Comparison of different classes of CpG-ODN in augmenting the generation of human epitope peptide-specific CTLs

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Received January 10, 2011; Accepted March 28, 2011

DOI: 10.3892/ijo.2011.1146

Abstract. Three distinct classes of CpG-oligonucleotides (ODN) (CpG-A, CpG-B and CpG-C) have been identified on the basis of differences in their structures and immune effects. To date, only CpG-B is applied for clinical treatments; however, it is still unknown which of the different CpG-ODN classes is most useful as an adjuvant for human cancer vaccine therapy. In the present study, we examined the activity of these 3 types of CpG-ODN in enhancing the induction of human peptide-specific CTLs. Our data showed that the specific cytotoxicity was augmented in the presence of CpG-A, -B and -C but not in the presence of control ODN, and the augmenting effect was most potent with CpG-A. Flow cytometric analysis showed the subpopulation of effector-memory cells in $CD8^+$ cells was most increased with CpG-A. Furthermore, depletion of PDCs from PBMCs before stimulation with peptide and CpG-ODN completely abrogated the augmenting effect of CpG-ODN. These data indicated that the stimulation of PDCs by CpG-ODN augmented the generation of peptide-specific CTLs, and CpG-A was superior to CpG-B and CpG-C in terms of augmenting the generation of human peptide-specific CTLs *in vitro*.

Introduction

The innate immune system is activated via exposure to pathogen-associated molecular patterns (PAMPs) that are expressed by a diverse group of infectious microorganisms. Subsequently, the host mounts an adaptive immune response directed against determinants that are uniquely expressed by the pathogen. The resultant antigen-specific immunity is characterized by the production of high-affinity antibodies and the generation of cytotoxic T cells that provide long-lasting protection (1). The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of what have been termed pattern-recognition receptors (PRRs). The best understood family of PRRs is the toll-like receptors (TLRs), of which 10 are known in humans.

In contrast to viruses and other pathogens, vaccines containing recombinant proteins or synthetic antigenic peptides usually fail to induce significant immune responses unless they are mixed with adjuvant (3,4). Because of their high efficacy, several recently identified TLR ligands are promising vaccine adjuvants. Bacterial unmethylated CpG-rich oligodeoxy-nucleotides (ODN), which bind to TLR-9, are one of the most promising candidates for a cancer vaccine adjuvant and are currently being tested in many human clinical trials (5-8).

In numerous murine models, TLR-9 activation enhances antigen-specific cellular responses to a wide variety of antigens. The mechanism that contributes to the potent adjuvant activity of CpG-ODNs is maturation and differentiation of dendritic cells resulting in the strong induction of CTLs, even in the absence of CD4 T-cell help (9). On the other hand, the cellular patterns of TLR expression vary between different species (2,10). B cells, monocytes and all DC subsets express TLR-9 in mice; however, only plasmacytoid dendritic cells (PDC) and B cells express TLR-9 in humans (11-14). Consequently, the murine immune system produces different actions from human systems when exposed to CpG-ODN. Therefore, it is impossible to extrapolate the experimental results from murine models to humans. Furthermore, little is known about the mechanism by which CpG-ODNs augment acquired cellular immunity in humans, although systemically administered CpG-ODNs have shown substantial evidence of augmenting the activity of anti-tumor immunity in human clinical cancer vaccine trials (5-8).

Three distinct classes of CpG-ODN have been identified on the basis of differences in their structures and immune-stimulating effects (9,15-17). CpG-A induces the production of high levels of IFN-α from PDC with relatively little B-cell stimulation. In contrast, CpG-B induces the production of low levels of IFN-α along with profound B-cell activation. CpG-C has intermediate immune effects with excellent *in vivo* stability and ease of formation. To date, only CpG-B has been applied for clinical treatments; however, the class of CpG-ODN

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Key words: CpG-ODN, peptide vaccine, effector-memory cells

that is most useful as an adjuvant for a human cancer vaccine is still unknown.

In the present study, we examined the immuno-modulatory activity of these 3 types of CpG ODN in terms of the generation of peptide-specific CTLs.

Materials and methods

Cell lines. A24+LCL cells (HLA-A24/24) were a generous gift of Takara Shuzo Co., Ltd. (Otsu, Japan). The A24LCL cells were used for peptide-mediated cytotoxicity assays. These cells were maintained in a tissue culture flask using RPMI and supplemented with antibiotics and 10% heat-inactivated fetal calf serum (Gibco BRL).

Oligodeoxynucleotides. CpG-A was synthesized by Gene Design (Osaka, Japan). CpG-B, CpG-C and GpC-ODN were synthesized by Hokkaido System Science (Sapporo, Japan); CpG-A, 5'-ggTGCAT**CG**ATGCAGGGGgg-3'; CpG-B, 5'-t**cgtc g**ttttgt**cg**ttttgtcgtt-3'; CpG-C, 5'-t**cg**t**cg**aa**cg**ttcgagagatgat-3'; GpC-ODN, the GC control to CpG-ODN, 5'-ggTGCATG CATGCAGGGGgg-3' (lower case letters indicate phosphorothioate linkage; capital letters, phosphodiester linkage 3' of the base; bold, CpG-dinucleotides).

Peptides. Peptide derived from the squamous cell carcinoma-associated differentiation antigen LY6K-177 (RYCNLEGPPI), influenza (flu) virus-derived peptide (RFYIQMCYEL) and HIV-derived peptide (RYLRDQQLL) with the HLA-A24 binding motif were purchased from Takara Bio Inc. (Otsu, Japan). The purity (>90%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. Peptides were dissolved in dimethylsulfoxide (DMSO) at 20 mg/ml and stored at -80˚C.

Cytokine assays. Human PBMCs from healthy volunteers (n=15) were isolated from freshly drawn peripheral blood by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Blood donors were negative for HIV, hepatitis B virus (HBV) and HCV infection.

Freshly isolated PBMC ($1x10^6$ in 500 μl of AIM-V media, Invitrogen) were incubated at 37˚C with 5% CO_2 in 48-well flat-bottom plates with each class of CpG-ODN at different concentrations. Cell supernatants collected after 48 h were stored at -80˚C until assayed. IFN-α in cell supernatants was measured by ELISA according to the manufacturer's instructions (PBL Biomedical Laboratories, Piscataway, USA). All assays were performed in triplicate.

Induction of flu peptide-specific CTLs. For this study, HLA-A24-positive donors were selected. PBMCs ($2x10^6$/ml) isolated from healthy volunteers were stimulated with the flu peptide at a concentration of 10 μg/ml in the presence of CpG-ODNs (20, 5 and 5 μg/ml for CpG-A, -B and -C, respectively) in 24-well culture plates in AIM-V containing 2% heat-inactivated autologous serum (AS). In some experiments, PBMCs were depleted of PDCs using BDCA4-coupled magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol (<0.01% PDCs identified as $BDCA2^+$ and $CD123^+$ after depletion) to investigate the role of PDCs on the adjuvant effect of CpG-ODNs. On day 7, the T cells were further stimulated with the peptide-pulsed adherent cells that were cultured with autologous irradiated PBMCs for 4 h. The cytotoxic activity of CTLs was tested against peptide-pulsed A24-LCL cells on day 14 as indicated.

Induction of LY6K peptide-specific CTLs. Monocyte-derived dendritic cells (DCs) were used as antigen-presenting cells to induce CTLs against peptides presented on HLA. DCs were generated *in vitro* as previously described (18). Briefly, PBMCs isolated from healthy volunteers (HLA-A*2402) were separated by adherence to a plastic tissue culture dish (Becton-Dickinson) so as to enrich them for the monocyte fraction. The monocyte-enriched population was cultured in the presence of 1,000 U/ml GM-CSF (Kirin) and 500 U/ml IL-4 (Ono) in AIM-V containing 2% heat-inactivated AS. After 5 days in the culture, TNF-α (10 ng/ml), IL-6 (1,000 U/ml), IL-1β (10 ng/ml) and PGE2 (1 μg/ml) were added to the culture to mature DCs. After 7 days, DCs were pulsed with 20 μg/ml of the synthesized peptides in the presence of 3 μg/ml β2-microglobulin (Sigma), pulsed on the cytokine-generated DCs for 4 h at 37˚C in AIM-V. These peptide-pulsed DCs were then inactivated by γ irradiation (50 Gy) and used as stimulator cells. 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, $CD8^+$ T cells and unseparated PBMCs were mixed at a 1:2 ratio and used as the responder cells. These cultures were set up in 48-well plates (Corning); each well contained $5x10^4$ stimulator cells and $1.5x10^6$ responder cells in the presence of CpG-ODNs in 0.5 ml of AIM-V/2% AS. In some experiments, PBMCs were depleted of PDCs using BDCA4-coupled magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol. Two days later, these cultures were supplemented with IL-2 to a final concentration of 20 IU/ml. On day 7, the T cells were further re-stimulated with the autologous peptide-pulsed DCs. The peptide-pulsed DCs were prepared in the same manner as described above. CTL activity was tested against peptide-pulsed A24-LCL cells on day 14 as indicated.

Cytotoxicity assay. Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Perkin-Elmer Life Sciences) for 1 h at 37˚C in a CO_2 incubator. Peptide-pulsed targets were prepared by incubating the cells with 20 μg/ml of the peptide for 16 h at 37˚C before labeling. Labeled target cells were rinsed and mixed with effector cells in a final volume of 0.2 ml in round-bottom microtiter plates. The plates were centrifuged (4 min at 800 x g) to increase cell-to-cell contact and placed in a CO_2 incubator at 37˚C. After 4 h of incubation, 0.1 ml of the supernatant was collected from each well and the radioactivity was determined with a γ counter. The percentage of specific cytotoxicity was determined by calculating the percentage of ^{51}Cr release in 4 h using the following formula: [(cpm of the test sample release - cpm of the spontaneous release)/(cpm of the maximum release - cpm of the spontaneous release)] x 100. Spontaneous release was determined by incubating the target cells alone, in the absence of effector cells, and the maximum release was obtained by incubating the targets with 1 N HCl. All measurements were carried out in triplicate and the standard

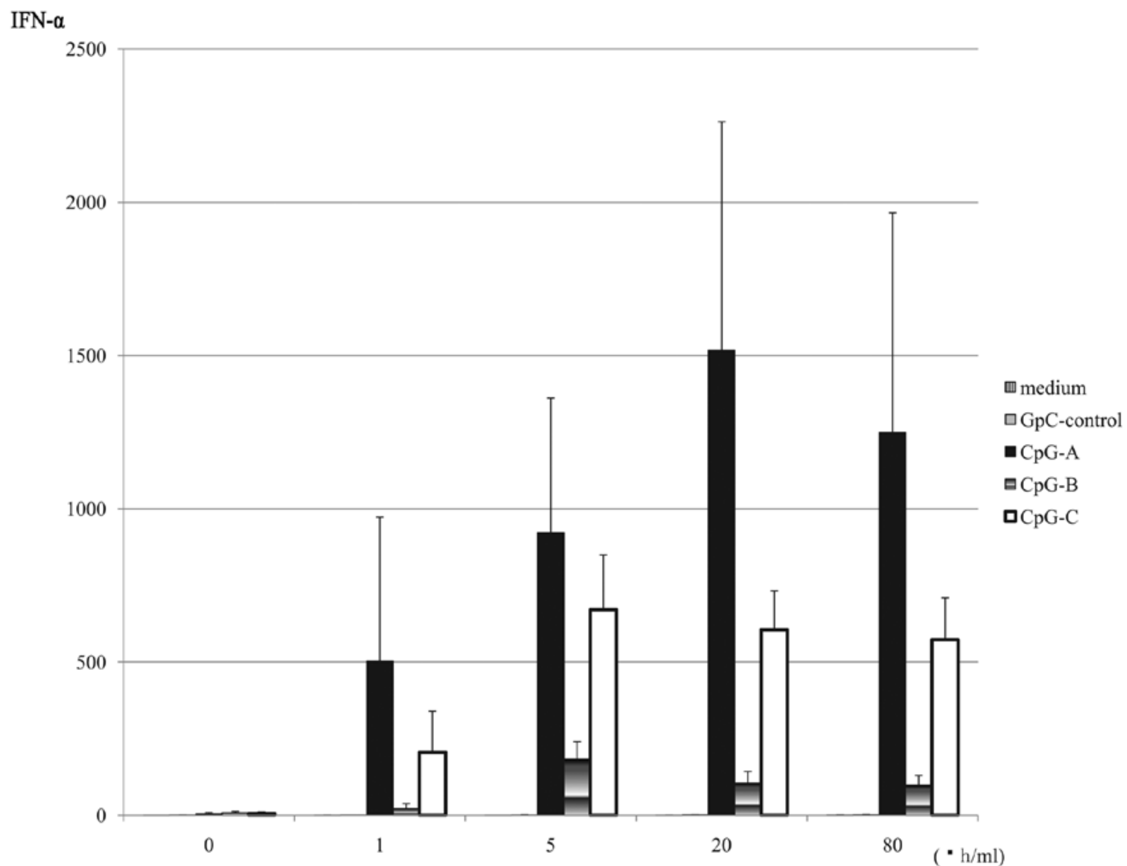

Figure 1. IFN-α secretion from PBMCs stimulated by CpG-ODNs. Levels of IFN-α secreted by PBMC from healthy donors (n=15) after 48-h culture with media, GpC control ODN, A-Class, B-Class or C-Class CpG (all ODN at 0, 1, 5, 20, 80 μg/ml). Bars show mean values and standard error of the means for each group of subjects.

errors of the means were consistently below 10% of the value of the mean.

Flow cytometric immunofluorescence analysis. Monoclonal antibodies against human CD8PerCP, CD45RAFITC and CCR7PE were purchased from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). Cells were incubated with specific antibodies in PBS for 30 min at 4˚C, then analyzed using a FACSCalibur with the Cell Quest software package (Becton-Dickinson).

Results

IFN-α secretion from PBMCs stimulated by CpG-ODNs. Distinct ODN classes were studied for their ability to stimulate human PBMCs to secrete IFN-α. Consistent with previous reports (15-17), IFN-α secretion from PBMCs was greatest with CpG-A, and it was moderate with CpG-C; it was lowest with CpG-B. The dosages of CPG-A, B and C that induced the maximum level of IFN-α were 20, 5 and 5 μg/ml, respectively (Fig. 1).

The effect of CpG-ODNs on the induction of the influenza peptide-specific CTL. The flu peptide-specific CTLs showed clear cytotoxicity against flu peptide-pulsed A24+LCL but not against HIV peptide-pulsed A24+LCL (Fig. 2A and B). The cytotoxicity against flu peptide-pulsed A24+LCL was augmented in the presence of CpG-A, -B and -C but not in the presence of control ODN, and the augmenting effect was greatest with CpG-A; it was moderate with CpG-C and low with CpG-B (Fig. 2A). The depletion of PDCs from PBMCs before stimulation with peptide and CpG-ODNs completely abrogated the augmenting effect of each class of CpG-ODN (Fig. 2C-E). These data indicated that the stimulation of PDCs by CpG-ODNs augmented the expansion and activation of flu peptide-specific CTL to increase the specific cytotoxicity.

Flow cytometric analysis showed the population of $CD8^+$ cells in flu peptide-specific CTLs. Interestingly, the subpopulation of effector-memory cells in $CD8^+$ cells was most increased with CpG-A, and moderately increased with CpG-C (Fig. 3).

CpG-A augmented the LY6K peptide-specific CTL induction in a PDC-dependent manner. The population of LY6K peptide-specific CTL precursor cells in healthy volunteers may be much smaller than that of flu peptide-specific CTLs because LY6K is a cancer-testis antigen (19). Therefore, we investigated whether CpG-ODNs could also affect the induction of LY6K-specific CTLs. Although the induction of influenza-specific CTL was augmented by all types of CpG-ODN (Fig. 2A), the LY6K peptide-specific CTLs were induced only with CpG-A from both donors 1 and 2, but not with CpG-B or CpG-C (Fig. 4A-D). Depletion of PDCs from PBMCs of donor 2 before stimulation with peptide and CpG-A completely abrogated the effect of CpG-A (Fig. 4E).

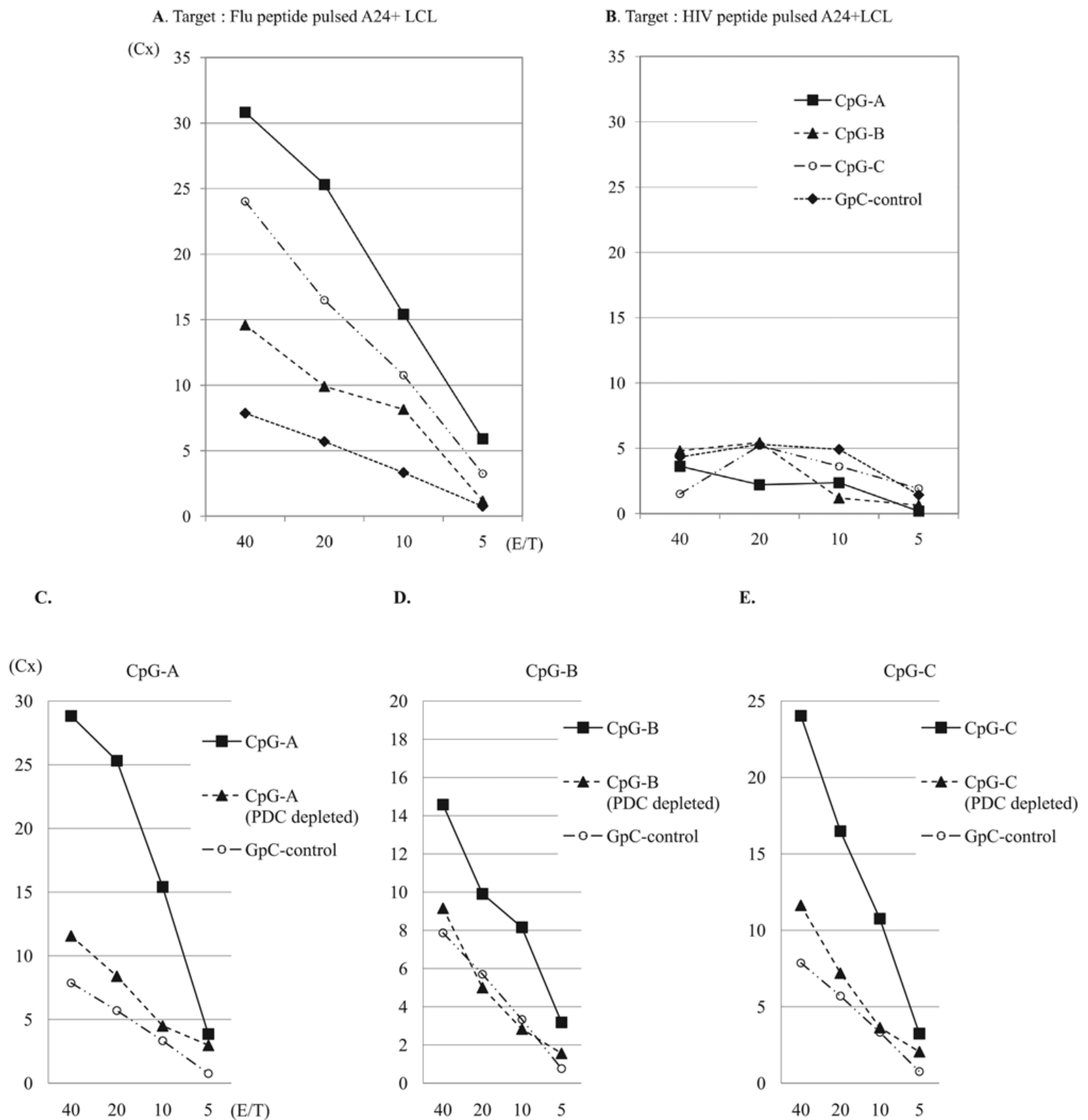

Figure 2. CpG-ODNs augments the influenza peptide-specific CTL induction in a PDC-dependent manner. PBMCs from HLA-A24-positive healthy volunteers were stimulated with the flu peptide in the presence or absence of each class of CpG-ODN (20, 5 and 5 μg/ml for CpG-A, -B and -C, respectively). After 7 days, effector cells were harvested and re-stimulated with the flu peptide pulsed on adherent cells of irradiated PBMCs. After another 7 days, the cytotoxicity of harvested cells against the HLA-A24-positive LCL-A24 cell line pulsed with the flu peptide (A) or a control HIV peptide (B) was assessed by standard ^{51}Cr release assay. Data shown are representative of three independent experiments. PDCs were depleted from PBMCs on day 0. After stimulation with peptide and CpG-ODNs, harvested cells were assessed by standard ^{51}Cr release assay in the same way (C-E).

These data indicated that the stimulation of PDCs by CpG-A augmented the expansion and activation of LY6K peptide-specific CD8$^+$ T cells.

Discussion

Recently, dozens of clinical trials of vaccine therapy for infectious diseases or cancers using CpG-ODNs as an adjuvant have been performed, and some of these trials have shown promising results (5-8). In contrast, there have been few studies that showed the augmenting effects of CpG-ODNs on acquired immunity as a vaccine adjuvant (20). Therefore, the mechanism by which CpG-ODNs augment the efficiency of a vaccine has yet to be clarified in sufficient detail.

In the present study, the efficiency of *in vitro* flu peptide-specific CTL induction by flu peptide was enhanced in the presence of each type of CpG-ODN, and CpG-A showed a more potent adjuvant effect than CpG-B and CpG-C. Moreover, the

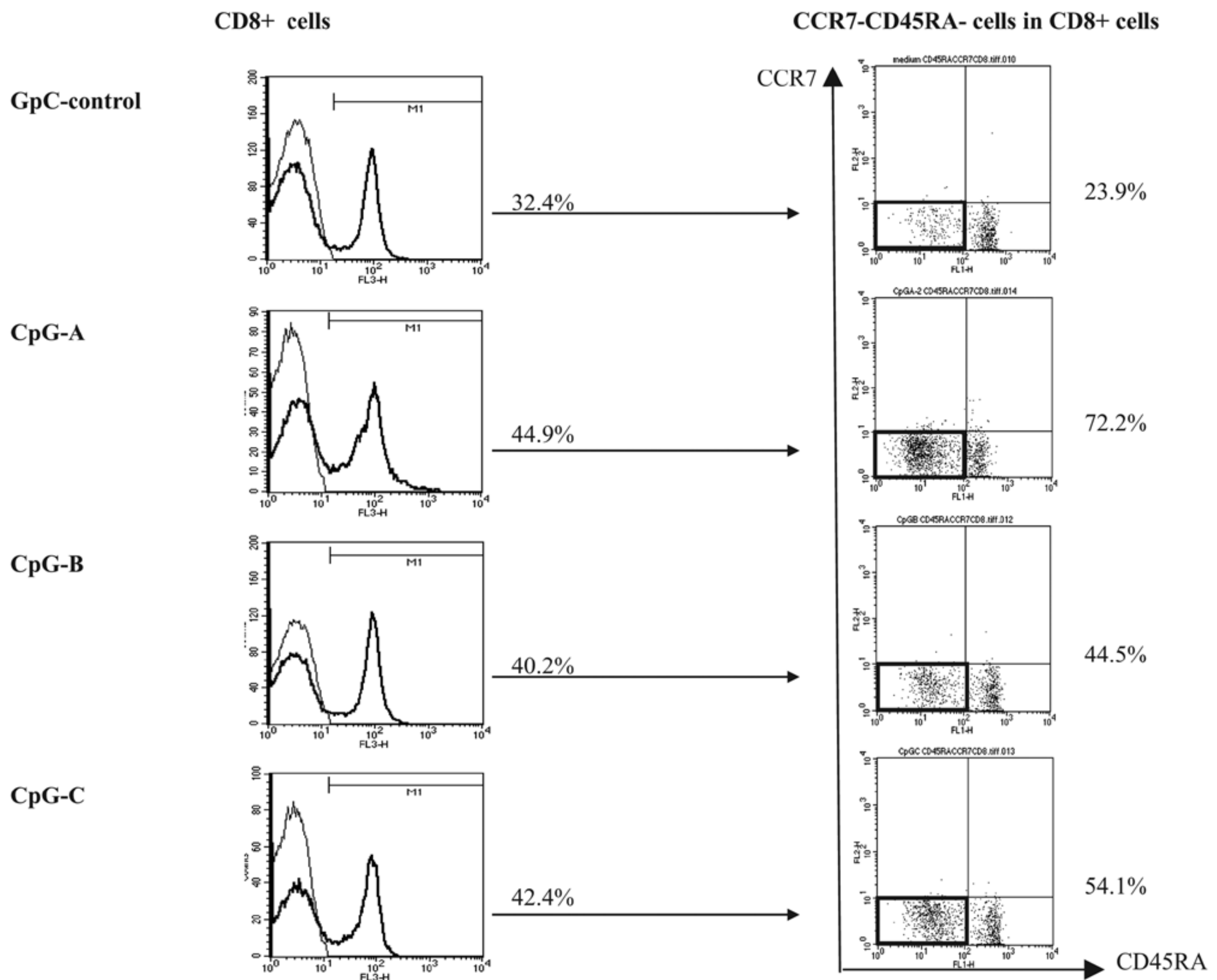

Figure 3. The subpopulation of effector-memory cells in $CD8^+$ cells was increased with CpG-ODN. Populations of $CD8^+$ cells in harvested cells and $CCR7^-$ $CD45RA^-$ effector-memory T cells among $CD8^+$ cells were assessed by flow cytometry.

Ly6K peptide-specific cytotoxicity was induced only with the coexistence of CpG-A, but not with CpG-B and CpG-C. It was suggested that the stimulation of CpG-B or CpG-C was insufficient to elicit Ly6K peptide-specific CTLs because the number of Ly6K peptide-specific precursor CTLs in healthy volunteers is much smaller than that of flu peptide-specific precursor CTLs. These data suggested that CpG-A might be more effective than CpG-B or CpG-C in terms of inducing peptide-specific CTLs *in vitro*.

Our data showed that this CpG-ODN-induced enhancement of cytotoxicity completely disappeared when PDCs were depleted from PBMCs, which means that PDCs were responsible for this enhancement effect. CpG-ODNs mature PDCs by up-regulating the expression of CD80, CD83 and CD86 (21). While most studies have indicated that MoDCs are better antigen-presenters than PDCs (22), many studies have demonstrated the ability of PDCs to function as APCs for both CD4- and CD8-positive cells (22-24). On the other hand, it is well known that CpG-B and CpG-C are more potent to mature PDCs than CpG-A (1,2). Because our data showed that CpG-A was superior to CpG-B and CpG-C in inducing peptide-specific CTLs, the maturation of PDCs by the stimulation of CpG-ODNs could not affect the results in view of our study design. Therefore, we considered that any cytokines produced from PDCs stimulated by CpG-ODNs must contribute to the enhancement of peptide-specific CTL induction. In addition to IFNs, PDCs also produced the pro-inflammatory cytokines TNF-α and IL-6 (data not shown). Type-1 IFN, TNF-α and IL-6 are known to drive the differentiation of DCs into mature antigen-presentation cells. Our data also showed that the supernatant of PBMCs stimulated by CpG-ODNs up-regulated the expression of CD80, 83 and 86 on monocyte-derived DCs, and the expression level was highest with CpG-A, less with CpG-C, and least with CpG-B (data not shown). However, the production levels of TNF-α and IL-6 from PBMCs stimulated by CpG-A were almost the same as those for CpG-B and CpG-C. Murine PDCs are known to produce IL-12, which induces Th1 differentiation by the stimulation of CpG-ODNs, but human PDCs do not induce IL-12 (25,26). In contrast, CpG-A produced a much higher level of IFN-α than CpG-B or CpG-C, and we considered that the reason why CpG-A has the most potent augmenting effect to induce peptide-specific CTLs is that CpG-A induces the highest level of type-1 IFN from PDCs.

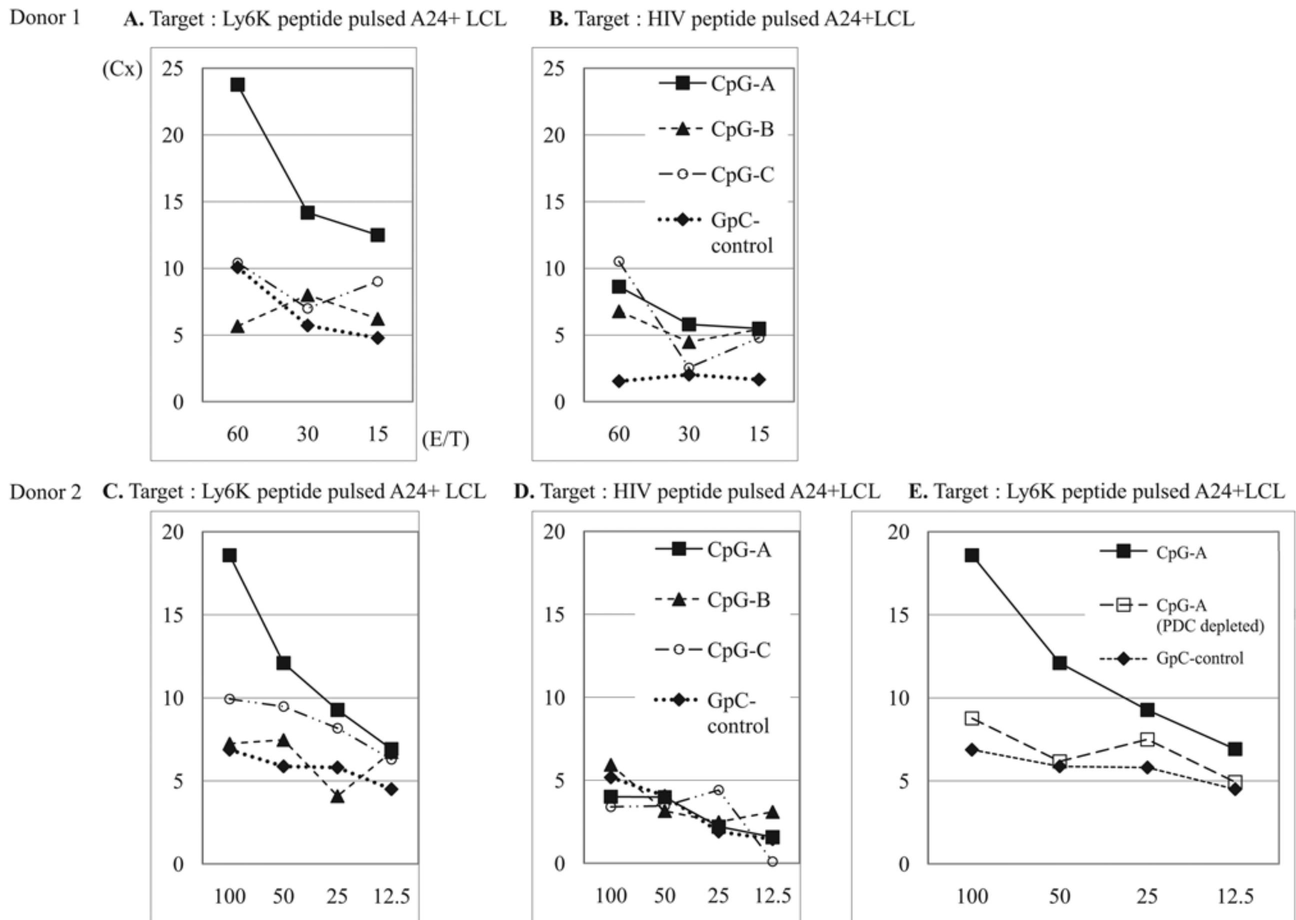

Figure 4. CpG-A augments the Ly6K peptide-specific CTL induction in a PDC-dependent manner. LY6K peptide-specific CTL was generated from 2 different donors in the presence or absence of each class of CpG-ODN (20, 5 and 5 μg/ml for CpG-A, -B and -C, respectively) as described in Materials and methods. The cytotoxicity of harvested cells against the HLA-A24-positive LCL-A24 cell line pulsed with the LY6K peptide (A and C) or a control HIV peptide (B and D) was assessed by standard ^{51}Cr release assay. PDCs were depleted from PBMCs on day 0. After stimulation with peptide and CpG-A, harvested cells were assessed by standard ^{51}Cr release assay in the same way (E).

Type-1 IFNs are known to activate NK cells (27,28) and induce activation of DCs (29-32). In murine models, it is known that type-1 IFNs promote Th1 cytokine production, effector differentiation, proliferation and contribute to the clonal expansion and formation of memory $CD8^+$ T cells (33-40).

Our data revealed that the population of $CD8^+$ cells and that of effector-memory cells in $CD8^+$ cells after induction of flu peptide-specific CTLs were increased the most with CpG-A, less with CpG-C and least with CpG-B. Memory cells persist for extended periods owing to antigen-independent homeostatic turnover and they respond rapidly upon re-encountering a pathogen (41). Two subsets of memory T cells were described on the basis of their anatomical location, expression of cell surface markers and effector functions (42). Memory T cells that express molecules such as CCR7, which allow efficient homing to lymph nodes (LN), are termed central memory cells (T_{CM}), whereas memory T cells that lack expression of these LN homing receptors and are located in no lymphoid tissues are termed effector memory cells (T_{EM}). Some studies have also shown that T_{EM} acquire effector functions, such as cytokine production and killing, more rapidly than T_{CM} (42-44). The mechanisms that contribute to the generation of memory cells are poorly understood. Previous studies suggested that infectious antigen-experienced $CD8^+$ T cells undergo programmed expansion for about 1 week after infection and then undergo programmed cell death (45-47). A majority of the daughter cells derived from antigen-experienced $CD8^+$ T cells undergo death in parallel with proliferation during the acute phase of viral infection and direct type-I IFN action rescues them from this death, thereby tilting the balance effectively toward clonal expansion (40). Therefore, the type-I IFN-mediated rescue from death during antigen-driven proliferation might be critical for the expansion of memory precursors.

In conclusion, our data showed that the stimulation of PDCs by CpG-ODN augmented the generation of effector-memory peptide-specific CTLs. Furthermore, CpG-A might be superior to CpG-B and CpG-C in augmenting the generation of human peptide-specific CTLs *in vitro*. Therefore, CpG-A could become a superior vaccine adjuvant rather than CpG-B or CpG-C in clinical application.

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