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CpG-A Oligonucleotides Induce a Monocyte-Derived Dendritic Cell-Like Phenotype That Preferentially Activates CD8 T Cells1

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Human B cells and plasmacytoid dendritic cells recognize CpG motifs within microbial DNA via Toll-like receptor 9. Two functionally distinct types of CpG motif containing oligonucleotides (CpG ODN) have been described, CpG-A and CpG-B. In contrast to CpG-B, CpG-A induces high amounts of type I interferon (IFN-α and IFN-β) in plasmacytoid dendritic cells. In the present study, we examined the effects of CpG-A on human primary monocytes. In PBMC stimulated with CpG-A and GM-CSF, monocytes showed excellent survival, increased in size and granularity, and within 3 days developed a dendritic cell-like phenotype that was characterized by down-regulation of CD14, partial up-regulation of CCR7, and an increased surface expression of costimulatory and Ag-presenting molecules. This effect could be inhibited by a combination of blocking Abs to type I IFN, and no such CpG-A-induced changes were observed in purified monocytes. Although IL-12 production by this dendritic cell-like phenotype required additional stimulation with CD40 ligand, this cell type spontaneously up-regulated IL-15 expression. Consistent with the known effect of IL-15 on effector and memory CD8 T cells, the frequency of CCR7−/CD45RA− CD8 T cells was selectively increased in allogeneic T cell assays. Furthermore, this dendritic cell type was more potent to support both the generation and the IFN-γ production of autologous influenza matrix peptide-specific memory CD8 T cells as compared with dendritic cells generated in the presence of GM-CSF and IL-4. In conclusion, monocytes exposed to the cytokine milieu provided by CpG-A rapidly develop a dendritic cell-like phenotype that is well equipped to support CD8 T cell responses. The Journal of Immunology, 2003, 170: 3468–3477.

Unmethylated CG dinucleotides within particular sequence contexts (CpG motifs) are responsible for the immunostimulatory activity of bacterial DNA (1). CG dinucleotides are underrepresented and selectively methylated in vertebrate DNA, while in microbial DNA, CG dinucleotides are present at the expected frequency and are usually unmethylated (2). Synthetic CpG oligonucleotides that contain CpG motifs mimic microbial DNA and elicit a coordinated set of immune responses, including innate and acquired immunity (3).

The CpG motif recognized by the murine and human immune system differs (4, 5). Based on the activation of primary B cells, a CpG motif was identified with potent activity in the human immune system (4). Nuclease-stable phosphorothioate ODN4 were developed that proved to be excellent vaccine adjuvants in primates (CpG-B; prototype ODN 2006 (6–8)). Besides B cells, the plasmacytoid dendritic cell (PDC) represents a primary target cell of CpG ODN in humans (9–14). The PDC is characterized by the production of extremely large amounts of type I IFN upon viral infection (15, 16). It has been reported that CpG-B ODN promote survival, activation, and maturation of PDC, but that the induction of type I IFN production was weak as compared with viruses (10–13). Based on the ability to stimulate type I IFN in PDC, a novel type of CpG ODN was identified (CpG-A; prototype ODN 2216) that induces maximal amounts of IFN-α and IFN-β in PDC (400 ng/ml IFN-α in the supernatant; 5 pg/single PDC (13)) and in this regard seems to mimic viral infection.

Toll-like receptor 9 (TLR9) is critically involved in the recognition of CpG motifs (5, 17, 18). Although considerable levels of TLR9 were found in B cells and PDC, TLR9 is weak or absent in monocytes and peripheral blood myeloid dendritic cells (DC) (12, 14). Consistent with TLR9 expression, monocyte-derived DC and myeloid DC are not sensitive to CpG ODN (5, 10, 12). Although TLR9 expression is weak in monocytes (14), monocytes have been described to respond to bacterial DNA and CpG ODN (11, 19, 20). Sensitivity of monocytes toward CpG motifs may change while monocytes enter distinct differentiation pathways depending on growth factors and cytokines to which they are exposed. There are several examples of different differentiation pathways that monocytes are capable to enter. In the presence of receptor activator of

4 Abbreviations used in this paper: ODN, oligodeoxynucleotides; CD40L, CD40 ligand; CpG-CM, CpG-conditioned medium; DC, dendritic cell; FSC, forward light scatter; MFI, mean fluorescence intensity; PDC, plasmacytoid DC; SSC, side light scatter; TLR, Toll-like receptor.
NF-κB ligand and M-CSF, multinucleated osteoclasts develop (21). Although GM-CSF alone leads to macrophage development, GM-CSF together with IFN-γ drives the formation of multinucleated Langhans’ giant cells (22), DC develop in the presence of GM-CSF and IL-4 (23) or during transendothelial migration (24).

Type I IFNs are an important link between innate and acquired immunity, and mediate a variety of immunoregulatory effects (25). Type I IFN have been demonstrated to modulate the development and the phenotype of monocyte-derived DC (26–30). Furthermore, type I IFN strongly activate NK cells, NK cells (31), and γδ T cells (32), and preferentially promote the expansion of memory CD8 T cells in vivo (33), an effect that is mimicked by IL-15 in vivo and in vitro (34). In addition, type I IFN directly prevent apoptosis of Ag-activated T cells in vivo (35). Direct type I IFN-mediated inhibition of Ag receptor-mediated apoptosis also occurs in B cells. In vivo, type I IFN lead to enhanced humoral immunity and to isotype switching of B cells (36).

Teleologically, it would make sense that the set of immune responses induced by a certain pathogen includes the generation of a DC phenotype that specifically promotes the type of T cell function appropriate to overcome the corresponding type of infection. In the present study, we examined the impact of a IFN-α-inducing CpG ODN (CpG-A, ODN 2216) on monocyte differentiation and DC development. We found that upon stimulation with this type of CpG ODN in the context of PBMC, monocytes acquired a DC phenotype that was characterized by spontaneous expression of IL-15, but not IL-12, and that preferentially supported CD8 T cells.

**Materials and Methods**

**Media and reagents**

RPMI 1640 culture medium (Biochrom, Berlin, Germany) supplemented with 5% (v/v) pooled human AB serum (BioWhittaker, Walkersville, MD), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, Munich, Germany) was used throughout (culture medium). All compounds were purchased endotoxin tested. Completely and partially phosphorothioate-modified ODN were provided by Coley Pharmaceuticals (Streatham Biotech; 10 ng/ml), IL-6 (R&D Systems, Hamburg, Germany; 50 U/ml), and 10 ng/ml streptomycin (Sigma-Aldrich). IFN-α neutralizing Abs were added at the beginning of the culture. Purified monocytes were cultured with 1×10⁶ cells/ml and incubated with GM-CSF, GM-CSF/IFN-α, or GM-CSF/IL-4. For some experiments, monocytes were resuspended in CpG-CM at 1×10⁶ cells/ml and cultured with GM-CSF. Immature DC were generated, as described, by culturing isolated monocytes (3×10⁶ cells/ml) with GM-CSF and IL-4 for 3–5 days; for maturation, maturation stimuli together with fresh medium containing GM-CSF and IL-4 were added for 48 h. Alternatively, two-thirds of the medium were replaced by CpG-CM, and cells were cultured with fresh GM-CSF and IL-4 for 48 h. At the indicated time points, supernatants were collected for detection of cytokines, and cells were harvested by vigorous pipetting with ice-cold PBS (no remaining cells on the cell culture plate) for flow cytometric analysis.

**Flow cytometry**

Surface Ag staining was performed, as previously described (37). Fluorescein-labeled Abs against CD8 (BD Pharmingen), CD11c, CD4, CD8, CD45RA, and appropriate isotype control Abs were purchased from BD Pharmingen (San Diego, CA). Anti-CD14 and anti-CD19 mAbs were provided from Miltenyi Biotec (Auburn, CA). CD40L transgenic BHK cells used in a ratio of 1:10; kindly provided by P. Guillemot (Ludwig Institute for Cancer Research, Lausanne branch, Epalinges, Switzerland) were synthesized, as previously described (38). Detection of intracellular cytokines, T cells were first labeled with FITC-labeled anti-CD8 and PerCP-labeled anti-CD3 mAbs, then fixed, permeabilized (Fix and Perm Kit; Caltag, Burlingame, CA), and stained with PE-labeled anti-IFN-γ or isotype control Abs (BD Pharmingen). Flow cytometric data were acquired on a BD Biosciences (Heidelberg, Germany) FACSCalibur equipped with two lasers (excitation at 488- and 635-nm wavelength). Analysis was performed on viable cells within a morphologic gate (forward light scatter (FSC), side light scatter (SSC), >97% of viable cells, as confirmed by propidium iodide staining). For analysis of monocyte-derived cells within PBMC, a gate was set on large granular cells (high FSC and SSC) that expressed the myeloid maker CD11c. For the measurement of cell yield and survival of monocyte-derived cells within PBMC, 105 μl of the resuspended PBMC was harvested and stained with FITC-labeled anti-CD11c mAb and TO-PRO-3 iodide (Molecular Probes, Eugene, OR) for dead cell exclusion. After staining, cells were resuspended in 200 μl, and the number of viable cells per 105 input concentrations of four different plasmids at log dilutions to the PCR analysis was performed on viable cells within a morphologic gate (forward light scatter (FSC), side light scatter (SSC), >97% of viable cells, as confirmed by propidium iodide staining). For analysis of monocyte-derived cells within PBMC, a gate was set on large granular cells (high FSC and SSC) that expressed the myeloid marker CD11c. For the measurement of cell yield and survival of monocyte-derived cells within PBMC, 105 μl of the resuspended PBMC was harvested and stained with FITC-labeled anti-CD11c mAb and TO-PRO-3 iodide (Molecular Probes, Eugene, OR) for dead cell exclusion. After staining, cells were resuspended in 200 μl, and the number of viable cells per 105

**Preparation and culture of cells**

Human PBMC were isolated from blood or buffy coats of healthy volunteers, as described. For preparation of highly purified untouched monocytes free of PDC, PBMC were first depleted of PDC by direct magnetic labeling with anti-blood DC Ag (BDCA)-4-coupled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The remaining cells were then depleted of T cells, NK cells, B cells, and basophils using the monocye isolation kit from Miltenyi Biotec, as described by the manufacturer. Untouched T cells (CD4⁺ and CD8⁺) were isolated from PBMC by depleting other cell types (pan T cell isolation kit; Miltenyi Biotec). CD8⁺ T cells were isolated using a positive selection with anti-CD8 microbeads (Miltenyi Biotec). After preparation of the different cell populations, viability was determined by trypan blue exclusion (96–99% viable).

PBMC were suspended in supplemented culture medium at 2×10⁶ cells/ml and incubated with GM-CSF alone or together with CpG ODN, IFN-γ, or IL-4 for 3–6 days. For neutralizing Abs were added at the beginning of the culture. Purified monocytes were cultured with 1×10⁶ cells/ml and incubated with GM-CSF, GM-CSF/IFN-α, or GM-CSF/IL-4. For some experiments, monocytes were resuspended in CpG-CM at 1×10⁶ cells/ml and cultured with GM-CSF. Immature DC were generated, as described, by culturing isolated monocytes (3×10⁶ cells/ml) with GM-CSF and IL-4 for 3–5 days; for maturation, maturation stimuli together with fresh medium containing GM-CSF and IL-4 were added for 48 h. Alternatively, two-thirds of the medium were replaced by CpG-CM, and cells were cultured with fresh GM-CSF and IL-4 for 48 h.

**Real time RT-PCR**

The protocol used for real time RT PCR was described in detail earlier (14). Briefly, cells were lysed, and RNA was extracted using the total RNA isolation kit (High Pure; RAS, Mannheim, Germany). An aliquot of 8.2 μl RNA was reverse transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT) as primer (First Strand cDNA Synthesis Kit; Roche, Mannheim, Germany). The obtained cDNA was diluted 1/25 with water, and 10 μl was used for amplification. An IL-15-specific primer set was optimized for the LightCycler (RAS) was developed by Search-LC GmbH (Heidelberg, Germany) and purchased from Search-LC. The PCR was performed with the LightCycler FastStart DNA Sybr GreenI kit (RAS), according to the protocol provided in the parameter-specific kits. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR cycle number (CP) at which the detected fluorescence intensity reaches a
fixed value. Using over 300 data points, the actual copy number per microgram of cDNA was calculated as follows: $X = a^{c-0.0535cF + 20.62}$. The data of two independent analyses for each sample and parameter were averaged. The copy number was normalized by the housekeeping gene cyclophilin B, and presented as number of transcripts per 10^3 copies of cyclophilin B.

T cell assays

To examine the effect of different monocyte-derived cell types on CD4 and CD8 T cell subsets, purified monocytes were incubated with GM-CSF alone or in combination with CpG-CM, IFN-α2a, or IL-4. After 3 days, cells were harvested, washed, counted, and used as stimulator cells in an allogeneic MLR. Pan T cells from a different donor were isolated. For measurement of T cell proliferation, T cells were stained with CFSE (Mo-specific IFN-$\gamma$ production, cells were harvested after 10 days, washed once, and restimulated in 96-well-bottom culture plates in 200 μl medium with 10 μM of their cognate peptide or the HIV-pol 476–58 matrix. For the expansion of in vitro-generated T cells, monocytes of HLA-A2-positive donors were incubated for 3 days with GM-CSF and IL-4 or GM-CSF and ODN 2216-conditioned medium. After 3 days, cells were harvested and washed, and 1 × 10^6 monocyte-derived cells were pulsed with 0.5 μM of the immunodominant HLA-A2-restricted peptide of the influenza matrix protein (GILGFVFTL referred to as Flu matrix<sub>54–65</sub>) and used to stimulate 1 × 10^5 autologous CD8+ T cells. After 10 days, the expansion of Ag-specific CD8+ T cells was analyzed by flow cytometry using PE-coupled HLA-A2/Flu matrix<sub>54–65</sub> tetramers. For the assessment of Ag-specific IFN-$\gamma$ production, cells were harvested after 10 days, washed once, and restimulated in 96-well-bottom culture plates in 200 μl medium with 10 μM of their cognate peptide or the HIV-pol<sub>476–58</sub> matrix peptide as a control. After 2-h incubation at 37°C, 1 μg/ml brefeldin A (Sigma-Aldrich) was added. After an additional 4-h incubation at 37°C, cells were harvested, and intracellular cytokine staining was performed.

Statistical analysis

Data are expressed as means ± SEM, unless otherwise indicated. Statistical significance of differences was determined by the paired two-tailed Student t test (p < 0.05). Statistical analyses were performed using StatView 4.5i software (Abacus Concepts, Calabasas, CA).

Results

CpG ODN induce a monocyte-derived DC-like phenotype

Our previous studies indicated that within PBMC, human monocytes are activated by bacterial DNA or CpG ODN to up-regulate ICAM-1 (CD54) and to produce inflammatory cytokines (40). We hypothesized that different types of CpG ODN may differ in their ability to impact on monocyte activation and differentiation. We screened a series of CpG ODN sequences for their activity to stimulate the expression of costimulatory molecules on monocytes. Among the CpG ODN tested, the recently described CpG-A sequence ODN 2216 was most active to up-regulate CD80 expression on monocytes within PBMC (data not shown). ODN 2216 not only activated monocytes, but also partially down-regulated CD14. Gating on monocytes (≥ 95% of these cells were CD14<sup>-</sup>), and their phenotype was analyzed by flow cytometry. As shown in Fig. 1A, ODN 2216 (CpG-A) strongly induced the expression of CD80, CD40, and CD54 in monocytes as compared with GM-CSF alone (mean fluorescence intensity [MFI] values ± SEM for CD80, 79 ± 18 vs 32 ± 12; for CD40, 133 ± 20 vs 58 ± 15; for CD54, 491 ± 98 vs 249 ± 50; n = 6, p < 0.05). Up-regulation of CD80 and HLA-DR by CpG ODN was less pronounced (CD80, 101 ± 22 vs 74 ± 12; HLA-DR, 131 ± 29 vs 89 ± 23; n = 6). Activation of monocytes in the presence of GM-CSF and IFN-α was similar as compared with stimulation with GM-CSF and ODN 2216. In contrast, monocytes cultured with GM-CSF and IL-4 exclusively up-regulated CD86 (148 ± 32 vs 74 ± 12; n = 6, p < 0.03). Consequently, the expression ratio of CD80 to CD86 on monocytes was higher in PBMC cultured with GM-CSF/CpG-A or GM-CSF/IFN-α than with GM-CSF/IL-4. The DC maturation marker CD83 was not significantly up-regulated on monocytes with any of the stimuli used. The majority of the increase of HLA-DR, CD80, and CD68 by CpG-A was found within the first 3 days of culture (Fig. 1B). CD14 was markedly down-regulated in all conditions by day 3. Most monocytes completely lost CD14 expression after culture of PBMC with GM-CSF and IL-4, whereas monocytes cultured with GM-CSF, GM-CSF/CpG-A, or GM-CSF/IFN-α still expressed low levels of CD14 (Fig. 1B, upper left panel).

Next, survival and yield of monocyte-derived cells generated within PBMC were examined. PBMC were incubated with different stimuli for 5 days, and the absolute number of viable monocyte-derived cells (CD11c<sup>+</sup>, FSC/SSC high, TO-PRO-3<sup>−</sup>) was counted by flow cytometry. In the presence of GM-CSF and ODN 2216, survival was much higher as compared with GM-CSF alone (Fig. 2, A and B). Increased survival was also seen for the combination of GM-CSF and rIFN-α, and for ODN 2216 alone. In contrast, the addition of IL-4 to GM-CSF did not enhance survival of monocyte-derived cells as compared with GM-CSF alone. The non-CG control ODN 2243 (CGs switched to GCs) was inactive. Furthermore, the CpG-B ODN 2006 did not significantly increase survival of monocyte-derived cells.

CpG ODN-induced monocyte-derived cells depend on type I IFN and express IL-15

To generate pure monocyte-derived cell populations, monocytes were isolated from PBMC by depleting other cell types, including plasmacytoid DC (>97% monocytes; <0.03% PDC). Monocytes purified by this method were not sensitive to stimulation with CpG ODN 2216 (no induction of CD80, CD86, HLA-DR, CD40, CD54), indicating that there is no direct effect of CpG ODN on monocytes, which is consistent with the lack of TLR9 expression in monocytes (14) (Fig. 3A, hatched bars). When PDC depletion was omitted from the purification protocol (~1% remaining PDC in monocyte preparation), partial activation of monocytes by CpG ODN alone was observed, indicating an indirect effect mediated by PDC-derived cytokines (not shown). When purified monocytes (no contaminating PDC) were incubated in the presence of supernatant derived from PBMC stimulated with ODN 2216 (CpG-CM) with or without GM-CSF, monocytes demonstrated marked up-regulation of CD80, CD86, HLA-DR, CD40, and CD54 (Fig. 3A, filled bars; Fig. 3B, left panel) similar to monocyte-derived cells generated with GM-CSF and ODN 2216 within PBMC (compare Fig. 1A). This effect was CpG specific because supernatants of PBMC stimulated with the non-CG control ODN 2243 (CGs switched to GCs) in combination with GM-CSF were negative (Fig. 3B). Furthermore, supernatants derived from PBMC stimulated with CpG-B ODN 2006 and GM-CSF showed no such effect. In addition to costimulatory molecules and MHC II, MHC I (HLA-A/B/C) also was up-regulated by supernatants generated in the presence of ODN 2216 (CpG-A), but not CpG-B ODN 2006 nor non-CpG-containing ODN (Fig. 3B, right panel).

Type I IFN has been described to affect the development of monocyte-derived DC (26–30). A combination of neutralizing
Abs to IFN-α, IFN-β, and IFN-α/β receptor to GM-CSF/CpG-A-stimulated PBMC abrogated CpG-A-induced expression of CD80 on monocytes (Fig. 4). Neutralization of IFN-β alone also markedly reduced the CpG-A-mediated activation of monocytes, whereas anti-TNF-α Ab showed no significant changes (Fig. 4).

It has been proposed that activation of memory CD8 T cells by type I IFN in vivo is mediated by the induction of IL-15 in myeloid cells (34). Because activation of CpG-induced monocyte-derived DC was type I IFN dependent, we hypothesized that CpG ODN would also up-regulate IL-15. Indeed, quantitative real time PCR revealed that purified monocytes exposed to the supernatant of ODN 2216-stimulated PBMC rapidly up-regulate the expression of IL-15 mRNA within 3 h (up to the copy number of the housekeeping gene cyclophilin B (Fig. 5)). Although IL-15 mRNA decreased over time, elevated levels of IL-15 mRNA were maintained at 15 h (56 ± 6), 3 days (21 ± 5), and 5 days (16 ± 6) (Fig. 5; copy number ± SEM per 1000 copies of cyclophilin B; n = 3). Increased IL-15 mRNA was also found when ODN 2216 was replaced by rIFN-α, but not in monocytes incubated in the presence of GM-CSF and IL-4 or with GM-CSF alone (Fig. 5).

**Phenotypic changes of CpG-induced monocyte-derived cells upon maturation**

In general, full maturation of monocyte-derived DC requires additional stimulation. We tested different maturation stimuli that are known to induce full maturation of monocyte-derived DC generated in the presence of GM-CSF and IL-4 (41). Monocytes were incubated with GM-CSF in combination with IL-4 or ODN 2216-conditioned medium. After 4 days, CD40L; poly(I:C); the combination of CD40L, poly(I:C), and IFN-γ; or a cytokine cocktail

**FIGURE 1.** CpG ODN induce a monocyte-derived DC-like phenotype within PBMC. A, PBMC were incubated with GM-CSF alone or with GM-CSF in combination with CpG-A ODN 2216, rIFN-α, or IL-4 for 3 days, as indicated. Expression of cell surface activation markers was analyzed by flow cytometry in monocyte-derived cells (MFI). Data are shown as means ± SEM of six independent experiments. *, Indicates p < 0.05. B, Expression of CD14, HLA-DR, CD80, and CD86 was analyzed at different time points. Data represent means ± SEM of three independent experiments.
consisting of IL-1β, TNF-α, IL-6, and PGE₂ was added as maturation stimuli. After another 2 days, the expression of surface markers and the production of IL-12 and IL-10 were examined.

In monocyte-derived cells generated in the presence of GM-CSF and ODN 2216-conditioned medium (GM-CSF/CpG-A-CM cells; Fig. 6, upper panel), elevated levels of CD80 without stimulus (medium) were further up-regulated by all maturation stimuli used. In monocyte-derived DC generated in the presence of GM-CSF and IL-4 (GM-CSF/IL-4 cells; Fig. 6, lower panel), baseline expression of CD80 was low, and the combination of CD40/IFN-γ/poly(I:C) or the cytokine cocktail TNF/IL-1β/IL-6/PGE₂ was required to achieve similarly high levels of CD80 expression as seen in GM-CSF/CpG-A-CM cells. Consistent with the literature, GM-CSF/IL-4 cells strongly up-regulated CD83 and CCR7 expression in response to maturation stimuli, while the slightly elevated baseline level of CD83 in GM-CSF/CpG-CM cells showed no or only minor further up-regulation upon stimulation. CCR7 expression in GM-CSF/CpG-A-CM cells was only increased in the presence of CD40L (CD40L alone or CD40L/IFN-γ/poly(I:C)). Upon stimulation with CD40L (CD40L alone or CD40L/IFN-γ/poly(I:C)), GM-CSF/CpG-A-CM cells produced high levels of IL-12 (>20 ng/ml with CD40L/IFN-γ/poly(I:C)) and considerable amounts of IL-10. IL-12 production was even higher in GM-CSF/IL-4 cells (>100 ng/ml IL-12 with CD40L/IFN-γ/poly(I:C)). Production of IFN-α could not be detected in both cell types.

*CpG-A-CM stimulates partial activation, but not maturation of GM-CSF/IL-4-generated immature DC*

It has been reported that immature human DC generated from purified monocytes in the presence of GM-CSF and IL-4 are not directly activated by CpG ODN (5, 10). We examined whether these DC are sensitive to indirect activation by CpG ODN. Immature DC were generated from isolated monocytes by using a standard protocol (GM-CSF and IL-4). After 5 days, CpG-A-CM was added, and after another 2 days, surface markers were studied by flow cytometry. As shown in Fig. 7A (hatched bars), in the presence of CpG-A-CM the expression of CD80, CD86, and HLA-DR was increased (mean MFI value ± SEM of CD80, 50 ± 8 vs 4 ± 3; CD86, 103 ± 19 vs 49 ± 16; HLA-DR, 77 ± 13 vs 54 ± 22; n = 3). The level of activation was lower as compared with a standard cytokine cocktail (filled bars). Furthermore, CD83 was strongly up-regulated by the standard cytokine cocktail (filled bar, MFI 73 ± 30), but was not increased by CpG-CM as compared with control (hatched bars, MFI 23 ± 9 vs 24 ± 7; n = 3). Although CpG-CM stimulated some IL-12 production of GM-CSF/IL-4-generated DC (Fig. 7B, hatched bar), IL-12 production was weak as compared with stimulation by the cytokine cocktail (Fig. 7B, closed bar).

Monocyte-derived cells generated in the presence of GM-CSF and ODN 2216 induce CD8 T cell proliferation and support the development of CD45RA⁻CCR7⁺ CD8 T cells

We compared the T cell stimulatory capacity of the different monocyte-derived cell types. Isolated monocytes were incubated with GM-CSF in combination with CpG-A-CM, IFN-α, or IL-4 for 3 days. Monocyte-derived cell types were co-cultured with CFSE-labeled CD3 T cells from a different donor. After 5 days, proliferation was determined by the intensity of CFSE staining (low CFSE staining indicates proliferation) on CD4 and CD8 T cells. As expected, proliferation of both CD4 and CD8 T cells was lowest in the presence of monocyte-derived cells generated in the

![FIGURE 2](http://www.jimmunol.org/)

Yield and survival of monocyte-derived cells. PBMC (2 × 10⁶/ml) were incubated for 5 days with different stimuli, as indicated. The absolute number of viable monocyte-derived cells (CD11c⁺, FSC/SSC high, TO-PRO-3⁺) was counted by flow cytometry. A, All viable cells within 100 µl of cell culture medium harvested after incubation under different conditions are depicted (FSC vs SSC). CD11c⁺ cells are shown in gray color (one representative experiment). B, The absolute numbers of viable CD11c⁺ cells per milliliter are shown as means ± SEM of three independent experiments.
The proliferation of both CD4 and CD8 T cells (Fig. 8, in the presence of GM-CSF and CpG ODN 2216 stimulated the cell growth of CD4 T cells and a smaller number of CD8 T cells compared with IFN-γ). This effect was higher for CpG ODN-derived cells as shown in three individual donors (1, 2, 3).

The development of CpG-A-induced monocyte-derived cells depends on type I IFN. PBMC were incubated with GM-CSF and the CpG-A ODN 2216 for 3 days. A combination of neutralizing Abs against total type I IFN (IFN-α, IFN-β, and type I IFN receptor), an anti-IFN-β Ab, or an anti-TNF-α Ab was added at the beginning of the cell culture. Expression of the costimulatory molecule CD80 on monocyte-derived cells was analyzed by flow cytometry. Means ± SEM of three independent experiments are shown. *, Indicates p < 0.05.

FIGURE 3. Purified monocytes are not activated by CpG-A, but by CpG-A-CM. A. Purified monocytes isolated from PBMC after depletion of plasmacytoid DC were incubated for 3 days with GM-CSF alone or in combination with the CpG-A ODN 2216 or with ODN 2216-conditioned medium (CpG-A-CM: supernatant of ODN 2216-stimulated PBMC). B. Purified monocytes were incubated for 3 days with CpG-A-CM generated with the CpG-A ODN 2216, the CpG-B ODN 2006, or the non-CpG control ODN 2243. Surface molecules were analyzed by flow cytometry. The means ± SEM of three independent experiments are shown. *, Indicates p < 0.05.

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To characterize the phenotype of developing T cells, the expression of CD45RA and of CCR7 on CD8 and CD4 T cells was examined. Cells derived in the presence of GM-CSF and IL-4 induced proliferation of CD4 T cells and a smaller number of CD8 T cells (Fig. 8, second panel from left). Monocyte-derived cells generated in the presence of GM-CSF and CpG ODN 2216 stimulated the proliferation of both CD4 and CD8 T cells (Fig. 8, third from left).

To characterize the phenotype of developing T cells, the expression of CD45RA and of CCR7 on CD8 and CD4 T cells was examined. Cells derived in the presence of GM-CSF and ODN 2216, but not GM-CSF and IL-4, selectively increased the percentage of CD45RA⁻/CCR7⁺ CD8 T cells (effector memory phenotype). This effect was higher for CpG ODN-derived cells as compared with IFN-α (Fig. 8C; mean percentage ± SEM, 45 ± 2% and 35 ± 6%, n = 4), but this difference did not reach statistical significance. Consistent with the increase in CD45RA⁻/CCR7⁺ CD8 T cells, the percentage of CD45RA⁻/CCR7⁺ CD8 T cells (naïve T cells) decreased (Fig. 8C). Unlike for CD8 T cells, no consistent changes of the CD45RA and CCR7 phenotype of CD4 T cells were observed (Fig. 8C).

Monocyte-derived cells generated in the presence of GM-CSF and CpG-A ODN support the expansion and IFN-γ production of influenza-specific memory CD8 T cells

Because GM-CSF/CpG-A-CM cells increased the frequency of CD45RA⁻/CCR7⁻ CD8 T cells (so-called effector memory T cells) in an allogeneic system, we studied the ability of this cell type to support an autologous peptide-specific CD8 T cell memory response. An HLA-A2-restricted peptide from the influenza matrix protein was used as a model Ag to study a recall response in adults that have experienced influenza infections during their lifetime. Monocytes of HLA-A2-positive donors were incubated with GM-CSF and IL-4 (GM-CSF/IL-4 cells) or GM-CSF and ODN 2216-conditioned medium (GM-CSF/CpG-A-CM cells). After 3 days, monocyte-derived cells were pulsed with the HLA-A2-restricted peptide Flu matrix 58–66 and used to stimulate autologous CD8 T cells. Tetramer staining revealed a frequency of 0.05–0.2% of influenza matrix peptide-specific CD8 T cells before stimulation. After 10 days of stimulation with peptide-pulsed GM-CSF/CpG-A-CM cells, a 40- to 150-fold increased frequency of influenza matrix peptide-specific CD8 T cells (7.8% of all CD8 T cells) was found (Fig. 9A). These T cells were functionally active, as revealed by intracellular IFN-γ staining upon restimulation with influenza matrix peptide (Fig. 9B). The background of IFN-γ-producing cells measured by restimulation with a HLA-A2-restricted control

FIGURE 4.

FIGURE 5. Expression of IL-15 mRNA in monocyte-derived cells incubated under different conditions. Purified monocytes were incubated with GM-CSF alone or with GM-CSF in combination with IL-4 or supernatant of CpG ODN-stimulated PBMC (CpG-A-CM; ODN 2216 at 3 μg/ml for 48 h) or with IFN-γ (500 U/ml). At the time points indicated, the expression of IL-15 mRNA was quantified by real time RT-PCR. The copy number per 1000 copies of the housekeeping gene cyclophilin B is shown in three individual donors (1, 2, 3).
peptide derived from HIV-pol was below 0.3% IFN-γ/H9253/H11001 cells for all conditions (data not shown). GM-CSF/CpG-A-CM cells were more active than GM-CSF/IL-4 cells to support the generation of peptide-specific CD8 T cells (Fig. 9B; p < 0.05).

Discussion

The immune system is equipped to elicit different sets of immune responses to attack a variety of pathogens. Several subtypes of DC exist that express distinct profiles of TLRs to recognize the type of infection and to initiate the appropriate type of immune response. CpG ODN represent a heterogeneous class of compounds that mimicks the presence of pathogen-associated DNA. Based on the type of DNA sequence, the immune system may enter into different immunological pathways. We hypothesized that a recently identified type of CpG ODN (CpG-A, prototype ODN 2216), based on its ability to induce large amounts of IFN-α, may mimic a molecular pattern used by the immune system to recognize the presence of virus, and thus may lead to an immune response that is appropriate to limit viral infection.

In the present study, we focused on the effect of CpG-A on monocytes. We found that CpG-A (ODN 2216), but not CpG-B (ODN 2006), has a strong impact on monocyte differentiation and function. In the presence of GM-CSF and CpG-A, monocytes within PBMC decreased CD14 expression and rapidly developed a DC-like phenotype (CpG-A DC). The development of CpG-A DC within PBMC depended on CpG-A-induced IFN-α. Purified monocytes were not sensitive to the CpG-A, but the development of CpG-A DC could be restored by the addition of PBMC-derived

FIGURE 7. DC generated from monocytes in the presence of GM-CSF and IL-4 are partially activated by CpG-A-CM. Immature DC were generated from isolated monocytes in the presence of GM-CSF and IL-4. After 5 days, DC were stimulated with CpG-A-CM (ODN 2216, hatched bars) or a combination of TNF-α, IL-1β, IL-6, and PGE2 for 48 h (filled bars). A, The expression of CD80, CD86, HLA-DR, and CD83 was analyzed by flow cytometry (means ± SEM, n = 3). B, IL-12 p40/p70 production was detected in the supernatant by ELISA (mean ± range, n = 2).
CpG-A DC expressed high levels of CD40, CD54, CD80, MHC I, and MHC II without an additional stimulus. CpG-A DC rapidly up-regulated high levels of IL-15 mRNA that were maintained at lower levels throughout differentiation. Further maturation and IL-12 production of CpG-A DC required additional CD40L-mediated stimulation. Even without such further stimulation, CpG-A DC supported the proliferation of allogeneic CD8 T cells from a different donor and stained by CFSE (see Materials and Methods). Monocyte-derived cells and T cells were coincubated at a ratio of 1:2. After 5 days, the amount of CFSE on CD4-positive vs CD4-negative cells was measured by flow cytometry. Decreased CFSE staining indicates proliferation. One of two experiments with similar results is shown. B and C, Monocyte-derived cells and pan CD3 T cells from a different donor were coincubated at a ratio of 1:10. After 5 days, the expression of CD45RA and CCR7 on T cells was analyzed by flow cytometry. B, Representative dot plots of CD45RA and CCR7 expression in CD8 T cells are shown. Numbers indicate the percentage of cells within the corresponding quadrant. C, The percentages of CD45RA⁻/CCR7⁻ (upper panel), of CD45RA⁺/CCR7⁻ (middle panel), and of CD45RA⁺/CCR7⁺ T cells (lower panel) are shown for CD8 T cells (left column) and CD4 T cells (right column). Data represent means ± SEM of four independent experiments. *, Indicates p < 0.05.

Comparing CpG-A DC with DC generated in the presence of GM-CSF and IL-4 (GM-CSF/IL-4 DC), an interesting feature of CpG-A DC is the spontaneous expression of high levels of costimulatory molecules and of IL-15 mRNA. This explains the high activity of CpG-A DC to promote a peptide-specific memory CD8 T cell response in the absence of a further stimulus. To achieve similar levels of the costimulatory molecule CD80, GM-CSF/IL-4 DC require an additional maturation stimulus. In the presence of such a stimulus, GM-CSF/IL-4 DC expressed higher levels of CD83, CCR7, and IL-12 than CpG-A DC. Therefore, it seems unlikely that CpG-A DC can be used instead of the gold-standard mature GM-CSF/IL-4 DC for ex vivo vaccination protocols. However, while GM-CSF/IL-4 DC require ex vivo protocols for their clinical use, CpG-A (also termed D type CpG ODN (20)) can be administered directly in vivo (42). Therefore, our study contributes to the understanding of the monocyte-differentiation pathway elicited by CpG-A rather than providing a new ex vivo DC generation protocol.
cells. IL-15 is known to bind to the IL-15 mRNA in CpG-A DC, as demonstrated in our study, may stimulation with the corresponding peptide Ag. Elevated levels of cubated in the presence of GM-CSF and type I IFN develop a potential of these DC to stimulate CD8 memory T cells was not examined in these two studies.

Expansion of in
FIGURE 9. Expansion of influenza matrix-specific CD8 T cells by CpG-A-induced monocyte-derived cells. Monocytes of three different HLA-A2-positive donors were incubated with GM-CSF and IL-4 or GM-SF and ODN 2216-conditioned medium. After 3 days, the cells were pulsed with the HLA-A2-restricted peptide Flu matrix55-66 and used to stimulate autologous CD8 T cells. A, After 10 days, the expansion of Ag-specific CD8 T cells was analyzed by flow cytometry using PE-coupled HLA-A2/Flu matrix55-66 tetramers (mean ± SEM). B. For the assessment of Ag-specific IFN-γ production, the cells were restimulated at day 10 with their cognate peptide, and intracellular cytokine staining was performed.

Our results on activation and differentiation of monocytes toward a DC-like phenotype by CpG-A are in agreement with recent results from the literature (20, 43, 44). Our study extends previous data by adding evidence that DC generated in the presence of this type of CpG ODN display a distinct activity regarding T cell differentiation. CpG-A DC were more potent than GM-CSF/IL-4 DC to expand peptide-specific memory T cells. Evidence for the functional activity of expanding CD8 T cells is provided by the capacity of these expanding CD8 T cells to produce IFN-γ upon re-stimulation with the corresponding peptide Ag. Elevated levels of IL-15 mRNA in CpG-A DC, as demonstrated in our study, may contribute to the activity of CpG-A DC to support memory CD8 T cells. IL-15 is known to bind to the β-chain of the IL-2R, which is preferentially expressed on memory CD8 T cells (34).

Based on the expression of CCR7, which is responsible for homing in secondary lymphoid organs, and of CD45RA, T cells can be divided in CD45RA+ naive T cells, as well as in two subsets of preactivated T cells (central memory T cells, CD45RA- /CCR7+; effector memory T cells, CD45RA+ /CCR7- ) (45). An intriguing feature of CpG-A DC (in contrast to GM-CSF/IL-4 DC) was its ability to increase the frequency of the CCR7+/CD45RA- subset of CD8 T cells, while no such selective increase was found for CD4 T cells. It has been proposed that CCR7+/CD45RA- effector memory T cells enter peripheral tissues and secrete inflammatory cytokines or exert effector function (45). In earlier studies, it has been shown that CpG-A (ODN 2216) potently stimulates the lytic activity and IFN-γ production of NK cells and γδ T cells within PBMC (32, 46). Together with the expansion of CD8 memory T cells, as shown in the present study, this type of CpG ODN seems to initiate a set of early immune responses useful to limit viral replication.

The induction of type I IFN plays an essential role in CpG ODN-mediated activation of NK cells and γδ T cells (32), as well as in the development of CpG-A DC, as demonstrated in the present study. It is known that type I IFN are responsible for the impaired IL-12-mediated immunity during viral infections (47). Type I IFN reduce the capacity of DC to produce IL-12 in response to microbial or CD40L-mediated stimulation (27, 48). As a consequence, DC are less active to promote naïve CD4 T cell proliferation and to induce IFN-γ-producing Th1 cells (48, 49). The potential of these DC to stimulate CD8 memory T cells was not examined in these two studies.

In another study, it has been demonstrated that monocytes incubated in the presence of GM-CSF and type I IFN develop a DC-like phenotype (28). Similar to our results, in that study DC showed only partial up-regulation of CD83 expression and an advanced stage of differentiation. IL-12 and IL-15 production in DC and IFN-γ production in DC-stimulated T cells were not examined. Another group reported the development of a monocyte-derived DC phenotype in the presence of GM-CSF and type I IFN (30). In this study, the authors observed an increased IFN-γ production in the supernatant of DC-stimulated PBMC without identifying the cell population responsible for this IFN-γ production. Consistent with our results, in their study, DC generated in the presence of GM-CSF and type I IFN lacked spontaneous IL-12 expression, but produced high amounts of IL-15 in the absence of a maturation stimulus. In another study, it has been reported that IL-15 is also expressed in mature IL-12-competent DC (50). Regarding the function of IL-15, it has been shown that IL-15 stimulates memory-phenotype CD8 T cells, but not CD4 T cells in vitro and in vivo (34). Furthermore, IL-15 production by DC correlated with expansion of Ag-specific preactivated CD8 T cells (51). Together, these studies and our own results are in agreement with the concept that type I IFN promote the development of a monocyte-derived DC-like phenotype that exhibits impaired IL-12 function, but via expression of IL-15, selectively supports CD8 memory T cells.

Differences between these studies regarding the DC phenotype, such as the level of CD83 expression, IL-12 expression, and final maturation, may be due to the source and concentration of type I IFN used (26–30). Recently, Biron (25) proposed a model in which the level of type I IFN induced by a pathogen determines the production of IL-12 and the type of immune responses. According to this model, one would assume that ODN 2216 as a potent inducer of IFN-α in plasmacytoid DC (13) is able to support a CD8 recall response and to activate NK cells, but would not promote priming of a CD8 T cell response. Indeed, our recent results demonstrate that CpG-A (ODN 2216) does not promote priming of melan A peptide-specific CD8 T cells in PBMC. In contrast, CpG-B, which supports IL-12 production in plasmacytoid DC, but is poor at inducing type I IFN (ODN 2006) (12), enhanced priming of CD8 T cells.5

In conclusion, our results revealed that soluble factors induced by CpG-A within PBMC drive monocytes toward a DC-like phenotype that preferentially promotes the expansion of memory CD8 T cells. It has to be considered that the results presented were obtained from circulating cell populations that might differ from the immunologic events in tissues. Furthermore, the CpG-A-induced DC type may be of limited value for ex vivo vaccination strategies, but it may improve our understanding of the immune responses elicited by CpG-A administered in vivo, and will help to appropriately design clinical studies testing CpG-A for the therapy of viral disease and cancer.

References