

Discovery of a Small Molecule TLR3 Agonist Adjuvant

Corresponding Author: Professor David Dowling

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Discovery of a TLR3 Small Molecule Agonist Adjuvant

In this manuscript, Lee et al., presents a novel TLR3 agonist denoted PVP-057 (a thiazole benzamide) identified in a screen of 200,000 small chemical compounds. Use of reporter cell lines (HEK-293 and THP-1), a TLR3 KO mouse, and a TLR3 inhibitor, revealed that PVP-057-mediated stimulation depends on TLR3.

Immunization with varicella-zoster virus (VZV) glycoprotein E (gE) together with PVP-057 intramuscularly (IM) or intradermally (ID) in mice, elicited anti-gE antibody responses (IgG, IgG1, IgG2c) and low numbers of T cells (cytokine producing CD4+ T, spectral flow cytometry; and IFN γ + T cells, ELISpot). PVP-057 was compared with the benchmark adjuvant AS01B, which induced antibody levels (IgG, IgG1, IgG2c) similar to PVP-057. The authors argue amongst other that PVP-057 increases the proportion of TNF expressing CD4+ T cells when compared to AS01B or gE alone. Given features such as highly efficient and scalable synthesis, robust adjuvanticity, and “the ability to act as a single-component vaccine adjuvant compatible with ID and IM administration”, the authors conclude that PVP-057 has prophylactic and therapeutic potential.

Development of potent adjuvants and identification of their mechanisms of action is an important research field, and successful adjuvants may greatly improve vaccine responses against infectious diseases and may also be applicable in therapeutic cancer treatment.

In order to prove the potential of PVP-057 as an efficient adjuvant, additional data are needed. Please see the major critique outlined below.

Major points

1. Identification of TLR3 as the receptor of PVP-057.

On page 9 and 10, line 178-180: “.. we investigated potential activation of two major pathways that interface strongly with IFN-based responses in a MyD88 independent fashion, namely, the STING and RIG-I-like Receptor (RLR)/MAVS-associated pathways.”

MDA5 is another RIG-I-like Receptor that senses dsRNA and which is associated with MAVS. Thus, if the authors want to exclude that PVP-057 can bind RIG-I-like receptors and mediate signaling via MAVS, the data should be strengthened.

Further, it is difficult to exclude that receptors potentially could act in concert with each other, i.e. TLR3 + another receptor. This could be mentioned in the Discussion.

2. Antibody and T cell responses versus protection against development of disease/treatment of disease.

a) The authors deserve credit for the inclusion of the VZV gE subunit-vaccine benchmark-adjuvant AS01B when investigating antibody and T cell responses in immunized mice.

But, elicitation of immune responses does not prove a functional and protective response in vivo. Thus, PVP-057 should be tested together with antigen in animal models (infectious and/or cancer models) in a prophylactic or therapeutic setting to

prove that PVP-057 promotes protection against the disease or promotes treatment in the model.

b) Number of animals.

The number of animals per group is in most experiments 6-7, and as far as I can understand, the experiments are performed once. This is not sufficient, in particular when comparing groups showing relatively small differences in response. The experiments should be repeated and each group should as a minimum consist of 8-10 mice.

c) Concerning the type of immune response.

The authors suggest TNF α and IL-2 as markers of CD4⁺ Th1 cells: Line 937-938 "gE-specific Th1 polarization was evaluated by targeting TNF⁺ (A) and IL-2⁺ (B) cells in the effector CD4⁺ T cell compartment."

However, the classical marker of CD4⁺ Th1 cells is IFN γ . Please explain/include references to document the reliability of TNF α and IL-2 as markers of Th1 cells.

Line 104-105: "Notably, PVP-057 demonstrated at least comparable results to AS01B for both Th1-polarized humoral and cell mediated outcomes when administered intradermally."

It is unclear whether the authors mean cell mediated outcomes as such or Th1-polarized cell mediated outcomes in line 104-105. Please clarify.

When reading the manuscript one gets the impression that the authors favor and are aiming for a Th1 response. But, the authors have not provided strong evidence for an IFN γ Th1-polarized cell response in the paper. The authors should take a more nuanced view on the type(s) of immune response(s) elicited after immunization. In Supplementary Table 2, antibodies against IL-4, IL-5, and IL-17A are listed. Which results were obtained when these antibodies were used?

3. The effect of PVP-057 on human cells.

There should be more data supporting that PVP-057 efficiently activate human primary cells.

PVP-057 did enhance CD80 and CD86 expression in monocyte-derived DCs from different donors (Supplementary Fig. 1), but many compounds can up-regulate CD80 and CD86 expression without being potent adjuvants. Thus, to show that PVP-057 has an effect on human primary cells, e.g. human monocyte-derived DCs, the authors should measure additional activation markers, for example quantitate secretion of type I IFN, TNF α , IL-1 β , IL-6, IL-12p70, CCL5, CCL17, CXCL10, and examine surface expression of CD40 and MHC class II molecules.

Reviewer #2

(Remarks to the Author)

The authors identified a novel TLR3 ligand PVP-057 by screening chemical libraries comprising about 200,000 small-molecules with a human monocytic cell line. They provide evidence demonstrating that PVP-057 stimulates TLR3 in vitro and in vivo models and possess adjuvant activity of some vaccine candidates. The studies in the manuscript appear to be of reasonable technical quality and provide novel and interesting results. The results might be useful for the further development of prophylactic vaccines or therapeutic drugs. However, there are some issues that might require the authors' attention.

Although they showed some in vitro and in vivo experimental results for the ability of PVP-057 to stimulate TLR3, it is not clear if PVP-057 directly binds to and stimulates TLR3 or indirectly activates TLR3 through other ways. Additional experiments are needed to clarify the molecular interaction between PVP-057 and TLR3 by some structural analysis technique such as surface plasmon resonance (SPR) analysis.

They provide solid data for proving the adjuvant activity of PVP-057. Nevertheless, it is not clear PVP-057 itself could be immunogenic in the animal immunization models used in the present study. Thus, additional experiments are necessary for testing the immunogenicity of PVP-057, for example, by measuring antibody against PVP-057 (with PVP-057 or its conjugate to carrier proteins).

More comprehensive study is needed to elucidate the action mechanisms for the adjuvant activity of PVP-057 at molecular and cellular levels including analyses of antigen-presenting cells in the draining lymph nodes, T cell subsets, memory cells, and so on.

Effective dose of PVP-057 appears to be too high (~ over ten micromolar concentrations) to be clinically applicable to prophylactic vaccines or therapeutic drugs. The authors are recommended to further screen the molecular derivatives with similar structure.

Reviewer #3

(Remarks to the Author)

The authors present a novel small molecule TLR3 agonist, have thoroughly investigated the targeting pathway and successfully identified it through various in vitro assays. In vivo proof of concept was afforded by demonstrating enhanced immunogenicity of the VZV gE. This is an exciting development for the small molecule medicinal chemistry and adjuvant

community and recommend this work be published. Please find below a few comments for the authors, as recommended minor changes.

1. Is it possible to provide more information on the origin of PVP-057? The authors mention that it was purchased from a screening library. Often screening libraries are sold in groups (kinase, GPCR, etc libraries), are custom made, or made at random. Curious if there is activity of PVP-057 on targets other than TLR3.
2. Authors need to mention CC50 values of PVP-057 on reported cell lines.
3. Authors need to state in the article if any weight loss was observed in mouse studies.
4. Authors could mention if the top 5 ranked compounds (figure 1C) were all from the same chemical series (that PVP-057 belongs too).
5. Its appreciated that PVP-057 is TLR3 selective. Authors could mention literature precedent of other small molecule (unselective) TLR3 agonists (J Med Chem 2017 60 (12), 5029-5044. DOI: 10.1021/acs.jmedchem.7b00419), structurally unrelated.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript has thoroughly addressed and resolved the majority of issues raised. However, if the authors want to present differences in T cell responses between immunization groups, the T cell data must be strengthened.

Major comment concerning the T cell data:

The number of detected T cells is very low, and the number of mice per group is low (n=6-8) (Figure 7, Supplementary Figure 12 and 13).

In order to present convincing results, an existing experiment should be repeated, or another type of experiment that can demonstrate T cell responses should be included with minimum 8-10 mice per group. An alternative is to use the existing data to show that gE + PVP-057 elicits a T cell response, while excluding the presentation and comparison with the gE alone and gE + AS01B groups.

In the Materials and Methods section, please include information about the peptides (PM-VZV-gE: length, number, defined MHC class II peptides) that were used before the measurement of T cell responses by flow cytometry and ELISpot assay.

Reviewer's specific comments to the authors' response regarding T cells:

"COMMENT 4: c) Concerning the type of immune response." and "COMMENT 6."

1) In the response, it is written:

"Also, in Supplementary Fig.12, while we only observed a biological trend towards gE-specific CD4⁺ IFN γ ⁺ T cells by spectral flow cytometry, as outlined in Supplementary Fig. 13, intradermally administered PVP-057 significantly increased the number of IFN γ SFCs by ELISPOT."

Reviewer's comment: To my understanding, the results presented in Figure 7 and Supplementary Figure 13 are from the same experiment, but present different parameters from an IFN-gamma ELISpot assay: SFC, well area, and spot intensity. The parameters SFC and well area (but not spot intensity) showed significance between the AS01B and PVP-057 adjuvanted groups. However, only 6-8 mice were included per group in the experiment. Further, the AS01B group ID appeared to induce minor if any T cell response to gE (Figure 7C). Was the immunization/ELISpot successful? Figure 7: The differences between the groups are too small and the number of mice per group is too few to draw firm conclusions.

2) Reviewer's understanding of Reference no. 2 and 3 listed in the response to COMMENT 4: In the review by Zhu, Yamane, and Paul (Annu Rev Immunol, 2009, 28:1) it is written "With regard to cytokine expression, Th1 cells make IFN- γ as their signature cytokine and also uniquely produce lymphotoxin. Th1 cells tend to be good IL-2 producers, and many make TNF- α as well. By contrast, Th2 cells fail to produce IFN- γ or lymphotoxin. Their signature cytokines are IL-4, IL-5, and IL-13. They also make TNF- α , and some produce IL-9. Although initially thought to be unable to make IL-2, later results indicated that Th2 cells could often produce relatively modest amounts of IL-2." TNF- α has been mentioned one more time in this publication: "Heterogeneity of cytokine production is also true for Th1 and Th17 cells. For example, the IFN- γ +TNF- α +IL-2+ multifunctional Th1 cells, but not the single cytokine producers, are likely to be the long-lasting memory cells (285)."

Moreover, Yamane and Paul (Nat Immunol, 2012, 13:11) write that IL-2 is important for differentiation of Th1 as well as Th2 cells. Yamane and Paul also present models for Th cell differentiation, e.g. differentiation into Th2 cells, which include IL-2 and TCR signal strength.

Reviewer's comment: As such, the references above (no 2 and 3) do not strongly support the arguments put forward in the response letter and the manuscript.

3) Line 414-417: "The incorporation of PVP-057 into a tailored delivery system may have the potential to further drive the relative polarization of PVP 057-based vaccines and immunotherapies towards a greater type 1 response, aiming for the Th1/Th2 balance seen in adjuvant formulations such as AS01B."

Reviewer's comment: Please include references that support the statement above ("Th1/Th2 balance seen in adjuvant formulations such as AS01B"). In a paper from the University of Oxford the authors argue that in comparison to viral vectors, AS01B promotes Tfh and Th2 cell responses (Nielsen et al., Protein/AS01B vaccination elicits stronger, more Th2-skewed antigen-specific human T follicular helper cell responses than heterologous viral vectors. Cell Rep Med. 2021 Feb 22;2(3):100207. doi: 10.1016/j.xcrm.2021.100207)

Statistics

1) Supplementary Figure 8: One-sided Mann-Whitney U test was applied to calculate statistics between PVP-057 and AS01B-adjuvanted groups; and two-sided Mann-Whitney U tests was used to examine significance against VZV gE alone. Reviewer's comment: Why was one-sided Mann-Whitney U used when comparing PVP-057 and AS01B-adjuvanted groups? Would it not be a more objective approach to use a two-sided test?

2) Reviewer's comment, Figure 7: Why were different tests used on the results in A, and B and C? According to the figure legend, t-test was used on the results presented in figure A, and Wilcoxon Rank-Sum test on the results in B and C?

Minor comments:

1) Reviewer's comment: Please include the results for the non-adjuvanted controls in Fig 5A, preferably also in Fig5B and C.

Figure legend 5A-C: "(A-C) Longitudinal antibody titers of IM and ID gE with PVP 057 are shown. Dotted line represents lower limit of detection."

Line 256-258: "...PVP-057 admixed with gE significantly enhanced anti-gE IgG titers as compared to the non-adjuvanted control even 184 days after the initial dose ($p < 0.05$ at all time points; Fig 5A).

2) Legend Figure 9:

"Mice were bled on Day 42, and anti-VZV gE (A), CMV gB (B), and HSV gD2 (C) IgG, IgG1, and IgG2c titers were assessed."

Reviewer's comment: Is it a typo? Results for IgG are presented, but not for IgG1 and IgG2c.

Reviewer #2

(Remarks to the Author)

The authors appropriately revised the manuscript according to the Reviewer's comments.

Reviewer #3

(Remarks to the Author)

Thank you for addressing the questions and providing more data in support of your work.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Dear authors,

Thank you for the thorough response.

Unfortunately, I remain of the opinion that if you want to present statistical differences in T cell responses between the immunization groups, it will be necessary to repeat experiments in order to reinforce the T cell data. (For my evaluation of the T cell data, see below.)

If you do not want to repeat experiments, I suggest presenting your current results on T cell responses without including p values and refraining from claiming statistical superiority of gE + PVP-057. Instead, explain that gE + PVP-057 elicits T cell responses that warrants further evaluation in the future. Moreover, for the sake of consistency in Figure 7, it appears more appropriate to report the percentage of effector CD4+IFN-gamma+ T cells (spectral flow) in Figure 7C rather than the number of IFN-gamma+ T cells (ELISpot).

Evaluation of the T cell data.

The T cell data presented in Figure 7 and Supplementary Figures 12 and 13 show some inconsistencies regarding the groups that are claimed to be significantly different: i) Supplementary Figure 13 which presents data on well area coverage and spot intensity obtained by IFN-gamma ELISpot, indicates significant p values for groups that do not fully correspond to

those identified in Figure 7C (number of gE-specific IFN-gamma+ splenocytes). ii) Supplementary Figure 12 which displays the percentages of effector CD4+IFN-gamma+ T cells identified by spectral flow, exhibits no significant differences among any of the groups.

Although the data for the T cell responses (Figure 7C, Supplementary Figures 12 and 13) derive from different parameters (numbers, well area, and intensity in IFN-gamma ELISpot) and methodologies (IFN-gamma ELISpot which may detect CD4 T and/or CD8 T cell responses; and spectral flow), the discrepancies in which groups are reported as significantly different, may suggest that the number of animals per group was too low.

Further, there are some inconsistencies when it comes to the choice of statistical tests in Figure 7, Supplementary Figures 12 and 13. In Supplementary Figure 12, the authors have used Kruskal-Wallis test with correction for multiple comparisons. This should also be an appropriate test to use for the T cell data presented in Supplementary Figure 13 and Figure 7B and C.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Reviewer #1

OVERALL COMMENT. In this manuscript, Lee et al., presents a novel TLR3 agonist denoted PVP-057 (a thiazole benzamide) identified in a screen of 200,000 small chemical compounds. Use of reporter cell lines (HEK-293 and THP-1), a TLR3 KO mouse, and a TLR3 inhibitor, revealed that PVP-057-mediated stimulation depends on TLR3.

Immunization with varicella-zoster virus (VZV) glycoprotein E (gE) together with PVP-057 intramuscularly (IM) or intradermally (ID) in mice, elicited anti-gE antibody responses (IgG, IgG1, IgG2c) and low numbers of T cells (cytokine producing CD4⁺ T, spectral flow cytometry; and IFN γ ⁺ T cells, ELISpot). PVP-057 was compared with the benchmark adjuvant AS01B, which induced antibody levels (IgG, IgG1, IgG2c) similar to PVP-057. The authors argue amongst other that PVP-057 increases the proportion of TNF expressing CD4⁺ T cells when compared to AS01B or gE alone. Given features such as highly efficient and scalable synthesis, robust adjuvanticity, and “the ability to act as a single-component vaccine adjuvant compatible with ID and IM administration”, the authors conclude that PVP-057 has prophylactic and therapeutic potential.

Development of potent adjuvants and identification of their mechanisms of action is an important research field, and successful adjuvants may greatly improve vaccine responses against infectious diseases and may also be applicable in therapeutic cancer treatment. In order to prove the potential of PVP-057 as an efficient adjuvant, additional data are needed. Please see the major critique outlined below.

RESPONSE: We appreciate the Reviewer’s overall summary. Of note, as Reviewer #3 has already recommended publication of our study, and Reviewer #2 provided very positive initial assessments, requesting only minor additions, as outlined in our response to all three reviewers, over the past ~6 months, we are happy to report we have now substantially expanded the re-submitted manuscript, including ~ 16 new major data additions, including one main figure and two supplementary figures targeted to address the Reviewer #1’s main suggestions.

COMMENT 1: Identification of TLR3 as the receptor of PVP-057. On page 9 and 10, line 178-180: “.. we investigated potential activation of two major pathways that interface strongly with IFN-based responses in a MyD88 independent fashion, namely, the STING and RIG-I-like Receptor (RLR)/MAVS-associated pathways.” MDA5 is another RIG-I-like Receptor that senses dsRNA and which is associated with MAVS. Thus, if the authors want to exclude that PVP-057 can bind RIG-I-like receptors and mediate signaling via MAVS, the data should be strengthened. Further, it is difficult to exclude that receptors potentially could act in concert with each other, i.e. TLR3 + another receptor. This could be mentioned in the Discussion.

RESPONSE: Thank you for your comment. As shown in our Supplementary Fig. 2, PVP-057 demonstrates little to no activation of hSTING or hRIG-I *in vitro* using (A) HEK-hSTING-overexpressing and (B) HEK-hRIG-I-overexpressing reporter cells, which were stimulated with 25 μ M PVP-057 and their respective positive controls, 25 μ M cGAMP for STING and 1 μ g/mL hpRNA for RIG-I. Furthermore, we agree that the complex nature of PRR activation and signaling necessitates the acknowledgement that the activation of TLR3 may not simply occur through direct binding. To address the possibility raised by Reviewer #1, we have added the following line to the Discussion (lines 663 ~ 669): “Furthermore, though our findings have established the activity of PVP-057 as being TLR3-dependent, this does not exclude the possibility that activity may be mediated by accessory proteins or be synergistic with the activation of other receptors. Small-

molecule and synthetic agonists with unique physical properties may induce activation of PRRs in ways that are often complex and may not follow canonical activation pathways seen with naturally derived agonists, despite phenotypic similarities in response⁴¹. Future mechanistic studies will further clarify the binding and signaling mechanisms of PVP-057.”

Comment 2: Antibody and T cell responses versus protection against development of disease/treatment of disease. a) The authors deserve credit for the inclusion of the VZV gE subunit-vaccine benchmark-adjuvant AS01B when investigating antibody and T cell responses in immunized mice. But, elicitation of immune responses does not prove a functional and protective response in vivo. Thus, PVP-057 should be tested together with antigen in animal models (infectious and/or cancer models) in a prophylactic or therapeutic setting to prove that PVP-057 promotes protection against the disease or promotes treatment in the model.

RESPONSE: We thank the Reviewer for their comment. We endeavor to strategically utilize clinically licensed vaccine formulations as a benchmark. Our study represents adjuvant discovery, demonstrating that PVP-057 is a novel small-molecule TLR3 agonist with immunomodulatory capabilities, as evidenced by this stage of experiments. Discovery research primarily focuses on identifying new drug candidates by highlighting their distinct activation of biological pathways, in contrast to development research, which focuses on the functionality of the drug candidates and prepares them for clinical use.

As such, our PVP-057 study identifies a novel TLR3 agonist through HTS using the human THP-1 cell line and demonstrates its immunomodulatory abilities both in vitro (Fig. 1 D-F, Fig. 2, Fig. 3, Supplementary Fig. 1, Supplementary Fig. 2) and in vivo (Fig. 5, Fig. 6, Fig. 7, Supplementary Fig. 5, etc.). In this study, we have opted to focus on the discovery of PVP-057 as a novel TLR3 adjuvant through 1) the elucidation of TLR3-specific pathway activation, and 2) the establishment of PVP-057 as an adjuvant via antigen-specific antibody elicitation and comparison to benchmark adjuvants (AS01B). To further highlight this point, we have added the following line to the Discussion (lines 756 ~ 759): *Furthermore, as elicitation of an immune response does not necessarily correlate to in vivo protection, future studies should include challenge models in*

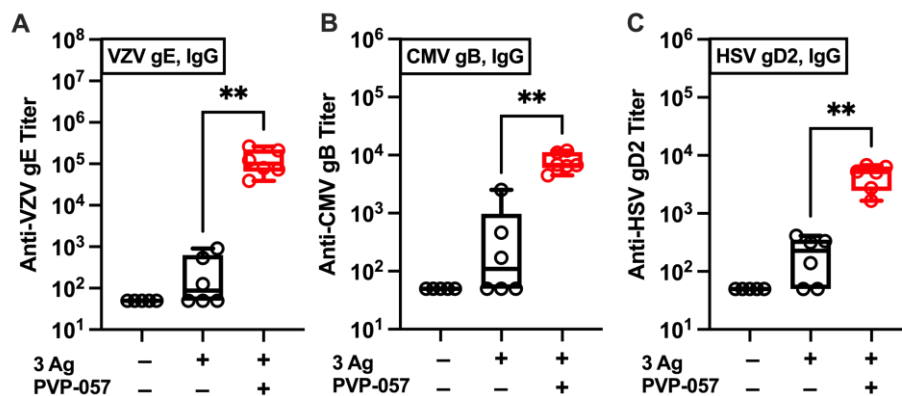


Figure 9. PVP-057 demonstrates adjuvantation of HHV antigens in a trivalent formulation. Adult C57BL/6J mice (N = 6 per group) were immunized intradermally on Day 0 and Day 14 with 0.5 µg VZV gE, 0.5 µg CMV gB, and 0.5 µg HSV gD2 +/- PVP-057 (100 nmol). Mice were bled on Day 42, and anti-VZV gE (A), CMV gB (B), and HSV gD2 (C) IgG, IgG1, and IgG2c titers were assessed. N = 6 per group. One-sided Mann-Whitney U tests were applied. ^{NS} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$.

order to test the prophylactic ability of antigen-specific antibody titers generated via adjuvantation with PVP-057.”.

Second, we have now made the addition of a **new main Figure 9**, in which we demonstrate that PVP-057 can adjuvant a trivalent, intradermally administered admixture of HHV2, 3, and 5 antigens. We have also added panels D-E to **Supplementary Figure 8**, in which we demonstrate that PVP-057 can separately adjuvant model HHV2 and HHV5 antigens. For these additions, we have made the following line edits:

(Lines 585 ~ 592): “As PVP-057 was able to consistently and robustly adjuvant the VZV gE antigen intradermally, we sought to evaluate the potential of this adjuvant response within a trivalent formulation of human herpesvirus (HHV) antigens. Six to eight-week old C57BL/6J mice were immunized ID following a 14-day prime-boost regimen with 0.5 μ g each of VZV gE (HHV3), HSV gD2 (HHV2), and CMV gB (HHV5), with or without 100 nmol PVP-057. PVP-057 enhanced IgG titers against all three antigens, up to Day 42 post-prime immunization (Fig. 9). This data reinforces the efficacy of PVP-057 as an ID adjuvant when admixed with multiple antigens and the potential for it to be used as an adjuvant for vaccines directed at HHVs.”

(Lines 453 ~ 454): “PVP-057 was also observed to adjuvant both the HSV gD2 and CMV gB antigens when administered IM (Supplementary Fig. 10D-E).”

For broader context, our AIDD Laboratory generally classifies early-stage projects into an “Innovation and Discovery stream”, as opposed to later-stage “Development” based projects. Many of our early-stage “Innovation and Discovery” streamed projects focus on use of globally accepted in vivo correlates of protection (**COPs**), and do not generally involve live-pathogen challenge (e.g., here are 10 examples of such projects - 25023392, 28343701, 28352660, 34783582, 36258023, 38959314, 36028220, 37884538, 37595760, 39047117). For instance, published work on the discovery of the TLR3 adjuvant NexaVant and our TLR7/8 adjuvant PVP-037 have effectively demonstrated the scope of adjuvant discovery research (**Table 1**).

Table 1. Examples of adjuvants discovery research and scope of projects.

Projects	NexaVant	PVP-037	PVP-057
PMID	36761735	38959314	Current
Screening compounds	N/A	HTS using PBMC	HTS using THP-1
Initial in vitro efficacy	HEK-hTLR3	PBMC, THP-1, BMDC, BMDM	THP-1, HEK-hTLR3
Initial in vivo efficacy	Ab titer, Flow cytometry	Ab titer	Ab titer, flow cytometry
MOA	RT-PCR, WB	ELISA	TLR3-selective

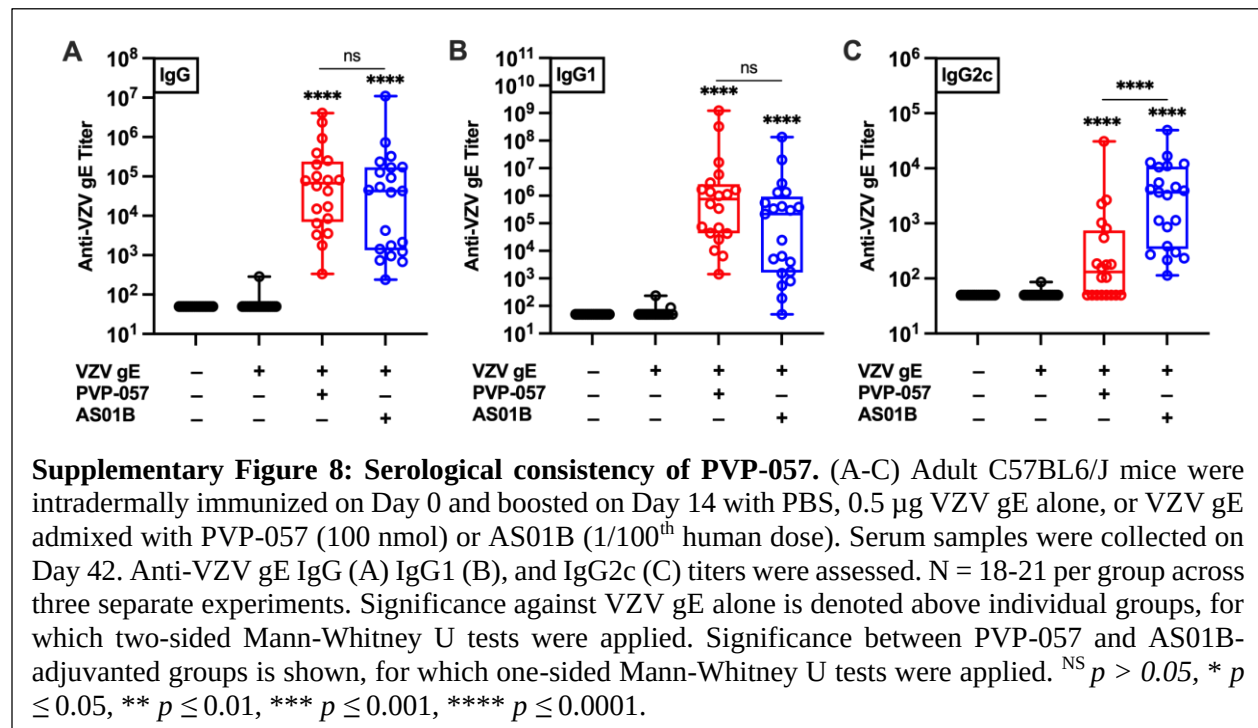
N/A: Not applicable, HTS: High-throughput screening, MOA: mechanism of action, WB: Western blot.

Lastly, we can ensure the reviewer that our current plan for this PVP-057 study is to move towards a “Development” stream. As such, we are activity working to obtain the grant funding needed and IACUC approval to establish a HSV-2 Guinea pig challenges model for use in the Therapeutic vaccine setting, alongside ongoing efforts to improve the potency, efficacy and formulation potential of PVP-057 pharmacophore analogs via structural activity studies (SAR), making it beyond the scope of this manuscript. We anticipate that access to these models will not occur until 2026. Additionally, the AIDD Laboratory does not currently have access to any in vivo cancer model assays, and the establishment of such models would likely only be used for this stand-alone single publication, making its use unsuitable to career development of current trainees in the lab.

COMMENT 3: b) Number of animals. The number of animals per group is in most experiments 6-7, and as far as I can understand, the experiments are performed once. This is not sufficient, in particular when comparing groups showing relatively small differences in response. The experiments should be repeated and each group should as a minimum consist of 8-10 mice.

RESPONSE: We appreciate the Reviewer's comment and can ensure that our study is robustly performed, with our in vivo studies repeatedly producing the same observations. For example, in the re-submitted manuscript, the demonstration of PVP-057-based adjuvanticity is shown via ~12 – 15 in vivo independent experiments, completed by multiple difference authors between 2019 and 2025, using multiple sources of PVP-057 (across Supplementary Figure 7, Supplementary Figure 10, Figure 5, Figure 6, Figure 7, Figure 8, and Figure 9), using 5 different antigens, including influenza, SARS, and multiple HHVs. Throughout, we employ the appropriate number of animals needed, with exploratory comparison of multiple dosing or route studies employing N = ~6 - 8 while benchmarking studies at a set dose employing N = ~20+, as now exemplified in **Supplementary Figure 8**.

Though slight experiment-to-experiment variability is inevitable, to address this comment, we would like to highlight the consistency of the trends (i.e. significance against antigen alone and relative Th1/Th2 polarization of adjuvants) that have been observed across our multiple smaller experiments. To do so, we are now including an additional **Supplementary Figure 8** in which we have collated data across three different experiments performed by one researcher, up to n=19-21 mice per group.



To address the reviewer's comment within the article, we have added the following sentence (lines 412 ~ 416): “Multiple repeated experiments comparing PVP-057 and AS01B-adjuvanted gE in a Day 0-Day 14 prime-boost ID immunization regimen have produced consistent signatures in their

relative antibody induction. While PVP-057 adjuvantation consistently induced IgG and IgG1 antibodies to a level non-significant against AS01B, AS01B outperformed PVP-057 in the induction of IgG2c (Supplementary Fig. 8)."

COMMENT 4: c) Concerning the type of immune response. The authors suggest TNF α and IL-2 as markers of CD4⁺ Th1 cells: Line 937-938 "gE-specific Th1 polarization was evaluated by targeting TNF⁺ (A) and IL-2⁺ (B) cells in the effector CD4⁺ T cell compartment.". However, the classical marker of CD4⁺ Th1 cells is IFN γ . Please explain/include references to document the reliability of TNF α and IL-2 as markers of Th1 cells.

RESPONSE: We appreciate the Reviewer's comment. For clarity, our interest is focused on signatures of polyfunctional T-cell responses, of which IFN γ and/or TNF may be canonically representative markers of Th1 polarized cells. This conceptual framework is also partly driven by our team's focus on immune ontogeny, in which we have demonstrated the role of TNF-producing polyfunctional T-cell responses at the extremes of life (see PMIDs 28352660, 34783582, 36028220, 36258023). Also, in **Supplementary Fig. 12**, while we only observed a biological trend towards gE-specific CD4⁺ IFN γ ⁺ T cells by spectral flow cytometry, as outlined in **Supplementary Fig. 13**, intradermally administered PVP-057 significantly increased the number of IFN γ SFCs by ELISPOT.

From a literature perspective, T cells consist of diverse subpopulations with varying levels of gene expression. The heterogeneity of T cells is influenced by factors such as tissue localization, phenotypic markers, and activation status. For instance, T cells are influenced by several factors during Th2 cell differentiation (1). Zhu et al. explains how individual T cells within the Th1 cell subpopulation utilize not only IFN γ but also TNF α and IL-2 as reliable markers (2). Furthermore, Yamane et al. illustrated that IL-2 is crucial for the proliferation of Th1 cells and is used to evaluate their activation status (3).

References:

1. BUETTNER, Florian, et al. Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells. *Nature biotechnology*, 2015, 33.2: 155-160.
2. ZHU, Jinfang; YAMANE, Hidehiro; PAUL, William E. Differentiation of effector CD4 T cell populations. *Annual review of immunology*, 2009, 28.1: 445-489
3. YAMANE, Hidehiro; PAUL, William E. Cytokines of the γ c family control CD4⁺ T cell differentiation and function. *Nature immunology*, 2012, 13.11: 1037-1044.

COMMENT 5: Line 104-105: "Notably, PVP-057 demonstrated at least comparable results to AS01B for both Th1-polarized humoral and cell mediated outcomes when administered intradermally." It is unclear whether the authors mean cell mediated outcomes as such or Th1-polarized cell mediated outcomes in line 104-105. Please clarify.

RESPONSE: Thank you for bringing this sentence to our attention. We are happy to add clarity. First, as outlined in **Supplementary Fig. 13**, intradermally administered PVP-057 significantly increased the number of IFN γ SFCs as measured by ELISPOT. Second, in our in vivo studies, we use IgG2c class switching as a humoral sign of Th1 polarization, and polyfunctional TNF and IL-2 producing CD4⁺ T cell induction as a cell-mediated measurement of Th1 polarization by flow cytometry.

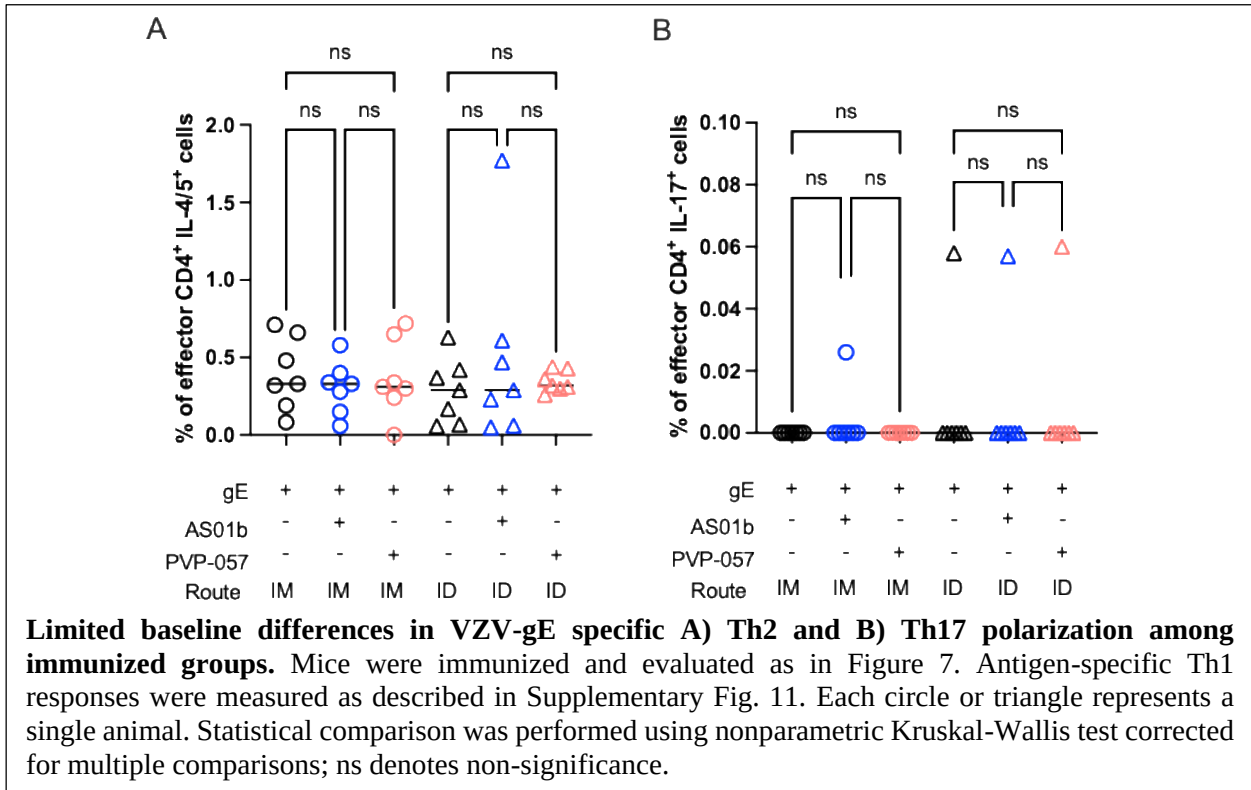
To help improve the clarity of this sentence we have revised it as follows (lines 139 ~ 142): "*Notably, when administered intradermally, PVP-057 demonstrated comparable results to AS01B for both type 1-polarized humoral (as measured via induction of IgG2c antibodies) and cell-*

mediated (as measured via polyfunctional CD4⁺ T cell induction via flow cytometry and ELISpot outcomes.”

COMMENT 6: When reading the manuscript one get the impression that the authors favor and are aiming for a Th1 response. But, the authors have not provided strong evidence for an IFN γ Th1-polarized cell response in the paper. The authors should take a more nuanced view on the type(s) of immune response(s) elicited after immunization. In Supplementary Table 2, antibodies against IL-4, IL-5, and IL-17A are listed. Which results were obtained when these antibodies were used?

RESPONSE: We thank the Reviewer for their comment and their careful examination of our immunophenotypic results and methods. As mentioned earlier, not only IFN γ but also TNF and IL-2 contribute to the characterization of individual T cells, including Th1 cell subpopulations. In addition, PVP-057 significantly increased the number of IFN γ SFCs after intradermal immunization in ELISpot assays. Furthermore, these results align with TNF and IL-2 data, as demonstrated by Spearman’s correlation (Figure 6, and Supplementary Fig. 13). We would also like to bring attention to Figure 6D, in which we demonstrate the positive correlation between IFN γ spot-forming cells and CD4⁺ T cells in stimulated splenocytes.

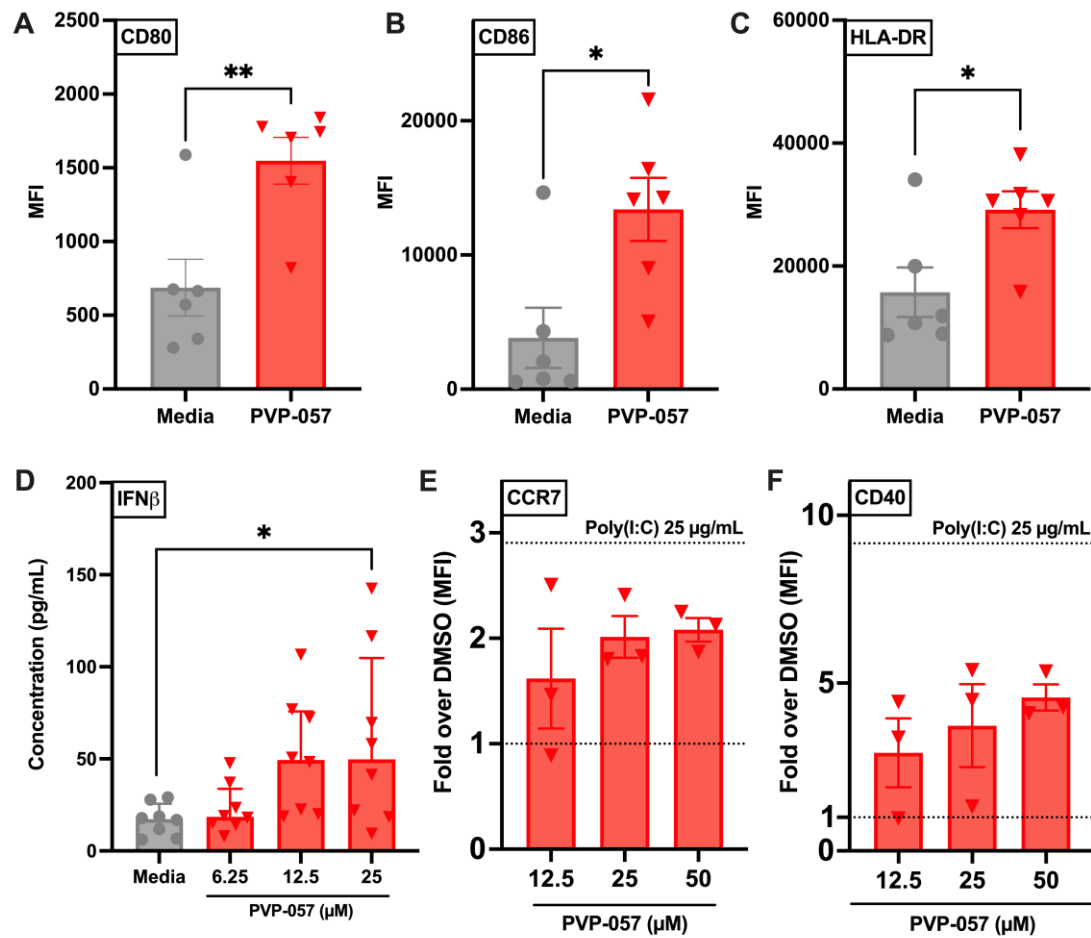
As per the concern raised by the reviewer, we have included the results related to IL-4/IL-5, and IL-17A below. VZV-gE specific Th2 and Th17 profiles were found to be limited and therefore we did not include the data in the manuscript. This method was validated and published as OMIP-111 in the journal Cytometry Part A (PMID 40095325).



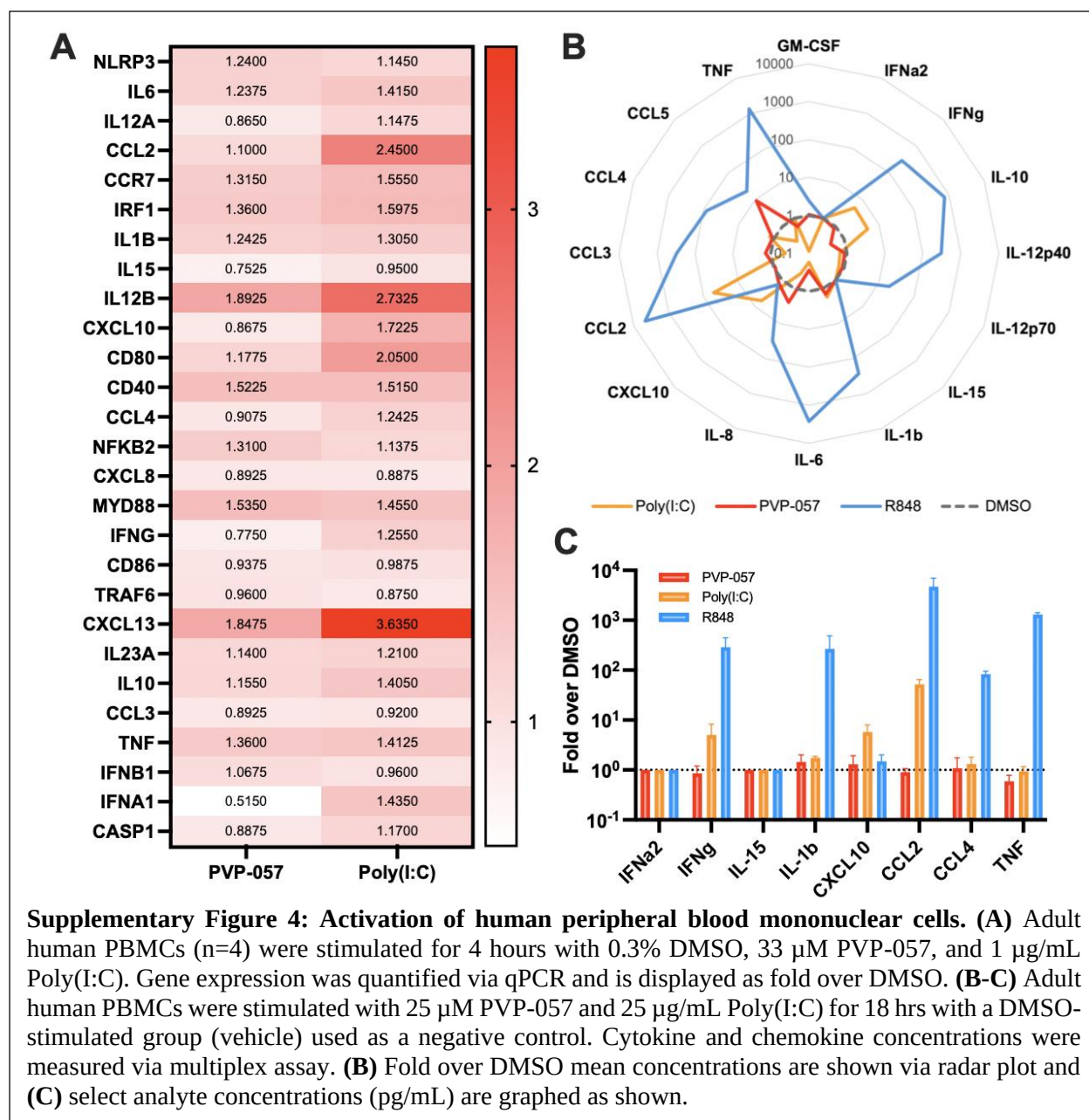
COMMENT 7: The effect of PVP-057 on human cells. There should be more data supporting that PVP-057 efficiently activate human primary cells. PVP-057 did enhance CD80 and CD86 expression in monocyte-derived DCs from

different donors (Supplementary Fig. 1), but many compounds can up-regulate CD80 and CD86 expression without being potent adjuvants. Thus, to show that PVP-057 has an effect on human primary cells, e.g. human monocyte-derived DCs, the authors should measure additional activation markers, for example quantitate secretion of type I IFN, TNF α , IL-1 β , IL-6, IL-12p70, CCL5, CCL17, CXCL10, and examine surface expression of CD40 and MHC class II molecules.

RESPONSE: We thank the Reviewer for their comment. To strengthen our data supporting the activation of human primary cells, we have added **Supplementary Figure 1C-F** in which we demonstrate the ability for PVP-057 to induce increased HLA-DR expression, the secretion of type I IFN β , and a dose-dependent induction of CCR7 and CD40, in human MoDCs. Finally, we have added **Supplementary Figure 4**, in which we highlight trends in both gene expression (via qPCR) and cytokine induction (via 16-plex assay) for human PBMCs stimulated with either PVP-057, Poly(I:C), or R848. To address the reviewer's comment within the article, we have added the following sentence to the Results (lines 257 ~ 266): *“After confirming the activity of PVP-057 as TLR3-dependent, we sought to evaluate the activity of PVP-057 in human primary cells using Poly(I:C) as a benchmark. We stimulated human monocyte-derived dendritic cells (MoDCs) with a dose-titration of PVP-057 and observed titratability in the quantitative expression levels of CCR7 and CD40, approaching the response induced by Poly(I:C) (Supplementary Fig. 1E-F). Furthermore, transcriptomic analysis of human peripheral blood mononuclear cells (PBMCs) stimulated with PVP-057 and Poly(I:C) demonstrated trends in the gene activation of key cytokines, and subsequent quantification of secreted cytokines corroborated this observation (Supplementary Fig. 4). Transcriptomic and cytokine responses for both PVP-057 and Poly(I:C) were generally observed to be muted, as compared to TLR7/8 agonist R848.”*



Supplementary Fig. 1: PVP-057 promotes human monocyte-derived dendritic cell maturation as measured by upregulation of co-stimulatory molecules, HLA-DR, and induction of IFN β . Adult monocyte-derived dendritic cells (MoDCs) were stimulated with 6.25, 12.5, or 25 μ M PVP-057 (red) for 24 hrs with untreated conditions (grey) used as a negative control. Quantitative expression levels, as measured through median fluorescence intensity (MFI), are visualized for (A) CD80, (B) CD86, and (C) HLA-DR. (D) IFN β was quantified via ELISA. Each dot represents a biological donor. N = 6 per condition. Two-sided Mann-Whitney U tests were applied. ^{NS} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$. (E-F) Adult monocyte-derived dendritic cells (MoDCs) were stimulated with 12.5, 25, or 50 μ M PVP-057, 25 μ g/mL Poly(I:C), or DMSO for 18 hrs. Quantitative expression levels, as measured through median fluorescence intensity (MFI), are visualized for (E) CCR7 and (F) CD40 as fold over DMSO-stimulated group.



Reviewer #2

OVERALL COMMENT. The authors identified a novel TLR3 ligand PVP-057 by screening chemical libraries comprising about 200,000 small-molecules with a human monocytic cell line. They provide evidence demonstrating that PVP-057 stimulates TLR3 in vitro and in vivo models and possess adjuvant activity of some vaccine candidates. The studies in the manuscript appear to be of reasonable technical quality and provide novel and interesting results. The results might be useful for the further development of prophylactic vaccines or therapeutic drugs. However, there are some issues that might require the authors' attention.

RESPONSE: We thank the Reviewer for their summary and for their positive remarks.

COMMENT 1: Although they showed some in vitro and in vivo experimental results for the ability of PVP-057 to stimulate TLR3, it is not clear if PVP-057 directly bind to and stimulate TLR3 or indirectly activate TLR3 through other ways. Additional experiments are needed to clarify the molecular interaction between PVP-057 and TLR3 by some structural analysis technique such as surface plasmon resonance (SPR) analysis.

RESPONSE: We thank the Reviewer for their comment and agree with the importance of clarifying the exact mechanism with which PVP-057 activates TLR3. We recognize that our current manuscript only goes so far as to demonstrate that PVP-057 operates in a TLR3-dependent manner without establishing the exact binding pattern. We plan to investigate direct binding with TLR3 in future publications. Because this paper concerns the discovery of the molecule and characterization as immunomodulatory, its binding and mechanism of action are prioritized in the future development of this small molecule. The use of CU-CPT4a as a selective inhibitor for TLR3 provides sufficient evidence of dependency on the TLR3-TRIF pathway. SPR analysis, along with full crystal structure determination, is the next step after the initial discovery is published. However, performing an SPR assay could take years to optimize and perform, and funding applications to achieve this have been requested. In the meantime, we have decided to prioritize dissemination of the discovery of the pharmacophore.

COMMENT 2: They provide solid data for proving the adjuvant activity of PVP-057. Nevertheless, it is not clear PVP-057 itself could be immunogenic in the animal immunization models used in the present study. Thus, additional experiments are necessary for testing the immunogenicity of PVP-057, for example, by measuring antibody against PVP-057 (with PVP-057 or its conjugate to carrier proteins).

RESPONSE: PVP-057 is a small-molecule TLR3 agonist that enhances the antibody response to antigens without generating immunogenicity itself. Small molecules are known to act as haptens – i.e., lack internal serology-based epitopes due to their structural simplicity, making it difficult for them to interact with B cells receptors. As a result, antibody responses specific to small molecule adjuvants, when simply admixed in solution, are extremely unlikely to be generated (i.e., we do not currently know of a published example).

Furthermore, we appreciate that carrier protein conjugation is often used in order to stimulate antibody production against molecules which are too small to induce antibodies against themselves (i.e. cannabinoids (PMID 34467269) and fentanyl (PMID 31173759)). However, we believe the value of PVP-057 lies in its immunomodulatory capability as it activates TLR3 and subsequent key immune pathways to successfully adjuvant an antigen-specific response. As such, we have not and do not intend to conjugate PVP-057 to a carrier protein and thus do not expect to induce antibodies specific to PVP-057.

COMMENT 3: More comprehensive study is needed to elucidate the action mechanisms for the adjuvanticity of PVP-057 at molecular and cellular levels including analyses of antigen-presenting cells in the draining lymph nodes, T cell subsets, memory cells, and so on.

RESPONSE: Thank you for the comment. First, our resubmission demonstrates the ability for PVP-057 to induce a) human monocytic cell maturation in vitro (**Figure 1**), b) primary human monocyte-derived dendritic cells (MoDCs) maturation in vitro (**Supplementary Figure 1**), and c) amplification of murine antigen-specific effector CD4⁺ T cells and IFN γ spot-forming cells in vivo (**Figure 7, Supplementary Figure 13**).

Second, as we continue to study and understand PVP-057 and its mechanism of action, we have since produced some transcriptomic and multiplex data, which have now been included. To both advance our work and address the feedback from the reviewer, we have added **Supplementary Figure 4**, where we demonstrate the Poly(I:C)-like transcriptomic and cytokine response of PVP-057-stimulated human peripheral blood mononuclear cells.

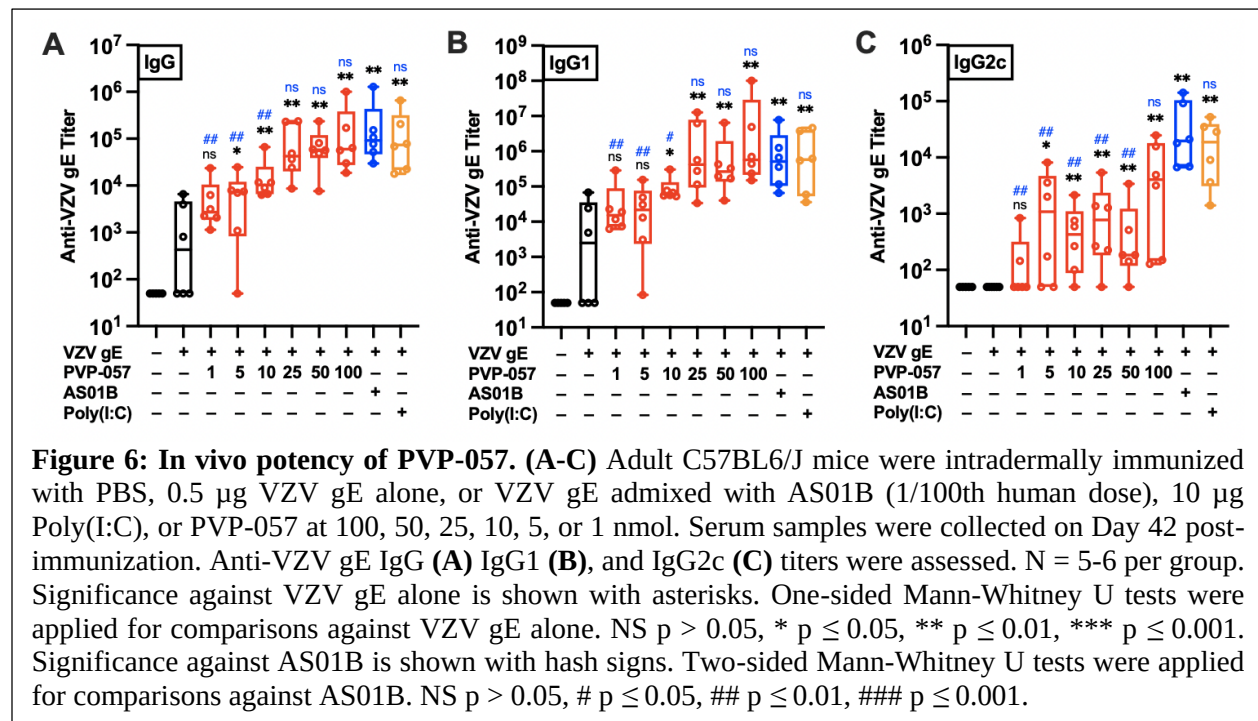
Third, as we illustrated in Comment 2 of Reviewer 1, as the high through-put (HTS) screen alone undertook ~3 years of work (between 2016 - 2019), in this manuscript, we have opted to outline the discovery of the PVP-057 pharmacophore via establishment of a broad MOA (putative TLR3 activation), demonstration of the activation of key innate immune pathways via ~7 different approaches, and demonstration of its immunomodulatory capabilities through its ability to enhance an adjuvant-specific response (again via the use of ~5 different antigens). While analysis of in vivo dLN-specific mechanism is planned, it will be matched with ongoing efforts to improve the potency, efficacy and formulation potential of PVP-057 pharmacophore analogs via structural activity studies (SAR), making it beyond the scope of this manuscript.

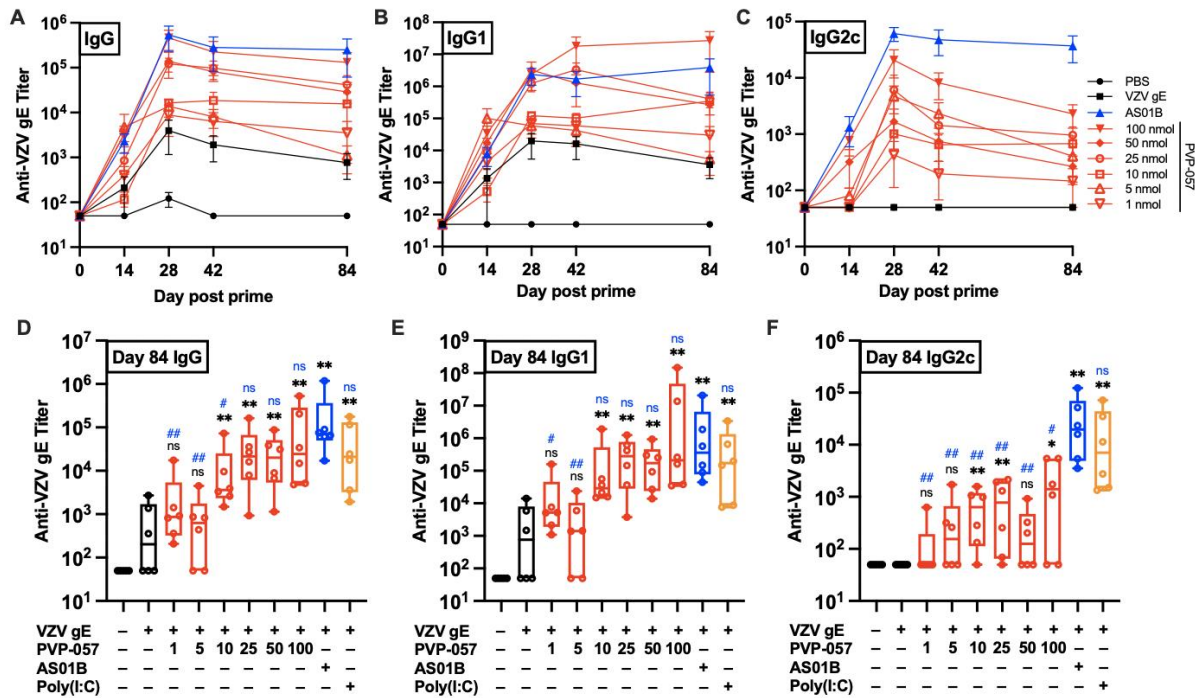
COMMENT 4: Effective dose of PVP-057 appears to be too high (~ over ten micromolar concentrations) to be clinically applicable to prophylactic vaccines or therapeutic drugs. The authors are recommended to further screen the molecular derivatives with similar structure.

RESPONSE: First, to clarify the effective dose of PVP-057, we have now included a new **Figure 6** in which we demonstrate the *in vivo* titratability and adjuvanticity of PVP-057 with an 8-point titration from our standard dose of 100 nmol down to a dose of 1 nmol. We found that PVP-057 displays significant adjuvantation of VZV gE with a dose as low as 5 nmol per mouse up to Day 42 post-immunization. Furthermore, we present longitudinal data on this titration out to Day 84 in a new **Supplementary Figure 7**. To address the reviewer's comment within the article, we have added the following sentence (lines 405 ~ 411): "*In a separate experiment, an in vivo dose-titration showed that PVP-057 can adjuvant gE at a dose as low as 5 nmol per mouse up to Day 42 (Fig. 6). This adjuvanticity was maintained up to Day 84 post-immunization at a dose of 10 nmol per mouse (Supplementary Fig. 7). Non-significance against AS01B was observed for gE-specific IgG and IgG1 at a dose as low as 25 nmol PVP-057 on Day 42, and this was sustained until Day 84 (Fig. 6A-B, Supplementary Fig. 7D-E). For IgG2c, non-significance against AS01B was only observed at the top concentration of 100 nmol PVP-057 on Day 42, and this effect waned on Day 84 (Fig. 6C, Supplementary Fig. 7F).*" This is accompanied by a new Supplementary Fig 11, demonstrating that "*6-8 week old mice immunized either IM or ID with PVP-057-adjuvanted gE demonstrated normal weight gain over the four days post-prime and four days post-boost immunizations (Supplementary Fig. 9)*" (lines 416 ~ 418).

Second, while we appreciate the Reviewer's recommendation to screen molecular derivatives in order to achieve a lower effective dose (in fact, the practice of structure activity relationship (SAR)

studies via the purchase and/or design of structurally similar compounds to find optimally performing analogs is commonly performed in our lab, as demonstrated in our recent publication outlining the discovery of PVP-037, a small-molecule TLR7/8 agonist adjuvant (PMID 38959314), we believe that the need to protect novel IP around such data would first need precede the inclusion in the current manuscript. As such, in this manuscript, we have chosen to focus on the discovery of the parent pharmacophore, establishing it as a TLR3 agonist with adjuvant potential across multiple indications, including influenza, SARS, and multiple HHVs.





Supplementary Figure 7: *In vivo* potency of PVP-057. (A-C) Adult C57BL6/J mice were immunized ID with PBS, 0.5 μ g VZV gE alone, or VZV gE admixed with AS01B (1/100th human dose), 10 μ g Poly(I:C), or PVP-057 at 1, 5, 10, 25, 50, or 100 nmol on Day 0 and Day 14. Serum samples were collected on Days 14, 28, 42, and 84 post-prime immunization. Anti-VZV gE IgG (A) IgG1 (B), and IgG2c (C) titers were assessed. (D-F) Titers shown for (D) IgG, (E) IgG1, and (F) IgG2c on Day 84. N = 5-6 per group. Significance against VZV gE alone is shown with asterisks. One-sided Mann-Whitney U tests were applied for comparisons against VZV gE alone. ^{ns} $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Significance against AS01B is shown with hash signs. Two-sided Mann-Whitney U tests were applied for comparisons against AS01B. ^{ns} $p > 0.05$, # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$.

Reviewer #3

OVERALL COMMENT. The authors present a novel small molecule TLR3 agonist, have thoroughly investigated the targeting pathway and successfully identified it through various in vitro assays. In vivo proof of concept was afforded by demonstrating enhanced immunogenicity of the VZV gE. This is an exciting development for the small molecule medicinal chemistry and adjuvant community and recommend this work be published. Please find below a few comments for the authors, as recommended minor changes.

RESPONSE: We thank the Reviewer for recommending publication of our study, with only minor edits. We have taken the resubmission process to significantly expand our study.

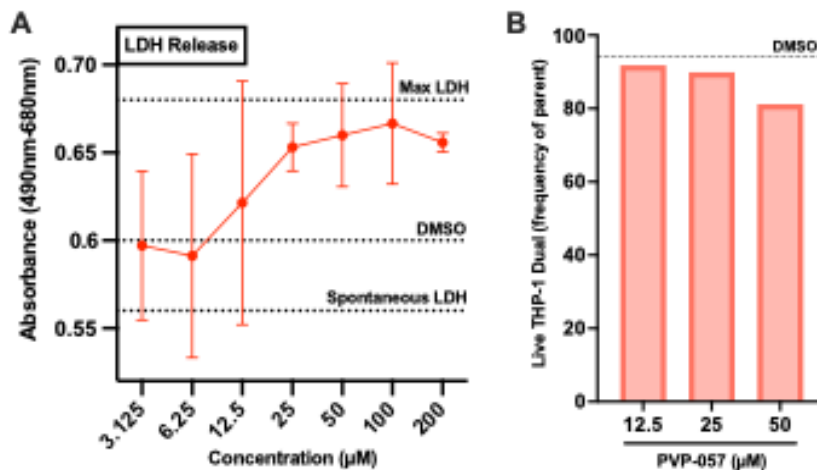
COMMENT 1: Is it possible to provide more information on the origin of PVP-057? The authors mention that it was purchased from a screening library. Often screening libraries are sold in groups (kinase, GPCR, etc libraries), are custom made, or made at random. Curious if there is activity of PVP-057 on targets other than TLR3.

RESPONSE: First, to directly address this question, to our knowledge, no other reported biological activity has been attributed to PVP-057. Second, the complete list of ~200,000 molecules screened in Figure 1A has already been recently reported by our team (Soni D et al., *From hit to vial: Precision discovery and development of an imidazopyrimidine TLR7/8 agonist adjuvant formulation*. Sci Adv. 2024, PMID: 38959314.). In sum, PVP-057 comes from the Chemdiv7 small-molecule library (non-patented molecules), which is part of a Targeted Diversity library of highly diverse drug-like compounds targeting various biological targets. The Harvard Medical School Screening Core (ICCB-L) purchased this library in 2013. Thus far, PVP-057 has been tested on the knock-in cell line targets outlined in this article (TLR3, RIG-I, STING). These targets were tested after having determined that the activity of PVP-057 is MyD88-independent, which ruled out MyD88-dependent targets such as TLR2, 4, 7, 8, etc.

COMMENT 2: Authors need to mention CC50 values of PVP-057 on reported cell lines.

RESPONSE: As our team has outlined in the past, adjuvants primarily function via PAMP and DAMP-mediated signaling and induction of an innate immune response (e.g., PMID 31904601 and 35222350). For specific examples, in PMIDs 25023392, 26933193, and 39437967, we outline how various adjuvant systems, including PCPPs, alum, and ILs, function primarily through DAMP-mediated signaling. As such, our team approaches the study of adjuvanticity via an agonistic approach to the cytotoxic nature of novel immunomodulators.

To address the Reviewer's comment, we have assessed LDH release from THP-1 cells stimulated with a dose titration of PVP-057. We intend to use cytotoxicity assays such as this as a metric to evaluate potent analogs and formulations in comparison to the parent pharmacophore in ongoing SAR studies. We have also assessed percentages of live THP-1 cells after stimulation with PVP-057 via flow cytometry. As such, we are including this data here for the purposes of the rebuttal letter, and we will expand on our analysis of cell cytotoxicity in future publications.

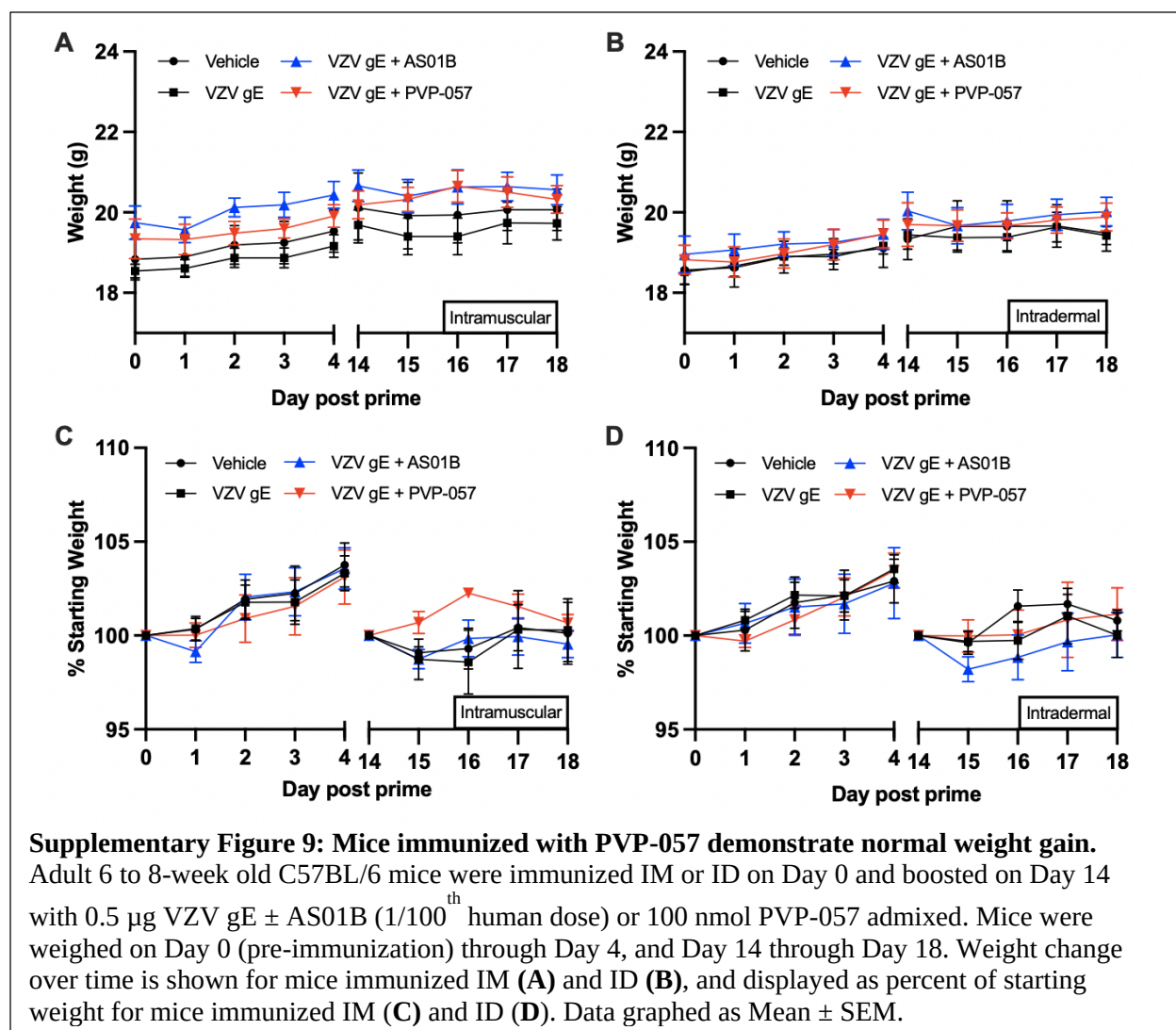


PVP-057-induced cell death. (A) THP-1 Dual cells were stimulated for 22 hours with PVP-057 then assessed for cytotoxicity based on the release of active lactate dehydrogenase (LDH) using a CyQUANT LDH Cytotoxicity Assay Kit (Invitrogen) according to the manufacturer's instructions. "Spontaneous activity" refers to LDH release from normal functioning, water treated cells, while "maximum activity" represents LDH release from lysed cells. (B) THP-1 Dual cells were stimulated for 18 hours with 12.5, 25, and 50 μM PVP-057, and DMSO (vehicle control). Live cells were quantified via flow cytometry as frequency of parent.

COMMENT 3: Authors need to state in the article if any weight loss was observed in mouse studies.

RESPONSE: We thank the Reviewer for their comment and for raising this metric of in vivo reactogenicity. We agree that evaluations of murine weight loss add value during preliminary discovery studies, as it may serve to help identify if an immunomodulatory/adjuvant may induce systemic inflammation. To address this Reviewer's comment, we are happy to include new data on weight loss post-immunization within the new **Supplementary Figure 9**. We can confirm that mice demonstrate normal weight gain following PVP-057-based vaccination.

In summary, mice were immunized with PVP-057 on Day 0 and boosted on Day 14, and weighed every 24 hours, starting on Day 0 (pre-immunization) through Day 4, as well as Day 14 through Day 18. Positive weight gain was noted for mice immunized both intramuscularly (**SFig. 9A, C**) and intradermally (**SFig. 9B, D**), with no notable weight loss observed in any groups immunized with PVP-057-adjuvanted VZV gE. To specifically address the reviewer's comment within the article, we have added the following sentence (lines 416 ~ 418): *"Furthermore, 6-8 week old mice immunized either IM or ID with PVP-057-adjuvanted gE demonstrated normal weight gain over the four days post-prime and four days post-boost immunizations (Supplementary Fig. 9)."*



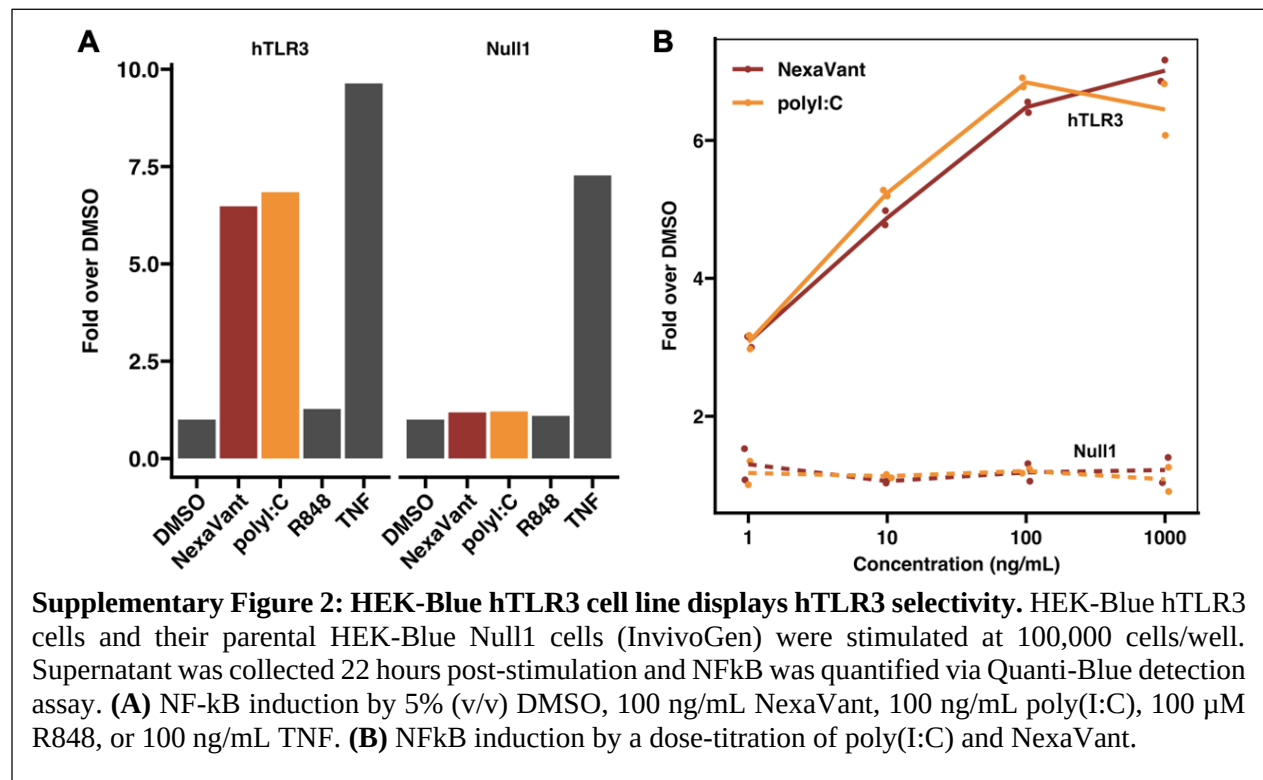
COMMENT 4: Authors could mention if the top 5 ranked compounds (figure 1C) were all from the same chemical series (that PVP-057 belongs to).

RESPONSE: Thank you for this interesting comment. We can confirm that none of the top 5 ranked molecules in the Chemdiv7 library belong to the same chemical series. This is the nature of this highly diverse library, where no compound is chemically the same as the others. To address the reviewer's comment within the article, we have added the following sentence (line 166): "*Of note, none of the top five ranked compounds had structural similarity to PVP-057.*"

COMMENT 5: It's appreciated that PVP-057 is TLR3 selective. Authors could mention literature precedent of other small molecule (unselective) TLR3 agonists (J Med Chem 2017 60 (12), 5029-5044. DOI: 10.1021/acs.jmedchem.7b00419), structurally unrelated.

RESPONSE: We appreciate the reviewer's reference (PMID 28537730). As the senior authors have previously outlined (e.g., PMID 22521247, 31022686), many small molecule immunomodulators and adjuvants have a) the reported capacity to display compound promiscuity (can interact with multiple biological targets), and/or b) lack evidence of in vivo activity. As such,

we have made the following line edit to include address the comment, while additionally highlighted the novelty and biological advancement around PVP-057 as a adjuvant (lines 652 ~ 655): “As compared to our study, limitations of many currently proposed TLR3-dependent small molecules include compound promiscuity, with the only previously reported agonist CU-CPT17e reported to simultaneously activate TLRs (such as 3, 8, and 9), along with unknown in vivo activity⁴²”. We have also now added **Supplementary Figure 2**, displaying the hTLR3 specificity of the HEK hTLR3 knock-in cell line used in this study, as compared to the parental HEK Null1 cell line (InvivoGen), along with the following line edit to the Results (lines 223 ~ 225): “The hTLR3-specific activity of the HEK-hTLR3 overexpressing cell line was validated with controls prior to use (Supplementary Fig. 2).”



Reviewer #1

OVERALL COMMENT. The revised manuscript has thoroughly addressed and resolved the majority of issues raised. However, if the authors want to present differences in T cell responses between immunization groups, the T cell data must be strengthened.

RESPONSE: We truly appreciate the Reviewer's attention to detail and dedication to improving the quality of our manuscript.

We would like to thank Reviewers #1 for highlighting that our prior manuscript had "thoroughly addressed and resolved the majority of issues raised", and given that Reviewers #2 and #3 have recommended acceptance of our manuscript, we hope our current responses address the final items needing clarity in the text.

Overall, in the latest round, Reviewer #1 has identified some minor errors and additional areas needing clarity in the manuscript, for which we appreciate the detailed reading, and which we have now been addressed. Most importantly, as shown in the updated **Figure 5A-C**, based on Reviewer #1's request, we have also included the results for the non-adjuvanted controls for the related samples in Figure 5A, B and C, as well as updated statistical comparisons and adjustments to the figure.

We have also provided additional rationale for the use of statistical approaches employed and have provided clarity about our current and prior approach to presenting T cell data, including reference to multiple similar examples of our work previously peer-reviewed studies on the characterization of novel adjuvants and formulations, including cellular responses. We hope this addresses these questions in full.

Lastly, it is worth noting that the Dowling laboratory at BCH has recently recruited Dr. JohnMark Awad as a Postdoctoral Research Fellow, arriving from our collaborate laboratory, Prof. Wei Zhang's at U. Mass Boston, where he completed his doctoral thesis on green chemistry of small molecule adjuvant. Over the coming 1-2 years, Dr. Awad's primary Postdoctoral project will continue to focus on optimizing the chemistry and formulations of 057-based pharmacophores. As such, via this planned first author paper from Dr. Awad, which we hope to submit for peer review in late 2026 (after BCH has had time to ensure appropriate intellectual property protection), we intend to explore in vivo dosing and expanding exploration of cellular response based on our recently published OMIP-111 (Barman et. al., *OMIP-111: Immune-Profiling of T Helper 1 (Th1), Th2, and Th17 Signatures in Murine Splenocytes by Targeting Intracellular Cytokines*, Cytometry A, PMID 40095325.), including into aged populations of mice.

COMMENT 1: Major comment concerning the T cell data: The number of detected T cells is very low, and the number of mice per group is low (n=6-8) (Figure 7, Supplementary Figure 12 and 13). In order to present convincing results, an existing experiment should be repeated, or another type of experiment that can demonstrate T cell responses should be included with minimum 8-10 mice per group. An alternative is to use the existing data to show that gE + PVP-057 elicits a T cell response, while excluding the presentation and comparison with the gE alone and gE + AS01B groups.

RESPONSE: We appreciate the Reviewer's perspective. First, we would like to highlight that the number of antigen-specific T cells detected in this study is consistent with the numbers we, and others, have observed in the past peer-reviewed publications. Specific examples of previously published peptide-specific T cell data presenting similar ranges of detected T cells include:

- **Grigoryan, L. et al. *NPJ Vaccines* (2022)** (PMID 35606518): In Figure 3, the % of CD4⁺ RBD-specific cells for adjuvant-immunized mice fell in the range of **0.0 - 1.0%** when looking at IFN γ ⁺, TNF⁺, and IL-2⁺ cell populations. This experiment contained 6-8 mice per group.
- **Barman, S. et al. *ACS Chem Bio* (2022)** (PMID 36028220): In Figure 4, the % of CD44⁺CD4⁺ rHA-specific cells for CL057 (TLR7/8) and 2'3'-cGAMP (STING)-adjuvanted rHA-immunized mice fell in the range of **0.0 - 1.5%** when looking at IFN γ ⁺, TNF⁺, and IL-2⁺ cell populations. This experiment contained 7 mice per group.
- **Phung, I. et al. *Nature Communications* (2023)** (PMID 37925510): In Figure 2, the % of CD40L⁺IL-2⁺ env-specific cells for adjuvanted and non-adjuvanted fell in the range of **0.0 - 0.2%**. This experiment contained 5-6 per group.

- **Brook, B. et al. *Science Translational Medicine* (2024)** (PMID 39047117): In Figure 3, the % of CD44⁺CD4⁺IFN γ ⁺ spike-specific cells for adjuvanted and non-adjuvanted BNT162b2-immunized mice fell in the range of **0.0 - 0.2%**. This experiment contained 6-8 mice per group.
- Other examples of our work on the characterization of novel adjuvants and formulations include PMIDs 28352660, 28343701, 29312305, 34783582, 39569372, 39437967, 39047117, 38959314, 37595760, 36788219, 36258023, 36028220, 35933439, 35148840, 32132997. In many of these studies, we have published ranges from **~0.0 – 2.0%** when investigating IFN γ ⁺, TNF⁺, and IL-2⁺ cell populations, across multiple species, and aged populations.

Second, we would like to emphasize that the purpose of this manuscript is to provide an initial immunological characterization of the activity of PVP-057, as a novel pharmacophore adjuvant, identified via HTS. As such, the T cell data is shown as an initial comparison to antigen alone and AS01B for the purposes of benchmarking and contextualizing the activity of PVP-057, whether the activity is significantly different from these groups or not. Therefore, while the reviewer may be correct in stating that “*An alternative is to use the existing data to show that gE + PVP-057 elicits a T cell response, while excluding the presentation and comparison with the gE alone and gE + AS01B groups.*”, given the fact that we would prefer to have this data published, it would necessitate a separate, which seems an inefficient use of our team resources, given the fact we are currently focused on Further optimization of this molecule (see next paragraph).

Further optimization of this molecule is recommended (and is currently ongoing) via examination of more potent molecular analogs and formulations, as highlighted in the Discussion (lines 417 ~ 419): “*Future studies may also need to examine molecular analogs and their potential to produce a heightened or more targeted response or be amenable to formulation*” and (lines 464 ~ 466): “*Given these attributes, PVP-057 is a promising immunomodulatory compound for further basic and translational investigation.*”

COMMENT 2: In the Materials and Methods section, please include information about the peptides (PM-VZV-gE: length, number, defined MHC class II peptides) that were used before the measurement of T cell responses by flow cytometry and ELISpot assay.

RESPONSE: We have revised the Materials and Methods section to include the information requested, as follows (lines 583 ~ 584): “*Rested splenocytes were stimulated in TCM with 1 μ g/mL of VZV gE protein (Dumas strain)-specific peptides mix (product code PM-VZV-gE; 153 peptides, 15mers with 11 aa overlap; 632 aa length; JPT Peptide Technologies, Berlin, Germany) with anti-mouse CD28/49d (BD, 1 μ g/mL) and 5 μ g/mL brefeldin A (BioLegend) for 6 hours.*”

COMMENT 3: Reviewer’s specific comments to the authors’ response regarding T cells: “COMMENT 4: c) Concerning the type of immune response.” and “COMMENT 6.”

1) In the response, it is written: “Also, in Supplementary Fig.12, while we only observed a biological trend towards gE-specific CD4⁺ IFN γ ⁺ T cells by spectral flow cytometry, as outlined in Supplementary Fig. 13, intradermally administered PVP-057 significantly increased the number of IFN γ SFCs by ELISPOT.”

Reviewer’s comment: To my understanding, the results presented in Figure 7 and Supplementary Figure 13 are from the same experiment, but present different parameters from an IFN-gamma ELISpot assay: SFC, well area, and spot intensity. The parameters SFC and well area (but not spot intensity) showed significance between the AS01B and PVP-057 adjuvanted groups. However, only 6-8 mice were included per group in the experiment. Further, the AS01B group ID appeared to induce minor if any T cell response to gE (Figure

7C). Was the immunization/ELISpot successful? Figure 7: The differences between the groups are too small and the number of mice per group is too few to draw firm conclusions.

RESPONSE: We thank the Reviewer for their thorough consideration of our ELISPOT data. Firstly, we would like to reiterate, as mentioned in response to Comment 1, that the purpose of this manuscript is to report the discovery of and characterize the activity profile of PVP-057 in vitro and in vivo via two routes of administration and in comparison to a known and commercially available adjuvant, in order to highlight its adjuvant potential.

Second, in researching a response to this comment, and noting that AS01B is formulated specifically for intramuscular delivery, we were unable to find any papers specifically describing the use of AS01B adjuvant via intradermal vaccination with VZV glycoprotein E (gE) antigen in mice or other animal models. So, our study may be the first published example of AS01B via intradermal vaccination with VZV gE, indicating a gap in the literature that our study now partially fills.

Third, as in our current and prior studies, AS01B has been used as a positive control and benchmark as it is readily available for purchase and is typically used to adjuvant the VZV gE antigen in the *Shingrix* vaccine, which is the antigen mainly used in this study. However, as the AS01B liposomal formulation is of an optimized GMP grade, we do not see it as a realistic goal to demonstrate overt improvement upon it. For this paper, our focus is on novel PVP-057 adjuvant discovery and its initial immunological characterization.

In sum, we have included this data in comparison to PVP-057 as we believe it highlights that a) PVP-057 can induce antigen specific cellular responses, and b) the route-specific activity of PVP-057, as PVP-057 demonstrated a uniquely heightened T cell response when administered intradermally (Fig. 7C and SFig. 13B-C), and c) we observed non-inferiority to AS01B at the doses tested. All of these observations are novel and innovative in the adjuvant field.

COMMENT 4: Reviewer's understanding of Reference no. 2 and 3 listed in the response to COMMENT 4. In the review by Zhu, Yamane, and Paul (Annu Rev Immunol, 2009, 28:1) it is written "With regard to cytokine expression, Th1 cells make IFN- γ as their signature cytokine and also uniquely produce lymphotoxin. Th1 cells tend to be good IL-2 producers, and many make TNF- α as well. By contrast, Th2 cells fail to produce IFN- γ or lymphotoxin. Their signature cytokines are IL-4, IL-5, and IL-13. They also make TNF- α , and some produce IL-9. Although initially thought to be unable to make IL-2, later results indicated that Th2 cells could often produce relatively modest amounts of IL-2." TNF- α has been mentioned one more time in this publication: "Heterogeneity of cytokine production is also true for Th1 and Th17 cells. For example, the IFN- γ +TNF- α +IL-2+ multifunctional Th1 cells, but not the single cytokine producers, are likely to be the long-lasting memory cells (285)." Moreover, Yamane and Paul (Nat Immunol, 2012, 13:11) write that IL-2 is important for differentiation of Th1 as well as Th2 cells. Yamane and Paul also present models for Th cell differentiation, e.g. differentiation into Th2 cells, which include IL-2 and TCR signal strength. Reviewer's comment: As such, the references above (no 2 and 3) do not strongly support the arguments put forward in the response letter and the manuscript.

RESPONSE: Again, we appreciate the Reviewer's attention to detail and dedication to improving the quality of our manuscript. We believe the Reviewer is correct in highlighting that much of the pre-clinical murine literature leans towards either TNF or IL-2 being *distinct* markers of Th1 polarization, unlike the classical marker IFN γ . However, we do believe that polyfunctional cellular responses (e.g., TNF/IL-2, TNF/IFN γ , IL-2/TNF/IFN γ expressing cells), especially in

immunological distinct populations, such as neonates, infants, older adults, and the immunocompromised, where IFN γ responses are known to be at the lowest, are also considered to be a marker of a type 1 response. Of note, this topic has been extensively reviewed in our prior review/perspectives, including:

- Nanishi E, Dowling DJ, Levy O. Toward precision adjuvants: optimizing science and safety. *Curr Opin Pediatr.* 2020 Feb;32(1):125-138. doi: 10.1097/MOP.0000000000000868. PMID: 31904601; PMCID: PMC6970548.
- Barman S, Soni D, Brook B, Nanishi E, Dowling DJ. Precision Vaccine Development: Cues From Natural Immunity. *Front Immunol.* 2022 Feb 10;12:662218. doi: 10.3389/fimmu.2021.662218. PMID: 35222350; PMCID: PMC8866702.

We have however, updated our language for the line in question, in the legend of Figure 7 (Lines 1094 ~ 1095) to now read: “*gE-specific TNF+ (A) and IL-2+ (B) cells in the effector CD4+ T cell compartment were quantified.*”

COMMENT 5: Line 414-417: “The incorporation of PVP-057 into a tailored delivery system may have the potential to further drive the relative polarization of PVP 057-based vaccines and immunotherapies towards a greater type 1 response, aiming for the Th1/Th2 balance seen in adjuvant formulations such as AS01B.”

Reviewer’s comment: Please include references that support the statement above (“Th1/Th2 balance seen in adjuvant formulations such as AS01B”). In a paper from the University of Oxford the authors argue that in comparison to viral vectors, AS01B promotes Tfh and Th2 cell responses (Nielsen et al., Protein/AS01B vaccination elicits stronger, more Th2-skewed antigen-specific human T follicular helper cell responses than heterologous viral vectors. *Cell Rep Med.* 2021 Feb 22;2(3):100207. doi: 10.1016/j.xcrm.2021.100207).

RESPONSE: We appreciate the Reviewer’s recommendation for greater evidence supporting the statements made. As highlighted in the below articles below, and based on the authors experience in the vaccine space, the AS01B system is canonically believed to promote an early IFN γ -skewed response and thus a Th1 phenotype, partly via a unique synergy between MPL and QS-21. We have now included references to the following articles highlighting the Type 1 induction seen with AS01B adjuvantation, for the sentence highlighted by the Reviewer:

- DENDOUGA, Najoua, et al. Cell-mediated immune responses to a varicella-zoster virus glycoprotein E vaccine using both a TLR agonist and QS21 in mice. *Vaccine*, 2012, 30(20)L 3126-35.
- COCCIA, Margherita, et al. Cellular and molecular synergy in AS01-adjuvanted vaccines results in an early IFN γ response promoting vaccine immunogenicity. *NPJ Vaccines*, 2017, 2:25.
- NANISHI, Etsuro, et al. An aluminum hydroxide:CpG adjuvant enhances protection elicited by a SARS-CoV-2 receptor binding domain vaccine in mice. *Science Translational Medicine*, 2022, 14.

If of interest, a detailed summary of each study, highlighting their relevance to the topic, is below:

Dendouga et al. (PMID 22326899) assessed the cell-mediated immune responses induced by AS01B-adjuvanted VZV gE, in comparison to other adjuvant systems such as Alum and AS01E (which contains half the amount of MPL and QS-21 as AS01B)), finding that “the gE-specific CD4+ T cell responses induced by gE/AS01B surpassed not only those induced by unadjuvanted gE, gE/alum and gE/AS02A (the latter with respect to IFN- expression by CD4+ T cells), but for the most part also those induced by lower doses of AS01 (AS01E and AS01F). Moreover, the

AS01B formulation tended to induce higher frequencies of CD4+ T cells positive for both IFN- and IL-2.”

Similarly, Coccia et al. (PMID 29263880) report that vaccines adjuvanted with the AS01 family of adjuvants (AS01B and AS01E) induce a Th1-biased response, owing to the induction of IFN γ - “Overall, these data indicate that IFN γ production in the early phase after vaccination is important for the induction of polyfunctional CD4+ T-cell response (mainly of a Th1 phenotype) and impact the quality of the antibody response to AS01-adjuvanted antigens” and “Overall, this indicates that innate IFN γ is required to induce CD4+ T-cell responses, with a Th1-bias after immunization with an AS01- adjuvanted vaccine, as further supported by the reduction in IgG2c antigen-specific antibodies in the absence of innate IFN γ signals.”

In Nanishi et al, (PMID 34783582), and an example of our own past published work in *Science Translational Medicine*, in which AS01B was used as a benchmark adjuvant. In this article, we observed heightened IgG2a antibody induction in mice intramuscularly immunized with AS01B-adjuvanted SARS-CoV-2 receptor binding domain, as compared to mice immunized with other pattern recognition receptor (PRR) agonists such as PHAD and CpG-2395 (Figure 1C-D). This is consistent with the production of IgG2c antibodies observed in mice immunized with gE + AS01B across multiple experiments within this manuscript (Figure 5F, Figure 6C, SFig. 7C, SFig. 7F, SFig. 8C). IgG2a and IgG2c production in mice is commonly correlated with Th1 polarization, as IFN γ promotes class-switching to these isotypes (Abbas et al. Cellular and Molecular Immunology, 9th edition; Chapter 10, pg. 333 – “IFN- γ acts on B cells to promote switching to certain IgG subclasses, notably IgG2a or IgG2c (in mice), and to inhibit switching to IL-4-dependent isotypes, such as IgE.”).

Lastly, in reference to the paper the Reviewer has highlighted (Nielsen et al.), we would like to point out that this paper compares AS01B-adjuvanted vaccines to non-adjuvanted viral vector vaccines. The relative skew of the AS01B response towards a Th2 phenotype is not accompanied by a complete lack of Th1 response, but rather less *in comparison* to the viral vectors being used. AS01B is still able to induce an early Th1 response. In our study, AS01B is compared to PVP-057, which exhibits a generally reduced Th1 response when looking at IgG2c readouts (Figure 6C, Supplementary Figures 7C,F & 8C). Thus, as a relative comparison, AS01B is able to induce a greater Th1 response than PVP-057, along with a similar Th2 response, resulting in the more relatively balanced Th1/Th2 readout observed.

COMMENT 6: Supplementary Figure 8: One-sided Mann-Whitney U test was applied to calculate statistics between PVP-057 and AS01B-adjuvanted groups; and two-sided Mann-Whitney U tests was used to examine significance against VZV gE alone. Reviewer’s comment: Why was one-sided Mann-Whitney U used when comparing PVP-057 and AS01B-adjuvanted groups? Would it not be a more objective approach to use a two-sided test?

RESPONSE: We thank the Reviewer for noticing this error. This was a simple typo, which we have now corrected within the figure legend. The statistical comparisons on the original figure reflect these comparisons, and we have cross-checked our data files to ensure this. We have corrected the figure legend as follows (lines 1206 ~ 1209): *“Significance against VZV gE alone is denoted above individual groups, for which one-sided Mann-Whitney U tests were applied. Significance between PVP-057 and AS01B-adjuvanted groups is shown, for which two-sided Mann-Whitney U tests were applied.”*

One-sided Mann-Whitney U tests were used when comparing PVP-057 with VZV gE alone, assuming adjuvanticity (our null hypothesis is a lack of statistical significance, thus we hypothesize some difference in antibody titer induction in one direction and opt for a one-sided test). Two-sided Mann-Whitney U tests were used when comparing PVP-057 and AS01B-adjuvanted groups, as our null hypothesis in this case is the opposite.

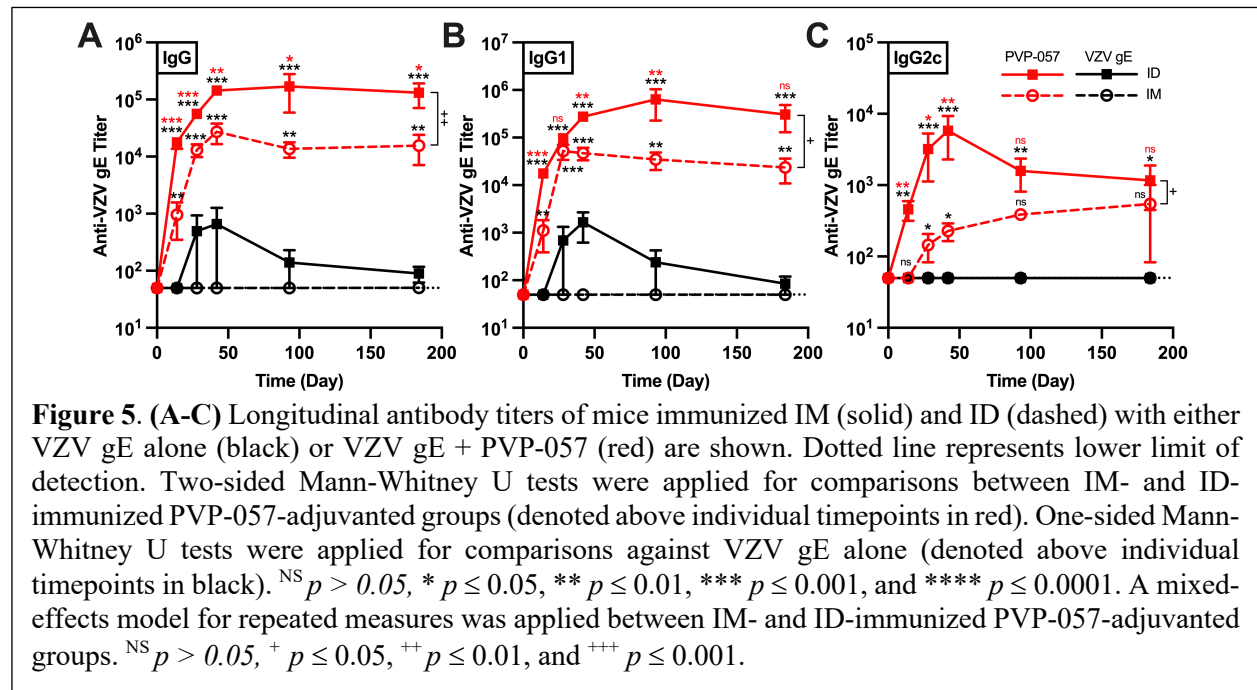
COMMENT 7: Figure 7: Why were different tests used on the results in A, and B and C? According to the figure legend, t-test was used on the results presented in figure A, and Wilcoxon Rank-Sum test on the results in B and C?

RESPONSE: Statistical tests were performed based on the normality of the results in question. The t-test, which assumes normal distribution, was performed on groups that demonstrated normal distribution, while Wilcoxon Rank-Sum was performed on non-normally distributed results. A Shapiro-Wilk normality test was initially run to determine which test would be run.

Of note, as part of our supplementary files, we have provided a complete Excel file of all the raw experimental data used for the data presented in the manuscript, allowing the community to complete secondary analysis at their pleasure after publication of our study.

COMMENT 8: Please include the results for the non-adjuvanted controls in Fig 5A, preferably also in Fig5B and C. Figure legend 5A-C: “(A-C) Longitudinal antibody titers of IM and ID gE with PVP 057 are shown. Dotted line represents lower limit of detection.” Line 256-258: “....PVP-057 admixed with gE significantly enhanced anti-gE IgG titers as compared to the non-adjuvanted control even 184 days after the initial dose (p < 0.05 at all time points; Fig 5A).

RESPONSE: We have included the requested longitudinal data for mice immunized with VZV gE alone (non-adjuvanted), for both routes of administration, in **Figure 5A-C**, as well as updated statistical comparisons, and adjustments to the figure legend.



COMMENT 9: Legend Figure 9 - “Mice were bled on Day 42, and anti-VZV gE (A), CMV gB (B), and HSV gD2 (C) IgG, IgG1, and IgG2c titers were assessed.”
Reviewer’s comment: Is it a typo? Results for IgG are presented, but not for IgG1 and IgG2c.
RESPONSE: We thank the Reviewer for the thoroughness of their review and for catching this typo. We have revised the legend as follows (lines 1125 ~ 1126): “*Mice were bled on Day 42, and anti-VZV gE (A), CMV gB (B), and HSV gD2 (C) IgG titers were assessed.*”

Reviewer #1

COMMENT. If you do not want to repeat experiments, I suggest presenting your current results on T cell responses without including p values and refraining from claiming statistical superiority of gE + PVP-057. Instead, explain that gE + PVP-057 elicits T cell responses that warrants further evaluation in the future. Moreover, for the sake of consistency in Figure 7, it appears more appropriate to report the percentage of effector CD4+IFN-gamma+ T cells (spectral flow) in Figure 7C rather than the number of IFN-gamma+ T cells (ELISpot).

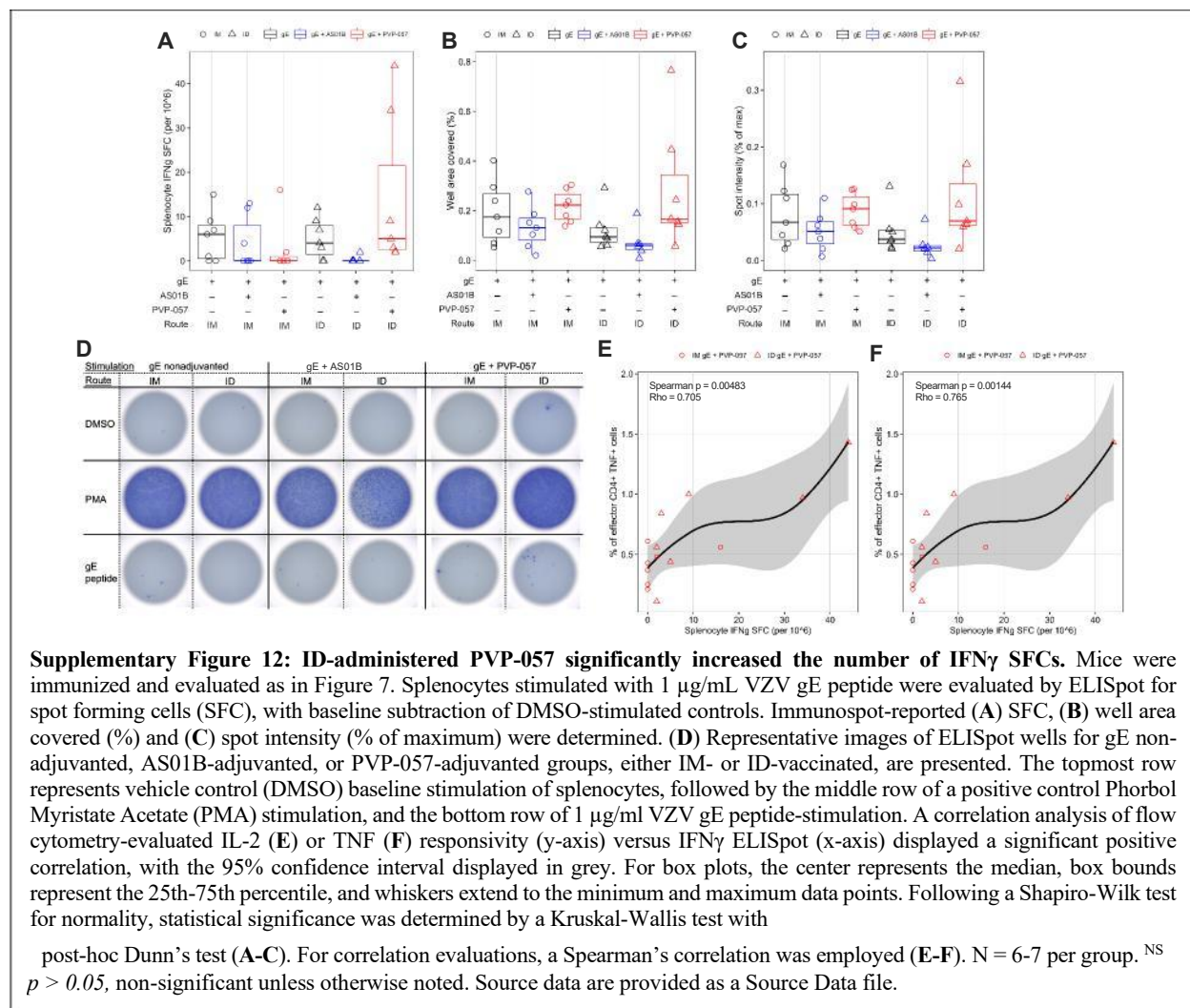
The T cell data presented in Figure 7 and Supplementary Figures 12 and 13 show some inconsistencies regarding the groups that are claimed to be significantly different: i)

Supplementary Figure 13 which presents data on well area coverage and spot intensity obtained by IFN-gamma ELISpot, indicates significant p values for groups that do not fully correspond to those identified in Figure 7C (number of gE-specific IFN-gamma+ splenocytes). ii)

Supplementary Figure 12 which displays the percentages of effector CD4+IFN-gamma+ T cells identified by spectral flow, exhibits no significant differences among any of the groups.

Although the data for the T cell responses (Figure 7C, Supplementary Figures 12 and 13) derive from different parameters (numbers, well area, and intensity in IFN-gamma ELISpot) and methodologies (IFN-gamma ELISpot which may detect CD4 T and/or CD8 T cell responses; and spectral flow), the discrepancies in which groups are reported as significantly different, may suggest that the number of animals per group was too low. Further, there are some inconsistencies when it comes to the choice of statistical tests in Figure 7, Supplementary Figures 12 and 13. In Supplementary Figure 12, the authors have used Kruskal-Wallis test with correction for multiple comparisons. This should also be an appropriate test to use for the T cell data presented in Supplementary Figure 13 and Figure 7B and C. **RESPONSE:** We have fully incorporated the Reviewer's suggestions and have updated Figure 7 to now report only spectral flow data, including percentages of CD4⁺ TNF⁺, IL-2⁺, and IFN⁺ cells as well as a correlation between CD4⁺ TNF⁺ and IL-2⁺ cells (originally in the Supplementary data). Furthermore, as per the Reviewer's recommendation, we have opted to pursue one-way ANOVA or Kruskal-Wallis (chosen based on normality) for our statistical analyses of both spectral flow and ELISpot data.

We have also moved the data on ELISpot IFN γ SFC to what is now **Supplementary Figure 12** (on next page), which encompasses all of the relevant ELISpot data. No new data has been added, only rearranged as recommended for consistency.



Lastly, we have also updated the manuscript text to match the updated statistical analysis as follows: In the Results section:

- Lines ~317-321: *“Mice immunized with PVP-057 showed significant increases in TNF expressing CD4⁺ T cells following ID immunization when compared to mice immunized with gE alone or with AS01B (Fig. 7A). Notably, mice immunized with gE adjuvanted with PVP-057 showed a significant increase in the percentage of CD4⁺ IL-2⁺ T cells vs. mice immunized with gE alone (Fig. 7B).”*
- Lines ~327-332: *“Mice immunized ID with PVP-057-adjuvanted gE demonstrated slightly higher induction of IFN γ spot-forming cells (SFC) compared to mice immunized ID with gE alone or AS01B-adjuvanted gE, with similar effects observed in percent well area and spot intensity, though these effects were non-significant (Supplementary Fig. 12A-C). A significant positive correlation was observed between TNF and IL-2 detected by spectral flow cytometry and IFN γ detected by ELISpot (Supplementary Fig. 12E-F).”*

In the Discussion:

- Lines ~477-479: *“Mice intradermally immunized with VZV gE admixed with PVP-057 displayed significant increases in the intracellular expression of IL-2 and TNF in the effector CD4⁺ T cell compartment.”*
- Lines ~480-484: *“A small increase in CD4⁺ IFN γ ⁺ T cells and IFN γ SFCs after ID administration of PVP-057 was also observed, though this effect was non-significant in both readouts. The ID-specificity of this T-cell activation suggests that there may be some unique skin-specific utility to the mechanism of action of PVP-057. This should be further evaluated in order to confirm and elucidate the modest trends observed here.”*