A limited CpG-containing oligodeoxynucleotide therapy regimen induces sustained suppression of allergic airway inflammation in mice

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ONLINE DATA SUPPLEMENT

SUPPLEMENTAL METHODS

Animal Procedures

Animal procedures were performed at Pacific Biolabs (Hercules, CA) or Murigenics (Vallejo, CA) and were Institutional and Animal Care Use Committee–approved following the "Guide for the Care and use of Laboratory Animals", National Research Council (1996). All mice were acclimatized to the facilities for at least one week between shipping and initiation of experimental procedures. In certain Tx Protocol 2 experiments, intraperitoneal injections of protein G-purified anti-IFN-γ antibody (2 mg, R46A2(ATCC) or XMG1.2) were concurrent with weekly low dose ragweed exposures after completion of 1018 ISS therapy. Hybridomas secreting XMG1.2 and GL113 (isotype control) were kind gifts from Dr Stephen Stohlman (Cleveland Clinic, OH).

Microarray Analysis

Microarray analysis was performed by Expression Analysis, Inc. (Durham, NC). RNA was extracted from individual mouse lung samples and profiled for quality on the Agilent 2100 bioanalyzer (Agilent Technologies) and for expression on Mouse WG-6 v2 BeadChips (Illumina). Of 45,281 probes targeting transcripts, 30,221 were detected in >95% of samples. Log-2 expression levels were further analyzed using R (The Comprehensive R Archive Network), specifically to perform T-tests with Welch's correction for unequal variance and to plot data.

T Cell Analysis

For stimulation of intracellular cytokine responses, isolated and enriched lung T cells ($1x10^6$/mL) were incubated in 96 well plates (200 μL/well) with irradiated splenic white blood cells (5×10^6/mL), anti-CD28 (1 μg/mL) and RW (250 μg/mL) for 6 hours with Brefledin A (5 μg/mL) added for the final 4 hours. Intracellular cytokines were quantified as a percentage of CD154 (CD40L) antigen-reactive T cells. In general, cells were pooled per treatment group to facilitate enrichment and/or because individual mice yield low cell numbers. Non-stimulated, enriched lung T cells were surface stained with indicated antibodies (BD Biosciences or eBiosciences). Samples were collected on a FACSCaliber or a LSRII flow cytometer (BD Biosciences). Detailed analysis was performed using Flow Jo software (Tree Star, Inc) with gating through light scatter-defined lymphocytes followed by $CD3^+CD4^+$ T cells. To evaluate T_{Reg} activity in vitro, enriched lung T cells (pooled/treatment group) were stained for CD4 and CD25 and sorted on a MoFlo high speed sorter (DakoCytomation). 200 μL triplicate cultures of $CD4^+CD25^+$ (T_{Reg}) cells from RW or RW+1018 ISS-treated mice and $CD4^+CD25^-$ (T_{Eff}) cells ($5 x10^4$) from RW-exposed mice were stimulated with anti-CD3 (0.5 μg/ml) in the presence of irradiated spleen cells (1×10^6) for 48 hours in 96-well U-bottom plates. Proliferation was measured following a final 6 hour pulse with 1 μCi of 3H-Thymidine.

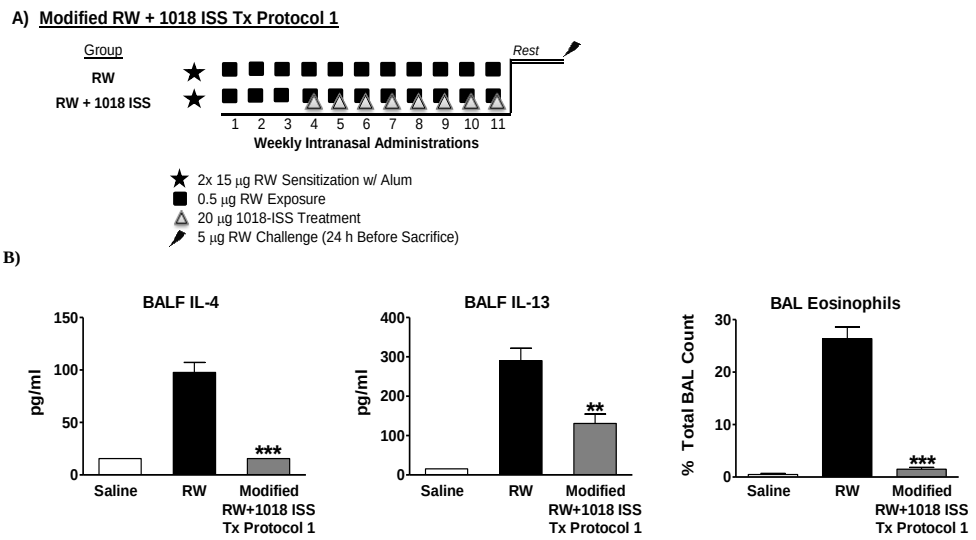

Figure S1 1018 ISS treatments initiated after 3 weekly RW exposures significantly suppress lungTh2 responses. (A) Modified Tx Protocol 1schematic: Mice received 3 weekly intranasal 0.5 μg RW, followed by 8 additional RW, or RW+ 20 μg1018 ISS treatments, 2 weeks rest, and a final 5 μg RW challenge 24 hours before sacrifice (control mice received only saline intranasally). (B) BALF cytokines and eosinophils (mean ± SEM, 8 mice/group). **P <0.01, or ***P <0.001 compared with the RW exposures only group. Data are representative of two independent experiments.

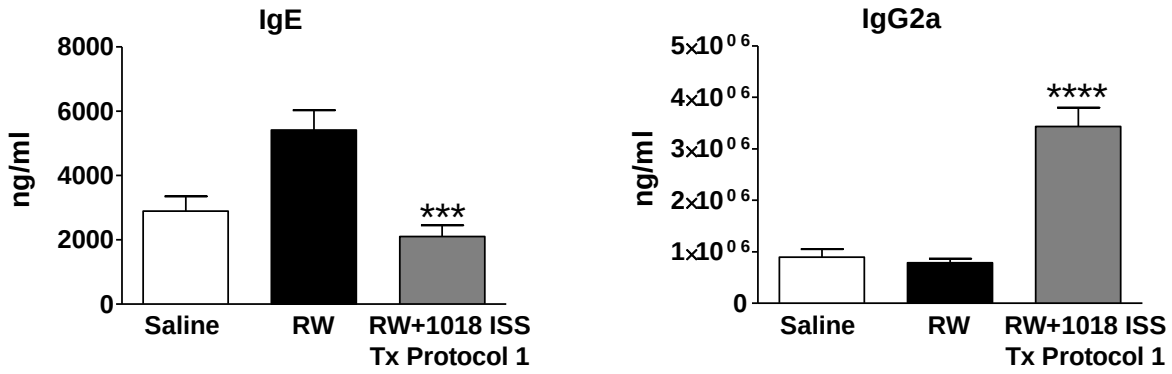

Figure S2 1018 ISS treatments induce a Th1-like shift in serum antibodies. Total serum IgE and IgG2a in mice that received 17 weeks of Tx protocol 1 RW+ 1018 ISS, RW or saline, followed by 2 rest and final RW challenge (mean ± SEM, 5 mice/group). ***P <0.001, ****P<0.0001, compared with the RW exposed only group. Data are representative of two independent experiments.

Table S1: Selected Th2-associated genes upregulated in ragweed (RW) allergic mice

Genes		With Final Challenge		Without Final Challenge	
		RW *versus* Saline		RW *versus* Saline	
Common Name	***mSymbol***	**Fold Induction**	**p-value**	**Fold Induction**	**p-value**
Anterior gradient 2 (*Xenopus laevis*)	***Agr2***	17.8	0.0017	1.6	0.4602
Arginase, liver	***Arg1***	102.6	< 0.0001	1.6	0.2370
Branched chain aminotransferase 1, cytosolic	***Bcat1***	13.0	0.0016	3.5	0.0233
Chemokine (C-C motif) ligand 7	***Ccl7***	31.7	0.0001	1.3	0.2655
Chemokine (C-C motif) ligand 8	***Ccl8***	22.5	< 0.0001	3.5	0.0024
Chemokine (C-C motif) ligand 9	***Ccl9***	4.5	0.0009	1.1	0.7067
Chemokine (C-C motif) ligand 11	***Ccl11***	20.0	< 0.0001	1.9	0.0457
Chemokine (C-C motif) ligand 17	***Ccl17***	2.2	0.0129	3.3	0.0044
Chemokine (C-C motif) receptor 8	***Ccr8***	2.8	0.0817	1.5	0.4973
CD209 antigen-like protein E	***Cd209e***	192.6	< 0.0001	2.1	0.2032
Chitinase, acidic	***Chia***	8.9	< 0.0001	1.4	0.0335
Calcium-activated chloride channel family member 3	***Clca3***	566.7	< 0.0001	449.8	0.0009
Eosinophil-associated, ribonuclease A family, member 11	***Ear11***	262.0	< 0.0001	21.8	0.0042
FXYD domain-containing ion transport regulator 4	***Fxyd4***	114.1	0.0004	3.0	0.0651
Glycine amidinotransferase	***Gatm***	17.1	< 0.0001	1.6	0.2127
Interleukin 4	***Il4***	24.2	0.0014	1.3	0.6631
Interleukin 5	***Il5***	3.3	0.0123	1.7	0.4084
Interleukin 13	***Il13***	1.7	0.0949	1.2	0.5898
Interleukin 13 receptor, alpha 2	***Il13ra2***	52.5	< 0.0001	1.4	0.1674
Integrin alpha X	***Itgax***	4.0	0.0066	1.9	0.3151
Matrix metallopeptidase 12	***Mmp12***	2.2	0.0084	1.5	0.0662
Homeobox, msh-like 3	***Msx3***	185.1	< 0.0001	1.5	0.3426
Periostin, osteoblast specific factor	***Postn***	2.4	0.0572	1.4	0.4992
Resistin like alpha	***Retnla***	5.2	0.0046	6.2	0.0047
Resistin like beta	***Retnlb***	7.8	0.0006	2.5	0.0754
Serum amyloid A 1	***Saa1***	8.8	0.0223	2.2	0.2086
Serum amyloid A 3	***Saa3***	5.4	0.0338	1.3	0.3834
Scinderin	***Scin***	20.8	< 0.0001	3.3	0.0232
Selectin, platelet	***Selp***	5.4	0.0002	1.2	0.0915
Solute carrier family 26, member 4	***Slc26a4***	19.1	0.0003	4.3	0.0014
Secreted phosphoprotein 1	***Spp1***	3.8	0.0014	1.2	0.0694

Mice were sensitized to ragweed pollen extract in alum by the i.p. route and were exposed weekly to low dose (0.5 µg) ragweed administered by the i.n. route for 16 weeks. Mice were then rested for 2 weeks and given a final challenge with a single high dose (5 µg) of RW 24 hours before sacrifice or were not challenged before sacrifice. Control mice were sensitized by i.p. injection with ragweed, but exposed to saline weekly thereafter. Gene expression was analyzed using Mouse WG-6 v2 BeadChips (Illumina). Values represent fold induction in RW-exposed mice in comparison to saline-exposed mice. Significance was evaluated by student's T test.

Table S2: Selected CpG-ODN-upregulated genes in ragweed (RW) allergic mice treated with 1018 ISS

Genes		With Final Challenge		Without Final Challenge	
		RW+1018 ISS *versus* Saline		RW + 1018 ISS *versus* Saline	
Common Name	**mSymbol**	**Fold Induction**	**p-value**	**Fold Induction**	**p-value**
Caspase 1	***Casp1***	2.3	< 0.0001	2.1	< 0.0001
Chemokine (C-C motif) ligand 4	***Ccl4***	2.9	0.0392	7.9	< 0.0001
Chemokine (C-C motif) ligand 5	***Ccl5***	2.7	0.0001	3.3	< 0.0001
Chemokine (C-C motif) ligand 19	***Ccl19***	14.9	< 0.0001	36.3	< 0.0001
Chemokine (C-C motif) receptor 5	***Ccr5***	10.3	< 0.0001	9.1	< 0.0001
CD72 antigen	***Cd72***	5.4	0.0003	6.1	0.0018
Chemokine (C-X-C motif) ligand 9	***Cxcl9***	226.4	< 0.0001	102.9	0.0002
Chemokine (C-X-C motif) ligand 10	***Cxcl10***	13.5	0.0030	10.0	0.0001
Chemokine (C-X-C motif) ligand 13	***Cxcl13***	4.9	0.0021	5.8	0.0008
Chemokine (C-X-C motif) receptor 3	***Cxcr3***	5.8	< 0.0001	6.6	< 0.0001
Guanylate binding protein 1	***Gbp1***	2.7	0.0110	1.0	0.5198
Glycosylation dependent cell adhesion molecule 1	***Glycam1***	14.1	0.0035	25.9	0.0078
Interferon-alpha 1	***Ifna1***	3.2	0.0314	2.8	0.0870
Interferon-gamma	***Ifng***	24.2	< 0.0001	16.8	< 0.0001
Interleukin 2	***Il2***	2.8	0.0549	2.5	0.0338
Interferon regulatory factor 1	***Irf1***	1.6	0.0463	1.2	0.0109
Jun-B oncogene	***Junb***	1.6	0.0025	1.1	0.3886
26S proteasome regulatory subunit p28	***Psmd10***	1.2	0.0123	1.2	0.0026
Pentraxin related gene	***Ptx3***	1.8	0.0531	1.6	0.1309
Src-like-adaptor 2	***Sla2***	6.3	0.0050	10.8	0.0002
Signal transducer and activator of transcription 1	***Stat1***	2.5	0.0109	1.5	0.0011
Signal transducer and activator of transcription 4	***Stat4***	1.6	0.0019	1.7	0.0006
Tumor necrosis factor	***Tnf***	2.8	0.0305	6.3	< 0.0001
Tumor necrosis factor receptor superfamily, member 1b	***Tnfrsf1b***	1.9	0.0003	2.4	< 0.0001

Mice were sensitized to ragweed pollen extract in alum by the i.p. route and were exposed weekly to low dose (0.5 μg) ragweed and 1018 ISS (20 μg) administered by the i.n. route for 16 weeks. Mice were rested for 2 weeks and given a final challenge with a single high dose (5 μg) of RW 24 hours before sacrifice or were not challenged before sacrifice. Control mice were sensitized by i.p. injection with ragweed, but exposed to saline weekly thereafter. Gene expression was analyzed using Mouse WG-6 v2 BeadChips (Illumina). Values represent fold induction in RW+1018 ISS-exposed mice in comparison to saline-exposed mice. Significance was evaluated by student's T test.