CpG-A and CpG-B oligonucleotides differentially enhance human peptide–specific primary and memory CD8$^+$ T-cell responses in vitro

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Two distinct types of CpG oligodeoxynucleotide (ODN) have been identified that differ in their capacity to stimulate antigen-presenting cells: CpG-A induces high amounts of interferon-α (IFN-α) and IFN-β in plasmacytoid dendritic cells (PDCs), whereas CpG-B induces PDC maturation and is a potent activator of B cells but stimulates only small amounts of IFN-α and IFN-β. Here we examined the ability of these CpG ODNs to enhance peptide-specific CD8$^+$ T-cell responses in human peripheral blood mononuclear cells (PBMCs). The frequency of influenza matrix–specific "memory" CD8$^+$ T cells was increased by both types of CpG ODN, whereas the frequency of Melan-A specific "naive" CD8$^+$ T cells increased on stimulation with CpG-B but not with CpG-A. The presence of PDCs in PBMCs was required for this CpG ODN-mediated effect. The expanded cells were cytotoxic and produced IFN-γ on peptide restimulation. Soluble factors induced by CpG-A but not CpG-B increased the granzyme-B content and cytotoxicity of established CD8$^+$ T-cell clones, each of which was IFN-α/-β dependent. In conclusion, CpG-B seems to be superior for priming CD8$^+$ T-cell responses, and CpG-A selectively enhances memory CD8$^+$ T-cell responses and induces cytotoxicity. These results demonstrate distinct functional properties of CpG-A and CpG-B with regard to CD8 T cells. (Blood. 2004;103:2162-2169)

Introduction

The induction of cytotoxic CD8$^+$ T cells (CTLs) in the absence of antigen-carrying living pathogens (viruses or intracellular bacteria) is a major goal of new vaccine strategies directed against established tumors and a range of infectious diseases. Since Freund's first report on mycobacterial extracts,[1] the use of vaccine adjuvants has become a widespread but incompletely understood practice to promote specific T- and B-cell responses. In recent years it has become clear that conserved microbial molecules are recognized by the innate immune system by Toll-like receptors, a new family of pattern recognition receptors eliciting specific signaling cascades that ultimately result in enhancing and guiding T- and B-cell responses (for reviews, see Akira et al[2] and Bendelac et al[3]).

Recognition of bacterial DNA by Toll-like receptor 9 (TLR9) is based on the presence of unmethylated CG dinucleotides in particular sequence contexts (CpG motifs). Synthetic oligodeoxynucleotides (ODNs) containing such CpG motifs (CpG ODNs) mimic bacterial DNA and induce a coordinated set of immune responses that comprise innate immunity and acquired T_H1-biased cellular and humoral immunity (for a review, see Krieg[4]). In mice and in nonhuman primates, CpG ODNs boost the efficacy of vaccines against bacterial, viral, and parasitic pathogens.[4,5] Several reports also demonstrate the potential of CpG ODNs to enhance CTL responses in mice.[6-10] However, thus far this has not been shown for humans. Because of evolutionary divergence, optimal CpG motifs[11-13] differ between mice and humans. In mice but not in humans, cells of the myeloid lineage, such as monocytes and myeloid dendritic cells, directly respond to CpG ODN,[14-17] leading to differences in the target cells activated and the cytokines induced by CpG ODN and limiting the extrapolation of results from mice to primates and humans.

Recently, 2 types of CpG ODN have been identified based on distinct immunologic activities on human plasmacytoid dendritic cells and B cells.[12,18,19] CpG-A (eg, ODN 2216 and ODN 1585) are characterized by poly G tails with phosphorothioate linkages flanking a central palindromic CpG motif-containing sequence with a phosphodiester backbone. CpG-A induces high amounts of interferon-α/-β (IFN-α/-β) in plasmacytoid dendritic cells (PDCs) (5 pg/PDC[18]) but is relatively weak at activating B cells. CpG-B (eg, ODN 2006), which has a phosphorothioate backbone and lacks poly-G tails, strongly promotes the maturation and activation of PDCs but induces only small amounts of IFN-α/-β. Unlike CpG-A, CpG-B is weak at activating natural killer (NK) cells but strongly stimulates B cells.[4] Similar types of CpG ODNs were described by Klinman et al[20] based on NK cell activation and IFN-γ production in peripheral blood mononuclear cells (PBMCs) (their D and K type CpG ODNs correspond to A and B type ODNs, respectively).[5]

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Recent results suggest that PDCs and B cells are the only cells within human PBMCs that are directly responsive to CpG ODN. Both NK cell activation and IFN-γ induction seem to be indirect effects of CpG ODN mediated through PDCs.[14,21]

In the present study we examined the activity of these 2 types of CpG ODN to enhance the induction of peptide-specific CTLs. Our studies demonstrate for the first time that CpG ODNs are capable of promoting peptide-specific $CD8^+$ T-cell responses in the human immune system. The results provide evidence that CpG-A and CpG-B ODNs differ in their ability to prime naive or to boost memory $CD8^+$ T cells and to confer cytotoxic activity.

Materials and methods

Oligodeoxynucleotides and synthetic peptides

Completely and partially phosphorothioate-modified ODNs were provided by the Coley Pharmaceutical Group (Wellesley, MA) (small letters indicate phosphorothioate linkage; capital letters, phosphodiester linkage 3′ of the base; bold, CpG-dinucleotides; ODN 2006, 5′ tcgtcgttttgtcgttttgtcgtt 3′; ODN 2137 the GC control to ODN 2006, 5′ tgctgcttttgtgcttttgtgctt 3′[12]; ODN 1585. 5′ ggGGTCAACGTTGAgggggG 3′[22]; ODN 2216, 5′ ggGGACGATCGTCgggggG 3′; ODN 2243 the GC control to ODN 2216, 5′ ggGGGAGCATGCTGgggggG 3′.[18] The HLA-A*0201–restricted peptides derived from the melanoma-associated differentiation antigen Melan-A/MART-1 ELAGIGILTV (A>L substitution at position 2 compared with its natural counterpart for higher immunogenicity[23] and referred to as Melan-$A_{26\text{-}35}$ A27L), from the influenza matrix protein GILGFVFTL (referred to as Flu $matrix_{58\text{-}66}$) and from the HIV pol protein ILKEPVHGV (referred to as HIV $pol_{476\text{-}484}$), were synthesized on a multiple peptide synthesizer (peptide synthesizer 433A; Applied Biosystems, Foster City, CA) by the core facility at the GSF Research Institute Munich (Dr Arnolds). All peptides were more than 90% pure, as indicated by high-performance liquid chromatography (HPLC) analysis. Lyophilized peptides were diluted in 30% dimethyl sulfoxide (DMSO) and were stored at −20°C. Pyrogen-free reagents were used for all dilutions. CpG ODN and peptides were found to be negative for endotoxin using the LAL assay (BioWhittaker, Walkersville, MD; lower detection limit, 0.1 EU/mL).

Preparation, culture, and expansion of peptide-specific $CD8^+$ T cells

Human PBMCs were isolated from buffy coats (provided by the Institute of Immunology and Transfusion Medicine, University of Greifswald, Germany) or freshly drawn peripheral blood by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Blood donors were 18- to 68-year-old healthy men and women who were negative for HIV, hepatitis B virus (HBV), and HCV infection. Informed consent was obtained from all donors. For this study HLA-A2–positive donors were selected after PBMCs were stained with an HLA-A2–specific antibody (BB7.2; hybridoma from ATCC) and analysis by flow cytometry. To increase the precursor frequency of peptide-specific cells, $CD8^+$ T cells were enriched by one round of positive selection using anti-CD8 antibody beads and MACS-technology according to the manufacturer's protocol (Miltenyi Biotec; Bergisch-Gladbach, Germany). Then 1×10^6 $CD8^+$ T cells together with 2×10^6 unseparated PBMCs were stimulated with the indicated peptide at a concentration of 5 μM (Melan-$A_{26\text{-}35}$ A27L) and 0.1 μM (Flu $matrix_{58\text{-}66}$), respectively, in the presence or absence of CpG ODN (6 μg/mL) in 24-well culture plates in 2 mL Iscove modified Dulbecco medium (IMDM) supplemented with 8% human AB serum (BioWhittaker), recombinant human interleukin-2 (IL-2) (10 U/mL), IL-7 (10 ng/mL) (both R&D Systems, Wiesbaden, Germany), 1.5 mM L-glutamine, and 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, Munich, Germany). In some experiments PBMCs were depleted of PDCs using BDCA4-coupled magnetic beads and of B cells using CD19-coupled beads (both from Miltenyi Biotec) according to the manufacturer's protocol (less than 0.02% PDCs identified as Lin^-, $CD123^+$, $HLA\text{-}DR^+$ and less than 0.1% B cells after depletion). Medium was changed every second day after day 5, but no further ODN or peptides were added. After 10 to 14 days, the cells were harvested and analyzed as indicated.

Flow cytometric immunofluorescence analysis

Monoclonal antibodies against human $CD3^{FITC}$ (clone UCHT1), $CD8^{PerCP}$ (clone RPA-T8), $CD28^{FITC}$ (clone CD28.2), $CD56^{APC}$ (clone B159), $CDw123^{PE}$ (clone 7G3), HLA DR^{PerCP} (clone L243), Fas-$ligand^{Biotin}$ (clone NOK1), and IFN-γ^{PE} (clone B27) were purchased from PharMingen/Becton Dickinson (Heidelberg, Germany). The antihuman $SLAM^{FITC}$ antibody (clone A12) was kindly provided by Lewis Lanier (University of California, San Francisco), and the anti–granzyme-B^{PE} antibody was from Hölzel Diagnostika (Cologne, Germany). Phycoerythrin (PE)–coupled HLA-A2/Melan-$A_{26\text{-}35}$ A27L, HLA-A2/Flu $matrix_{58\text{-}66}$, and HLA-A2/HIV $pol_{476\text{-}484}$ tetramers (kindly provided by Philippe Guillaume, Ludwig Institute for Cancer Research, Lausanne branch, Epalinges, Switzerland) were synthesized as previously described.[24] For surface analysis, cells were harvested and stained in 50 μL phosphate-buffered saline (PBS)/2% human serum albumin (HSA) with the indicated tetramers for 40 minutes at 20°C, and then the other fluorescence-labeled antibodies were added. After another 20 minutes of incubation on ice, the cells were washed once and analyzed on a FACSCalibur (Becton Dickinson). In 3-color stainings, TO-PRO-3 iodide (Molecular Probes, Eugene, OR), a DNA intercalating dye with fluorescence characteristics similar to that of allophycocyanin (APC), was added immediately before analysis to exclude dead cells. To assess antigen-specific IFN-γ production, cells were harvested after 10 to 14 days, washed once, and restimulated in 96-well, round-bottom culture plates in 200 μL medium with 10 μM cognate peptide or the HIV $pol_{476\text{-}484}$ peptide as a control. After 2-hour incubation at 37°C, 1 μg/mL brefeldin A (Sigma, Munich, Germany) was added. After 4-hour incubation at 37°C, cells were harvested, and intracellular cytokine staining was performed as previously described.[18] Data were analyzed using CellQuest software (Becton Dickinson).

Generation of peptide-specific $CD8^+$ T-cell clones

Melan $A_{26\text{-}35}$ A27L and Flu $matrix_{58\text{-}66}$ peptide-specific $CD8^+$ T-cell clones were generated from PBMCs of HLA-A2–positive healthy volunteers. PBMCs were stimulated in vitro with Melan $A_{26\text{-}35}$ A27L and Flu $matrix_{58\text{-}66}$ peptides as described. After 14 days, Melan $A_{26\text{-}35}$ A27L and Flu $matrix_{58\text{-}66}$ peptide-specific $CD8^+$ T cells were labeled with HLA-A2/Melan $A_{26\text{-}35}$ A27L tetramers and HLA-A2/Flu $matrix_{58\text{-}66}$ tetramers, respectively, and anti-CD8 antibodies, subsequently sorted directly into 96-well plates using a $FACstar^{Plus}$ flow cytometer (Becton Dickinson, Heidelberg, Germany) at a frequency of 1 cell per well and expanded as previously described.[25] All T-cell clones were grown in IMDM supplemented with 8% human AB serum, 100 IU/mL IL-2, 0.25 μg/mL phytohemagglutinin (PHA; Sigma, Munich, Germany), and irradiated feeder cells (3×10^4 allogeneic PBMCs plus 1.5×10^4 721 LCL cells per well). At 2- to 4-week intervals, the clones were passaged with feeder cells and PHA. The phenotype and the expression of an HLA-A2/Melan $A_{26\text{-}35}$ A27L- and an HLA-A2/Flu $matrix_{58\text{-}66}$-specific T-cell receptor (TCR), respectively, were confirmed by flow cytometry for each clone used in subsequent experiments. For the activation assays, clones were washed, incubated for 18 hours in the cell-free supernatant derived from CpG ODN-stimulated PBMCs (2×10^6/mL; 6 μg CpG ODN for 24 hours; no peptide added), washed again, and then used as effectors in chromium Cr 51 release assays. In some experiments blocking antibodies to interferon type 1 (a combination of polyclonal rabbit anti–IFN-α (5000 neutralizing U/mL) and rabbit anti–IFN-β (2000 neutralizing U/mL) antibodies, together with 20 μg/mL monoclonal mouse antihuman IFN-α/-β receptor chain 2 antibody (all from PBL, New Brunswick, NJ) or IL-12 (clone C8.6, 3 μg/mL; BD/PharMingen), were added at the beginning of the 18-hour incubation time of the T-cell clones in CpG-induced supernatants.

Assay of in vitro cytolytic activity

Antigen-specific lytic activity was measured by performing a standard ^{51}Cr release assay using peptide-pulsed HLA-A2–positive, TAP-negative T2 cells (lymphoblast cell line, ATCC CRL-1992) as target cells. Briefly, 2 to 5×10^6 T2 cells were pulsed in 150 μL medium with 10 μM cognate or control peptide for 2 hours at 37°C and subsequently were labeled with 100 μCi (3.7 MBq) ^{51}Cr (NEN Life Science Products, Köln, Germany) for another hour at 37°C. Cells were washed 4 times and were used as target cells (3×10^3 cells/well) for effector T cells (E/T ratios as indicated) in 96-well, round-bottom plates. After 4-hour incubation at 37°C, 50 μL supernatant/well were harvested, and radioactivity was measured on a gamma counter. Maximum release was assessed by the addition of Triton X 0.01% (Sigma, Munich, Germany). Spontaneous release was determined in wells with labeled targets in the absence of effectors. The mean of triplicate measurements is expressed as percentage specific lysis according to the formula: [(experimental counts per minute − spontaneous counts per minute)/(maximum release − spontaneous counts per minute)] × 100%. The peptide-specific percentage specific lysis shown in the figures represents the mean of triplicate measurements from which the nonpeptide-specific lytic activity, defined as percentage-specific lysis of T2 cells pulsed with the control peptide, was subtracted, unless indicated otherwise. The lysis of T2 cells pulsed with the control peptide was lower than 10% in conditions containing PBMCs and lower than 5% in cytolytic assays with peptide-specific T-cell clones. There was no consistent difference in unspecific background lysis regardless of whether the T cells were activated with CpG ODN- or CpG ODN-conditioned supernatants.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance of differences was determined by the paired Wilcoxon signed-rank test. Differences were considered statistically significant for $P < .05$. Statistical analyses were performed using StatView 4.51 software (Abacus Concepts, Calabasas, CA). Asterisks indicate $P < .05$ and $P < .01$ for differences between medium control and stimulation with CpG ODN or between the indicated conditions.

Results

Circulating $CD8^+$ T cells specific for HLA-A2–restricted peptides derived from the melanoma-associated differentiation antigen Melan-A/MART-1 and from the influenza matrix protein have been shown to carry distinct phenotypes in healthy adult donors: influenza-specific $CD8^+$ T cells carry the features of antigen-experienced memory T cells; Melan-A specific $CD8^+$ T cells in melanoma-free healthy persons have all the characteristics of naive cells.[26,27] Based on these results we used the 2 corresponding HLA-A*0201–restricted peptides as model antigens to test the effect of CpG ODN on $CD8^+$ T-cell responses: the influenza matrix protein–derived peptide Flu $matrix_{58-66}$ and the Melan-A protein–derived peptide Melan-A_{26-35} A27L. The Melan-A_{26-35} A27L analog peptide substituted at position 2 (A>L) was used because it has been shown to be more immunogenic than its natural counterpart.[23]

In the human immune system PDCs and B cells express TLR9 and thus are sensitive to CpG ODN, whereas monocytes, myeloid DCs, T cells, and NK cells are indirectly activated through stimulated PDCs and B cells.[14,28] To integrate direct and indirect effects of CpG ODN, we studied the effect of CpG ODN on $CD8^+$ T-cell responses in unseparated PBMCs. The frequency of antigen-specific precursors was increased by adding purified $CD8^+$ T cells from the same donor to the PBMCs tested (final concentration, 30%-40% $CD8^+$ T cells). Before peptide stimulation less than 0.75% of the $CD8^+$ T cells stained positive for the HLA-A2/Flu $matrix_{58-66}$ (0.13% ± 0.04%; n = 20) and less than 0.1% for the HLA-A2/Melan A_{26-35} A27L tetramers (0.04% ± 0.01%; n = 10) in all donors tested.

CpG-A and CpG-B enhance the expansion of influenza peptide–specific memory $CD8^+$ T cells that produce IFN-γ and are cytotoxic

PBMCs from HLA-A2–positive donors were stimulated with the Flu $matrix_{58-66}$ peptide in the presence or absence of CpG-B (ODN 2006) or CpG-A (ODN 1585; ODN 2216). After 10 to 14 days, Flu $matrix_{58-66}$ peptide–specific $CD8^+$ T cells were detected by tetramer staining (Figure 1A). Adding CpG ODN increased the frequency of Flu $matrix_{58-66}$ peptide–specific $CD8^+$ T cells compared with stimulation with peptide alone. The mean frequency of HLA-A2/Flu $matrix_{58-66}$ $tetramer^+$ cells of all $CD8^+$ T cells from 16 donors tested was 12.2% for ODN 2006, 11.8% for ODN 1585, 10.4% for ODN 2216, and 5.8% for the control without CpG ODN (Figure 1B). There was no significant difference between the 3 CpG ODNs used ($P > .5$). However, the effect was CpG-dependent because the GC control ODN to ODN 2006 (ODN 2137) and the GC control ODN to ODN 2216 (ODN 2243) showed no increase in the frequency of $tetramer^+$ cells (Figure 1C).

In addition to tetramer staining, we measured the number of IFN-γ–producing cells on Flu $matrix_{58-66}$ peptide restimulation (Figure 1D-E). Similar to the results obtained by tetramer staining, both types of CpG ODN increased the frequency of Flu $matrix_{58-66}$ peptide–specific IFN-γ–producing cells (from 1.6% IFN-γ^+ cells of all $CD8^+$ T cells without CpG ODN to 4.4% with ODN 2006, 3.1% with ODN 1585, 2.9% with ODN 2216; n = 13). Stimulation with a control peptide (HIV $pol_{476-484}$) showed less than 0.2% IFN-γ–positive $CD8^+$ T cells. The number of peptide-specific $CD8^+$ T cells detected by intracellular IFN-γ staining on peptide restimulation correlated with tetramer staining, but on a lower level. On average, approximately one third of tetramer-positive cells produced IFN-γ in response to peptide restimulation. There was a trend for ODN 2006 to induce higher numbers of peptide-specific IFN-γ–producing cells compared with the other CpG ODNs, but this difference was not statistically significant. ($P = .27$ for differences between ODN 2006 and ODN 1585; $P = .34$ for differences between ODN 2006 and ODN 2216). For some donors we assessed the cytotoxic capacity of $CD8^+$ T cells within PBMCs against the HLA-A2–positive T2 cell line pulsed with the Flu $matrix_{58-66}$ peptide or a control peptide. In agreement with the higher number of peptide-specific $CD8^+$ T cells, the Flu $matrix_{58-66}$ peptide-specific cytotoxicity of PBMCs was enhanced in the presence of CpG-A and CpG-B but not in the presence of a control ODN to 2006 (ODN 2137) (Figure 1F). The percentage specific lysis ± SEM increased from 17.8% ± 3.5% without CpG ODN to 44% ± 8.8% (ODN 2006) and 54% ± 10.1% (ODN 2216) in the presence of CpG-ODN (E/T ratio 27:1; n = 4; $P < .05$ for differences between medium and CpG ODN-stimulated conditions). Together these results demonstrate that both CpG-A and CpG-B increase the frequency of influenza peptide–specific $CD8^+$ T cells and that these T cells are functionally active in terms of IFN-γ production on peptide restimulation and peptide-specific cytolytic activity.

CpG-B but not CpG-A supports priming of naive Melan-A–specific $CD8^+$ T cells toward IFN-γ–producing cytotoxic CTLs

As a model for priming of naive $CD8^+$ T cells, we tested the ability of different CpG ODNs to enhance the induction of Melan A_{26-35}

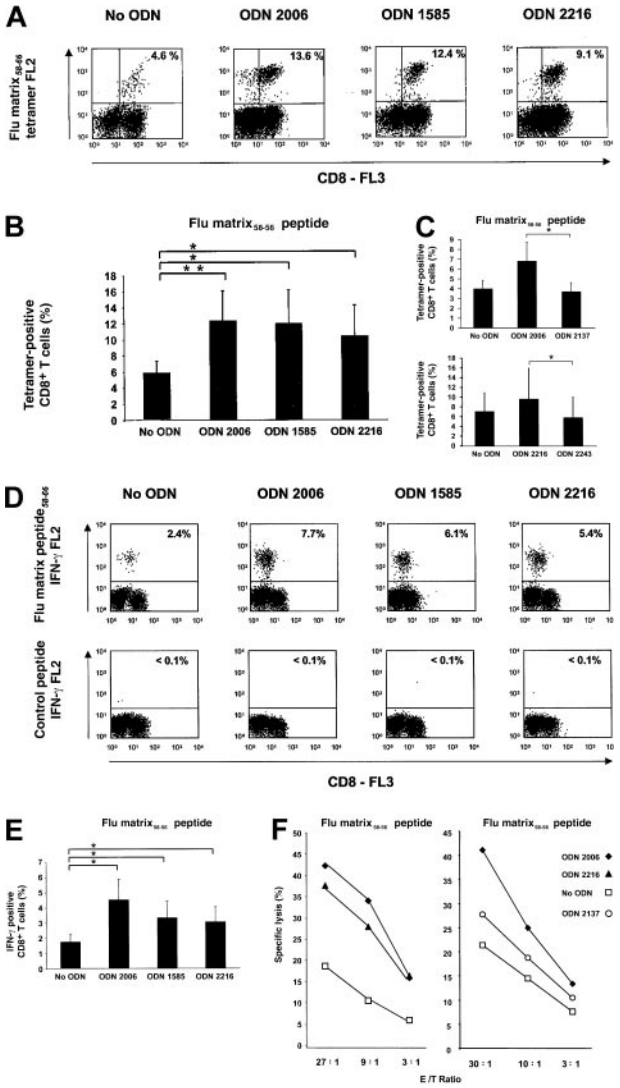

Figure 1. CpG-A and CpG-B enhance the expansion of IFN-γ–producing, cytotoxic influenza peptide–specific CD8⁺ T cells. PBMCs (3×10^6) from HLA-A2–positive donors were enriched for $CD8^+$ T cells and stimulated with the HLA-A0201–restricted Flu $matrix_{58-66}$ peptide in the presence or absence of CpG ODN (6 μg/mL). After 10 to 14 days, cells were harvested and further analyzed. (A-C) After staining with HLA-A2/Flu $matrix_{58-66}$ tetramers-PE, anti-$CD8^+$ PerCP, and Topro-3 (for exclusion of dead cells), the percentage of peptide-specific cells within all $CD8^+$ cells was determined by flow cytometry (upper right quadrant: numbers indicate the percentage of peptide-specific CD8 T cells). Results of one exemplary experiment (A) and the mean ± SEM of 16 donors (B), respectively, are depicted. $^*P < .05$; $^{**}P < .01$. (C) ODN 2137 and ODN 2243 are GC control ODN to ODN 2006 and ODN 2216, respectively. Mean ± SEM of 7 (ODN 2137) and 5 (ODN 2243) donors are shown. $^*P < .05$. (D-E) After restimulation with the Flu $matrix_{58-66}$ peptide or an HLA-A2–restricted control peptide (HIV Pol), the percentage of IFN-γ–producing cells within all $CD8^+$ cells was measured by intracellular cytokine staining and flow cytometry (indicated by the numbers on the dot plots). Results from a representative experiment (D) and the mean ± SEM (E) of 13 donors are shown. $^*P < .05$. (F) Cells were harvested and used as effector cells in a standard ^{51}Cr lysis assay against T2 cells pulsed either with the Flu $matrix_{58-66}$ peptide or a control peptide derived from the HIV pol protein. The results in peptide-specific percentage specific lysis represent the mean of triplicate measurements from which the nonpeptide-specific lytic activity, defined as percentage specific lysis of T2 cells pulsed with the control peptide, was subtracted. Results from 1 of 4 experiments for the comparison of ODN 2006 and ODN 2216 and 1 of 2 experiments for the comparison of ODN 2006 and ODN 2137 are shown. $^*P < .05$.

$A27L$–specific $CD8^+$ T cells in PBMCs of HLA-A2–positive donors. Although the expansion of influenza-specific $CD8^+$ T cells was increased by both types of CpG ODN (see Figure 1B), only CpG-B (ODN 2006) increased the frequency of Melan A_{26-35} $A27L$–specific naive $CD8^+$ T cells (Figure 2A-B), and this effect was CpG dependent (Figure 2C). In contrast, CpG-A (ODN 1585; ODN 2216) was unable to enhance the frequency of Melan A_{26-35} $A27L$–specific $CD8^+$ T cells (from 1.1% HLA-A2/Melan A_{26-35} $A27L$ $tetramer^+$ cells of all $CD8^+$ T cells without ODN to 1.4% with ODN 1585 and 1.1% with ODN 2216; n = 16). Consistent with the number of $tetramer^+$ cells, IFN-γ^+ $CD8^+$ T cells increased from 0.5% without CpG ODN to 1.5% with ODN 2006, 0.5% with ODN 1585, and 0.4% with ODN 2216; n = 12; Figure 2D). Stimulation with a control peptide (HIV $pol_{476-484}$) showed less than 0.2% IFN-γ–positive $CD8^+$ T cells. In agreement with the higher numbers of Melan-A–specific $CD8^+$ T cells with CpG ODN 2006 in PBMCs, cytotoxicity toward peptide-loaded target cells was also increased (Figure 2E). The percentage specific lysis ± SEM increased from 38.7% ± 15.6% without CpG ODN to 66.7% ± 21.2% in the presence of ODN 2006 (E/T ratio 20:1; n = 3; $P = .16$). When we used HLA-A2–restricted peptides derived from the HIV pol or gag proteins as model antigens for a primary immune response in HIV-negative donors, unlike for Melan-A, we could not detect peptide-specific $CD8^+$ T cells within

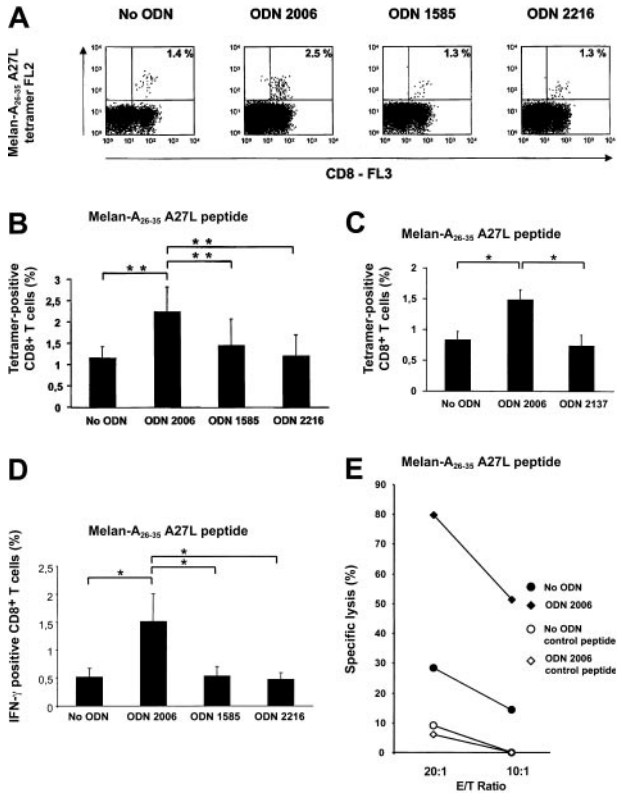

Figure 2. CpG-B but not CpG-A promotes priming of IFN-γ–producing, cytotoxic Melan-A peptide–specific CD8⁺ T-cells. PBMCs (3×10^6) from HLA-A2–positive donors were enriched for $CD8^+$ T cells and stimulated with the HLA-A*0201–restricted Melan A_{26-35} $A27L$ peptide in the presence or absence of CpG ODN (6 μg/mL). After 10 to 14 days, the cells were harvested and further analyzed. (A-C) After staining with HLA-A2/Melan $A_{26-35}$$A27L$ tetramers-PE, anti-CD8 PerCP, and Topro-3 (for exclusion of dead cells), the percentage of peptide-specific cells within all $CD8^+$ cells was determined by flow cytometry. Results of a representative experiment (A; numbers indicate frequency of peptide-specific CD8 T cells in the upper right quadrant), the mean ± SEM of 16 donors (B) and the mean ± SEM of 3 donors (C), respectively, are depicted. $^{**}P < .01$. ODN 2137 is a GC control ODN to ODN 2006. (D) After restimulation with the Melan $A_{26-35}$$A27L$ peptide or an HLA-A2–restricted control peptide derived from HIV Pol, the percentage of IFN-γ–producing cells within all $CD8^+$ cells was measured by intracellular cytokine staining and flow cytometry. Data from 12 donors are presented as mean ± SEM. $^*P < .05$. (E) Cells were harvested and used as effector cells in a standard ^{51}Cr lysis assay against T2 cells pulsed with Melan $A_{26-35}$$A27L$ peptide or a control peptide derived from the HIV pol protein (1 of 3 experiments).

PBMCs (neither by tetramer-staining nor by IFN-γ production; data not in figure). The frequency of naive T cells specific for these HIV antigens seemed to be too low to allow efficient priming and expansion in our in vitro system.

PDCs are required for the CpG ODN-induced enhancement of peptide-specific CD8+ T-cell responses

Next we studied whether PDCs and B cells, the only TLR9-expressing cell subsets in PBMCs, contribute to the expansion of peptide-specific CD8+ T cells in response to CpG ODN. PBMCs were depleted of PDCs and B cells before stimulation with peptide and CpG ODN. In a first set of experiments, PBMCs depleted of PDCs or of B cells were stimulated with Flu matrix$_{58\text{-}66}$ peptide in the presence or absence of CpG-A (Figure 3). In the absence of CpG-A, the depletion of PDCs or B cells did not significantly change the frequency of peptide-specific T cells (frequency of Flu-peptide–specific T cells: PBMCs, 13% ± 7.5%; PBMCs depleted of B cells, 14% ± 4.8%; PBMCs depleted of PDCs, 12% ± 8.4%; $P > .6$ depleted vs undepleted; not in figure). In contrast, depletion of PDCs abrogated the CpG-A–induced increase of Flu matrix$_{58\text{-}66}$–specific T cells. The depletion of B cells had no significant effect on the frequency of Flu matrix$_{58\text{-}66}$–specific T cells (Figure 3, left panel). In contrast, the depletion of B cells enhanced the frequency of Melan-A–specific T cells (Figure 3, right panel). However, the depletion of B cells and PDCs completely abrogated the CpG-mediated effect (Figure 3, right panel). Again, in the absence of CpG, the depletion of PDCs or B cells did not significantly change the frequency of peptide-specific T cells (frequency of Melan-A–specific T cells: PBMCs, 0.18% ± 0.11%; PBMCs depleted of B cells, 0.27% ± 0.21%; PBMCs depleted of B cells and PDCs, 0.15% ± 1.2%; $P > .1$ depleted vs undepleted; not in figure). Together these data indicated that the presence of PDCs in PBMCs is required for the CpG-mediated enhancement of Flu- and Melan-A peptide–specific T cells.

CD8+ T cells generated in the presence of CpG ODN express a phenotype associated with cytotoxicity and terminal differentiation

An effective CD8+ T-cell response depends on the quantity and the quality of the T cells generated. Antigen-specific CD8+ T cells expanded in vitro *or* in vivo may lack effector functions and cytotoxicity.[29,30] To monitor the functional activity of CD8+ T cells in vaccination studies with tumor antigen–derived peptides, the down-regulation of CD28 and CD45RA and the up-regulation of signaling lymphocytic activation molecule (SLAM) have been described to correlate with the differentiation toward effector cells.[31,32] The expression of CD56 is reported to correlate with lytic activity of ex vivo–analyzed CD8+ T cells.[33]

To examine whether CpG ODNs not only increase the frequency of peptide-specific CD8+ T cells but also affect their phenotype, we measured the expression of CD28, CD56, and SLAM on Flu matrix$_{58\text{-}66}$ peptide–specific cells expanded in the absence or presence of different CpG ODNs. Compared with nonantigen-specific CD8+ T cells, Flu matrix$_{58\text{-}66}$ tetramer+ cells showed a lower expression of CD45RA (not in figure) and CD28 and a higher expression of SLAM ($P < .05$ comparing the expression on antigen-specific and antigen-nonspecific T cells for each condition) (Figure 4A-B). Thus, antigen-specific T cells carried a phenotype compatible with that of terminally differentiated effector cells. No difference was found regardless of whether peptide-specific T cells were generated in the presence or absence of CpG ODN, and there was no significant change in the expression of these markers between the different types of CpG ODNs. The situation was different for CD56, which is reported to be associated with cytolytic activity. Although there was no significant difference in CD56 expression between antigen-specific and antigen-nonspecific CD8+ T cells without CpG ODN ($P = .29$), the presence of CpG ODNs during CD8+ T-cell expansion led to increased CD56 expression on peptide-specific CD8+ T cells ($P < .01$ for differences between no ODN and any of the CpG ODNs; Figure 4B). Within the 2 types of CpG ODNs used, CpG-A (CpG ODN 2216 and ODN 1585) was more potent than CpG-B (ODN 2006) to up-regulate CD56 expression ($P < .01$ for differences between ODN 2006 and ODN 2216, and $P = .05$ for differences between ODN 2006 and ODN 1585). Experiments with GC control ODN to ODN 2006 and ODN 2216 demonstrated the CpG specificity of that effect (Figure 4C). Similar changes were seen on Melan-A–specific CD8+ T cells (data not shown).

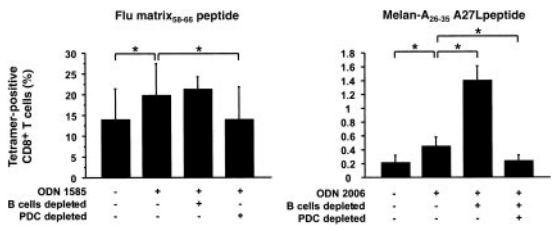

Figure 3. PDC depletion but not B-cell depletion abrogates the CpG ODN-induced enhancement of peptide-specific CD8+ T-cell responses. PBMCs from HLA-A2–positive donors were depleted of PDCs, B cells, or both; enriched for CD8+ T cells; and then stimulated with the HLA-A*0201–restricted Flu matrix$_{58\text{-}66}$ or Melan A$_{26\text{-}35}$ A27L peptide in the presence or absence of ODN 1585 and ODN 2006, respectively. After 12 days the cells were stained with the corresponding tetramers, and the percentage of peptide-specific cells within all CD8+ cells was determined by flow cytometry. Mean ± SEM of 3 individual donors are depicted. *$P < .05$.

CpG-A ODN–induced IFN-α/-β increases the lytic activity and the granzyme-B content of pre-established CD8+ T-cell clones

The increased CD56 expression on Flu matrix$_{58\text{-}66}$–specific CD8+ T cells in response to CpG ODN suggested that CpG not only increases the frequency of peptide-specific CD8+ T cells but also enhances their cytotoxic activity. To test the effect of CpG ODN on the cytotoxic activity of T cells on a per cell basis, we generated Melan A$_{26\text{-}35}$ A27L–specific CD8+ T-cell clones. Established T-cell clones were incubated in the presence of supernatants derived from PBMCs stimulated with ODN 2006, ODN 2216, or ODN 2243 or without ODN. After 18 hours, the cytotoxic activity of T-cell clones against Melan A$_{26\text{-}35}$ A27L–pulsed T2 cells was determined. As seen in Figure 5A-B, supernatant derived from CpG-A (ODN 2216)–, but not from CpG-B (ODN 2006)–, stimulated PBMCs increased the lytic activity of the T-cell clones ($P = .001$). This effect was CpG-specific, as demonstrated by the non-CpG control ODN 2243 (Figure 5B-C). Increased cytotoxic activity of T-cell clones in response to CpG-A–derived supernatant correlated with the higher induction of CD56 expression (compare Figure 4B) by CpG-A compared with CpG-B ODN. Unlike CpG ODN–induced PBMC supernatant, neither CpG-A nor CpG-B was able to directly activate T-cell clones (data not shown). These results indicate that soluble factors induced by CpG-A confer increased cytotoxic activity to peptide-specific CD8+ T cells and that the direct cell-to-cell contact between T cells and APC within PBMCs is not required for this activity.

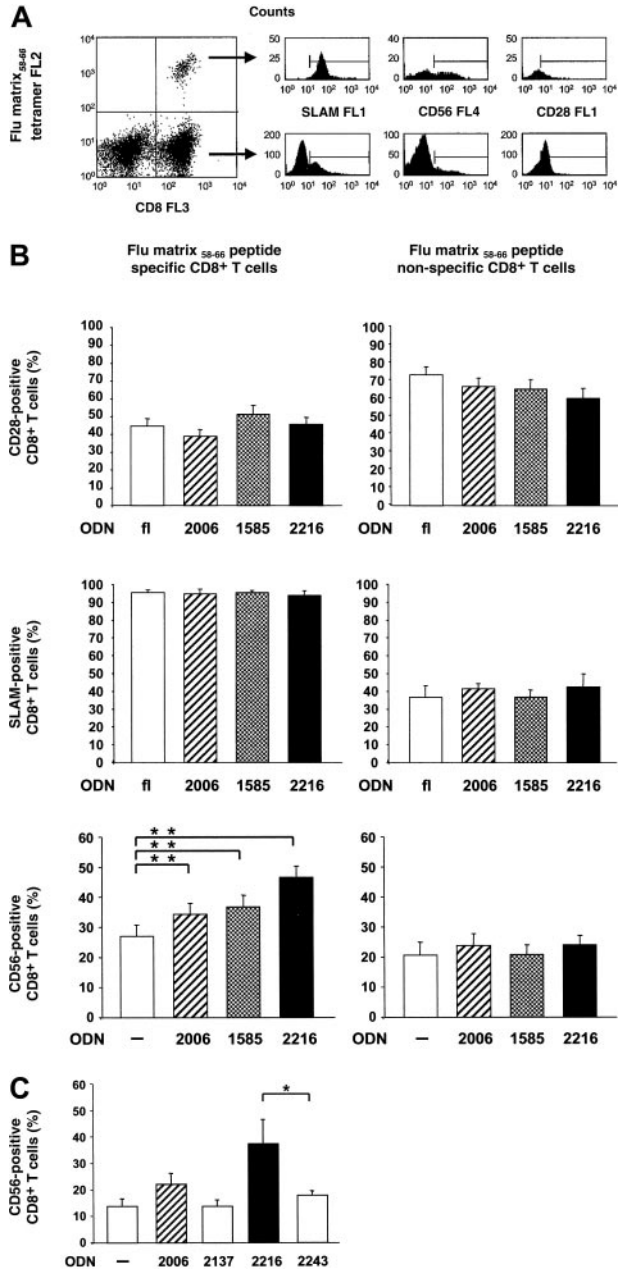

Figure 4. Expression of CD28, SLAM, and CD56 by CD8$^+$ T cells expanded in the presence of CpG PBMCs were stimulated in the presence or absence of CpG ODN (6 μg/mL) with the HLA-A*0201–restricted Flu matrix$_{58\text{-}66}$ peptide. After 10 to 14 days cells were harvested and stained with HLA-A2/Flu matrix$_{58\text{-}66}$ tetramers-PE, anti-SLAM^{FITC}, anti–PerCP, and Topro-3, or with HLA-A2/Flu matrix$_{58\text{-}66}$ tetramers-PE, anti–CD28-FITC, anti–CD8-PerCP, and anti-CD56 APC and were analyzed on a flow cytometer. HLA-A2/Flu matrix$_{58\text{-}66}$ tetramers$^+$ CD8$^+$ cells and HLA-A2/Flu matrix$_{58\text{-}66}$ tetramers$^-$ CD8$^+$ were gated, and the expression of the indicated surface antigen on these subpopulations was determined. Markers were set according to isotype controls, and the percentage of cells positive for the indicated marker is depicted. (A) Results from 1 representative experiment are shown. Upper arrow indicates peptide-specific CD8 T cells in upper right quadrant; lower arrow, peptide-nonspecific CD8 T cells in lower right quadrant. (B) Data from different donors (n = 14 [CD28]; n = 14 [CD56]; n = 6 [SLAM]) are presented as mean ± SEM. $^{**}P < .01$. (C) Mean ± SEM from 3 different donors are shown. ODN 2137 and ODN 2243 are the GC control ODN to ODN 2006 and ODN 2216, respectively. $^*P < .05$.

Cytotoxic T cells kill their target cells mainly by 2 different mechanisms, the Fas/Fas-ligand pathway and through the effects of perforin and granzymes. To further characterize the mechanisms that lead to enhanced T-cell lytic activity, we measured the intracellular granzyme-B content and the expression of Fas-ligand on T-cell clones after incubation in ODN 2216-induced supernatant by flow cytometry. Compared with the isotype control, no expression of Fas-ligand could be detected on the clones even if they were not incubated with CpG-conditioned supernatant. However, increased cytolytic activity of the T-cell clones was associated with an increase in intracellular granzyme-B content (Figure 5D).

Because IFN-α is described to increase the cytotoxicity of activated CD8$^+$ T cells and NK cells,[34,35] we further examined the role of IFN-α/-β in the ODN 2216-induced supernatants for the increased cytotoxicity of peptide-specific clones. Indeed blocking interferon type 1 during the incubation time in supernatants derived from PBMCs stimulated with ODN 2216 reversed the cytotoxicity and increase of granzyme-B content almost to the level of the control without CpG ODN (Figure 5C-D). Blocking antibodies to IL-12 led only to a small reduction in the cytotoxicity (not in figure) and of granzyme-B content (Figure 5 D). Blocking antibodies to IFN-γ had no effect (data not shown).

Discussion

Progress in the immunotherapy of cancer and of infections with intracellular pathogens and viruses depends on vaccine strategies that induce a large number of antigen-specific CD8$^+$ T cells with high cytotoxic activity. Today vaccines that meet these criteria are mainly based on viable pathogens. Activation of the innate immune

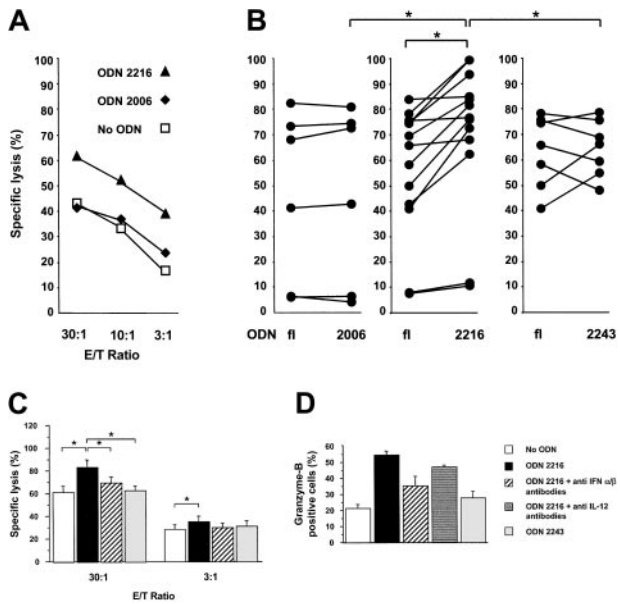

Figure 5. ODN 2216 (CpG-A) increases the granzyme-B content and lytic activity of peptide-specific T-cell clones through IFN-α/-β. HLA-A2–restricted Melan A$_{26\text{-}35}$ A27L peptide-specific T-cell clones were generated as described in "Materials and methods" and were incubated in the presence of supernatants derived from PBMCs stimulated with 6 μg/mL ODN 2006, ODN 2216, ODN 2243, or with medium. After 18 hours, the clones were harvested, washed, and used as effector cells in a standard ^{51}Cr release assay against T2 cells pulsed with their cognate peptide or as a control peptide derived from the HIV pol protein (A-C) or stained for their intracellular granzyme-B content and analyzed by flow cytometry (D). Results in percentage specific lysis represent the mean of triplicate measurements from which the nonspecific lytic activity (percentage specific lysis of T2 cells pulsed with the control peptide) has been subtracted. (A) Results of 1 representative experiment of a Melan A$_{26\text{-}35}$A27L–specific clone at different E/T ratios are shown. (B) Data from different Melan A$_{26\text{-}35}$A27L–specific clones at an E/T ratio of 30:1 are depicted. $^*P < .05$. (C) Data from 4 different clones are depicted as mean ± SEM. $^*P < .05$. (D) Percentages of granzyme-B–positive cells from 2 clones are shown as mean ± SEM. Blocking antibodies were added at the beginning of the 18-hour incubation period in ODN 2216-induced supernatant.

system by conserved microbial products triggers a cascade of events that have profound effects on adaptive immunity.[3] The present study provides evidence that CpG ODNs as molecular mimics of bacterial DNA may serve as vaccine adjuvants for the generation of peptide-specific CTL in humans even in the absence of a live vaccine.

We demonstrate that CpG ODN improves the generation and cytotoxic activity of peptide-specific human $CD8^+$ T cells within PBMCs in vitro. Interestingly, 2 types of CpG ODN, CpG-A and CpG-B, showed distinct functional profiles depending on the type of $CD8^+$ T cells exposed to CpG ODN. Although CpG-B (ODN 2006) enhanced the frequency of Melan-A– and influenza matrix–specific $CD8^+$ T cells, CpG-A (ODN 2216, ODN 1585) only expanded influenza matrix–specific $CD8^+$ T cells. Within PBMCs both types of CpG ODN conferred peptide-specific cytotoxicity and IFN-γ production to T cells; however, only CpG-A induced a $CD8^+$ T-cell phenotype associated with increased lytic activity. Quantitative analysis of cytotoxicity in peptide-specific T-cell clones revealed that CpG-A was more active than CpG-B in supporting cytotoxic activity and granzyme-B content on a per cell basis in $CD8^+$ T cells and that this was mediated mainly by IFN-α/β.

The distinct response of Melan-A– and influenza matrix–specific $CD8^+$ T cells could be attributed to the distinct differentiation stages of T cells. Evidence in the literature shows that Melan-A–specific $CD8^+$ T cells carry a naive phenotype, whereas influenza matrix–specific $CD8^+$ T cells carry a memory phenotype.[36] The concept of differentiation dependence of the effect of CpG-A compared with CpG-B is supported by our finding that CpG-A failed to activate naive Melan-A–specific T cells but induced the cytotoxic activity of established Melan-A–specific T-cell clones even though the same antigenic peptide was used. However, it cannot be excluded that different molecular characteristics of the 2 antigenic peptides also contribute to the observed differences.

Neither CpG-A nor CpG-B activates $CD8^+$ T cells directly. Previous studies identified PDCs and B cells as the only 2 cell types within primary human PBMCs that express TLR9 and are sensitive to CpG ODN.[14,19] The differences between CpG-A and CpG-B with regard to $CD8^+$ T-cell expansion must, therefore, be mediated by PDCs and B cells. Depletion of PDCs revealed that the presence of PDCs within PBMC was necessary to mediate the effects of CpG-A and CpG-B. Although the presence of PDCs was essential, the expansion of $CD8^+$ T cells was higher within PBMCs than with isolated PDCs as antigen-presenting cells (V.H., unpublished results, 2003), suggesting that other cell subsets contribute to $CD8^+$ T-cell expansion. In contrast to PDCs, the depletion of B cells did not decrease the expansion of influenza-reactive T cells, and it even increased the expansion of Melan-A–specific T cells. It is interesting that though dendritic cells can activate naive and memory T cells, B cells have been reported to activate memory T cells but to render naive T cells tolerant.[37-39] In our in vitro model, peptide presentation takes place simultaneously on DCs and B cells, which could explain the enhanced CTL response of naive Melan-A–specific cells, when the B cells were depleted. Similar increases in CTL responses after B-cell depletion have been described in tumor and transplantation models in mice.[40,41]

Besides cell contact–dependent mechanisms between T cells and antigen-presenting cells, CpG-induced cytokines impact on T-cell function. CpG-A is known to stimulate PDCs to release large amounts of IFN-α. IFN-α activates NK cells and induces partial activation of memory $CD8^+$ T cells[14] and proliferation, IFN-γ production and lytic activity of γδ T cells[21] known to carry a memory phenotype.[42] This is consistent with the role of IFN-α to promote preactivated or memory T cells, an effect which is partly mediated via the induction of IL-15 in myeloid cells.[43-48] Interestingly, CpG-A (also called D-type) through IFN-α has been reported to induce monocyte-derived dendritic cells that produce IL-15.[28,49] In addition to memory T-cell expansion, IFN-α has been shown to increase the cytotoxicity of activated $CD8^+$ T cells and NK cells.[34,35] Together, the present study and the previous results suggest that CpG-A recruits innate effector cells (NK cells and γδ T cells), selectively expands memory $CD8^+$ T cells, and confers cytotoxicity through IFN-α.

Unlike the effects of IFN-α on memory T cells, IFN-α is known to have a strong antiproliferative effect on naive T cells and to inhibit the progress from G_1 to the S phase of the cell cycle after T-cell receptor stimulation[50] and to inhibit IL-12 production in human monocytes and myeloid DCs.[51,52] The antiproliferative effect of IFN-α known from the literature is in agreement with our observation that priming of $CD8^+$ T cells carrying a naive phenotype (Melan-A–specific $CD8^+$ T cells) was not supported by CpG-A (inducing high amounts of IFN-α in PDCs). However, priming of $CD8^+$ T cells was mediated by CpG-B, which is weak at inducing IFN-α in PDCs. In previous studies, we found that CpG-B in combination with CD40L is a potent stimulus for the production of IL-12 in PDCs and that it promotes PDC-dependent priming and differentiation of naive $CD4^+$ T cells toward T_H1.[28] Results from the present study now indicate that CpG-B also supports the priming of functionally active antigen-specific $CD8^+$ T cells.

In conclusion, our results suggest that CpG-B may be the superior vaccine adjuvant when the induction of a primary CTL immune response is needed, such as in prophylactic vaccines. On the other hand, CpG-A may be superior to expand preexisting T cells and to regain T-cell responsiveness and cytotoxicity. However, the activity of CpG ODN in vivo, especially in patients with cancer, cannot be predicted based on in vitro data. In vivo, in a recent study Verthelyi et al[5] compared 2 types of CpG ODN (K-type ODN, similar to CpG-B; D-type ODN, similar to CpG-A) as adjuvants for a heat-killed leishmania vaccine in rhesus macaques.[5] In agreement with our results, K-type ODN (CpG-B) induced more antigen-specific IFN-γ–producing T cells than D-type ODN (CpG-A) concerning a primary immune response. Ongoing clinical trials will provide further insight into the activity of CpG ODNs as immune adjuvants for peptide vaccines against cancer.[53]

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