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Eduardo Davila and Esteban Celis

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Repeated Administration of Cytosine-Phosphorothiolated Guanine-Containing Oligonucleotides Together with Peptide/Protein Immunization Results in Enhanced CTL Responses with Anti-Tumor Activity

Eduardo Davila and Esteban Cels

The development of therapeutic anti-cancer vaccines designed to elicit CTL responses with anti-tumor activity has become a reality thanks to the identification of several tumor-associated Ags and their corresponding peptide T cell epitopes. However, peptide-based vaccines, in general, fail to elicit sufficiently strong CTL responses capable of producing therapeutic anti-tumor effects (i.e., prolongation of survival, tumor reduction). Here we report that repeated administration of synthetic oligonucleotides containing foreign cytosine-phosphorothiolated guanine (CpG) motifs increased 10- to 100-fold the CTL response to immunization with various synthetic peptides corresponding to well-known T cell epitopes. Moreover, repeated CpG administration allowed the induction of CTL to soluble protein even in the absence of additional adjuvant. Our results indicate that the potentiating effect of CpG in CTL responses required the participation of Th lymphocytes. Repeated CpG administration resulted in overt splenomegaly and lymphadenopathy with a significant increase in the numbers of CTL precursors and dendritic cells. Protein vaccination in combination with repeated CpG therapy was effective in delaying tumor cell growth and extending survival in mice bearing melanoma tumors. These findings support the contention that repeated administration of CpG-oligonucleotides enhances the effect of peptide and protein vaccines leading to potent anti-tumor responses, presumably through the induction of Th1 and dendritic cells, which are essential for optimal CTL responses. The immunostimulatory properties of CpG motifs may be key in inducing a consistent long term immunity to tumor-associated Ags when using peptides or proteins as T cell-inducing vaccines. The Journal of Immunology, 2000, 165: 539–547.
the use of live cell vaccines for the treatment of cancer. However, vaccination with proteins or synthetic peptides representing discrete CTL epitopes in most instances fails to induce sufficiently strong CTL responses, offering minimal therapeutic benefit against tumors expressing the relevant TAA. Moreover, in some circumstances peptide vaccination has shown to inhibit T cell responses, possibly by deleting or tolerizing the peptide-reactive T cells (14).

The most likely explanation for these unsatisfactory results with peptide vaccination is that the immunizing peptides are not delivered to activated professional APC such as DC and when these epitopes are presented to CTL by cells that lack costimulatory activity, a state of tolerance or anergy is induced. Furthermore, it has been suggested and substantiated that the appropriate inflammatory and/or other danger signals are necessary to activate DC to stimulate naive CTL to attain their effector function (15, 16).

Among the various types of danger signals to which the immune system responds are bacterial and viral products, such as toxins, LPS, and DNA, particularly those DNA sequences containing abundant cytosine guanine motifs (17, 18).

With the purpose of developing peptide-based vaccines for the therapy of cancer, we have initiated studies aimed at eliciting strong CTL responses effective against tumors. Herein, we report that repeated daily administration of synthetic oligonucleotides (ODN) containing cytosine-phosphorothiolated guanine (CpG) motifs significantly enhanced CTL responses to poorly immunogenic peptides representing CTL epitopes even in the absence of additional adjuvants. Surprisingly, CpG therapy was also effective in inducing strong CTL activity to soluble protein, resulting in significant prophylactic and therapeutic anti-tumor responses. The enhancement of CTL responses by CpG appears to be mediated by a large increase in the numbers of DC and CTL precursors in peripheral lymphoid organs and to depend, to some extent, on the presence of Th lymphocytes (HTL). These results should facilitate the utilization of well-characterized peptide- or protein-based vaccines for the therapy of cancer.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA). The OT-I TCR transgenic mice (19), developed by Dr. F. Carbone (Monash Medical School, Victoria, Australia) were obtained from Dr. M. Mescher (University of Minnesota, Minneapolis, MN). These mice are in the C57BL/6 background (H-2b) and express a transgenic TCR that reacts with the OVA-expressing EG.7 cell line (22), were obtained from American Type Culture Collection (Manassas, VA). The SV40 T Ag-expressing cell lines, C57SV and 1803.1, both derived from C57BL/6 mice (23, 24), were provided by Dr. B. Knowles (The Jackson Laboratory). The OVA-transfected B16 melanoma cell line, B16-OVA (25), was obtained from E. Lord (University of Rochester Medical Center, Rochester, NY). All these cell lines were maintained in tissue culture in DMEM or RPMI containing 10% FBS, t-glutamine, and antibiotics.

Immunogens and CpG therapy

All experiments were routinely performed in groups of three to five mice each. Mice received nine daily injections of 100 μg of CpG-1826 (9X-GpCpG) in PBS or PBS alone. Synthetic OVA-derived peptides SIINFEKL (OVA257–264), the SV40-IV CTL epitope (VVYDFLKC), was provided by guest on May 30, 2017 http://www.jimmunol.org/ Downloaded from...
FIGURE 1. Single injection of CpG-1826 augments the Ag-specific CTL response to peptide vaccination. C57BL/6 mice (three mice per group) were immunized s.c. with SV40-IV peptide and PADRE HTL epitope emulsified in IFA together with CpG-1826 (■) or without CpG-1826 (□). The cytolytic activity of splenocytes was determined in a 4-h cytotoxicity assay as described in Materials and Methods against the following targets: EL4 (A), peptide-pulsed EL-4 (B), and 1803.1, a SV40 T-expressing cell line (C).

Evaluation of anti-tumor effects in vaccinated mice

The effect of protein vaccination (with and without 9X-CpG) on tumor growth and survival was evaluated in both prophylactic and therapeutic modes. For prophylaxis, groups of five mice were immunized with OVA protein (or vehicle/PADRE peptide alone) with or without 9X-CpG regimens as described above. Seven days later mice were challenged s.c. with 5 × 10⁶ live B16-OVA cells in the rear leg flank. Mice were observed daily or every other day, and when tumors became evident, their perpendicular diameters were measured using a set of calipers. Although most tumor-bearing mice died on their own, some animals were euthanized when tumors became ulcerated or surpassed 600 mm². Significance analysis for the prevention and therapeutic use of 9X-CpG in tumor growth results was performed using log-rank tests (26).

Results

CpG is a potent adjuvant for CTL induction using peptide immunogens

For our initial experiments, we selected a well-characterized H-2Kd-restricted CTL epitope derived from the SV40 T Ag, peptide SV40-IV (VYDFLKC) (27). Normal C57BL/6 mice received a single injection of 50 μg of peptide SV40-IV mixed with 140 μg of HTL epitope peptide, PADRE (20) emulsified in IFA. This vaccine was administered s.c. either with or without 100 μg of CpG-1826. The data presented in Fig. 1 demonstrate that a single injection of peptide in the presence of CpG-1826 increased severalfold the CTL response to peptide immunization. Similar results were obtained when this peptide was administered without IFA, although the levels of cytotoxicity were 10–20% lower than those observed with IFA (data not shown).

It has been reported that ODN containing CpG motifs increase DC activity, which could help explain its ability to enhance CTL responses (18, 28, 29). Also, there are reports that some immuno-stimulatory factors, such as soluble Flt-3 ligand, increase DC numbers and their activity, but require repeated daily administration (for 9 days) for optimal effects to be achieved (30). Thus, we explored whether a 9-day regimen of CpG therapy (referred to here as 9X-CpG) would further increase its CTL immunostimulatory activity to a single injection of peptide SV40-IV. The results from three separate experiments presented in Table I show that repeated administration of CpG was capable of further enhancing CTL responses, as measured by lytic units (LU), to both peptide-sensitized targets (EL4 plus SV-40IV) and 1803.1 cells, which express the SV40 T Ag. These results were substantial when comparing the values of LU that were calculated per spleen in each animal (shown in parentheses).

To determine whether CpG-ODN would also be effective in enhancing CTL responses to poorly immunogenic peptides, we tested the effect of 9X-CpG in mice immunized with a well-known CTL epitope derived from OVA (SIINFEKL) (31), which in our hands routinely fails to elicit CTL when administered in IFA even in the presence of HTL peptides. The results presented in Fig. 2 demonstrate that only those mice receiving the peptide vaccine in combination with 9X-CpG produced significant CTL responses to the SIINFEKL epitope.

Repeated administration of CpG increases the numbers of DC and T cell precursors

Throughout these experiments, we observed that the spleens from 9X-CpG mice that were removed 6 or 7 days after the last CpG administration were ~3–4 times larger than spleens derived from

Table I. Repeated administration of CpG-1826 enhances CTL responses to peptide vaccination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LU₃₀ Against EL4 + SV40-IV Targets</th>
<th>LU₃₀ Against 1803.1 (SV40T⁺) Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expt. 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;1</td>
<td>16 (1.2 × 10³)</td>
</tr>
<tr>
<td>9X-CpG</td>
<td>200 (7.1 × 10⁴)</td>
<td>345 (1.2 × 10³)</td>
</tr>
<tr>
<td><strong>Expt. 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CpG-1826 (single injection)</td>
<td>6 (1.3 × 10³)</td>
<td>13 (2.8 × 10³)</td>
</tr>
<tr>
<td>9X-CpG</td>
<td>11 (4.1 × 10³)</td>
<td>89 (3.4 × 10³)</td>
</tr>
<tr>
<td><strong>Expt. 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CpG-1826 (single injection)</td>
<td>20 (5.9 × 10³)</td>
<td>29 (8.2 × 10³)</td>
</tr>
<tr>
<td>9X-CpG</td>
<td>50 (6.7 × 10⁴)</td>
<td>174 (2.3 × 10⁵)</td>
</tr>
</tbody>
</table>

* Mice (three per group) were immunized with peptide SV40-IV mixed with PADRE in IFA with and without CpG therapy. CTL activity was determined 7–10 days after the immunization as described in Materials and Methods, and LU₃₀ values for each target were calculated after subtracting the lysis of unpulsed EL4 cells. Numbers represent the LU₃₀ per 1 × 10⁶ splenocytes. Numbers in parentheses are the LU₃₀ calculated per spleen.
normal animals (or from mice that received a single CpG injection). When we proceeded to quantify the total number of splenocytes in mice that received one to nine daily s.c. injections of CpG, the results showed a continuous increment of cells, which reached a 5-fold increase in cell numbers with nine doses of CpG, reaching up to \(3.7 \times 10^8\) cells/spleen by day 9, whereas control mice receiving saline injections had \(~7 \times 10^7\) cells/spleen (data not shown). The dramatic increase in lymphoid cells was also observed in most other lymphoid organs, especially those draining near the injection sites, such as the axillary lymph nodes (not shown). The numbers of DC, measured by cells expressing both the DEC-205 and CD11c markers, in the spleens of mice that received 9X-CpG were \(~30\)-fold higher than those in mice injected with PBS \((41\) million/spleen in 9X-CpG vs \(1.2\) million/spleen in 9X-PBS). Similarly, the total number of CD3+ T cells obtained from spleens of mice treated with 9X-CpG was significantly higher than that in animals that did not receive this treatment \((71\) million/spleen in 9X-CpG vs \(13\) million/spleen in nontreated mice).

The large increase in DC numbers in spleens of CpG-treated mice could result in the capacity of these APC to prime CTL in vitro during Ag restimulation step of the T cell cultures. If this were the case, the in vitro CTL priming could explain to some extent the magnitude of the responses presented in Figs. 1 and 2 and Table I. However, this possibility seems unlikely, because CTL responses to the SIINFEKL peptide from OVA were not observed in spleen cell cultures derived from mice that received 9X-CpG and were vaccinated with the PADRE HTL epitope in IFA (without the CTL peptide), although these cultures were restimulated with irradiated EG.7 cells, which express OVA (data not shown). Nevertheless, to establish whether peptide vaccination combined with 9X-CpG or a single injection of CpG-1826 results in the in vivo expansion of CTL, precursor frequency analyses were performed. The results presented in Table II indicate that a single injection of CpG-1826 increased the number of CTL precursors to this CTL epitope 5- to 15-fold (per spleen) compared with that in mice that did not receive CpG. Furthermore, the effect of 9X-CpG was strikingly greater, because it increased 42–270 times the number of CTL precursors (per mouse). These results indicate that the potentiating effect of CpG requires the presence of Ag in vivo, and as a result of CpG administration, Ag-specific CTL expand more effectively in vivo than in mice receiving Ag alone.

Although the numbers of Ag-specific CTL increased significantly in the spleens of mice receiving nine daily doses of CpG, these numbers are relatively small compared with the increase in the total number of T cells that results from this therapy. Therefore, it is likely that CpG also stimulates the expansion of other T cells in addition to those activated by the immunizing Ag. To analyze the non-Ag-driven T cell stimulatory activity of CpG and to further assess the in vivo requirement of Ag for CTL induction, OT-1 TCR transgenic mice were treated with the 9X-CpG regimen in the presence and the absence of a single injection of the SIINFEKL peptide (which is recognized by the T cells from this mouse strain). Nine days after the last injection of CpG (or PBS in control mice), the draining axillary lymph nodes were examined to determine whether the OT-1 CTL were activated as the result of the various modes of treatment. Lymph node T cells expressing the Vα2 TCR (present on the OT-1 T cells) were studied for their increase in expression of several activation markers (CD44, CD25, and CD69) by cytofluorometric analysis. The results presented in Table III indicate that the percentage of OVA-specific CTL (as determined by Vα2+ T cells) in lymph nodes of OT-1 mice did not vary significantly in response to 9X-CpG or peptide vaccination. However, when taking into account the overall increase in lymph node size (and cell numbers), the mice that received 9X-CpG had \(~3\) times more Vα2+ T cells than the nontreated mice. Analyses of T cell activation molecules (CD44, CD25, and CD69) revealed that the expression of these markers was increased only in Vα2+ T cells from mice vaccinated with peptide plus 9X-CpG therapy and not in the mice that received the 9X-CpG treatment alone or the control untreated mice.

The cytotoxic activity of the lymph node-derived OT-1 cells was measured after placing these cells in tissue culture for 4 days in the presence of IL-2 without further addition of Ag. The data shown in Fig. 3 demonstrate that the CTL derived from the peptide-immunized OT-1 mice had significant cytolytic activity against peptide-pulsed EL4 cells and OVA-expressing EG.7 target cells. On the other hand, considerably less activity was observed in the CTL from the mice that did not receive peptide immunization. Taken together, these results suggest that although repeated CpG
administration induces an overall expansion of T cells, Ag is required to fully activate these cells into mature effector CTL.

Role of HTL in the potentiating effect of CpG therapy in CTL responses

Because HTL are believed to play an important role in CTL responses mainly by producing growth factors for CTL (IL-2) and by conditioning DC for presentation of Ag to CTL precursors, we routinely include in our vaccination protocols a peptide that induces strong HTL responses. To directly examine the role of HTL in the CTL responses enhanced by 9X-CpG therapy, we performed experiments in CIIKO mice, which lack CD4⁺ HTL. The results presented in Fig. 4 show that, as in past experiments, 9X-CpG treatment increased CTL responses in normal mice immunized with either SV40T or SIINFEKL peptide epitopes. On the other hand, 9X-CpG therapy did not potentiate the CTL response of CIIKO mice to peptide SV40-IV immunization. Nevertheless, a small enhancing effect of 9X-CpG was observed on the response of CIIKO mice to SIINFEKL peptide epitopes. These results indicate that HTL participate in the potentiating effect of CpG in CTL responses to peptide vaccination.

9X-CpG therapy augments CTL responses to soluble protein

In general, CTL responses are difficult to generate when intact soluble proteins are used as immunogens. Nevertheless, there are reports that insoluble vaccine formulations, such as proteins bound to polymer beads (32) or to ISCOMS (33), can elicit CTL responses. It appears that the particle-bound protein may become immunogenic for CTL by targeting the Ag into specialized phagocytic cells that are able to process and present these Ags within the MHC class I pathway. Because the particulate form of protein Ag tend to be difficult to produce and characterize, we evaluated whether 9X-CpG therapy would facilitate the induction of CTL to a soluble protein. The results presented in Fig. 5 show that 9X-CpG therapy was successful in enhancing the generation of CTL to OVA when this protein was administered in either IFA or PBS. The responses in IFA were moderately higher than those obtained in PBS. On the other hand, immunization with OVA protein in IFA in the absence of 9X-CpG failed to generate any detectable CTL. Because the CTL responses to EL4 cells pulsed with the OVA peptide (SIINFEKL) were almost identical with the responses observed to the EG.7 targets (which express the whole OVA molecule), it is likely that the majority of the CTL induced by the Ag were directed toward this immunodominant epitope. Thus, from these results it is evident that repeated administration of CpG up-regulates the immune system to allow the generation of CTL after the administration of vaccines prepared containing soluble Ag. We have recently obtained similar results using another protein (VP2 from Theiler’s virus; data not shown), suggesting that this effect of CpG may extend various types of soluble proteins in the absence of additional adjuvants.

![FIGURE 3](http://www.jimmunol.org)
Anti-tumor activity of CTL induced with 9X-CpG therapy

To evaluate the physiological role of CTL induced by protein vaccination in combination with 9X-CpG therapy, groups of five mice were immunized once with OVA protein plus PADRE in IFA with and without the 9X-CpG regimen. In addition, a third group was immunized with the PADRE peptide alone in IFA in combination with 9X-CpG therapy, and a fourth group received no treatment. One week after vaccination, the splenocytes were restimulated in tissue culture with irradiated C57SV cells (A and B) or EG.7 cells (C and D), and 1 wk later the cytolytic activity was determined in a 4-h assay against the following targets: EL4 plus SV40-IV (A and B), 1803.1 (B), EL4 plus SIINFEKL (C), and EG.7 (D). The cytolytic activity of all effector cell populations against unpulsed EL4 cells was <5% in all cases (data not presented).

Anti-tumor activity of CTL induced with 9X-CpG therapy

To evaluate the physiological role of CTL induced by protein vaccination in combination with 9X-CpG therapy, groups of five mice were immunized once with OVA protein plus PADRE in IFA with and without the 9X-CpG regimen. In addition, a third group was immunized with the PADRE peptide alone in IFA in combination with 9X-CpG therapy, and a fourth group received no treatment. One week after vaccination all mice were challenged s.c. with $4 \times 10^5$ live B16 melanoma cells that express the OVA protein (B16-OVA). The data presented in Fig. 4 indicate that the animals that were not treated and those that received 9X-CpG with PADRE in IFA in the absence of OVA rapidly developed tumors (by day 12), and all died by day 20. Similarly, three of the mice that were injected with OVA and PADRE in IFA without 9X-CpG therapy developed tumors by day 17 and were dead by day 20. Nevertheless, two mice from this group had a delay in their tumor growth, but died by day 50. In contrast, four of five animals that received 9X-CpG treatment in conjunction with protein vaccine remained tumor free and survived until day 60, when the experiment was terminated. Furthermore, the only animal that developed a tumor in this group survived for a significant longer period than the mice that were not treated or those that received 9X-CpG and HTL peptide without protein vaccination.

Next, we proceeded to ascertain whether protein immunization in combination with 9X-CpG would be capable of exhibiting any therapeutic effect in established s.c. tumors. For this experiment, groups of five mice were injected s.c. with $4 \times 10^5$ live B16-OVA cells, and 3 days later 9X-CpG therapy was initiated. Protein vaccine in IFA was administered 7 days after tumor inoculation. The
In addition to the production of high numbers of potent APC, the results presented herein show that a 9-day repeated administration of synthetic ODN containing CpG motifs significantly enhanced CTL responses to peptide and protein immunogens and that this effect depends to some extent on the participation of MHC class II-restricted T cells. Furthermore, the 9X-CpG regimen that we used to enhance CTL responses produced a substantial increase in the number of DC, similar to what has been reported with the administration of Flt-3 ligand (30). In addition to the increase in DC numbers, 9X-CpG therapy generated an Ag-independent proliferative response of T and B lymphocytes, which resulted in splenomegaly and lymphadenopathy (34). However, the activation of T cells to become effector CTL appears to require the in vivo presentation of either peptide or protein because target cells (EL-4) incubated overnight with a high concentration of OVA protein (1 mg/ml) could not be sensitized for lysis by OVA-specific CTL (data not shown). Peptide vaccination, administered usually in IFA emulsions, is commonly thought to be an effective way to induce CTL responses, which in some cases provides protection against live tumor challenges (36). However, the magnitude and effectiveness of the CTL responses derived from peptide vaccination vary significantly depending on the specific peptide used and possibly due to other factors that are more difficult to control, such as the presence of stimulatory factors such as endotoxin (LPS) in the vaccine formulation or the presence of microorganisms or their products in the animal's living environment, which could provide the necessary “danger signals” to the immune system. Most importantly, it has been reported that in some circumstances peptide vaccination, even in the presence of adjuvant, may result in the induction of T cell tolerance/anergy, which would have the opposite effect desired for tumor immunotherapy (14). The most likely explanation of these variable effects is that to obtain an effector CTL response, peptides may need to be presented to the naive CTL precursors by a professional APC in the context of a danger signal (15). On the other hand, peptide presentation by non-APC or even by professional APC in the absence of such danger signals could result in T cell anergy or inactivation (37). Our results suggest that the repeated administration of CpG not only increases the total number of professional APC (DC), but also may provide the necessary danger signal to facilitate the activation and expansion of CTL to vaccination with poorly immunogenic peptides. In fact, it has been reported that CpG can induce activation and maturation of DC (28, 29), which is one of the effects caused by danger signals (LPS, TNF, necrosis) necessary for effective CTL induction (16).

It is thought that the ability of some peptides to induce CTL may be due to the lack of a processing requirement that may allow the peptides to bind directly to cell surface MHC molecules that are temporarily empty or by displacing low affinity binding peptides. In fact, the capacity of peptides to induce CTL responses derived from vaccination correlates with their MHC binding affinity (38). On the other hand, vaccines containing intact soluble proteins usually fail to elicit CTL responses, because the peptide epitopes need to be generated intracellularly through the MHC class I Ag-processing pathway. Furthermore, exogenous Ags that are endocytosed by most APC are more likely to generate MHC class II epitopes than class I CTL epitopes. Despite this, it has been reported that proteins in the form of particles (conjugated to beads, incorporated into immune-stimulating complexes (ISCOMS), liposomes, or in detergent micelles) are capable of inducing CTL responses, possibly by targeting the Ags to specialized APC that can ingest the particles, process the proteins, and target the epitopes to the class I MHC pathway (32, 33, 39, 40). Our results show that vaccination with soluble protein using the 9X-CpG regimen results in strong CTL responses that are not be observed in the absence of CpG. Thus, it appears that the APC that are generated and stimulated by 9X-CpG treatment are capable of capturing and processing soluble proteins into class I MHC CTL epitopes. In support of this, there are various examples that DC pulsed in vitro with intact soluble proteins are capable of inducing CTL responses when injected into mice (41). It is currently unknown whether the soluble protein is processed intracellularly by the APC in our system. The possibility that the OVA preparation used here may contain some peptide fragments including the SINFEKL peptide was considered, but seems unlikely, because target cells (EL-4) incubated overnight with a high concentration of OVA protein (1 mg/ml) could not be sensitized for lysis by OVA-specific CTL (data not shown).

In addition to the production of high numbers of potent APC, 9X-CpG therapy results in a substantial increase in T lymphocytes in peripheral lymphoid organs. However, these T cells do not appear to be fully activated, as determined by the presence of several cell surface activation markers (CD25, CD44, and CD69). Furthermore, our results show that Ag (in the form of either peptide or protein) is required in vivo to produce CTL with lytic activity. The
observed that the administration of blocking anti-CTLA-4 mAbs potentially improved the efficacy of therapeutic peptide vaccination combined with 9X-CpG treatment (E. Davila, manuscript in preparation).

One final point worth discussing is that the capacity of synthetic peptides and soluble proteins to induce CTL responses when administered together with 9X-CpG has significant practical and economical implications for the production of vaccines. First, although insoluble proteins (coupled into latex or biodegradable beads, incorporated into immune-stimulating complexes or liposomes) can induce CTL, these vaccine formulations tend to be complicated and expensive to produce. Furthermore, particle-based vaccines require extensive quality control testing with respect to those parameters that may contribute to their immunogenicity, such as particle size, composition, and quantity of Ag per particle mass. Lastly, the stability of particulate forms of protein/peptide Ags is likely to be an issue, in contrast to pure soluble proteins or peptides that in most instances can be stably preserved in a lyophilized form for long periods of time. The results presented here indicate that soluble proteins may be employed as well-characterized and stable vaccine preparations to generate effective CTL responses when repeated CpG administration is used to generate large numbers of DC capable of capturing and processing soluble Ags into MHC class I T cell epitopes.

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