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Identification and Characterization of Stimulator of Interferon Genes As a Robust Adjuvant Target for Early Life Immunization

Francesco Borriello1,2,3,4,5†, Carlo Pietrasanta1,2,3,6†, Jacqueline C. Y. Lai1,2,3,7, Lois M. Walsh8, Pankaj Sharma1,2,3, David N. O’Driscoll1,3, Juan Ramirez1,3, Spencer Brightman1,3, Lorenza Pugni6, Fabio Mosca6, David J. Burkhart8, David J. Dowling1,2*‡ and Ofer Levy1,2,3*‡

1 Division of Infectious Diseases, Department of Medicine, Boston Children’s Hospital, Boston, MA, United States, 2 Harvard Medical School, Boston, MA, United States, 3 Precision Vaccines Program, Division of Infectious Diseases, Boston Children’s Hospital, Boston, MA, United States, 4 Department of Translational Medical Sciences, Center for Basic and Clinical Immunology Research (CISI), University of Naples Federico II, Naples, Italy, 5 WAO Center of Excellence, Naples, Italy, 6 Neonatal Intensive Care Unit, Department of Clinical Sciences and Community Health, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy, 7 Department of Physiology, Institute of Neuroscience and Physiology, University of Gothenburg, Gothenburg, Sweden, 8 Biomedical & Pharmaceutical Science Skaggs School of Pharmacy, University of Montana, Missoula, MT, United States

Immunization is key to preventing infectious diseases, a leading cause of death early in life. However, due to age-specific immunity, vaccines often demonstrate reduced efficacy in newborns and young infants as compared to adults. Here, we combined in vitro and in vivo approaches to identify adjuvant candidates for early life immunization. We employed newborn and adult bone marrow-derived dendritic cells (BMDCs) to perform a screening of pattern recognition receptor agonists and found that the stimulator of interferon genes ligand 23-cGAMP (hereafter cGAMP) induces a comparable expression of surface maturation markers in newborn and adult BMDCs. Then, we utilized the trivalent recombinant hemagglutinin (rHA) influenza vaccine, Flublok, as a model antigen to investigate the role of cGAMP in adult and early life immunization. cGAMP adjuvantation alone could increase rHA-specific antibody titers in adult but not newborn mice. Remarkably, as compared to alum or cGAMP alone, immunization with cGAMP formulated with alum (Alhydrogel) enhanced newborn rHA-specific IgG2a/c titers ~400-fold, an antibody subclass associated with the development of IFNγ-driven type 1 immunity in vivo and endowed with higher effector functions, by 42 days of life. Highlighting the amenability for successful vaccine formulation and delivery, we next confirmed that cGAMP adsorbs onto alum in vitro. Accordingly, immunization early in life with (cGAMP+alum) promoted IFNγ production by CD4+ T cells and increased the proportions and absolute numbers of CD4+ CXCR5+ PD-1+ T follicular helper and germinal center (GC) GL-7+ CD138+ B cells, suggesting an enhancement of the GC reaction. Adjuvantation effects were apparently specific for IgG2a/c isotype switching without effect on antibody affinity maturation, as there was no effect on rHA-specific IgG avidity. Overall, our studies suggest that cGAMP when formulated with alum may represent an effective adjuvantation system to foster humoral and cellular aspects of type 1 immunity for early life immunization.

Keywords: vaccines, adjuvants, newborn, antigen-presenting cells, germinal centers, T follicular helper cells, antibodies, stimulator of interferon genes
INTRODUCTION

Infectious diseases represent a major cause of morbidity and mortality in neonates and young infants (1, 2). For example, each year in the US ~20,000 children <5 years old are hospitalized due to influenza complications and flu-related death may occur, especially among those with underlying chronic illness (3). Immunization strategies are fundamental to prevent infectious diseases. However, due to age-specific immunity, vaccines often demonstrate reduced efficacy in newborns and young infants compared to adults (4, 5). Newborn innate immune cells exhibit distinct activation profiles in response to pattern recognition receptor (PRR) agonists (6, 7), and only certain PRR agonists (e.g., TLR7/8 agonists) (8–14) or their combinations (15, 16) are able to induce an adult-like response. The newborn adaptive immune compartment presents distinct features that may also limit vaccine efficacy. Neonatal B cells can produce immunoregulatory cytokines (e.g., IL-10) (17–20), and the magnitude and persistence of the antibody response are reduced (21). Several mechanisms may contribute to distinct immunity in early life, including distinct activity of B and plasma cells (22, 23), the presence of maternal antibodies, impaired CD4+ CXCR5+ PD-1+ T follicular helper (Tfh) cell differentiation and lymph node germinal center (GC) reaction (24, 25) that may adequately support the antigen-specific B cell response. Moreover, neonatal CD4+ T cells produce lower amounts of IFNγ and are skewed toward Th2, Th17, and Treg polarization (6, 7). Of note, adjuvants exhibit age-specific patterns of Th polarization (16) such that adjuvantanation systems that boost adult immune responses do not necessarily lead to enhanced vaccine efficacy in newborns or young infants (26). Therefore, identification of vaccine adjuvants capable of activating neonatal and infant immune responses may inform development of adjuvanted vaccine formulations that enhance early life immunization (8, 9).

Dendritic cells (DCs) play a pivotal role in activating T cells and instructing the adaptive immune response. They express a high diversity of PRRs, whose activation leads to DC migration to lymph nodes and enhancement of immune-stimulatory functions (27). Recently, a systems vaccinology analysis of young infants vaccinated with trivalent inactivated influenza vaccine (28). Agonists of the intracellular receptors TLR7/8, that recognize viral single-stranded RNAs, potentiate Th1-polarizing responses, including expression of interferons (IFNs), production of IL-12p70 and upregulation of co-stimulatory molecules in newborn DCs in vitro and enhance vaccine efficacy in newborn non-human primates in vivo (8–14). Moreover, adjuvantation with the TLR9 agonist CpG increases CG Tfh and B cell responses in newborn mice (25). Among intracellular PRRs, the stimulator of interferon genes (STING) is an agonist of the TLRs (29, 30). It binds cyclic dinucleotides (CDNs) derived from bacteria (i.e., c-di-AMP, c-di-GMP), and 3′-3′-cGAMP) or synthesized in mammalian cells by cGAMP synthase in response to double-stranded DNA in the cytoplasm (i.e., 2′3′-cGAMP). Upon activation, STING induces the TBK-1-mediated phosphorylation of IRF3, which in turn modulates the expression of type I IFNs, IFN-stimulated genes, and also promotes DC maturation and type 1 (i.e., IFNY-driven) immunity (31). Accordingly, STING agonists have demonstrated promising adjuvanticity in adult experimental models of parenteral and mucosal immunization as well as cancer immunotherapy (32–49). However, to our knowledge, STING has not yet been investigated as an adjuvant target for early life immunization.

Here, we took an unbiased approach to identify PRR-based agonists for early life immunization. We employed adult and neonatal bone marrow-derived DCs (BMDCs) to screen the activity of a comprehensive panel of PRR agonists and adjuvants, and found that the STING ligand 2′3′-cGAMP is a potent activator of newborn BMDCs. Strikingly, we found that 2′3′-cGAMP formulated with alum induces antibody isotype switching toward IgG2a/c, a subclass endowed with higher effector functions, appears to enhance the GC reaction and also promotes Th1 polarization in immunized newborn mice. Altogether, our study supports the use of STING ligands and their formulations for enhancement of early life immunization.

MATERIALS AND METHODS

Ethics Statements

All experiments involving animals were approved by the Animal Care and Use Committee of Boston Children’s Hospital and Harvard Medical School (protocol numbers 15-11-3011 and 16-02-3130).

Animals

C57BL/6 and BALB/c mice were obtained from Taconic Biosciences or Charles River Laboratories and housed in specific pathogen-free conditions in the animal research facilities at Boston Children's Hospital. For breeding purposes, mice were housed in couples, and cages checked daily to assess pregnancy status of dams and/or the presence of pups. When a new litter was discovered, that day was recorded as day of life (DOL) 0. Both male and female pups were used for experiments.

Generation of Neonatal and Adult Murine Bone Marrow-Derived Dendritic Cells (BMDCs)

BMDCs were generated from newborn (5–7 days old) and adult (6–12 weeks old) C57BL/6 mice with an adaptation of previously described methods (50, 51). Briefly, mice were sacrificed and legs removed; bones were surgically cleaned from surrounding tissue, extremities of tibiae and femurs were trimmed with sterile scissors and bone marrow flushed through a 70-µm nylon mesh strainer (Corning Life Sciences). Cell number and viability was determined by trypa blue exclusion. Whole bone marrow cells were plated into non-tissue culture-treated 100 mm Petri dishes (Corning Life Sciences) at a density of 0.3 × 10⁶ cells/ml in 10 ml total volume/plate of complete culture medium (RPMI 1640 plus 10% heat-inactivated fetal bovine serum [FBS, GE Healthcare.
HyClone], 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin [Gibco ThermoFisher Scientific] supplemented with 20 ng/ml of recombinant murine GM-CSF (rmGM-CSF, R&D systems). Plates were incubated in humidified atmosphere at 37°C, 5% CO2 for 6 days, with one supplement of 10 ml of complete culture medium and rmGM-CSF on day 3. On day 6, non-adherent and loosely adherent cells were harvested by washing the plate gently with culture medium. Adherent cells were discarded. For flow cytometry analysis, BMDCs were stained and analyzed by flow cytometry as indicated above. For experiments involving blocking antibodies, BMDCs were pre-incubated for 20 min at 37°C with anti-mouse cGAMP adsorption to aluminum hydroxide (Alhydrogel) we mixed 100 μg/100 μl of 2’3’-cGAMP with 1000 μg/100 μl of alum (a 1:10 cGAMP:alum mass ratio) plus 300 μl of 0.9% saline. After vortexing for 10 s the sample was placed in a 37°C incubator. Every 15 min, the sample was vortexed for an additional 5 s and placed back into the incubator. Aliquots were taken at t = 0.25, 0.5, 1, 2, 4 and 24 h and centrifuged at 3,000 RPM (rcf = 664 g) to separate the alum from the supernatant. Supernatant was immediately removed and placed into an autosampler vial undiluted for analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) to determine adsorption as a function of time. RP-HPLC samples were run on a Waters 2695 HPLC equipped with a 2996 photodiode array detector at a wavelength of 254 nm. A gradient was performed using a two mobile phase system of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, on an Agilent Zorbax Eclipse Plus C18, 4.6 × 150 mm, 5 μm column at 25°C. The response (peak area) of the samples were compared against a 50 μl 2’3’-cGAMP plus 200 μl 0.9% saline control and a separate 100 μl alum plus 400 μl saline control.

Quantification of 2’3’-cGAMP Adsorption onto Alum
To quantify the extent of 2’3’-cGAMP adsorption to aluminum hydroxide (Alhydrogel) we mixed 100 μg/100 μl of 2’3’-cGAMP with 1000 μg/100 μl of alum (a 1:10 cGAMP:alum mass ratio) plus 300 μl of 0.9% saline. After vortexing for 10 s the sample was placed in a 37°C incubator. Every 15 min, the sample was vortexed for an additional 5 s and placed back into the incubator. Aliquots were taken at t = 0.25, 0.5, 1, 2, 4 and 24 h and centrifuged at 3,000 RPM (rcf = 664 g) to separate the alum from the supernatant. Supernatant was immediately removed and placed into an autosampler vial undiluted for analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) to determine adsorption as a function of time. RP-HPLC samples were run on a Waters 2695 HPLC equipped with a 2996 photodiode array detector at a wavelength of 254 nm. A gradient was performed using a two mobile phase system of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile, on an Agilent Zorbax Eclipse Plus C18, 4.6 × 150 mm, 5 μm column at 25°C. The response (peak area) of the samples were compared against a 50 μl 2’3’-cGAMP plus 200 μl 0.9% saline control and a separate 100 μl alum plus 400 μl saline control.

In Vitro Restimulation of rHA-Specific T Cell Responses
Splenocytes from immunized mice were harvested 10 days post-boost (DOL 24) as previously reported (25, 52, 53) and restimulated in vitro to assess cytokine production by flow cytometry. Spleens were mashed through a 70 μM strainer, washed with PBS, and erythrocytes were lysed with 2 min of incubation in ammonium chloride-based lysis buffer (BD Biosciences). Cells were then counted and plated 2 × 10^6 per well (round bottom
RESULTS

Phenotypic and Functional Characterization of Neonatal BMDCs

Murine BMDCs represent a widely used model to study DC function in vitro. Adult BMDCs represent a heterogeneous population composed of CD11c+ macrophage-like and DC-like cells with distinct phenotypic and functional profiles (54). However, murine neonatal BMDCs have never been characterized in depth. Therefore, we first sought to define the phenotypic and functional properties of neonatal BMDCs. Although the cell yield from neonatal bone marrow was lower compared to adult ones (Figures S1A,B in Supplementary Material), neonatal immature BMDCs generated from 7-day-old mice grew in culture similarly to adult cells (Figure S1C in Supplementary Material), and once fully differentiated they expressed similar levels of CD11c compared to adult cells but significantly lower levels of MHCII (Figures S1D,E in Supplementary Material). To further characterize phenotypic differences between newborn and adult BMDCs, we assessed by flow cytometry the expression of different macrophage and DC markers. As previously reported for adult BMDCs (54), neonatal BMDCs also comprised CD11c+ MHCII-low and CD11c+ MHCII-high cells. Of note, the percentage of MHCII-low cells was higher in neonatal BMDCs compared to adult BMDCs. Neonatal MHCII-low BMDCs also expressed higher levels macrophage-associated markers (CD64, CD115, CD11b, F4/80) compared to MHCII-high BMDCs, while this population expressed higher levels of CD117. No significant differences in surface marker expression were found between corresponding neonatal and adult MHCII-high and -low populations, except for neonatal MHCII-low BMDCs that expressed higher levels of F4/80 and neonatal MHCII-high BMDCs that expressed higher levels of CD117 compared to their adult counterparts (Figures S2A,B in Supplementary Material).

To characterize a functional response of newborn BMDCs, we next assessed cytokine production and upregulation of co-stimulatory molecules in response to the TLR4 agonist smooth LPS. While newborn BMDC production of IL-6 and TNF was, respectively, comparable or slightly lower than adult BMDCs, IL-12p70 production, albeit detectable, was markedly reduced compared to adult BMDCs (Figure S3A in Supplementary Material). The latter result might be consistent with a more macrophage-like phenotype of newborn BMDCs. As previously reported, both adult and newborn BMDCs produced IL-1β in response to rough but not smooth LPS (55), with newborn BMDCs producing slightly higher amounts of IL-1β (Figure S3B in Supplementary Material). Finally, newborn BMDCs expressed lower levels of MHCII, CD40, and CD86 in response to smooth LPS (Figures S3C,D in Supplementary Material).

Statistical Analyses and Graphics

Data were analyzed and graphed using Prism for Macintosh v. 7.0 (GraphPad Software). Tests used for statistical comparisons are indicated in figure legends. p-value <0.05 was considered significant.

Analysis of the GC Reaction

Draining (inguinal) lymph nodes (dLNs) from immunized mice were harvested 10 days post-boost (DOL 24) as previously reported (25, 52, 53). To prepare a single-cell suspension, dLNs were pressed using the plunger end of a syringe. Then, cells were washed and stained with the following antibodies: for GC Tfh cells, anti-CD45, anti-CD4, anti-CD28 (Biolegend) and cultured for 18 h. Wells were then washed twice with wash buffer (PBS + Tween-20 0.05%) and once with distilled water. Wells were blocked with 200 µl of complete medium supplemented with recombinant mouse IL-2, IL-4, and IL-17. Data were analyzed and graphed using Prism for MacIntosh v. 7.0 (GraphPad Software). Tests used for statistical comparisons are indicated in figure legends. p-value <0.05 was considered significant.

IFNγ ELISPot

Draining lymph nodes from immunized mice were harvested 3 days post-boost (DOL 17). Nitrocellulose 96-microwell plates (Millipore) were coated with 75 µl/well of anti-mouse IFNγ (10 µg/ml in PBS, clone R4-6A2, BD Pharmingen) overnight at 4°C. Plates were incubated with 100 µl of biotinylated anti-mouse IFNγ (5 µg/ml in PBS + FBS 10%, clone XMG1.2, BD Pharmingen) for 2 h at RT, washed again and incubated with 100µl of streptavidin-alkaline phosphatase (1:1000 dilution in PBS + FBS 10%, MabTech) for 1 h prior to color development using BCIP/NBT substrate (Biorad) as per manufacturer’s protocol. Spots on air-dried plates were counted on an ImmunoSpot Analyzer.
panel of PRR agonists and adjuvants (Table S2 in Supplementary Material). As readouts we measured cytokine production (TNF, IL-1β, IL-6, and IL-12p70) and surface expression of maturation markers (CD40, CD80, and CD86). At the most effective, non-toxic (as established in preliminary experiments, data not shown) concentration of each agonist (in bold in Table S2), neonatal BMDCs produced similar amounts of TNF, IL-6, and IL-1β compared to adult BMDCs in response to different TLR7/8 agonists, namely R848 (Resiquimod, imidazoquinoline), CLO75 (thiazoloquinolone) or CL264 (9-benzyl-8 hydroxyadenine), but again failed to produce IL-12p70 (Figure 1A). Remarkably, the upregulation of surface maturation marker expression on neonatal BMDCs was much lower than adult BMDCs upon any PRR stimulation, with the exception of the STING agonist 2′,3′-cGAMP (hereafter cGAMP) (Figure 1B). To assess in depth the response to STING and TLR7/8 agonists, we stimulated neonatal and adult BMDCs with different concentrations of cGAMP and R848. We confirmed that R848 induced higher production of TNF and IL-12p70 (the latter only in adult BMDCs), while cGAMP was more effective than R848 at upregulating the expression of surface maturation markers (Figure 1C). cGAMP also induced dose-dependent IFNγ production in both newborn and adult BMDCs (Figure 1C). Of note, the response of neonatal and adult BMDCs to cGAMP was comparable (Figure S4 in Supplementary Material). Using neutralizing antibodies against TNF or type I IFN receptor (IFNAR), we demonstrated that the expression of maturation markers by neonatal BMDCs mostly relies on type I IFN signaling (Figure S5 in Supplementary Material).

cGAMP Formulated with Alum Enhances Anti-rHA IgG2a/c Antibody Titers in an Early Life Immunization Model

The in vitro results obtained so far supported further investigation of cGAMP as adjuvant candidate for early life immunization. Therefore, we proceeded to test this hypothesis in vivo. We immunized newborn (7-day old) and adult (8- to 10-week old) C57BL/6 mice using a prime-boost schedule (Figure 2A) and employing trivalent recombinant hemagglutinin (rHA) influenza vaccine Flublok as clinically relevant model antigen that is devoid of adjuvant, alone, or formulated with alum [Alhydrogel, Al(OH)3], cGAMP or (cGAMP + alum) (Figure 2B). Mice were bled 14, 21, 28, and 35 days post-prime (respectively, day of life (DOL) 21, 28, 35, and 42 for newborn mice) to assess the magnitude and kinetic of the antibody response. As expected, both alum and cGAMP increased anti-rHA IgG titers in adult mice. We also investigated the titers of the IgG subclasses IgG1 and IgG2c, respectively associated with type 2 and type 1 (IFNγ-driven) immunity (56, 57). In keeping with previously published data, alum preferentially increased anti-rHA IgG1 titers (median anti-rHA IgG1 titers at Day 35 post-prime: 5.02 × 10^6 for alum, 0.77 × 10^6 for cGAMP), while cGAMP was more effective than alum at enhancing anti-rHA IgG2c titers (median anti-rHA IgG2c titers at day 35 post-prime: 0.16 × 10^6 for alum, 0.82 × 10^6 for cGAMP). (cGAMP + alum) was as effective as alum at increasing anti-rHA IgG and IgG1 titers [median anti-rHA IgG and IgG1 titers at day 35 post-prime: 4.77 × 10^6 and 4.46 × 10^6 for (cGAMP + alum)], and even more effective than cGAMP alone at enhancing anti-rHA IgG2c titers [median anti-rHA IgG2c titers at day 35 post-prime: 3.27 × 10^5 for (cGAMP + alum)] (Figure 2B, upper panels and Figure S6 in Supplementary Material). In newborn mice, we unexpectedly found that cGAMP was much less effective at increasing anti-rHA IgG, IgG1, and IgG2c titers [median anti-rHA IgG, IgG1, and IgG2c titers at day 35 post-prime (DOL 42): respectively, 20.57 × 10^5, 24.51 × 10^5, and 0.23 × 10^6 for cGAMP]. Alum enhanced anti-rHA IgG and IgG1 titers, but in marked contrast from adult mice it did not induce anti-rHA IgG2c titers [median anti-rHA IgG, IgG1, and IgG2c titers at day 35 post-prime (DOL 42): respectively, 48.35 × 10^5, 143.23 × 10^5, and 0.00 × 10^5 for alum]. Surprisingly, (cGAMP + alum) adjuvantage matched or exceeded alum at increasing anti-rHA IgG and IgG1 titers [median anti-rHA IgG and IgG1 titers at Day 35 post-prime (DOL 42): respectively, 329.19 × 10^5 and 167.83 × 10^5 for (cGAMP + alum)], and, remarkably, also induced relatively high titers of anti-rHA IgG2c as early as 14 days post-prime (DOL 21) [median anti-rHA IgG2c titers at day 14 (DOL 21) and day 35 post-prime (DOL 42): respectively, 0.14 × 10^6 and 4.23 × 10^5 for (cGAMP + alum)] (Figure 2B, lower panels and Figure S7 in Supplementary Material). Therefore, the addition of cGAMP to alum markedly enhanced anti-rHA antibody production (in particular IgG2c), with a more prominent effect in newborn than adult mice (~400 as compared to ~150-fold increase, respectively) (Figure 2C). Interestingly, newborn mice immunized at DOL 7 and 14 (as indicated in Figure 2B) with (cGAMP + alum) still display the highest anti-rHA IgG and IgG2c titers at DOL 90 compared to saline and alum groups (Figure S8 in Supplementary Material). Enhancement of anti-rHA IgG and IgG2a titers induced by (cGAMP + alum) was also demonstrable in the Th2-skewed mouse strain BALB/c (Figure S9 in Supplementary Material).

In light of the robust adjuvanticity of the (cGAMP + alum) formulation, we quantified cGAMP adsorption to alum by RP-HPLC (Table 1). We observed a rapid initial adsorption of cGAMP onto alum (63% of total cGAMP) within 15 min from the incubation. The adsorption rate dropped quickly, with the overall adsorption reaching a plateau (75.33% of total cGAMP) after 24 h of incubation. No significant degradation products were observed over this time window.

Altogether, our in vivo results demonstrate that (cGAMP + alum) is an effective formulation to enhance antigen-specific antibody titers (especially of the IgG2a/c subclass) for early life immunization.

(cGAMP + Alum) Fosters Th1 Polarization and GC Reaction

IgG2a/c isotype switching is driven by IFNγ in vivo (58), and reduced in early life, since newborns display reduced IFNγ production and Th1 polarization to many stimuli (6, 7). Therefore, we investigated whether (cGAMP + alum) was able to modulate the polarization and cytokine production of antigen-specific T cells. Accordingly, newborn mice were immunized as indicated in Figure 2A with alum or (cGAMP + alum). Ten days
|       | TNF | IL-1β | IL-6 | IL-12p70 |
|-------|-----|-------|------|----------|
| CTRL  |     |       |      |          |
| Ad    | 12000 | 8000  | 27000| 200      |
| N     |     |       |      |          |
| R848  |     |       |      |          |
| CL264 |     |       |      |          |
| CL075 |     |       |      |          |
| MPLA  |     |       |      |          |
| Curdlan |   |       |      |          |
| TDB   |     |       |      |          |
| Pam3CSK4 |   |       |      |          |
| FSL-1 |     |       |      |          |
| Pam2CSK4 |   |       |      |          |
| Chitosan |  |       |      |          |
| TL8-506 |   |       |      |          |
| ODN 2395 |  |       |      |          |
| cGAMP |     |       |      |          |
| Furfuran |   |       |      |          |
| L-18  |     |       |      |          |
| 5'-ppp-dsRNA | |   |      |          |
| Poly (dA:dT) | |   |      |          |
| Adju-Phos |   |       |      |          |
| C12-IE-DAP |   |       |      |          |
| Poly(I:C) HMW | |   |      |          |
| Flagellin |   |       |      |          |
| Alhydrogel | | |      |          |

|       | CD40 | CD80 | CD86 |
|-------|------|------|------|
| CTRL  |      |      |      |
| Ad    | 54   | 38   | 18   |
| N     |      |      |      |
| R848  |      |      |      |
| LPS R595 |      |  |      |
| LPS O55:B5 |   |      |      |
| Chitosan |   |      |      |
| Poly(I:C) HMW | |   |      |
| TDB  |      |      |      |
| Pam3CSK4 |   |      |      |
| Pam2CSK4 |   |      |      |
| Chitosan |  |      |      |
| TL8-506 |   |      |      |
| ODN 2395 |  |      |      |
| cGAMP |      |      |      |
| Furfuran |   |      |      |
| Curdlan |   |      |      |
| TDB   |      |      |      |
| Adju-Phos |   |      |      |
| Flagellin |   |      |      |
| C12-IE-DAP |   |      |      |
| L-18  |      |      |      |

**Figure 1** | Screening of pattern recognition receptor (PRR) agonists on neonatal and adult BMDCs. (A–C) Newborn (N) and adult (Ad) BMDCs were stimulated with the indicated PRR agonists or adjuvants for 20–24 h. Cytokine production (A,C) and MFI of surface marker expression (B,C) were, respectively, assessed by ELISA and flow cytometry. (A,B) Color intensities of the heatmaps are proportional to (A) mean cytokine levels (expressed as pg/ml) or (B) mean co-stimulatory molecule levels (expressed as fold change of median fluorescence intensity over CTRL) of 6–8 (A) or 3 (B) independent experiment. (C) Results are expressed as mean ± SEM of 4–5 (cytokine production) or 3 (surface marker expression) independent experiments. *p < 0.05, **p < 0.01 determined by repeated measures two-way ANOVA with Sidak post hoc test.
post-boost, splenocytes were harvested, re-stimulated with rHA in the presence or absence of the co-stimulus αCD28, and cytokine production by CD4+ T cells was measured by flow cytometry (Figure 3A). While IL-2- and IL-4-producing cells were observed in both groups, IFNγ+ CD4+ T (Th1) cells were only detected among splenocytes isolated from mice immunized
with (cGAMP + alum) [median percentages of IFNγ+ CD4+ T cells upon rHA re-stimulation: 0.000 for saline, 0.031 for alum, and 0.295 for (cGAMP + alum) groups; upon rHA + αCD28 re-stimulation: 0.009 for saline, 0.021 for alum, and 0.280 for (cGAMP + alum) groups]. No IL-17 production was observed in any of the tested conditions (Figure 3B). To corroborate this evidence, upon in vitro re-stimulation with rHA + αCD28 we found by ELISPOT a higher number of IFNγ-producing cells in the dLNs of mice immunized with (cGAMP + alum) 3 days post-boost (Figure 4).

T cell-dependent antibody generation is initiated in GCs and guided by Tfh cells (59, 60). Since GCs are major sites for isotype switching, we reasoned that immunization of newborn mice with (cGAMP + alum) might promote the GC reaction, thereby inducing IgG2a/c switching. To this aim, we assessed by flow cytometry the percentages and absolute numbers of GC Tfh and B cells (respectively, identified as viable singlet CD45+ B220− CD3+ CD4+ CXCR5+ PD-1+ and CD45+ CD3− B220+ GL−7+ CD138− cells) in dLNs 10 days post-boost of newborn mice immunized with (cGAMP + alum) 3 days post-prime (Figure 5A).

The GC is also the site where the processes of somatic hypermutation of antibody variable region genes and generation of high-affinity antibodies take place (60). To verify whether cGAMP modulates antibody affinity maturation, we measured rHA-specific IgG avidity of newborn mice immunized with alum or (cGAMP + alum) as indicated in Figure 2A. Although we observed a steep increase in antibody avidity 21 days post prime (DOL 28) which reached a plateau later on [28 (DOL 35) and 35 (DOL 42) days post-prime], no differences between the two groups were detected at any time point (Figure 6).

Overall, these results demonstrate that the addition of cGAMP to alum promoted the induction of IFNγ-producing T cells and appeared to foster the GC reaction, which might in turn drive IgG2a/c isotype switching in our early life immunization model.

### Single-Dose Immunization with (cGAMP + Alum) Induces rHA-Specific IgG2c Antibodies

The results obtained so far supported the efficacy of (cGAMP + alum) as an adjuvantication system in a prime/boost model of neonatal murine immunization. Of note, a single-dose immunization strategy capable of enhancing antigen-specific antibody titers would be highly desirable early in life. To this end, we immunized newborn mice with rHA formulated with alum, cGAMP, or (cGAMP + alum). Distinct from its effects in prime/boost immunization, cGAMP without alum did not induce detectable anti-rHA IgG, IgG1, and IgG2c titers. Alum and (cGAMP + alum) significantly increased anti-rHA IgG and IgG1 titers [median anti-rHA IgG and IgG1 titers: respectively, 26.74 × 10^4 and 52.08 × 10^4 for alum; respectively, 1.07 × 10^5 and 1.48 × 10^5 for (cGAMP + alum)]. Interestingly, only (cGAMP + alum) induced detectable levels of anti-rHA IgG2c (median: 571.9), albeit at lower levels compared to prime/boost immunization (Figure 7). Altogether, these results demonstrate that (cGAMP + alum) is an effective adjuvantication system also for single dose early life immunization.

### DISCUSSION

Over the past decades, many PRRs and their agonists have been identified, and the molecular definition of their mechanisms of action and immunostimulatory properties has paved the way for new classes of adjuvants (26, 61). For example, the TLR4 agonist monophosphoryl lipid A is employed in different FDA-approved vaccine formulations. Despite this wealth of knowledge, the portfolio of adjuvants approved or in clinical development for...
the newborn and the young infant is much narrower, in part due to our limited knowledge of the immune system early in life (6, 7, 62). Notwithstanding these limitations, in vitro and pre-clinical in vivo studies have shown that targeting some PRRs, in particular TLR7/8 (8–14), potently activates newborn immune cells and markedly enhances vaccine efficacy early in life. Here, by combining an in vitro analysis of newborn BMDC activation in response to PRR agonists and an in vivo immunization models, we identify the STING agonist cGAMP as adjuvant candidate for early life immunization. In particular, we demonstrate that immunization of newborn mice with cGAMP formulated with alum appears to foster the GC reaction as well as features of IFNγ-driven type 1 immunity, namely switching toward IgG2a/c subclass and Th1 polarization.

Although there is no comprehensive consensus on whether and how in vitro models can predict the in vivo effect of candidate adjuvants, the use of DCs has some advantages for assessing their activity in vitro (6, 27, 63, 64). First, DCs are the most prominent subset of antigen-presenting cells. Second, they express many PRRs. Third, DCs can be employed to recapitulate age-specific differences. Although isolating primary DCs from spleen and lymph nodes of neonatal mice would be ideal, this approach is cumbersome if not impossible due to low cell yield (65, 66). Therefore, we developed and characterized a neonatal BMDC...
In conclusion, we demonstrate that cGAMP is a promising and robust adjuvant candidate for early life immunization. We also show that cGAMP formulated with alum potently enhances humoral and cellular aspects of type 1 immunity in early life. Since we employed the rHA influenza vaccine throughout our work,
Immunization with (cGAMP + alum) fosters the germinal center (GC) reaction. Newborn mice were immunized with alum or (cGAMP + alum) as indicated in Figure 2A. Ten days after boost [day of life (DOL) 24] cells were isolated from draining lymph nodes and the percentages and absolute numbers of CD4+ T cells, B cells, GC Tfh, and B cells were assessed by flow cytometry. (A,B) Representative gating strategies. CD4+ T cells were defined as viable singlet CD45+ B220− CD3+ CD4+ cells. GC Tfh cells were defined as viable singlet CD45+ B220− CD3+ CD4+ CXCR5+ PD-1+ cells. B cells were defined as viable singlet CD45+ B220+ CD3− GL-7− CD138− cells. GC B cells were defined as viable singlet CD45+ B220+ CD3− CD19+ GL-7+ CD138− cells. B cells were defined as viable singlet CD45+ B220+ CD3− GL-7− CD138− cells. Results are shown as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 9–10 mice per group. *p < 0.05, **p < 0.01 determined by one-way ANOVA with Holm–Sidak’s post hoc test.

FIGURE 5 | Immunization with (cGAMP + alum) fosters the germinal center (GC) reaction. Newborn mice were immunized with alum or (cGAMP + alum) as indicated in Figure 2A. Ten days after boost [day of life (DOL) 24] cells were isolated from draining lymph nodes and the percentages and absolute numbers of CD4+ T cells, B cells, GC Tfh, and B cells were assessed by flow cytometry. (A,B) Representative gating strategies. CD4+ T cells were defined as viable singlet CD45+ B220− CD3+ CD4+ cells. GC Tfh cells were defined as viable singlet CD45+ B220− CD3+ CD4+ CXCR5+ PD-1+ cells. B cells were defined as viable singlet CD45+ B220+ CD3− GL-7− CD138− cells. GC B cells were defined as viable singlet CD45+ B220+ CD3− GL-7+ CD138− cells. Results are shown as the median, the 25th and 75th percentiles (boxes) and the 5th and 95th percentiles (whiskers) of 9–10 mice per group. *p < 0.05, **p < 0.01 determined by one-way ANOVA with Holm–Sidak’s post hoc test.

FIGURE 6 | Continued
our results may be applicable to influenza immunization. Use of (cGAMP + alum) may also represent a general strategy to elicit type 1 immunity toward protein antigens for early life immunization.

ETHICS STATEMENT

All experiments involving animals were approved by the Animal Care and Use Committee of Boston Children’s Hospital and Harvard Medical School (protocol numbers 15-11-3011 and 16-02-3130).

AUTHOR CONTRIBUTIONS

FB, CP, DD, and OL designed the study. CP, DD, and FB conducted the in vitro experiments. FB, CP, and JL conducted the in vivo experiments. LW and DB conducted the adsorbance experiments. FB and CP wrote the manuscript. DD and OL provided overall mentorship and assisted in writing the manuscript. FB, CP, JL, LW, PS, DO, JR, SB, LP, FM, DB, DD, and OL contributed to helpful discussions, review, and approval of the final manuscript. All the authors have given final approval for the version submitted for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01772/full#supplementary-material.
REFERENCES

1. Bhutta ZA, Black RE. Global maternal, newborn, and child health—so near and yet so far. N Engl J Med (2013) 369(23):2226–35. doi:10.1056/NEJMa1111853
2. Liu L, Johnson HL, Coussens S, Perin J, Scott S, Lawn JE, et al. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet (2012) 379(9832):2151–61. doi:10.1016/S0140-6736(12)60560-1
3. Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, et al. Influenza-associated hospitalizations in the United States. JAMA (2004) 292(11):1333–40. doi:10.1001/jama.292.11.1333
4. Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, Levy O. Protecting the newborn and young infant from infectious diseases: lessons from immune ontology. Immunity (2017) 46(3):350–63. doi:10.1016/j.immuni.2017.03.009
5. Levy O, Goriely S, Kollmann TR. Immune response to vaccine adjuvants during the first year of life. Vaccine (2013) 31(21):2500–5. doi:10.1016/j.vaccine.2012.10.016
6. Dowling DJ, Levy O. Ontogeny of early life immunity. Trends Immunol (2014) 35(7):299–310. doi:10.1016/j.ti.2014.04.007
7. Zhang X, Zhivaki D, Lo-Man R. Unique aspects of the perinatal immune system. Nat Rev Immunol (2017) 17(8):495–507. doi:10.1038/nri.2017.54
8. Dowling DJ, Scott EA, Scheid A, Bergelson I, Joshi S, Pietrasanta C, et al. Toll-like receptor 8 agonist nanoparticle mimic immunomodulating effects of the live BCG vaccine and enhance neonatal innate and adaptive immune responses. J Allergy Clin Immunol (2017) 140(5):1339–50. doi:10.1016/j.jaci.2017.12.985
9. Dowling DJ, van Haren SD, Scheid A, Bergelson I, Kim D, Mancuso CJ, et al. TLR7/8 adjuvant overcomes hyporesponsiveness to pneumococcal conjugate vaccine at birth. JCI Insight (2017) 2(6):e91020. doi:10.1172/jci.insight.91020
10. Dowling DJ, Tan Z, Prokopowicz ZM, Palmer CD, Matthews MA, Dietsch GN, et al. Ultra-potent and selective TLR8 agonist VTX-294 activates human newborn and adult leukocytes. PLoS One (2013) 8(3):e58164. doi:10.1371/journal.pone.0058164
11. Ganapathi I, Van Haren S, Dowling DJ, Bergelson I, Shukla NM, Malladi SS, et al. The imidazoquinoline toll-like receptor-7/8 agonist hybrid-2 potently induces cytokine production by human newborn and adult leukocytes. PLoS One (2015) 10(8):e0134640. doi:10.1371/journal.pone.0134640
12. Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood (2006) 108(4):1284–90. doi:10.1182/blood-2005-12-4821
13. Levy O, Zarenber KA, Roy RM, Cywes C, Godowski PJ, Wessels MR. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol (2004) 173(7):4627–34. doi:10.4049/jimmunol.173.7.4627
14. Phull BM, Dowling DJ, Gallington LC, Cortes G, Tan Z, Suter EE, et al. Imidazoquinoline toll-like receptor 8 agonist activate human neonatal monocytes and dendritic cells through adenosine-rafactory and caspase-1-dependent pathways. J Allergy Clin Immunol (2012) 130(1):195–204.e9. doi:10.1016/j.jaci.2012.02.042
15. Lemoine S, Jaborn B, Tabka S, Ettreiki C, Deriaud E, Zhivaki D, et al. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. Immunity (2016) 44(3):397–408. doi:10.1016/j.immuni.2016.02.004
16. Corrales L, Glickman LH, McWhirter SM, Kanne DB, Sivick KE, Katibah GE, et al. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. Cell Rep (2015) 11(7):1018–30. doi:10.1016/j.celrep.2015.04.031
17. Currin E, Chen X, Corrales L, Kline DE, Duttagupta TW Jr, Bopp BH, et al. STING pathway activation stimulates potent immunity against acute myeloid leukemia. Cell Rep (2015) 15(11):2357–66. doi:10.1016/j.celrep.2015.06.023
18. Ehbens D, Libanova R, Schulze K, Yevsa T, Moro M, Guzman CA. Bis-(3’-5’) cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. Vaccine (2011) 29(32):5210–20. doi:10.1016/j.vaccine.2011.05.026
19. Ehbens T, Schulze B, Ries P, Link C, Moro M, Guzman CA. The bacterial second messenger cyclic diGMP exhibits potent adjuvant properties. Vaccine (2007) 25(8):1464–9. doi:10.1016/j.vaccine.2006.10.033
40. Hanson MC, Crespo MP, Abraham W, Moynihan KD, Szeto GL, Chen SH, et al. Nanoparticulate STING agonists can cure established tumors resistant to PD-1 blockade. Sci Transl Med (2015) 7(283):283ra252. doi:10.1126/scitranslmed.aac4306

41. Karaiskis DK, Means TK, Yang D, Takahashi M, Yoshimura T, Muraille E, et al. Bacterial c-di-GMP is an immunostimulatory molecule. J Immunol (2007) 178(4):2171–81. doi:10.4049/jimmunol.178.4.2171

42. Li XD, Wu J, Gao D, Wang H, Sun L, Chen ZJ. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. Science (2013) 341(6152):1390–4. doi:10.1126/science.1244474

43. Libanova R, Ebensen T, Schulze K, Bruhn D, Norder M, Y evsa T, et al. The AMP induces Th17 + Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-GMP vaccine. 2017.02.064

44. Libanova R, Ebensen T, Schulze K, Bruhn D, Norder M, Y evsa T, et al. The AMP induces Th17 + Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-GMP vaccine. 2017.02.064

45. Madhun AS, Haaheim LR, Nostbakken JK, Ebensen T, Chichester J, Hanson MC, Crespo MP, Abraham W, Moynihan KD, Szeto GL, Chen SH, et al. Nanoparticulate STING agonists can cure established tumors resistant to PD-1 blockade. Sci Transl Med (2015) 7(283):283ra252. doi:10.1126/scitranslmed.aac4306

46. Madhun AS, Haaheim LR, Nostbakken JK, Ebensen T, Chichester J, Yusibov V, et al. Intranasal c-di-GMP-adjuvanted plant-derived HSV induces Th17+ Th1 specific immune responses and confers protection against Trypanosoma cruzi. PLoS Negl Trop Dis (2017) 11(2):e005300. doi:10.1371/journal.pntd.005300

47. Nakamura T, Miyabe H, Hyodo M, Sato Y, Hayakawa Y, Harashima H. Liposomes loaded with a STING pathway ligand, cyclic di-GMP, enhance cancer immunotherapy against metastatic melanoma. J Control Release (2015) 216:149–57. doi:10.1016/j.jconrel.2015.08.026

48. Ogunniyi AD, Paton JC, Kirby AC, McCullers JA, Cook J, Hyodo M, et al. Protective neutralizing influenza antibody response in the absence of T follicular helper cells. Nat Immunol (2016) 17(12):1447–58. doi:10.1038/nai.3563

49. Ogunniyi AD, Paton JC, Kirby AC, McCullers JA, Cook J, Hyodo M, et al. Protective neutralizing influenza antibody response in the absence of T follicular helper cells. Nat Immunol (2016) 17(12):1447–58. doi:10.1038/nai.3563

50. Ogunniyi AD, Paton JC, Kirby AC, McCullers JA, Cook J, Hyodo M, et al. Protective neutralizing influenza antibody response in the absence of T follicular helper cells. Nat Immunol (2016) 17(12):1447–58. doi:10.1038/nai.3563

51. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. Development of the dendritic cell system during mouse ontogeny. J Immunol (2004) 172(10):12180–8. doi:10.4049/jimmunol.172.2.12180

52. Bagnoli F, Fontana MR, Soldaini E, Mishra RP, Fiaschi L, Cartocci E, et al. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against Staphylococcus aureus. Proc Natl Acad Sci U S A (2015) 112(12):3680–5. doi:10.1073/pnas.1424924112

53. Lofano G, Mancini F, Salvatore G, Cantisani R, Monaci E, Carrisi C, et al. Oil-in-water emulsion MF59 increases germinal center B cell differentiation and persistence in response to vaccination. J Immunol (2015) 195(4):1617–27. doi:10.4049/jimmunol.1402604

54. Left J, Bottcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c−/CD11c+ macrophages and dendritic cells. Immunity (2015) 42(6):1197–211. doi:10.1016/j.immuni.2015.05.018

55. Zanoni I, Bodio C, Broggi A, Otsuni R, Caccia M, Collini M, et al. Similarities and differences of innate immune responses elicited by smooth and rough LPS. Immunol Lett (2012) 142(1–2):41–7. doi:10.1016/j.imlet.2011.12.002

56. Bournaud N, Ravetch JV. Fc gamma receptor function and the design of vaccination strategies. Immunity (2017) 47(2):224–33. doi:10.1016/j.immuni.2017.07.009

57. Gunn BM, Alter G. Modulating antibody functionality in infectious disease and vaccination. Trends Mol Med (2016) 22(11):969–82. doi:10.1016/j.molmed.2016.09.002

58. Miyazaki K, Sugimoto-Ishige A, Harada Y, Adachi Y, Usami Y, Kaji T, et al. Bacterial c-di-GMP is an immunostimulatory molecule. J Immunol (2007) 178(4):2171–81. doi:10.4049/jimmunol.178.4.2171

59. Wang J, Li P, Wu MX. Natural STING agonist as an “Ideal” adjuvant for cutaneous vaccination. J Invest Dermatol (2016) 136(1):2183–91. doi:10.1016/j.jid.2016.05.010

60. Dowling D, Hamilton CM, O’Neill SM. A comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with TLR ligands. Cytokine (2008) 41(3):254–62. doi:10.1016/j.cytob.2007.11.020

61. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. Advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods (1999) 223(1):77–92. doi:10.1016/S0022-1759(98)00204-X

62. Bagnoli F, Fontana MR, Soldaini E, Mishra RP, Fiaschi L, Cartocci E, et al. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against Staphylococcus aureus. Proc Natl Acad Sci U S A (2015) 112(12):3680–5. doi:10.1073/pnas.1424924112

63. Lofano G, Mancini F, Salvatore G, Cantisani R, Monaci E, Carrisi C, et al. Oil-in-water emulsion MF59 increases germinal center B cell differentiation and persistence in response to vaccination. J Immunol (2015) 195(4):1617–27. doi:10.4049/jimmunol.1402604

64. Kreutz M, Tacken PJ, Figdor CG. Targeting dendritic cells – why bother? Blood (2013) 121(15):2836–44. doi:10.1182/blood-2012-09-452078

65. Dacic A, Shao QX, D’Amico A, O’Keefe M, Chen WF, Shortman K, et al. Neutrophil influx promotes survival of CD24low dendritic cells in response to pneumococcal infection. J Immunol (2015) 195(4):1617–27. doi:10.4049/jimmunol.195.4.1617

66. Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R. Ontogeny and innate properties of neonatal dendritic cells. J Immunol (2004) 172(10):12180–8. doi:10.4049/jimmunol.172.2.12180

67. Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R. Ontogeny and innate properties of neonatal dendritic cells. J Immunol (2004) 172(10):12180–8. doi:10.4049/jimmunol.172.2.12180

68. Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R. Ontogeny and innate properties of neonatal dendritic cells. J Immunol (2004) 172(10):12180–8. doi:10.4049/jimmunol.172.2.12180

69. Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R. Ontogeny and innate properties of neonatal dendritic cells. J Immunol (2004) 172(10):12180–8. doi:10.4049/jimmunol.172.2.12180

70. Sun CM, Fiette L, Tanguy M, Leclerc C, Lo-Man R. Ontogeny and innate properties of neonatal dendritic cells. J Immunol (2004) 172(10):12180–8. doi:10.4049/jimmunol.172.2.12180