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CpG oligodeoxynucleotides activate dendritic cells in vivo and induce a functional and protective vaccine immunity against a TERT derived modified cryptic MHC class I-restricted epitope

Sébastien Cornet [a], Jeanne Menez-Jamet [a], François Lemonnier [b], Kostas Kosmatopoulos [a,*], Isabelle Miconnet [a]

[a] Vaxon Biotech, Génopole bat G2, 2 rue Gaston Crémieux, 91057 Evry, France
[b] Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, 28 rue du docteur Roux, 75015 Paris, France

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Abstract

The use of synthetic peptides derived from tumor-associated Ags is attractive for the development of antitumoral vaccines as far as strong adjuvants are found to render them immunogenic. Here, we investigated the possibility to enhance the CD8 response against the human and mouse shared $TERT_{572Y}$ HLA-A*0201 restricted modified cryptic peptide by using ODN-CpG as adjuvant. Humanized transgenic mice were immunized with the $TERT_{572Y}$ modified cryptic peptide in the presence of ODN-CpG and compared to mice immunized in IFA. By contrast with IFA, we first showed that, in vivo, ODN-CpG leads to the recruitment of dendritic cells in the lymph nodes draining the injection site. Those cells and especially the $CD11c^+$ $CD11b^-$ $CD8a^+$ lymphoid and the $CD11c^+$ $B220^+$ plasmacytoid dendritic cells were activated as shown by up-regulation of CD40 at their cell surface. Immunization against $TERT_{572Y}$ peptide in the presence of ODN-CpG rather than IFA led to a strong CD8 response and can delayed mortality in an induced tumor model. Study of the CD8 response obtained after antigenic challenge suggested that a functional memory response is induced upon vaccination with ODN-CpG. Thus, MHC class I-restricted epitope in combination of ODN-CpG is a promising and rather simple cancer vaccine formulation.
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1. Introduction

The identification of CTL-defined tumor-associated Ag has allowed for the development of new strategies for cancer therapy based on the use of synthetic peptides. Since numerous tumor Ag are self proteins involved in the negative selection of the T cell repertoire [1], the efficiency of this approach depends on the capacity of the vaccination strategy to bypass the immune tolerance against self tumor antigens. In this regard, the tumor Ag-derived peptide has to be carefully selected and the conditions for vaccination optimized.

We previously found that low affinity tumor Ag-derived peptides (so-called cryptic peptides) which are weakly presented at the cell surface and thus weakly involved in the T cell negative selection are better candidates than high affinity ones (so-called dominant peptides) [2,3]. The substitution of the first amino acid of these low affinity peptides, involved in their interaction with the MHC class I molecule, importantly increased their immunogenicity [4]. The CD8 T cells generated against the modified cryptic peptides are able to cross recognize their low affinity native counterpart [2].

Among the different actors involved in the expansion of a functional CD8 T cell response, the appropriate presentation of antigen by activated dendritic cells (DC) is required. DC mature upon different danger sig-

* Corresponding author. Tel.: +33 160789210; fax: +33 160789219.
E-mail address: kkosmatopoulos@vaxon-biotech.com (K. Kosmatopoulos).

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nals and adjuvants are thought to mimic these danger signals. Besides IFA which has been so far commonly used in experimental models of vaccination, new potent bacterial derived adjuvants such as ODN-CpG have been identified [5]. Moreover, their strong DC activating effect [6] suggests that a concomitant T helper CD4 T cell response could be avoided which would be of particular interest in humans in view of simplifying vaccine formulation.

In this paper, we investigated the possibility of enhancing the CD8 response against the human and mouse shared $TERT_{572Y}$ HLA-A*0201 restricted modified cryptic peptide by using ODN-CpG as an adjuvant. HLA-A*0201 transgenic HHD mice [7] were immunized with the $TERT_{572Y}$ modified cryptic peptide in the presence of ODN-CpG and compared to HHD mice immunized in IFA. We first assessed the influence of the two different adjuvants on the recruitment and activation status of APC in the lymph node draining the injection site. A strong activation of lymphoid and plasmacytoid dendritic cells was only observed in mice injected with ODN-CpG. Then, we directly monitored the peptide-specific $CD8^+$ T cell response in both conditions of vaccination by tetramer staining ex vivo and examined the functionality of specific T cells by measuring their IFN-γ production. ODN-CpG are more effective than IFA to recruit specific $CD8^+$ T cells against $TERT_{572Y}$ peptide and to induce a functional memory response. This conclusion is confirmed in vivo thanks to results obtained in a tumoral challenge which prove that two vaccinations of HHD mice with ODN-CpG and $TERT_{572Y}$ delayed significatively mortality as compared with vaccinations with IFA.

2. Materials and methods

2.1. Cell lines

Previously described EL4/HHD cells [7] were kindly provided by Pr. François Lemonnier (Institut Pasteur, Paris, France) and maintained in DMEM medium supplemented with 1% HEPES, 1% strepto-penicillin and 10% heat inactivated fetal calf serum (FCS).

2.2. Synthetic peptides, ODN-CpG

$TERT_{572}$ (RLFFYRKSV), $TERT_{572Y}$ (YLFFYRKSV) and the control $gp100_{209}$ (ITDQVPFSV) were made by Epytop (Nîmes, France). The immunostimulatory synthetic ODN-CpG 1826 optimized for stimulation of the mouse immune system (TCCATGA<u>CG</u>TTCCTGA<u>CG</u>TT) were used (CpG motifs are underlined) (Sigma-Genosys, Haverhill, UK). The backbone for these ODN was sulfur-modified phosphorothioate to protect it from nucleases. ODN were formulated as a sterile phosphate buffered saline solution and stored at −20 °C.

2.3. Immunization

HHD transgenic mice were kindly provided by Pr François Lemonnier (Institut Pasteur, Paris, France). HHD mice were immunized subcutanously (s.c.) at the base of the tail with 100 μg peptide emulsified in IFA or mixed with 50 μg ODN-CpG in a volume of 100 μl. To study the secondary response, mice were boosted under the same conditions 1 month after the first immunization.

2.4. Tumor challenge

HHD transgenic mice were immunized s.c. with 100 μg peptide emulsified in IFA or mixed with 50 μg ODN-CpG in a volume of 100 μl. Fourteen days later, they were injected s.c. with 25000 EL4/HHD tumor cells. Tumor growth was monitored weekly. For ethical reasons and according to the good laboratory practices defined by the animal experimentation rules in France, mice were euthananized when the tumor size reached 300 mm^2. Statistical analysis performed using the logrank test of the Kaplan–Meier model ($p \leq 0.05$).

2.5. Flow cytometry immunofluorescence analysis

For tetramer staining, cells from peripheral blood, inguinal and paraaortic lymph nodes (LN) and spleen from immunized mice were stained with 15 μg/ml of PE-coupled HLA-A2/$TERT_{572Y}$ tetramer synthesized as previously described [8] in the presence of anti-Fc receptor Ab (clone 2.4 G2) in 20 μl PBS 2% FCS for 1 h at room temperature. Cells were washed once in PBS 2% FCS and then stained with anti-CD44-FITC (clone 1M.178), anti-TCRβ-Cychrome (clone H57) and anti-CD8α-APC (clone 53.6.7) (BD Biosciences, Le Pont de Claix, France) in 50 μl of PBS 2% FCS 30 min at 4 °C. The phenotype and activation status of antigen presenting cells recruited in the draining lymph nodes 18 h after immunization were also studied before and after enrichment of $CD11c^+$ dendritic cells. Briefly, inguinal and paraaortic LN were cut in small pieces and incubated in PBS containing 0.5% FCS and 1 mg/ml collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) at 37 °C for 45–60 min. Digested fragments were filtered through a stainless-steel sieve and cell suspensions washed in PBS 0.5% FCS. Cells were preincubated with anti-Fc receptor Ab (clone 2.4 G2) and submitted to CD11c enrichment by using MACS CD11c micro beads according to the manufacturer's instructions (Miltenyi Biotec, Paris, France). The phenotype of LN cells obtained before and after enrichment was determined by using anti-F4/80-FITC, anti-CD11c-PE, anti-CD11b-biotin Abs and their activation status by staining them with anti-CD40-biotin Ab (BD Biosciences, Le Pont-de-Claix, France). The biotin conjugated Abs were revealed with FITC- or Cy5-conjugated streptavidin (BD Biosciences, Le Pont-de-Claix, France). Cells were washed once in PBS 2% FCS and immediately analyzed using a FACSCalibur® (Becton Dickinson, San Jose, CA, USA).

2.6. *Intracellular IFN-γ staining*

Splenocytes or lymph node cells (2×10^6) from immunized HHD mice were stimulated with 1 μM of $TERT_{572Y}$ modified cryptic peptide or third-party $gp100_{209}$ modified cryptic peptide in the presence of 10 μg/ml brefeldin A (Sigma, Oakville, Canada). Six hours later, cells were washed, stained with anti-CD8a-PE mAb (BD Biosciences, Le Pont-de-Claix, France) in PBS for 25 min at 4 °C, washed and fixed with 4% paraformaldehyde. Then cells were permeabilized with PBS containing 0.2% saponin and 0.5% BSA and stained with anti-IFN-γ-APC (BD Biosciences, Le Pont-de-Claix, France). Cells were analyzed on a FACSCalibur® (Becton Dickinson, San Jose, CA, USA).

2.7. *ELISpot assay*

Peptide-specific T cells from immunized mice were evaluated in IFN-γ ELISpot assay as previously described [21]. Mice were immunized twice at 1 month intervals with 100 μg of $TERT_{572Y}$ in the presence of either IFA or ODN-CpG as mentioned above. Mouse IFN-γ ELISpot PVDF-Enzymatic kit (Diaclone, Besançon, France) was used according to the manufacturer's recommendation. After activation of PVDF plates and coating with IFN-γ capture antibody, 3×10^5 splenocytes from immunized mice were distributed in each well and 1 μM of $TERT_{572}$ native or $TERT_{572Y}$ modified peptide was added. Concanavalin A (5 μg/ml) and $gp100_{209}$ third part peptide stimulation served as positive and negative control, respectively. After 18 h, plates were washed three times with PBS-Tween and then PBS, incubated with biotinylated anti-mouse IFN-γ detection antibody and then with alkaline phosphatase-conjugated streptavidin (Roche Molecular Biochemicals, Mannheim, Germany). Spots were developed by adding peroxidase substrates 5-bromo-4,3-indolyl phosphate and nitroblue tetrazolium (Promega Corp., Madison, Wisconsin, USA) and counted using the automated image analysis system Bioreader 2000 (BIO-SYS, Karben, Germany).

3. Results

3.1. *ODN-CpG induce the recruitment and the activation of dendritic cells in the lymph nodes draining the injection site*

We compared the absolute number of cells recruited in the draining lymph nodes of mice injected with 50 μg ODN-CpG, IFA or PBS 18 h before (Table 1). A significant increase in the absolute number of cells was defined by the mean value in the experimental group higher than the mean value plus 3 S.D. in PBS injected mice. First, variability between individuals was observed. Two out of six mice injected with ODN-CpG and one out six mice injected with IFA presented an increase in the absolute number of lymph node cells.

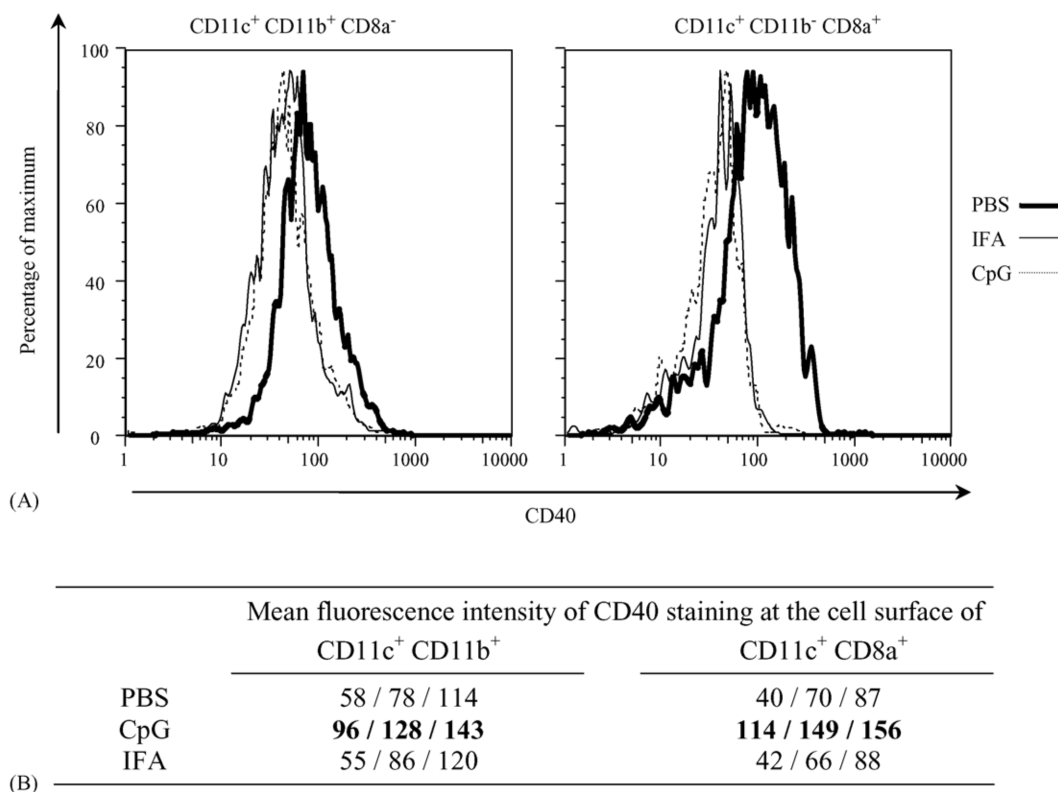
Table 1
Absolute numbers of whole lymph node cells, dendritic cells and macrophages in the draining lymph node of adjuvant injected or naive HHD mice

Experimental group	Absolute number of cells		
	Lymph node cells ($\times 10^{-6}$)	$CD11c^+$ $F4/80^-$ ($\times 10^{-3}$)	$CD11c^-$ $F4/80^+$ ($\times 10^{-3}$)
PBS	10.0	117	63
	11.7	139	93
	9.5	134	92
	7.0	84	69
	12.0	143	230
	8.5	110	119
ODN-CpG	12.5	125	207
	15.4	**288**	209
	7.7	81	36
	20.0	**208**	232
	19.5	**243**	**320**
	10.0	80	69
IFA	9.5	101	66
	8.2	74	33
	20.0	**234**	**350**
	12	165	169
	14	78	91
	15	115	89

HHD mice were injected s.c. at the base of the tail with PBS, 50 μg ODN-CpG or IFA. Draining lymph nodes were collected 18 h after injection and analyzed for their composition in dendritic cells ($CD11c^+$ $F4/80^-$) and macrophages ($CD11c^-$ $F4/80^+$). Values in bold represent a significative increase of the absolute number of positive cells (mean + 3 S.D.) as compared with values obtained in mice injected with PBS.

This increase was associated with a significant increase in the absolute number of $F4/80^-$ $CD11c^+$ dendritic cells in ODN-CpG injected mice. A significant recruitment of $F4/80^+$ $CD11c^-$ macrophages was not systematically observed in these mice. Only one of these two mice injected with ODN-CpG also showed a significant increase in the number of $F4/80^+$ $CD11c^-$ macrophages. The IFA injected mouse in which an increase of $F4/80^-$ $CD11c^+$ dendritic cell number was observed, also presented a significant increase of $F4/80^+$ $CD11c^-$ macrophages. Thus, the injection of ODN-CpG led to a more efficient recruitment of $CD11c^+$ $F4/80^-$ dendritic cells as compared with IFA.

We further investigated the activation status of the different dendritic cell subsets upon ODN-CpG or IFA stimulation. For that purpose, we measured CD40 expression by $CD11c^+$ $CD11b^+$ $CD8a^-$ myeloid and $CD11c^+$ $CD11b^-$ $CD8a^+$ lymphoid dendritic cells (Fig. 1A). As it is shown in Fig. 1B, neither myeloid nor lymphoid dendritic cells from IFA injected mice up-regulated CD40 compared to dendritic cells from PBS injected control mice. In contrast, $CD11c^+$ dendritic cells enriched from the draining lymph nodes of mice injected with ODN-CpG up-regulated CD40 molecule. Furthermore, the increase of CD40 expression appeared stronger in the $CD11c^+$ $CD11b^-$ $CD8a^+$ lymphoid than in the $CD11c^+$ $CD11b^+$ $CD8a^-$ myeloid subset of dendritic cells. A third type of dendritic cells called plasmacy-



	Mean fluorescence intensity of CD40 staining at the cell surface of	
	$CD11c^+$ $CD11b^+$	$CD11c^+$ $CD8a^+$
PBS	58 / 78 / 114	40 / 70 / 87
CpG	**96 / 128 / 143**	**114 / 149 / 156**
IFA	55 / 86 / 120	42 / 66 / 88

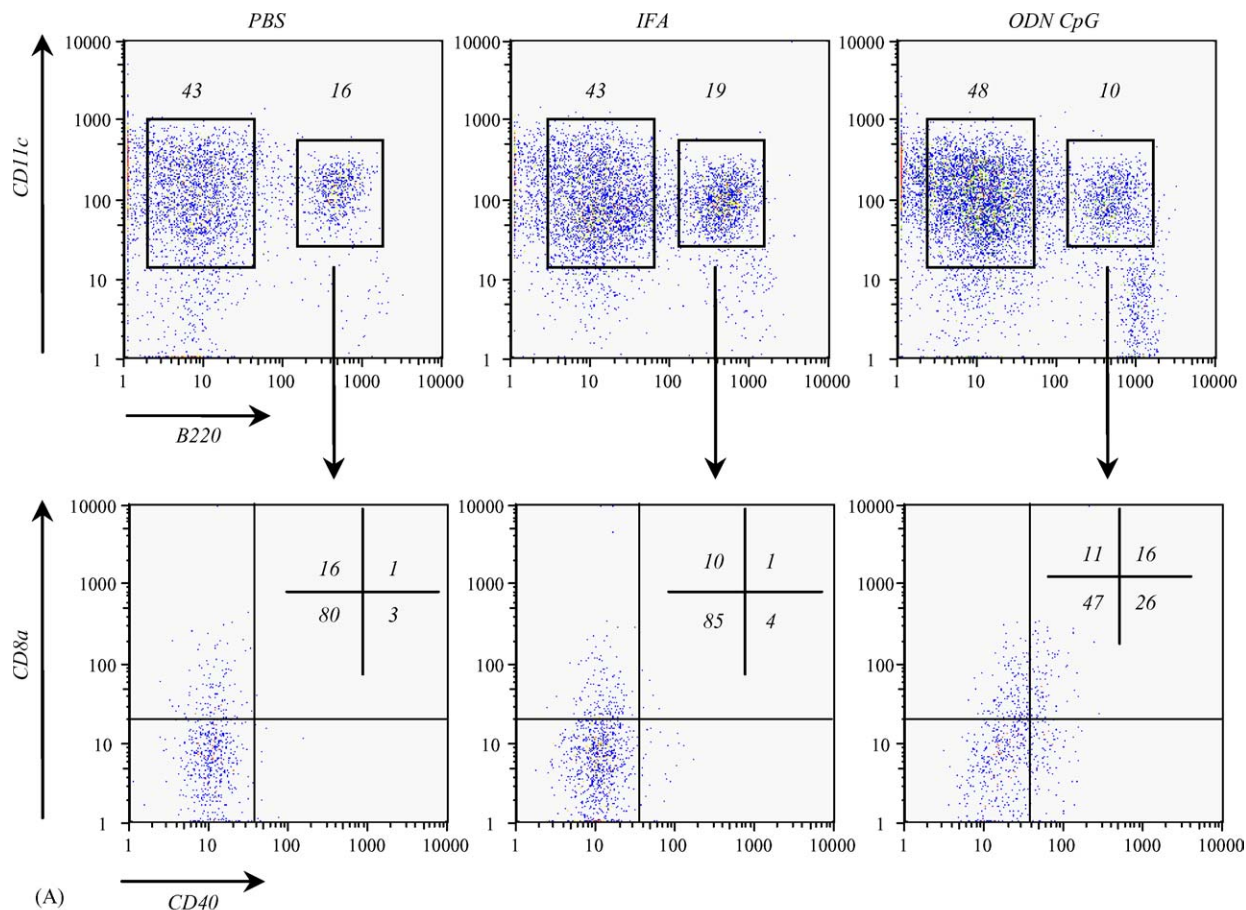
Fig. 1. Activation of myeloid and lymphoid dendritic cell subsets upon CpG or IFA stimulation. HHD mice were injected s.c. at the base of the tail with PBS, 50 μg ODN-CpG or IFA. Draining lymph nodes were collected 18 h after injection. $CD11c^+$ dendritic cells were enriched by using anti-CD11c magnetic microbeads and analyzed for their activation status by staining with anti-CD40 mAb. (A) A typical experiment is shown. (B) The data from three experiments are summarized. Values in bold indicate an increase of CD40 mean fluorescence intensity as compared to PBS injected mice.

toid cells was also studied. These cells have been recently described as the mouse counterpart of the human plasmacytoid cells which are the natural IFN-α-producing and present a $CD11c^{low}$ $CD11b^-$ $B220^+$ $CD8a^{low}$ $GR1^+$ phenotype when unstimulated [9]. First, the percentage of $CD11c^+$ $B220^+$ dendritic cells varied between PBS, IFA and ODN-CPG injected mice. Indeed, a significant reduction ($p < 0.01$) in the percentage of $B220^+$ cells within the $CD11c^+$ dendritic cells subset was observed in ODN-CpG mice (10 ± 2.0) as compared with PBS (18.6 ± 3.0) or IFA (19.6 ± 2.1) injected mice (Fig. 2A and data not shown). Moreover, as compared with PBS injected control mice, the data presented in Fig. 2B indicated an increase of the percentage of $CD8a^+$ within $CD11c^+$ $B220^+$ plasmacytoid cells upon ODN-CpG stimulation as well as an up-regulation of the expression of CD40 molecule at the cell surface of both $CD11c^+$ $B220^+$ $CD8a^+$ and $CD11c^+$ $B220^+$ $CD8a^-$ plasmacytoid cells. This subpopulation was not altered in IFA injected mice.

Altogether, the stimulation with ODN-CpG in vivo induced a specific recruitment of $F4/80^-$ $CD11c^+$ dendritic cells. Both $CD11b^+$ $CD8a^-$ myeloid and $CD11b^-$ $CD8a^+$ lymphoid dendritic cells exhibited an increase of CD40 expression at the cell surface suggesting their activated status. In parallel, a lower percentage of $CD11c^+$ $B220^+$ plasmacytoid cells also presenting an up-regulation of CD40 at the cell surface was observed upon ODN-CpG injection. In contrast, IFA stimulation in vivo led to non-systematic recruitment of dendritic cells exhibiting a non-activated phenotype.

3.2. Comparison of ODN-CpG and IFA as adjuvants of the CD8 response against telomerase derived peptide

HHD mice were immunized with the $TERT_{572Y}$ modified cryptic peptide in combination with either ODN-CpG or IFA. The frequency of specific $CD8^+$ T lymphocytes was measured in PBL between 7 and 14 days after immunization using HLA-A2/$TERT_{572Y}$ tetramers and compared to the frequency of $tetramer^+$ cells in naive HHD mice. The tetramer used was validated for its specificity and sensitivity as previously described [8], by using a mouse CTL line specific for $TERT_{572Y}$ obtained by repeated stimulation of lymph node cells from HHD mice immunized with the peptide emulsified in IFA (data not shown). The percentage of $tetramer^+$ lymphocytes was determined within the $CD8^+$ $TCR\beta^+$ subset. These $tetramers^+$ T cells displayed an activated phenotype as demonstrated by the up-regulation of the CD44 activation marker at their surface (not shown). A significant response was defined as the frequency of $tetramer^+$ cells higher than the mean value of $tetramer^+$ cell frequency calculated in naive mice + 3 S.D. (i.e. 0.04%). As demonstrated for Melan-A



	Percentage of $CD8a^+$ positive within $CD11c^+ B220^+$	CD40 Mean fluorescence intensity at the cell surface of	
		$B220^+ CD8a^+$	$B220^+ CD8a^-$
PBS	17 / 12 / 27	13 / 38 / 61	11 / 34 / 54
CpG	**27 / 30 / 32**	**41 / 75 / 93**	**26 / 59 / 81**
IFA	11 / 11 / 21	13 / 36 / 61	11 / 34 / 55

(B)

Fig. 2. Activation of $CD11c^+$ $B220^+$ dendritic cell subset upon ODN-CpG or IFA stimulation. HHD mice were injected s.c. at the base of the tail with PBS, 50 μg ODN-CpG or IFA. Draining lymph nodes were collected 18 h after injection. $CD11c^+$ dendritic cells were enriched by using anti-CD11c magnetic microbeads and analyzed for their phenotype and activation status by staining with anti CD40 mAb. (A) A typical experiment is shown. The numbers shown in the upper panel indicate the percentages of each dendritic cell subset ($CD11c^+$ $B220^-$ and $CD11c^+$ $B220^+$) within the CD11c enriched fraction. The numbers in the lower panel correspond to the percentages of $CD11c^+$ $B220^+$ dendritic cell subsets defined on the basis of their CD8a and CD40 expression. (B) The data from three independent experiments are summarized. Values in bold indicate an increase of CD40 mean fluorescence intensity as compared to PBS injected mice.

derived peptide [8], $TERT_{572Y}$ specific CD8 T cell response were detectable in PBL of immunized mice by using specific tetramer staining. Typical profiles of tetramer staining of PBL derived from naive mice and mice immunized against $TERT_{572Y}$ in the presence of ODN-CpG and IFA are shown in Fig. 3A.

In the period between 7 and 14 days after immunization which corresponds to the peak of the CD8 T cell response, 5 out of 15 mice immunized against the $TERT_{572Y}$ peptide in the presence of ODN-CpG presented a significant response (Fig. 3B). The frequency of $tetramer^+$ $CD8^+$ T cells was variable between individuals. The CD8 T cell response obtained in $TERT_{572Y}$ immunized mice was specific for the immunizing peptide since less than 0.01% of T cells were stained with an irrelevant tetramer (data not shown). Only 1 out 29 mice immunized with $TERT_{572Y}$ in the presence of IFA responded. After 14 days, the frequency of specific CD8 T cells decreased to reach the naive level at 1 month after immunisation in both groups of mice (not shown).

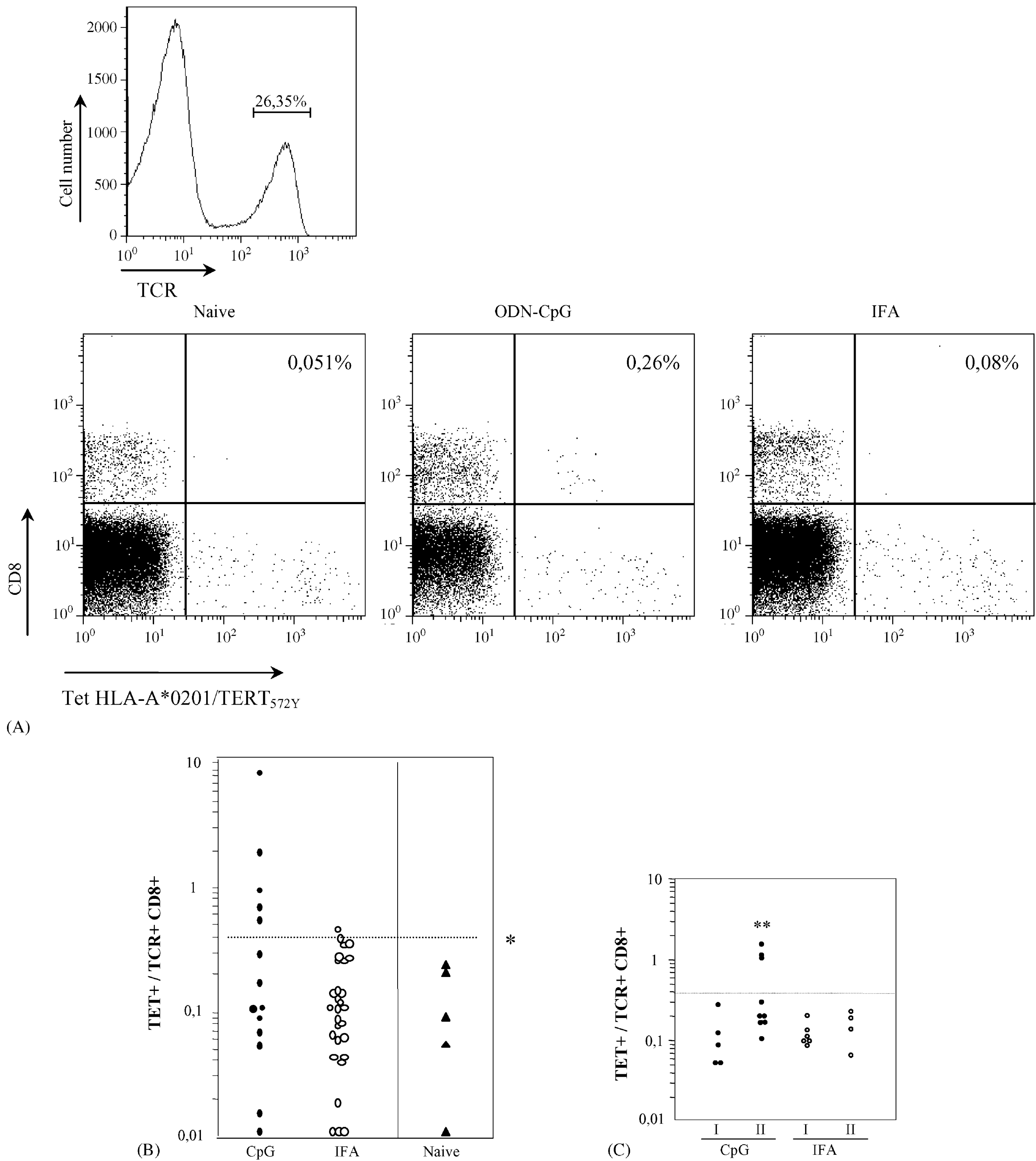


Fig. 3. HHD mice were immunized with $TERT_{572Y}$ modified peptide together with ODN-CpG or IFA. Naive mice were used as controls. PBLs were prepared 7–14 days after immunization and stained ex vivo with specific HLA-A2/$TERT_{572Y}$ tetramer as detailed in Section 2. (A) Typical tetramer staining profile. The percentages of HLA-A2/$TERT_{572Y}$ tetramer$^+$ CD8$^+$ cells indicated in dot blots were determined within the TCR$^+$ gated cells represented in histogram. (B) Monitoring of the CD8 response in immunized HHD mice. The percentages of tetramer$^+$ cells within CD8$^+$ TCRβ$^+$ lymphocytes are represented for each individual mouse from the different experimental groups. *The line indicates the cut-off value calculated as the mean of CD8$^+$ tetramer$^+$ T lymphocytes analyzed from naive mice + 3 S.D. (0.04%). (C) Monitoring of the CD8 response after antigenic challenge of HHD mice. HHD mice were immunized twice at 1 month interval with $TERT_{572Y}$ peptide together with ODN-CpG or IFA. PBLs were isolated 5 days after the first (I) and the second immunization (II), stained ex vivo with specific HLA-A2/$TERT_{572Y}$ tetramer and analyzed as above. Statistical analysis was performed using the Mann–Whitney test. "**" Indicates a significative difference ($p \leq 0.05$).

3.3. A peptide-specific secondary T cell response is induced following immunization in the presence of ODN-CpG

One month after the first immunization, HHD mice were submitted to antigenic challenge under the same conditions. The kinetics of the specific CD8 T cell response after antigenic challenge was different than the one obtained after one immunization with a peak of $CD8^+$ T cell response at day 5 (data not shown). Therefore, we analyzed the specific CD8 T cell response 5 days after recall with $TERT_{572Y}$ peptide in the presence of IFA or ODN-CpG (Fig. 3C). The CD8 T cell response specific for the $TERT_{572Y}$ peptide observed in the blood 5 days after antigenic challenge in the presence of ODN-CpG was stronger than the one observed at day 5 after the first immunization (according to Student's test $p \leq 0.05$). By contrast, the frequencies of HLA-A2/$TERT_{572Y}$ $tetramer^+$ $CD8^+$ within T lymphocytes measured 5 days after antigenic challenge in the presence of IFA were not different from the frequencies observed 5 days after the first immunization. This data suggest the development of a memory CTL response against $TERT_{572Y}$ peptide in the presence of ODN-CpG.

3.4. Functionality of peptide-specific T cell response induced following immunization in the presence of ODN-CpG

The functionality of the specific T cells recruited in the presence of ODN-CpG after an antigenic recall was assessed by measuring their IFN-γ production in ELISpot assay (Fig. 4). Splenocytes, collected at day 5 after antigenic challenge were incubated in the presence of 10 μM of $TERT_{572}$ native or $TERT_{572Y}$ modified peptide. Four out of five (80%) mice immunized with ODN-CpG showed a specific response for the injected $TERT_{572Y}$ peptide and cross recognized the $TERT_{572}$ native peptide. Three of these mice presented a mean number of IFN-γ-producing cells of 8 and 10 for 10,000 CD8 cells for stimulation with modified and native peptide, respectively; one present a four time higher response compared to the other with 43 and 36 positive cells with modified peptide and native peptide, respectively. By contrast, only one out of four (25%) mice immunized in the presence of IFA was able to mount an IFN-γ-producing CD8 T cell response with 15–20 IFN-γ-producing cells for 10,000 CD8 cells for modified or native peptide stimulation, respectively, indicating that ODN-CpG are more efficient than IFA in inducing an IFN-γ-producing functional CD8 T cell response.

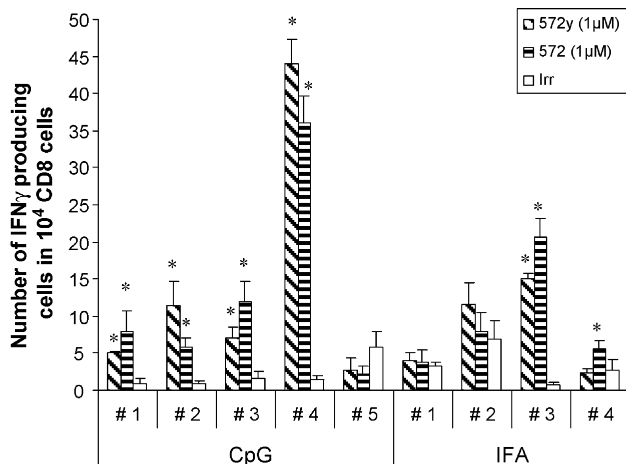


Fig. 4. Functionality of CD8 T cell response. Splenocytes of mice immunized twice with 100 μg of $TERT_{572Y}$ peptide in the presence of either ODN-CpG or IFA were tested in ELISpot assay for their ability to produce IFN-γ in response to $TERT_{572Y}$ and $TERT_{572}$ specific and to $gp100_{209}$ third-party peptides. Histograms represent the number of IFN-γ-producing cells for 10,000 CD8 cells. "*" represents a significative difference between native or modified peptide and the third-party peptide ($p \leq 0.05$ according to the Student's test for each triplicate).

3.5. Effect of the immunization against telomerase derived peptide in the presence of ODN-CpG or IFA on tumor protection

HHD mice were immunized once with the $TERT_{572Y}$ modified peptide in the presence of either ODN-CpG or IFA 14 days before EL4/HHD tumor challenge. Control mice were immunized under the same conditions with the irrelevant $gp100_{209}$ peptide. Mice immunized against the third-party $gp100_{209}$ peptide in the presence of either ODN-CpG or IFA developed a tumor with a median survival of 49 and 35 days, respectively. Moreover, mice immunized against the $TERT_{572Y}$ modified peptide in the presence of IFA did not present any significant protection (median survival of 35 days), compared with the two negative control groups. By contrast, mice immunized against the $TERT_{572Y}$ modified peptide in the presence of ODN-CpG where partially protected. Although 94% mice developed a tumor, the median survival in this group was significantly increased compared to the survival rate observed in the group of mice immunized against $TERT_{572Y}$ in the presence of IFA (63 days in $TERT_{572Y}$ + ODN-CpG versus 35 days in $TERT_{572Y}$ + IFA, $p \leq 0.05$) (Fig. 5).

4. Discussion

The use of synthetic peptides derived from tumor-associated Ags is attractive for the development of antitumoral vaccines. However, these compounds are usually not very immunogenic unless they are administered in combination with a strong adjuvant. In the present study, we demonstrate the potent role of ODN-CpG as an adjuvant of the CD8 response directed against TERT-derived modified cryptic peptide. By contrast with IFA, we first show that, in vivo, ODN-CpG leads to the recruitment of dendritic cells in the lymph nodes draining the injection site. Dendritic cells and especially the $CD11c^+$ $CD11b^-$ $CD8a^+$ lymphoid ones are activated as shown by CD40 up-regulation at their cell surface. In agreement with previous report [10], the $CD11c^+$

B220+ plasmacytoid dendritic cells are also activated upon ODN-CpG stimulation in vivo, but their frequency in draining lymph nodes is lower as compared to the PBS control treatment. Immunization against $TERT_{572Y}$ peptide in the presence of ODN-CpG rather than IFA leads to a strong CD8 response and can delayed death in an induced tumor model. The superiority of ODN-CpG over IFA as adjuvant of the CD8 T cell response specific for TERT-derived modified cryptic peptide confirms previous studies on the CD8 response specific for melanoma tumor antigens performed in both mouse model [8] and melanoma patients [30]. Finally, the study of the CD8 response obtained after antigenic challenge suggests that a functional memory response is induced upon vaccination with ODN-CpG.

ODN-CpG interact with Toll-like receptor (TLR) 9 present in the endocytic vesicles of plasmacytoid dendritic cells and thus can directly induce activation of those cells. Although myeloid dendritic cells do not express TLR9, we observed that these cells are recruited and activated upon ODN-CpG stimulation in vivo. This could be explained by an indirect effect of cytokines and chemokines secreted in inflamed draining lymph nodes. In this regard, we report an activation of plasmacytoid dendritic cells upon ODN-CpG stimulation, as shown by the up-regulation of both CD40 and CD8a at their cell surface [11,12]. These activated plasmacytoid dendritic cells via a secretion of type I IFN contribute to the regulation of immune response and especially to the differentiation, maturation and immunostimulatory function of myeloid dendritic cells. The recruitment and the activation of myeloid DC is critical, since those cells are more efficient than plasmacytoid dendritic cells in presenting Ag and in inducing T cell expansion [12,13]. Moreover, it has been clearly shown that activated mouse plasmacytoid dendritic cells are able to produce IL12 that contribute to a Th1 polarization of the T cell response [14]. This is obviously particularly relevant with the objective to generate an efficient anti-tumor T cell response.

The impact of ODN-CpG on mouse plasmacytoid dendritic cells we reported is also of particular interest, since an equivalent subset has also been described in humans. Plasmacytoid dendritic cells from both human and mouse origin produce high amounts of type I IFN (reviewed in [15]). However, the production of IL12 by human plasmacytoid dendritic cells has been controversial [16,17]. This may be explained by the differences in isolation and stimulation protocols used by the different groups. In line with this, a proper activation of plasmacytoid dendritic cells is critical in the balance between the induction of tolerogenicity and immunogenicity. Indeed plasmacytoid dendritic cells activated in the presence of IL-3 and CD40-ligand although expressing high level of MHC molecules and costimulatory molecules and exhibiting potent T cell stimulatory capacity induce naive CD8 T cell differentiation into IL-10 producing anergic CD8 T cells, unable to respond to an antigenic challenge [18]. By contrast, activation of plasmacytoid dendritic cells with ODN-CpG and CD40L give rise to maturation into immunogenic plasmacytoid dendritic cells [17,12]. The choice of adjuvant is thus critical in view of designing an efficient vaccine against tumors.

We showed that a CD8 response against $TERT_{572Y}$ MHC class I-restricted epitope can be induced in the presence of ODN-CpG and in the absence of any CD4 T helper epitope. The stronger and more rapid CD8 response obtained after antigenic challenge led us to propose that a memory response is induced by this immunization protocol. Moreover, this CD8 response is functional as seen by the IFN-γ production. This suggests that a CD8 response independent on an Ag-specific CD4 response can be generated in the presence of TLR9 signalling. The CD4 independency of the CD8 response has been rather controversial. Report on cell based antigens first showed that CD4 T cells are required during the in vivo phase to produce CD8 T cell priming [19–21]. CD4 T cells recognize MHC class II-restricted Ag at the surface of dendritic cells and then license them to stimulate the response of naive CD8 cells, the role of CD40-CD40L interaction being determinant in this process [22–24]. In contrast with the requirement of CD4 cells in the induction of a CD8 response to non-inflammatory Ag, a strong primary CD8 response can be mounted to infectious agents independently of CD4 [25–27]. However, the generation of long lived, functional memory CD8 T cells needs CD4 activity [28,29]. These data have been obtained in experimental model where CD4-deficient mice were immunized. This is different from our protocol where CD4 wild type mice were immunized against CD8 MHC class I-restricted peptide in the presence of ODN-CpG. Besides the efficiency of ODN-CpG to activate dendritic cells as demonstrated, we cannot exclude the activity of bystander CD4 cells that could also contribute in helping CD8 T cells (Fig. 5).

The demonstration of the efficiency of ODN-CpG to recruit and activate dendritic cells and to behave as potent

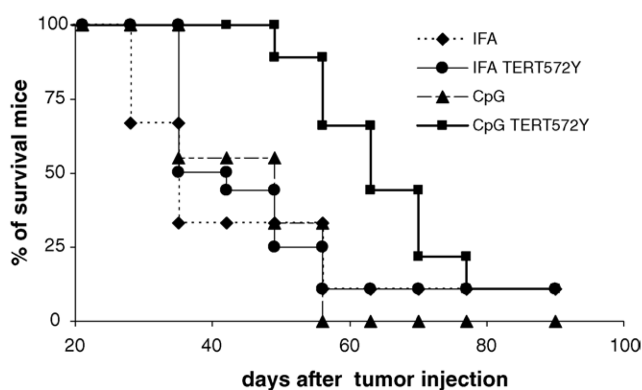


Fig. 5. . In vivo antitumor immunity induced in HHD mice vaccinated against $TERT_{572y}$ in the presence of IFA or ODN-CpG. HHD mice were vaccinated with 100 μg $TERT_{572Y}$ modified peptide emulsified in IFA or mixed with 50 μg ODN-CpG. Fourteen days after vaccination mice were challenged s.c. with 2×10^4 EL4/HHD cells. Mice were observed daily and survival was recorded. Mice were euthananized when the tumor size reached 300 mm^2. A pool of two experiments of 10 mice per group is presented. Statistical analysis was performed using the logrank test of the Kaplan–Meier model ($p \leq 0.05$).

adjuvant for a functional CD8 response against TERT derived modified cryptic peptide has important implications for the design of a cancer vaccine. The fact that MHC class I-restricted epitope combined with ODN-CpG is able to induce a functional CD8 response without the requirement of a T helper CD4 epitope is promising in view of simplifying vaccine formulation.

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