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Short Communication:

Treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 leads to reduction of autoantibody production and amelioration of disease symptoms

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The presence of autoantibodies specific for nucleic acid-associated antigens is the hallmark of systemic lupus erythematosus (SLE). We have recently developed a specific inhibitor of TLR7 and TLR9, called immunoregulatory sequence (IRS) 954, and showed that it inhibits the induction of IFN-α by human plasmacytoid dendritic cells in response to DNA and RNA viruses and isolated immune complexes from lupus patients. In this study, we show that IRS 954 can prevent progression of disease when injected in the lupus prone (NZB x NZW)F_1 mice. Following treatment, we observed a significant reduction of serum levels of nucleic acid-specific autoantibodies as well as decreased proteinuria, reduced glomerulonephritis, end-organ damage and increased survival. These data demonstrate that in addition to its ability to block IFN-α, IRS 954 can reduce symptoms in a lupus model and thus represents a promising therapeutic agent for the treatment of SLE.

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Introduction

Systemic lupus erythematosus (SLE) is a relapsing, remitting disease with extensive and variable symptoms that affects over a million people in the United States alone, primarily young and middle-aged women. The presence of autoantibodies specific for nucleic acids is diagnostic for SLE and is thought to play an important role in the pathogenesis of the disease [1]. A growing body of evidence suggests that IFN-α promotes lupus [2], as many patients have elevated serum IFN-α levels [3] and PBMC from patients exhibit an IFN-α-induced gene expression signature that correlates with disease severity [4, 5]. Recent findings in both human and mouse models suggest that TLR7 and TLR9 may play a central role in maintenance and progression of the disease by promoting elevated IFN-α levels from human plasmacytoid dendritic cells (PDC) [6, 7] and by activating B cells to produce autoantibodies [8, 9]. The (NZB x NZW)F1 mouse is one of the best-characterized models of lupus and several studies have suggested a role for IFN-α in the development of disease in this model [10–12]. We have recently described an oligonucleotide, called immunoregulatory sequence (IRS) 954 that can block both TLR7 and TLR9 activation of B cells and IFN-α production by PDC in response to viruses and immune complexes [6]. To demonstrate the potential for treating SLE with IRS 954, we have tested its efficacy in the (NZB x NZW)F1 model of this disease.

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Abbreviations: **IRS:** immunoregulatory sequence · **PDC:** human plasmacytoid dendritic cells · **SLE:** systemic lupus erythematosus

Results and discussion

(NZB x NZW)F1 mice treated with IRS 954 have reduced levels of nuclear antigen-specific autoantibodies

To evaluate the effect of TLR7 and 9 inhibition on progression of disease, mice were injected subcutaneously twice weekly, beginning at the onset of disease (4 months of age) with two doses of IRS 954 (15 and 45 μg) or left untreated. At 9 months, we observed in both treated groups a significant reduction of anti-dsDNA, anti-nucleosomes, anti-smith and anti-nRNP (Fig. 1A–D) autoantibodies. Reduced levels of the autoantibodies were observed over the course of the experiment, suggesting a continuous effect of the inhibition (Fig. 1E). The observed effect was not due to an overall reduction in total IgG, IgG1 or IgG2a in the treated group, as compared to the untreated group (Supporting Fig. 1A). An inert control ODN with similar backbone composition was tested in similar protocol at 45 μg and had no effect (Supporting Fig. 1B), demonstrating the specificity for TLR7 and TLR9 of the observed effect with IRS 954. Although we have shown previously in single-dose experiments that the 15- and 45-μg doses might produce suboptimal and optimal effects, respectively [13], in this treatment setting both doses reduced disease progression to a similar extent. ODN accumulation over time in tissue may explain these results. These data clearly show that simultaneously inhibiting TLR7 and 9 in adult mice can inhibit the development of pathogenic autoantibodies to both DNA- and RNA-containing autoantigens in these lupus-prone mice.

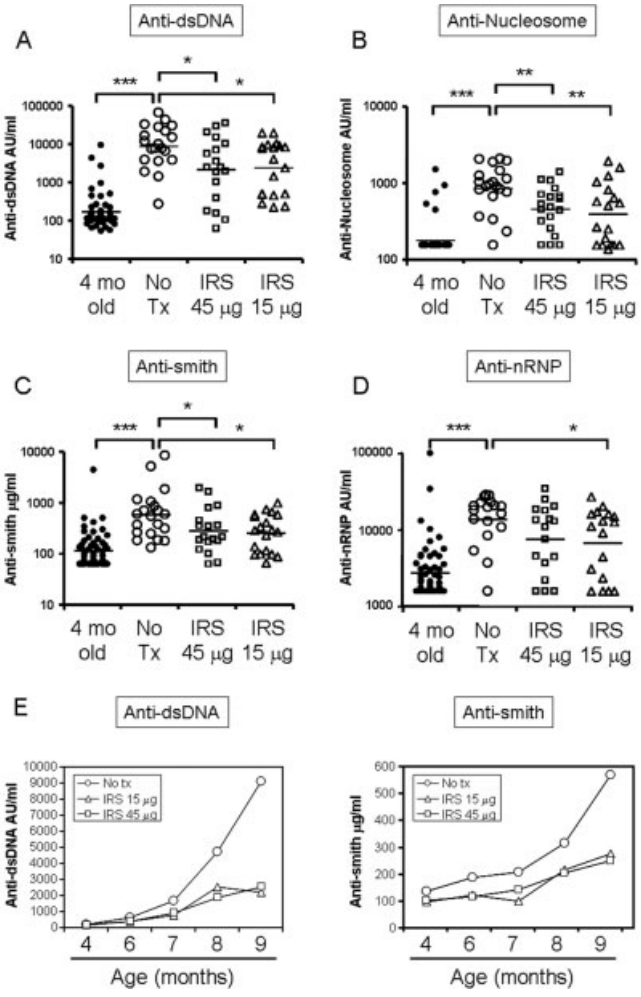

Figure 1. IRS 954 treatment leads to reduced levels of autoantibodies in (NZB x NZW)F1 mice. Female (NZB x NZW) F1 mice were treated beginning at 4 months of age with IRS 954, with 15 (n = 19 mice, triangle) and 45 (n = 18 mice, square) μg/injection, two injections weekly, or left untreated (n = 20 mice, circle) and levels of autoantibodies in the serum were measured at 9 months of age. Levels of (A) anti-dsDNA, (B) anti-nucleosome, (C) anti-smith and (D) anti-RNP autoantibodies are shown. Increase in autoantibody levels in the untreated group was significantly higher as compared with levels at the start of the experiment (4 months old, filled dot). (E) Effect of IRS on anti-dsDNA and anti-smith over the course of the experiment is shown as well. The geometric mean of the levels of autoantibodies is represented for each group. The figure represents one of three similar experiments. Significance is represented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Reduction of proteinuria and glomerulonephritis and increased survival in IRS 954-treated (NZB x NZW)F1 mice

At 9 months of age, both groups of IRS 954-treated mice showed a significant reduction in proteinuria as compared to the untreated group (Fig. 2A). Of note, in both IRS 954-treated groups only about half of the mice showed evidence of proteinuria (9/19 in the 15-μg group; 10/18 in the 45-μg group), whereas all mice developed proteinuria in the untreated group (20/20) (Fig. 2A). Both groups of IRS 954-treated mice also had reduced kidney damage (Fig. 2B) with statistically significant reductions in glomerulonephritis, glomerular changes and interstitial changes, although no change in the lymphoplasmacytic infiltration in the kidney was observed (Fig. 2C). These data show that IRS 954 is effective at suppressing the production of autoantibodies, occurrence of proteinuria and end-organ pathology in the lupus-prone (NZB x NZW)F1 mice. In addition, the IRS 954-treated group had a significant reduction of mortality, with 13/20 mice dead by the end of the experiment in the untreated group compared to 4/18 ($p = 0.023$) and 5/19 ($p = 0.037$) in the IRS 954-treated (15 and 45 μg) groups (Fig. 3A). Increased survival was also observed when the treatment was initiated in mice with already established disease. Untreated 10-month-old mice with severe symptoms (high proteinuria levels)

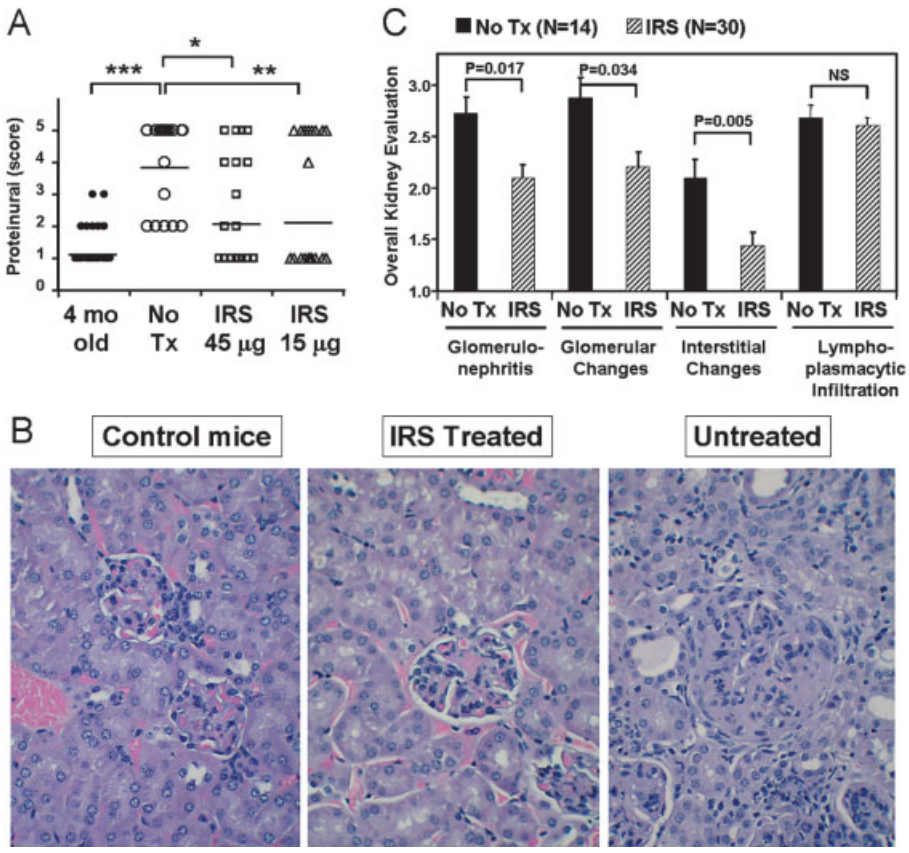

Figure 2. Female (NZB × NZW) F1 mice were treated as in Fig. 1 and proteinuria was evaluated using the Multiplex strips at 9 months of age and scored 1–5 based on protein levels. 4 months old mice (filled dot), untreated (circle), IRS 954 treated 45 µg/injection (square) and IRS 954 treated 15 µg/injection (triangle) are shown. (B) At 10 months of age, kidneys from treated and untreated mice as well as a control animal were harvested, preserved in 10% formalin and histological evaluation was performed by H&E staining. (C) Kidneys from 10 months old treated (15 and 45 µg) and untreated (NZB x NZW) F1 mice were graded by an experienced pathologist in a blinded fashion for overall glomerulonephritis, glomerular and interstitial changes as well as lymphoplasmacytic infiltration using a 1 to 4 scale as described in the *Materials and methods*. Results represent an average of 14 untreated mice and 30 mice combined from the two IRS 954-treated groups. Significance is represented as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

were treated with IRS 954 or control inert ODN and after 9 weeks of treatment, 83% of the mice from the control group had died while only 45% had died from the IRS 954-treated group (Fig. 3B). Although larger studies would need to be done to draw definitive conclusion, these data in addition to the reduced mortality in the long-term treatment experiment suggest an effect on mortality by treatment with IRS 954.

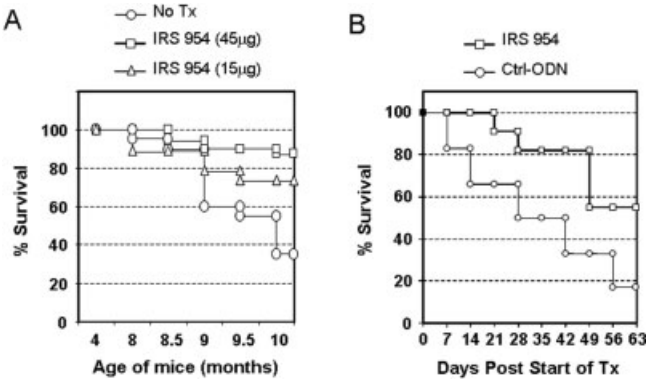

Figure 3. IRS 954-treated (NZB x NZW)F1 mice have increased survival. (A) Female (NZB x NZW) F1 mice treated as described in Fig. 1 were monitored for survival during the course of the experiment. (B) Untreated 9–10-month-old females (NZB x NZW)F1 mice scoring 4+ proteinuria for three consecutive weeks were treated with IRS 954 (n = 11 mice) or an inactive control (n = 6 mice), 100 µg/injection subcutaneous, two injections weekly and monitored for survival.

Concluding remarks

Substantial evidence has suggested that TLR7 and TLR9 activation could lead to abnormal function of two key cell types in lupus – B cells and PDC [14]. Stimulation through these receptors promotes autoantibody production by B cells [8, 9] and leads to high levels of IFN-α production by PDC [15]. A TLR7 and 9 antagonist is predicted to have therapeutic benefit for lupus by (i) inhibiting the major source of IFN-α contributing to the pathogenesis of lupus without blocking low levels of IFN-α and IFN-β induced by other pathways in many cell types and (ii) by inhibiting activation of anti-DNA and anti-RNP-specific B cells and consequent production of anti-nucleic acid autoantibodies.

We have recently shown that the dual inhibitor IRS 954 can inhibit human PDC and B cell in response to TLR7 and 9 activation by synthetic ligands, viruses, as well as immune complexes isolated form lupus patients [6]. In order to evaluate this approach in a mouse model of lupus, we have selected to test IRS 954 in the (NZB x NZW)F1 mice, as this well-characterized model shares with the human lupus evidence for a pathogenic role for

IFN-α. As in the human disease [4, 5], these mice constitutively express high levels of some IFN-α-regulated genes that correlate with disease severity [10]. Treatment of these mice with an adenovirus secreting IFN-α greatly accelerates disease progression [12]. In addition, NZB mice have less severe disease with delayed onset when made deficient for the IFN-α receptor [11]. The use of IRS 954 is unique because of its specificity for TLR7 and 9 [6], as compared to other inhibitory ODN, the specificity of which is not as defined [16] and because it allowed us to intervene at onset of disease and thus avoid the use of mice deficient for both TLR7 and 9. We cannot exclude that activation of these two nucleic acid-specific receptors during an inflammatory response could have opposite effect. Therefore, it will be important to better understand their respective role in other autoimmune models such as rheumatoid arthritis or psoriasis, as such inhibitors are advancing toward clinical development.

In summary, we have observed that simultaneously blocking TLR7 and 9 signaling in (NZB x NZW)F1 mice using IRS 954 leads to the reduction of autoantibody levels, proteinuria and kidney damage. Our data support the notion that blocking TLR7 and TLR9 in both B cells and PDC is an attractive approach for the treatment of lupus.

Materials and methods

Oligonucleotides and mice

Phosphorothioate IRS 954 were prepared as previously described [17]. The composition of IRS 954 is: 5′- TGC TCC TGG AGG GGT TGT - 3′. Control ODN used is 5′- TCC TGC AGG TTA AGT - 3′. ODN were diluted in saline for injection.

Treatment of (NZB x NZW) F1 mice (Jackson Laboratory, Bar Harbor, ME) started at onset of disease (4 months of age) when 25% of the mice began showing proteinuria. Mice received subcutaneous injections of IRS 954 (15μg or 45μg) twice a week up to the end of the experiment. At 9 months of age, proteinuria and autoantibody levels were measured. At 10 months of age, kidneys were harvested for histology evaluation.

Proteinuria and autoantibody level measurements

Urine protein levels were measured using the Multistix 9 urinalysis strips (Bayer, Leverkusen, Germany). Autoantibody levels were quantified by ELISA. All protocols used a goat anti-mouse IgG (Fc) HRP (Jackson Immunoresearch, West Grove, PA) as secondary reagents. Serum from retired MLR/MPJ-tnfrsf6^{lpr} breeder mice was used as a positive control to standardize the amount observed. Autoantibodies were detected by adding serum to 96-well plates coated with their respective antigens. Poly(dAdT): poly(dAdT) (Sigma); Sm antigen of calf thymus origin, purified nRNP antigens (Immunovision, Springdale, AR) and nuclesomes (Euroimmun, Luebeck, Germany) were used.

Kidney histology

Formalin preserved tissues were sectioned and stained with hematoxylin and eosin (H&E) and scored by a veterinary pathologist that was blinded throughout the experiment. Scoring is described as 1= normal, 2= mild, 3= moderate, 4=severe and correspond to the severity of damage of the entire section (Glomerulonephritis), for the glomeruli exclusively (Glomerular changes), for damages in spaces between glomeruli; i.e.: tubules, protein casts, etc. (Interstitial changes) as well as the severity of lymphoplasmacytic infiltration into the kidney.

Statistical analysis

Autoantibody levels, proteinuria and symptom scores were analyzed using a 2-tailed Student's *t* test using unpaired non-parametric test (Mann-Whitney). Significance is represented as $p < 0.05$ (*), $p < 0.01$ (**) and $p<0.001$ (***).

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Conflict of interest: The authors are all full-time employees of Dynavax Technology.

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