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CpG Are Efficient Adjuvants for Specific CTL Induction Against Tumor Antigen-Derived Peptide

Isabelle Miconnet,[1]* Sylvain Koenig,* Daniel Speiser,[†] Arthur Krieg,[‡§] Philippe Guillaume,* Jean-Charles Cerottini,* and Pedro Romero[2†]

The identification of CTL-defined tumor-associated Ags has allowed the development of new strategies for cancer immunotherapy. To potentiate the CTL responses, peptide-based vaccines require the coadministration of adjuvants. Because oligodeoxynucleotides (ODN) containing CpG motifs are strong immunostimulators, we analyzed the ability of CpG ODN to act as adjuvant of the CTL response against tumor-derived synthetic peptide in the absence or presence of IFA. Mice transgenic for a chimeric MHC class I molecule were immunized with a peptide analog of MART-1/Melan-A_{26-35} in the presence of CpG ODN alone or CpG ODN emulsified in IFA. The CTL response was monitored ex vivo by tetramer staining of lymphocytes. In blood, spleen, and lymph nodes, peptide mixed with CpG ODN alone was able to elicit a stronger systemic CTL response as compared with peptide emulsified in IFA. Moreover, CpG ODN in combination with IFA further enhanced the CTL response in terms of the frequency of tetramer$^+$CD8$^+$ T cells ex vivo. The CTL induced in vivo against peptide analog in the presence of CpG ODN are functional, as they were able to recognize and kill melanoma cells in vitro. Overall, these results indicate that CpG ODN by itself is a good candidate adjuvant of CTL response and can also enhance the effect of classical adjuvant. ***The Journal of Immunology,*** **2002, 168: 1212–1218.**

The identification of CTL-defined tumor-associated Ags has allowed the development of new strategies for cancer therapy based on the use of synthetic peptides corresponding to the CTL epitopes from these tumor Ags. To elicit a CTL response, peptides need to be presented by professional APCs from which dendritic cells are the most efficient (1). Therefore, one strategy consists of generating dendritic cells (DCs)[3] ex vivo, pulsing, and transferring them back to the patient. However, this procedure involves steps of cell purification and culture and requires a standardization of criteria to define the various DCs preparations (2). In addition, the use of immature or mature DCs is still under debate. In this regard, cell-free vaccines would be more suitable for clinical purposes. To that end, peptides need to be administered in combination with adjuvants of which the most common used so far in experimental models is IFA. IFA has also been successfully used in human immunotherapy against melanoma involving gp100 peptide immunization (3). However, this adjuvant is not widely used in human vaccination protocols due to its undesirable side effects, such as erythema and induration at the injection site. In addition, specific CTL tolerance rather than immunity against immunizing peptide in IFA has also been reported in an experimental model (4). For these reasons, alternative potent and safe adjuvants need to be identified.

The curative potential of bacteria in the treatment of malignancies was proposed a very long time ago (reviewed in Ref. 5), and more recently it has been suggested that bacterial DNA could be responsible for immunostimulation and antitumor effect (6). More specifically, the unmethylated CpG dinucleotides in a certain base context (CpG motifs) contained in synthetic oligodeoxynucleotides (ODN) are able to stimulate B cells and NK cells (7). They also activate DCs and induce their maturation into professional APC (8–12), thereby enhancing their ability to stimulate Ag-reactive T cells in vitro and in vivo. ODN-containing CpG motifs (hereafter referred to as CpG ODN) also stimulate macrophages to secrete Th1 cytokines, which are important in the development of a CTL response (13). In addition, CpG ODN have been shown to behave as adjuvant of Ab (14) and CTL response directed against liposome-entrapped whole protein or class I-restricted peptides (15). When coadministered with whole protein and IFA, CpG ODN provide a signal to switch on specific Th1 response to Ag (16). They are efficient for the induction of a protective antiviral immune response after T cell peptide vaccination (17). Repeated administration of CpG ODN potentiates the CTL response against CTL peptide or protein emulsified in IFA and promotes the survival in response to tumor challenge in both prophylactic and therapeutic vaccination protocols (18). So far, one study provided evidence for the induction of a specific CTL response against a CD8$^+$ T cell peptide in the presence of CpG ODN without additional adjuvant by assessing the cytolytic activity of lymph node cells after in vitro stimulation (12).

In this report, the ability of CpG to act as an adjuvant of the CTL response against a tumor-derived synthetic peptide in the absence or presence of an additional adjuvant was directly studied by identifying and enumerating peptide-specific CTLs in different body

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[3] Abbreviations used in this paper: DC, dendritic cell; ODN, oligodeoxynucleotide; HHD, human human D^b.

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compartments ex vivo. The HLA-A*0201-restricted peptide used was an analog of the decapeptide Melan-$A_{26–35}$ (EAAGIGILTV, referred to as $EAA_{26–35}$) derived from the melanoma-associated differentiation Ag MART-1/Melan-A (hereafter referred to as Melan-A) (19, 20). This peptide analog substituted at position 2 (Melan-$A_{26–35}$ A27L peptide analog referred to as $ELA_{26–35}$) has been shown to be more immunogenic than its natural counterpart (21, 22). We took advantage of human human D^b (HHD) mice derived from a strain deficient for the endogenous β_2-microglobulin and MHC class I H-$2D^b$ molecules and transgenic for a chimeric MHC class I molecule, HLA-A*0201/D^b, linked to the human β_2-microglobulin (23). In these mice, only chimeric MHC class I molecule can be detected at the cell surface. By monitoring the CTL response with peptide-specific tetramer staining in immunized HHD mice, we show that peptide mixed with CpG ODN elicits a systemic CTL response. Whereas CpG ODN alone appeared to be more efficient than IFA alone, a combination of both CpG ODN and IFA led to the strongest recruitment of peptide-specific $CD8^+$ T cells. The high frequency of specific CTLs recruited after immunization with $ELA_{26–35}$ suggests that, like in the human situation (24), a large T cell repertoire is available against the Melan-A Ag. In addition, the CTL induced in vivo against $ELA_{26–35}$ in the presence of CpG ODN were cytolytic against human melanoma target cells in vitro. These data demonstrate that CpG ODN is a good candidate adjuvant for the induction of a CTL response, especially when used in combination with IFA, and illustrate the power of using human tetramers to study the CTL response directed against peptides derived from human tumor-associated Ags in a preclinical model.

Materials and Methods

Cell lines

Mouse EL-4 cells transfected with *HLA-A*0201/K^b* gene (EL-4.A2/K^b transfectants) (25) were kindly provided by Dr. L. Sherman (The Scripps Clinic and Research Foundation, La Jolla, CA) and maintained in DMEM medium supplemented with 1% HEPES, 1% strepto-penicillin, 10% heat-inactivated FCS, and 0.5 mg/ml G418. Human melanoma cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS.

Synthetic peptides and CpG ODN

Peptides were synthesized by standard solid phase chemistry on a multiple peptide synthesizer (Applied Biosystems, Foster City, CA) by using F-moc for transient NH_2-terminal protection and analyzed by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at −20°C. The immunostimulatory synthetic CpG ODN 1826 optimized for stimulation of the mouse immune system (TCCATGACGTTCCTGACGTT) and the control ODN 1982 (TCCAGGACTTCTCTCAGGTT) were used (CpG motifs are underlined). The backbone for these ODN was sulfur-modified phosphorothioate to protect from nucleases. ODN were formulated as a sterile PBS solution (Coley Pharmaceutical Group, Wellesley, MA) and stored at −20°C.

Immunization

HHD transgenic mice were kindly provided by Dr. F. Lemonnier (Institut Pasteur, Paris, France). Different protocols of immunization were used. Transgenic mice were immunized s.c. at the base of the tail with 50 μg of peptide emulsified in IFA or mixed with 50 μg of ODN in a volume of 100 μl. In experiments where a combination of IFA and CpG ODN was used as adjuvant, mice received 50 μg of peptide together with 50 μg of CpG ODN emulsified in IFA in a volume of 100 μl.

Flow cytometry immunofluorescence analysis

Cells ($0.5–1 \times 10^6$) were prepared from peripheral blood, inguinal and paraaortic lymph nodes, and spleen from immunized mice and were stained with PE-coupled HLA-A2/$ELA_{26–35}$ (ELAGIGILTV) tetramer synthesized as previously described (26) at doses indicated in the text in the presence of anti-FcR Ab (clone 2.4 G2) in 50 μl of PBS, 2% FCS for 1 h at room temperature. As a control of the specificity of T cell response, we used an HLA-A2 tetramer made with the peptide 157–165 derived from the tumor-associated NY-ESO-1 (SLLMWITQC, HLA-A2/NY-ESO-$1_{157–165}$) tetramer (27). Cells were washed once in PBS, 2% FCS and then stained with anti-CD44-FITC (clone 1 M.178), anti-TCRβ-CyChrome (clone H57), and anti-CD8α-allophycocyanin (clone 53.6.7; BD PharMingen, San Diego, CA) in 50 μl of PBS, 2% FCS for 30 min at 4°C. Cells were washed once in the same buffer as before and immediately analyzed in a FACSCalibur (BD Biosciences, San Jose, CA).

Generation of specific mouse CTL by in vitro stimulation

Lymph node cells ($4–5 \times 10^6$) from immunized HHD mice were cultured with $2–5 \times 10^5$ irradiated (100 Gy) EL-4 A2/K^b cells, prepulsed with 1 μM relevant peptides for 1 h at 37°C, in six-well cell culture plates in 5 ml of DMEM medium supplemented with 10 mM HEPES, 50 μM 2-ME, 10% FCS, and EL-4 cell culture supernatant containing 30 U/ml IL-2. After one or more rounds of weekly stimulation, the cultured cells were tested for cytolytic activity.

Assessment of in vitro cytolytic activity

Cytolytic activity of CTL lines or of $CD8^+$ T lymphocytes enriched from the spleen of immunized HHD mice by one round of positive selection using the MiniMACS system (Miltenyi Biotec, Sunnyvale, CA) was determined in a ^{51}Cr release assay. The Melan-A protein expression in melanoma target cells was assessed by Western blotting with A103 Ab (28). Target cells were labeled with ^{51}Cr for 1 h at 37°C in the presence or absence of tested peptides, then washed and coincubated with effector cells at the indicated lymphocyte:target cell ratio in V-bottom 96-well plates in a total volume of 200 μl of DMEM medium. Chromium release was measured in 100 μl of supernatant harvested after 4–6 h of incubation at 37°C. Percentage of specific lysis was calculated as follows:

$$\%\ \text{specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Results

Validation of the tetramer staining method

To assess the specificity and the staining efficiency of mouse lymphocytes by human HLA-A2 tetramers, we first established a mouse CTL line specific for $ELA_{26–35}$ CTL peptide by repeated in vitro stimulation of lymph node cells from HHD mice immunized with $ELA_{26–35}$ emulsified in IFA. The specificity of CTL line was verified in cytolytic assays against specific and third party Ags (data not shown). As shown in Fig. 1*A*, human HLA-A2 tetramers bound to mouse CTLs that were positively selected in the thymus in the context of a chimeric HLA-A2/D^b class I molecule. The specificity of tetramer staining correlated with the specificity of the cytolytic activity displayed by CTL lines against target cells pulsed with their cognate peptide. The mean fluorescence intensity increased proportionally with the concentration of tetramer added. Based on these observations, tetramer stainings were performed at a final concentration of 14 μg/ml throughout the study.

We then evaluated the sensitivity of detection of Ag-specific cells by HLA-A2 tetramers in defined mixtures containing tetramer$^+$ cells from the reference CTL line diluted with splenocytes from naive C57BL/6. Data shown in Fig. 1*B* represent the percentage of tetramer$^+$ cells in the cell mixture. First, the background level of tetramer$^+$ cells within $CD8^+$ T cells in the spleen of naive C57BL/6 mice was relatively low ($0.17 \pm 0.03\%$ HLA-A2/$ELA_{26–35}$ tetramer$^+$) (Fig. 1*B*). Second, the percentages of tetramer$^+$ cells measured within the $CD8^+TCR\beta^+$ lymphocytes correlated well with the theoretical percentage of HLA-A2/$ELA_{26–35}$ tetramer$^+$ cells added to naive C57BL/6 splenocytes ($y = 0.876x + 0.261$; $r = 0.999$). Together, these data indicated that human HLA-A2 tetramer can be used for the detection of mouse lymphocytes selected on chimeric HLA-A2/D^b molecules. Moreover, the HLA-A2/$ELA_{26–35}$ tetramer was able to accurately detect tetramer$^+$ cells within $CD8^+TCR\beta^+$ lymphocytes.

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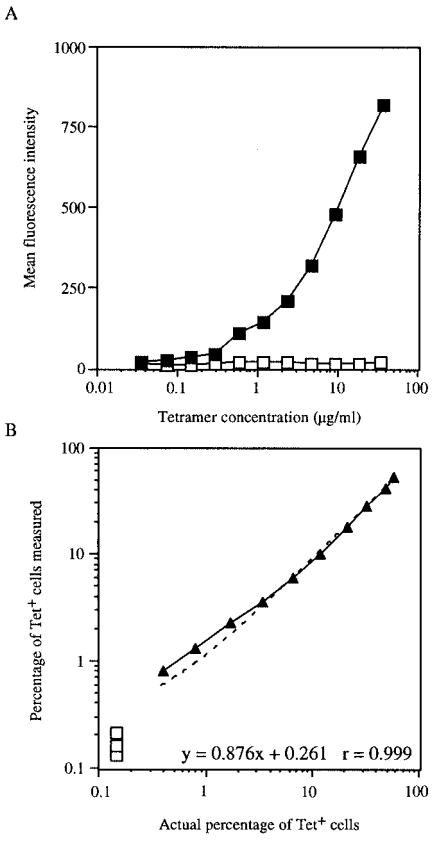

FIGURE 1. Validation of tetramer staining. *A*, CTL line specific for ELA_{26-35} was established in vitro and stained in the presence of different amounts of HLA-A2/ELA_{26-35} tetramer (■), or of HLA-AZ/NY-ESO-$1_{157-165}$ tetramer (□). The mean fluorescence intensity of cells is shown at each tetramer concentration. *B*, Tetramer$^+$ CTLs from CTL line were mixed at different ratios with splenocytes from naive C57BL/6 mice (*x*-axis) and stained with HLA-A2/ELA_{26-35} tetramer at a final concentration of 14 μg/ml. The percentage of tetramer$^+$ cells measured in the cell mixtures within the CD8$^+$TCRβ^+ lymphocytes is shown on the *y*-axis. The percentages of tetramer$^+$ cells measured within the CD8$^+$TCRβ^+ lymphocytes of splenocytes from naive C57BL/6 mice is represented by triangles. The correlation curve is represented by a dashed line.

CpG ODN as adjuvant of the CTL response against tumor Ag-derived peptides

To test the ability of CpG ODN to behave as an adjuvant for the induction of specific CTL following peptide inoculation, we chose a Melan-A peptide analog with enhanced immunogenicity as shown previously (21, 22). This peptide is a candidate vaccine actively tested in phase I clinical trials. Mice of the HHD line were immunized with peptide ELA_{26-35} in combination with either CpG ODN 1826 or ODN control 1982 that did not contain CpG motifs. The peptide-specific CTL response was measured ex vivo 7 days after immunization using tetramers and flow cytometry in the peripheral blood of these mice. A representative experiment is shown in Fig. 2*A*. In the population of viable cells (gated in G1), we selected the CD8$^+$TCRβ^+ T cells (gate G2) in which the percentage of tetramer$^+$ cells was determined (Fig. 2*A*, *lower panels*). This type of analysis was used in all additional experiments. A strong CTL response directed against ELA_{26-35} was specifically elicited in mice immunized with peptide in the presence of CpG ODN 1826 (6.6% of CD8$^+$ T cells were HLA-A2/ELA_{26-35} tetramer$^+$) but not in the presence of the control ODN 1982 (0.2% of CD8$^+$ T cells). As expected, nearly all tetramer$^+$ cells displayed an activated phenotype as shown by the up-regulation of the CD44 activation marker at their surface. The data demonstrate that CpG ODN are required for strong CTL activation detectable ex vivo. In addition, the CTL response obtained in ELA_{26-35}-immunized mice is specific for the immunizing peptide because <0.1% of CD8$^+$ T cells were stained with an irrelevant tetramer (HLA-A2/NY-ESO-$1_{157-165}$). Thus, both CTL peptide and CpG ODN 1826 are required to induce a strong and specific CTL response.

The kinetics of the CTL response against ELA_{26-35} peptide mixed with CpG ODN 1826 was determined in the peripheral blood of immunized HHD mice. The level of tetramer$^+$ CD8 lymphocytes peaked 7 days after immunization in four of five mice with a mean proportion of 9.2 ± 2.3% tetramer$^+$ within CD8$^+$TCRβ^+ T cells (Fig. 2*B*). One mouse presented a weaker and delayed tetramer$^+$ CD8 lymphocyte wave with 0.8% ELA_{26-35} tetramer$^+$ within CD8$^+$TCRβ^+ T cells at day 7 and 2.8% at day 11.

The unexpected high frequency of tetramer$^+$CD8$^+$ cells detected in mice immunized against ELA_{26-35} peptide in presence of CpG ODN led us to investigate the response in larger groups of immunized HHD mice and to compare it to the response obtained in mice immunized with peptide emulsified in IFA (Fig. 3, *A* and *B*). Based on the results presented in Fig. 2*B*, we analyzed the circulating tetramer$^+$CD8$^+$ lymphocyte response 7 days after immunization. As compared with naive mice, an 8-, 10-, and 9-fold increase of the mean frequency of HLA-A2/ELA_{26-35} tetramer$^+$ cells were respectively observed in blood, spleen, and draining lymph nodes of mice immunized in the presence of CpG ODN. It is noteworthy that the differences of CTL frequency between immunized and naive mice were significant in blood ($p < 0.01$) and spleen ($p < 0.05$) but not in draining lymph nodes ($p < 0.2$), suggesting the development of a systemic CTL response. In addition, no increase in the percentage of irrelevant HLA-A2/NY-ESO-$1_{157-165}$ tetramer$^+$ cells was observed as compared with naive mice. This indicates that the CTL response induced in the presence of CpG ODN was specific for the immunizing peptide. Likewise, no difference in the frequency of HLA-A2/ELA_{26-35} tetramer$^+$ cells was detected between naive mice and mice immunized with CpG ODN alone (0.18 ± 0.09 in blood, 0.15 ± 0.05 in lymph nodes, and 0.51 ± 0.36 in spleen). By contrast, the percentage of HLA-A2/ELA_{26-35} tetramer$^+$ cells was not significantly increased in peptide/IFA-immunized mice as compared with naive mice. Indeed, the highest frequency of HLA-A2/ELA_{26-35} tetramer$^+$ cells detectable in the spleen of those mice was also associated with a non-negligible background, as shown by the significant increase of the mean percentage of HLA-A2/NY-ESO-$1_{157-165}$ tetramer$^+$ cells in this organ ($p < 0.05$). Thus, immunization in the presence of IFA was significantly less efficient than immunization in the presence of CpG ODN in recruiting HLA-A2/ELA_{26-35} tetramer$^+$ cells.

Finally, we investigated whether the coadministration of both IFA and CpG ODN would elicit a stronger CTL response specific for ELA_{26-35}. For that purpose, we analyzed the frequency of CTL specific for the immunizing peptide in HHD mice immunized 7 days earlier with a combination of IFA and CpG ODN (Fig. 3*C*). A specific CTL response was elicited in those mice as indicated by the significant difference in the percentages of HLA-A2/ELA_{26-35} and HLA-A2/NY-ESO-$1_{157-165}$ tetramer$^+$ cells within the CD8$^+$TCRβ^+ T cell population. As compared with naive mice, the frequency of HLA-A2/ELA_{26-35} tetramer$^+$ cells was highly significantly increased in blood ($p < 0.007$), spleen ($p < 0.007$), and draining lymph nodes ($p < 0.007$). As mentioned above, this was not the case in mice immunized against peptide mixed with CpG ODN alone where the frequency of HLA-A2/ELA_{26-35} tetramer$^+$ cells was significantly increased only in blood ($p < 0.01$) and spleen ($p < 0.05$) and in mice immunized with the peptide

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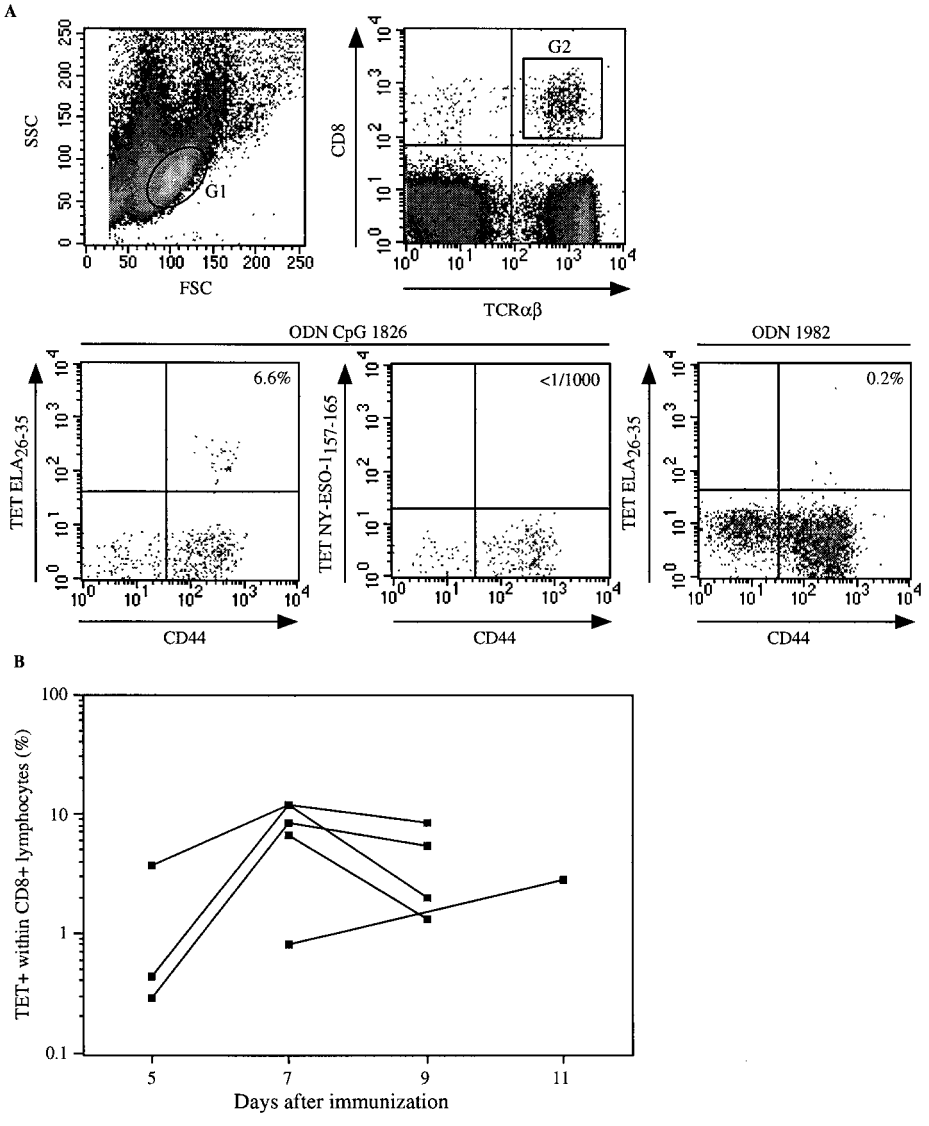

FIGURE 2. Monitoring peptide-induced CTL responses with tetramer. *A*, Visualization of specific $CD8^+$ T cells in PBMCs freshly isolated from immunized HHD mice. PBMCs were prepared from HHD mice immunized 7 days before with ELA_{26-35} mixed with CpG ODN 1826 or ODN control 1982. Cells were then stained with specific HLA-A2/ELA_{26-35} or third party HLA-A2/NY-ESO-$1_{157-165}$ tetramers coupled to PE together with anti-CD8-allophycocyanin, anti-TCRβ-Cy-Chrome, and anti-CD44-FITC. Viable cells were selected (gate G1) and the percentage of tetramer$^+$CD44$^+$ cells within $CD8^+TCR\beta^+$ lymphocytes (gate G2) were shown (*lower panels*). *B*, Kinetics of the CTL response in peripheral blood of HHD mice immunized with ELA_{26-35} in the presence of CpG ODN 1826. The percentage of tetramer$^+$CD44$^+$ cells within the $CD8^+TCR\beta^+$ cell subset is represented for each individual mouse.

emulsified in IFA (increase of the frequency of specific HLA-A2/ELA_{26-35} and nonspecific HLA-A2/NY-ESO-$1_{157-165}$ tetramer$^+$ cells in spleen). In addition, the frequency of circulating HLA-A2/ELA_{26-35} tetramer$^+$ cells in mice immunized in presence of both CpG ODN and IFA was higher than the one detectable in the peripheral blood of mice immunized with peptide mixed with CpG ODN ($p < 0.005$) or in the blood ($p < 0.001$), the spleen ($p < 0.001$), and the draining lymph nodes ($p < 0.007$) of mice immunized with the peptide emulsified in IFA. It should be noted that the number of splenocytes, lymph node cells, and TCR$^+$ and $CD8^+$ T lymphocytes in lymphoid organs were not significantly different between groups of mice immunized with the dose of CpG used (50 μg) or IFA and naive mice (data not shown). However, as compared with naive mice, a significant increase of the absolute number of tetramer$^+$CD8$^+$ T cells were detected in lymph nodes and spleen of mice immunized with ELA_{26-35} mixed with CpG. This increase was even greater in both organs of mice immunized with peptide in the presence of CpG and IFA (Table I). Altogether, these data demonstrated that the use of CpG ODN as adjuvant led to a strong systemic and specific CTL response against peptide. Whereas CpG ODN appeared more efficient than IFA, a combination of both adjuvants further enhanced the recruitment of peptide-specific CTLs.

CTL elicited in vivo in the presence of CpG ODN are functional

To determine whether the CTLs generated against ELA_{26-35} mixed with CpG ODN were functional, we assessed their cytotoxic activity against the EL4-A2/K^b mouse cell line pulsed with exogenous peptide. Fig. 4*A* showed that CTLs specific for ELA_{26-35} peptide lysed EL4-A2/K^b mouse cells pulsed with ELA_{26-35}. Because CTL generated against ELA_{26-35} emulsified in IFA cross-recognize the natural EAA_{26-35} peptide endogenously processed and presented at the cell surface of Melan-A$^+$ human melanoma cells (21, 22), we investigated the ability of CTLs elicited against ELA_{26-35} in the presence of CpG ODN to kill this type of target. As expected, the HLA-A*0201$^+$Melan-A$^-$ Na8 and SK-Mel-37 melanoma cells were not killed in the absence of exogenous peptide. By contrast, the two HLA-A*0201$^+$Melan-A$^+$ melanoma cell lines tested were efficiently killed by the CTL line specific for ELA_{26-35} in the absence of exogenous peptide. Me 290 melanoma cells were more efficiently killed than SK-Mel-23 cells, which were poorly lysed by CTL line even in the presence of exogenous peptide.

We also investigated ex vivo the cytolytic activity of $CD8^+$ T lymphocytes enriched from the spleen of HHD mice immunized 7 days before with ELA_{26-35} mixed with CpG ODN. A representative

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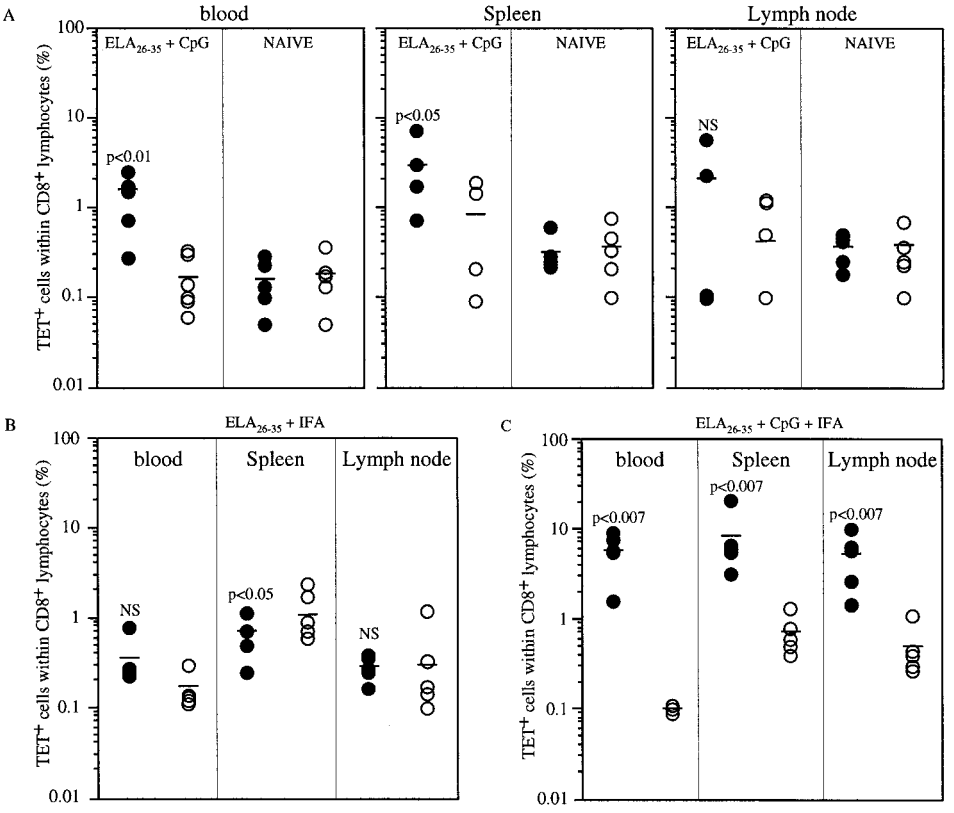

FIGURE 3. Monitoring of the CTL response in peptide-immunized HHD mice. HHD mice were immunized with $ELA_{26–35}$ CTL peptide together with CpG ODN 1826 (*A*) or IFA (*B*), respectively. Naive HHD mice are also shown as control. PBMCs, splenocytes, and draining lymph node cells were prepared 7 days after immunization and stained ex vivo with either specific HLA-A2/$ELA_{26–35}$ (●) or third-party HLA-A2/NY-ESO-$1_{157–165}$ (○) tetramers coupled to PE together with anti-CD8-allophycocyanin, anti-TCRβ-CyChrome, and anti-CD44-FITC. The percentages of tetramer$^+$CD44$^+$ cells within CD8$^+$TCRβ^+ lymphocytes are represented for each individual mouse. Four to six mice per group were analyzed. *C*, Adjuvant effect of CpG ODN and IFA on the specific CTL response. HHD mice were immunized with $ELA_{26–35}$ CTL peptide mixed with both CpG ODN 1826 and IFA and analyzed as above. The difference of CTL frequency found in immunized and naive mice was statistically analyzed by using the Student *t* test.

experiment is shown in Fig. 4*B*. Tetramer$^+$CD8$^+$ T lymphocytes were able to specifically kill EL4-A2/K^b mouse cells pulsed with $ELA_{26–35}$ or $EAA_{26–35}$ peptide. A ratio of two tetramer$^+$CD8$^+$ T lymphocytes to one $ELA_{26–35}$-pulsed target cell led to a 25% specific lysis. Altogether, these data demonstrated the functionality of CTL recruited by immunization against peptide in the presence of CpG.

Table I. *Absolute numbers of tetramer$^+$CD8$^+$ T cells detected in lymphoid organs of immunized or naive HHD mice*[a]

Experimental Group	Tetramer$^+$CD8$^+$ T Cell Number ($\times 10^{-3}$)	
	Spleen	Lymph node
$ELA_{26–35}$ + CpG		
1	**12.7**	<0.2
2	**4.2**	<0.1
3	**18.9**	**17.1**
4	**12.9**	**5.1**
$ELA_{26–35}$ + IFA		
1	0.6	<0.1
2	**2.2**	0.3
3	**3.9**	0.7
4	**1.0**	0.1
5	**7.2**	0.1
$ELA_{26–35}$ + CpG + IFA		
1	**14.3**	**11.8**
2	**28.6**	**10.9**
3	**18.0**	**6.3**
4	**9.3**	**5.6**
5	**80.3**	**12.7**
Naive		
1	0.4	1.3
2	0.7	0.7
3	0.3	0.3
4	0.1	0.1

[a] The absolute numbers of tetramer$^+$CD8$^+$ T lymphocytes were calculated in lymphoid organs of mice analyzed in Fig. 3. The numbers of tetramer$^+$CD8$^+$ T lymphocytes corresponding to a significant difference as compared to naive mice (higher than mean value + SD) are typed in bold.

Discussion

This study demonstrates the role of CpG ODN as adjuvant for CTL induction against peptide derived from tumor Ag when used alone or in combination with IFA. The direct assessment of CTL frequency by tetramer staining confirms and extends previous data reported by others (12, 18) by showing for the first time the strong activation and the resulting high frequency of CTL specific for peptide derived from tumor Ag in both peripheral blood and lymphoid organs.

We report that human HLA-A*0201 tetramers are able to efficiently stain mouse CD8$^+$ T lymphocytes. This observation may appear contradicting to the demonstration of the critical role of the CD8 molecule in class I/peptide complexes binding to TCR and in T cell activation (29, 30). However, this is in agreement with our previous study showing that mouse CTL lines are able to lyse human melanoma cells via a CD8-dependent interaction (22), suggesting that mouse CD8 does interact with human class I molecules. The regions involved in these interactions remain to be mapped. The use of human tetramers to enumerate specific mouse T cells ex vivo without introducing any culture bias has potential implications in preclinical models of vaccination to investigate the kinetics and the localization of T cell response and thus to define the best protocol of immunization.

The relative high frequency of CTLs specific for $ELA_{26–35}$ recruited in immunized mice is in agreement with data obtained in the metastatic lymph nodes of melanoma patients (24). This might reflect the high frequency of CTL precursors circulating in the periphery due to a partial tolerance against endogenously expressed Melan-A Ag. Indeed, a homolog of the human Melan-A gene is endogenously expressed in the mouse (31). The mouse putative protein contains 113 amino acids with 68.8% identity to

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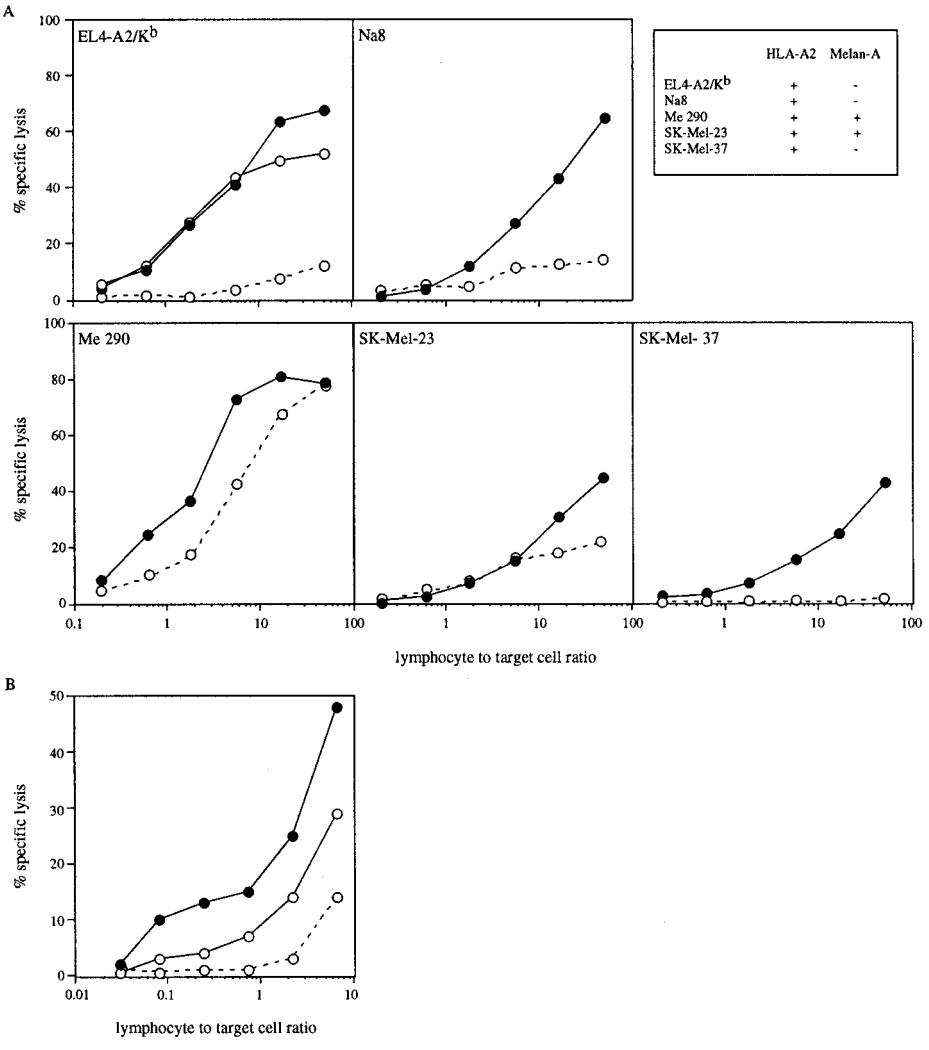

FIGURE 4. Recognition of human melanoma cells by mouse CTLs specific for ELA_{26-35}. *A*, The CTL line specific for ELA_{26-35} was tested for its ability to lyse mouse EL4-A2/K^b cell line or human melanoma cells in the presence (solid line) of ELA_{26-35} (●) or EAA_{26-35} (○), or in the absence of exogenous peptide (dashed line) in a 4-h ^{51}Cr release cytotoxicity assay. The expression of Melan-A was determined by Western blotting as described in *Materials and Methods*. *B*, $CD8^+$ T cells were purified from a pool of three spleens of HHD mice immunized 7 days before with ELA_{26-35} in the presence of CpG ODN 1826 and analyzed by tetramer staining. The cytolytic activity was directly tested against mouse EL4-A2/K^b cell line pulsed with ELA_{26-35} (solid line, ●) or EAA_{26-35} (solid line, ○) or against EL4-A2/K^b cell line alone (dashed line) in a 4-h ^{51}Cr release cytotoxicity assay at different tetramer$^+CD8^+$ T lymphocytes: target cell ratio. One representative experiment of three is shown.

its human homolog. In addition, an HLA-A*0201-restricted epitope mapping to residues 24–33 is highly homologous to the human Melan-A_{26-35}, with only one amino acid difference.

The high frequency of ELA_{26-35}-specific CTLs allowed us to demonstrate their cytolytic activity directly ex vivo without in vitro stimulation and thus without culture bias. These cells are able to kill human melanoma cell lines which endogenously express Melan-A and present the natural EAA_{26-35} epitope at the cell surface, confirming our previous data on the T cell cross-recognition of analog and natural Melan-A peptides (21, 22). It is noteworthy that the susceptibility to lysis by mouse CTLs differs between human melanoma cells, possibly reflecting a differential expression of T cell epitope at the cell surface. Alternatively, we cannot exclude an overall difference at the level of interactions between mouse and human costimulatory molecules because certain human cells were weakly lysed by mouse CTLs even in the presence of exogenous peptide (e.g., SK-Mel-23 and SK-Mel-37 as compared with Me 290).

An important characteristic of the CTL response induced in the presence of CpG ODN is its systemic distribution. Exposure to CpG ODN leads to extramedullary splenic hemopoiesis (32) and to a lymphadenopathy restricted to the drainage field from the injection site (33, 34). The T cell proliferation observed in the spleen is probably associated with the down-regulation of the lymph node homing receptor, CD62L, and could favor the efficient recirculation of specifically activated T cells to the tumor site. There remains, finally, the question of the possibility to induce a long-lasting CTL response in the presence of CpG ODN, which would be required to control tumor growth. This could be solved by the combination of both CpG ODN and conventional adjuvants. Indeed, adjuvants such as IFA are responsible for a "depot" effect, leading to a progressive release of Ag, which could be associated with the maintenance of specific CTL responses. Besides their "depot" effect, these adjuvants have the ability to induce inflammatory processes, potentiating further specific T cell responses. In line with this, we have shown that a mixture of both CpG ODN and IFA led to a stronger specific response as compared with CpG ODN or IFA alone. Previous reports showed that CpG ODN synergizes with alum for Ab production (14) and with IFA for CTL response and T cell proliferation against whole protein (16, 18, 34). Based on the recent report of Kaech and Ahmed (35) suggesting that the most effective T cell vaccines will be those that recruit the largest number of Ag-specific $CD8^+$ T cells, the peptide mixed with CpG ODN and IFA would be a good candidate as antitumor vaccine.

The strong expansion of peripheral $CD8^+$ T lymphocytes is probably not due to a direct effect of CpG ODN on T cells. Apart from two reports (36, 37), CpG ODN do not seem to be a T cell mitogen (33, 38). The most likely hypothesis is that T cell expansion is the consequence of CpG ODN-mediated DCs proliferation (33), activation, and maturation. In vivo, the coinjection of peptide and CpG ODN leads to the maturation of DCs and the presentation

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of peptide by CD11c$^+$ DCs (12). Once maturated, DCs could activate CTL precursors in a Th cell-independent manner (39–41). This might explain why, in our study, no Th peptide is required to elicit a strong CTL response. In human, CpG ODN also induces the survival, maturation, and secretion of cytokines by plasmacytoid precursor DCs (42). Recently, the Toll-like receptor-9 has been evidenced as an essential factor in immune activation by CpG ODN (43).

In conclusion, the demonstration of the adjuvant effect of CpG ODN on the induction of a CTL response directed against melanoma Ag-derived peptide and its effect on the recruitment of a high frequency of CTL precursors when combined with IFA have important implications in vaccine development. In addition, the validation of a quantitative study of CTL response by using human tetramers in mouse experimental systems might be useful to design the most efficient vaccination procedure in a preclinical model.

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