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Enhanced Murine Macrophage TNF Receptor Shedding by Cytosine-Guanine Sequences in Oligodeoxynucleotides

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The immunomodulatory role of unmethylated cytosine-guanine sequences (CpG) in bacterial DNA has been well documented. We have previously demonstrated that murine macrophage-like RAW 264.7 cells respond to CpG DNA with an increase in the proinflammatory cytokine, TNF-α, in both a dose-dependent and time-dependent manner. In addition, CpG DNA stimulates a significant, though delayed, secretion of the anti-inflammatory cytokine IL-10. Because TNF-α and TNFR (TNFRI and II) expression are tightly regulated responses, we hypothesized that CpG containing oligodeoxynucleotide (CpG ODN) would also affect TNFRI and II shedding. Using both murine peritoneal macrophages and RAW 264.7 cells, we demonstrated a significant, time-dependent increase in soluble TNFRI and TNFRII production with CpG ODN stimulation. RAW 264.7 cells treated with CpG ODN had a transient increase in membrane TNFRII expression, but not TNFRI. Both types of TNFR mRNA were also up-regulated by CpG ODN, and addition of the transcriptional inhibitor actinomycin D abrogated the effect of CpG ODN on TNFR mRNA and protein expression. Addition of anti-IL-10 and anti-TNF-α Abs did not change these results. The addition of plate-bound anti-TNF receptor Abs to this system increased the amount of bioactive TNF, implying that these receptors are acting as inhibitors of TNF activity. These results suggest that the de novo, non-IL-10- and non-TNF-α-dependent transcription, translation, and shedding of TNFRs are additional potential counterinflammatory effects of CpG DNA. The Journal of Immunology, 2000, 165: 5153–5160.

Recently, there has been increased recognition that bacterial DNA, but not vertebrate DNA, can have significant immunostimulatory effects on B cells, NK cells (1–3), and macrophages (4), inducing the production of a variety of proinflammatory cytokines in vitro and in vivo, including TNF-α, IFN-γ, IL-6, and IL-12. Bacterial and vertebrate DNA differ in the frequency and methylation of cytosine-guanine sequences (CpG)3 dinucleotides (5–7). CpG dinucleotides are expressed nearly 20 times more frequently in bacterial than in vertebrate DNA (8). In addition, synthetic CpG-containing oligonucleotide motifs can mimic bacterial DNA, stimulating a Th1-like inflammatory response dominated by the release of IL-12 and IFN-γ. Meanwhile, CpG motifs also stimulate the production and secretion of IL-10, a potent immunosuppressive cytokine (9, 10). The immune-stimulatory potential of CpG motifs is primarily restricted to 12- to 20-base sequences containing CpG dinucleotides with selective flanking bases, 5'-Pu-Pu-CpG-Pyr-Pyr-3' (11). The profound immunomodulatory effects of CpG DNA suggest that the vertebrate immune system recognizes structural patterns specific to non-cell wall microbial molecules.

TNF-α is the predominant cytokine in the early septic response to acute extracellular infection as well as an important mediator of apoptosis (12). There are two major cell surface receptors for TNF-α, TNFR type I (TNFRF; CD120a) and TNFR type II (TNFRII; CD120b), both of which belong to the TNFR superfamily and bind TNF-α with high affinity (13). Cellular activation by agents such as LPS, anti-CD3 Abs, phorbol esters, and TNF-α itself induces rapid shedding of membrane TNFR in different types of cells, including monocyte/macrophages (14), T lymphocytes (15, 16), and granulocytes (17). The shedding of TNF receptors may function as a potent inhibitor of the binding and activity of TNF-α by competing for ligand with membrane-bound TNFRs (18, 19). Soluble TNFR (sTNFR) is 5–30 times more potent than sTNFR in vitro and a better inhibitor of TNF-α activity in vivo (18–21). Despite significant data concerning the function of sTNFRs as inhibitors of TNF activity, other reports show that when sTNFR concentrations are low, they may increase TNF activity by stabilizing TNF trimeric structures and prolonging their availability for binding to membrane receptors (22, 23).

Although bacterial DNA and CpG motifs have significant effects on TNF-α secretion, little information exists regarding their influence on TNFR expression. In the present study we demonstrate that in murine macrophages, CpG ODN increases TNFR transcription, translation, and shedding in a non-IL-10- and non-TNF-α-dependent fashion. In addition, we further show in this system that sTNFRs neutralize the bioactivity of TNF, suggesting the de novo shedding of TNFR may be an additional potential counterinflammatory effect of the CpG motif.

Materials and Methods

Cell culture techniques and reagents

Experiments using mice were approved by the University of Virginia animal care and use committee and were performed in accordance with National Research Council guidelines. Female BALB/c mice (Hilltop Laboratories, Scottsdale, PA), weighing 20–25 g, were housed in a pathogen-free environment and injected i.p. with 1 ml of sterilized 3% Brewer thioglycolate medium (Difco Products, Becton Dickinson, Mountain View,
CA) with 1% penicillin and streptomycin 3 days before macrophage harvest. Mice were sacrificed with halothane anesthesia and cervical dislocation, and peritoneal macrophages were harvested and pooled from multiple mice. Cells were resuspended at the desired concentration. An α-naphthyl acetate esterase assay (Sigma, St. Louis, MO) was performed on a sample of the cell suspension to confirm the purity of macrophages within the cell population (>80%).

For in vitro experiments, the murine macrophage cell line RAW 264.7 (ATCC TIB 71, American Type Culture Collection, Manassas, VA) was used. Cells were cultured in 170-ml sterile culture flasks in DMEM with 4 mM l-glutamine and 4.5 g/L glucose supplemented with 1.0 mM sodium pyruvate and 5% FBS (Life Technologies, Grand Island, NY). The cells were incubated at 37°C in 5% CO₂, washed twice with PBS, and resuspended in medium before each experiment.

Abs used in various experiments included rat anti-TNF-α mAb (clone G281-2626, PharMingen, San Diego, CA), isotopic control rat IgG1 (clone R3-34, Pharmingen), rat anti-IL-10 mAb (clone JES5-16E3, PharMingen), isotype control rat IgG2b (clone A95-1, PharMingen), goat anti-TNFRI polyclonal Ab (R&D Systems, Minneapolis, MN), goat anti-TNFRII polyclonal Ab (R&D Systems), goat IgG for control (R&D Systems), PE-labeled rat anti-mouse TNFRI (clone IOT-CD120a, Coulter Immunotech, Miami, FL), PE-labeled rat anti-mouse TNFRII (clone IOT-CD120b, Coulter Immunotech), and isotype control Ab IgG2a (clone L3DONP-16, Coulter Immunotech).

CpG and non-CpG-containing oligonucleotides and LPS

CpG-containing oligonucleotide 5′-TTC ATT AGC TCG TTC AGG TT and non-CpG containing sequence 5′-TCC AGG ACT TTC CTC AGG TT were synthesized on a DNase-resistant phosphorylate backbone (Bio-Synthesis, Lewisville, TX) as previously described (24–26). Before each experiment, the oligonucleotides were dissolved in medium to the desired concentration and left to incubate overnight in the entirety of the oligonucleotides. Measured with the Limulus amebocyte lysate endotoxin-knockout (EKC) kit (Charles River Endosafe, Charleston, SC), was <0.5 pg/ml of culture medium. LPS O182:B12 (Sigma) was resuspended in medium before each experiment.

Measurement of TNF-α and TNFR

TNF-α secreted from RAW 264.7 cells was measured using a TNF-α ELISA minikit (Endogen, Woburn, MA). Total TNF secretion was also measured by bioassay. L929 cells (American Type Culture Collection, CRL-2148; 4 × 10⁶) were added to each well in a 96-well plate and incubated overnight at 37°C in 5% CO₂. After confluent growth was assured, cells were resuspended in 50 μl of DMEM containing test samples or TNF standards (Endogen). Another 50 μl of medium containing actinomycin D (10 μM) and 50 μl of 0.05% Tween 20 and 1% Triton-X were added to each well. The plates were washed and dried overnight, 100 μl of 100% methanol was added to each well, and plates were read at 95 nM on an automated plate reader and compared with those with medium alone and TNF standards to determine TNF activity. Although this assay does not differentiate between TNF-α and TNF-β, the use of cell lines of monocyte origin and highly purified primary monocyte/macrophage cells was believed to minimize the contribution of TNF-β (27–33).

Soluble TNFRI and TNFRII were measured using TNFRI and TNFRII Quantikine kits (R&D Systems). In some experiments RAW 276.4 cells were cultured with CpG ODN for various time periods in plates precoated with 5 μg/ml goat anti-TNFRI Ab, goat anti-TNFRII, or goat IgG. For some in vitro experiments, 5 μg/ml actinomycin D (Sigma) was used as a transcriptional inhibitor.

CpG ODN increases sTNFR secretion

To measure relative changes in cell surface TNFRI and TNFRII in vitro, RAW 264.7 cells were incubated for various time intervals with CpG ODN, non-CpG ODN, or medium alone. At each designated time interval, the cells were washed and labeled with PE-labeled rat anti-mouse TNFRI or PE-labeled rat anti-mouse TNFRII or the appropriate isotype control Ab. IgG2a. After labeling, flow cytometric analysis was performed using a FACStar flow cytometer system (Becton Dickinson). Unstained cells were washed and treated in similar manner to measure the level of autofluorescence.

Results

CpG ODN enhances the secretion of sTNFRs

CpG DNA increases proinflammatory cytokine TNF-α secretion by the mouse macrophage cell line RAW 264.7 (4). Because cellular activation increases soluble TNF receptor secretion (14–17), and high levels of TNF-α secretion also increase the production of its own soluble receptor (15), we postulated CpG ODN would stimulate soluble TNF receptor secretion. RAW 264.7 cells (1.5 × 10⁶) were treated with 1.5 μg/ml CpG ODN, non-CpG ODN, or medium alone for various time periods (1–9 h), with subsequent measurement of supernatant sTNFRI and sTNFRII. A concentration of 1.5 μg/ml CpG ODN is the lowest capable of drawing consistent maximal TNF-α secretion in RAW 264.7 cells (our unpublished observations). Soluble TNFRII secretion after incubation with CpG ODN, but not with non-CpG, increased steadily through the 9-h period (Fig. 1). Soluble TNFRII secretion occurred earlier, but to a lesser degree (Fig. 1A), than sTNFRII secretion (Fig. 1B). Compared with 1 μg/ml LPS, CpG ODN induced slightly less sTNFRII, but more sTNFRII (Fig. 1).

CpG ODN enhances the secretion of sTNFRs in mouse peritoneal macrophages

To confirm that the above results were not limited to immortalized cell lines, similar experiments were performed using elicited mouse peritoneal macrophage. Peritoneal macrophages were harvested and resuspended in DMEM complete medium at 3 × 10⁶/ml; 1.5 × 10⁶ cells were incubated with 1.5 μg/ml CpG ODN, non-CpG ODN, or medium alone for different time intervals, and sTNFRI and sTNFRII were measured in the supernatant. As shown in Fig. 2A, the secretion of sTNFRII was stimulated, but occurred later and to a lesser extent compared RAW 264.7 cells. In contrast, CpG ODN stimulated earlier, and greater sTNFRII secretion by peritoneal macrophage compared with RAW 264.7 cells (Fig. 2B).
The secretion of sTNFRs is not significantly regulated by IL-10 or TNF-α

Because previous studies have shown that IL-10 and TNF-α up-regulate TNFRII expression and shedding in activated monocytes and T lymphocytes (15, 34), we postulated that the CpG ODN increase in sTNFRII secretion might also be regulated by cytokines. To investigate these effects, RAW 264.7 cells were treated with CpG ODN, non-CpG ODN, or medium alone in the presence of anti-IL-10 or anti-TNF-α Ab (both at 10 μg/ml) for various time periods. Soluble TNF receptors in the supernatant were measured. Values represent the means of at least three experiments. *p < 0.05; **p < 0.01 (vs non-CpG ODN). A, Time response of CpG ODN stimulation of sTNFRI secretion by RAW 264.7 cells. B, Time response of CpG ODN stimulation of sTNFRII secretion by RAW 264.7 cells. Assays with medium alone were performed at 9 h only.

CpG ODN stimulates the expression of membrane TNFRII, but not TNFRI

Cellular activation frequently induces an up-regulation of the expression of membrane cytokine receptors. To elucidate the role of CpG ODN in the expression of membrane TNFRs, we performed FACS analysis of cell surface TNFRI and TNFRII expression. RAW 264.7 cells were treated with CpG ODN or non-CpG ODN for 1–9 h. No change in surface expression of TNFRI was found at any time (Fig. 4A). Compared with non-CpG ODN, surface expression of TNFRII started to increase after 3 h of treatment with CpG ODN (data not shown), peaked at 6 h (Fig. 4B), then returned to baseline after 9 h (data not shown).

The increased shedding of TNFRs is due to CpG ODN-stimulated de novo TNFR production

The enhanced shedding of TNFRs with CpG ODN stimulation could be from preformed TNFRs and/or from newly produced and secreted protein. To examine this question, we stimulated cells with CpG ODN in the presence or the absence of the transcriptional inhibitor actinomycin D for different time periods. Actinomycin D abrogated the CpG ODN-stimulated increase in sTNFRI and sTNFRII to less than basal levels (Fig. 5). Although by 9 h of CpG ODN/actinomycin D treatment cells had significantly decreased viability, even 3–6 h of incubation rendered cells incapable of secreting sTNFR. Together with the fact that RAW 264.7 cells expressed minimal amounts of TNFRI and TNFRII on their
surface even when stimulated with CpG ODN (Fig. 4), we concluded that the enhanced TNFR shedding with CpG ODN stimulation is primarily due to enhanced de novo TNFR formation and shedding.

CpG ODN stimulation up-regulates mRNA levels for TNFRs

To confirm the hypothesis that CpG ODN increases sTNFR secretion by stimulating de novo synthesis, we next examined the effect of CpG ODN treatment on TNFR mRNA expression. RAW 264.7 cells were stimulated with CpG ODN, non-CpG ODN, or medium alone for different time periods, and changes in TNFRI and TNFRII mRNA levels were examined by RPA. CpG ODN increased the mRNA levels of both receptors (Fig. 6). CpG ODN-mediated stimulation of TNFRI mRNA (Fig. 6, A and B) was slower and less profound than the effects on TNFRII mRNA (Fig. 6, A and C). The addition of 5 μg/ml actinomycin D eliminated detectable TNFR mRNA (data not shown), again suggesting that CpG ODN-increased TNFR shedding occurs through de novo transcription rather than stabilization of preformed mRNA. Because TNF-α is known to stimulate the production of its receptor, similar experiments were conducted in the presence of anti-TNF-α Ab or isotype control Ab. There was no difference in TNFR mRNA level in cells stimulated with CpG ODN in the presence or the absence of anti-TNF-α Ab (Fig. 6), suggesting that regulation of TNFR mRNA by CpG ODN is not an autocrine effect of TNF-α secretion.

Shed TNFRs neutralize TNF activity from CpG ODN-stimulated RAW cells

Although sTNFRs are frequently considered natural inhibitors of TNF-α activity (20, 21, 35), they may also potentiate TNF activity by stabilizing the trimeric structure of physiologic TNF, serving as carriers (22, 23). To better understand the function of sTNFR in our system, bioassays and TNF-α ELISAs were simultaneously performed in the presence of anti-TNFRI or anti-TNFRII Abs. RAW 264.7 cells were cultured in plates precoated with anti-TNFRI or anti-TNFRII polyclonal Abs to deplete secreted TNFRs in supernatants. Cells were simultaneously stimulated with CpG ODN for various time periods, and supernatants were collected and assayed. As shown in Fig. 7A, the presence of anti-TNFRI Abs did not affect the amount of TNF-α protein secreted by cells. Bioassays of the same supernatants, however, show that TNF activity was increased in the presence of anti-TNFRI Abs, most notably at 6 h (Fig. 7B). Together, these data suggest that sTNFRs partially neutralize TNF-α activity from CpG ODN-stimulated cells in vitro.

FIGURE 3. Effects of anti-IL-10 and anti-TNF-α Ab on CpG ODN-induced production of TNFRs. RAW 264.7 cells were treated with 1.5 μg/ml CpG ODN, 1.5 μg/ml non-CpG ODN, or medium containing neutralizing anti-IL-10 Ab (10 μg/ml) or isotype control Ab (rIgG2b; A and C), or anti-TNF-α Ab (10 μg/ml) or isotype control Ab (rIgG1; B and D) for the designated time periods. The culture supernatant levels of sTNFRI (A and B) and sTNFRII (C and D) were measured. Values represent the means of at least three experiments. Assays with medium or Abs alone (without CpG ODN or non-CpG ODN) were performed at 9 h only.
Discussion

Multiple studies over the past decade have demonstrated the immunomodulatory activity of prokaryotic DNA, especially CpG motifs. In this paper we demonstrate that CpG ODN increases TNFR shedding and up-regulates TNFR mRNA transcription in a time-dependent manner. We further show that enhanced TNFR shedding is due to TNFR formation de novo and is not predominantly mediated by IL-10 and TNF-α, because the addition of anti-IL-10 and anti-TNF-α did not change the results. Moreover, we show that shed TNFRs neutralize the bioactivity of TNF-α, implying that they serve as a physiologic regulator of TNF activity.

Like TNF-α, the transcription, translation, and expression of the two main TNFRs are tightly regulated. Mediators known to affect their expression include LPS, anti-CD3 Abs, phorbol esters, and TNF-α. Our data imply a possible direct effect of CpG ODN on shedding of TNFR in a time-dependent manner. It has been reported that TNF-α stimulates TNFR secretion (15), and the amount and speed of shedding of TNFR were proportional to the serum TNF levels (36). In our system, the addition of anti-TNF-α Ab does not change TNF-α secretion, although it does increase TNF-α secretion a small amount (data not shown). IL-10, one of the most prominent anti-inflammatory cytokines, inhibits monocyte/macrophage TNF-α secretion in vitro (37–39), yet also up-regulates the synthesis and release of sTNFR II by LPS-stimulated monocytes (34). Because we (manuscript in preparation) and Anitescu et al. (10) have demonstrated stimulation of late IL-10 secretion in vitro and in vivo by CpG ODN, we hypothesized that the shedding of TNFR by CpG ODN would be up-regulated by IL-10 in macrophages. Surprisingly, the addition of anti-IL-10 Ab did not significantly change sTNFR secretion, although it did increase TNF-α secretion (data not shown). Because CpG ODN-induced IL-10 secretion occurs later than TNF-α secretion, the regulatory effect of IL-10 in the stimulation of sTNFR shedding could occur in a later phase. Other potential mechanisms involved in TNFR shedding could also be active, such as increased receptor cleavage, or more proximal mechanisms such as changes in signal transduction known to be activated by CpG ODN, including p38 and c-Jun N-terminal kinase (40, 41).

CpG DNA was initially believed to be a predominantly proinflammatory molecule, increasing TNF-α, IL-6, IL-12, and IFN-γ secretion and polarizing lymphocytes toward a Th1 response. More recently, however, some anti-inflammatory properties, including decreased NO production from macrophages in response to endotoxin (42), decreased pulmonary inflammation in response...
to endotoxin after systemic exposure to CpG motifs (8) and decreased TNF-α secretion in response to endotoxin in vitro (our manuscript in preparation) have been reported. Our data showing that CpG ODN increases sTNFR secretion is further evidence of this anti- or counterinflammatory property. These effects are almost certainly dependent on the CpG ODN dose and duration of exposure as well as the cell type studied, and additional experiments in these areas are underway.

Membrane TNFRI is the predominant mediator of TNF-α responses in vitro and in vivo (43–55). TNFRFII has been shown to have a secondary role in signaling, possibly serving a ligand-passing role (46, 56). Unlike TNFRI and some other TNFR family members, TNFRII lacks the death domain motif critical for the cytocidal effect of TNFRI in its cytoplasmic domain (57). In contrast, sTNFRs generated by proteolytic cleavage of the cell surface receptors have been shown to bind TNF with high affinity (18, 58, 59). Both receptor molecules can compete with cell surface receptors for TNF and block its availability and activity, thus functioning as TNF antagonists (18, 19, 60–62). Alternatively, sTNFRs can enhance TNF activity by stabilizing the trimeric structure of TNF molecules and prolonging its availability for binding to cell

FIGURE 6. CpG ODN up-regulates sTNFR mRNA production in a TNF-α-independent manner. RAW 264.7 cells (2.0 × 10⁶) were treated with 1.5 μg/ml CpG ODN, 1.5 μg/ml non-CpG ODN, or medium alone in the presence or the absence of anti-TNF-α mAb (10 μg/ml) for various time periods. At the end of the incubation period, cells were harvested, RNA was isolated, and levels of sTNFRI (A and C) and sTNFRII (B and D) mRNA were determined by RPA. A. Representative RPA gels from three experiments. Each experiment was performed under identical conditions with similar results. B and C. Graphic depiction of sTNFR receptors as a function of time, normalized to GAPDH [³²P]mRNA expression. *, p < 0.05 vs non-CpG ODN.

FIGURE 7. Soluble TNFRs neutralize TNF activity from CpG ODN-stimulated RAW cells. RAW 264.7 cells (1.5 × 10⁶) were incubated with 1.5 μg/ml CpG ODN on plates precoated overnight with 5 μg/ml goat anti-sTNFRI, 5 μg/ml goat anti-sTNFRII polyclonal Ab, or 5 μg/ml goat IgG to deplete sTNFRs in the supernatant. After the designated incubation periods, supernatants were harvested. A. TNF-α protein level measured by ELISA. B. TNF bioactivity, measured using L929 cells. Values represent the means of at least three experiments. *, p < 0.05 for plates precoated with anti-sTNFRs vs plates precoated with control goat IgG.
surface receptors (22, 23). Our TNF bioassay data indicate that both shed TNFRs neutralize the bioactivity of TNF to relatively equivalent degrees. Because RAW cells produce sTNFR in a TNFRII to TNFRRII ratio of 1:15–20, these data are consistent with previous reports that sTNFR is 5–30 times more potent than sTNFRII in vitro and is a better inhibitor of TNF-α activity in vivo (18–21).

The roles of bacterial DNA and CpG motifs in in vivo experiments and in clinical infections remain largely unknown. Because the response to CpG motifs appears to be relatively nonspecific and evolutionarily conserved, it might be expected to most profoundly affect the early innate immune response. The possibility that prokaryotic DNA could simultaneously or sequentially elicit both a proinflammatory and a counter- or anti-inflammatory response, similar to our in vitro data, is at least plausible. This hypothesis, however, requires further whole animal and clinical studies.

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