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In Vivo Impact of CpG1826 Oligodeoxynucleotide on CD8 T Cell Primary Responses and Survival

Laurent Beloeil,* Martine Tomkowiak,* Georgi Angelov,* Thierry Walzer,2* Patrice Dubois,3† and Jacqueline Marvel3,4,*

CpG oligodeoxynucleotide (ODN)5 CG-containing molecules (CpG ODNs) have been extensively studied for their ability to stimulate activation of the innate and adaptive immune responses (1). Toll-like receptor 9, a member of the Toll-like receptor family, has recently been identified as the receptor for CpG ODNs (2). CpG ODNs trigger activation and maturation of dendritic cells (DC) resulting in increased cell surface expression of CD40, CD80, and CD86 (3–5). DC exposure to CpG ODNs induces expression of proinflammatory cytokines including IL-1, IL-6, TNF-α, and type I IFNs as well as the Th1-promoting cytokine IL-12 (3, 6). CpG has been shown to promote both B and T cell responses when combined with protein or peptide Ags. In addition, this adjuvant induces large populations of Ag-specific effector CD8 T cells capable of expressing IFN-γ and TNF-α and displaying cytotoxic activity (7–9). The ability of CpG to promote CD8 T cell responses following vaccination with proteins appears to bypass the need for CD4 T cells (8, 10, 11). This could be related to CpG-induced DC maturation and expression of costimulatory molecules such as CD40 which are essential for induction of CD8 T cell responses in vivo (12–14).

Many models have investigated the properties of CpG ODN on CD8 T cell responses by investigating protection against bacteria and rejection of tumors (15, 16). In this study, we sought to characterize the effects of CpG1826 on a population of influenza nucleoprotein (NP)-68-specific TCR transgenic CD8 T cells. In the F5 TCR transgenic mouse model, NP68 peptide priming leads to the generation of peptide-specific CD8 T cells that have the hallmark of memory CD8 T cells. Primed F5 CD8 T cells have a lower activation threshold, produce more IFN-γ and more rapidly than naive T cells (17, 18), and their RANTES secretion is augmented compared with naive CD8 T cells (19). These cells also express higher levels of cell surface CD44, Ly-6C, and CD122 which are associated with memory T cell populations (20, 21). Our data show that the adjuvant effect of CpG1826 could not be identified in F5 TCR transgenic mice. However, following adoptive transfer which dilutes the F5 peptide-specific T cell population, we found that CpG1826 induced more in vivo cell divisions, increased ex vivo IFN-γ expression and in vivo cytotoxic effector functions. In addition, CpG1826 promoted long-term survival of a small population of Ag-specific CD8 T cells. Together these data show that CpG1826 can have a direct impact on the expansion, effector functions, and survival of an Ag-specific CD8 T cell population in a system which does not involve CD4 T cells.

Materials and Methods

Mice

F5 TCR transgenic were a gift from D. Kioossi (National Institute for Medical Research, London, U.K.). F5 mice express a TCR recognizing an H2-Dd–restricted A/NT/60/68 influenza virus NP epitope (AS3NE MDAM). F5-perforin knockout (KO) mice were generated by crossing F5 mice with perforin KO mice (a kind gift of D. Kāgi (Institute of Pathology, University of Bern, Bern, Switzerland)). C57BL/10 were purchased from Charles River Breeding Laboratories (Les Oncins, France). C57BL/6 Lpr mice were purchased from the Centre de Distribution, Typage and Archivage Animal (Orléans, France). All mice were bred at the Institute’s animal facility under specific pathogen-free conditions. For all experiments, mice were used at ages ranging from 6 to 10 wk.

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Vaccination protocol

Mice were immunized i.p. once or twice at 24-h intervals with 10 or 50 nmol NP68 peptide (Neosystem, Strasbourg, France) in saline or together with 60 μg of the CpG1826 ODN (22) which is a 20 mer containing two CpG motifs (TTCCATGACGTTCCTCTACGTT) in a final volume of 200 μl. Peptide and CpG1826 were free of endotoxin contamination.

CFSE labeling of F5 TCR transgenic cells and adoptive transfers

CFSE labeling was modified from a previously described protocol (23). Cell suspensions of spleens and lymph nodes from F5 TCR transgenic mice were incubated with 0.75 μM CFSE (Molecular Probes, PooRgebour, The Netherlands) at a concentration of 5–10 × 10^6 cells/ml at 37°C for 15 min. Cells were then washed once with cold DMEM (Life Technologies, Cergy-Pontoise, France), 6% FCS and twice with PBS. C57BL/10 mice were adoptively transferred by i.v. injection with 2 × 10^6 CFSE-labeled F5 CD8 T cells in a final volume of 200 μl into the retro-orbital sinus. Immunizations were performed 24 h after adoptive transfer.

Cell staining and flow cytometry

Splenocytes from C57BL/10 recipients were washed in PBS, 0.09% NaN_3, 1% FCS. Cell surface expression markers were stained by incubating 5 × 10^6 splenocytes with CyChrome-coupled anti-CD8 (clone 53-6.7; BD PharMingen, Le Pont de Claix, France) and anti-CD122 PE (clone Tm-β1; BD PharMingen), or biotinylated anti-Ly6C (clone AL-21; BD PharMingen) or anti-CD44 PE (clone IM7; BD PharMingen) for 20 min at 4°C. The control population was incubated for the same amount of time without peptide at 37°C. At the end of the labeling reaction, cells were washed twice and resuspended in 500 μl of PBS, 0.09% NaN_3, 1% FCS. Three-color flow cytometry was performed on a FACS Calibur (BD Immunocytometry Systems, San Jose, CA). A total number of 40,000 CD8 T cells were acquired for each sample.

Intracellular staining

For all intracellular cytokine detection assays, 5 × 10^6 splenocytes from recipient mice were stimulated in a 96-well plate with monensin (0.67 μM GolgiStop; BD PharMingen) with or without 10 nM NP68 peptide for 5 h. Cells were stained with anti-CD8-TC for 20 min at 4°C washed and incubated with 80 μl of Cytofix/Cytoperm (BD PharMingen) for 15 min at 4°C. The control population was incubated for the same amount of time without peptide at 37°C. At the end of the incubation, cells were washed twice and resuspended in 500 μl of PBS, 0.09% NaN_3, 1% FCS. Three-color flow cytometry was performed on a FACS Calibur (BD Immunocytometry Systems, San Jose, CA). A total number of 40,000 CD8 T cells were acquired for each sample.

Ex vivo cytotoxic assay

CTL were generated after two injections of NP68 peptide (50 nmol) with or without CpG1826 (60 μg) into F5 mice. Thirty six hours after the first immunization, splenocytes were removed and plated into Micro Tubes-Bulk (Biorad, Hercules, CA) in the presence of CFSE-labeled peptide-pulsed EL-4 target cells at different E:T ratios for 4 h at 37°C. EL-4 target cells were pulsed with 100 nM NP68 for 90 min then labeled with CFSE (0.25 μM) for 13 min and washed twice in DMEM 6% FCS. At the end of the incubation 10 μl of propidium iodide (20 μg/ml) were added to each tube. Tubes were then carefully pipetted to separate aggregated effectors and target cells and the target lysis was analyzed on a FACSCalibur. Cytotoxic activity was determined by calculating the percentage of EL-4 target cells stained with PI.

In vivo cytotoxic assay

This assay was performed as described previously (24). Targets were prepared from C57BL/10 spleen and lymph node cell suspensions. Cells were divided into two populations, one of which was pulsed with 1 μM F5 peptide for 90 min at 37°C. The control population was incubated for the same amount of time without peptide at 37°C. After the incubation, cells (5 × 10^5 cells/ml) were labeled with CFSE (see above) at 0.75 μM (peptide-pulsed population) or 75 nM (control population). When mentioned, a third population of C57BL/6Lpr cells was pulsed with 1 μM peptide. In this case, CFSE concentrations used for labeling the different target populations were 0.75 μM (C57/BL10 cells pulsed with NP68 peptide), 94 nM (C57/BL6-Lpr pulsed with NP68 peptide), and 14 nM (control C57/BL10 cells), respectively. The same number of cells (8 × 10^5) from each population was mixed and injected i.v. into C57BL/10 recipients previously transferred with nonlabeled F5 cells. Spleen cells from recipients were collected 15 h after target injection and 10,000 CFSE + cells were acquired on the FACSCalibur. In vivo cytotoxicity was assessed by calculating the ratio of nonpulsed targets/pulsed targets.

Results

Induction of effector CD8 T cells in F5 TCR transgenic mice

To study the effects of CpG1826 on the induction of CD8 T cell primary responses, we immunized the F5 TCR transgenic mice i.p. with NP68 peptide ± CpG1826. Two parameters of the effectors...
response, i.e., IFN-γ production and cytotoxic activity, were investigated 36 h after immunization. Both functions have been described to be increased following vaccination with CpG ODN (8, 25). As shown in Fig. 1A, no differences were observed in the percentages of IFN-γ-positive CD8 T cells following peptide immunization with or without CpG1826 after a short ex vivo restimulation. We then investigated the ability of F5 CD8 T cells to perform ex vivo cytolytic activity. Results presented in Fig. 1B show that CpG1826 did not increase the CTL activity observed after NP68 peptide immunization. These results clearly show that CpG1826 does not increase effector functions in F5 TCR transgenic mice.

This lack of CpG1826 adjuvant activity could be explained by the fact that a high frequency of CD8 T cell is stimulated simultaneously and can develop strong effector functions in the absence of exogenous in vivo stimulation of the innate immune system. This observation confirms recent studies indicating that in vivo activation of a high frequency of CD8 T cells leads to DC maturation which might in turn provide costimulatory signals to T cells (26, 27).

CpG1826 enhances peptide-induced in vivo proliferation of adoptively transferred CD8 T cells

As injection of peptide into F5 TCR transgenic mice did not allow us to investigate the effects of CpG1826 on the induction of effector CD8 T cell functions, we transferred F5 CD8 T cells into C57BL/10 syngeneic recipients. Under these conditions, F5 CD8 T cells are diluted into the polyclonal CD8 T cell population of the host and should enable us to investigate the impact of CpG1826 on the induction of primary responses.

Naïve C57BL/10 mice were adoptively transferred with $2 \times 10^6$ CFSE-labeled F5 CD8 T cells and immunized 24 h later with 10 nmol of NP68 peptide alone or combined with 60 μg of CpG1826. F5 CD8 T cell proliferation in the spleen was analyzed at days 1, 2, and 5 post immunization (Fig. 2A). Results presented in Fig. 2B show that division peaks can be detected when mice were immunized with peptide alone or cojected with CpG1826, while cells from control animals did not proliferate. In addition, we also found that CpG1826 injection in the absence of peptide did not induce cell divisions (data not shown). Although divisions could be detected when peptide was injected with or without CpG1826 at all time points, the proportion of F5 cells having divided once 24 h after injection was greater when mice were vaccinated with peptide and CpG1826 (Fig. 2B). At day 2, F5 cells harvested from mice vaccinated with peptide and CpG1826 contained a larger proportion of cells having performed four or five divisions compared with mice vaccinated with peptide alone. The CFSE profiles at day 5 suggest that without CpG1826, CD8 T cell have stopped proliferating as we were not able to detect significant numbers of cells having accomplished more than three divisions (Fig. 2B). Cell division analysis of CFSE-labeled CD8 T cells in the spleen, lymph nodes, and liver showed similar division patterns (data not shown). This suggests that cells that have performed four or five divisions have not migrated into other lymphoid organs and hence are likely to have selectively died. In contrast, in mice vaccinated with peptide and CpG1826, CD8 T cells have further divided with some cells having performed at least seven divisions.

The total number of F5 CD8 T cells was also analyzed at different time points. Twenty-four hours after injection (Fig. 3), the number of F5 CD8 T cells initially showed a decrease compared with naïve cells. This Ag-driven cell death was followed by a significant increase in the number of CD8 T cells detected in the spleens 48 h after vaccination. The increase in the number of F5 CD8 T cells was more prominent when mice were vaccinated with peptide and CpG1826 (Fig. 3). At day 5, F5 CD8 T cell numbers in the spleen began to decrease but still remained superior in the group vaccinated with peptide and CpG1826 (Fig. 3).

These results argue that in contrast to what was observed in F5 mice where the adjuvant effect of CpG1826 could not be detected, dilution of F5 CD8 T cells into a polyclonal syngeneic host highlights the adjuvant effects of CpG1826. Under these experimental conditions, CpG1826 augments in vivo expansion of the Ag-specific F5 CD8 T cell population. This could result, at least in part, from the different division patterns and the increased survival of dividing F5 CD8 T cells observed when peptide is injected in the presence of CpG1826.

CpG1826 enhances peptide induced up-regulation of CD122 and Ly6C on CD8 T cells

As CpG1826 enhanced expansion of F5 CD8 T cells transferred into a polyclonal host, we then sought to investigate the impact of

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**FIGURE 2.** CpG1826 enhances F5 CD8 T cell proliferation in vivo. A. Immunization protocol. CFSE-labeled F5 cells were adoptively transferred into syngeneic C57BL/10 recipients. Twenty-four hours later mice were immunized with 10 nmol NP68 influenza peptide with or without CpG1826 (60 μg). Read-outs to detect CD8 T cell divisions were performed 24 h, 48 h, or 5 days after immunization. B. In vivo CD8 divisions during the primary response. Splenocytes were stained with anti-CD8 at different times after immunization and cell divisions were detected by measuring the decrease in CFSE fluorescence emission. Data are representative of at least six individual mice. Legend: PBS (thin line); NP68 peptide (shaded profile); NP68 peptide plus CpG1826 (black line).

**FIGURE 3.** Number of adoptively transferred CFSE $^+$ F5 CD8 T cells in the spleen after vaccination. F5 CFSE-positive cells were adoptively transferred into C57BL/10 syngeneic recipients. Mice were immunized with NP68 peptide ± CpG1826 24 h after adoptive transfer. The total number of F5 CD8 T cells (CFSE $^+$) at different times after peptide priming was calculated using the following equation: (number of viable cells × % total CD8 T cells) × % CFSE $^+$ CD8 T cells within total CD8 population. Data are presented as mean values ± SD of four independent experiments with at least three mice per group.
this adjuvant on the expression of cell surface markers which are usually associated with CD8 T cell activation.

Expression of CD122, Ly6C, and CD44 (20, 21, 28) which are expressed on activated/memory T lymphocytes were monitored at different times after immunization. Results presented in Fig. 4 show that CD122-positive CD8 T cells were observed in mice that were vaccinated with peptide in the presence of CpG1826. This observation applied to days 2 (Fig. 4A) and 5 (Fig. 4B) after vaccination. However, a slight up-regulation of CD122 was detected at day 2 postimmunization with peptide alone.

When looking at Ly6C, we found that expression of this marker was heterogeneous on naive CD8 T cell populations. However, upon antigenic stimulation, F5 CD8 T cells expressed higher levels of Ly6C in mice vaccinated with peptide and CpG1826 (Fig. 4) and two populations, Ly6C_{int} and Ly6C_{high}, of CD8-positive cells could be discriminated. The latter of this subset became more prominent as cells divided in vivo and was mainly found in peptide plus CpG1826-vaccinated mice (Fig. 4B). The level of CD44 was also markedly increased following immunization (Fig. 4) with expression levels that increased with each cell division. However, we observed no differences when looking at CD44 expression levels following immunization with or without CpG1826.

Together, these results indicate that cells that are generated in the presence of CpG1826 express higher levels of CD122 and Ly6C. By contrast, up-regulation of CD44 was roughly equivalent in mice vaccinated with or without CpG1826.

CpG1826 increases the frequency of IFN-γ^{+} F5 CD8 T cells

Induction of effector CD8 T cells expressing IFN-γ can occur in the absence of CpG1826 in peptide-vaccinated F5 TCR transgenic mice (Fig. 1). Therefore, we investigated whether this also applied to F5 T cells transferred into a host where they are diluted into a polyclonal T cell population. IFN-γ expression by CD8 T cells was assessed following a 5-h NP68 peptide ex vivo restimulation. Data presented in Fig. 5 show that IFN-γ^{+}-expressing F5 CD8 T cells were only detectable at days 2 and 5 postvaccination in mice injected with peptide and CpG1826 (Fig. 5). In this group, the frequency of IFN-γ^{+}-positive cells was highest among cells having performed more than three divisions (Fig. 5A). No CD8 T cells expressing IFN-γ were detected in control mice or mice vaccinated with peptide alone (Fig. 5). Likewise, CpG1826 alone did not elicit IFN-γ expression by CD8 T cells (data not shown).

These results confirm numerous reports showing increased IFN-γ production by CD8 T cells following vaccination with CpG DNA. In F5 mice, where a high frequency a CD8 T cells can be activated, peptide alone is capable of inducing expression of IFN-γ by F5 CD8 T cells (Fig. 1). In contrast, CpG1826 is necessary for the induction of an Ag-specific IFN-γ^{+} CD8 T cell population in a context where F5 cells are less abundant because they are diluted in a polyclonal host.

CpG1826 allows CD8 transgenic T cells to differentiate into cytotoxic effector cells

As CpG1826 is a key for inducing proliferation and differentiation into IFN-γ^{+}-expressing effector cells, we sought to determine whether this adjuvant was also capable of inducing differentiation of adoptively transferred F5 CD8 T cells into CTL. We used an in vivo CTL assay to determine the capacity of CD8 T cells to differentiate into cytotoxic effectors. Mice were injected with peptide or peptide and CpG1826. A mix of control and peptide-pulsed target cells was then injected i.v. at times ranging from 2 to 7 days post vaccination (Fig. 6A). Targets were then analyzed by flow cytometry 15 h later. Our results show that a single peptide immunization is unable to induce significant CTL activity in vivo when targets were injected at day 3 (Fig. 6C). This was true when mice were vaccinated both with peptide or peptide and CpG1826 (Fig. 6C). When peptide priming was sustained by a second peptide injection 24 h later, a strong in vivo cytotoxic activity was detected in mice vaccinated with peptide and CpG1826 (Fig. 6).

We then studied the kinetics of cytotoxic activity induction after two immunizations with peptide and CpG1826. Data presented in Fig. 6D show that specific cytotoxic activity was detectable 2 days after the first injection, peaked at day 5 and was still detectable at day 7. As seen in the experiment reported in Fig. 6C, cytotoxic activity could also be detected in mice vaccinated with two injections of peptide alone. However, this activity was always transient and much lower that the one observed in the presence of CpG1826.

Together these results argue that induction of cytotoxic activity requires both sustained exposure to Ag and the adjuvant activity of CpG1826.

CpG1826-induced CTL use the CD95 and perforin pathways to kill targets

At least two mechanisms, the CD95 and the perforin pathways, are involved in CD8-mediated cytotoxicity (29, 30). Other mechanisms such as TNF-mediated apoptosis could also contribute to in vivo target elimination (31, 32). In this study, we have investigated the role of CD95 and perforin in cell-mediated cytotoxicity. To evaluate the role of perforin and CD95, F5, or F5 perforin^{−/−} cells were adoptively transferred into syngeneic C57BL/10 recipients.

FIGURE 4. F5 CD8 T cells up-regulate CD122 and Ly6C upon peptide plus CpG1826 priming. A. Spleen and lymph node from F5 mice were labeled with CFSE and injected i.v. into C57BL/10 recipients. Twenty-four hours after adoptive transfer, recipients were immunized i.p. with 10 nmol NP68 peptide with or without 60 μg of CpG1826. Splenocytes were surface-stained 48 h after immunization for CD8 and CD122, Ly6C, or CD44. B. The same protocol was followed as described in A. Splenocytes were stained at day 5 after a single immunization.
who were then vaccinated twice with peptide ± CpG1826. Target cells from wild-type C57BL/10 or C57BL/6-Lpr mice were injected 5 days after the last vaccination.

A strong cytotoxic response was observed in mice adoptively transferred with F5 CD8 T cells and vaccinated with peptide and CpG1826 (Fig. 7A). However, cytotoxic activity was partially reduced when C57BL/6-Lpr targets pulsed with peptide were injected into the same animals indicating that CD95 contributes to in vivo cytotoxic activity (Fig. 7A). When looking at cytotoxic responses obtained in recipient mice adoptively transferred with perforin-/- F5 T cells, specific cytotoxic activity was also partially reduced when compared with the activity mediated by F5 T cells (Fig. 7). In contrast, the cytotoxic activity became undetectable when C57BL/6-Lpr targets were injected into vaccinated mice adoptively transferred with F5 perforin-/- T cells (Fig. 7B). Together, these data indicate that both pathways, CD95 and perforin, contribute to the bulk of the cytotoxic response induced by vaccination with peptide and CpG1826.

**CpG1826 promotes CD8 T cell survival in vivo**

As shown above, CpG1826 is able to modulate CD8 T cell proliferation, alter the surface phenotype of responding Ag-specific T cells, induce F5 T cells to express IFN-γ and augment cytotoxic effector functions. We also sought to determine whether CpG1826 had an impact on the survival of Ag-specific T cells in vivo. Recipient mice were immunized twice with NP68 peptide plus CpG1826 and the presence of F5 CD8 T cells 49 days after immunization was measured using a Db tetramer loaded with the NP68 peptide. F5 CD8 T cells were detected only in mice immunized with peptide plus CpG1826 (Fig. 8). No tetramer-positive CD8 T cells were detectable in recipient mice immunized with NP68 peptide only. We were able to detect tetramer-positive cells until at least 5 mo after immunization (not shown). These data clearly show that coinjection of CpG1826 and peptide is able to activate naïve T cells and to drive them into fully differentiated effector CD8 T cells that can persist in vivo.

**Discussion**

In this study, we have compared CD8 T cell responses induced by peptide immunization with or without CpG1826. Our data show that CpG1826 augments the expansion of Ag-specific CD8 T cells and modifies the expression of activation and differentiation markers such as CD122 and Ly6C. It also triggers differentiation of activated F5 CD8 T cells into effector cells which rapidly express IFN-γ and/or exert direct CD95- and perforin-mediated cytotoxic activity upon in vivo challenge. Finally, our data also indicate that CpG1826 promotes survival of Ag-specific CD8 T cells in vivo.

We first began to investigate the effect of CpG1826 on CD8 T cells responses in F5 mice. The effector responses obtained with peptide alone show a strong CD8 T cell response that the adjuvant activity of CpG1826 was unable to increase (Fig. 1). The example of F5 mice where all CD8 peripheral T cells bear the same TCR suggests that a high frequency of Ag-specific T cells may compensate for the lack of adjuvant. These results are in agreement with recent studies showing that costimulation-independent activation of a high frequency of T cells may induce the maturation of DC in vivo (26, 27) and could therefore bypass the need for adjuvant. In addition, the fact that an elevated CD8 frequency substitutes for CD4 T cell help to generate cytotoxic effectors (33) also supports a model where adjuvant may not be required for the induction of T cell proliferative and effector responses in vivo. Although the mechanisms responsible for APC maturation have not been addressed in this study, we have previously reported TNF-α expression by a large subset of CD8 T cells from naive and peptide-primed F5 mice (17). As TNF-α can act as a maturation factor for DCs in vitro and in vivo (34, 35), it is possible that F5 CD8 T cell-derived TNF-α can substitute for the lack of adjuvant in vivo. Taken together these data could explain the strong immunogenicity of NP68 peptide in the absence of exogenous adjuvant observed in F5 TCR transgenic mice.

Adoptive transfer of F5 cells into syngeneic recipient allows the F5 CD8 frequency to drop from almost 100% in F5 mice to

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** NP68 peptide and CpG1826 induce IFN-γ expression by CD8 transgenic T cells. A. Spleens from C57BL/10 mice adoptively transferred with F5 cells were removed 48 h after immunization and the accumulation of IFN-γ in F5 cells was measured as described in Materials and Methods. IFN-γ production by CD8 T cells was inferior to 0.5% when incubated without F5 peptide. Nondividing F5 CD8 T cells are indicated by an arrow. B. Percentages of CFSE CD8 T cells expressing IFN-γ at days 2 and 5 after peptide re-stimulation. Data are presented as mean values ± SD of six independent experiments with at least three mice per group.
2–3% of the peripheral CD8 T cell population in the host 24 h after adoptive transfer. Under these experimental conditions, addition of CpG1826 enhanced expansion of the Ag-specific CD8 T cells. A number of different mechanisms could explain this observation. First, it is possible that vaccination in the presence of CpG1826 allows T cells to perform more divisions (Fig. 2B). This could be related to the ability of CpG1826 to modulate DC expression of CD80 and CD86 (3). These costimulatory molecules engage CD28 which increases IL-2 expression by T cells. In addition, T cells stimulated in vivo in the presence of CpG1826 may have a survival advantage. Again, CD28 could be a key player in promoting survival of activated T cells in vivo in that it also regulates expression of the survival factor Bcl-xL (36). In addition, when looking at surface markers usually associated with memory T cells, we found that CD122, which is the 8-chain shared by IL-2 and IL-15 receptors, is expressed at higher levels when mice are vaccinated with peptide and CpG1826. IL-15 is thought to play a key role in maintaining CD8 T cells in vivo in that it mediates homeostatic proliferation thus maintaining populations of memory CD8 T cells over long periods in vivo (37). Our own data shows that vaccination in the presence of CpG1826 allows a population of Ag-specific CD8 T cells to survive in vivo for several weeks. In support of this, in vitro induction of CD122 expression on CD8 T cells has been shown to correlate with long-term persistence of T cell populations adoptively transferred into syngeneic hosts (38). Whether IL-15 is also involved in the maintenance of T cells for the first few days after antigenic stimulation remains to be established.

We show that CpG1826 increases Ly6C on CD8 T cells (Fig. 4). This result could be explained by the fact that CpG sequences are known to induce type I IFN production by DC (39–41) and that Ly6C expression is increased by type I IFN (42). Finally, the strong up-regulation of Ly6C seems to correlate with the generation of CD8 memory T cells because almost all CD8 T cells have up-regulated Ly6C following vaccination with peptide and CpG1826. Strong Ly6C up-regulation by CD8 memory T cells is also observed in F5 mice immunized only with peptide (20).

When investigating CD8 T cell effector functions following immunization with peptide, we found that CpG1826 was critical for```
the induction of IFN-γ production by F5 CD8 T cells. The mechanisms by which CpG ODN enhances IFN-γ expression by CD8 T cells are not fully understood. At least two mechanisms could contribute to IFN-γ expression by CD8 T cells which have been stimulated with peptide and CpG1826 in vivo. First, as pointed out in the introduction, CpG1826 induced CD80 and CD86 up-regulation and could modulate T cell expansion. Because IFN-γ expression associated with priming in the presence of CpG1826 only occurs after F5 CD8 T cells have undergone at least four divisions (Fig. 5), one could speculate that the presence of CpG1826 augments the population of CD8 T cells which survive in vivo and have performed a number of divisions which is compatible with IFN-γ expression. Another factor which is likely to contribute to IFN-γ expression by F5 T cells is the ability of CpG1826 to induce IL-12 expression by APCs (43–45). Although IL-12 expression was not investigated in this study, this cytokine promotes IFN-γ expression by T cells. Although we cannot rule out other mechanisms to explain the role of CpG1826 in promoting IFN-γ expression by F5 CD8 T cells, it is likely that survival of CD8 T cells combined to the induction of a critical number of divisions and the modulation of T cell functions by IL-12 contribute to the establishment of a population of effector CD8 T cells expressing IFN-γ.

In our system, peptide immunization is strictly class I-restricted. This means that only CD8 T cells are activated in response to peptide injection. In this context, no CD4 T cell help is provided following immunization. This study confirms previous observations showing that signals provided by CpG1826 can lead to differentiation of CD8 effector populations in the absence of CD4 T cell help (8, 10, 11). However, in these studies the evaluation of cytotoxic effector functions relies on in vitro T cell restimulation before monitoring CTL activity. It is thus possible that an Ag-specific T cell population is induced to differentiate into cytotoxic effectors in vitro. Our own results clearly indicate that F5 CD8 effector T cells induced in the presence of CpG1826 can exert direct cytotoxic effector functions in vivo without CD4 help (Figs. 6 and 7). Our data also show that a single peptide immunization with or without CpG1826 is not sufficient to induce strong cytotoxic activity. However, when T cells are exposed to a sustained antigenic signal provided by two injections of peptide and CpG1826, strong cytotoxic activity was observed in vivo. The kinetics of cytotoxic activity shows that it appears as soon as 48 h, peaks at day 5, and is still detectable at day 7. The precise mechanisms which promote CD8 T cell differentiation into effector cells remain poorly understood. CD4 T cell help, which is mediated by CD154 engagement of CD40 on the surface of APCs, provides signals that are critical to the generation of CD8 effector populations (12–14). As CD8 T cells can express CD154 (46) and CpG ODN mediate up-regulation of CD40 on DC (3, 5), it is reasonable to speculate that CD40 engagement is likely to play a significant role in the induction of F5 T cell effector functions. However, this does not rule out that other mechanisms contribute to the induction of CD4-independent CTL functions. Thus, CpG1826 provides a key signal which promotes induction of cytotoxic effector cells.

When investigating the relative contribution of perforin and CD95 in the T CD8-dependent cytotoxic activity observed in vivo, we found that both pathways play a significant role in the elimination of specific targets indicating that these two pathways are used by effector CD8 T cells generated with peptide and CpG1826.

Recent studies have investigated the dependence of CD4 help on memory CD8 T cell generation (47–49). These reports show that CD4 T cell help is critical to the maintenance of an effector CD8 T cell population. In this study, we show that vaccination with peptide and CpG1826 leads to the generation of effector CD8 T cells and to a population of memory cells with can be detected until 5 mo after the last exposure to Ag. Whether memory cells induced following vaccination with peptide and CpG1826 are capable of displaying rapid effector functions such as IFN-γ expression and in vivo cytotoxic activity is currently under investigation.

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References


In *Materials and Methods*, in the first sentence under the heading *Vaccination protocol*, an extra T was added to the 5′ end of the CpG1826 sequence. The correct sequence is 5′-TCC ATG ACG TTC CTG ACG TT-3′. The authors wish to underline that CpG1826 has a phosphorothioate backbone.

Also in this section, in the second sentence under the heading *CFSE labeling of F5 TCR transgenic cells and adoptive transfers*, there is a mistake in the CFSE concentration. For adoptive transfer, the CFSE concentration used is 7.5 μM (instead of 0.75 μM). Under the heading *In vivo cytotoxic assays*, in the fifth sentence, CFSE concentrations for target cells are 7.5 μM (pulsed B10) and 0.75 μM (non-pulsed B10), and in the seventh sentence when three targets are used: 7.5 μM (pulsed B10), 0.94 μM (pulsed B6-Lpr) and 0.14 μM (non-pulsed B10).


In *Results*, the legends for Figures 2 and 3 are inverted. The legend with Figure 2 should have been published with the image for Figure 3, and vice versa.


The second author’s first name was misspelled. The correct spelling is Hsi-Hsien.