Supplemental Information for:

Natural STING agonist as an "ideal" adjuvant for cutaneous vaccination

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Supplemental Material and Methods

Animals

Female outbred Swiss Webster mice and C57BL/6J and IFNAR-/- mice (Muller et al, 1994) were purchased from Charles River Laboratories and Jackson Laboratories, respectively. MHC II-GFP mice expressing MHC class II molecule infused into enhanced green fluorescent protein (GFP) and STING-/- mice were kindly gifts of Dr. Hidde Ploegh, Massachusetts Institute of Technology and Dr. Glen Barber, University of Miami (Boes et al, 2002, Ishikawa et al, 2009). MHC II-GFP STING-/- mice were generated by breeding MHC II-GFP mice with STING-/- mice. Male Yorkshire pigs at 4 months of age were obtained from the Teaching and Research Resources at Tufts University. The animals were housed in the pathogen-free animal facilities of Massachusetts General Hospital (MGH) in compliance with institutional, hospital, and NIH guidelines. All studies were reviewed and approved by the MGH Institutional Animal Care and Use Committee.

Influenza viruses and vaccines

Pandemic A/California/7/2009 H1N1 influenza virus was obtained from American Type Culture Collection (ATCC, #FR-201). The reassortant H5N1 virus (SJRG-161052) was obtained from Dr. Richard Webby, St. Jude Children's Research Hospital, which is comprised of HA and NA genes from A/Vietnam/1203/2004 H5N1 but other genes from model A/Puerto Rico/8/1934 H1N1 virus. To further detoxify the virus, the stretch of six basic amino acids at the cleavage site between HA1 and HA2 were replaced with an avirulent avian sequence. The resulting virus was antigenically identical to the wild-type A/Vietnam/1203/2004 virus, but was apathogenic in chickens (Treanor et al, 2006). The viruses were expanded in 10-day-old embryonated chicken eggs at 35°C for 3 days, harvested, purified by sucrose gradient ultracentrifugation, and frozen at -80^{o}C until use. To challenge mice, 2009H1N1 virus was adapted in mice for three cycles of intranasal instillation-lung homogenate preparation and their infectivity in mice was assayed by a 50% lethal dose (LD_{50}) following a standard protocol (Cottey et al,

2001). Monovalent 2009 H1N1 influenza vaccine (NR-20347, Sanofi Pasteur, Inc.) and monovalent H5N1 pandemic influenza vaccine (NR-4143, Aventis Pasteur, Inc.) were obtained from BEI Resources, NIAID, NIH.

Immunizations and challenges

For ID immunization, the lower dorsal skin of mice was hair removed, into which H1N1 or H5N1 vaccine at 300 ng HA content was ID administered in a total volume of 20 μl, either alone or together with 20 μg 2'3'-cGAMP, cdGMP or 3'3'-cGAMP (all from Invivogen). The same amount of vaccines was prepared in 50 μl PBS, mixed with 50 μl PBS, AddaVax (Invivogen) or AddaVax plus 20 μg MPL (Sigma), and IM injected for comparison. To compare the adjuvant effect of cGAMP with CpG or MPL, H5N1 vaccine at 300 ng HA content was ID administered with varying doses of cGAMP (2.3, 8.1 or 20 μg), 20 μg CpG (CpG-ODN 1826, TriLink Biotechnologies) or 20 μg MPL. The blood samples were collected via tail vein 7, 14, and 28 days after immunization for evaluating cellular and humoral immune responses. Additional blood samples were also collected 8 and 40 weeks after immunization to follow up for long term immunity. The immunized and control mice were challenged by intranasal instillation of 10× LD_{50} mouse-adapted A/California/7/2009 H1N1 virus in five weeks after immunization. Body weight and survival were monitored daily for 14 days after the infection.

For swine immunization, the animals were anesthetized by IM injection of telazol (2.2 mg kg-1)/xylazine (2.2 mg kg-1)/atropine (0.04 mg kg-1) and maintained under isoflurane (2-3%) inhalation during hair removal and immunization. H5N1 vaccine at 6 μg HA content per animal was ID injected into exterior hind leg skin alone or along with 200 μg 2'3'-cGAMP. The pigs received a booster immunization similarly on the contralateral leg 14 days after priming immunization. Seasonal influenza vaccine at 6 μg HA per animal (2012-2013 formulation, CSL Biotherapies) was ID immunized similarly but only once to compare skin reactions. Blood samples were collected 14 days after priming or 7 days after boosting vaccination, respectively.

Hemagglutination inhibition (HAI) assays

Serum samples were prepared and incubated with receptor-destroying enzyme (RDE) (Denka Seiken) at 37°C for 20 hours followed by inactivation of the enzyme at 56°C for 30 minutes. The resultant serum samples were serially diluted and incubated with 4 hemagglutination units (HAU) of influenza virus (A/California/7/2009 H1N1 or the reassortant H5N1 virus SJRG-161052) at 37°C for 1 hour. Then the mixture was incubated with 0.5% chicken red blood cells (for H1N1) or horse red blood cells (for H5N1) at room temperature for 30 minutes. The HAI titer was defined as the reciprocal of the highest serum dilution that inhibited 4 HAU.

The enzyme-linked immunosorbent assay (ELISA)

Influenza-specific IgG, IgG1, IgG2a, and IgG2c antibody titers were measured by ELISA. In brief, 1 μg/ml recombinant HA was coated onto ELISA plates in $NaHCO_3$ buffer, pH9.6 overnight. After incubation with serially diluted serum samples, HRP-conjugated goat anti-mouse IgG (NA931V, GE healthcare, dilution 1:6000), IgG1 (1073-05, Southern Biotech, 1:4000), or IgG2a (1083-05, Southern Biotech, 1:4000) antibody was added to measure specific subtypes. Anti-mouse IgG2c (1079-05, Southern Biotech, 1:5000) antibody was used for C57BL/6 mice in place of anti-IgG2a antibody.

Cellular immune responses

Peripheral blood mononuclear cells (PBMCs) were obtained from blood after lysis of red blood cells. PBMCs at 10^6 cells per ml were incubated with inactivated influenza virus (1 μg/ml HA content) and 4 μg/ml anti-CD28 (clone 37.51, BD Pharmingen) antibody overnight. Golgi-Plug (BD Pharmingen, 1:1000) was added to the culture and continuously incubated for another 5 hours. The stimulated cells were surface stained with fluorescence-conjugated antibodies against CD4 (clone RM4-5, Biolegend, dilution 1:100) and CD8 (clone 53-6.7, Biolegend, dilution 1:200), followed by intracellular staining with anti-IFNγ (clone XMG1.2, Biolegend, 1:100) and anti-IL4 (11B11, Biolegend, dilution 1:100) antibodies and flow cytometric analysis as previously described (Hong et al, 2010).

Histological examination

Mice were ID injected with PBS, 20 μg cGAMP, 20 μg resiquimod (IMGENEX), 2009 H1N1 influenza vaccine (300 ng HA) or the vaccine plus 20 μg cGAMP. The inoculation sites were photographed on indicated days. The tissue at the inoculation site was dissected 2 days after injection, fixed, and stained by a standard H&E procedure. The slides were scanned and analyzed by NanoZoomer and Digital Pathology software (Hamamatsu).

Intravital confocal imaging

The ears of MHC II-GFP mice or MHC II-GFP STING-/- mice were hair removed and injected with 20 μg cGAMP, followed by imaging by intravital two-photon confocal microscopy (Olympus FV-1000) for 12 hours (Chen et al, 2010). The velocity of migrating MHC II+ cells was analyzed by MTrackJ plugin of Image J software (Meijering et al, 2012).

cGAMP distribution analysis

C57BL/6J mice were injected ID or IM with 20 μg fluorescein conjugated cGAMP (FITC-cGAMP, c[8-Fluo-AET-G(2',5')pA(3',5')p]) from Biolog. The tissue of the injection site and draining lymph nodes were collected in 0, 3, 6, 12 and 24 hours after injection, homogenized, and centrifuged at 18,000 rcf for 15 minutes. The concentration of cGAMP in supernatants was measured by a Fluorescent Microplate Reader (SpectraMax M5).

Quantitative real-time RT-PCR

The skin or muscle was administered with PBS or 20 μg cGAMP. The injection sites were excised in six hours and total RNA was extracted, reverse-transcribed, and amplified by real-time PCR using an SYBR Green PCR kit (Roche). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were listed in **Supplementary Table 1**.

Bone marrow derived dendritic cell (BMDC) culture and stimulation

Bone marrow cells were harvested from tibiae and femurs of C57BL/6 mice at 4-6 weeks of age. Cells at a concentration of 1×10^6 / ml were cultured with 10 ng/ml Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) for 7 days (Boonstra et al, 2003). BMDCs were sorted from the culture by flow cytometry on the basis of CD11c expression. The sorted BMDCs were cultured for 6 hours with different concentrations of cGAMP ranging from 0.002 to 40 μg/ml or with 20 μg/ml cGAMP for varying times from 1 to 24 hours. Cytokine and chemokine expressions in BMDCs were quantified by real-time RT-PCR as above.

Statistical analysis

Data are presented as mean ± s.e.m. Two-tailed t-test (t-test) was used to analyze a difference between two groups, and one way ANOVA was used among multiple groups. Log-rank test was used to analyze the survival. p value was calculated by PRISM software (GraphPad) and a difference was regarded significant if p value was less than 0.05.

Supplementary Table 1 Primers for real-time PCR Analysis

Gene	Forward	Reverse
Gapdh	atcaagaaggtggtgaagca	agacaacctggtcctcagtgt
Ccl2	ggctcagccagatgcagttaa	cctactcattgggatcatcttgct
Cxcl9	ggagttcgaggaaccctagtg	gggatttgtagtggatcgtgc
Cxcl10	ccaagtgctgccgtcattttc	tccctatggccctcattctca
Ifnb1	agctccaagaaaggacgaaca	gccctgtaggtgaggttgat
Tnfa	cctgtagcccacgtcgtag	gggagtagacaaggtacaaccc
Il12	ctgtgccttggtagcatctatg	gcagagtctcgccattatgattc
Il6	tagtccttcctaccccaatttcc	ttggtccttagccactccttc
Ifng	atgaacgctacacactgcatc	ccatccttttgccagttcctc
Il1b	acatcagcacctcacaagca	ttagaaacagtccagcccata
Tgfb1	tgacgtcactggagttgtacgg	ggttcatgtcatggatggtgc

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