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Non-standard viral genome-derived RNA activates TLR3 and type I IFN signaling to induce cDC1-dependent CD8+ T-cell responses during vaccination in mice

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There is a critical need to develop vaccine adjuvants that induce robust immune responses able to protect against intracellular pathogens, including viruses. Previously, we described defective viral genome-derived oligonucleotides (DDOs) as novel adjuvants that strongly induce type 1 immune responses, including protective Th1 CD4+ T-cells and effector CD8+ T-cells in mice. Here, we unravel the early innate response required for this type 1 immunity induction. Upon DDO subcutaneous injection, type 1 conventional dendritic cells (cDC1s) accumulate rapidly in the draining lymph node in a Toll-like receptor 3 (TLR3)- and type I interferon (IFN)-dependent manner; cDC1 accumulation in the lymph node is required for antigen-specific CD8+ T-cell responses. Notably, in contrast to poly I:C, DDO administration resulted in type I IFN expression at the injection site, but not in the draining lymph node. Additionally, DDOs induced an inflammatory cytokine profile distinct from that induced by poly I:C. Therefore, DDOs represent a powerful new adjuvant to be used during vaccination against intracellular pathogens.

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1. Introduction

Over the last two centuries, vaccines have saved countless lives [1]. However, recent epidemics and pandemics have highlighted the continued need for vaccines against newly emerging pathogens. The development of new vaccines is constrained by safety issues and by our ability to tailor them to induce the right type of immune response capable of controlling and eliminating the target pathogen. Attenuated vaccines, while capable of inducing robust immune responses that mimic those generated during natural infections, present challenges due to their ability to revert to virulence or cause disease in a growing population of immunocompromised people [2]. Messenger RNA (mRNA) vaccines have been instrumental in efforts to mitigate the COVID-19 pandemic, however the extent of lasting immunity remains to be determined [3]. Inactivated or subunit vaccines offer a safer alternative to live attenuated vaccines but they require an adjuvant to induce protective immune responses. Adjuvants provide a necessary stimulus for the immune response to vaccinated antigens, and shape the induced immune response [4].

Type 1 immunity that includes the development of T helper 1 (Th1) CD4+ cells and robust CD8+ T-cell responses, is critical for clearance of many intracellular pathogens. However, currently we lack adjuvants capable of inducing robust CD8+ T-cell responses, thereby limiting our ability to develop effective vaccines against many pathogens. Type 2 immunity is commonly induced by existing adjuvants and consists of antibodies and Th2 CD4+ T-cells. While the optimal response for extracellular pathogens, this type of response can be detrimental to the host during many infections, especially from respiratory viruses. Early preclinical studies for SARS-CoV vaccines included alum, a type 2 immunity-inducing adjuvant, and resulted in worsened morbidity after viral challenge [5]. Similarly, type 2 immune responses induced upon respiratory syncytial virus (RSV) vaccination or infection led to worsened disease or even death [6–8]. Comparable findings were observed with rhinovirus infections in infants [9,10]. These observations highlight the importance of developing safe adjuvants that induce type 1 immune responses upon vaccination against these pathogens. However, to develop effective type 1 immunity-inducing adjuvants, we first must understand what early steps are required during vaccination that prompt a CD8+ T-cell response.

We have shown that a virus-derived RNA adjuvant named defective viral genome-derived oligonucleotide (DDO) induces a
robust type 1 immune response during vaccination against influenza virus in mice. This response synergizes with other licensed adjuvants and induces effector CD8+ T-cells leading to faster recovery from heterosubtypic influenza virus challenge [11]. DDOs are synthetic and replication incompetent RNAs derived from the 546nt-long Sendai virus (SeV) non-standard viral genome (NsVG) that is the primary immunostimulatory molecule during SeV infections [12,13].

In this study, we asked how a 268nt-long DDO was able to induce an antigen-specific CD8+ T-cell response. We investigated the type of dendritic cells (DCs) activated in response to DDO injection and if those DCs were critical for the CD8+ T-cell response. We next investigated the local cytokine response at the injection site and the draining lymph node to better understand the early response that leads to type 1 immunity. Finally, we uncovered how DDO-268 was sensed by cells to induce an immune response to DDO-adjuvanted vaccines. Together, this study helps build our understanding of how the early innate immune response to a type 1 immunity-inducing adjuvant leads to robust adaptive immunity.

2. Methods

2.1. Ethics statement

All described studies carefully adhered to the recommendations in the Guide for the Care and Use of Laboratory Animal of the National Institute of Health. Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01 approved protocol 804,691 and Washington University in St. Louis approved protocol 20–0120.

2.2. Mice

C57BL/6 mice were obtained from The Jackson Laboratory. Iftnari+ mice were a kind donation of Dr. Thomas Moran (Icahn School of Medicine at Mount Sinai) [14] and were used with sex and age matched C57BL/6 mice (Jackson Laboratory bred in house). Mavs+ mice (B6:129-Mavs+tm1Zjc/J) and WT controls (B6129SF2/J) were purchased from The Jackson Laboratory. Tlr7tm1Flv/J and WT controls (B6129SF2/J) and WT controls (C57BL/6NJ) were purchased from The Jackson Laboratory and WT controls (B6129SF2/J mice) were used with sex-matched C57BL/6 mice (Jackson Laboratory bred in house). Mavs+ mice were a kind donation of Dr. Thomas Moran (Icahn School of Medicine at Mount Sinai) [14] and were used with sex and age matched C57BL/6 mice (Jackson Laboratory bred in house). Mavs+ mice (B6:129-Mavs+tm1Zjc/J) and WT controls (B6129SF2/J) were purchased from The Jackson Laboratory. Tlr7tm1Flv/J and WT controls (C57BL/6NJ) were purchased from The Jackson Laboratory. Ifnafs+ mice [15] were a kind gift from Dr. Kenneth Murphy (Washington University in St. Louis). All experiments were performed using male and female mice.

2.3. Vaccine formulation

Inactivated-disrupted influenza A virus (distIAV) vaccine: Influenza A/Puerto Rico/8/1934 H1N1 (IAV PR/8) was harvested 40 h post-inoculation from the allantoic fluid of 10 day old embryonated eggs and purified through a 35% sucrose cushion. Virus was inactivated with UV light (254 nm at 6-inch distance) for 40 min as previously described [11]. Inactivated virus was disrupted by freezing on dry ice and ethanol, thawing at 37 °C, and vortexing for 1 min. The freeze–thaw cycle was repeated three times. Inactivation was confirmed by the inability of the virus to replicate in MDCK cells (Madin-Darby canine kidney cells, gift from Dr. Scott Hensley, University of Pennsylvania) in the presence of 2 mg/ml trypsin. DDO is a single stranded 268nt in vitro-transcribed RNA that contains the immunostimulatory motif of a DVG and was previously characterized [16]. DDO was produced, stored, characterized, and quality controlled as described [11,12].

2.4. Mice immunization and injection

For immunization, mice were anesthetized with isoflurane and injected subcutaneously (s.c.) into the rear footpad with 10 µg dis-IAV vaccine diluted in PBS adjuvanted with 5 µg DDO, 5 µg Low Molecular Weight polynoisine-polycytidylic acid (poly L:C, Invivogen), or Alum (Alhydrogel 2 %, InvivoGen) at 50% v/v at final volume of 30 µl per dose. Mice were primed and boosted 14 days later with the same vaccine formulation. For adjuvant only experiments, mice were anesthetized with isoflurane and injected s.c. into the rear footpad with PBS, 5 µg DDO, or 5 µg poly I:C diluted with PBS to a final volume of 30 µl.

2.5. RT-qPCR from footpads and lymph nodes

The flesh of injected foot pads and draining lymph nodes were harvested at 4, or 24 h post-injection and placed in TRIzol (Invitrogen). Two lymph nodes were pooled to increase RNA yield and quality. One microgram of RNA isolated by TRIzol was reversed transcribed using high-capacity RNA to cDNA reagents (Applied Biosystem). cPCR assays were performed using SYBR Green PCR Master Mix (Applied Biosystem) in a Viia7 (University of Pennsylvania) Applied Biosystem Lightcypher and transcripts were measured using the delta-delta Ct method. Primers used in the assay were:

B actin for-5'-AGTGTCACAGGTGCTCTCTG-3' and rev-5'-GCTGCC TCAACACCTAAC-3', Ifnb1 for-5'-AGATTGTCCTCAACGTGCTCTC-3' and rev-5'-AGATTGATCACCAGTCCAC-3', Ifna1 for-5'-GGACAGTGTCCTCAACTT-3' and rev-5'-GGAGGAGCTGTACAGTCA-3', Ccl5 for-5'-GGCAGAAGTGTGCTGACT-3' and rev-5'-GGTGACCAGCGATGTCCTA-3'.

2.6. Flow cytometry

Single-cell suspensions of spleen and popliteal lymph nodes were prepared and stained with fluorochrome-labeled antibodies as previously described [17]. Popliteal lymph node cells used for DC staining were digested using DNase (1 µg/ml) and Liberase (5 µg/ml) in Roswell Park Memorial Institute (RPMI) 1640 Medium (Life Technologies) for 20 min at 37 °C. Fixable Viability Dye eFluo506, monoclonal antibody specific for mouse IFNγ (clone XMG1.2), Ly6c (clone HK1.4), CD3 (clone 17A2), CD19 (clone eBio1D3), B220 (clone RA3-6B2), NK1.1 (clone PK136), CD11b (clone N418), XCR1 (clone XMG1.2), Ly6c (clone HK1.4), CD3 (clone 17A2), CD19 (clone eBio1D3), B220 (clone RA3-6B2), NK1.1 (clone PK136), CD11b (clone M1/70), and TNFα (clone MP6-XT22) were obtained from eBioscience. Monoclonal antibodies specific for mouse CD3 (clone CD3E), CD4 (clone GK1.5), CD11a (clone H11578), CR1 (clone ZET), PDCD1 (clone 129 cl), CD11c (clone N418), SLRPs (clone P84), CD64 (clone x54/7.1), and MHC-II (clone M5/114.15.2) were obtained from Biolegend. Monoclonal antibodies specific for mouse CD8 (clone 53–6.7) and CD8α (clone 53–6.7) were obtained from BD BioSciences. IAV-specific tetramers: H-2Db tetramers (QVYSLIRPNENPAHK) were obtained from the NIH Tetramer Core Facility at Emory University. Samples were collected on
a LSRFortessa (BD Bioscience) cytometer and analyzed using the FlowJo Software (TreeStar).

2.7. Quantification of influenza-specific serum antibodies

Sera from immunized mice were analyzed for anti-IAV IgG1 and IgG2c antibodies on day 14 post-boost using ELISA [11]. ELISA plates (Immulon, 4 HBX Extra High Binding) were coated with 5 μg purified IAV and treated with pre-diluted sera (1:100, 1:1000, 1:10000) in triplicate, followed by HRP-conjugated anti-mouse IgG1 or IgG2c (Sourthen Biotech) and TMB substrate (Sera Care).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 and 9 for Mac (GraphPad Software). RT-qPCR, antibody quantification, and flow cytometry data were analyzed by two-way ANOVA followed by Tukey’s multiple comparison test.

3. Results

3.1. Subcutaneous injection of a DDO-adjuvanted vaccine induces CD8+ T-cell responses

We previously showed that upon intramuscular immunization, DDO-268 (DDO from now on) induces a robust type 1 immune response in mice that includes antigen-specific IgG2c antibodies, TNFα- and IFNγ-producing CD4+ T-cells, and CD8+ T-cells [11]. To confirm that DDO is effective in generating antigen-specific CD8+ T-cells when used during subcutaneous (s.c.) immunization, and to compare its activity with the gold standard RNA adjuvant CD8+ T-cells when used during subcutaneous (s.c.) immunization, and to compare its activity with the gold standard RNA adjuvant CD8+ T-cells were examined using IAV-specific tetramer staining. DDO induced twice as many antigen-specific CD8+ T-cells by number and percentage than all other groups, including those treated with inert antigen, such as that found during killed or subunit vaccines 

3.2. DDO induces cDC1 accumulation in the draining lymph node

DCs are critical translators of the innate immune response into adaptive immunity [19]. DCs recruited to the draining lymph node at the time of T-cell priming direct and determine the adaptive immune response bias towards type 1 or type 2 immunity [20]. DCs are divided into three main subtypes, each with different functions. Type 1 conventional DCs (cDC1s) are especially equipped to take inert antigen, such as that found during killed or subunit vaccination, and cross-present it to activate CD8+ T-cells [20]. In contrast, cDC2s more readily activate CD4+ T-cells [21]. Finally, plasmacytoid DCs (pDCs) express high levels of virus sensors and are specially equipped to produce type 1 interferon (IFN) upon sensing viral nucleic acids but are not typically activators of naive T-cells [22].

To examine the timing of DC recruitment to the draining lymph node and the changes that occurred in the DC composition of the draining lymph node upon s.c. injection, mice were injected with PBS, DDO, or poly I:C, and draining lymph nodes were harvested 12, 24, or 36 h post-injection. At baseline, the conventional DC population composition in the lymph node is about 60% cDC2 (XCR1+, SIRPα+), and 40% cDC1 (XCR1+, SIRPα+) (Fig. 2A, C). Both DDO and poly I:C injection increased the percentage and number of cDC1s in the lymph node which peaked at 12 h post-injection (Fig. 2A-B). cDC2s were reduced by percentage in the lymph node due to the early influx of cDC1s, with a maximal reduction in percentage at 12 h post-injection (Fig. 2C-D). The composition of the cDCs within the lymph node returned to baseline percentages by 36 h post-injection, as shown by the ratio of cDC1 to cDC2 returning to the level of PBS (Fig. 2E). In addition, we observed that the percentage of pDCs (B220+, PDCA1+) was increased in the lymph node reaching a maximum at 24 h post infection upon injection of both DDO and poly I:C (Fig. 2F-G). Overall, s.c. injection of DDO induces a rapid accumulation of cDC1s and pDCs in the draining lymph node, similar to poly I:C.

3.3. cDC1s are required for DDO-induced CD8+ T-cell responses

cDC1s are excellent at cross-presenting antigen and are therefore important for the generation of CD8+ T-cell responses to subunit and killed vaccines [20]. Development of cDC1s, but not cDC2s or pDCs, depends on the activation of an irf8 enhancer (+32 kb irf8) [15]. To investigate the requirement for cDC1s in the immune response mounted by DDO, mice lacking cDC1 (irf8 +32−/) and WT mice were injected as described above and analysis of the composition of DCs in the lymph node 12 h after-injection confirmed that the enhancer+32 kb irf8 is required for cDC1 accumulation, but not for cDC2 (Fig. 3A-B). To assess the role of cDC1s in the induction of antigen-specific CD8+ T-cells responses to DDO-adjuvanted vaccines, mice lacking cDC1s and WT mice were vaccinated against IAV as described above. Spleens were harvested 7d post-boost for T-cell analysis. Antigen-specific CD8+ T-cells were only generated in response to DDO-adjuvanted vaccines in WT mice, but not in irf8 +32−/ mice lacking cDC1s (Fig. 3C). In addition, DDO significantly enhanced the type-1 immunity-associated IgG2c response in WT mice, while irf8 +32−/ mice showed a type 2 immunity-associated IgG1 response (Fig. 3D). Overall, these data show that DDO injection induced a cDC1 response that is required for antigen-specific CD8+ T-cell responses.

3.4. DDO induces a local type 1 IFN response

To investigate the early response to DDO injection, which leads to the accumulation of cDC1s in the draining lymph node, mice were injected s.c. with PBS, DDO, or poly I:C and the injected footpad and draining lymph node were harvested at 4, 12, and 24 h post-injection for cytokine expression analysis by RT-qPCR. At 4 h post-injection, Ifnb1 transcripts peaked in the footpad and quickly returned to near baseline levels for both DDO and poly I:C (Fig. 4A). Transcription of IFN-stimulated genes (ISGs), such as Mx1 and the chemokine Ccl5 peaked at 12 h post-injection for both DDO and poly I:C (Fig. 4B-C). Mx1 returned to baseline expression levels by 24 h but Ccl5 remained elevated. Both DDO and poly I:C induced transient Il6 expression at 4 h post-injection (Fig. 4D). Interestingly, DDO induced greater Il1b expression than poly I:C in the injection site, indicating differences in the inflammatory response between these RNA molecules (Fig. 4E).

Greater differences in response to treatments were revealed in the draining lymph node where only injection with poly I:C resulted in detectable Ifnb1 transcripts at any time point (Fig. 4F). Both DDO and poly I:C injection induced ISG transcription at 4 h in the draining lymph node, however, poly I:C induced sustained ISG expression that lasted at least 12 h post-injection (Fig. 4G-H). These data show the induction of type 1 IFN expression in the
draining lymph nodes upon poly I:C injection, a response not observed in DDO-injected mice. The inflammatory cytokines Il6 and Il1b were not significantly induced over PBS injected mice in either condition, indicating a primarily local inflammatory response at these time points (Fig. 4 I-J).

In both the footpad and the lymph node, other type I IFN gene transcripts were examined including Ifna1, Ifna2, Ifna4, Ifna5, Ifna6, Ifna12, and Ifna13. Both DDO and Poly I:C elicited small increases in Ifna4 at 4 hr in the footpad. However, unlike poly I:C, DDO did not induce upregulation of any of the Ifna genes examined in the draining lymph node (Figure S1). As Ifnb1 transcript copy numbers were the only interferon copy numbers that were elevated at the injection site, we determined that IFNβ was the primary IFN produced at the site of injection.

Together, these data show that DDO induces a more focused type I IFN response than poly I:C, a known inducer of systemic type I IFN responses [23]. In addition to the magnitude of IFN responses, DDO induces a stronger expression of inflammatory cytokines than poly I:C. These data underscore the differences between the local responses to two seemingly similar RNAs, one a natural virus-
derived pathogen molecular pattern (PAMP) and one a synthetic dsRNA, confirming previous observations with a prototype DDO at a single time point [13].

3.5. Type I IFN is required to initiate immune responses to DDO-adjuvanted vaccines

Type I IFN is a cytokine family with broad functions that include preparing cells for a viral challenge and helping to shape the adaptive immune response. Type I IFN can aid in the activation of DCs [24,25] and lead to the activation and type 1 immunity polarization of T-cells [26,27]. As we have previously observed a role for type I IFN in inducing T-cell responses upon intramuscular (i.m.) immunization with DDO-adjuvanted vaccines [11], we tested if type I IFN was necessary for cDC1 accumulation in the draining lymph node upon s.c. immunization. WT mice and mice deficient in type I IFN signaling (Ifnar-/-) were injected in the footpad with PBS or DDO and the draining lymph nodes were harvested at 12 h post-injection to analyze the DC composition. In contrast to WT mice, upon DDO injection, there was no increase in the percentage of cDC1s in the draining lymph node in Ifnar-/- mice (Fig. 5A-B) indicating a dependence on type I IFN signaling for the accumulation of cDC1s in the draining lymph node. Additionally, there was no increase in the percentage of pDCs in the draining lymph nodes of DDO Ifnar-/-mice (Fig. 5C).

To determine if the dependence on type I IFN signaling for DC accumulation resulted in a reduction in CD8+ T-cell responses in this system, mice were vaccinated against IAV as described above. Ifnar-/-mice vaccinated with DDO-adjuvanted vaccines were unable to generate IAV-specific CD8+ T-cells (Fig. 5D), indicating a complete reliance on type I IFN for response to DDO-adjuvanted vaccines.

3.6. TLR3 is required for the accumulation of DCs in the draining lymph node and the subsequent CD8+ T-cell response in response to DDO

Adjuvants, including DDO and poly I:C, act as PAMPs [28]. PAMPs are sensed through various pattern recognition receptors.
Fig. 4. DDO activates a rapid type I IFN response with a different cytokine profile than poly I:C. WT mice were injected s.c. with PBS, 5 μg DDO, or 5 μg poly I:C (pIC) in the rear footpad. Injected footpads and draining lymph node were harvested 4, 12, and 24 h post-injection. A-E Expression of transcripts in the footpad are relative to the housekeeping gene β-actin. (n = 6/group). Mean ± SEM of each group is shown. A Relative Ifnb1. B Relative Mx1. C Relative Ccl5. D Relative Il6. E Relative Il1b. F-J Two lymph nodes/group were pooled into one sample for better RNA quality. Expression of transcripts are relative to the housekeeping gene β-actin. (n = 3/group). Data correspond to mean ± SEM of each group. F Relative Ifnb1. G Relative Mx1. H Relative Ccl5. I Relative Il6. J Relative Il1b. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001. Data represent one representative experiment out of 2 independent repeats.
and adaptive immune response induced by DDO, we chose to examine the accumulation of cDC1s in the draining lymph node and the subsequent T-cell response to DDO-adjuvanted vaccines. WT (black) and Ifnar\(^{-/-}\) (red) mice were injected s.c. with PBS or 5 \(\mu\)g DDO in the rear footpad. Draining lymph nodes were harvested 12 h post-injection and processed into a single cell suspension for analysis by flow cytometry. (n = 4–6/group) Mean ± SEM of each group is shown.

(A-C) A Percent of conventional DCs that are cDC1s characterized live, CD3\(^{-}\), B220\(^{-}\), CD11c\(^{hi}\), CD64\(^{hi}\), Ly6C\(^{hi}\), CD8\(^{+}\), CD4\(^{+}\), tetramer+, and Irf8\(^{+}\). B Percent of conventional DCs that are cDC1s characterized live, CD3\(^{-}\), NK1.1\(^{-}\), B220\(^{-}\), CD11c\(^{hi}\), CD64\(^{hi}\), Ly6C\(^{hi}\), CD8\(^{+}\), CD4\(^{+}\), tetramer+, and Irf8\(^{+}\). C Percent of cells in the lymph nodes that are pDCs that are characterized as live, PDCA1\(^{-}\), B220\(^{-}\), CD4\(^{+}\), CD8\(^{-}\), CD11c\(^{-}\), Irf7\(^{+}\), and Irf8\(^{+}\). WT and Ifnar\(^{-/-}\) mice were immunized twice, 14 d apart, with 10 \(\mu\)g disIAV alone (−) or adjuvanted with 5 \(\mu\)g DDO. Spleens were harvested 7 d post-boost, processed into a single cell suspension, and analyzed by flow cytometry (n = 3–4/group). Mean ± SEM of each group is shown. D Number of tetramer\(^{+}\) CD8\(^{+}\) T-cells were defined as Live, CD3\(^{-}\)-CD4\(^{-}\)-CD8\(^{-}\)-CD4 tetramer\(^{-}\). **p < 0.01, ****p < 0.0001. Data represent one representative experiment out of 2 independent repeats. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(DOO) including endosomal RNA sensors TLR7 [29] and TLR3 [28,30] and cytosolic RNA sensors RIG-I and MDA5, which signal through the common adaptor MAVS for the production of type I IFN and protect from lethal challenge upon i.m. injection is critical for targeted vaccine design. DDO is a 268nt-long RNA derived from the primary immunostimulatory molecule of SeV [12,13]. Here we show that DDO induces a local type I IFN and inflammatory response that leads to the accumulation of cDC1s in the draining lymph node. cDC1 recruitment to the lymph node relies on TLR3 and type I IFN signaling, and loss of cDC1 accumulation in the draining lymph nodes prevents the development of antigen-specific CD8\(^{+}\) T-cell responses.

Intriguingly, to trigger RIG-I, RNA would have to be internalized to the cytoplasm after injection [36]. Work from the Mossman lab has demonstrated the need for class-A scavenger receptors for the internalization and subsequent sensing of dsRNA through both TLR3 and RLRs [37,38]. Additionally, proteins such as SiDT2 have been identified as RNA transporters from endosomes into the cytosol for RLR sensing [39]. Further studies are required to determine if class-A scavenger receptors or SiDT2 are used by DDO and if differences between the full-length DVG-adjuvanted vaccinations and DDO-adjuvanted vaccinations change how these related RNAs interact with proteins involved with RNA internalization and lead to sensing by TLRs or RLRs.

4. Discussion

Understanding the innate immune response needed to instruct a CD8\(^{+}\) T-cell response to vaccination is critical for targeted vaccine design. DDO is a 268nt-long RNA derived from the primary immunostimulatory molecule of SeV [12,13]. Here we show that DDO induces a local type I IFN and inflammatory response that leads to the accumulation of cDC1s in the draining lymph node. cDC1 recruitment to the lymph node relies on TLR3 and type I IFN signaling, and loss of cDC1 accumulation in the draining lymph nodes prevents the development of antigen-specific CD8\(^{+}\) T-cell responses.

TLR3 is a known poly I:C sensor [28] and we show here that it is also required for DDO-induced immune responses during vaccination in mice. However, the cytokine response to these PAMPs is not identical. DDO does not induce type I IFN expression in the draining lymph node and triggers higher expression of Il1b than poly I:C. The lack of IFN at the draining lymph node demonstrates that DDO is not as inflammatory as poly I:C and the cytokine secretion is more restricted to the site of injection. We observed that IFN is not needed at the draining lymph node, but at the site of injection to trigger cDC1 accumulation in the lymph node. These intriguing results raise questions about how these differences arise and whether additional sensors or cell types are engaged by DDO at the injection site. Studies with licensed adjuvants, alum and MF59, have shown that additional innate cells such as natural killer cells and monocytes are critical for antigen transport to the lymph node [35]. Perhaps DDO and poly I:C interact with different cells to induce this differential cytokine response. The diversity in sequence lengths of poly I:C, in contrast to the uniformity of DDO, may also play a role. Further examination of the exact cell types interacting with DDO, as well as analysis of half-life of this RNA molecule is needed to fully understand its adjuvancy mechanism.

Another group using a full-length version of the SeV DVG (546nt-long) as a vaccine adjuvant showed that its ability to induce type I IFN and protect from lethal challenge upon i.m. injection is dependent on RIG-I [36]. We show that DDO, which is a truncated version of the full-length DVG [12], induced cDC1 accumulation in the draining lymph node and antigen specific CD8\(^{+}\) T-cells in a TLR3-dependent manner, despite that DDO is sensed through TLR3-dependent manner, despite that DDO is sensed through TLR3 and RLRs [37,38]. Additionally, proteins such as SiDT2 have been identified as RNA transporters from endosomes into the cytosol for RLR sensing [39]. Further studies are required to determine if class-A scavenger receptors or SiDT2 are used by DDO and if differences between the full-length DVG-adjuvanted vaccinations and DDO-adjuvanted vaccinations change how these related RNAs interact with proteins involved with RNA internalization and lead to sensing by TLRs or RLRs.

**Fig. 5.** Type I IFN is required for cDC1 accumulation and the subsequent T-cell response to DDO-adjuvanted vaccines. WT (black) and Ifnar\(^{-/-}\) (red) mice were injected s.c. with PBS or 5 \(\mu\)g DDO in the rear footpad. Draining lymph nodes were harvested 12 h post-injection and processed into a single cell suspension for analysis by flow cytometry. (n = 4–6/group) Mean ± SEM of each group is shown.

(A-C) A Percent of conventional DCs that are cDC1s characterized live, CD3\(^{-}\)NK1.1\(^{-}\)B220\(^{-}\)CD19\(^{-}\)MHCII\(^{hi}\)CD64\(^{hi}\)Ly6c\(^{hi}\)CD11c\(^{hi}\)XCR1\(^{-}\)SIRPa\(^{-}\). B Percent of conventional DCs that are cDC1s characterized as live, CD3\(^{-}\)NK1.1\(^{-}\)B220\(^{-}\)CD19\(^{-}\)MHCII\(^{hi}\)CD64\(^{hi}\)Ly6c\(^{hi}\)CD11c\(^{hi}\)XCR1\(^{-}\)SIRPa\(^{-}\). C Percent of cells in the lymph nodes that are pDCs that are characterized as live, PDCA1\(^{-}\)B220\(^{-}\)CD4\(^{+}\)CD8\(^{-}\)CD11c\(^{-}\)Irf7\(^{+}\)Irf8\(^{+}\). WT and Ifnar\(^{-/-}\) mice were immunized twice, 14 d apart, with 10 \(\mu\)g disIAV alone (−) or adjuvanted with 5 \(\mu\)g DDO. Spleens were harvested 7 d post-boost, processed into a single cell suspension, and analyzed by flow cytometry (n = 3–4/group). Mean ± SEM of each group is shown. D Number of tetramer\(^{+}\) CD8\(^{+}\) T-cells were defined as Live, CD3\(^{-}\)-CD4\(^{-}\)-CD8\(^{-}\)-CD4 tetramer\(^{-}\). **p < 0.01, ****p < 0.0001. Data represent one representative experiment out of 2 independent repeats. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Our data show that DDO relies on cDC1s to induce CD8+ T-cell responses. Any loss of cDC1 accumulation in the draining lymph node, through loss of Ifrb +32/-, type I IFN signaling, or TLR3, abrogated DDO's adjuvancy. It is clear that cDC1s are a potent driver of CD8+ T-cell responses in this system and many viral infections, including West Nile virus [22], cytomegalovirus [40], influenza virus [41], cowpox virus [42], and others. However, the exact mechanism of cDC1 activation and PAMP sensing during many vaccinations is still unclear. Studies from the Kedl lab indicate the IFN-stimulated gene IL-27 production from cDC1s as an indicator of subsequent CD8+ T-cell responses after subunit vaccination [43,44], however they did not examine the signals that lead to the activation of and IL-27 production by cDC1s. To gain a comprehensive picture of how CD8+ T-cell responses are induced, we must fully understand how the cells directly instructing CD8+ T-cells are activated.

We have shown DDO act as a type 1 immunity inducing adjuvant using an influenza vaccination model [11]. In that report, we also demonstrated that in a heterosubtypic challenge, DDO reduced disease severity and induced long lasting protective CD8+ T-cell responses without type-2 immunity-associated pathology. DDO was derived from a different virus than it was used to vaccinate against, therefore is likely that DDO would be beneficial in vaccines for other intracellular pathogens. Intracellular parasites remain a difficult target for vaccination and pathogens such as plasmodium, toxoplasma, and leishmania rely on type 1 immune responses for their clearance and control [45–47]. Recent advances in epitope discovery, such as T-scan [48], are allowing for more targeted vaccine antigen design. By pairing appropriate antigens with DDO, tailored vaccinations against diverse pathogens could be tested.

Great strides have been made in understanding the difference between responses to infection and vaccination with a shift in the focus on the role of innate responses. We have shown an adjuvant derived from a natural PAMP is sensed by TLR3 and induces cDC1 accumulation in the draining lymph node and that this process is necessary for antigen specific CD8+ T-cell development. This study has uncovered the early innate immune response to DDO and has identified potential targets for type 1 immunity inducing adjuvants.

5. Importance

There is a paucity of vaccine adjuvants able to trigger effective and safe protective responses to many intracellular pathogens. Defining the minimal requirements to achieve type 1 (Th1) immunity, which includes antigen specific CD8+ T-cells capable of eliminating infected cells, is essential for the development of adjuvants that induce optimal protective immune responses during vaccination against intracellular pathogens. We used a virus-derived immunostimulatory molecule, defective viral genome-derived oligonucleotide (DDO), to provide insights into how type 1 immune responses are triggered during vaccination using an inactivated influenza vaccine model. Understanding the mechanism of action of vaccine adjuvants not only aids in the advancement of vaccine development, but also in understanding specific immune pathways required for efficient induction of adaptive immune responses to infections.

Authors contributions

Conceived experiments: D.G.F., and C.B.L.; performed experiments and collected data: D.G.F., V.G., D.J.H. Wrote the original draft: D.G.F. and C.B.L.; Supervised research activities: C.B.L.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The University of Pennsylvania and C.B.L. have a patent for Methods and Compositions For Stimulating Immune Response Using Potent Immunostimulatory RNA Motifs.]

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.10.052.

References


Fig. 6. TLR3 is required for cDC1 migration in response to DDO injection. A-C WT (black) and Mavs (-/-) (cyan) mice were injected s.c. with PBS or 5 μg DDO in the rear footpad. Draining lymph nodes were harvested at 12 h post-injection, processed into a single cell suspension, and analyzed by flow cytometry. (n = 4–6/group) Mean ± SEM of each group is shown. A Percent of conventional DCs that are cDC1s characterized live, CD3-CD11c+CD11b+CD14-CD16-CD49b-. B Percent of conventional DCs that are cDC2s characterized as live, CD3-CD11c+MHCII+CD64-CD14+CD16+. C Percent of cells in the lymph nodes that are pDCs that are characterized as live, PDCA1+BDCA2-. D-F WT (black) and Tlr3 (-/-) (green) mice were injected s.c. with PBS or 5 μg DDO in the rear footpad. Draining lymph nodes were harvested at 12 h post-injection, processed into a single cell suspension, and analyzed by flow cytometry. (n = 6/group) Mean ± SEM of each group is shown.