Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles

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ABSTRACT

Unmethylated CpG dinucleotides present within certain specific sequence contexts in bacterial and synthetic DNA stimulate innate immune responses and induce cytokine secretion. Recently, we showed that CpG DNAs containing two 5'-ends, immunomers, are more potent in both regards. In this study, we show that an immunomer containing a synthetic CpR motif (R = 2'-deoxy-7-deazaguanosine) is a potent immunostimulatory agent. However, the profile of cytokine induction is different from that with immunomers containing a natural CpG motif. In general, a CpR immunomer induced higher interleukin (IL)-12 and lower IL-6 secretion. Compared with conventional CpG DNAs, both types of immunomers showed a rapid and enhanced activation of the transcription factor NF-kB in J774 cells. NF-kB activation by CpG DNA corresponded to degradation of IkBα in J774 cells. All three immunostimulatory oligonucleotides activated the p38 mitogen-activated protein kinase pathway as expected. Immunomers containing CpG and CpR motifs showed potent reversal of the antigen-induced Th2 immune response towards a Th1 type in antigen-sensitized mouse spleen cell cultures. Immunomers containing a CpR motif showed significant antitumor activity in nude mice bearing MCF-7 human breast cancer and U87MG glioblastoma xenografts. These studies suggest the ability for a divergent synthetic nucleotide motif recognition pattern of the receptor involved in the immunostimulatory pathway and the possibility of using synthetic nucleotides to elicit different cytokine response patterns.

INTRODUCTION

The presence of CpG dinucleotides in certain sequence contexts in bacterial and synthetic oligodeoxyribonucleotides (CpG DNAs) is known to activate vertebrate innate immune cells and B cells (1–4). Various cytokines are secreted in the process, including interferon-γ (IFN-γ), interleukin (IL)-12, tumor necrosis factor-α (TNF-α) and IL-6, and the levels of co-stimulatory surface molecules increased (5–8). These activities depend on the sequences flanking the CpG dinucleotide (3,9–11), and we have investigated the structural and chemical characteristics of CpG DNA required for immune stimulation (12–14).

Although direct evidence for physical binding is lacking, it is believed that Toll-like receptor 9 (TLR9) recognizes CpG dinucleotides in bacterial and synthetic DNA and activates intracellular immune signaling pathways (15). Our structure–immunostimulatory activity studies showed that the receptor is highly specific for deoxyribonucleotides in CpG, as ribonucleotides or 2'-O-alkyl ribonucleotides abrogate immunostimulation (12). We have identified critical structural features in the pentose sugar (13,16–19), phosphate backbone (13,20), nucleobases (21,22) and nucleosides (23) required for activity. Moreover, through the use of synthetic pyrimidine (Y) and purine (R) bases in YpG and CpR motifs, we showed requirements for a functional group on cytosine and guanine in receptor recognition (24).

More importantly, our recent studies suggest that the receptor reads CpG DNA sequence from the 5'- to the 3'-end, and modifications that block the 5'-end abrogate immunostimulation (25–29). In contrast, CpG oligonucleotides with two accessible 5'-ends (immunomers) show enhanced activity (25–29). Immunomers containing phosphodiester backbones show enhanced nuclease resistance and immunostimulation without the need for palindromic or guanine-rich sequences (28). The size of ligand conjugated at the 5'-end determines accessibility. For example, fluorescein conjugation at the 5'-end does not effect cellular uptake compared with 3'-conjugate, but prevents immunostimulation (26). Immunomers containing as few as 5 or 6 nt in each

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MATERIALS AND METHODS

Synthesis and purification

CpG DNA [1, 5′-d(CTATCTGACGGTCTCTGT)-3′] and immunomers [2, 5′-d(TCTGACGGTCT-X-TCTTGCAG-TCT)-5′; 3, 5′-d(TCTGACGGTCT-X-TCTTGCAG-TCT)-5′ where X and R are glyceryl linker and 2′-deoxy-7-deazaguanosine, respectively] were synthesized on a 1–2 μmol scale using β-cyanoethylphosphoramidite chemistry on a PerSeptive Biosystem’s 8909 Expedite DNA synthesizer as described earlier (27). The phosphoramidites of dA, dG, dC and T were obtained from Applied Biosystems. 2′-Deoxy-7-deazaguanosine phosphoramidite and the solid support attached with di-4,4′-dimethoxytrityl-protected glyceryl linker were obtained from ChemGenes. Beaucage reagent was used as an oxidant to obtain the phosphorothioate backbone modification (30). After the synthesis, immunomers were deprotected using standard protocols, purified by HPLC, and dialyzed against USP quality sterile water for irrigation (Braun). The immunomers were lyophilized and dissolved in TE buffer (10 mM Tris±HCl pH 7.5, 1 mM EDTA) was added to a final concentration of 0.03, 0.1, 0.3, 1.0, 3.0 or 10.0 μg/ml to the cell cultures. The cultures were then incubated at 37°C for 24 h and the supernatants were collected for enzyme-linked immunosorbent assays (ELISAs). The experiments were performed two or three times for each CpG DNA in triplicate for each concentration. The secretion of IL-12 and IL-6 was measured by sandwich ELISA as described previously (27). The required reagents, including cytokine antibodies and standards, were purchased from PharMingen.

Cytokine ELISAs

Mouse spleen or J774 cells were plated in 24-well dishes using 5 × 10⁶ or 1 × 10⁶ cells/ml, respectively. The CpG DNA dissolved in TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) was added to a final concentration of 0.03, 0.1, 0.3, 1.0, 3.0 or 10.0 μg/ml to the cell cultures. The cells were then incubated at 37°C for 24 h and the supernatants were collected for enzyme-linked immunosorbent assays (ELISAs). The experiments were performed two or three times for each CpG DNA in triplicate for each concentration. The secretion of IL-12 and IL-6 was measured by sandwich ELISA as described previously (27). The required reagents, including cytokine antibodies and standards, were purchased from PharMingen.

Mouse splenomegaly assay

Female BALB/c mice (4–6 weeks, 19–21 g) were divided into groups of two or three mice. CpG DNAs were dissolved in sterile phosphate-buffered saline (PBS) and administered intraperitoneally (i.p.) to mice at a dose of 10 mg/kg. The mice were sacrificed after 40 h, the spleens were harvested and weighed as described previously (12,32) and the number of mononuclear cells was determined. Blood was withdrawn from animals by retro-orbital puncture before sacrificing for cytokine assays. The IL-12 levels in serum samples were determined by ELISA as described above.

Sensitivity of mice with conalbumin

Four- to six-week-old BALB/c female mice were obtained from Tacomic (Germantown, NY). The mice were given i.p. injections of 200 μg of conalbumin (Sigma) in 100 μl of PBS mixed with 100 μl of ImjectAlum adjuvant (Pierce) on days 0 and 7, and intranasally challenged on days 14 and 21. The mice were sacrificed 72 h after the last challenge by CO₂ inhalation. Spleens were excised and single cell suspensions were prepared as described above. Spleen cells were treated with immunomers at different concentrations for 2 h followed by treatment with 50 μg/ml of conalbumin. Supernatants were harvested after 72 h, and IL-5, IL-6 and IL-12 levels were measured by ELISA as described above.

Preparation of J774 cell nuclear extracts and EMSA

NF-κB activation in J774 cells treated with CpG DNAs was carried out and analyzed by EMSA as described previously (27).
RESULTS

CpG DNA containing two accessible 5¢-ends shows greater immune stimulation

Activation of mouse (BALB/c, C57BL/6 or C3H/HeJ) spleen cells in cultures with CpG DNA for 24 h resulted in cytokine induction. In the present studies, two typical cytokines, IL-12 and IL-6, are measured. Both 1 and 2 induced concentration-dependent IL-12 and IL-6 secretion in all three strains of mouse spleen cell cultures (Table 1). Figure 2 shows a typical concentration-dependent IL-12 and IL-6 secretion in BALB/c mouse spleen cell cultures. In general, 3¢–3¢-linked immunomer 2 induced higher IL-12 secretion than conventional CpG DNA 1. A control DNA molecule lacking CpG induced similar levels of IL-12 and IL-6 secretion to background values observed with medium alone (data not shown). Previously we showed that two CpG DNAs covalently attached through 5¢–5¢ linkages induce minimal cytokine secretion (25–28). We also demonstrated that two CpG DNAs attached through 5¢–3¢ linkage (containing two immunostimulatory motifs) showed no greater activity than a shorter CpG DNA containing one immunostimulatory motif (27). An undecanucleotide that corresponds to one of the segments of immunomer 2 showed minimal activity in previous studies (27). Therefore, 5¢–5¢-linked and 5¢–3¢-linked oligonucleotides were not tested further in the present study.

Preparation of J774 cell lysates and western blotting

The levels of phosphorylated and total p38 mitogen-activated protein kinases (MAPKs) in J774 cells following CpG DNA treatment were measured by western blotting as described previously (28). All antibodies required for the assay were purchased from Cell Signaling Technology.

In vivo nude mice human cancer models and treatment plan

The animal use and care protocols were approved by the Institutional Committee on Animal Use and Care of the University of Alabama at Birmingham. Female athymic nude mice (nu/nu, 4–6 weeks old) were obtained from the Frederick Cancer Research and Development Center (Frederick, MD, USA) and inoculated with MCF-7 or U87MG cells as described previously (33,34). Tumor-bearing animals were divided randomly into various treatment groups (five/group) and treated by subcutaneous injection with immunomer 3 at a dose of 0.5 mg/kg/injection or saline (control) 3 days per week (days 1, 3 and 5). The MCF-7 study was terminated on day 15 and the U87MG study was terminated on day 42 when the tumor size reached 3000 mg in control group mice. Animals in the treatment group in the MCF-7 study received a total of six injections and those in the U87MG study received a total of 15 injections. The mice were monitored by general clinical observations, body weight and tumor growth. Tumor growth was recorded with the use of calipers, by measuring the long and short diameters of the tumor. Tumor mass (in g) was calculated using the formula 1/2a × b², where a and b are the long and short diameters (in cm), respectively.

Table 1. Induction of cytokine secretion by immunomers in mouse spleen cell cultures

<table>
<thead>
<tr>
<th>CpG DNA</th>
<th>IL-12 (pg/ml) ± SD</th>
<th>0.1 µg/ml 1.0 µg/ml 3.0 µg/ml 5.0 µg/ml</th>
<th>IL-6 (pg/ml) ± SD</th>
<th>0.1 µg/ml 1.0 µg/ml 3.0 µg/ml 5.0 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100 ± 3</td>
<td>546 ± 23</td>
<td>1313 ± 69</td>
<td>1775 ± 139</td>
</tr>
<tr>
<td>2</td>
<td>1142 ± 99</td>
<td>3330 ± 324</td>
<td>3735 ± 180</td>
<td>3640 ± 186</td>
</tr>
<tr>
<td>3</td>
<td>219 ± 9</td>
<td>1151 ± 30</td>
<td>2601 ± 197</td>
<td>2823 ± 134</td>
</tr>
<tr>
<td>C</td>
<td>57 ± 4</td>
<td>7 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>130 ± 8</td>
<td>682 ± 2</td>
<td>787 ± 19</td>
<td>892 ± 71</td>
</tr>
<tr>
<td>2</td>
<td>1526 ± 171</td>
<td>1989 ± 272</td>
<td>1771 ± 41</td>
<td>1698 ± 38</td>
</tr>
<tr>
<td>3</td>
<td>454 ± 23</td>
<td>1523 ± 56</td>
<td>1478 ± 90</td>
<td>1487 ± 46</td>
</tr>
<tr>
<td>C</td>
<td>71 ± 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>206 ± 23</td>
<td>781 ± 63</td>
<td>880 ± 53</td>
<td>931 ± 148</td>
</tr>
<tr>
<td>2</td>
<td>1394 ± 97</td>
<td>2183 ± 96</td>
<td>1935 ± 115</td>
<td>1770 ± 33</td>
</tr>
<tr>
<td>3</td>
<td>487 ± 35</td>
<td>1124 ± 160</td>
<td>1428 ± 55</td>
<td>1414 ± 71</td>
</tr>
<tr>
<td>C</td>
<td>25 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, no added immunomer control; ND, not detected.
To evaluate the immunostimulatory effects of CpG DNAs on splenomegaly and serum IL-12 levels in BALB/c mice, we administered three different doses of CpG DNAs (1–3) to BALB/c mice i.p. at a dose of 10 mg/kg (single dose). The increases in spleen weight, splenocyte number and IL-12 levels in serum were determined 40 h post-administration (Table 2). A significant increase in spleen weights was observed in mice injected with CpG DNA compared with mice injected with PBS (86 ± 10 mg) (Fig. 4). The mice treated with CpG DNAs 1–3 showed an average increase of 300–330% in their spleen weights compared with the control group. There were also more mononuclear cells in spleens of treated mice (Table 2). After 40 h, serum IL-12 levels increased with all three CpG DNAs (Table 2). Moreover, as in cell cultures, greater IL-12 induction resulted with immunomers 2 and 3 than with 1.

### Activation of NF-κB in J774 cells

The transcription factor NF-κB is activated by CpG DNA treatment of macrophages (35) and is known to induce cytokine secretion. We compared the activation of NF-κB by CpG DNAs 1–3 in J774 cells. Figure 5A shows an EMSA of J774 nuclear extracts treated with an NF-κB-binding oligonucleotide probe. All three CpG DNAs activated the transcription factor, as shown in Figure 5B. However, significant differences are seen in the kinetics of activation of NF-κB (Fig. 5A) by 1 (lane 2) and immunomers 2 (lane 3) and 3 (lane 4). Immunomers 2 and 3 showed strong activation of NF-κB within 30 min, while 1 showed minimal activation in this time. After 1 h, significant activation of NF-κB was found with all three CpG DNAs 1–3. However, immunomers 2 and 3 showed relatively more potent activation of NF-κB at this time also compared with 1 (Fig. 5A). After 3 h, all three CpG DNAs showed a similarly strong NF-κB binding presence in J774 cell nuclear extracts. Importantly, both 2 and 3, containing natural CpG and synthetic CpR motifs, respectively, showed similar levels of NF-κB activation.

### Degradation of IκBα in J774 cells

By interacting with NF-κB, IκBαs inhibit the translocation of NF-κB to the nucleus and thereby its binding to DNA (36,37). Therefore, the presence of NF-κB in nuclear extracts in...
general should correspond to the degradation of IκBα, although recent studies indicate that NF-κB activation could also occur without IκB degradation (38). We evaluated whether CpG DNA-mediated activation of NF-κB correlates with an enhanced and sustained degradation of IκBα in J774 cells. A western blot assay was performed on cytoplasmic extracts using specific antibodies against murine IκBα. As shown in Figure 5B, IκBα levels were only marginally affected by treatment with 1 for 30 min, corresponding to the minimal activation of NF-κB (Fig. 5A). After 1 h, IκBα had completely disappeared, but it reappeared after 3 h. In the case of treatment with immunomers 2 and 3, IκBα was rapidly degraded within 30 min and reappeared after 1 h.

**Activation of the p38 MAPK pathway in J774 cells**

CpG DNA is also known to activate the stress-activated p38 MAPK pathway (39,40). To evaluate whether immunomers 2 and 3 activate the p38 MAPK pathway similarly to 1, we measured the status of p38 phosphorylation in J774 cells following treatment with CpG DNAs for 30 min. All three CpG DNAs induced phosphorylation of p38 in J774 cells to similar extents (Fig. 6), although significant differences were observed in NF-κB activation.

Consistent with rapid and strong activation of NF-κB by immunomers 2 and 3, higher levels of IL-12 and IL-6 induction were found in J774 cell cultures (Fig. 5C).

**Effect of immunomers on cytokine induction in conalbumin-sensitized mouse spleen cell cultures**

CpG DNA induces predominantly Th1-type immune responses. It has been shown that CpG DNA treatment reverses established antigen-induced Th2 responses to Th1-type responses (41,42). To assess the effects of treatment of immunomers on Th2 cytokines associated with allergic airway responses, we measured IL-5, IL-12 and IL-6 secreted in spleen cell cultures obtained from BALB/c mouse sensitized and challenged with conalbumin. In the absence of immunomer treatment, conalbumin-sensitized mouse spleen cells secreted markedly higher levels of IL-5, and low IL-12, suggesting predominantly a Th2 polarization (Table 3). When the allergen-primed spleen cells were treated with immunomers, a concentration-dependent decrease in IL-5 and increase in IL-12 secretion was observed. Table 3 shows the levels of IL-5, IL-12 and IL-6 induced following treatment with 1.0 μg/ml concentration of immunomers. These data suggest that immunomers not only induce Th1-type cytokine secretion but potently reverse antigen-induced Th2 responses. Significantly, in these assays, immunomer 3 containing the CpR motif also induced lower levels of IL-6 compared with immunomer 2 containing the natural CpG motif.

**Antitumor activity in nude mice bearing human tumor xenografts**

CpG DNAs have been shown to induce antitumor activity in vivo (28,29,43). Since the immunomer containing the synthetic CpR motif (3) induced potent immunostimulatory activity in cell culture assays, we evaluated its antitumor activity in vivo. Compound 3 was administered subcutaneously three times a week at a dose of 0.5 mg/kg to nude mice bearing MCF-7 human breast tumor or U87MG human glioblastoma xenografts that express wild-type p53. Growth of MCF-7 tumors was inhibited by 60% on day 15 compared with the saline control (Fig. 7). At the same dose, 3 inhibited growth of the glioblastoma by 63% on day 42 compared with the saline control (Fig. 7). At this dose, 3 did not cause any treatment-related toxicity in mice. There was no difference in body weight gains between treated animals and controls (data not shown). These preliminary antitumor studies suggest that immunomers containing synthetic CpR motifs exhibit potent antitumor activity in vivo.
Table 3. Cytokine induction by CpG DNA and immunomers in conalbumin-sensitized BALB/c mouse spleen cell cultures

<table>
<thead>
<tr>
<th>CpG DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IL-5 (pg/ml ± SD)</th>
<th>IL-12 (pg/ml ± SD)</th>
<th>IL-6 (pg/ml ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>567 ± 148</td>
<td>647 ± 102</td>
<td>1246 ± 109</td>
</tr>
<tr>
<td>2</td>
<td>131 ± 36</td>
<td>1660 ± 56</td>
<td>9567 ± 940</td>
</tr>
<tr>
<td>3</td>
<td>325 ± 7</td>
<td>1508 ± 98</td>
<td>4382 ± 694</td>
</tr>
<tr>
<td>Medium</td>
<td>1345 ± 437</td>
<td>45 ± 9</td>
<td>396 ± 12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Conalbumin-sensitized BALB/c mouse spleen cells were cultured, treated with different concentrations of CpG DNAs and the culture supernatants were assayed for the cytokines by ELISA as described in Materials and Methods. Data shown are for 1 µg/ml concentration of CpG DNA and immunomers. Each value is an average of three readings.

DISCUSSION

Bacterial and synthetic CpG DNAs promote B cell proliferation, immunoglobulin production and the secretion of a number of cytokines, including IL-12, IFN-γ, IL-6 and TNF-α, from B cells, monocytes/macrophages, dendritic cells and natural killer cells. It has been shown that a receptor, TLR9, is engaged in the recognition of CpG DNA and subsequent initiation of the signaling cascade leading to the activation of the transcription factor NF-κB. In this study, we show that immunomers containing two accessible 5'-ends are more immunostimulatory than the natural CpG DNA, and the receptor recognizes a synthetic CpR motif in immunomers similarly to the natural CpG motif.

Our recent studies showed that covalent conjugation of molecules such as fluorescein at the 5'-end of a CpG DNA molecule abrogated the immunostimulatory activity, leaving uptake unaffected compared with the 3'-conjugate (26). Earlier studies of CpG DNAs containing multiple copies of CpG motifs showed no increased activity (25–27). From these results and those reported here, we conclude that the increased activity of 2 and 3 is not due to the presence of two copies of the CpG or CpR dinucleotide motif but to the presence of two 5'-accessible ends in the molecule. If this were not so, then similar cytokine induction would be expected if 1 were used at double the concentration of 2, giving the same concentration of CpG dinucleotide motifs. However, this is not the case. Additionally, immunomers linked 5'-5' are inactive or much less active than 3'-3'-linked analogs or conventional CpG DNAs (25–27).

Figure 7. In vivo antitumor activity. Nude mice bearing MCF-7 human breast cancer (left panel) or U87MG glioblastoma (right panel) xenografts were treated with saline or immunomer 3 as specified in Materials and Methods. Data are shown for days 0, 9 and 15 for MCF-7, and for days 0, 28 and 42 for U87MG studies. Each group had six mice. Immunomer was given subcutaneously on every other day starting at day 0. *Represents a statistically significant value (P < 0.01).

Only unmethylated CpG dinucleotide motifs have been shown to induce immunostimulatory effects. We and others have shown that the presence of a methyl group at the 5-position of cytosine abrogates immunostimulatory activity (12). Through the use of synthetic pyrimidines in place of cytidine, we showed that 5-hydroxyl and N4-ethyl substituents do not interfere with receptor recognition and immunostimulation (24). Similar studies with guanosine analogs showed the need for hydrogen bond donors at positions 1 and 2, and a hydrogen bond acceptor at position 6. However, the hydrogen bond-accepting N7 atom is not essential, and a 2'-deoxy-7-deaza-guanosine can be used in the synthetic immunostimulatory motif CpR (see Fig. 1 for structures) (24).

Here, we provide the first evidence that the CpR motif in immunomers is not only recognized by the receptor but induces a different cytokine secretion profile from that of the natural CpG motif. In both in vitro and in vivo studies, CpG immunomer 2 induced higher IL-12 and IL-6 than did conventional CpG DNA 1. Importantly, compared with 2, the CpR immunomer 3 consistently induced significantly lower levels of IL-6, but similar levels of IL-12 in vitro and in vivo. Elevated levels of IFN-γ expected with increased IL-12 expression were also observed [(27), and data not shown]. In allergen-sensitized mouse spleen cell cultures, both immunomers showed a potent inhibition of IL-5 secretion.
compared with CpG DNA. Immunomodulators showed similar IL-12 and IL-6 secretion profiles in Th2 polarized mouse spleen cells to those found in normal mouse spleen cell cultures and in vivo. In addition to different cytokine secretion profiles, immunomunes 3 showed a greater antigen-specific IgG2a/IgG1 antibody ratio compared with immunomune 2 (D.Wang and S.Agrawal, unpublished data), suggesting that the CpR motif is recognized by the receptor but results in distinct downstream effects compared with the normal CpG motif-containing immunomune. The kinetics of activation of NF-kB is similar with both immunomers, and it is not clear how they produce distinct cytokine expression profiles. Detailed mechanistic studies are required with different subsets of immune cells to distinguish the downstream events. Nonetheless, recognition of both natural CpG and synthetic CpR motifs suggests alternative NMRPs for the receptor, although the involvement of a related receptor cannot be ruled out. TLR3 that belongs to the same family specifically recognizes viral dsRNA and synthetic poly(I)-poly(C) (47). Greater IL-12 induction by immunomones could be of importance for cancer therapy (43,48) as well as for adjuvant action (49,50) with vaccines, allergens and monoclonal antibodies in therapy and prophylaxis. Induction of lower IL-6 and TNF-α levels ([27], and data not shown) by synthetic CpR immunomones may permit development of immunotherapeutic agents with reduced cytokine-related toxicities (51,52).

In conclusion, we have shown that immunomones induce rapid and sustained immunomunostimulation with distinct cytokine secretion profiles both in vitro and in vivo. Their increased activity results from two accessible 5’-ends and improved metabolic stability in vivo. For the first time, we have shown that the receptor recognizes a synthetic CpR motif and that alternative NMRPs produce different cytokine secretion profiles. The ability of the novel synthetic motif to induce immune stimulation resulted in potent antitumor activity in nude mice bearing MCF-7 human breast tumor and U87MG human glioblastoma xenografts. Additionally, no treatment-related toxicity was observed in mice at the dose studied. A number of other purine and pyrimidine analogs that are recognized by the receptor are under study currently for their distinct immunomunological properties. These studies will not only permit development of tailor-made immunomunotherapeutic agents for specific diseases but also illuminate receptor–CpG DNA recognition and signaling at the molecular level.

REFERENCES


