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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 TNF receptor (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.

Recent evidence has shown 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) (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 (TNFR1; CD120a) and TNFR type II (TNFR2; 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 TNFRs (sTNFR) is 5–30 times more potent than membrane-bound TNFRs (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 TNF 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 that the de novo shedding of TNF 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, Scottdale, 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,
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 then 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 μL glucose supplemented with 1.0 mM sodium pyruvate and 10% FBS (Life Technologies, Grand Island, NY). The cells were incubated at 37°C in 5% CO₂. Cells were washed in saline and stained with 50 μl of 0.05% crystal violet in 20% ethanol for 10 min. The plates were washed to remove excess probe. A standard curve was plotted to establish the identity of the RNase-protected bands in experimental samples. Films were developed using photodensitometry to quantify ³²P activity associated with the TNFR mRNA in each sample. Levels were reported as the ratio of TNFR/GAPDH to control for the amount of RNA loaded into each sample on the gel. GAPDH mRNA is constitutively expressed in these cells.

**Statistical analysis**

Values for protein concentration and mRNA levels were compared using ANOVA and post-hoc Tukey’s honestly significance difference (HSD) test to compare the means. p < 0.05 was considered significant. Values are reported as the mean ± SE. All calculations were performed using statistical software (Statistica; Statsoft, Tulsa, OK).

**Results**

**CpG ODN enhances the secretion of TNFRs**

CpG DNA increases proinflammatory cytokine TNF-α secretion by the mouse macrophage cell line RAW 264.7 (4). Because cellular activation increases soluble TNFR 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 TNFR 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 TNFR secretion after incubation with CpG ODN, but not with non-CpG, increased steadily through the 9-h period (Fig. 1). Soluble TNFR 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 sTNFRI (Fig. 1).

**CpG ODN enhances the secretion of TNFRs 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 × 10⁸ cells/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 sTNFRII 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 for various time periods, and supernatant sTNFRs 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 sTNFRII 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 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, this suggests that the increased shedding of TNFRs observed with CpG ODN treatment might be due to de novo TNFR production.

FIGURE 1. Effect of CpG ODN on sTNFR secretion from RAW 264.7 cells. RAW cells (1.5 × 10⁶) were treated with 1.5 μg/ml CpG ODN, 1.5 μg/ml non-CpG ODN, 1 μg/ml LPS, or medium alone for the designated time periods, and supernatant sTNFRs 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 sTNFRII 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.

FIGURE 2. Effect of CpG ODN on sTNFR secretion from elicited mouse peritoneal macrophages. Peritoneal macrophages (1.5 × 10⁶) were stimulated with 1.5 μg/ml CpG ODN, 1.5 μg/ml non-CpG ODN, or medium for various time periods, and supernatant sTNFRs were measured. Values represent the means of at least three experiments. A, Time course of CpG ODN stimulation of sTNFRII secretion from peritoneal macrophages. *, p < 0.05; **, p < 0.01 (vs non-CpG ODN). Assays with medium alone were performed at 9 h only.
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-TNFR 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.
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 TNFRII were proportional to the serum TNF levels (36). In our system, the addition of anti-TNF-α Ab does not change TNFR 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 sTNFRII 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 secretion 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

**FIGURE 4.** Effect of CpG ODN on membrane expression of TNFRs in RAW 264.7 cells. RAW 264.7 cells (2 × 10⁶) were incubated in medium alone or in medium containing 1.5 μg/ml CpG ODN or 1.5 μg/ml non-CpG ODN for 6 h. Cells were then labeled with PE-conjugated rat anti-mouse TNFRI mAb (A) or PE-conjugated rat anti-mouse TNFRII mAb (B) and were analyzed by FACS. Results are representative of three experiments with similar results.

**FIGURE 5.** Actinomycin D abrogates TNFR secretion. RAW 264.7 cells were incubated with 1.5 μg/ml CpG ODN, 1.5 μg/ml non-CpG ODN, or medium alone in the presence or the absence of actinomycin D (5 μg/ml) for various time periods. At the end of incubation, the supernatants were harvested, and sTNFRI (A) and sTNFRII (B) protein levels were measured by ELISA. *, p < 0.05; **, p < 0.01 (vs CpG ODN plus actinomycin D). There is no 9-h point in the actinomycin D group due to reduced cell viability.
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). TNFRII 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 sTNF 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 TNFRF to TNFRFII ratio of 1:15–20, these data are consistent with previous reports that sTNFR is 5–30 times more potent than sTNFRFII in vitro and is a better inhibitor of TNFα activity in vivo (18–21).

The roles of bacterial DNA and CpG motifs 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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