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Inhibitors of TLR-9 Act on Multiple Cell Subsets in Mouse and Man In Vitro and Prevent Death In Vivo from Systemic Inflammation

Omar Duramad,1 Karen L. Fearon, Bonnie Chang, Jean H. Chan, Josh Gregorio, Robert L. Coffman, and Franck J. Barrat2

In parallel with the discovery of the immunostimulatory activities of CpG-containing oligodeoxynucleotides, several groups have reported specific DNA sequences that could inhibit activation by CpG-containing oligodeoxynucleotides in mouse models. We show that these inhibitory sequences, termed IRS, inhibit TLR-9-mediated activation in human as well as mouse cells. This inhibitory activity includes proliferation and IL-6 production by B cells, and IFN-α and IL-12 production by plasmacytoid dendritic cells. Our studies of multiple cell types in both mice and humans show the optimal IRS to contain a GGGG motif within the sequence, and the activity to require a phosphorothioate backbone. Although the GGGG motif readily itself leads to formation of a tetrameric oligodeoxynucleotide structure, inhibitory activity resides exclusively in the single-stranded form. When coinkjected with a CpG oligodeoxynucleotide in vivo, IRS were shown to inhibit inflammation through a reduction in serum cytokine responses. IRS do not need to be injected at the same site to inhibit, demonstrating that rapid, systemic inhibition of TLR-9 can be readily achieved. IRS can also inhibit a complex pathological response to ISS, as shown by protection from death after massive systemic inflammation induced by a CpG-containing oligodeoxynucleotide. The Journal of Immunology, 2005, 174: 5193–5200.

Toll-like receptors are among the most widely expressed recognition receptors of the innate immune system (1). Of the 10 TLRs identified in humans, four recognize nucleic acids: dsRNA (TLR-3), ssRNA (TLR-7 and TLR-8), or unmethylated CpG motifs in viral and bacterial DNA (TLR-9). This demonstrates the fundamental importance of microbial DNA and RNA in response to pathogenic microorganisms (2, 3). Signaling through TLR-9, which is expressed primarily by subsets of B cells and dendritic cells in humans, can be accomplished with high efficiency with small, synthetic oligodeoxynucleotides (ODN) containing optimized CpG motifs (termed immunostimulatory sequences (ISS)). ISS show great promise as potent vaccine adjuvants, inducers of immune deviation toward a Th1 response, and inhibitors of Th2-mediated allergic responses (2, 4). Inappropriate TLR-9 signaling may also be involved in some diseases, in particular the generation of B cell hyper-reactivity and anti-DNA Ab production in lupus (5, 6). Thus, specific inhibitors of TLR-9 function would be quite valuable as tools to understand TLR-9-mediated responses and, potentially, as therapeutics for some autoimmune diseases.

Natural and synthetic DNA sequences have also been identified that are able to inhibit activation through TLR-9. These sequences are derived from diverse sources, including viral sequences, mutated CpG sequences, and repeats of the TTAGGG motif present in mammalian telomeres (7–12). We have termed these immunoregulatory DNA sequences (IRS), because they act to regulate immunostimulatory TLR-9 ligands. The mechanism of action of these IRS is not known; however, evidence to date suggests that IRS do not interfere with cellular uptake, which is critical for ISS activity, nor do they simply compete for binding of the receptor for ISS, TLR-9. Recently, it was shown that IRS can affect Th1 priming through the inhibition of STAT1, -3, and -4 (12, 13). However, much remains to be determined about the mechanisms of action of IRS. The sequence and structural requirements for optimum IRS activity have not been fully defined. Furthermore, studies have been almost exclusively limited to mouse models (7–12, 14, 15), and it is not known whether IRS have similar activities in humans. We describe in this report IRS activity with respect to base composition, secondary structures, as well as backbone requirement for optimal activity. We present evidence that IRS effectively inhibits ISS activation of both major populations of TLR-9-bearing cells in human blood. In particular, we show that IRS can block IFN-α production from plasmacytoid dendritic cells (PDC) in response to all three classes of ISS (CpG-A, -B, and -C) and IL-12 production when additional signal through CD40 was added. Furthermore, we demonstrate that IRS are active in vivo and do not need to be present at the site of inflammation to be active. Finally, we evaluate the potency of IRS in vivo in preventing death after massive systemic inflammation induced by TLR-9 activation.

Materials and Methods

Oligonucleotide synthesis

Phosphorothioate ODNs were prepared as previously described (16). The prototypes for the ISS classes used were: D19, 5′-GGGcactagag cagGGGGG (CpG-A ISS); 1018, 5′-TGCAGTGAACGGTGAGATGA (CpG-B ISS); and C274, 5′-TCCGCAACGGTGAGATG (CpG-C ISS).
and T2G4T2 to form a 10 mg/ml solution in Dulbecco's PBS. The sample mixture of ODNs was obtained by adding equal molar amounts of 2088 turing and flash cooling 0.33 mg/ml samples in 1/1000 sodium phosphate, pH 7.2, at a flow rate of 0.75 ml/min. Detection was at 260 nm. The tetrameric form of 2088 was purified under the same conditions, except that detection was at 290 nm.

Size exclusion chromatography

Analytical SEC was performed using a Superdex 200 HR 10/30 column (GE Healthcare) in 10 mM sodium phosphate/150 mM sodium chloride, pH 7.2, at a constant 200 mV for 3 h. Bands were visualized with Stains-All (Eastman Kodak) and allowed to develop overnight before being scanned on a densitometer (Molecular Devices).

Native gel analysis

Single-stranded 2088 and the T5G3T3 ODN were prepared by heat denaturing and flash cooling 0.33 mg/ml samples in 1/10 Dulbecco’s PBS. The mixture of ODNs was obtained by adding equal molar amounts of 2088 and T5G3T3 to form a 10 mg/ml solution in Dulbecco’s PBS. The sample was then heat denatured at 95°C and cooled to room temperature before being removed to 4°C overnight to allow G-tetrad formation. DNA (0.05 OD) was loaded in 10 μl of a 20% glycerol/water solution onto a nondeaturing 20% polyacrylamide gel (19/1, acrylamide/N,N'-methylenebisacrylamide) with 1× Tris-borate-EDTA and was run at ambient temperature at a constant 200 mV for 3 h. Bands were visualized with Stains-All (Eastman Kodak) and allowed to develop overnight before being scanned on a densitometer (Molecular Devices).

In vitro stimulation of mouse cell subsets

Spleens from 6- to 12-wk-old BALB/c mice were harvested, and the splenocytes were isolated using standard techniques. Cells were stimulated with 0.7 μM ISS.

Isolation and in vitro stimulation of purified human cell subsets

Buffy coats were obtained from the Stanford Blood Center. PBMC were isolated by centrifugation through a Ficoll (Pharmacia Biotech) density gradient. B cells were isolated using CD19 enrichment as previously described (16). Purity was routinely 99%. Experiments were conducted with 2–4 × 10^5 B cells/well cultured in 96-well, flat-bottom plates. Stimulation was performed with 0.7 μM ISS or selective concentrations of IRS ranging from 0.0875 to 2.8 μM for 24–72 h.

ELISA and measurement of proliferation

All cytokines were measured by ELISA. Human IFN-α production was assayed with reagents from PBL Biomedical Laboratories. Human IL-6 and IL-12 were assayed using CytoSetAb pairs from BioSource International, and human IL-10 was assayed using Abs from BD Pharmingen. Mouse IL-6 and IL-12 were assayed using Abs from BD Pharmingen, whereas we used the Quantikine Immunoassay (R&D Systems) was used for measurement of mouse TNF-α. All kits and Ab pairs were used according to the manufacturers’ instructions. To measure B cell proliferation, the cells were pulsed with [3H]thymidine (1 mCi/well; Amersham Biosciences) and incubated for an additional 8 h during the activation. The cells were then harvested, radioactive incorporation was determined using standard liquid scintillation techniques, and data were expressed as cpm.

Mice and in vivo experiments

Six- to 12-wk-old BALB/c mice were used for all in vivo experiments. Mice were injected s.c. with 25 μg of 1018 ISS and variable quantities of IRS as mentioned in the figure legends. In some cases, IRS were injected using the i.p. or i.v. route. All injections used ODN in saline. Two hours after the injections, blood was harvested, and serum was prepared using standard procedures.

Induction of a lethal TLR-9-dependent systemic inflammation

Six- to 12-wk-old BALB/c mice were injected i.p. with 20 mg of n-galactosamine (n-Gal; Sigma-Aldrich) in saline and s.c. with 50 μg of 1018 ISS and variable quantities of IRS or control ODN as described in the figure legends. Mice were then evaluated for survival over a 7-day period.

Results

G-rich IRS have strong inhibitory properties in mouse splenocytes

Various ODNs with suppressive properties have been described, but the basis for their activity is still unrevealed. To compare the inhibitory activities of multiple types of IRS, splenocytes from BALB/c mice were stimulated with 1018 ISS in combination with each type of IRS at a 1:1 molar ratio, and cytokine production was monitored. Although GG-containing (1019), GC-rich (1955, 1895, and 311), and G-rich (2088, 2114, 533, and 708) ISS were all

![FIGURE 1. G-rich containing IRS have strong inhibitory properties in mouse splenocytes. Splenocytes from BALB/c mice were stimulated for 48 h with 1018 ISS alone or in the presence of various IRS-like or inactive control sequences at a 1:1 molar ratio. ODNs were premixed before being used to stimulate the splenocytes. IL-6 and IL-12 production were evaluated by immunoassay and were plotted as a percentage of 1018 ISS alone. Averages of three independent experiments are shown. *** p < 0.001.](http://www.jimmunol.org/2)
able to inhibit IL-6 and IL-12 induced by 1018 ISS, the G-rich IRS were the most effective IRS (Fig. 1).

IRS can inhibit ISS-activated human B cells independently of IL-10

To explore the potential clinical application of IRS, we investigated whether these sequences were active in man by examining their ability to block human B cells activation by 1018 ISS. We used IL-6 production and B cell proliferation as readouts, because these are the most robust responses induced by ISS. Testing a representative sequence from each type of IRS, the G-rich sequence again proved clearly superior in its inhibition of 1018 ISS-induced IL-6 production in a dose-dependent manner (Fig. 2A). Similar effects were observed by measurement of proliferation (data not shown). In addition to IL-6, B cells make the inhibitory cytokine IL-10 in response to ISS stimulation. IL-10 induction by 1018 ISS was also inhibited by 2088 (Fig. 2B) and other IRS (data not shown), suggesting that IRS do not act by enhancing the production of IL-10.

Presence of four contiguous guanosines is optimal for IRS activity

To define more precisely the sequence requirements for IRS activity, defined alterations were made in the G-rich motif (GG C GGGG) of IRS 2088, and sequences were tested for inhibition of proliferation and IL-6 production in 1018 ISS-stimulated human B cells. Removing the first two Gs did not alter the IRS activity (Fig. 3, C827). However, reducing the number of sequential G residues led to reduction (G3 motif, 2243) and ultimately loss (G2 motif, C826) of inhibitory capacities of the ODN. Breaking the G4 motif with a single base substitution (C824) also led to total loss of activity, suggesting that four contiguous Gs are required for optimal IRS activity in human B cells (Fig. 3). One complication with IRS 2088 is the presence of a CpG located in front of the G4 motif. This CpG is not part of a classical CpG motif known to stimulate TLR-9, but it may favor binding with TLR-9. Interestingly, replacing this CG with an AG motif (C824) did not affect IRS function, suggesting again the G4 motif, but not the CpG, to be important (Fig. 3).

IRS containing four contiguous guanosines can form parallel stranded G-tetrads that are stable under assay conditions

ODNs containing four contiguous guanosine residues are known to self-assemble into four-stranded helices stabilized by planar Hoogsteen base-paired quartets of guanosine (18, 19). The structure and stability of these tetraplex ODNs are influenced by surrounding sequence composition, ODN concentration, and the nature and concentration of monovalent cations, such as sodium and/or potassium, in the ODN diluent (20, 21). Evaluation of 2088 and most other G4-containing IRS dissolved in PBS by SEC, a method previously shown to be able to resolve G-tetrad and monomeric G4-containing ODNs (22, 23), showed the presence of...
two distinct peaks (Fig. 4A). This allowed us to isolate, characterize, and evaluate the activity of the two physical forms of 2088. Both the monomeric and G-tetrad forms of 2088 remained stable for >3 mo under conditions of concentration, pH, and temperature similar to those used in biological assays (data not shown). Evidence that the larger form of 2088 is a G-tetrad was provided by native PAGE. 2088 (a pentadecamer) was annealed with the octamer 5'-T2G4T2 before being run on the polyacrylamide gel. 5'-T2G4T2 is an ODN that readily forms the G-tetrad. If 2088 itself -T2G4T2 ODN at high H11032 stranded G-tetrad. FIGURE 4. IRS containing four contiguous guanosines form parallel-stranded tetrameric complexes (Fig. 4B).

Single-stranded, but not G-tetrad, form contains IRS activity

To test whether the G-tetrad structure is required for the activity of G4-containing IRS, stably purified monomeric and G-tetrad forms of the IRS 2088 (see Fig. 4A) were tested in the human B cell assay as described in Fig. 3. Surprisingly, only the monomeric form of IRS 2088 inhibited, in a dose-dependent manner, both IL-6 production (Fig. 5A) and proliferation (data not shown) by 1018 ISS-activated B cells, whereas the G-tetrad form had no effect (Fig. 5A). A mixture of both forms (2088 mixed, as shown in Fig. 4A) exhibited an intermediate inhibition. These data demonstrate clearly that only the monomeric form is active as an IRS. Similar results were obtained in mouse splenocytes (data not shown). The role of G-tetrad formation was independently tested by the sequence IRS 2296, which contained a 7-deaza guanosine base that prevents the G-tetrad formation of 2088 (data not shown). As previously described in mice (10), IRS 2296 showed similar activity to single-stranded 2088 in the human B cell assay (Fig. 5B).

Phosphorothioate backbones are important for full IRS activity

It has been widely reported that in addition to sequence composition, the backbone of the CpG-containing ODN influences ISS activity. PO backbones are the natural backbone of bacterial DNA, but ODNs used in clinical trials are produced with PS backbones to increase resistance to nucleases and, thus, stability. We examined backbone composition requirements of different G-rich IRS in the ISS-stimulated human B cell assay. Two different G4-containing IRS (2088 and 708) and the recently described telomeric-based sequence containing TTAGGG repeats (IRS 888) were synthesized with either PS or PO backbones. A version of 2088 sequence with alternate PO and PS bases was also evaluated (2088 PO/PS). 2088 PS, 708 PS, and 888 PS were active, whereas their PO version had almost no activity (Fig. 6). Interestingly, the 2088 PO/PS had a somewhat intermediate activity compared with pure PO or PS. Similar results were obtained in mouse splenocytes (data not shown).

FIGURE 5. The single-stranded, not the G-tetrad, form contains IRS activity. A, Human B cells were stimulated with 1018 ISS (0.7 μM), either alone or in the presence of various concentration of 2088 (from an IRS:ISS ratio of 2:1 up to 1:8). G-tetrad and monomeric forms of 2088 were purified as shown in Fig. 4A and tested as compared with 2088. B, Comparison of 2088 and 2296, which is the 2088 sequence with the second G replaced by a 7-deaza guanosine. IL-6 production by B cells was evaluated by immunoassay. Averages of eight donors are shown. *** , p < 0.001.

IRS inhibits ISS-induced IFN-α from human PDC

Given the robust ability of IRS to inhibit mouse splenocytes and human B cells, we examined whether these sequences could inhibit the other important TLR-9-positive APC type, the PDCs. In human peripheral blood, B cells and PDCs are the two main subsets of cells that express TLR-9, although ISS elicit quite different cytokine responses from each. In response to ISS stimulation, B cells secrete proinflammatory cytokines such as IL-6, whereas PDCs secrete predominantly IFN-α (4, 17, 24). In addition, when stimulated with ISS and CD40L, PDC can produce moderate amounts of IL-12 (17, 25). We stimulated PDCs with the three different classes of ISS (CpG-A, -B, and -C) and IRS at a 1:1 ratio (Fig. 7A). These ISS classes produce distinctly different responses from PDC (17), including different maximum levels of IFN-α; however, IRS 2088 (and IRS 869; data not shown) effectively inhibited IFN-α production induced by each type of ISS. In addition, IRS was
capable of inhibiting both IFN-α and IL-12 production in ISS-stimulated PDCs in the presence of CD40L (Fig. 7B). Inactive control ODN were unable to block cytokine responses. These findings suggest that IRS can inhibit TLR-9 signaling in different cell types, and they may be useful for potential treatments in IFN-α-associated diseases.

**IRS are active in vivo and act systemically to block TLR-9 stimulation**

When mice are injected with ISS, they respond very quickly, with substantial amounts of IL-6, IL-12, and TNF-α detectable in the serum after 2 h. To explore whether these IRS are active in vivo, mice were coinjected s.c. with 1018 ISS and IRS 869 at varying ratios, and proinflammatory cytokines levels were measured in serum collected 2 h later (Fig. 8A). IRS 869 coinjection caused a dose-dependent inhibition of these cytokines, whereas the inactive control ODN did not inhibit (Fig. 8A). Furthermore, IRS, to function, did not need to be injected at the same site as ISS, suggesting that its effect was systemic in this short 2-h assay. This was demonstrated by injecting 1018 ISS s.c. on the left side and an equal amount of IRS at different anatomical locations. Serum was collected for cytokines measurement 2 h postinjection. Strikingly, injection of IRS 869 at different sites either s.c. on the opposite flank i.p. or i.v. were able to systemically block IL-6 and IL-12 production from 1018 ISS-treated mice with the same efficiency as coinjection at the same site (Fig. 8B). This suggests that IRS are able to inhibit inflammation systemically and highlights their potential for therapeutic application.

**IRS prevent death of mice after lethal TLR-9-dependent systemic inflammation**

To provide additional support for the idea that IRS can function as an inhibitor of a complex inflammatory response, we used a previously described TLR-9-dependent murine sepsis model (26). In normal mice, ISS stimulation does not induce death; however, when mice are pretreated with D-Gal, they become highly susceptible to inflammation and die rapidly upon injection of ISS or very low doses of LPS. Neither IRS (200 μg) nor inactive control ODN (200 μg) caused death in D-Gal-sensitized mice, whereas 1018 ISS caused death in 90% of the mice by 24 h (Fig. 9 and data not shown), demonstrating that inducing death requires an active CpG-containing ODN. Injection of IRS 869 prevented death of D-Gal-sensitized mice in a dose-dependent manner, whereas inactive control-ODN did not. At the highest dose (200 μg of IRS), 100% of the mice were protected, almost 90% with 100 μg of IRS and still >70% at a 1:1 ratio. These data thus support a strong in vivo activity of IRS.

**Discussion**

A strong innate immune activation is an essential component of the response to pathogens such as bacteria or viruses (1). TLRs are important components of this system and recognize a broad range
of ligands derived from pathogens (1, 2). In particular, TLR-9 has been shown to be activated by bacterial DNA and oligonucleotides containing a CpG motif. These CpG-ODNs promote a Th1-like response via the secretion of inflammatory cytokines and the activation of B cells and PDC (4, 17, 24, 26–31). These activities of CpG-ODN are the basis for novel vaccine adjuvants as well as therapies for asthma, infectious diseases, and cancer (2–4). It is thus extremely important to better understand how TLR-9 is activated and regulated.

Innate immune responses to microbial DNA are of fundamental importance (2, 3). Many viruses have evolved so that they have a reduced content of CpG, and it was shown that they even contain sequences that would reduce the reactivity of TLR-9 to expand (7). It was also reported that sequences within the telomeres of the mammalian chromosome contain a motif that can down-regulate TLR-9 activation and that this could be an important mechanism to prevent autoimmunity (12). These observations have been strengthened by the identification of oligonucleotides that can effectively inhibit responses to CpG-containing sequences (7–12). These ODNs are able to regulate TLR-9 activation; thus, we have named them IRS. Despite the potential for IRS to treat autoimmune disease, little is known about the optimal motif and structure required for IRS activity or their mechanism of action. To date, the activity of IRS has been demonstrated in mouse systems only. Therefore, it is essential to better understand what are the necessary characteristics for IRS to envisage using these sequences in clinical settings.

In this study we present evidence that IRS have activity in both mice and humans and act systemically in vivo. B cells and PDC, the two cell types in human blood that express TLR-9, can be inhibited with IRS in response to ISS activation despite their very different responses to ISS activation. Human B cells proliferate and produce the cytokines IL-6 and IL-10, whereas PDC will respond with a vigorous production of IFN-α (17, 24). The different classes of ISS will also trigger very different quantitative responses in these cell subsets. CpG-A ISS induces very strong IFN-α production from PDC, but a very limited B cell activation; CpG-B ISS induces low IFN-α production from PDC, but strong B cell activation; whereas CpG-C ISS combine a strong induction of IFN-α with a robust B cell activation (4, 16). Strikingly, IRS can inhibit both B cells and PDC in response to all classes of ISS in both mice and humans. In addition, responses to the newly defined chimeric immunomodulatory compound (32) were inhibited by IRS (data

FIGURE 8. IRS are active in vivo and act systemically to block TLR-9 stimulation. A, BALB/c mice were injected by the s.c. route with saline, 1018 ISS alone, or 1018 ISS in the presence of inactive control ODN or decreasing amounts of IRS 869. Two hours after the injections, serum was collected and IL-6, IL-12, and TNF-α were measured by immunoassay. Averages of a group of 10 mice are shown. B, BALB/c mice were injected by the s.c. route in the left flank with 25 µg of 1018 ISS (s.c.L). IRS (25 µg) was injected using different routes, s.c. in the left (s.c.L), or in the right (s.c.R) flank i.p. or i.v. Two hours after the injections, the serum was collected, and cytokines were measured by immunoassay. Averages of a group of 10 mice are shown. *** \( p < 0.001 \).

FIGURE 9. IRS prevent the death of mice after a TLR-9-induced inflammatory response. All BALB/C mice were injected i.p. with 20 mg of α-Gal in saline. Lethal shock was then induced by injecting the mice s.c. with 50 µg of 1018 ISS. To prevent death in these mice, either inactive control ODN (200 µg) or IRS 869 at three doses (200, 100, and 50 µg) was injected simultaneously with 1018 ISS, and mouse survival was assessed over a 7-day period. Averages of 25–35 mice/group are shown.
not shown). Interestingly, we were not able to detect any activity when using IRS alone in any of our experiments. However, broader assays, such as microarray analysis, will be necessary to determine whether IRS can induce specific sets of genes. Previous studies have suggested that IRS are not simple competitive antagonists for TLR-9 binding. They act even when added to cells much later than ISS (10) (data not shown), and the in vivo experiments presented in our study also strengthen this idea. Although these data cannot formally exclude direct binding of IRS to TLR-9, they suggest that IRS most likely interfere with components of the very early events of TLR-9 signaling. The precise mechanism of action is still unclear, but probably does not involve uptake (8, 10) through a scavenger receptor. Additional studies will be necessary to fully understand how IRS inhibit TLR-9.

To better understand the relationship between IRS structure and function, we focused on the definition of the optimal motif necessary for IRS activity and found that the presence of four contiguous guanosines was critical for optimal activity in both mice and humans. Disrupting this motif by reducing the number of guanosines or substituting one with a different base led to a reduction or loss of IRS activity. As previously suggested, IRS containing four contiguous guanosines can self-assemble into four-stranded helices stabilized by planar Hoogsteen base-paired quartets of guanosine known as G-tetrads (18, 19). This particular secondary structure is indeed formed by the G-rich IRS, and the importance of this G4 motif suggested that the G-tetrad form might be the biologically active form. The opposite proved true, however. Once purified and tested separately, the single-stranded form, but not the G-tetrad form, contained all measurable IRS activity. This was independently confirmed by replacing one of the four guanosines with a 7-deaza guanosine to prevent formation of the G-tetrad. This variant of the 2088 sequence, IRS 2296, retained the activity of the parent molecule. These results contrast with the report by Gursel et al. (12) showing that the presence of 7-deaza guanosine in a different G-rich IRS led to a complete loss of IRS activity, suggesting that only the G-tetrad form was active. In their study, however, the ODNs containing 7-deaza guanosine were prepared with a PO backbone, whereas the parent ODN was synthesized with PS linkage (12). As we show in Fig. 6, the presence of a PS backbone is absolutely necessary for IRS activity, which probably explains the discrepancy between these studies. Finally, despite containing series of three contiguous guanosines, the IRS derived from telomeric ends (IRS 888 in Fig. 6) do not readily form G-tetrad when evaluated by SEC (data not shown).

We show that when co-injected in vivo with 1018 ISS at different ratios, IRS could block the production of serum cytokines in a dose-dependent manner, and extensive inhibition occurred at IRS: ISS ratios of much <1. It is known, however, that some ISS activities, such as adjuvant effect, are confined to the local site of injection. Thus, it was important to test whether IRS needed to act in close proximity to the TLR-9 stimulus. We show in this study that IRS activity in vivo is not exclusively local, but can affect ISS-induced inflammation systemically. To test IRS in an inflammatory model with significant associated pathology, we turned to a model in which injection of CpG-ISS creates a lethal inflammation. As previously described, mice pretreated with d-Gal become extremely sensitive to normally nontoxic doses of ISS and die of massive systemic inflammation (26). Coinjection of IRS with ISS was quite effective in preventing death, demonstrating that IRS are sufficiently potent in vivo to provide extensive control of ISS-induced inflammation. IRS have recently been tested in vivo in autoimmune models and have been proven to have some efficiency (11, 14, 15). However, some of these models required the use of CFA, which includes mycobacterial DNA. Thus, the effect of IRS in these models may be by inhibiting the adjuvant and not the resulting pathology. Additional experiments using CFA-free models will be necessary to address the potential of IRS to prevent autoimmunity. The interaction of IRS with other innate immunity ligands should be evaluated as well. No inhibition of TLR-4 stimulation in response to LPS was observed (data not shown), and potential inhibition of other TLR ligands is still being evaluated.

In summary, our data provide a better understanding of the requirements for IRS activity in terms of motif, backbone composition, and secondary structures. We also demonstrate that these IRS are active in man and that they can block TLR-9 activation by all defined classes of ISS in B cells and PDC. Finally, we reported that these sequences have a strong systemic effect in vivo, because they protected mice from lethal ISS-induced inflammation. These data strengthen the potential for such ODNs in therapeutic settings with respect to sepsis as well as IFN-α-mediated diseases, such as systemic lupus erythematosus.

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Disclosures
All authors of the manuscript (O. Duramad, K. L. Fearon, B. Chang, J. H. Chan, I. Gregorio, R. L. Coffman, and F. J. Barrat) were employees of Dynavax Technologies at the time the work was performed.

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