failure of CpG and cytc methods, our data here have no bearing on the presentation pathways used by the various native epitopes of VACV used in

our study. We do note that our experiments using UV/psoralen inactivated WR suggest that $CD8^+$ T cell responses to MVA very much resembles those of WR if differences in replication are controlled.

In conclusion, we show that much caution is required when interpreting experiments in which cross presentation is targeted by manipulating or killing DC subsets. This may be in part due to the complex outcome of treatments that affect DC function. However, we also suggest that direct and cross presentation of virus antigens are not functions that are separated according to DC subsets or their maturation state. For example $CD8\alpha^+$ DCs are likely to be required both for cross and direct presentation. This notion has ramifications for the interpretation of experiments where CpG or cytc have been used, but also for data obtained using mice lacking genes involved in the development or function of DCs (47, 48).

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Figure legends

Figure 1

CpG pretreatment inhibits cross presentation. CpG-treated or control mice were immunized i.p. with $1x10^6$ WR-OVA-infected and heat inactivated 293A cells and $CD8^+$ T cell responses to the peptides shown were measured in spleens 7 days later. (A) Representative FACS plots gated on $CD8^+$ events. The numbers indicate the percentages of IFN-γ^+ of $CD8^+$ events. (B)Data represent results from three independent experiments, each with groups of three mice.(C) Extent to which responses to B8-20, OVA or the sum of 13 other epitopes (others) were suppressed in CpG compared with control treated mice (based on the data in panel B). * denotes statistical significance ($p<0.05$).

Figure 2

CpG treatment suppresses anti-VACV $CD8^+$ T cell immunity after dermal immunization. (A) CpG-treated or control mice were immunized with $2x10^6$ PFU VACV WR (A, C left) or MVA (B, C right) and $CD8^+$ T cell responses to the peptides shown were measured in spleens 7 days later. (A and B) Means and SEM of the percent of $CD8^+$ T cells that made IFN-γ in a short stimulation with the peptides shown. (C) Extent to which responses to B8-20 or the sum of other epitopes (others) were suppressed in CpG compared with control treated mice for WR (left) and MVA (right). Data were compiled from two experiments, each with groups of three mice. * denotes statistical significance ($p<0.05$).

Figure 3

CpG treatment suppresses direct priming after dermal immunization. CpG-treated or control mice were immunized with $2x10^6$ PFU of (A) WR-SIIN or (B) MVA-SIIN. Graphs on the left show B8-20- and OVA-specific responses measured in the spleen after 7 days. Graphs on the right show the extent to which responses to B8-20 and OVA were suppressed in CpG compared with control treated mice, based on the data

on the left. Data were compiled from two experiments, each with groups of three mice. * denotes statistical significance ($p<0.05$).

Figure 4

The choice of antigen and virus replication dictate the inhibitory effect of CpG treatment on $CD8^+$ T cell responses. CpG-treated or control mice were immunized i.p. with $2x10^6$ PFU of (A) WR-SIIN, (B) MVA-SIIN or (C) UV/psoralen-inactivated WR-SIIN. In each case, $CD8^+$ T cell responses to the peptides shown were measured in spleens 7 days later. (D) Extent to which responses to B8-20, OVA or the sum of other epitopes (others) were suppressed in CpG compared with control treated mice after immunization with WR-SIIN (left), MVA-SIIN (middle), and UV/psoralen-treated WR-SIIN (right), based on the results from A, B and C respectively. N.S.: not significant. * denotes statistical significance ($p<0.05$). Results were compiled from three experiments, each with groups of three mice.

Figure 5

CpG treatment reduces VACV loads, but lower virus doses prime typical $CD8^+$ T cell responses. A) and B) CpG-treated or control mice were immunized with $2x10^6$ PFU VACV WR by i.d. (A) or WR-SIIN by i.p. (B) routes. Virus titers from organs as shown were determined by standard plaque assay 1 and 3 days later (C. LN, cervical LN; M. LN, mediastinal LN). Data in (A) are representative of two experiments with groups of 3 mice. Data in (B) are combined from two experiments. C) and D) Mice were immunized i.d. with $2x10^3$ PFU WR and virus titres in LN and ear (C) or $CD8^+$ T cell responses in the spleen (D) were measured 1 and 3 or 7 days later, respectively. Data in (C) are representative of two experiments with 2 mice and in (D) represent results of four mice from two experiments. * denotes statistical significance $p<0.05$.

Figure 6

Increased antigen dose overcomes the inhibitory effect of CpG treatment on $CD8^+$ T cell responses. (A) Mice were immunized i.p. with $2x10^8$ PFU of MVA-SIIN on day 0 and day 2 one day after PBS or CpG treatments. (B) CpG-treated and untreated mice were immunized i.p. with $2.5x10^7$ WR-OVA-infected heat-inactivated 293A cells. Graphs on the left show the $CD8^+$ T cell responses to OVA and B8-20 measured in spleens 7 days after immunization. Graphs on the right show the extent to which responses were suppressed in CpG compared with control treated mice, based on the data presented on the left. Results were compiled from two experiments, each with groups of three mice.* denotes statistical significance ($p<0.05$).

Figure 7

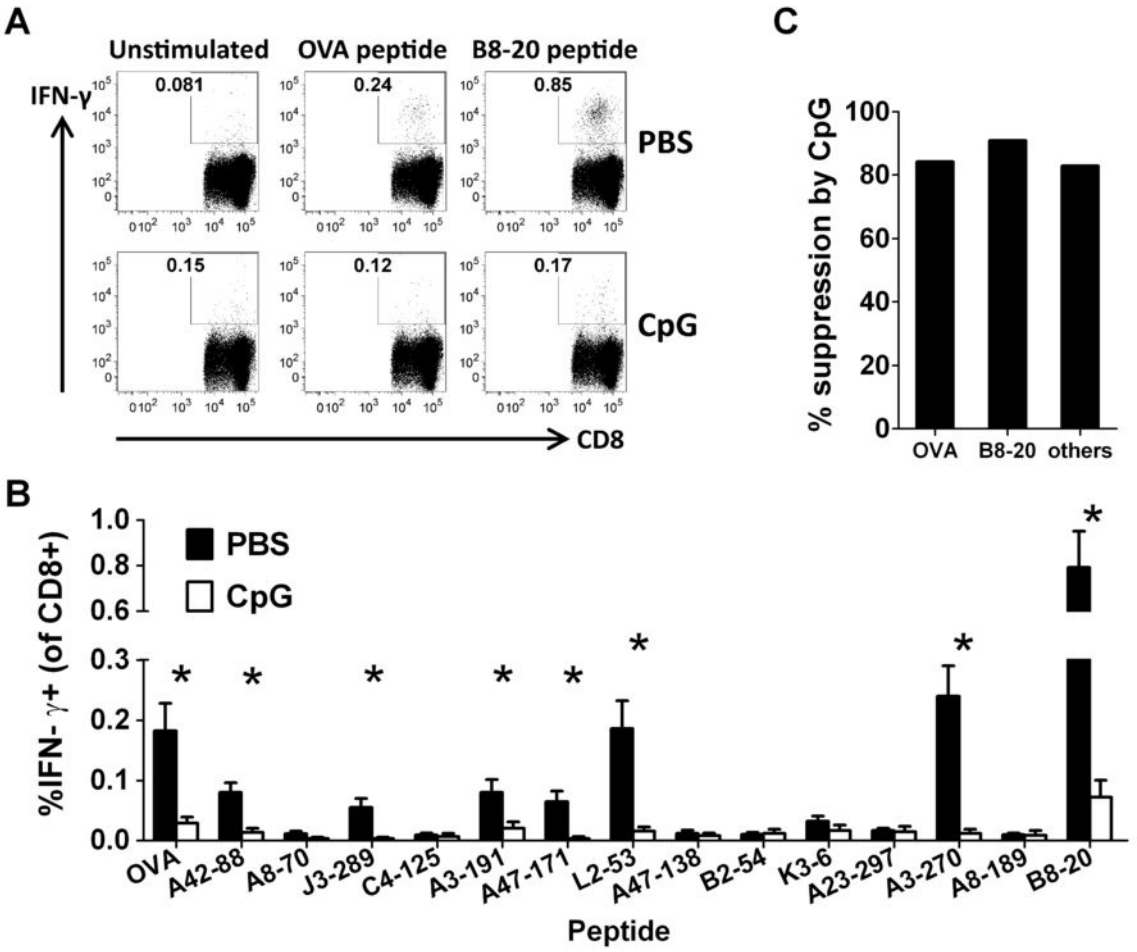
Cytc treatment does not dissect $CD8^+$ T cell priming pathway for VACV WR and MVA. Cytc-treated or control mice were immunized i.p. with 1×10^6 WR-infected, heat killed cells (A) or 2×10^6 PFU of WR-SIIN (B), MVA-SIIN (C) or UV/psoralen treated WR-SIIN (D). $CD8^+$ T cell responses to the peptides shown were measured in spleens 7 days later. Data are combined from three (B) and two (A, C, D) experiments to give a total of 6-9 mice per treatment, except for (A): n=2 and 5 for PBS and cytc respectively. * denotes statistical significance ($p<0.05$).

Table I. Peptides used in the study

Name	Origin of peptide	Sequence
OVA	Chicken $OVA_{257-264}$	SIINFEKL
A42-88	VACV $A42_{88-96}$	YAPVSPIVI
A8-70	VACV $A8_{70-77}$	IHYLFRCV
J3-289	VACV $J3_{289-296}$	SIFRFLNI
C4-125 *	VACV $C4_{125-132}$	LNFRFENV
A3-191	VACV $A3_{191-199}$	YSPSNHHIL
A47-171	VACV $A47_{171-180}$	YAHINALEYI
L2-53	VACV $L2_{53-61}$	VIYIFTVRL
A47-138	VACV $A47_{138-146}$	AAFEFINSL
B2-54 *	VACV $B2_{54-62}$	YSQVNKRYI
K3-6	VACV $K3_{6-15}$	YSLPNAGDVI
A23-297	VACV $A23_{297-305}$	IGMFNLTFI
A3-270	VACV $A3_{270-277}$	KSYNYMLL
A8-189	VACV $A8_{189-196}$	ITYRFYLI
B8-20	VACV $B8_{20-27}$	TSYKFESV

* MVA does not express C4-125 and B2-54 peptides

Figure 1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Unstimulated
OVA peptide
B8-20 peptide
IFN-γ
0.081
0.24
0.85
PBS
0.15
0.12
0.17
CpG
CD8
C
% suppression by CpG
100
80
60
40
20
0
OVA
B8-20
others
B
%IFN-γ+ (of CD8+)
PBS
CpG
1.0
0.8
0.6
0.3
0.2
0.1
0.0
OVA
A42-88
A8-70
J3-289
C4-125
A3-191
A47-171
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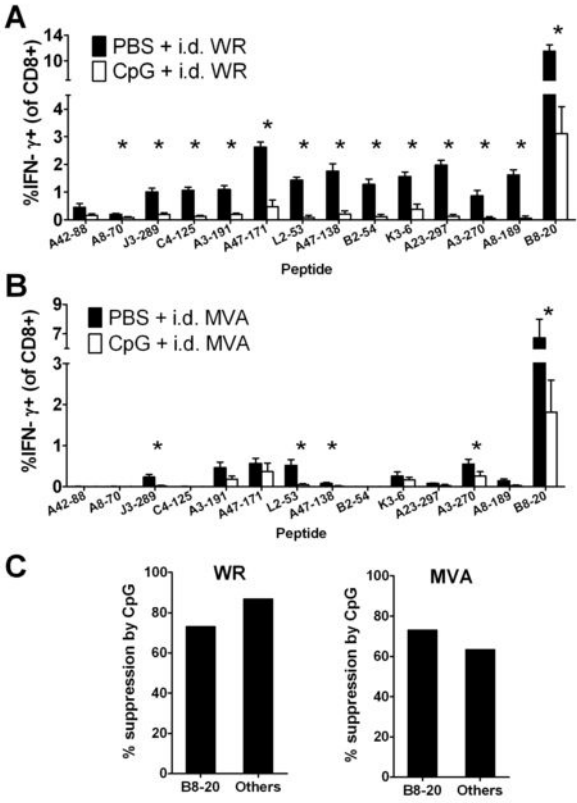
B8-20
Peptide

Figure 2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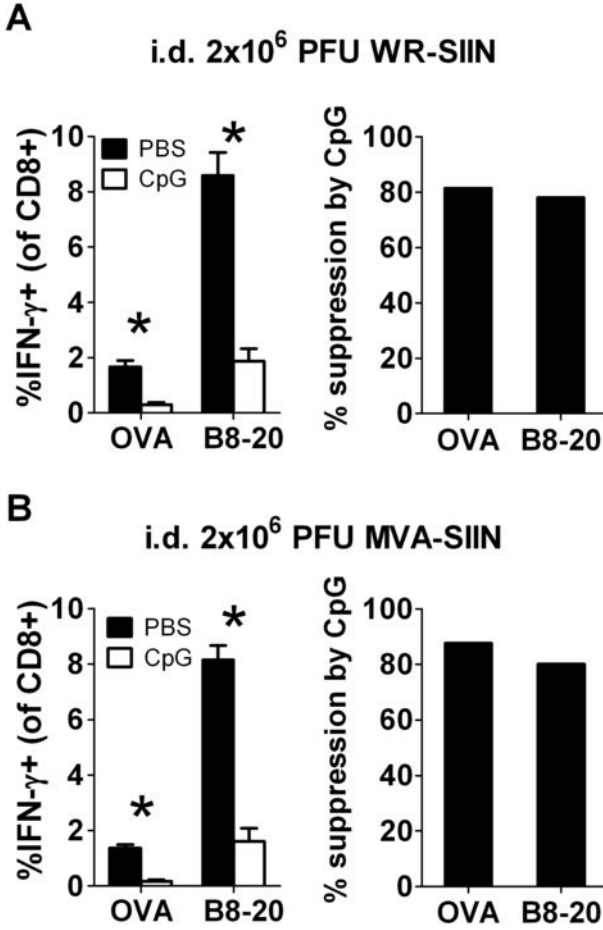
PBS + i.d. WR
CpG + i.d. WR
%IFN- γ+ (of CD8+)
14
10
4
3
2
1
0
*
A42-88
A8-70
J3-289
C4-125
A3-191
A47-171
L2-53
A47-138
B2-54
K3-6
A23-297
A3-270
A8-189
B8-20
Peptide
B
PBS + i.d. MVA
CpG + i.d. MVA
%IFN- γ+ (of CD8+)
9
7
3
2
1
0
*
A42-88
A8-70
J3-289
C4-125
A3-191
A47-171
L2-53
A47-138
B2-54
K3-6
A23-297
A3-270
A8-189
B8-20
Peptide
C
WR
% suppression by CpG
100
80
60
40
20
0
B8-20
Others
MVA
% suppression by CpG
100
80
60
40
20
0
B8-20
Others

Figure 3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i.d. 2x10^6 PFU WR-SIIN
%IFN-γ+ (of CD8+)
PBS
CpG
OVA
B8-20
% suppression by CpG
B
i.d. 2x10^6 PFU MVA-SIIN

Figure 4

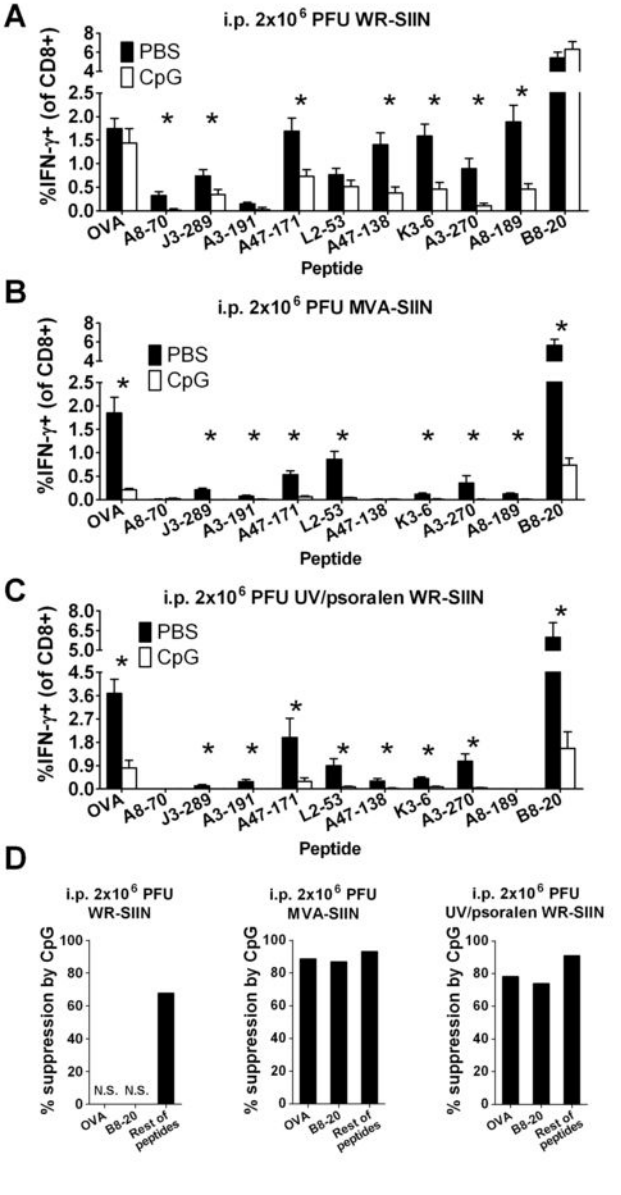
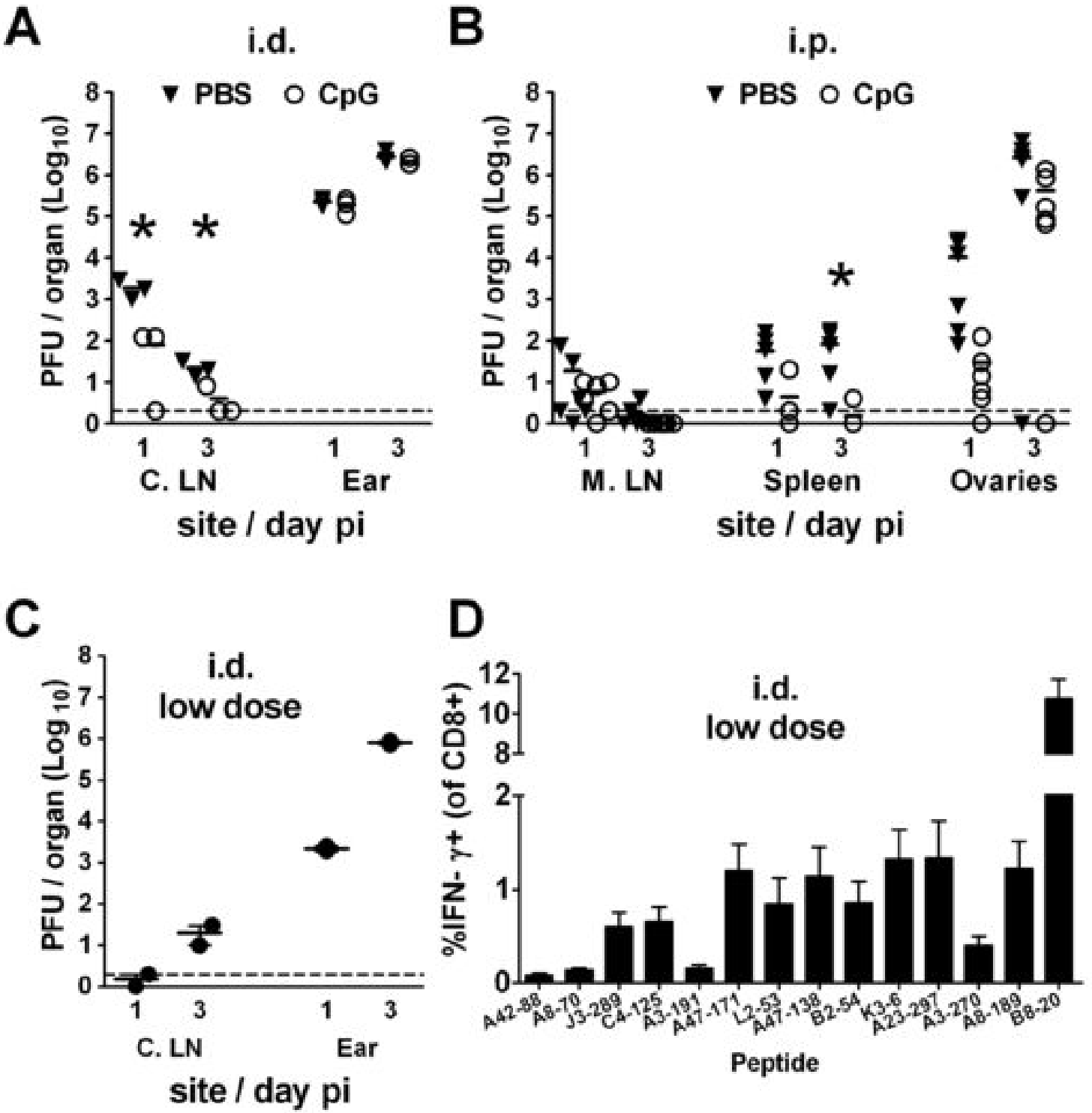

A
i.p. 2x10^6 PFU WR-SIIN
%IFN-γ+ (of CD8+)
PBS
CpG
OVA
A8-70
J3-289
A3-191
A47-171
L2-53
A47-138
K3-6
A3-270
A8-189
B8-20
Peptide
B
i.p. 2x10^6 PFU MVA-SIIN
%IFN-γ+ (of CD8+)
PBS
CpG
OVA
A8-70
J3-289
A3-191
A47-171
L2-53
A47-138
K3-6
A3-270
A8-189
B8-20
Peptide
C
i.p. 2x10^6 PFU UV/psoralen WR-SIIN
%IFN-γ+ (of CD8+)
PBS
CpG
OVA
A8-70
J3-289
A3-191
A47-171
L2-53
A47-138
K3-6
A3-270
A8-189
B8-20
Peptide
D
i.p. 2x10^6 PFU WR-SIIN
% suppression by CpG
N.S.
N.S.
OVA
B8-20
Rest of peptides
i.p. 2x10^6 PFU MVA-SIIN
% suppression by CpG
OVA
B8-20
Rest of peptides
i.p. 2x10^6 PFU UV/psoralen WR-SIIN
% suppression by CpG
OVA
B8-20
Rest of peptides

Figure 5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i.d.
PBS
CpG
PFU / organ (Log10)
C. LN
Ear
site / day pi
B
i.p.
PBS
CpG
PFU / organ (Log10)
M. LN
Spleen
Ovaries
site / day pi
C
i.d.
low dose
PFU / organ (Log 10)
C. LN
Ear
site / day pi
D
i.d.
low dose
%IFN- γ+ (of CD8+)
A42-88
A8-70
J3-289
C4-125
A3-191
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B8-20
Peptide

Figure 6

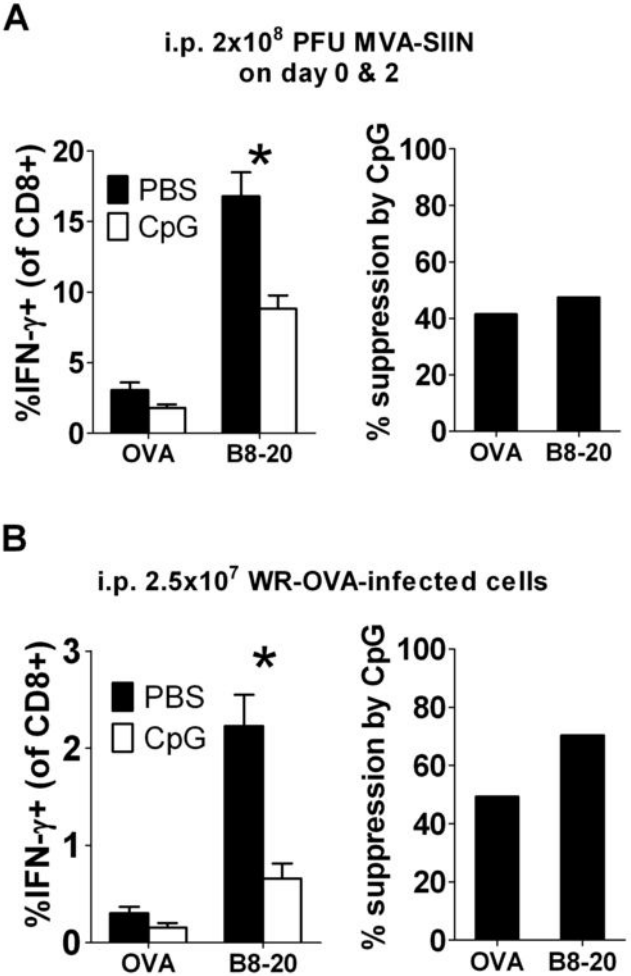
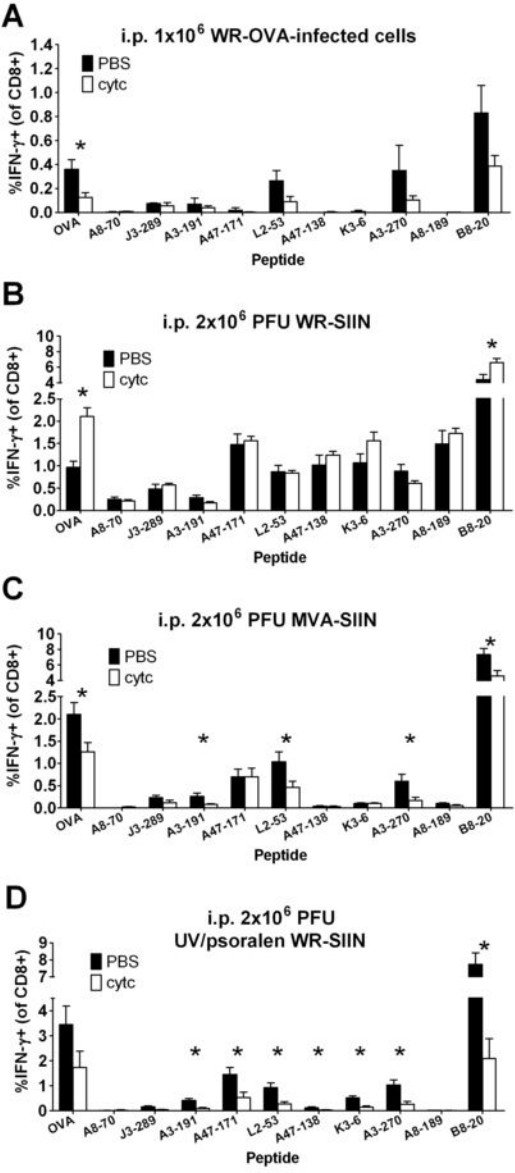

A
i.p. 2x10^8 PFU MVA-SIIN
on day 0 & 2
%IFN-γ+ (of CD8+)
PBS
CpG
*
OVA
B8-20
% suppression by CpG
OVA
B8-20
B
i.p. 2.5x10^7 WR-OVA-infected cells
%IFN-γ+ (of CD8+)
PBS
CpG
*
OVA
B8-20
% suppression by CpG
OVA
B8-20

Figure 7


A
i.p. 1x10^6 WR-OVA-infected cells
PBS
cytc
%IFN-γ+ (of CD8+)
OVA
A8-70
J3-289
A3-191
A47-171
L2-53
A47-138
K3-6
A3-270
A8-189
B8-20
Peptide
B
i.p. 2x10^6 PFU WR-SIIN
C
i.p. 2x10^6 PFU MVA-SIIN
D
i.p. 2x10^6 PFU
UV/psoralen WR-SIIN