CpG-Independent Synergistic Induction of β-Chemokines and a Dendritic Cell Phenotype by Orthophosphorothioate Oligodeoxynucleotides and Granulocyte-Macrophage Colony-Stimulating Factor in Elutriated Human Primary Monocytes

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CpG-Independent Synergistic Induction of β-Chemokines and a Dendritic Cell Phenotype by Orthophosphorothioate Oligodeoxynucleotides and Granulocyte-Macrophage Colony-Stimulating Factor in Elutriated Human Primary Monocytes

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Chemokines attract leukocytes bearing the relevant chemokine receptors and regulate innate immune responses. CpG oligodeoxynucleotides (ODN) and GM-CSF are potent vaccine adjuvants and in combination induce enhanced Th1 responses by mechanisms yet to be determined. We have examined combinations of CpG- or non-CpG-ODN and GM-CSF for effects on the production of chemokines and the differentiation of monocytes to dendritic cells. High levels of the Th1-attracting, HIV-1-inhibitory chemokines, CCL3/MIP-1α and CCL4/MIP-1β, were induced in human primary monocytes when CpG- or non-CpG-ODN was combined with GM-CSF, but not with IL-4 or IFN-γ. The synergistic induction of β-chemokines by non-CpG-ODN was phosphorothioate (PS) chemistry dependent and inhibited by blocking endosome maturation/acidification and ERK1/2 activation. Chemokine and TLR9 mRNAs were induced by PS-ODN. Cells treated with non-CpG PS-ODN and GM-CSF expressed dendritic cell marker CD83 and high levels of HLA-DR and costimulatory molecules, and were CD14+ or CD14dim, consistent with monocyte differentiation into a dendritic cell phenotype. The induction of CD83 and β-chemokines was tyrosine phosphorylation dependent. Secreted CCL3 and CCL4 were detected as a heterodimer. Our results indicate the CpG-independent synergy between PS-ODN and GM-CSF mediated through chemokine and dendritic cell induction. In addition, our observations suggest that PS-ODN plus GM-CSF may be useful as potent in vivo dendritic cell differentiation/maturation agents for dendritic cell therapy and as vaccine adjuvants for tumor and infectious microorganisms, including HIV-1.
Chemokines regulate innate as well as adaptive immune responses (20, 21). CCR5 and CXCR3 are preferentially expressed in human Th1s. Th2s preferentially express CCR4 (22). The differential expression of chemokine receptors may contribute to Th1 or Th2 responses. Ligands for CCR5 may chemotact Th1-biased cells to sites of immune response. Th1-attracting chemokines CCL3 and CCL4 are induced by IL-12 and IL-2 cytokines from PBLs and by LPS from monocytes (23–25). CCL3 and CCL4 have potent anti-HIV-1 activity (26–28).

DC are considered to be the most potent professional APCs (29). Monocytes migrate from peripheral blood into tissues and become immature DC. After capturing Ags, these immature DC mature and migrate to lymph nodes, where they present captured Ag as peptide fragments to T cells, stimulating T cell-dependent immunity. DC efficiently activate not only T cells, but also B cells, NK, and NK T cells. DC represent the interface between foreign or tissue-specific Ags and T lymphocytes and, thus, are key players in the regulation of cell-mediated immunity. DC-based cancer immunotherapy has been shown to be safe and can elicit tumor-specific immune response in cancer patients (30). However, the source and preparation of DC are critical issues in achieving effective therapy.

CpG-ODNs have been reported to have greater adjuvant activity when combined with GM-CSF. This has been shown for tumor immunity in murine lymphoma and neuroblastoma models (31, 32). Adding CpG-ODN to Id-GM-CSF fusion proteins potentiated both therapeutic and prophylactic antitumor responses. IgG2a isotype anti-Id Abs were induced, indicating a Th1-type response. However, the mechanism for this effect is not known. The activity of non-CpG-ODN combined with cytokines has also not been studied. In murine macrophages, CpG-ODN has been shown to up-regulate mRNA for CCL3, CCL4, CCL2/MCP-1, and CXCL10/IFN-γ-inducible protein-10 (IP-10) (33), and to induce the secretion of CCL3 and CCL4 proteins when conjugated to Ag (34). In human pDC and macrophages, CpG-ODN stimulate IP-10 production (35), but no induction of CC chemokines has yet been published. In this study, we report that, in elutriated human primary monocytes, CpG or non-CpG-ODN and GM-CSF cooperate in inducing Th1-type chemokines CCL3/MIP-1α and CCL4/MIP-1β and in promoting monocyte differentiation to a DC-like phenotype expressing CD83; high levels of CD86, CD40, and HLA-DR; but little or no CD14.

Materials and Methods

Cells and reagents

Human monocytes were isolated by countercurrent centrifugal elutriation from single-donor preparations of peripheral blood leukocytes, as described previously (16), and were cultured in macrophage serum-free medium (Invitrogen Life Technologies). IL-4 was purchased from R&D Systems. GM-CSF and IFN-γ were from Amgen and Genentech, respectively. Human rCCL4/MIP-1β was purchased from Sigma-Aldrich, and human (4–rCCL3/MIP-1α and affinity-purified Abs to chemokines from R&D Systems. PD98059, SB203580, and herbimycin A were purchased from Calbiochem, and chloroquine from Sigma-Aldrich. Sytox-Green was purchased from Molecular Probes.

Table I. ODNs synthesized by the CBER Core Facility

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>ODN Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>T+cG+cT+cG+cT+T+T+T+T+G+cT+cG+T+T+G+cT+cG+T+T+G+cT+cG+T+T</td>
<td>PS-ODN</td>
</tr>
<tr>
<td>2006a</td>
<td>G+cT+cG+cT+G+cT+G+cT+cG+T+T+G+cT+cG+T+T+G+cT+cG+T+T</td>
<td>PS-ODN</td>
</tr>
<tr>
<td>2006b</td>
<td>T+cG+cT+cG+cT+T+T+T+T+G+cT+cG+T+T+G+cT+cG+T+T</td>
<td>PO-ODN</td>
</tr>
<tr>
<td>2006c</td>
<td>T+cG+cT+cG+cT+T+T+T+G+cT+cG+T+T+G+cT+cG+T+T</td>
<td>PO-ODN</td>
</tr>
<tr>
<td>2006d</td>
<td>T+cG+cT+cG+cT+T+T+T+T+G+cT+cG+T+T+G+cT+cG+T+T</td>
<td>PO-ODN</td>
</tr>
</tbody>
</table>

*PS linkage
CpG-ODN2006 contains three optimal CpG motifs (GTCGTT) for hTLR9.

To study the effect of CpG-ODN and GM-CSF on monocyte/macrophage expression of Th1-attracting CC chemokines CCL3 and CCL4, human primary monocytes were cultured in the presence or absence of CpG-ODN2006 and GM-CSF and monitored for chemokine secretion. As shown in Fig. 1A, CCL3 and CCL4 were not detected in cells cultured in medium alone or with GM-CSF. CpG-ODN2006 stimulated only a low level of CCL4, and no CCL3 was detected. In the presence of GM-CSF, high levels of CCL3 and CCL4 were induced by CpG-ODN2006. These data indicate that neither GM-CSF nor CpG-ODN was a strong inducer of CCL3 and CCL4, while in combination GM-CSF and CpG-ODN were potent stimulators of CCL3 and CCL4 production.

Synergistic induction of CCL3 and CCL4 by non-CpG-ODN in combination with GM-CSF

Various cytokines can prime monocytes for subsequent inflammatory cytokine secretion and a few induce differentiation of monocytes into DC. For example, IFN-γ has been shown to prime for IL-12 production in human monocytes/macrophages (38), and monocytes cultured in IL-4 and GM-CSF differentiate to DC (39). To determine whether induction of CCL3 and CCL4 by CpG-ODN and GM-CSF is specific, IFN-γ, IL-4, and non-CpG-ODN were tested. We found that neither IFN-γ nor IL-4 could prime monocytes to produce CCL3 and CCL4 in response to ODNs (Fig. 1B).

In the presence of GM-CSF, not only CpG-ODN2006, but also non-CpG-ODN2006a induced high levels of CCL3 and CCL4. Interestingly, the addition of IL-4 to cultures of ODNs and GM-CSF inhibited the production of chemokines.

PS chemistry-dependent induction of CCL3 and CCL4

To assess PS-ODN dependence in chemokine production, five ODNs were synthesized and tested. These included PS CpG-ODN2006, PS non-CpG-ODN2006a with inverted CpG, and PS non-CpG-ODN2006c containing neither CpG nor inverted CpG (Fig. 2). No CCL3 or CCL4 was induced by non-CpG PO-ODN2006b and non-CpG PO-ODN2006d. Our results indicated that the synergistic induction of CCL3 and CCL4 by ODNs and GM-CSF was PS chemistry dependent, and does not require the CpG motif.
Transcriptional induction of CCL3 and CCL4 by PS-ODN and GM-CSF

To test whether induction of CCL3 and CCL4 occurred at the transcriptional level, total RNA was isolated and amplified by RT-PCR with CCL3- and CCL4-specific primers. Very low levels of CCL3 and CCL4 mRNA were detected in cells cultured in medium alone (Fig. 3A). GM-CSF induced a low level of CCL4 and CCL3 mRNA. High levels of CCL4 and CCL3 messages were induced by CpG-ODN2006 and non-CpG PS-ODN2006a in the absence or presence of GM-CSF.

Induction of TLR9 by GM-CSF, CpG, or non-CpG-ODN

TLR9 has been identified as the receptor for CpG-ODN. Prior studies suggested that human monocytes express very low level of TLR9 (8). To determine whether TLR9 can be induced in this system, monocytes were cultured in the presence of GM-CSF and CpG, or non-CpG-ODN, and then assayed for TLR9 expression by RT-PCR. Monocytes expressed low levels of TLR9 message, consistent with previous reports. However, as shown in Fig. 3B, TLR9 mRNA was induced by GM-CSF, and strongly induced by CpG-ODN2006 and non-CpG-ODN2006a in elutriated monocytes. Our results indicated that TLR9 mRNA was inducible in human primary monocytes.

Endosome maturation/acidification is required for the optimal induction of CCL3

A requirement for endosome maturation/acidification for CpG-ODN activity has been reported (40). To examine whether the effect of PS-ODN and GM-CSF was mediated through endosome maturation/acidification, we precultured monocytes with chloroquine, an inhibitor of endosomal maturation/acidification, and then stimulated monocytes with PS-ODN and GM-CSF. The production of CCL3 was partially inhibited by chloroquine in two donors and totally blocked in the third donor (Fig. 4).

Induction of high levels of CD83, CD86, CD40, and HLA-DR with down-regulation of CD14 consistent with DC maturation

CD86 and CD40 are important costimulatory molecules during Ag presentation. To study the effects of ODNs and GM-CSF on the expression of costimulatory molecules, monocytes were cultured in the absence or presence of CpG- or non-CpG-ODN and GM-CSF and analyzed by flow cytometry. As shown in Fig. 5A, cells cultured in medium alone expressed moderate amounts of CD86 and low levels of CD40 and HLA-DR. HLA-DR expression was increased in GM-CSF-treated cultures, further up-regulated in GM-CSF plus non-CpG-ODN2006a-treated cultures, but decreased in CpG-ODN2006-treated cells compared with GM-CSF-only cultures. CD86 expression was increased in cultures of GM-CSF plus non-CpG-ODN2006a-treated cultures, but decreased in CpG-ODN2006-treated cells compared with GM-CSF-only cultures. CD86 expression was increased in GM-CSF-only cultures and further enhanced by adding 2006 or 2006a into GM-CSF cultures.

CD83 is a marker of blood DC (15). To test whether a mature DC phenotype has been induced, the expression of CD83 was examined after the treatment of CpG-ODN2006, non-CpG-ODN 2006a and 2006c, and non-CpG PO-ODN 2006b and 2006d in the presence or absence of GM-CSF. As shown in Fig. 5B, CD83 was not induced by any of the PO-ODN or GM-CSF alone. Low levels of CD83 were induced by PS-ODN 2006, 2006a, and 2006c. In the presence of GM-CSF, CD83 was induced by PS CpG-ODN-2006, non-CpG-ODN 2006a and 2006c, but not by PO-ODN 2006b and 2006d. These results show that the induction of DC maturation
marker CD83 is PS chemistry dependent. CD14 expression was dramatically reduced in GM-CSF plus ODN 2006a or 2006c (Fig. 5C), but not 2006b- nor 2006d-treated cells, and CD83^+CD14^- cells increased from 2% in medium cultures to >30% in GM-CSF plus 2006a or 2006c cultures (Fig. 5D). Cells treated with PS-ODN and GM-CSF displayed DC phenotype with dendritic processes (data not shown).

**ERK1/2-dependent induction of CCL3**

MAPKs regulate a variety of cellular activities, including gene expression, movement, and programmed cell death (41). Phosphorylation of ERKs is induced by PS-ODN in macrophages (42), and PS-ODN-mediated chemotaxis requires the activation of ERKs and p38 MAPK (43). To examine whether the induction of chemokine CCL3 is mediated by p38 MAPK and ERK1/2, monocytes were pretreated with or without SB203580, an inhibitor of p38 MAPK, and PD98059, an inhibitor of ERK1/2 activation, then stimulated with GM-CSF and PS-ODN. CCL3 production was not reduced in SB203580-treated cells. In PD98059-treated cells, CCL3 secretion was significantly inhibited (Fig. 6). These data indicate that ERK1/2 activation is important in the induction of CCL3 by GM-CSF and PS-ODN.

**FIGURE 5.** Differentiation into a DC phenotype. Elutriated monocytes were cultured in the presence or absence of GM-CSF (20 ng/ml) or indicated PS-ODN 2006, 2006a, 2006c or PO-ODN 2006b, 2006d (10 μg/ml) for 24 h. Cells were stained with indicated labeled Abs and analyzed on flow cytometry. A, Expression of CD86, CD40, and HLA-DR. B, Induction of CD83. C, Down-modulation of CD14. Cells were cultured in medium containing GM-CSF and indicated ODNs. D, Induction of CD83^+CD14^- cells. The numbers in upper quadrants are percentages of cells. The results are representative of two similar experiments.
Tyrosine phosphorylation-dependent induction of CD83, CCL3, and CCL4

GM-CSF transduces signals through JAK2 and tyrosine-phosphorylated STAT5a in human monocytes (44). To investigate whether tyrosine phosphorylation is critical for the induction of CD83 and β-chemokines by GM-CSF and PS-ODN, monocytes were pretreated with the tyrosine phosphorylation inhibitor herbimycin A, then stimulated with PS-ODN and GM-CSF. CD83 was also examined by flow cytometry. The expression of CD83 was completely blocked by herbimycin A (Fig. 7, A and B). This induced expression was inhibited strongly by herbimycin A (Fig. 7C). Cell death as assessed by Cytotox-green staining, a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes, but will not cross the membrane of live cells, was not increased in herbimycin A-pretreated, GM-CSF plus ODN2006a- or ODN2006-stimulated cells compared with GM-CSF plus ODN2006a- or 2006-treated cells. These results indicate that tyrosine phosphorylation is critical in the induction of CD83 and β-chemokines by GM-CSF and PS-ODN.

CCL3 and CCL4 are secreted as a complex

To examine the molecular state of CCL3 and CCL4 induced by GM-CSF and PS-ODN, supernatants of GM-CSF and CpG-ODN2006 or PS-ODN2006c-stimulated monocytes were immunoprecipitated with anti-CCL3 or anti-CCL4 Ab, and then the immunoprecipitate was analyzed by MALDI-TOF mass spectrometry. The specificity of the Abs has been shown previously (25). As shown in Fig. 8, the two Abs precipitated identical complexes from the culture supernatant of GM-CSF and CpG-ODN2006 or GM-CSF and PS-ODN2006c-stimulated monocytes. The complexes consisted predominantly of two polypeptides with molecular masses of 7459 and 7826 Da, which correspond to CCL3 lacking four NH2-terminal residues ((-4)CCL3) and a full-length CCL4, respectively. These results indicated that CCL3 and CCL4 induced by CpG-ODN through TLR9 and by non-CpG-ODN are secreted as a heterocomplex.

Discussion

Immune responses can be enhanced by increasing Ag presentation to T cells. Chemoattraction of T cells and APCs to sites of inflammation can also improve these responses. Prior studies have demonstrated that either GM-CSF or PS-ODN alone can augment Ag presentation. In this study, we show that the combination of GM-CSF and PS-ODN potently induces monocytes to differentiate into a DC phenotype expressing high levels of CD83, CD86, CD40, and HLA-DR and low levels of CD14, and secreting CCL3 and CCL4-β-chemokines in a CpG-independent fashion. Synergistic activation is associated with TLR9 induction and involves transcriptional and translational regulation of chemokine expression.

In the presence of GM-CSF, both CpG- and non-CpG-ODN are active in stimulating differentiation and chemokine production. Although these effects are not CpG dependent, DNA activation does require a phosphothioate backbone. Recently, non-CpG high thymidine PS-ODNs have been shown to induce CD86 expression on B cells (45). The CCL3- and CCL4-inducing PS-ODNs all have high levels of thymidine content, consistent with this previous report (45).

Our experiments, designed to elucidate the mechanism of synergy between ODNs and GM-CSF, reveal an interplay between chemokine mRNA transcriptional induction and regulation of translation or secretion. High amounts of CCL3 and CCL4 mRNA...
were induced by CpG- and non-CpG-ODNs in the absence of significant CCL3 and CCL4 protein production. GM-CSF also induced some chemokine message, which was not accompanied by protein secretion. This suggests either a transcription to translation block, or alternatively, a block in protein secretion. A similar phenomenon has been reported in memory T cells in which RANTES mRNA was detected in T cells without expression of RANTES protein. Stimulation of T cells through the TCR leads to translation and secretion of RANTES (46). Similarly, GM-CSF may provide signals to promote efficient translation and secretion in monocytes. The mechanism of synergy has yet to be determined, but may involve either regulation of RNA stability, availability of translation initiation factors, or expression of inhibitors of translation.

It has been shown that expression of TLR9 on responder cells is essential for CpG-ODN- as well as non-CpG-ODN-mediated activation (47, 48). The ability of chloroquine to partially inhibit ODN effects in this study indicates the involvement of endosome maturation in mediating the PS-ODN interaction with monocytes. This is consistent with previous results showing PS-ODN-mediated chemotaxis requires endosome maturation (43). The cells used in our study are elutriated monocytes with 95% CD14-positive cells. Low level of TLR9 mRNA is present in unstimulated elutriated monocytes. TLR9 mRNA was induced by CpG or non-CpG PS-ODN directly and was also weakly induced by GM-CSF. Therefore, it is likely that ODNs mediate continued signaling by inducing TLR9 expression. Similarly, some cytokines induce autologous receptors, such as IL-4, which then activates expression of the IL-4Rα chain (49). The induction of TLR9 suggests that, under certain conditions, monocytes, and possibly other cells, may express low levels of TLR9, which upon engagement enhance receptor expression as a positive feedback loop. Another example is the induction of TLR9 in naive B cells by BCR signaling that allows naive B cells to proliferate and differentiate into Ig-secret ing cells in response to CpG (50). Others report that microbial stimuli and CpGs affect the expression of their cognate TLR (51, 52).

It has been reported that type A or D ODNs induce monocyte production of IP-10 (35) and differentiation into DC (53), which require production of type 1 IFN (mainly IFN-α) from plasmacytoid DC. ODN2006 is a type B ODN, and only induced very low levels of IFN-α in elutriated monocytes with/without GM-CSF, and no IFN-α was induced by PS-ODN2006a with/without GM-CSF (data not shown). It is therefore unlikely that the effects we observed in this study were due to production of IFN-α by pDC.

Tyrosine phosphorylation is essential for the induction of CD83, CCL3, and CCL4 by PS-ODN and GM-CSF. GM-CSF has a profound impact on monocytes and activates cells through a tyrosine phosphorylation-dependent pathway (16). We have demonstrated previously that GM-CSF induces CXCR4 endocytosis in a tyrosine phosphorylation-dependent fashion (16). In this study, we demonstrated that tyrosine phosphorylation is a paramount link in the induction of CD83, CCL3, and CCL4 by PS-ODN and GM-CSF in human monocytes. It has yet to be determined whether STAT5, the key transducer of GM-CSF, is essential for the induction of these molecules. ERK1 activation is required for non-CpG-ODN effects, as it is in CpG-ODN stimulation.

The potent activity of CpG- and non-CpG-ODN with GM-CSF on chemokine production suggests that this combination would be active in attracting CCR5-expressing Th1 cells, macrophages, and DC to sites of immune response through induction of CCL3 and CCL4. This should sustain Ag uptake and presentation and further support a Th1-type immune response. Consistent with this hypothesis is the recent finding that CCL3 is a potent immunostimulator when coexpressed with GM-CSF in a leukemia/lymphoma vaccine that improved survival in treated mice (54). DC differentiation induced by non-CpG-ODN and GM-CSF may be more efficient in Ag sampling and presentation compared with CpG-ODN and GM-CSF because cells treated with the former combination express higher levels of HLA-DR more quickly. Differentiation of monocytes to immature DC (CD83) by GM-CSF and IL-4 requires a 5-day culture and needs to be treated further with TNF-α, IL-1β, and IL-6 for maturation.

We have also observed that the addition of IL-4 in our culture system reduced secretion of CC chemokines. We previously published that IL-4 inhibited CCR5 and CXCR4 expression in human primary monocytes, and this inhibited chemotaxis of monocytes toward their ligands (16, 55). In comparison with GM-CSF and IL-4, we believe that the combination of GM-CSF and PS-ODN is a more efficient and potent strategy for stimulating cell maturation and activation.

Although CC chemokines adapt an elongated or cylindrical structure, CXC chemokines have a globular structure. Analysis reveals a highly conserved topology for each chemokine that includes a flexible N-terminal segment that precedes the first cysteine, a long loop, three strands that form an antiparallel β-sheet, and a C-terminal α-helix. CC chemokines such as CCL5/RANTES, CCL2/MCP-1, or CCL8/MCP-2 have similar elongated or cylindrical structure and can form homodimers on their own. In a previous study, we demonstrated that a CCL3 and CCL4 complex was induced by LPS through TLR4 (25). We demonstrated in this study that CCL3 and CCL4 induced by CpG-ODN and by non-CpG-ODN, which mediate their effects through TLR9, are also secreted as a heterocomplex. The biological importance of the heterodimer of chemokines has yet to be determined.

At this time, clinical data in humans are not available on the combination of CpG-ODN adjuvants with tumor vaccines. In the mouse, immunization of a peptide analog of MART-1/Melan-A (26–35) mixed with CpG-ODN was able to elicit a systemic CTL response that was able to kill melanoma cells in vitro (56). Data from our group and from others showing PS-ODN enhancement of costimulatory molecules on DC and induction of IL-12, IFN-α, chemokines, and Ag-induced IgG2a indicate that PS-ODN may enhance human cellular and humoral immune responses in vivo. Our novel findings demonstrate that CpG and non-CpG PS-ODN synergize with GM-CSF to induce DC maturation and stimulate Th1-type chemokines CCL3 and CCL4 that are capable of blocking entry of macrophage tropic HIV-1 through CCR5. We suggest...
that combinations of CpG- or non-CpG-ODN with GM-CSF should be further investigated not only as potent ex vivo DC differentiation/maturagen agents for DC therapy, but also as in vivo adjuvants for tumor vaccines and vaccines for infectious microorganisms, including HIV-1.

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Disclosures

The authors have no financial conflict of interest.

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