Title: The Mucosal Adjuvant Cyclic di-GMP Enhances Antigen Uptake and Selectively Activates Pinocytosis-efficient Cells in vivo

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Running Title: In vivo mechanisms of mucosal adjuvant cyclic di-GMP
Disclosures

The authors have no conflicting financial interests.
Abstract

Effective mucosal adjuvants enhance the magnitude and quality of the vaccine response. Cyclic di-GMP is a promising mucosal vaccine adjuvant. However, its in vivo mechanisms are unclear. Here, we showed, in mice, that cyclic di-GMP elicits stronger Ab and T_H responses than the mammalian 2’3’-cyclic GMP-AMP, and generated better protection against *Streptococcus pneumoniae* infection than 2’3’-cyclic GMP-AMP adjuvanted vaccine. We identified two in vivo mechanisms of cyclic di-GMP. First, intranasally administered cyclic di-GMP greatly enhances Ag uptake, including pinocytosis and receptor-mediated endocytosis in vivo. The enhancement depends on MPYS (STING, MITA) expression in CD11C^+ cells. Second, we found that cyclic di-GMP selectively activated pinocytosis-efficient-DCs, leading to T_H polarizing cytokines IL-12p70, IFNγ, IL-5, IL-13, IL-23 and IL-6 production in vivo. Notably, cyclic di-GMP induces IFNλ, but not IFNβ, in vivo. Our study revealed previously unrecognized in vivo functions of MPYS and advanced our understanding of cyclic di-GMP as a mucosal vaccine adjuvant.
Introduction

Most pathogens enter the body via mucosal surfaces. Immunization by mucosal routes is more effective at inducing protective immunity against mucosal pathogens than systemic immunization. Moreover, mucosal vaccines have the benefits of low cost and ease of administration, which make mucosal vaccines particularly suitable for developing countries and during emergency. Currently, only a dozen mucosal vaccines are approved for human use. This is largely due to problems with developing safe and effective mucosal adjuvants.

Cyclic di-GMP is a promising mucosal vaccine adjuvant candidate. It is ubiquitously found in bacteria, but is absent in higher eukaryotes. Yan et al. found that intranasal administration of cyclic di-GMP, along with the pneumococcal Ag PsaA, elicits a comparable Ag-specific Ab response, and reduces bacterial colonization to those mice immunized with cholera toxin and PsaA. Cholera toxin is the most potent experimental mucosal adjuvant. Cyclic di-GMP also exhibits balanced Th1, Th2, and Th17 immune responses. A recent study found that cyclic di-GMP is a more potent activator of both Th1 and Th2 immune responses than LPS, CpG oligonucleotides (ODN), and aluminum salt based adjuvant in mice. Thus, cyclic di-GMP is an excellent mucosal vaccine adjuvant candidate promoting both strong humoral and cellular immune responses.

The mechanism by which cyclic di-GMP acts as a mucosal adjuvant in vivo is not known. We previously showed that MPYS-deficient mice (Tmem173−/−) completely lost cyclic di-GMP induced Ag-specific Ab and Th responses. MPYS, also known as STING, MITA and TMEM173, is a type I IFN stimulator. However, we found that...
type I IFN signaling is not required for the mucosal adjuvant activity of cyclic di-GMP in vivo\(^9\). Cyclic di-GMP activates both type I IFN and NF-κB signaling\(^{13}\). While MPYS is required for both cyclic di-GMP induced type I IFN and NF-κB activations\(^{14,15}\), we found that these two pathways can be uncoupled in dendritic cells (DCs) and macrophages\(^9\). Of note, it is still unknown which cell type responds to mucosal adjuvant cyclic di-GMP \textit{in vivo}.

In this study, we investigated how cyclic di-GMP promotes its mucosal adjuvant activity in vivo. We found that cyclic di-GMP enhances Ag uptake in vivo, and selectively activates pinocytosis-efficient DCs in vivo. Furthermore, we demonstrated that these cyclic di-GMP activities depend on the expression of MPYS in DCs in vivo.
Results

Cyclic di-GMP is a better mucosal pneumococcal vaccine adjuvant than the mammalian cyclic dinucleotide 2’3’-cyclic GMP-AMP in mice

Cyclic di-GMP is a potent mucosal vaccine adjuvant with activity similar to that of cholera toxin, the gold standard of a mucosal vaccine adjuvant. The 2’3’-cyclic GMP-AMP is a newly discovered mammalian cyclic dinucleotide that also has mucosal adjuvant activity in vivo. Both cyclic di-GMP and 2’3’-cyclic GMP-AMP can bind MPYS in vitro. The 2’3’-cyclic GMP-AMP has a much better binding affinity to MPYS than cyclic di-GMP. Furthermore, 2’3’-cyclic GMP-AMP induces stronger type I IFN production than cyclic di-GMP does in mammalian cells. We, thus, asked if the 2’3’-cyclic GMP-AMP exhibits superior mucosal adjuvant activity to cyclic di-GMP in vivo.

We intranasally administered BALB/C mice with cyclic di-GMP plus OVA Ag, or 2’3’-cyclic GMP-AMP, plus OVA Ag three times at two weeks interval. The serum anti-OVA IgG1, IgG2A and nasal IgA were quantified. Surprisingly, cyclic di-GMP adjuvanted vaccine induced higher Ag-specific IgG1 and IgA production than the 2’3’-cyclic GMP-AMP adjuvanted vaccine (Fig 1A). The production of OVA-specific IgG2A was similar in both vaccines (Fig 1A).

As a mucosal adjuvant, cyclic di-GMP generates balanced $T_{H1}$, $T_{H2}$ and $T_{H17}$ responses. We next performed the ex vivo recall assay in splenocytes from immunized mice, and examined the $T_{H}$ cytokine production. Again, cyclic di-GMP adjuvanted vaccine generated better IL-13, a $T_{H2}$ cytokine, and IL-17 production than the 2’3’-cyclic GMP-AMP adjuvanted vaccine (Fig 1B). The $T_{H1}$ cytokine, IFNγ, was similarly
produced by both cyclic dinucleotides (Fig 1B).

We then replaced OVA Ag with PspA (pneumococcal surface protein A), a protein Ag extensively tested in various pneumococcal vaccines. We also used a different mouse strain, C57BL/6, to repeat the immunization experiment. We found that cyclic di-GMP adjuvanted PspA based pneumococcal vaccine generated higher titers of PspA-specific IgG1 and nasal IgA (Fig 1C). Additionally, they had stronger IL-13 (T H2) and IL-17 (T h17) responses in the ex vivo recall assay than the 2’3’-cyclic GMP-AMP adjuvanted pneumococcal vaccine (Fig 1D). The IgG2C and IFNγ (T h1) responses were similar between cyclic di-GMP and 2’3’-cyclic GMP-AMP adjuvanted vaccine (Fig 1C and 1D).

Last, we examined the protective immunity against pneumococcal infection in cyclic di-GMP plus PspA versus 2’3’-cyclic GMP-AMP plus PspA immunized mice. We found that mice immunized with cyclic di-GMP adjuvanted pneumococcal vaccine have a lower bacterial burden in the spleens and lungs than mice immunized with 2’3’-cyclic GMP-AMP adjuvant pneumococcal vaccine (Fig 1E). We concluded that, in mice, cyclic di-GMP, as a mucosal adjuvant, generated better Ag-specific Ab production as well as stronger T h responses than the mammalian cyclic dinucleotide 2’3’-cyclic GMP-AMP. This translated into better protection against pneumococcal infection in vivo.

**Intranasal administered cyclic di-GMP does not cause lung injury**

Next, we examined the safety profile of cyclic di-GMP adjuvant. At the dose of cyclic di-GMP used in Fig 1 (5μg), we saw only very mild neutrophil infiltration in Bronchoalveolar lavage fluid (BALF) (Fig 2A) and lungs (Fig 2D). We also determined lung permeability by serum albumin level in BALF. There was no significant difference
in samples from saline or cyclic di-GMP treated mice (Fig 2B). Last, lung histology also
did not reveal any lung damage in cyclic di-GMP treated mice (Fig 2C). We concluded
that intranasally administered cyclic di-GMP, at the dose used as an effective mucosal
adjuvant, did not cause lung injury.

**Intranasal administered cyclic di-GMP does not cause excessive inflammatory**
**responses**

Next, we examined cyclic di-GMP induced cellular responses *in vivo*. Besides a mild
increase in the number of neutrophils in lung, there was also a ~2-fold increase in Ly6C$^{\text{hi}}$
monocytes in the lung after intranasal cyclic di-GMP administration (Fig 2D). There
were no significant increases in numbers of Mast cells or eosinophils in lungs at the
vaccine adjuvant dose of cyclic di-GMP used (5$\mu$g) (Fig 2D). There were also no
increases in the number of B cells or NK cells (Fig 2D and 2E).

Ly6C$^{\text{hi}}$ monocytes could differentiate into dendritic cells (DCs), mainly CD11B$^+$
myeloid DCs, in situ. We did not find any difference in total DC number, or CD103$^+$,
CD11B$^+$ DCs subset numbers in the lungs after cyclic di-GMP treatment (Fig 2F and
2G).

Cyclic di-GMP induces the production of the proinflammatory cytokines TNF$\alpha$ and
IL-1$\beta$ *in vitro*. We confirmed this *in vivo* (Fig 2H). However, we found that cyclic di-
GMP also induced potent IL-10 production, an anti-inflammatory cytokine, *in vivo* (Fig
2I). Furthermore, cyclic di-GMP induced strong IL-22 production *in vivo* (Fig 2I), which
is important for lung epithelium repair. The balanced production of inflammatory and
anti-inflammatory cytokines by cyclic di-GMP likely explains the absence of excess
inflammatory responses *in vivo*.

**Cyclic di-GMP induces potent type II (IFNγ) and III IFN (IFN λ) production *in vivo***

While cyclic di-GMP-induced TNFα and IL-22 production were completely dependent on the expression of MPYS, IL-1β and IL-10 production in *vivo* were only partially dependent on MPYS (Fig 2H and 2I). This was surprising considering that MPYS was the proposed direct receptor for cyclic di-GMP in mammalian cells. We then investigated the cytokine milieu in the lungs after cyclic di-GMP administration in WT and *Tmem173*<sup>−/−</sup> mice.

We first examined the production of type I IFN, the signature cytokine stimulated by MPYS/STING, in the lungs. Although we detected low-level background IFNβ production in the lungs, cyclic di-GMP treatment did not increase IFNβ levels above the background (Fig 3A). This was consistent with our previous observation that the mucosal adjuvant activity of cyclic di-GMP is type I IFN independent<sup>9</sup>.

Surprisingly, we detected potent type III IFN (IFN λ) production in the lungs after intranasal administration of 5μg cyclic di-GMP (Fig 3A). Type III IFN activates similar groups of interferon stimulating genes (ISGs) as type I IFN. However their receptors are mainly expressed on lung epithelial cells<sup>25</sup>. Furthermore, neutralizing IFNλ in *vivo* did not affect the adjuvant activity of cyclic di-GMP (Fig 3-figure supplemental 1).

We also detected strong cyclic di-GMP induced type II IFN (IFN γ) *in vivo* (Fig 3B). Both type II and III IFN production by cyclic di-GMP were absent in MPYS<sup>−/−</sup> mice (Fig 3A and 3B). We concluded that intranasally administered cyclic di-GMP, at the dose used as an effective mucosal adjuvant, induces potent type II and III IFN, but not type I
IFN production *in vivo*.

**Cyclic di-GMP induces T\(_{H1}\), T\(_{H2}\) and T\(_{H17}\) polarizing cytokines *in vivo***

Cyclic di-GMP immunization generates T\(_{H1}\), T\(_{H2}\) and T\(_{H17}\) responses. Type II IFN is a T\(_{H1}\) polarizing cytokine. We examined if cyclic di-GMP induced other T\(_{H}\) polarizing cytokines in the lungs. Indeed, intranasally administered cyclic di-GMP induced T\(_{H1}\) polarizing cytokine IL-12p70, T\(_{H2}\) polarizing cytokine IL-5, to a lesser degree IL-4 and IL-13, and T\(_{H17}\) polarizing cytokines IL-23, IL-6, and TGF-β1 (Fig 3B, 3C and 3D). Except for IL-6 production, all these cyclic di-GMP induced cytokines were absent in *Tmem173*\(^{-/-}\) mice (Fig 3B, 3C and 3D).

**Cyclic di-GMP induces potent lung epithelium-derived cytokines *in vivo* that is only partially dependent on the expression of MPYS**

Lung epithelial cells generate unique cytokines when activated, and their *in vivo* roles in modulating immune responses have been appreciated recently\(^{26}\). We examined lung epithelium-derived cytokines during *in vivo* cyclic di-GMP activation. Indeed, cyclic di-GMP induced potent IL-33 and, to a lesser degree, IL-1α and TSLP production (Fig 3E). Distinct from many of the cytokines examined above, these cyclic di-GMP induced lung epithelium cytokines were only partially dependent on the expression of MPYS (Fig 3E).

Noticeably, all cytokines were detected at both 6hrs and 24hrs post cyclic di-GMP administration (Fig2 and Fig 3). In fact, we could detect these cytokine as early as 4hrs post cyclic di-GMP administration *in vivo*. The rapid production of these cytokines by cyclic di-GMP *in vivo* suggested that cyclic di-GMP induced cytokines were a primary response rather than a secondary effect.
Cyclic di-GMP generates IL-12p70 producing DC \textit{in vivo}

The rapid generation of T_{H1}, T_{H2}, and T_{H17} polarizing cytokines in the lungs from cyclic di-GMP treated mice led us to hypothesize that cyclic di-GMP directly activated pulmonary DCs \textit{in vivo} that generated T_{H} polarizing cytokines, leading to differentiated T-helper cell responses.

To test this hypothesis, we performed intracellular cytokine staining in pulmonary DC from cyclic di-GMP treated mice. We focused on detecting T_{H1} promoting DCs as defined by IL-12p35 or IFN\gamma production. Unlike IL-12p40, IL-12p35 is unique to IL-12p70. We gated MHC II^{hi} CD11C^{+} DCs from total lung and looked for IL-12p35^{+} or IFN\gamma^{+} DC (Fig 3F). IL-12p35^{+} DC accounted for \sim 0.035\% of DCs, which amounted to less than 500 of these cells in a lung from a cyclic di-GMP treated mouse (Fig 3G). The percentage of IL-12p35^{+} IFN\gamma^{+} DC was \sim 0.01\% (Fig 3F and 3G). As a control, no IL-12p35^{+} DCs were detected in saline treated mice (Fig 3F).

Cyclic di-GMP enhances Ag uptake in APCs and non-APCs \textit{in vivo}

Next, we investigated how cyclic di-GMP affects DCs \textit{in vivo}. We used Alexa Fluor® 647 conjugated OVA Ag (OVA-647) to examine Ag uptake and DQ-OVA for Ag processing (Fig 4A\&4B). DQ-OVA is a self-quenched conjugate of OVA that exhibits bright, photostable, and pH insensitive green fluorescence upon proteolytic degradation (DQ-Green) (Fig 4A). Furthermore, when digested fragments of DQ-OVA accumulate in organelles at a high concentration, it forms excimers emitting red fluorescence (DQ-Red) (Fig 4A).

We intranasally administered mice with the OVA-647 plus DQ-OVA in the presence
or absence, of cyclic di-GMP. After 24 hrs, we examined OVA-647+ and DQ+ cells in the lung. We found that including cyclic di-GMP in the immunization dramatically improved Ag uptake, as indicated by the increased number of OVA-647+ cells in lung (Fig 4C). Furthermore, ~34% of these OVA-647+ cells were DQ+, which indicated that only a portion of OVA-647+ cells have the ability to process Ag (Fig 4C). The DQ+ cells included both DQ-Green and DQ-Red cells (Fig 4C).

Of note, the cyclic di-GMP induced OVA-647+ cells included both MHC II+ APCs and MHC II− non-APCs (Fig 4C). We focused on MHC II+ APCs. There are three populations of APCs from WT mice: MHC IIhiCD11C+ (i.e. DCs), MHC IIlowCD11C+ and MHC IIintCD11C− (Fig 4D). Notably, the majority of OVA-647+ MHC IIlowCD11C+ cells were OVA-647hi cells, while the majority of OVA-647+ MHC IIhiCD11C+ and OVA-647+ MHC IIintCD11C− cells were OVA-647low cells (Fig 4F). A previous study established that OVA-647hi cells were generated via receptor-mediated endocytosis while OVA-647low cells were a result of pinocytosis-mediated Ag uptake. Thus, cyclic di-GMP predominantly enhanced receptor-mediated endocytosis in MHC IIlowCD11C+ and pinocytosis in MHC IIhiCD11C+ and MHC IIintCD11C− cells.

**MHC IIhiCD11C+ and MHC IIintCD11C− cells are activated by cyclic di-GMP in vivo**

Cyclic di-GMP treatment activates cells *in vitro*, which depends on MPYS15, 23. We next wanted to know which APCs were activated during intranasal administration of cyclic di-GMP. APCs increase CD86 expression during activation. In the OVA-647+ MHC IIlowCD11C+ population, there was no increase of the activation marker CD86 (Fig 4E). In the remaining two APC populations, MHC IIhiCD11C+ and MHC IIintCD11C−, the OVA-647+ cells had increased CD86 expression (Fig 4E). Thus, cyclic di-GMP activates
MHC II$^{\text{hi}}$CD11C$^+$ and MHC II$^{\text{int}}$CD11C$^-$, but not MHC II$^{\text{low}}$CD11C$^+$ APCs in vivo. The total numbers of CD86$^+$MHC II$^{\text{hi}}$ activated OVA-647$^+$ cells were similar between MHC II$^{\text{hi}}$CD11C$^+$ and MHC II$^{\text{int}}$CD11C$^-$ APCs (Fig 4G).

Of note, while cyclic di-GMP selectively activated different APCs, it did enhance Ag uptake in all three APCs populations in vivo (Fig 4D). This suggested that cell activation is not a prerequisite for cyclic di-GMP enhanced Ag uptake in vivo.

**Cyclic di-GMP enhance Ag processing in APCs in vivo**

Cyclic di-GMP also dramatically increased numbers of DQ$^+$ cells in vivo (Fig 5A). As shown in Fig 4C, only a third of OVA-647$^+$ cells were able to process Ag (DQ$^+$). We, thus, focused on DQ$^+$ cells, where Ag was processed. Gated on the DQ$^+$ lung cells, we found that the vast majority of DQ$^+$ cells (~94%) were OVA-647$^+$ cells (Fig 5A). Since cells have to take up Ag (OVA-647$^+$) before processing it (DQ$^+$), the small percentage of DQ$^+$OVA-647$^+$ cells (~5%) could represent cells that lost the OVA-647 signal during the Ag process. Alternatively, DQ-OVA signal could be more sensitive than the OVA-647 signal.

The DQ$^+$OVA$^+$ consisted of two populations: OVA-647$^{\text{hi}}$ and OVA-647$^{\text{low}}$ cells (Fig 5A). OVA-647$^{\text{hi}}$ cells were generated via receptor-mediated endocytosis while OVA-647$^{\text{low}}$ cells were a result of pinocytosis-mediated Ag uptake$^{27}$. The DQ$^+$OVA-647$^{\text{hi}}$ cells had a strong DQ-Red signal, indicating that processed Ag concentration was high in these cells (Fig 5B). The DQ$^+$OVA-647$^{\text{low}}$ cells were DQ-Red negative, though they still processed Ag as they were DQ-Green$^+$ (Fig 5B). Thus, the receptor-mediated Ag endocytosis generates DQ-Green$^+$DQ-Red$^+$ cells, while pinocytosis-mediated Ag uptake generates DQ-Green$^+$DQ-Red$^-$ cells.
DQ⁺ Lung cells are APCs

We found that the DQ⁺ cells were almost exclusively APCs (MHC II⁺ cells) (Fig 5C). This was different from the OVA-647⁺ cells, which included both APC and non APCs (Fig 4C). Furthermore, the vast majority of the DQ⁺ lung cells (>90% of DQ⁺ cells) were MHC IIlow CD11C⁺ APC (Fig 5C). The MHC IIhi CD11C⁺ and MHC IIint CD11C⁻ APCs accounted for ~1% and 2% of DQ⁺ cells, respectively (Fig 5C). The MHC IIlowCD11C⁺DQ⁺ cells were Siglec F⁺ (Fig 5C) cells, which should be characterized as alveolar macrophages. This suggested that alveolar macrophages are the dominant Ag uptake and processing cells during intranasal cyclic di-GMP administration. Indeed, ~26% of total lung Siglec F⁺ alveolar macrophages were DQ⁺ cells (Fig 5D). In comparison, only ~1% of total lung cells were DQ⁺ cells (Fig 5A).

Cyclic di-GMP administration generates mature DCs (DQ⁺MHC IIhiCD11C⁺)

We then investigated which DQ⁺ APCs were activated by cyclic di-GMP in vivo. Studies done in OVA-647⁺ cells revealed that OVA-647⁺ MHC IIlowCD11C⁺ cells were not activated (Fig 4E). Only OVA-647⁺MHC IIhiCD11C⁺ and OVA-647⁺MHC IIintCD11C⁻ APCs were activated (Fig 4E). However, in DQ⁺ cells, the only CD86⁺ APC was MHC IIhi DCs (Fig 5E). These cells also had increased CD80 expression (Fig 5E).

The CD86⁺CD80⁺DQ⁺ DCs are DQ-Red⁺ pinocytosis-efficient DCs

Cyclic di-GMP predominantly activated OVA-647low APCs (Fig 4E, 4F), which took up Ag via pinocytosis. We found that OVA-647low cells were all DQ-Red⁻ while OVA-647hi cells were all DQ-Red⁺ (Fig 5B). Similarly, the vast majority of MHC IIhiCD86⁺CD80⁺DQ⁺ cells were DQ-Red⁻ cells (Fig 5F). We concluded that during
intranasal administration of cyclic di-GMP, the only APCs that took up Ag (OVA-647\(^+\)), processed Ag (DQ-Green\(^-\)) and activated (CD86\(^+\)CD80\(^+\)), were MHC II\(^{hi}\) pinocytosis-efficient (DQ-Red\(^-\)) DCs.

Cyclic di-GMP is a 690Da small molecule with two phosphate groups that cannot directly cross cell membrane (Fig 10, red arrows). Thus, during intranasal administration, cyclic di-GMP is likely brought into the cytosol by pinocytosis, and stimulates DCs.

**Cyclic di-GMP enhances Ag uptake, processing, and cell activation in both CD103\(^+\) and CD11B\(^+\) pulmonary DCs in vivo**

Pulmonary DCs include CD103\(^+\)DCs and CD11B\(^+\)DCs. By co-administration of DQ-OVA and cyclic di-GMP, we found that cyclic di-GMP enhanced Ag uptake and processing, as indicated by increased numbers of DQ\(^+\) cells, in both CD103\(^+\) and CD11B\(^+\) DCs (Fig 6A, 6D). We did notice that CD103\(^+\)DCs had a higher percentage of DQ\(^+\) cells than the CD11B\(^+\) DCs (Figure 6A and 6D). Both DC subsets had DQ-Red\(^+\) and DQ-Red\(^-\) populations (Fig 6A and 6D).

**Cyclic di-GMP activates and mobilizes pulmonary CD103\(^+\) DC in vivo**

Activated DCs express high MHC II and co-stimulator factor CD86. Furthermore, they migrate to draining lymph nodes (DLN), where they encounter naïve T cells and stimulate diversified T cell responses. We first examined the actions of CD103\(^+\) DC. A significant portion of lung CD103\(^-\)DQ\(^+\) DCs (~35\%) from cyclic di-GMP treated mice were MHC II\(^{hi}\) CD86\(^+\) activated DCs (Fig 6B). The absolute number of CD103\(^-\)DQ\(^-\)CD86\(^+\) cells was also recorded (Fig 6G). Interestingly, these activated DQ\(^-\)CD103\(^-\)CD86\(^+\) DCs were all DQ-Red negative cells (Fig 6B). In fact, it appeared
that all DQ-Red$^+$ cells were CD86$^+$ DCs and all DQ-Red$^+$ cells were CD86$^-$ (Fig 6B and 6C).

We then examined migratory CD103$^+$ DC in lung DLN. Cyclic di-GMP treatment increased total CD103$^+$ DCs numbers in DLN (Fig 6H and 6L). However, only a very small percentage of the migratory CD103$^+$ DCs (~1.8%) were DQ$^+$ (Fig 6H and 6M). This indicated that a large portion of CD103$^+$ DCs were migratory, likely activated by cyclic di-GMP, but did not take up the DQ-OVA Ag. Among those DQ$^+$CD103$^+$ migratory DCs, the vast majority of them were MHC II$^{hi}$ CD86$^+$ cells (~89%) (Fig 6I), which indicated that these migratory DQ$^+$CD103$^+$DCs were indeed activated DCs. Consistent with the finding in the lungs, all these migratory DQ$^+$CD103$^+$ in DLN were DQ-Red$^-$ cells (Fig 6I). The number of DQ$^+$ CD86$^+$ migratory CD103DCs was recorded (Fig 6N).

Cyclic di-GMP activates and mobilizes pulmonary CD11B$^+$ DC in vivo

We next examined the activation of CD11B$^+$ DCs by cyclic di-GMP in vivo. Similar to CD103$^+$DCs, we found that 1) a portion of lung CD11B$^+$DQ$^+$ DCs were MHC II$^{hi}$ CD86$^+$ activated DCs (Fig 6E); 2) these activated DQ$^+$CD11B$^+$CD86$^+$ DCs were all DQ-Red negative cells (Fig 6E); 3) all the DQ-RedDC-Green$^+$CD11B$^+$ DCs were CD86$^+$ (Fig 6E); 4) total CD11B$^+$ DCs numbers were increased in DLN (Fig 6J and 6L); 5) only a very small percentage of these CD11B$^+$ DCs (~0.8%) were DQ$^+$ (Fig 6J and 6M); 6) the vast majority of DQ$^+$CD11B$^+$ migratory DC were MHC II$^{hi}$ CD86$^+$ cells (~82%) (Fig 6K and 6N); 7) all these migratory DQ$^+$CD11B$^+$ were DQ-Red$^-$ cells (Fig 6K).

Cyclic di-GMP differentially mobilizes pulmonary DC in vivo based upon their endocytosis ability
Our investigation, so far, revealed that cyclic di-GMP differentially mobilized two major types of Ag-loaded pulmonary DCs: DQ-Green$^+$DQ-Red$^-$CD86$^+$ and DQ-Green$^+$DQ-Red$^+$CD86$^-$ DCs. DQ-Red$^-$ DQ-Green$^+$ cells represented pinocytosis-efficient DCs (Fig 6A and 6B). The fact that these were the only CD86$^+$ and DQ$^+$ migratory DCs found in DLN after cyclic di-GMP treatment suggested that cyclic di-GMP only activated pinocytosis-efficient DCs in vivo. It did not matter whether they were CD103$^+$ or CD11B$^+$ DCs (Fig 6H and 6J).

In contrast, all the DQ-Green$^+$DQ-Red$^+$ cells were CD86$^-$ and non-migratory, suggesting that though cyclic di-GMP enhanced Ag uptake in these cells (Fig 4), it did not lead to cell activation. It further strengthened the notion that activation of these cells is not a prerequisite for cyclic di-GMP enhanced Ag uptake (Fig 4E).

**MPYS is critical for cyclic di-GMP enhanced Ag uptake in vivo**

Mucosal adjuvant activity of cyclic di-GMP requires MPYS in vivo$^9$. We next asked how MPYS regulated cyclic di-GMP enhanced Ag uptake and processing in vivo. Upon co-administration of OVA-647 and cyclic di-GMP, lung cells from Tmem173$^{-/-}$mice had no increased OVA-647$^+$ cells (Fig 7A).

Since DQ-OVA may be more sensitive than OVA-647 in detecting Ag-loaded APCs (Fig 5A), we examined DQ-OVA signals in lung cells from cyclic di-GMP treated Tmem173$^{-/-}$mice. As expected, Tmem173$^{-/-}$mice had significantly less cyclic di-GMP induced DQ-OVA$^+$ cells than WT mice (Fig 7B).

Both CD103$^+$DC (Fig 7C) and CD11B$^+$ DC (Fig 7D) from cyclic di-GMP treated Tmem173$^{-/-}$mice, had dramatically decreased DQ$^+$ cells (Fig 7E). This included both the
DQ-Green$^+$DQ-Red$^+$ receptor-mediated endocytosis and DQ-Green$^+$DQ-Red$^-$ pinocytosis cells. We concluded that MPYS is critical for cyclic di-GMP induced DC Ag endocytosis and pinocytosis in vivo.

**MPYS is critical for the generation of activated DQ$^+$ DCs by cyclic di-GMP in vivo**

We next examined activated DQ$^+$ DCs in Tmem173$^{-/-}$ mice. As expected, no CD86$^+$CD80$^+$DQ$^+$ MHC II$^{hi}$ cells can be detected in cyclic di-GMP treated Tmem173$^{-/-}$ mice (Fig 7F). This was consistent with the finding that Tmem173$^{-/-}$ mice had a severe defect on cyclic di-GMP induced cytokine production in vivo (Fig 3). Cyclic di-GMP is likely brought into cells by pinocytosis in vivo (Fig 4E, 5F, 6B and 6E) and MPYS is critical for cyclic di-GMP enhanced pinocytosis (Fig 7A). Thus, the reasons for the lack of overall activation by cyclic di-GMP in Tmem173$^{-/-}$ mice could be two-fold. On one hand, cyclic di-GMP cannot efficiently get into MPYS-deficient cells by pinocytosis; on the other hand, the small amount of cyclic di-GMP that does get in can’t activate MPYS-deficient cells.

**Cyclic di-GMP/PspA immunization did not induce protective immunity in Tmem173$^{-/-}$ mice**

Next, we examined cyclic di-GMP/PspA vaccine induced protective immunity in the Tmem173$^{-/-}$ mice. CDG/PspA immunization significantly lowered the lung bacterial burden in the WT mice (Fig 7G). However, the bacterial burden in lungs from CDG/PspA and PspA immunized Tmem173$^{-/-}$ were not significantly different (Fig 7G). We concluded that the mucosal pneumococcal vaccine adjuvant activity of cyclic di-GMP requires MPYS.
Interestingly, PspA immunized Tmem173<sup>−/−</sup> mice had significantly lower lung bacterial burden than the PspA immunized WT mice (Fig 7G). We further found that Tmem173<sup>−/−</sup> mice, without PspA immunization, are much more resistant to S. pneumoniae infection than the WT mice (unpublished data). Currently, we are dissecting the in vivo mechanism underlying this MPYS-mediated susceptibility to S. pneumoniae infection.

**Generation of Itgα<sup>C</sup>Cre<sup>Tmem173</sup>F<sub>lox/Fl</sub>ox mice**

Our investigation revealed two mechanisms by which cyclic di-GMP promotes its adjuvant activity in vivo: 1) enhances Ag uptake in vivo; 2) activates and mobilizes DCs in vivo, specifically, the pinocytosis-efficient DQ-Green<sup>+</sup>DQ-Red<sup>−</sup> DCs. MPYS expression is required for both actions. We then asked whether this MPYS requirement was DC-intrinsic. To achieve that, we generated Itgα<sup>C</sup>Cre<sup>Tmem173</sup>F<sub>lox/Fl</sub>ox mice (Fig 8-figure supplemental 1). Since essentially all DQ<sup>+</sup> (Ag-processing) cells were CD11C<sup>+</sup> (Fig 5C), the Itgα<sup>C</sup>Cre<sup>Tmem173</sup>F<sub>lox/Fl</sub>ox mice will eliminate MPYS expression in the vast majority of DQ<sup>+</sup> cells except for the CD11C<sup>−</sup>MHC II<sup>int</sup> APCs, which accounts for ~2% of DQ<sup>+</sup> cells (Fig 5C).

We detected MPYS expression by Flow cytometry intracellular staining. We used the same type of cell from Tmem173<sup>−/−</sup> mice as a negative control and the same type of cell from WT mice as a positive control. BALF cells, which are overwhelmingly CD11C<sup>hi</sup> alveolar macrophages, had dramatically decreased MPYS expression (>90%) in Itgα<sup>C</sup>Cre<sup>Tmem173</sup>F<sub>lox/Fl</sub>ox mice (Fig 8-figure supplemental 1B). MPYS expression in spleen B cells (IgD<sup>+</sup>) or T cells (CD4<sup>+</sup> or CD8<sup>+</sup>) did not change in Itgα<sup>C</sup>Cre<sup>Tmem173</sup>F<sub>lox/Fl</sub>ox mice (Fig 8-figure supplemental 1C).

There were two major CD11C<sup>hi</sup> populations in lung cells: CD11C<sup>−</sup>MHC II<sup>low</sup> and
CD11C\(^+\)MHC II\(^{hi}\) (Fig 8-figure supplemental 1D). MPYS expression was dramatically
decreased in both populations in \(Itgax^{Cre}Tmem173^{Flox/Flox}\) mice (Fig 8-figure
supplemental 1D). When we separated the DC population (CD11C\(^+\)MHC II\(^{hi}\) ) into
CD103\(^+\) and CD11B\(^+\) DCs, we found that MPYS expression was eliminated in both DCs
subsets (Fig 8-figure supplemental 1D).

The MPYS expression was down ~40% in MHC II\(^{int}\) CD11C\(^-\) cells from
\(Itgax^{Cre}Tmem173^{Flox/Flox}\) mice (Fig 8-figure supplemental 1D). NK cells also showed
~40% decreased MPYS expression in \(Itgax^{Cre}Tmem173^{Flox/Flox}\) mice (Fig 8-figure
supplemental 1E). We wanted to see if the partial decrease of MPYS expression affected
cyclic di-GMP adjuvant activity. MPYS heterozygous mice, which had ~50% decreased
MPYS expression (Fig 8-figure supplemental 1F), were immunized with cyclic di-GMP
and OVA. The total OVA-specific IgG production was similar in WT as in the
\(Tmem173^{+/−}\) mice (Fig 8-figure supplemental 1G). Thus, the partial decreased MPYS
expression (~50%) does not affect cyclic di-GMP adjuvant activity.

**Cyclic di-GMP induced Ag uptake requires MPYS expression in CD11C\(^+\) cells**

We then examined cyclic di-GMP generated DQ-OVA\(^+\) cells in the lungs of
\(Itgax^{Cre}Tmem173^{Flox/Flox}\) mice. The total numbers of DQ\(^+\) cells were dramatically
decreased in \(Itgax^{Cre}Tmem173^{Flox/Flox}\) mice (Fig 8A,8B). The decreased number of DQ\(^+\)
cells was seen in both CD103\(^+\)DC (Fig 8C, 8D) and CD11B\(^+\) DC (Fig 8C, 8E) from
\(Itgax^{Cre}Tmem173^{Flox/Flox}\) mice. The numbers of decreased DQ\(^+\) DCs in
\(Itgax^{Cre}Tmem173^{Flox/Flox}\) /\(\text{lox}\) was comparable to that of \(Tmem173^{+/−}\) mice (Fig 8B). Thus,
cyclic di-GMP induced DC Ag uptake requires MPYS expression in CD11C\(^+\) cells.

**Cyclic di-GMP induced cytokine productions in lung requires MPYS expression in**
CD11C$^+$ cells

Intranasally administered cyclic di-GMP generated a lung cytokine milieu that is dependent on the expression of MPYS (Fig 3). We then examined the cytokine milieu in $\text{Itgax}^{\text{Cre}}\text{Tmem173}^{\text{Flx/Flox}}$ mice. Cyclic di-GMP induced T$_{H1}$ polarizing (IL-12p70 and IFN$\gamma$) and T$_{H17}$ polarizing (IL-23 and IL-6) cytokines were significantly decreased in $\text{Itgax}^{\text{Cre}}\text{Tmem173}^{\text{Flx/Flox}}$ mice (Fig 8F and 8H). Surprisingly, we did not see much of a decrease in the T$_{H2}$ polarizing cytokines (IL-5, IL-13) (Fig 8G). Thus, MPYS expression in CD11C$^+$ cells is critical for T$_{H1}$ and T$_{H17}$ polarizing cytokine production in vivo.

Cyclic di-GMP induced IFN-$\lambda$, IL-22, TNF-$\alpha$ and IL-1$\beta$ productions were also dramatically decreased in $\text{Itgax}^{\text{Cre}}\text{Tmem173}^{\text{Flx/Flox}}$ mice (Fig 8I and 8J). Since the CD11C$^+$MHC II$^{hi}$ DCs were the only activated CD11C$^+$ cells by cyclic di-GMP in vivo (Fig 4E, 5E), we concluded that DCs expression of MPYS was critical for the generation of T$_{H1}$ and T$_{H17}$ polarizing cytokine during intranasal administration of cyclic di-GMP.

The lung epithelial cytokine TSLP was slightly lower in cyclic di-GMP treated $\text{Itgax}^{\text{Cre}}\text{Tmem173}^{\text{Flx/Flox}}$ mice than in the $\text{Tmem173}^{\text{Flx/Flox}}$ mice, but it was not statistically significant (Fig 8K). However, the lung epithelial cytokine IL-33 production was significantly lower in cyclic di-GMP treated $\text{Itgax}^{\text{Cre}}\text{Tmem173}^{\text{Flx/Flox}}$ mice than in the $\text{Tmem173}^{\text{Flx/Flox}}$ (Fig 8K). We favored the idea that there is a crosstalk/communication between lung epithelial cells and CD11C$^+$ cells during cyclic di-GMP induced immune response.

$\text{Itgax}^{\text{Cre}}\text{Tmem173}^{\text{Flx/Flox}}$ mice had impaired Ab responses to cyclic di-GMP adjuvanted vaccine
The \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice are defective in cyclic di-GMP induced DCs Ag uptake and activation \textit{in vivo}. To determine if these mice were defective in cyclic di-GMP adjuvanted immune responses, we immunized these mice with cyclic di-GMP plus OVA and measured anti-OVA Ab productions. \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice exhibited significantly decreased production of anti-OVA IgG1, IgG2C, and nasal IgA (Fig 9A). Noticeably different from the \( \text{Tmem173}^{-/-} \) mice, where no anti-OVA Ab could be detected, cyclic di-GMP/OVA immunized \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice still generated decent amounts of anti-OVA Ab (Fig 9A).

We next immunized \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice with pneumococcal vaccine consisting of cyclic di-GMP and PspA and examined their Ab responses. Again, \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice showed decreased anti-PspA IgG1, IgG2C, and nasal IgA production in comparison to the immunized WT mice (Fig 9B). Similar to the cyclic di-GMP/OVA immunization, \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice still had elevated anti-PspA Ab responses compared to the MPYS^{-/-} mice (Fig 9B).

\( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice had impaired \( \text{T}_1 \) responses to cyclic di-GMP/PspA immunization.

The cyclic di-GMP/PspA immunized \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice also showed dramatically decreased \( \text{T}_1 \text{H1}, \text{T}_1 \text{H2}, \text{and T}_1 \text{H17} \) responses in the ex vivo recall assay on splenocytes (Fig 9C). Lungs can form Bronchus associated lymphoid tissue (BALT) after immunization and initiate an adaptive immune response in situ. We did the recall assay on the lung cells from immunized mice to examine the local immune responses. Similar to the responses in splenocytes, \( \text{T}_1 \text{H1}, \text{T}_1 \text{H17} \) and, to a lesser degree, \( \text{T}_1 \text{H2} \) responses were decreased in lung cells from \( \text{Itgax}^{\text{Cre}} \text{Tmem173}^{\text{Flox/Flox}} \) mice (Fig 9D).
Cyclic di-GMP/PspA immunization did not induce protective immunity in the \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice

Next, we examined the cyclic di-GMP/PspA vaccine induced protective immunity in the \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice. While CDG/PspA immunization significantly lowered the lung bacterial burden in the \textit{Tmem173}^{\text{Flox/Flox}} mice, it did not alter the bacterial burden from lungs of the \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice (Fig 9E). We concluded that the mucosal pneumococcal vaccine adjuvant activity of cyclic di-GMP requires MPYS expression in CD11C$^+$ cells. Noticeably, unlike the \textit{Tmem173}^{-/-} mice, PspA immunized \textit{Tmem173}^{\text{Flox/Flox}} and \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice had similar lung bacterial burden (Fig 9E).

The impaired adjuvant activity of cyclic di-GMP in \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice is not due to the overexpression of \textit{Cre} gene in the CD11C$^+$ cells

The \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice also overexpressed the \textit{Cre} gene in the CD11C$^+$ cells. To exclude the possibility that the defect seen in the \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice was due to the \textit{Cre} overexpression, we compared \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice with the \textit{Itgax}^{\text{Cre}}-C57BL/6 mice upon intranasal cyclic di-GMP/PspA immunization. Similar to the observation in Fig 9, cyclic di-GMP/PspA immunized \textit{Itgax}^{\text{Cre}} \textit{Tmem173}^{\text{Flox/Flox}} mice had a severe defect in anti-PspA Ab production compared to immunized \textit{Itgax}^{\text{Cre}}-C57BL/6 mice (Fig 9-figure supplemental 1A). Their T$_{H}$ responses in spleen cells were largely non existent, except for IL-5 (Fig 9-figure supplemental 1B). A similar observation was made in lung recall assay (Fig 9-figure supplemental 1C).
Our study revealed two novel in vivo mechanisms of action of the mucosal vaccine adjuvant cyclic di-GMP (Fig 10). First, cyclic di-GMP enhances Ag uptake in APCs and non-APCs in vivo. Second, cyclic di-GMP activates pinocytosis-efficient cells in vivo. Cyclic di-GMP has two phosphate groups preventing it from directly passing through the cell membrane. The mammalian receptor for cyclic di-GMP, MPYS, is located inside cells. Thus, though intranasally administered cyclic di-GMP enhances Ag uptake in all types of cells, only cells that efficiently take up cyclic di-GMP, via pinocytosis, will be activated (Fig 10A).

How does cyclic di-GMP, as a mucosal adjuvant, enhance Ag uptake in vivo? Three observations in this study may shed light on the mechanism. First, cyclic di-GMP enhances Ag uptake by both pinocytosis and receptor mediated endocytosis (Fig 4); Second, while cyclic di-GMP enhances Ag uptake in all types of cells (Fig 4), deletion of MPYS in only CD11C+ cells severely impaired that (Fig 8); Third, MPYS expression in CD11C+ cells is mainly responsible for the cyclic di-GMP induced cytokine milieu in lungs (Fig 8). We propose that cyclic di-GMP enhances MPYS-dependent Ag uptake in cells directly taking up cyclic di-GMP (pinocytosis-efficient, OVA-647Low, DQ-Green+DQ-Red- cells). In cells that do not take up cyclic di-GMP (OVA-647hi, DQ-Green+DQ-Red+ cells), Ag uptake is enhanced by the cytokine milieu generated mainly by cyclic di-GMP activated CD11C+ cells (Fig 10B).

We favored the hypothesis that intranasally administered cyclic di-GMP directly primed pulmonary DCs, leading to MPYS-dependent production of Th polarizing cytokines in vivo (Fig 10C). Two pieces of data support this hypothesis. First, we
detected IL-12 and IFNγ producing DCs in vivo as early as 5hrs post treatment (Fig 3F&3G). Second, the *Itgax*^Cre^*Tmem173*^Flax/Flox^ mice had dramatically decreased cyclic di-GMP-induced T\(_H\)1 and T\(_H\)17 cytokine in vivo (Fig 8F&8H). There are two CD11C\(^+\) populations in the lung: MHC II\(^{hi}\) and MHC II\(^{low}\). Among Ag positive (OVA-647\(^+\)) cells, only the MHC II\(^{hi}\)CD11C\(^+\) population (i.e. DCs) were activated by cyclic di-GMP in vivo (Fig 4E). This suggested that the deletion of MPYS in MHC II\(^{hi}\) CD11C\(^+\) cells (DCs) was responsible for the impaired T\(_H\)1 and T\(_H\)17 polarizing cytokine production in vivo.

Intriguingly, the production of T\(_H\)2 polarizing cytokines IL-5 and IL-13 were less dependent on the expression of MPYS in DCs (Fig 8G). Indeed, unlike *Tmem173*\(^{-/-}\) mice, *Itgax*^Cre^*Tmem173*^Flax/Flox^ mice still have some Ab and T\(_H\) responses after cyclic di-GMP immunization (Fig 9). Therefore besides DCs, MPYS expression in other cells contributes to the adjuvant activity of cyclic di-GMP in vivo. We found that the OVA-647\(^+\)CD11C\(^-\)MHC II\(^{int}\) APC were activated by cyclic di-GMP in vivo (Fig 4E). The total number of OVA-647\(^+\) activated cells in this CD11C\(^-\)MHC II\(^{int}\) population are comparable to that of the CD11C\(^+\) MHC II\(^{hi}\) population (Fig 4G). Thus, these CD11C\(^-\) OVA-647\(^+\) MHC II\(^{int}\) CD86\(^+\) cells may contribute to the adjuvant activity of cyclic di-GMP in *Itgax*^Cre^*Tmem173*^Flax/Flox^ mice.

How does cyclic di-GMP enhance MPYS-mediated Ag uptake in pinocytosis-efficient cells in vivo? Cyclic di-GMP activates MPYS-TBK1-IRF3-Type I IFN signaling in vitro. However, intranasally administered cyclic di-GMP did not induce Type I IFN production in vivo. Instead, it generates type II, type III IFN and various cytokines that depend on NF-κB activation. We previously showed, in vitro, that cyclic di-GMP induced type I IFN and NF-κB activation can be uncoupled in DCs and macrophages\(^9\).
Thus, MPYS is not just a type I IFN stimulator. New molecular mechanisms by which cyclic di-GMP enhances MPYS-dependent Ag uptakes as well as activation of Type II, III IFN and NF-κB signaling in pinocytosis-efficient cells in vivo remains to be discovered.

*Tmem173*−/− mice still made several cytokines, namely IL-1α, IL-1β, IL-6, IL-10, IL-33 and TSLP, after cyclic di-GMP treatment in vivo. This indicates that cyclic di-GMP can activate MPYS-independent signaling in vivo. A previous study showed that cyclic di-GMP activated NLRP3 inflammasome independent of STING/MPYS. Cyclic di-GMP also bound to hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) and inhibited cAMP regulated heart rate. Very recently, it was found that cyclic di-AMP, a similar cyclic dinucleotide for STING/MPYS, induces human monocyte apoptosis independent of STING/MPYS. Thus, other mammalian receptors for cyclic di-GMP exist.

We showed that cyclic di-GMP is a superior mucosal pneumococcal vaccine adjuvant than the 2′3′-cGAMP in mice (Fig 1). As a mammalian cyclic dinucleotide, 2′3′-cGAMP can be hydrolyzed by the ecto-nucleotide phosphodiesterase (ENPP1) found in mammalian cells. On the contrary, as a bacterial cyclic dinucleotide, cyclic di-GMP may be more resistant to hydrolysis when introduced into mammalian cells. Further study is needed to determine if cyclic di-GMP is a better human adjuvant than 2′3′-cGAMP.

The anti-tumor molecules 10-carboxymethyl-9-acridanone (CMA) and 5,6-dimethylxanthenone-4-acetic acid (DMXAA) activate mouse, but not, human MPYS signaling. Cyclic di-GMP, on the other hand, is functional in human cells as well. In fact, cyclic di-GMP binds to mouse MPYS/STING and human MPYS/STING with
similar dissociation constant ($K_d$: 2–5µM). Nevertheless, we first discovered that human TMEM173 gene displays great heterogeneity\textsuperscript{39}. We further identified a loss-of-function human TMEM173 variant HAQ (R71H-G230A-R293Q) that is carried by ~20% of Americans\textsuperscript{39}. In vitro studies demonstrated that many of these human TMEM173 variants are functionally different from the R232 (wild type) TMEM173 allele.\textsuperscript{34,40-42} To develop cyclic di-GMP or other cyclic dinucleotide as a human mucosal vaccine adjuvant, it becomes critical to determine if the adjuvant activity of cyclic dinucleotides is influenced by human TMEM173 variations in vivo.

In summary, we found that cyclic di-GMP enhances Ag uptake and selectively activates pinocytosis-efficient cells in vivo. These qualities should be explored further for the development of cyclic di-GMP as an effective human mucosal vaccine adjuvant.
Materials and Methods

Mice

Six to twelve week old mice were used for all experiments. *Tmem173^+/^-* mice (Tmem173<tm1Camb>) have been described previously. The *Itgax^Cre-Tmem173^Flox/Flox* mouse was generated as in Figure S4A. All mice are on a C57BL/6 background. Mice were housed and bred in the Animal Research Facility (ARF) at Albany Medical College. All experiments with mice were performed in accordance to the regulations and approval of Albany Medical College (Albany, NY) and the Institutional Animal Care and Use Committee.

Reagent

The following reagent was obtained through BEI Resources, NIAID, NIH: Streptococcus *pneumoniae* Family 1, Clade 2 Pneumococcal Surface Protein A (PspA UAB055) with C-Terminal Histidine Tag, Recombinant from *Escherichia coli*, NR-33178.

Intranasal Immunization

Mice were immunized with three doses (14 days apart) of OVA (20 μg, Invivogen, cat# vac-efova) or PspA (2 μg, BEI Resources) with, or without, cyclic di-GMP (5 μg, Invivogen, cat# vac-cdg) or 2’3’-cyclic GMP-AMP (5 μg, Invivogen, cat# vac-cga23). Groups of mice (4 per group) were intranasally vaccinated with adjuvanted protein Ag, or Ag alone. For intranasal vaccination, animals were anaesthetized using isoflurane in an E-Z Anesthesia system (Euthanex Corp, Palmer, PA). Ag, with or without cyclic di-GMP, was administered. Sera and nasal washes were collected 14 days after the last immunization.
Detection of Ag-specific Ab

The Ag-specific Abs were determined by ELISA. The anti-IgG-HRP used were anti-mouse IgG1-HRP (Southern Biotech, cat#1070-05), anti-mouse IgG2C-HRP (Southern Biotech, cat#1079-05), and anti-mouse IgA-HRP (Southern Biotech, cat#1040-05). Total anti-OVA IgG2A, IgG1 and IgA were quantified using a mouse anti-OVA IgG2A kit (Chondrex, Redmond, WA, cat#3015), anti-OVA IgG1 kit (Chondrex, Redmond, WA, cat#3013) and anti-OVA IgA kit (Chondrex, Redmond, WA, cat#3018).

Bronchoalveolar Lavage

Mice were sacrificed at the indicated time by CO2 asphyxiation and lungs were lavaged with 0.8ml ice-cold PBS. The lavage fluid was centrifuged at 2000xg for 1min. Collected cells were analyzed by Flow cytometry.

Detection of Lung Cytokine Production

Mice were intranasally administered 5μg cyclic di-GMP (vaccine grade), then sacrificed at the indicated time by CO2 asphyxiation. BALF was collected and the lungs were subsequently perfused with cold PBS. The harvested lungs were washed in PBS once, then stored in 0.7ml Tissue protein extraction reagent (T-PER) (Thermo Scientific, cat#78510) containing protease inhibitors (Roche, cat#11836153001) at -80°C. Later, the lung was thawed on ice and homogenized on ice in the T-PER homogenate buffer in a 2ml homogenizer. Lung homogenates were transferred to a 1.5ml tube and spun at 14,000g for 30min at 4°C. Supernatant was collected and analyzed for cytokine production.

Streptococcus pneumoniae infection
S. pneumoniae D39 (serotype 2; ATCC) were grown in Todd-Hewitt broth containing 0.5% yeast extract (THY; BD Biosciences) to an optical density (OD) of 0.4 (~10^8 cfu/ml). Mice were intranasally administered ~5 x 10^6 cfu. CFUs were confirmed by colony counting of log_{10} serial dilutions of bacteria cultured overnight on a TSA II with 10% sheep blood agar plate (BD Bioscience, cat#221162).

Flow Cytometry Analysis of in vivo Ag Uptake and Processing

Mice were intranasally administered 20μg DQ™-Ovalbumin (DQ-OVA) (Life technologies, D12053) or 20μg Ovalbumin Alexa® Fluor 647 (OVA-647) (Life technologies, O34784) with, or without, the adjuvant cyclic di-GMP (5μg, vaccine-grade). After 20hrs, the lungs were lavaged and perfused with ice-cold PBS. Excised lungs were digested in DMEM contain 200μg/ml DNase I (Roche, 10104159001), 25μg/ml Liberase TM (Roche, 05401119001) at 37°C for 3hrs. Red blood cells were then lysed and a single cell suspension was prepared and analyzed by BD™ LSR II and FACScan flow cytometry.

The following Abs from Biolegend were used in the flow cytometry: CD80 (16-10A1), CD86 (GL1), Ly6C (HK1.4), CD11B (M1/70), Ly6G (1A8), IgD (11-26c.2a), CD11C (N418), FcεRIa (MAR-1), NK-1.1 (PK136), MHC II (M5/114.15.2), CD103(2E7). The following Abs from BD Biosciences were used: Siglec F (E50-2440), c-Kit (2B8), and CD68(FA-11).

Intracellular IL-12p35 and IFNγ staining

The intracellular cytokine staining was performed using the Cytofix/Cytoperm™ kit from BD Biosciences (cat#555028). Briefly, mice were intranasally administered saline or
cyclic di-GMP (5µg, vaccine-grade). The lungs were lavaged, perfused, and harvested at
5hr post treatment. Excised lungs were washed in PBS and digested in DMEM containing
200µg/ml DNase I (Roche, 10104159001), 25µg/ml Liberase TM (Roche, 05401119001),
and Golgi-plug at 37°C for 6hrs. The single lung cell suspension was fixed in
Cytofix/perm buffer (BD Biosciences) in the dark for 20min at RT. Fixed cells were then
washed and kept in Perm/wash buffer at 4°C. Golgi-plug was present during every step
before fixation. The following Abs from eBioscience were used: IL-12p35 (4D10P35)
and IFNγ (XMG1.2).

Cytokine ELISAs

Cytokine concentrations were measured using ELISA kits from eBioscience according to
the manufacturer’s instructions. The ELISA kits used were IL-1α (cat#88-5019), IL-1β
(cat#88-7013), IL-4 (cat#88-7044), IL-5 (cat#88-7054), IL-6 (cat#88-7064), IL-10
(cat#88-7105), IL-12/p70 (cat#88-7921), IL-13 (cat#88-7137), IL-17A (cat#88-7371),
IL-22 (cat#88-7422), IL-23 (cat#88-7230), IL-33 (cat#88-7333), TNF-α (cat#88-7324),
TGF-β1 (cat#88-8350), TSLP (cat#88-7490), IFN-λ (cat#88-7284), and IFN-γ (cat#88-
7314). The IFNβ ELISA kit was from PBI InterferonSource (cat#42410-1).

Statistical Analysis

All data are expressed as means ± SEM. Statistical significance was evaluated using
Prism 5.0 software to perform a Student’s t test (unpaired, two tailed) for comparison
between mean values.

Online Supplemental Figures

The online supplemental materials include 3 supplemental figures.
Acknowledgements

We thank the Flow Cytometry Core Facility in the Center for Immunology and Microbial Diseases for the assistance.


This work was supported by Albany Medical College New faculty startup funds (to L.J.) and National Institute of Allergy and Infectious Diseases Grant 1R56AI110606-01 (to L.J.), 1R01AI110606-01A1 (to L.J.)

Abbreviations: CDG, cyclic-di-GMP; cGAMP, 2’-3’-cyclic-GMP-AMP; PspA, pneumococcal surface protein A; BALF, Bronchoalveolar lavage fluid; i.n., intranasal; APC, antigen presenting cells; DCs, dendritic cells; DLN, draining lymph node
Figure 1. Cyclic di-GMP is a better mucosal pneumococcal vaccine adjuvant than the mammalian cyclic dinucleotide 2’3’-cyclic GMP-AMP in mice. A. BALB/c mice were intranasally (i.n.) immunized with three doses (14 days apart) of OVA (20μg) alone or together with 5μg cyclic di-GMP (CDG) or 5μg 2’3’-cyclic GMP-AMP (cGAMP). Each group consisted of four mice. Sera or nasal washes from the 4 mice in the same group were pooled. Blood and nasal washes samples were collected 14 days after the last immunization. Anti-OVA IgG1, IgG2A and IgA were quantified by ELISA. n=3. B. Splenocytes from immunized BALB/c mice were stimulated with 50μg/ml OVA for 4 days in culture. Supernatants from the same group were pooled together. Cytokines were measured in the supernatant by ELISA. n=3. C. C57BL/6 mice were immunized with 3 doses of PspA (2μg) alone or together with 5μg cyclic di-GMP (CDG) or 5μg 2’3’-cyclic GMP-AMP (cGAMP) as in A. Blood and nasal washes were collected 14 days after the last immunization. Anti-PspA IgG1, IgG2C and IgA were measured by ELISA as in A. n=3 D. Splenocytes from immunized C57BL/6 mice were stimulated with 5μg/ml PspA for 4 days in culture. Cytokines were measured in the supernatant by ELISA as in B. n=3 E. Immunized mice were infected (i.n.) with S.pneumoniae (~ 5.0x10⁶ c.f.u.). At 48hrs post infection, lung and spleen bacterial burden were determined. n=2. Graph present means ± standard error from three independent experiments. Significance is represented by an asterisk, where p<0.05.

Figure 2. Cyclic di-GMP does not cause lung injury or excess inflammatory
**Figure 3. Cyclic di-GMP induces a variety of cytokines in lung that is dependent on the expression of MPYS.**

A-C. C57BL/6 and Tmem173^-/- mice were treated (i.n.) with saline or CDG (5μg) for the indicated time. Cytokines were determined in lung homogenates by ELISA. n>3. F-G. C57BL/6 mice were treated (i.n.) with saline or CDG (5μg) for 5hrs. IL-12-p35 and IFNγ positive DCs were identified by intracellular cytokine stains and quantified. n=3. Graph present means ± standard error from three independent experiments. Significance is represented by an asterisk, where p<0.05.

**Figure 4. Cyclic di-GMP enhances Ag uptake and activates pinocytosis-efficient APCs in vivo.**

A-B. A cartoon showing mechanism of action of DQ-OVA (A) and OVA-647 (B). C. Flow cytometry analysis of lung cells from C57BL/6 mice treated with saline,
DQ-OVA(20μg)+OVA-647(20μg) or 5μg CDG + DQ-OVA(20μg)+OVA-647(20μg) for 20hrs. Live cells were gated. n>3.  

D. Flow cytometry analysis of lung cells from C57BL/6 mice treated with OVA-647(20μg) or 5μg CDG + OVA-647(20μg) for 20hrs. Live cells were gated. n>3.  

E. Flow cytometry analysis of lung APCs from C57BL/6 mice treated with 5μg CDG + OVA-647(20μg) for 20hrs. Live cells were gated. n>3.  

F. Histogram of OVA-647 signals from OVA-647+ APCs. n>3.  

G. Cell numbers of activated OVA-647+ APCs were quantified. n>3. Graph present means ± standard error from three independent experiments.

**Figure 5. Cyclic di-GMP generates mature DCs in vivo.**  
A. Flow cytometry analysis of lung cells from C57BL/6 mice treated with saline, DQ-OVA(20μg)+OVA-647(20μg) or 5μg CDG + DQ-OVA(20μg)+OVA-647(20μg). Live cells were gated. n>3.  

B. Histogram of DQ-Red and DQ-Green signals from indicated populations. n>3  

C. Flow cytometry analysis of DQ+ lung cells from CDG + DQ-OVA treated (i.n.) C57BL/6 mice. Live DQ+ cells were gated. n>3.  

D. Flow cytometry analysis of lung cells from CDG + DQ-OVA treated (i.n.) C57BL/6 mice. Live cells were gated. n=3.  

E. Flow cytometry analysis of DQ+ lung cells from DQ-OVA or CDG+DQ-OVA treated (i.n.) C57BL/6 mice. Live were gated. n>3.  

F. Flow cytometry analysis of the indicated population from lung cells of CDG+DQ-OVA treated (i.n.) C57BL/6 mice. Gated on live DQ+ CD80+MHC II+ or live DQ+ CD86+MHC II+ cells. n>3.

**Figure 6. Cyclic di-GMP activates pinocytosis-efficient CD103+ and CD11B+ DCs in**
**vivo. A&D.** Flow cytometry analysis of lung cells from C57BL/6 mice treated (i.n.) with DQ-OVA (20μg) or CDG (5μg)+ DQ-OVA (20μg) for 20hrs. Live cells were gated. n>3.

**B&E.** Flow cytometry analysis of DQ⁺ DCs from lung of CDG + DQ-OVA treated C57BL/6 mice. Cells were gated on live DQ⁺CD103⁺MHC II⁺ or live DQ⁺CD11B⁺MHC II⁺ cells. n>3. **C&F.** Histogram of DQ-Green and DQ-Red signals from cell populations in B&E. n>3. **G.** Total cell number of CD86⁺DQ⁺ DCs in lung. n=3. **H&J.** Flow cytometry analysis of lung draining lymph nodes (DLN) from DQ-OVA or CDG + DQ-OVA treated C57BL/6 mice. Live cells were gated. n=3. **I&K.** Flow cytometry analysis of DQ⁺ DCs in DLN of CDG+DQ-OVA treated C57BL/6 mice. Cells were gated on live DQ⁺CD103⁺MHC II⁺ or live DQ⁺CD11B⁺MHC II⁺ cells. n=3. **L-M.** Total cell numbers of DCs, DQ⁺DCs and CD86⁺DQ⁺DCs in DLN from DQ-OVA or CDG+DQ-OVA treated mice. n=3. Graph present means ± standard error from three independent experiments. Significance is represented by an asterisk, where p<0.05.

**Figure 7. MPYS is critical for cyclic di-GMP induced Ag uptake and activation in vivo. A.** Flow cytometry analysis of lung cells from OVA-647 (20μg) or OVA-647(20μg) +CDG(5μg) treated (i.n.) C57BL/6 or Tmem173⁻/⁻ mice. Live cells were gated. n>3. **B.** Flow cytometry analysis of lung cells from DQ-OVA (20μg) or DQ-OVA(20μg) +CDG(5μg) treated (i.n.) C57BL/6 or Tmem173⁻/⁻ mice. Live cells were gated. n>3. **C&G.** Flow cytometry analysis of lung cells from C57BL/6 or Tmem173⁻/⁻ mice treated with saline, DQ-OVA(20μg) or 5μg CDG + DQ-OVA(20μg). Live cells were gated. n>3. **E.** DQ⁺CD103⁺DCs and DQ⁺CD11B⁺DCs numbers from DQ-OVA or CDG + DQ-OVA treated C57BL/6 and Tmem173⁻/⁻ mice. n=3. **F.** Flow cytometry
analysis of DQ+ lung cells from DQ-OVA(20μg) +CDG(5μg) treated (i.n.) C57BL/6 or  
*Tmem173*+ mice. Live DQ+ cells were gated. n>3. G. One month after the last  
immunization, CDG/PspA or PspA immunized WT and *Tmem173*+ mice were infected  
(i.n.) with *S.pneumoniae* (D39 strain, ~ 5.0x10^6 c.f.u.). At 48hrs post infection, lung  
bacterial burden were determined. n=2. Graph present means ± standard error from three  
independent experiments. Significance is represented by an asterisk, where p<0.05.  

**Figure 8. Cyclic di-GMP induced DC Ag uptake and activation requires MPYS**  
expression in CD11C+ cells. A. Flow cytometry analysis of lung cells from DQ-OVA  
(20μg) or DQ-OVA(20μg) +CDG(5μg) treated (i.n.) *Tmem173*+ or *Itgax*Cre  
*Tmem173*Flox/Flox mice. Live cells were gated. n=3. B. Total DQ+ lung cells from DQ-  
OVA or CDG + DQ-OVA treated *Tmem173*Flox/Flox, *Itgax*Cre *Tmem173*Flox/Flox and  
*Tmem173*+ mice. n=3 C. DQ+ DCs numbers from DQ-OVA or CDG+DQ-OVA treated  
cytometry analysis of DQ+ DCs from lung of DQ-OVA or CDG+DQ-OVA treated mice  
*Tmem173*Flox/Flox, *Itgax*Cre *Tmem173*Flox/Flox. Live cells were gated. n=3. F-K.  
*Tmem173*Flox/Flox, *Itgax*Cre *Tmem173*Flox/Flox or *Tmem173*+ mice were treated with saline or  
CDG ((5μg) for 20hrs. Indicated cytokines were measured in lung homogenates by  
ELISA. n=3. Graph present means ± standard error from three independent experiments.  
Significance is represented by an asterisk, where p<0.05.  

**Figure 9. MPYS expression in CD11C+ cells is required for the optimal mucosal**
adjuvant activity of cyclic di-GMP. A. *Tmem173*\textsuperscript{Flox/Flox}, *Itgax*\textsuperscript{Cre} *Tmem173*\textsuperscript{Flox/Flox} or *Tmem173*\textsuperscript{-/-} mice were intranasally administered OVA (20μg) alone or together with 5μg CDG as in Figure 1A. Anti-OVA IgG1, IgG2C and IgA were determined by ELISA. n=3. B. *Tmem173*\textsuperscript{Flox/Flox}, *Itgax*\textsuperscript{Cre} *Tmem173*\textsuperscript{Flox/Flox} or *Tmem173*\textsuperscript{-/-} mice were immunized with PspA (2μg) alone or together with 5μg CDG as in Figure 1C. Anti-PspA IgG1, IgG2C and IgA were measured by ELISA. n=3. C-D. Splenocytes and lung cells from PspA or cyclic di-GMP + PspA immunized *Tmem173*\textsuperscript{Flox/Flox}, *Itgax*\textsuperscript{Cre} *Tmem173*\textsuperscript{Flox/Flox} or *Tmem173*\textsuperscript{-/-} mice were stimulated with 5μg/ml PspA for 4 days in culture. Cytokines were measured in the supernatant by ELISA as in Figure 1D. n=3. E. One month after the last immunization, CDG/PspA or PspA immunized *Tmem173*\textsuperscript{Flox/Flox} and *Itgax*\textsuperscript{Cre} *Tmem173*\textsuperscript{Flox/Flox} mice were infected (i.n.) with *S.pneumoniae* (D39 strain, ~5.0x10\textsuperscript{6} c.f.u.). At 48hrs post infection, lung bacterial burden were determined. n=2. Graph present means ± standard error from three independent experiments. Significance is represented by an asterisk, where p<0.05.

Figure 10. In vivo Mechanisms of the Mucosal Vaccine Adjuvant Cyclic di-GMP. A. The formula of cyclic di-GMP. Red arrows indicate the phosphate groups that prevent cyclic di-GMP from directly crossing the cell membrane. B. **Mechanism I: cyclic di-GMP enhances Ag uptakes in APCs and non-APCs.** Among OVA647\textsuperscript{+}APCs, only a portion of MHC II\textsuperscript{hi}CD11C\textsuperscript{+} (DCs) and MHC II\textsuperscript{int} CD11C\textsuperscript{-} cells up-regulate CD86 expression in vivo. They are mainly OVA647\textsuperscript{low} cells, which take up Ag by pinocytosis. The activation of these cells generate a cytokine milieu that acts on other cells leading to enhanced Ag uptake (OVA647\textsuperscript{hi} cells) but not cell activation. Cyclic di-GMP also
activates lung epithelial cells (LEC), leading to TSLP and IL-33 production. But this is only partially dependent on MPYS and is not sufficient to enhance Ag uptake in vivo (Fig 8). C. **Mechanism II: cyclic di-GMP selectively activates pinocytosis-efficient DCs in vivo.** After administering DQ-OVA together with cyclic di-GMP, the DQ⁺MHC Ⅱ⁺CD11C⁺ Ag-loading DCs can be separated into two distinct populations: DQ-Green⁺DQ-Red⁻CD86⁺ and DQ-Green⁺DQ-Red⁺CD86⁻. DQ-Green⁺DQ-Red⁻ cells are OVA-647low cells, while DQ-Green⁺DQ-Red⁺ cells are OVA-647hi cells. Only the DQ-Green⁺DQ-Red⁻CD86⁺ cells migrated to lung draining lymph nodes.
Fig 3-figure supplemental 1: IFNλ production is dispensable for the mucosal adjuvant activity of cyclic di-GMP.

Fig 8-figure supplemental 1: Generation of Itgax\textsuperscript{Cre}\textsuperscript{\textminus}Tmem173\textsuperscript{Flox/Flox} Mouse.

Fig 9-figure supplemental 1: The impaired cyclic di-GMP response in Itgax\textsuperscript{Cre}\textsuperscript{\textminus}Tmem173\textsuperscript{Flox/Flox} mice is not due to the over-expression of Cre in CD11C\textsuperscript{\textplus} cells.
A  

**DQ-Red**  

**DQ™ Ovalbumin**  
(DQ-OVA, pH insensitive)  

protease  

Highly concentrated  
digested DQ-OVA  

**DQ-Green**  

OVA-647 (pH insensitive)  

protease  

Digested  
DQ-OVA  

Cells take up OVA-647 exhibiting  
far-red fluorescence

---

C  

Untreated  

DQ-OVA+OVA647  

DQ-OVA+OVA647+CDG  

OVA-647*cells  

DQ-Red  

DQ-Green  

CD11C  

MHC II  

FSC  

OVA-647  

OVA647  

OVA647+CDG  

OVA647*cells  

Total CD11C^+ MHC II\textsubscript{low}  

CD11C^+ MHC II\textsubscript{low}  

CD86  

Total CD11C^+ MHC II\textsubscript{int}  

CD11C^+ MHC II\textsubscript{int}  

CD86  

Total CD11C^+ MHC II\textsubscript{hi}  

CD11C^+ MHC II\textsubscript{hi}  

CD86  

OVA-647^+CD86^+MHC II\textsubscript{int} cells
A) **Cyclic di-GMP**

B) **Mechanism I: Direct and indirect enhance Ag uptake in APCs and non-APCs by cyclic di-GMP in vivo**

C) **Mechanism II: Selectively activate pinocytosis-efficient cells by cyclic di-GMP in vivo**

**Pinocytosis-efficient DC**

- DQ-Green only
- DQ-Red CD86
- CD86+ migratory DCs
- Mature DC: IL-6, IL-23, IL-10, IFN-γ, IL-5
- IL-17, IL-1, IL-2

**Receptor-mediated endocytosis DC**

- DQ-Green
- DQ-Red CD86
- CD86- and non-migratory DCs
- DC: LEC Ag