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Guanine Modification of Inhibitory Oligonucleotides Potentiates Their Suppressive Function

Franziska Römmler,* Marion Jurk,†,1 Eugen Uhmann,†,2 Monika Hammel,* Anna Waldhuber,* Lavinia Pfeiffer,* Hermann Wagner,* Jörg Vollmer,†,3 and Thomas Miethke‡

Inhibitory TLR7 and/or TLR9 oligonucleotides (inhibitory oligonucleotide [INH-ODN]) are characterized by a phosphorothioate backbone and a CC(T)XXX3–5GGG motif, respectively. INH-ODN 2088 is a prototypic member of this class of INH-ODN and acts as a TLR7 and TLR9 antagonist. It contains a G quadruple that leads to higher order structures by the formation of G tetrads. These structures are unfavorable for the prediction of their pharmacological and immunological behavior. We show in this study that modification of Gs within the G quadruple by 7-deaza-guanine or 7-deaza-2′-O-methyl-guanine avoids higher order structures and improves their inhibitory potential. Whereas TLR9-induced TNF-α secretion of bone marrow–derived macrophages and conventional dendritic cells was equally inhibited by INH-ODN 2088 and G-modified INH-ODNs such as INH-ODN 24888, TLR7-induced TNF-α release and TLR7- and TLR9-induced IL-12p40 release were significantly more impaired by G-modified INH-ODNs. Similarly, the IL-6 release of B cells from wild-type and autoimmune MRL/Mp-lpr/lpr mice was more efficiently impaired by G-modified INH-ODNs. Surprisingly, INH-ODN 2088 stimulated B cells to proliferate when used in higher doses. Finally, in vivo, in wild-type and autoimmune MRL/Mp-lpr/lpr mice, G-modified INH-ODN 24888 was significantly more efficient than unmodified INH-ODN 2088. In summary, G modification allows the development of INH-ODNs with superior inhibitory potency for inflammatory diseases with high medical need such as systemic lupus erythematosus. The Journal of Immunology, 2013, 191: 000–000.
INH-ODNs contain G triplets or quadruples that form higher order structures, also called G stacks, which make their immunological and pharmacological behavior unpredictable. For example, G-rich oligonucleotides (ODNs) also interfere with STAT3 and were proposed to act as anti-cancer drugs (12). Furthermore, treatment of Salmonella enterica subsp. enterica serovar Typhimurium-infected bone marrow–derived macrophages (BMDM) with INH-ODN 2114, a TLR9 inhibitor that contains a G quadruple, enhanced intracellular bacterial numbers in a TLR9-independent manner (13). However, INH-ODN 2114 also suppressed TLR2-dependent responses and impaired NF-κB activation and IL-12p40 secretion (13). Thus, a more generalized immunosuppression was induced, and this may hamper the use of similar INH-ODNs in the treatment of autoimmune diseases. It was further reported that the G stack forming INH-ODN 2088 was only inhibitory as a linear molecule, whereas its G-tetrad form was inactive (10). These data indicate that the avoidance of higher order structures of INH-ODNs may substantially increase their efficacy.

Sequence motifs that characterize TLR7-specific INH-ODNs are less well established. However, Barrat et al. (14) described the TLR7-specific INH-ODN 661 containing the sequence 5′-TG-TGGCCAAGCTTGAAGC-3′. Upon further modification, they obtained INH-ODN 954, which blocked TLR7 and TLR9. It appeared that the minimal requirement for TLR7 inhibition was represented by a TGC motif at the 5′ terminus of the INH-ODN (14). In contrast to these results, others reported that TLR7 was impaired by INH-ODNs in a sequence-independent, but backbone-dependent manner. Thus, a phosphorothioate backbone was inhibitory, whereas a phosphodiester was not (9, 15).

B- and R-class INH-ODNs were successfully used in strains of mice suffering from experimental lupus: B-class INH-ODN INH18 and R-class INH-ODN INH11 prolonged survival of MRL-Fas−/− mice. However, only R-class INH-ODN INH11 reduced, in addition, the enlargement of lymph nodes, the level of anti-DNA, and each of Smith/ribonucleoprotein Abs, and the extent of kidney damage (9). Similarly, INH-ODN 954 delayed progression of spontaneous lupus in the New Zealand Black/White-F1 strain and also reduced the production of several autoantibodies (16).

Although INH-ODNs are quite effective inhibitors of TLR7 and TLR9 in vitro and in vivo, their mechanism of inhibition remains obscure to date. Inhibition could occur at different levels such as the following: 1) TLR7 or TLR9 ligand uptake by receptor-mediated endocytosis or phagocytosis; 2) TLR trafficking or processing; or 3) competition of the ligand by the inhibitor to bind to TLR7 or TLR9. Thus, it was demonstrated that phosphorothioate 2-deoxyribose bond to TLR7 and TLR9 and competitively interfered with TLR9 ligand binding (15).

To improve the efficacy of INH-ODNs and increase their specificity for TLR7 or TLR9 or both TLRs, we synthesized several INH-ODNs, which were modified in the first or second guanine within the G quadruplex, to prevent the formation of higher order structures, which are presumably involved in off-target effects. In this study, we tested the inhibitory potential of this new series of INH-ODNs in comparison with the well-studied G quadruplex containing INH-ODN 2088 in vitro and in vivo.

Materials and Methods

**Ethics statement**

All animal experiments were reviewed and approved by the local authorities (Regierung von Oberbayern, file 55.2-1-54-2531-89-10).

**Strains of mice**

C57BL/6, BALB/c, and MRL/Mp-lpr/lpr mice were purchased from Harlan Winkelmann (Borchern, Germany). TLR9−/− mice came from S. Akira (Osaka University). All mice were kept on their own in the animal facility of the Institut für Medizinische Mikrobiologie, Immunologie und Hygiene der Technischen Universität München under specific pathogen-free conditions.

**Reagents**

The mAbs specific for CD45RB220, CD11b, CD11c, and CD19 were provided by BD Biosciences (Heidelberg, Germany); F4/80 was from eBioscience (Frankfurt, Germany). INH-ODNs were provided by Coley Pharmaceutical or purchased from BioSpring (Frankfurt/Main, Germany). CFSE was purchased from Molecular Probes (Eugene, OR). Imiquimod was bought from InvivoGen (San Diego, CA), oligoribonucleotide (ORN) 22075 from Coley Pharmaceutical (Düsseldorf, Germany), and DOTAP from Sigma-Aldrich (Munich, Germany).

**Injection protocol**

Mice were treated s.c., i.p., or intranasally with INH-ODNs and subsequently s.c. challenged with the stimulatory ODN 1826 or imiquimod. In case of intranasal application of INH-ODNs, mice were anesthetized. Three hours after challenge, mice were sacrificed by cervical dissection, serum was prepared, and IL-12p40 levels were determined.

**Preparation of immune cells**

Conventional bone marrow–derived dendritic cells (BMDCs) were generated according to Inaba et al. (17) with slight modifications. Mice were sacrificed, and femora and femurs were removed, cleaned, and flushed with cell culture medium. They were plated on bacterial petri dishes overnight in culture medium (RPMI 1640, 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin [PAA Laboratories, Pasching, Austria]), and 50 μg/ml 2-ME [Invitrogen, Carlsbad, CA] to remove adherent cells. Nonadherent cells were plated at a density of 7 × 107 cells/dish, and cultured for another 6 d in complete medium in the presence of GM-CSF (15% v/v).

Plasmacytoid BMDCs were generated as above with the exception that FLT3 ligand (R&D Systems Europe, Abingdon, U.K.) was used instead of GM-CSF to mature the cells and medium was additionally supplemented (1% sodium pyruvate, 1% nonessential amino acids, and 1% L-glutamine from PAA Laboratories, Pasching, Austria). Cells were directly plated on 6-well plates at a density of 4.5 × 105 cells/well and cultivated for 7–8 d. FACS analysis demonstrated that the majority of cells obtained were CD45RB220 high and CD11b low.

BMDCs were generated according to Rutschman et al. (18). Briefly, femora and tibiae of mice were rinsed with cell culture medium applied through a 27-gauge syringe. Bone marrow cells were cultured in petri dishes at a density of 2 × 108 cells/dish in the presence of L cell–conditioned medium as a source of M-CSF (15% v/v). The medium used was very low endotoxin DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin (PAA Laboratories), and 50 μg/ml 2-ME. Cells were washed vigorously, and only adherent macrophages were used 6–7 d after plating. FACS analysis showed that these BMDCs were F4/80+ and CD11b+, as described previously (data not shown) (19).

B cells were prepared from spleen. After lysis of erythrocytes with ammonium chloride, B cells were isolated by depletion of CD43+ cells via MACs (Milteny Biotec). FACS analysis demonstrated that 95% of the cells obtained were CD45RB220+.

**TLR-reporter assay**

Stably transfected HEK293 cells expressing human TLR9, human TLR7, or murine TLR9 were described before (20, 21). Stable transfectants (3 × 105 cells/well) were incubated with the respective agonist, for human TLR9, 0.5 μM ODN 10103 (22); for murine TLR9, 0.5 μM ODN 1826 (23); and for human TLR7, 2 μM R-848 (21), with increasing amounts of INH-ODN for 16 h at 37°C in a humidified incubator. Each data point was done in duplicate. Cells were lysed and assayed for luciferase gene activity (using Promega, Madison, WI). Kinetic analysis indices were calculated in reference to reporter gene activity of medium without addition of ODN. Activity of TLR agonist alone was set to 100%, and inhibition of activity in the presence of inhibitory ODN was calculated accordingly.

**Inhibition assay**

BMDCs, BMDCs, plasmacytoid dendritic cells (pDCs), and B cells were stimulated with the TLR9 agonists CpG-ODN 1826 or TLR7 agonists RNA-ORN 22075 (R-1075, C*C*G*U*C*U*G*U*U*G*U*G*A*C*U*C) (24) or imiquimod in the presence of 10-fold titrated amounts of INH-ODNs (0.001–10 μM). pDCs were also stimulated with the TLR9 agonist CpG-ODN 2216. The medium used was RPMI 1640 or DMEM supple-
mented with 10% FCS, (100 IU/ml penicillin, 100 μg/ml streptomycin [PAA Laboratories], and 50 μM 2-ME). Cytokine levels in the supernatant were determined after 24–72 h of culture in 96-well microtiterplates (Falcon).

**Determination of cytokine and intracellular ATP levels**

The cytokines TNF-α, IL-12p40 (R&D Systems Europe, Abingdon, U.K.), and IL-6 (eBioscience, Frankfurt, Germany) were determined using commercially available ELISA kits. IFN-α was measured using Abs from Tebubio (Offenbach, Germany) and Jackson ImmunoResearch Europe (Suffolk, U.K.). The assays were performed according to manufacturer’s manual. Intracellular ATP levels were measured by CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). Briefly, cells were washed and subsequently lysed with CellTiter-Glo Buffer. The ATP content of the lysate was measured via luminometer (Berthold Tittertek Instruments, Pforzheim, Germany). Data were analyzed using SigmaPlot 10.0 (Systat Software).

**Table I. Characteristic of INH-ODNs**

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<tr>
<th>INH-ODN</th>
<th>Sequence 5’–3’</th>
<th>Inhibits</th>
<th>% G Tetrade</th>
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<td>TLR7 + TLR9</td>
<td>96</td>
</tr>
<tr>
<td>21595</td>
<td>T<em>C</em>C<em>T</em>G<em>G</em>G<em>G</em>G<em>A</em>A<em>G</em>T</td>
<td>TLR7 + TLR9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>20844</td>
<td>T<em>C</em>C<em>T</em>G<em>G</em>G<em>G</em>G<em>A</em>A<em>G</em>T</td>
<td>TLR7 + TLR9</td>
<td>6</td>
</tr>
<tr>
<td>24888</td>
<td>T<em>C</em>C<em>T</em>G<em>G</em>G<em>G</em>G<em>A</em>A<em>G</em>T</td>
<td>TLR7 + TLR9</td>
<td>&lt;1</td>
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<tr>
<td>21158</td>
<td>C<em>C</em>T<em>G</em>G<em>G</em>G<em>G</em>G<em>A</em>A<em>G</em>T</td>
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<tr>
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<td>24991</td>
<td>C<em>C</em>T<em>G</em>G<em>G</em>mE<em>G</em>G<em>A</em>A<em>G</em>T</td>
<td>TLR9</td>
<td>13</td>
</tr>
</tbody>
</table>

Analysis of secondary and tertiary structures of INH-ODN by size-exclusion chromatography-HPLC.

*Phosphorothioate binding.

E, 7-deaza-guanine; mE, 7-deaza-2’,O-methyl-guanine.

**FIGURE 1.** G4-stack formation by G-rich INH-ODN. (A) Structure of guanine and 7-deaza-guanine. (B) G4-stack formation via Hoogsten hydrogen bonding. (C) Unmodified INH-ODN 2088 forms G4 stacks, as analyzed by gel filtration. In contrast, higher order structures due to G4 stacks are not visible in the case of G-modified INH-ODN 20844. Arrow denotes the position of higher order structures. Numbers indicate the exact position of individual peaks in minutes.

**B cell proliferation**

Proliferation of B cells was measured by labeling the cells with CFSE (2 μM) and cultivating them for 72 h. Cells were harvested, and the remaining CFSE was measured by FACS.

**Flow cytometry**

Flow cytometry was performed with a Calibur instrument (BD Biosciences, San Jose, CA), and the data were analyzed using the FloJo software (Tree Star).

**Statistics**

More than two equally treated groups were tested for significant differences with one-way ANOVA, post hoc test Holm–Sidak, or, in case the data were not equally distributed, with one-way ANOV A on Ranks post hoc Student–Newman–Keul. Statistical analysis was performed with SigmaStat (SPSS).
FIGURE 2. INH-ODNs impair TLR7- or TLR9-stimulated BMDMs. BMDMs (2 × 10^5 cells/well) were pretreated for 15 min with titrated doses (10^{-3}, 10^{-2}, 10^{-1}, 10^0, 10^1 μM) of INH-ODN, as indicated. Cells were either stimulated with TLR9 ligand CpG-ODN 1826 (100 nM) (A) or TLR7 ligand imiquimod (1 μg/ml) (B, C) for 24 h. To evaluate whether INH-ODNs influence the cells per se, the highest dose (10^1 μM) of each INH-ODN was also evaluated without TLR-mediated stimulation. INH-ODNs were grouped according to their TLR specificity (TLR7 + TLR9, TLR9, or TLR7), as indicated (see also Table I). TNF-α (A, B) and IL-12p40 (C) levels were determined in the culture supernatant. Error bars represent SD of three individual cell cultures. The experiment was repeated once with similar results. ^p < 0.05, ANOVA compared with CpG-ODN 1826 or imiquimod. *p < 0.05, ANOVA compared with INH-ODN 2088 for TLR7/9 INH-ODNs and TLR7 INH-ODNs or to INH-ODN 21158 for TLR9 INH-ODNs.
**Results**

Modification of guanine residues within the G quadruple prevents stack formation of INH-ODNs

Based on the sequence of the potent INH-ODN 2088, which impedes TLR9 and to some extent TLR7 (7, 8, 25), we generated three different INH-ODNs containing a modified guanine within the G quadruple of 2088, as shown in Table I and illustrated in Fig. 1A. The first or the second guanine was replaced by either a 7-deaza-guanine or 7-deaza-2′-O-methyl-guanine to suppress tetrad formation. As analyzed via gel filtration and demonstrated in Fig. 1C and Table I, this modification largely reduced G4-stack formation via Hoogsten hydrogen bonding (Fig. 1B). In contrast and as expected, G4 stacks were formed by the INH-ODN 2088 (Fig. 1C). In addition, we similarly modified INH-ODN 21158, which contained the TLR9-inhibitory motif CCTXXX3–5GGG, is specific for TLR9, and also forms complex structures (Table I). Finally, we created two TLR7 antagonists with G modifications (Table I). The rationale for their design relied on our finding that phosphorothioate INH-ODNs inhibited TLR7 responses in a sequence-independent manner (25). Thus, we used the G-modified INH-ODNs 21595 and 24888 as basis and replaced their TLR9-inhibiting motif CCTXXX3–5GGG by AATXXX3–5GGG.

Inhibitory efficacy of G-modified INH-ODNs on BMDMs and BMDCs from healthy C57BL/6 mice is significantly increased

It was noted earlier that G modification of INH-ODN 2088 appeared not to reduce its inhibitory capacity (7, 10). This encouraged us to explore the functional consequences of G modification and hence the avoidance of G4-stack formation. In a first set of experiments, we tested our series of G-modified INH-ODNs extensively on BMDMs and conventional BMDCs from healthy C57BL/6 mice.

Inhibitory efficacy of G-modified INH-ODNs on BMDMs and BMDCs from healthy C57BL/6 mice is significantly increased

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**FIGURE 3.** Unmodified INH-ODNs are significantly less effective than G-modified INH-ODNs to inhibit IL-6-release by B cells from lupus-prone MRL/MP-lpr/lpr mice. B cells prepared from MRL/MP-lpr/lpr mice (3.0 × 10^5 cells/well) were pretreated for 15 min with titrated doses (10^-3, 10^-2, 10^-1, 10^0, 10^1 μM) of INH-ODN, as indicated, and stimulated with TLR9 ligand CpG-ODN 1826 (100 nM) (A) or TLR7 ligand imiquimod (1 μg/ml) (B) for 48 h. IL-6 levels were determined in the culture supernatant. Error bars represent SD of three individual cell cultures. The experiment was repeated once with similar results. *p < 0.05, ANOVA compared with CpG-ODN 1826 or imiquimod. *p < 0.05, ANOVA compared with INH-ODN 2088 for TLR7/9 INH-ODNs and TLR7 INH-ODNs or to INH-ODN 21158 for TLR9 INH-ODNs. †p < 0.05, ANOVA compared with mock in the absence of a TLR stimulus.
influenced TLR9-mediated stimulation of BMDMs only at higher doses (Fig. 2A). TNF-α secretion of BMDMs stimulated by the TLR7-agonist imiquimod was impaired by these INH-ODNs to different extents (Fig. 2B). Whereas the inhibitory activity of unmodified TLR7/9-specific INH-ODN 2088 appeared comparatively weak, the G-modified INH-ODNs 21595, 20844, and 24888, as well as the TLR7 agonists INH-ODN 105870 and 105871 were significantly more effective (Fig. 2B). The unmodified TLR9-specific INH-ODN 21158 was, as expected, the weakest inhibitor of TLR7-mediated TNF-α secretion. Surprisingly, its G-modified derivatives 24987 and 24991 were efficient inhibitors of the TLR7-induced TNF-α response (Fig. 2B). The analysis of the imiquimod-induced IL-12p40 response of BMDMs revealed that unmodified INH-ODN 2088 failed to impair the secretion of this cytokine at the concentrations tested, whereas G-modified INH-ODNs 21595, 20844, 24888, as well as 105870 and 105871 showed a significantly higher activity (Fig. 2C). As observed above, INH-ODNs 24987 and 24991 derived from the TLR9-specific antagonist 21158 inhibited TLR7-mediated IL-12p40 release and were significantly more efficient than the unmodified INH-ODN 21158, which showed again only a very weak inhibitory activity.

Almost identical inhibition results were obtained when our series of G-modified INH-ODNs was used to impair conventional BMDCs (Supplemental Fig. 1). In contrast, unmodified INH-ODN 2088 impaired imiquimod-induced IL-12p40 release of BMDCs, but not of BMDMs (Supplemental Fig. 1, Fig. 2C). The reason for this difference is at present unclear. We conclude that G-modified INH-ODNs 21595, 20844, 24888, 24987, and 24991 are at least equal to unmodified INH-ODNs 2088 and 21158 in their ability to inhibit TLR9-driven TNF-α responses by BMDMs and BMDCs. However, all G-modified INH-ODNs are significantly more efficient to impair TLR7-induced IL-12p40 responses by BMDMs and BMDCs.

**G-modified INH-ODNs block activation of B cells from lupus-prone mice, whereas unmodified INH-ODNs stimulate this cell type at higher doses**

B cells are known to be involved in the pathogenesis of SLE by the production of autoantibodies (26). We therefore isolated B cells from autoimmune MRL/Mp-lpr/lpr mice, which develop a lupus-like syndrome. CpG-ODN 1826–stimulated B cells were efficiently impaired by TLR7/9-specific G-modified INH-ODNs 21595, 20844,

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** G-modified INH-ODNs are significantly more efficient than unmodified INH-ODNs to inhibit CpG- or imiquimod-induced intracellular ATP levels by B cells from lupus-prone MRL/Mp-lpr/lpr mice. In (A) and (B), the intracellular ATP content was evaluated upon stimulation with CpG-ODN 1826 or imiquimod, respectively, as described in Fig. 3A and 3B. Error bars represent SD of three individual cell cultures. The experiment was repeated twice with similar results. *p < 0.05, ANOVA compared with CpG-ODN 1826 or imiquimod. **p < 0.05, ANOVA compared with INH-ODN 2088 for TLR7/9 INH-ODNs and TLR7 INH-ODNs or with INH-ODN 21158 for TLR9 INH-ODNs. ***p < 0.05, ANOVA compared with mock in the absence of a TLR stimulus.
and 24888 (Fig. 3A). Unmodified INH-ODN 2088 was significantly less effective and even stimulated MRL/Mp-lpr/lpr B cells at higher doses to release IL-6 in the absence of CpG-ODN 1826 (Fig. 3). Also, TLR9-specific G-modified INH-ODNs 24987 and 24991 were significantly more efficient than the parent unmodified INH-ODN 21158, and were as capable as G-modified TLR7/9 INH-ODNs, whereas TLR7-specific INH-ODNs were less efficient (Fig. 3A). Imiquimod-activated MRL/Mp-lpr/lpr B cells were inhibited by TLR7/9- and TLR7-specific G-modified INH-ODNs (Fig. 3B). Again, compared with G-modified INH-ODNs, unmodified INH-ODN 2088 was significantly less efficient (Fig. 3B). As expected, TLR9-specific INH-ODNs with the exception of G-modified INH-ODN 24991 inhibited less potently (Fig. 3B).

We then quantified intracellular ATP levels of B cells, which is a marker for cellular proliferation, but also of cellular activation (27). The experiments revealed that both unmodified INH-ODNs 2088 and 21158 significantly increased this parameter in the absence of another stimulus (Fig. 4A). Presumably, as a consequence, both INH-ODNs failed to lower the ATP content upon stimulation with CpG or imiquimod (Fig. 4). In contrast, G-modified INH-ODN 21595, 20844, 24888, 24987, and 24991 lowered CpG-ODN 1826–induced ATP content to the prestimulation level, whereas TLR7-specific G-modified INH-ODNs were almost without effect (Fig. 4A). In case of imiquimod-stimulated B cells, G-modified INH-ODNs 21595, 20844, 24888, 24991, 105870, and 105871 reduced the ATP content to the pretreatment level and were significantly more effective than unmodified INH-ODNs 2088 and 21158 (Fig. 4B). TLR9-specific INH-ODNs, with the exception of INH-ODN 24991, were not active (Fig. 4B). Interestingly, we also noted that G-modified INH-ODNs 21595, 24888, 24987, and 24991 reduced prestimulation ATP levels of B cells, and this difference was in some experiments significant (Fig. 4B), indicating that the spontaneous ATP level of B cells from MRL/Mp-lpr/lpr mice is at least in part influenced by nucleotide-recognizing TLR signals. Almost identical results regarding IL-6 release and ATP levels were obtained with wild-type B cells (Supplemental Fig. 2).

We also explored the mechanism, how unmodified INH-ODN 2088 stimulates B cells at higher doses. Using TLR7/9−/− mice, we show that TLR9 was crucial for INH-ODN 2088–mediated stimulation of B cells to secrete IL-6 and to increase intracellular ATP levels (Fig. 5).

As expected, CpG-ODN 1826 stimulated B cells from autoimmune MRL/Mp-lpr/lpr mice to proliferate as revealed by a reduction of CFSE fluorescence intensity (compare Fig. 6A and 6B). Unexpectedly, incubation of these cells with unmodified INH-ODN 2088 was in the absence of CpG-ODN 1826 also significantly stimulated proliferation (Fig. 6C, 6D). Whereas G-modified INH-ODNs 24888 and 105870 had no effect (Fig. 6D, 6E, 6F). Interestingly, only G-modified TLR7/9-specific INH-ODN 24888 almost completely impaired CpG-ODN 1826–induced B cell proliferation, whereas unmodified INH-ODN 2088 was only partially effective and significantly less effective than G-modified INH-ODN 24888 (Fig. 6F, 6G, 6H). TLR7-specific G-modified INH-ODN 105870 showed no effect, as expected (Fig. 6H, 6I).

In summary, G-modified INH-ODNs were clearly more effective to impair B cells from autoimmune MRL/Mp-lpr/lpr and wild-type mice. Paradoxically, unmodified INH-ODNs even stimulated B cells at high doses.

G-modified INH-ODNs inhibit pDCs as efficiently as unmodified INH-ODNs

The signature cytokine observed in SLE is IFN-α produced by pDCs. The analysis of pDCs from MRL/Mp-lpr/lpr mice revealed that IFN-α secretion of these cells induced by CpG-ODN 1826 was impaired by all INH-ODNs tested, even by the TLR7-specific INH-ODNs (Fig. 7A). Although not statistically significant, unmodified INH-ODN 2088 and TLR7-specific INH-ODNs appeared to be less effective.

These results were confirmed by the analysis of wild-type pDCs (Supplemental Fig. 3). Because type A/D oligonucleotides stimulate pDCs to produce significantly higher amounts of IFN-α (28), we stimulated wild-type pDCs with CpG-ODN 1826 (100 nM), imiquimod (1 μg/ml), or titrated doses (10−10, 10−9, 10−8 μM) of INH-ODNs, as indicated, for 48 h, and IL-6 levels were determined in the culture supernatant. The experiment was performed twice with similar results. (B) Wild-type and TLR9−/− B cells were stimulated, as described in (A), and intracellular ATP levels were determined. The experiment was performed twice with similar results. *p < 0.001, ANOVA compared with mock in the absence of a TLR stimulus.
which interact with nonnucleotide PAMPs. IL-12p40 release by BMDMs induced by the TLR4 ligand endotoxin or the TLR2 ligand Pam2Cys was not impaired by any of the INH-ODNs tested (Fig. 8A, 8B). However, high doses of the unmodified INH-ODN 2088 amplified significantly the response to endotoxin (Fig. 8A). In contrast, G-modified INH-ODN 24888 did not influence the endotoxin response (Fig. 8A). Because the endotoxin content of all INH-ODNs used in this study was below 0.005 EU/ml and the INH-ODN did not stimulate BMDM in the absence of PAMPs, it is highly unlikely that contaminating substances were responsible.

**FIGURE 6.** Proliferation of B cells from lupus-prone MRL/Mp-lpr/lpr mice is suppressed by G-modified INH-ODNs, but stimulated by unmodified INH-ODNs at high doses. B cells from MRL/Mp-lpr/lpr mice (3.5 × 10⁶ cells/well) were labeled with CFSE (2 µM) and left either untreated (A) or incubated with CpG-ODN 1826 (1 µM) (B), INH-ODN 2088 (10 µM) (C), INH-ODN 24888 (10 µM) (D), INH-ODN 105870 (10 µM) (E), INH-ODN 2088 (10 µM) plus CpG-ODN 1826 (1 µM) (F), INH-ODN 24888 (10 µM) plus CpG-ODN 1826 (1 µM) (G), or INH-ODN 105870 (10 µM) plus CpG-ODN 1826 (1 µM) (H). Cell cultures were harvested after 3 d and analyzed by FACS. Numbers in each graph designate percentage of proliferating B cells. The experiment was repeated twice with similar results. In (I), trittrated doses (10⁻² 10⁻¹, 10⁻µ M) of INH-ODN 2088, INH-ODN 24888, or INH-ODN 105870 were used to inhibit the proliferation of B cells from MRL/Mp-lpr/lpr mice. The experiment was performed, as described above. Data shown are from three individual experiments. *p < 0.05, ANOVA compared with CpG-ODN 1826. **p < 0.05, ANOVA compared with INH-ODN 2088.
for the augmented endotoxin response. Interestingly, IL-12p40 release triggered by the TLR3 ligand poly(I:C) was impaired by all INH-ODNs tested (Fig. 8C). Thus, in addition to TLR7 and 9, also the nucleic acid-recognizing TLR3 was impaired by these INH-ODNs.

We conclude from these data that G modification of INH-ODNs improved their inhibitory ability with respect to several, but not all immune responses, and never resulted in a loss or decrease of the inhibitory activity, but reduced off-target effects. INH-ODNs 20844 and 24888 were among the most efficient INH-ODNs within this series.

G-modified INH-ODNs are more efficient to impair a systemic TLR7- or TLR9-mediated IL-12p40 release in vivo

To further explore the inhibitory potential of G-modified INH-ODNs, we analyzed their ability to interfere with TLR9- or TLR7-induced immune responses in vivo. Because G-modified INH-ODN 24888 belonged to the most potent INH-ODNs, we compared this INH-ODN with unmodified INH-ODN 2088. We first determined a dose of CpG-OGN 1826 that potently induced IL-12p40 in vivo and found that s.c. injection of 50 μg/mouse strongly increased the level of this cytokine (Fig. 9A). As demonstrated in Fig. 9B, IL-12p40 levels in the serum of mice injected s.c. with CpG-ODN 1826 were significantly reduced by s.c. pretreatment with unmodified INH-ODN 2088; however, G-modified INH-ODN 24888 was more effective. TLR7-specific INH-ODN 105870 used as negative control for TLR9 stimulation was inactive.

We repeated this experiment with the modification that injection sites for stimulus and inhibitor were separated. Thus, mice were injected s.c. with CpG-ODN 1826, but pretreated i.p. with unmodified INH-ODN 2088 and G-modified INH-ODN 24888. Both INH-ODNs reduced IL-12p40 serum levels significantly (Fig. 9C).

Next, we evaluated whether mucosal application of INH-ODNs would also efficiently impair a systemic inflammatory response. Mice were treated intranasally with unmodified INH-ODN 2088, G-modified INH-ODN 24888, or TLR7-specific INH-ODN 105871, and were challenged s.c. with CpG-ODN 1826 2 h later. In this case, G-modified INH-ODN 24888 was significantly more efficient than unmodified INH-ODN 2088 to reduce IL-12p40 serum levels (Fig. 9D). TLR7-specific INH-ODN 105871 showed again no effect.

To further examine TLR specificity of INH-ODNs in vivo, we administered the TLR7 ligand imiquimod s.c. and pretreated the
mice i.p. with TLR7/9-specific, unmodified INH-ODN 2088 and G-modified INH-ODN 24888, or TLR9-specific INH-ODN 21158. Whereas the latter INH-ODN and INH-ODN 2088 showed only weak inhibition, the TLR7/9-specific G-modified INH-ODN 24888 significantly impaired IL-12p40 release (Fig. 9E).

Furthermore, we explored whether G-modified INH-ODNs also inhibit CpG-ODN 1826–induced immune responses in autoimmune MRL/Mp-lpr/lpr mice in vivo. IL-12p40 serum levels induced by s.c. injected CpG-ODN 1826 were reduced by i.p. pretreatment with unmodified INH-ODN 2088 and G-modified INH-ODN 24888 (Fig. 10). However, the latter was significantly more active. The TLR7-specific INH-ODN 105871 showed in these experiments no effect.

All together these data suggest that G-modified INH-ODNs were at least as or even more effective in vivo as unmodified INH-ODN 2088.

**Inhibition of human TLR7 and TLR9 by G-modified INH-ODNs**

Finally, we evaluated whether human TLR7 and TLR9 are impaired by G-modified INH-ODNs. Using HEK293 cells transfected with human TLR7 or TLR9 together with a NF-κB luciferase reporter construct (20, 21), we show that activation of the cells with the TLR9 ligand CpG-ODN 10103 was impaired by all INH-ODNs tested (Supplemental Fig. 4A). HEK293 transfectants expressing human TLR7 and stimulated with the TLR7 ligand R-848 were impaired by TLR7/9-specific INH-ODN 2088, 21595, 20844, and 24888, whereas TLR9-specific INH-ODNs were less effective, as expected (Supplemental Fig. 4B). Taken together, G modification of INH-ODNs neither negatively nor positively influenced their inhibitory activity for human TLRs as analyzed by this reporter system. Further work is required to explore whether G-modified INH-ODNs will inhibit primary human immune cells more efficiently compared with unmodified INH-ODNs.
INH-ODNs were demonstrated to suppress inflammatory immune responses in murine models of autoimmune diseases such as SLE (9, 16, 29–31) and were described to contain G stretches forming higher ordered structures. However, as shown in this study, prevention of G4 stacks by G modification of INH-ODNs considerably increased the inhibitory ability of INH-ODN in a cell type- and immune response–specific way. Thus, G modification significantly increased

**Discussion**

INH-ODNs were demonstrated to suppress inflammatory immune responses in murine models of autoimmune diseases such as SLE (9, 16, 29–31) and were described to contain G stretches forming higher ordered structures. However, as shown in this study, prevention of G4 stacks by G modification of INH-ODNs considerably increased the inhibitory ability of INH-ODN in a cell type– and immune response–specific way. Thus, G modification significantly increased
impairment of IL-12p40 secretion by murine BMDMs and conventional BMDCs stimulated with TLR7 ligands, although it did not reduce the inhibition of TLR9-mediated cytokine responses. Moreover, B cell responses such as IL-6 release, accumulation of intracellular ATP, and proliferation induced by TLR7 or TLR9 ligands were significantly more reduced by G-modified INH-ODNs. Furthermore, it avoided the TLR9-dependent stimulation of B cells upon treatment of the cells with higher doses of the inhibitor and the augmentation of TLR4 responses. In vivo, G-modified INH-ODN 24888 reduced IL-12p40 serum levels in TLR9-stimulated mice with similar efficiency to the suppression of responses stimulated by poly(I:C). The inhibitory effects were observed at similar doses and with similar efficiency to the suppression of responses stimulated by TLR7 or TLR9. Therefore, such INH-ODNs appear to be even more useful in the treatment of autoimmune diseases due to their suppressive effect on TLR7, TLR9, and TLR3.

G modification of INH-ODNs also avoided the unexpected stimulation of B cells from lupus-prone MRL/Mp-lpr/lpr and wild-type mice in the absence of TLR stimulation. B cells are involved in the pathogenesis of the lupus-like syndrome in MRL/Mp-lpr/lpr mice (39), and endogenous ligands such as chromatin-IgG complexes can trigger rheumatoid factor–positive B cells, further underlining their role in autoimmune diseases (5). Therefore, the activation of B cells by unmodified INH-ODNs at high doses might be particularly cumbersome and argues even further for the application of G-modified INH-ODN. Interestingly, IRS 954, an INH-ODN containing a G quadruplex, appeared to increase anti-dsDNA IgG1 and IgG2a levels as well as anti-Smith IgG Abs, whereas IRS 661, an INH-ODN lacking a G quadruplex, lowered the levels of these Abs (29).

An important consideration for the application of INH-ODNs to treat autoimmune diseases is their potential for TLR-unrelated side effects, which may be imposed by the formation of G tetrad. INH-ODN 2114, which is identical to INH-ODN 2088, except that a cytosome at the seventh position of the sequence is replaced by an adenine, is a potent TLR9 antagonist in vitro (7) and in vivo (31), and also forms G4 stacks (8). Infection of BMDM with *S. enterica* subsp. *enterica serovar* Typhimurium induced TLR9 expression, and treatment of the cells with INH-ODN 2114 unexpectedly resulted in increased intracellular bacterial burden (13). Moreover, the same effect was induced in TLR9-deficient cells, and a suppression of TLR2-mediated responses was observed, which may explain the lack of control of intracellular bacterial growth. In our studies, although not reducing TLR2-mediated effects, INH-ODN 2088 augmented TLR4 responses of BMDMs. Thus, the inherent characteristic of an unmodified INH-ODN to form G4 stacks may lead to unpredictable off-target effects imposed by effects on other than the nucleic acid–recognizing TLRs, which will potentially impair its further development and clinical use. The avoidance of the formation of higher ordered structures by applying specific G modifications therefore may reduce the risk for the occurrence of potential off-target effects, which should be further investigated.
Current treatments for lupus are limited. Besides chloroquine, corticosteroids represent a standard therapy for SLE. However, low-dose oral prednisone is often not sufficient to control disease activity, and high prednisone is required to combat flares. Interestingly, the latter treatment correlates with a downregulation of IFN-regulated genes (33), underlining the relevance of these cytokines in SLE. Glucocorticoids fail to prevent IFN-α release by human pDCs stimulated with TLR7 or TLR9 ligands or with immune complexes from SLE patients (40). Glucocorticoids also fail to affect the viability of TLR-7 or TLR-9-stimulated pDCs because they are unable to impair TLR-driven NF-κB activation in this cell type (40). That study also demonstrated that an inhibitory ODN, IRS 954, was not only able to reduce the production of IFN-α in these cells, but also reduced NF-κB activation (40). Thus, INH-ODNs might replace or at least spare the use of corticosteroids in SLE, and a combination with G-modified INH-ODNs may be more effective compared with unmodified INH-ODNs.

Our results also show that mucosal application of INH-ODN 2088 and 24888 reduced serum cytokine levels, although only INH-ODN 24888 induced a significant reduction. This route of application appeared to be as efficient as i.p. administration, and both INH-ODNs prevented CpG-ODN 1826–induced IL-12p40 release by B cells, but also reduced NF-κB activation (40). Thus, INH-ODNs might replace or at least spare the use of corticosteroids in SLE, and a combination with G-modified INH-ODNs may be more effective compared with unmodified INH-ODNs.

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Disclosures

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