Understanding T-cell dependence in the mouse immune response to glycosylated outer membrane vesicles

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ABSTRACT

While engineered *Escherichia coli* outer membrane vesicles expressing glycans have been previously characterized as efficient vaccine delivery platforms capable of producing a glycan-specific immune response, the mechanisms behind this immune response are largely unknown. Furthermore, the modification of the native structure of lipid A on the OMVs from a hexa- to a penta-acylated form has been routinely used to decrease the toxicity of OMVs under the assumption that it does not have an effect on the adaptive immune response to the antigen displayed on the OMV. Here, we immunized C57BL/6 and T-cell deficient mice with hexa- and penta-acylated OMVs expressing polysialic acid (polySia), a repeating glyco-polymer of sialic acid. Results demonstrate that the immune response to penta-acylated polySia-OMVs relies on the presence of T cells, is driven by B2 cells in the peritoneal cavity, and fails to activate or create a polySia-specific memory B cell response. Alternatively, the response to hexa-acylated polySia-OMVs has a T-independent component that is driven by B1 cells in the spleen and peritoneal cavity, and generates a class-switched, polySia-specific memory B cell population. These results raise key insights for the use of glycosylated outer membrane vesicles (glycOMVs) in vaccination, as considering induction of a T-dependent or T-independent immune response may be beneficial for patients with differing immune competencies.

INTRODUCTION

In the human humoral immune response, antibodies produced by B cells can prevent the spread of infection by destroying extracellular pathogens [14]. Antigens that activate B cells are typically divided into two types based on their interaction or lack-of-thereof with T cells into T-dependent or T-independent, respectively [9].

Although some current vaccines — such as the vaccine that targets the polysaccharide in meningococcal meningitis [7] — produce a T-independent antibody response, most other vaccines based on protein antigens induce T cell-dependent responses [15]. In addition, immunization with pure polysaccharide antigens generally fails to produce an antibody response to the glycan, marking glycan antigens as nonimmunogenic, especially when compared to protein antigens [7]. This issue has been mitigated through the conjugation of the polysaccharide to a protein, resulting in increased immunogenicity and a T-dependent immune response [7]. However, the design of these glycoconjugates needs to be adapted to each glycan of interest, with some glycans failing to become immunogenic with this approach [6, 12]. Also, while conjugate vaccines have proven to be safe and effective, their manufacture is costly and low-yielding [2].

To address these issues, the DeLisa lab has engineered glycosylated outer membrane vesicles (glycOMVs) derived from *Escherichia coli*, and demonstrated that this approach can produce a robust humoral immune response to the glycan being expressed [2]. Outer membrane vesicles (OMVs) are nonpathogenic, naturally-occurring nanostructures that resemble the outer membrane and periplasm of gram-negative bacteria, and are used by bacteria in the wild to

deliver pathogenic agents to host cells. To construct these glycOMVs, our lab first uses recombinant DNA technology in a hypervesiculating strain of *E. coli* to synthesize the specific glycan of interest. The synthesized glycan is then attached to the core of lipid A, a strongly immunogenic bacterial lipid, and then transferred to the outer membrane of the bacteria [2].

These OMVs have been previously harnessed by the DeLisa lab to demonstrate the efficacy of this platform as a vaccine candidate for eliciting an immune responses to a myriad of glycans, including the *O*-linked polysaccharide from *Franscicella Tularensis*, polysialic acid (polySia), and the Thomsen-Friedenreich antigen [2, 21]. Some microbial antigens, such as the highly repetitive capsular polysaccharides on bacterial cell walls, are T-independent antigens that can cross-link the B-cell receptor and elicit a B-cell response in the absence of helper T cells [14]. In these cases, one of the necessary signals for B-cell activation can be provided by a toll-like receptor (TLR) signaling pathway that recognizes a common microbial pattern, resulting in activation of further signaling pathways to promote B cell survival and proliferation [14]. Lipid A is one such activator of TLR4 [23] that has shown to contribute to the T-independent activation of B cells [16]. Meanwhile, glycans conjugated to proteins elicit T-dependent responses because the glycoconjugate can be processed within the cell, allowing for the peptide portion to bind to MHC II while the carbohydrate portion can be recognized by T-cells [2]. It is currently unknown whether the humoral response against glycOMVs is T-dependent or T-independent, or if the role of T cells depends on the characteristics of the displayed glycan or the OMV itself [6]. Thus, this study aims to determine whether the glycan-specific antibody response to glycOMVs expressing polySia is T-dependent or T-independent.

The DeLisa lab has previously employed the use of penta-acylated OMVs, in which a mutation in the structure of lipid A in *E.coli* elicits a mild innate immune response, allowing for mice to be immunized with higher doses of OMVs without the threat of septic shock. Penta-acylated OMVs contain a mutation in the *lpxM* gene that encodes for an acetyltransferase, changing lipid A from a hexa- to a penta-acylated structure. This change dramatically decreases the affinity of lipid A and the TLR-4 by 80% [2]. Here, we characterize the effects that changing the structure of lipid A from hexa- to penta-acylated on glycOMVs expressing polySia has on the mechanisms that drive the immune response to the glycan. Our results show that although both types of glycOMVs produce comparable levels of polySia-specific IgG titers in blood sera, the immune response after immunization with hexa-acylated polySia-OMVs contains a T-cell independent component that is completely absent after immunization with penta-acylated OMVs. Moreover, we found that the population B cells driving the immune response to the glycan is dependent on the structure of the lipid A in the glycOMVs. Namely, we think that the polySia-specific IgGs after immunization with hexa-acylated polySia-OMVs are driven by a population of B1 cells in the spleen and peritoneum, while the polySia-specific IgGs after immunization with penta-acylated polySia-OMVs are driven by a population of B2 cells that is activated in the peritoneal cavity.

RESULTS

Immunization with hexa-acylated polySia-OMVs induces a T-independent antibody response to polySia

Following immunization of wild type (WT, C57BL/6) and T cell-deficient (TCR-KO) mice with glycOMVs (Fig. 1A), several blood titers were performed to determine the extent of the antibody

response to polySia, as seen in Figure 1B. Since antibodies are produced by mature B cells, measuring antibody titers can indicate the activation of B cells against antigens, as well as whether B cells have been class-switched to produce different isotypes of antibodies. B cells can undergo this class-switch DNA recombination with engagement of CD40 on B cells by CD40L on CD4+ T cells, stimulating the B cells to diversify and increase the affinity of their antibodies in the germinal center [5]. However, class switch recombination can also more rapidly occur independent of CD40 in response to pathogen-associated molecular patterns (PAMPs), allowing splenic marginal zone and mucosal B cells to generate lower-affinity IgG, IgA and IgE in addition to IgM [5]. Thus, by measuring IgG and IgM levels for both WT and T-deficient mice, we can gain further insight into whether antibody production against the polySia-lipidA antigen relies on innate or adaptive pathways, and investigate possible mechanisms for these pathways.

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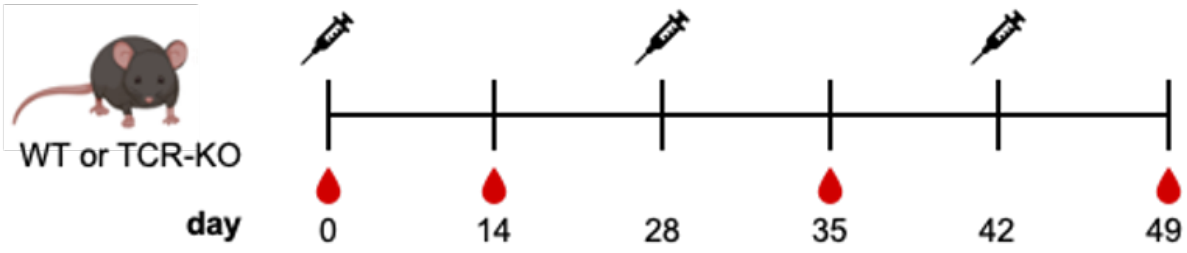

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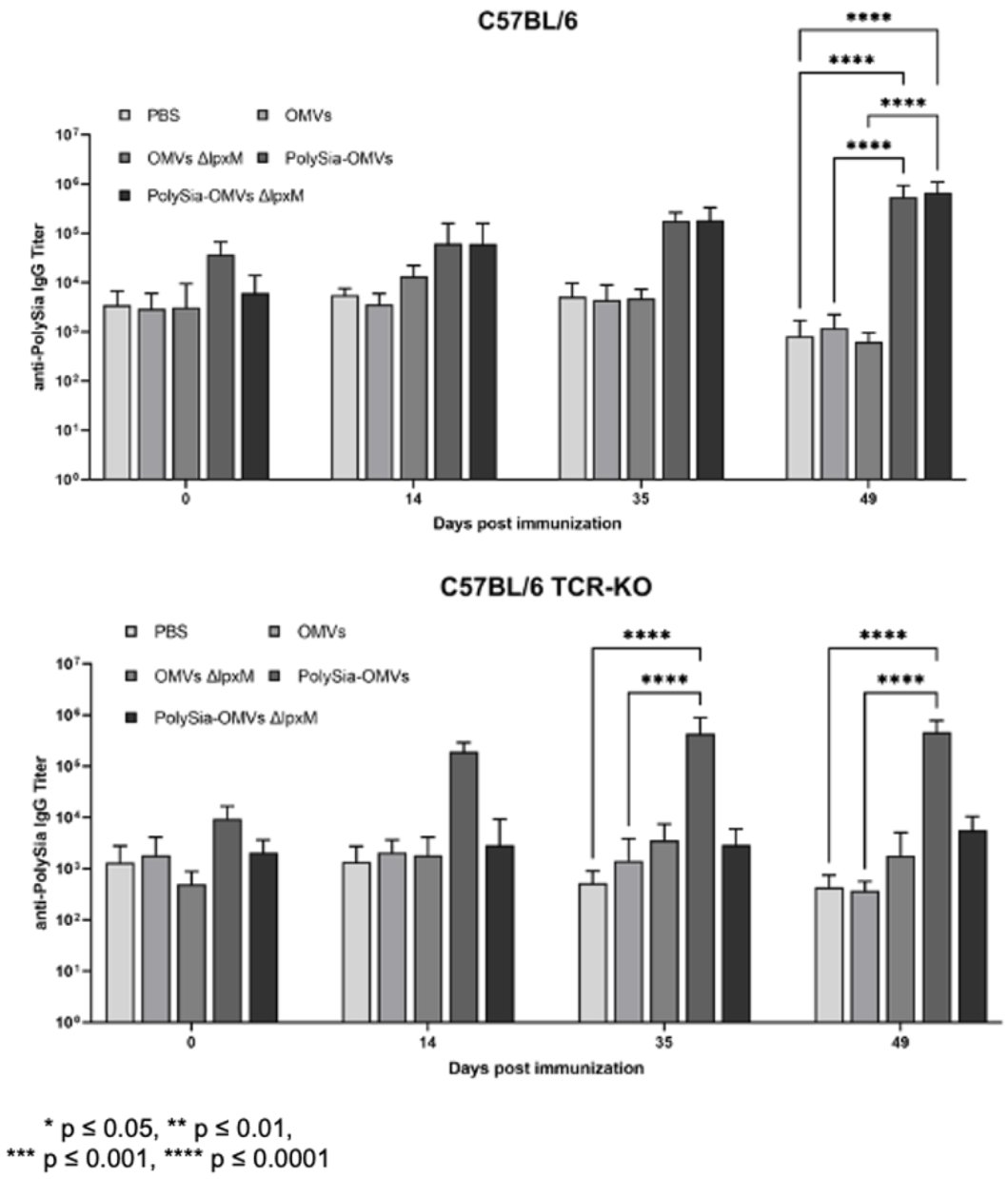
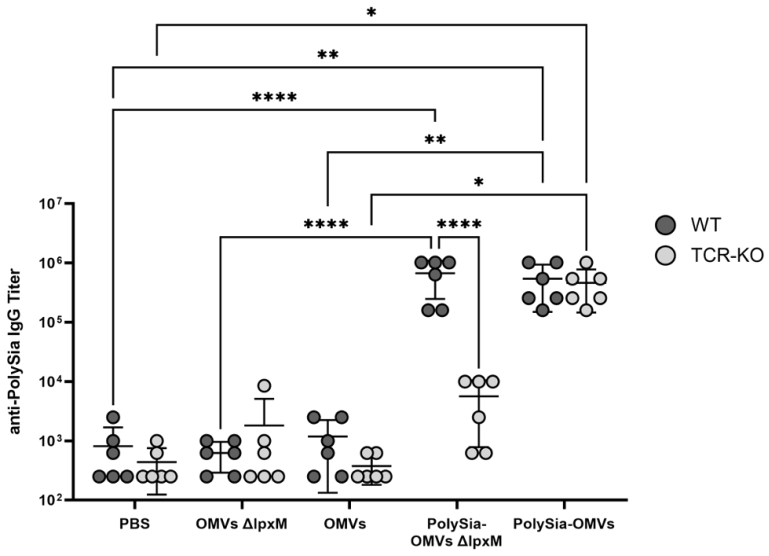


Figure 1. Anti-polySia IgG titers for wild type (WT, C57BL/6) and TCR-KO mice post-immunization with glycOMVs. ***(A)*** Immunization schedule. 30 wild type C57BL/6 and 30 C57BL/6 TCR-KO mice, each divided into 5 groups of 6 mice, were immunized subcutaneously with the following: Phosphate Buffer Saline (PBS), empty JC8031 OMVs, empty JC8033 OMVs (OMVs ΔlpxM), JC8031 polySia-glycOMVs, or JC8033 polySia-glycOMVs (polySia-OMVs ΔlpxM). Mice were boosted 28 and 42 days after the initial immunization. Blood was drawn from the jaw of the mice to analyze serum titers on the day of initial immunization, as well as 14 days, 35 days, and 49 days after the initial dose. ***(B)*** Anti-polySia IgG titers from blood serum pre- and post-immunization. Although WT mice showed a statistically significant difference in their immune response to polySia independently of the type of OMV, mice lacking T-cells only mount a polySia-specific immune response after immunization with JC8031 OMVs. Asterisks are used to indicate statistical significance in an unpaired t-test, with four asterisks (****) indicating statistical significance of $P < 0.0001$.

The data show that for immunization with hexa-acylated glycOMVs, TCR-KO mice mount an IgG response similar to that of WT mice, indicating that isotype switching from IgM to IgG can occur even without the presence of helper T cells. This finding may indicate the strong immunogenicity of polySia conjugated to the native structure of lipid A in a T-independent manner. On the contrary, penta-acylated glycOMVs only produced polySia-specific IgG titers in WT mice, suggesting that changing lipid A to a penta-acylated structure shifts the immune response to be entirely T-cell dependent. This change in the role of T cells during the immune response to polySia might be related to the change in affinity between lipid A and TLR4 after shifting from a hexa- to a penta-acylated structure [2, 11].

At the end of the trial, final IgG titers (Fig. 2) showed a limited antibody response for the PBS group, as well as groups immunized with OMVs not displaying polySia. However, there was no significant difference in the WT mice IgG response for either penta-acylated or hexa-acylated polySia-OMVs.



* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001

Figure 2. The T-cell dependent, glycan immune response after immunization with penta-acylated OMVs. WT mice immunized with hexa-acylated polySia-OMVs mounted a similar IgG response to TCR-KO mice, while the IgG response was significantly reduced for TCR-KO mice immunized with penta-acylated (Δ*lpxM*) polySia-OMVs compared to WT mice.

Splenic B1 cell population contributes to a T-Independent immune response to polySia

Analysis of the splenic B cell populations via flow cytometry (Fig. 3) showed that immunization with hexa-acylated polySia-OMVs promotes the activation of polySia-specific B cells in the spleen, which was virtually absent in mice immunized with penta-acylated polySia-OMVs. Moreover, surface markers of the B cell population indicated that the immune response to polySia in the spleen was independent of T cells and dominated by B1 cells. Our results also suggest that the activation of glycan-specific B2 cells in the spleen, which is the dominant B cell population in this lymph node, was not achieved by either hexa- or penta-acylated OMVs, regardless of the presence or absence of T cells.

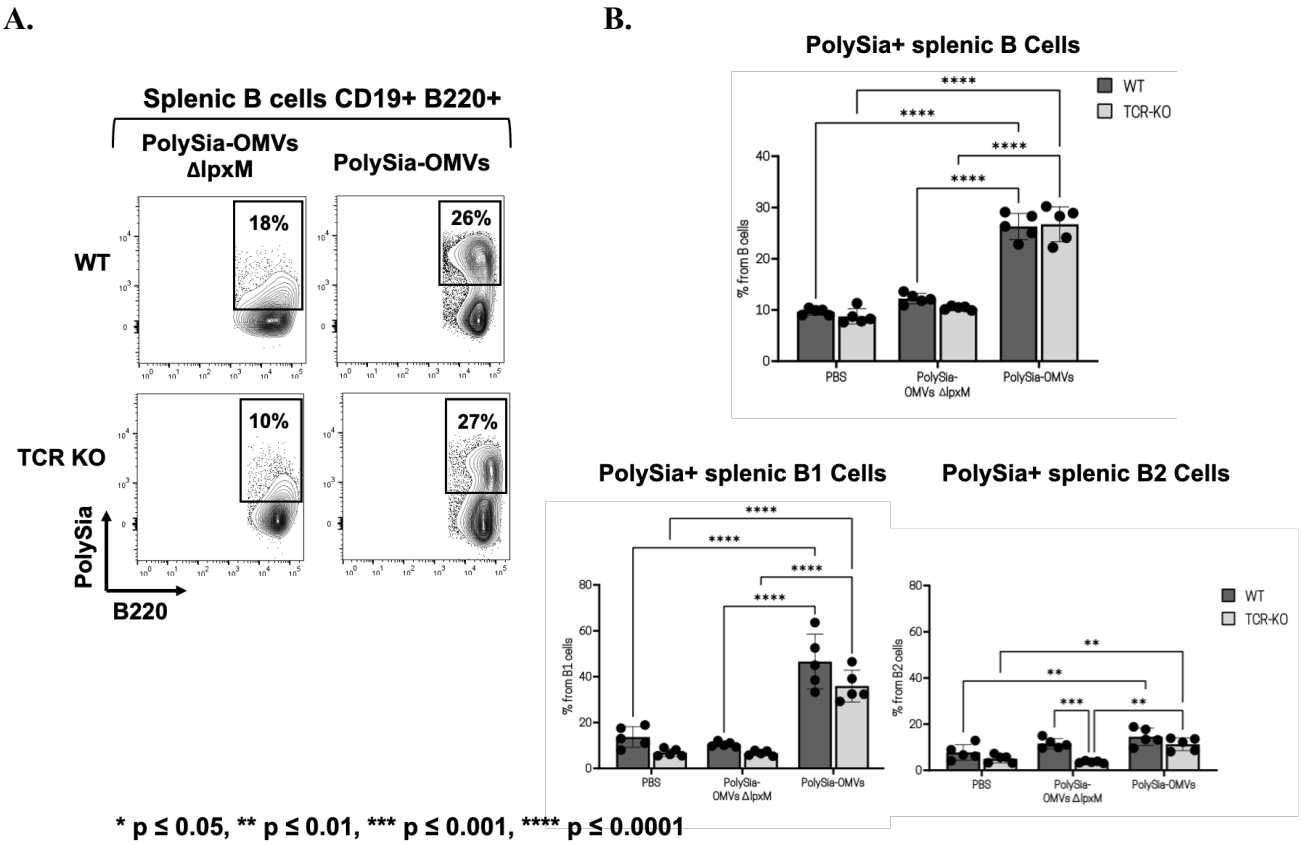


Figure 3. Flow cytometry analysis of mature splenic B cell populations. B cell populations from the spleen were gated based on their expression of CD19 and B220 and their lack of TCRa/b. Binding to polySia was defined by the population of B cells with fluorescence above controls. ***(A)*** Representative contour plots of total B cells from the spleen of one mouse from each group. Square gates represent the population of B cells that recognize polySia. ***(B)*** Percentage of total B cells that recognize polySia from the spleen of five mice per group. B cells that recognize polySia are further characterized by their phenotype as B1 or B2 cells. Bars represent the statistical mean, error bars the standard deviation, and circles the value for each mouse within the group. Statistical significance was determined for p values < 0.05 using the Turkey-Kramer post hoc honest significant difference (HSD).

Hexa-acylated polySia-OMVs increase the activation of splenic memory B cells

Splenic memory B cells were defined by the expression of CD38 and the lack of GL7 on their surface. The splenic B cell population was further analyzed for their binding to polySia (Fig. 4), and hexa-acylated polySia OMVs were found to significantly increase the activation of new or pre-existing memory B cells independent of the presence or absence of T cells (Fig. 4B). Since WT and TCR-KO mice appeared to have similar polySia+ memory B cell activation after immunization with hexa-acylated polySia-OMVs, we wondered if these had undergone class-switching.

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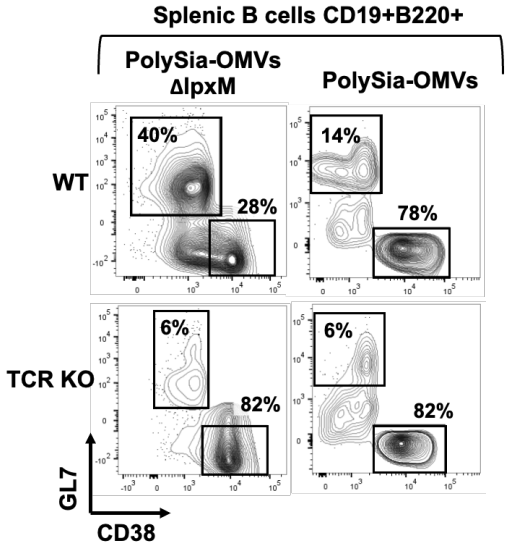

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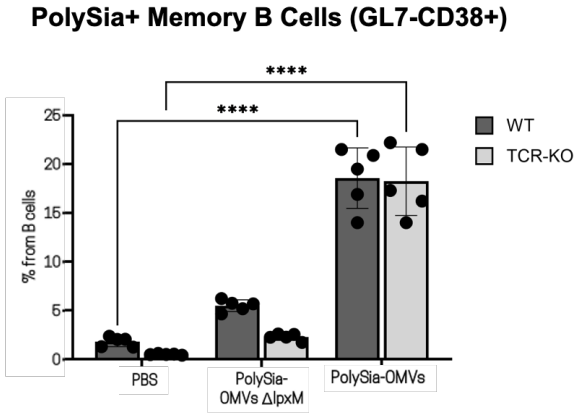

*** p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001**

Figure 4. Flow cytometry analysis of polySia-specific splenic memory B cells. Splenic memory B cells were defined by their lack of GL7 and the presence of CD38. ***(A)*** Representative contour plots of total B cells from the spleen of one mouse from each group. Square gates show the percentage of B cells that have a germinal center (GL7+CD38-) or a memory (GL7-CD38+) phenotype. ***(B)*** Percentage of total memory B cells that recognize polySia from the spleen of five mice per group. Bars represent the statistical mean, error bars the standard deviation, and circles the value for each mouse within the group. Statistical significance was determined for p values < 0.05 using the Turkey-Kramer post hoc honest significant difference (HSD).

Figure 5 shows that these memory cells are class-switched and expand in greater numbers in the presence of T cells, suggesting that the immune response to hexa-acylated OMVs might also contain a T-dependent component. Further experiments are needed to determine why penta-acylated polySia-OMVs fail to activate new or pre-existing memory B cells in the spleen.

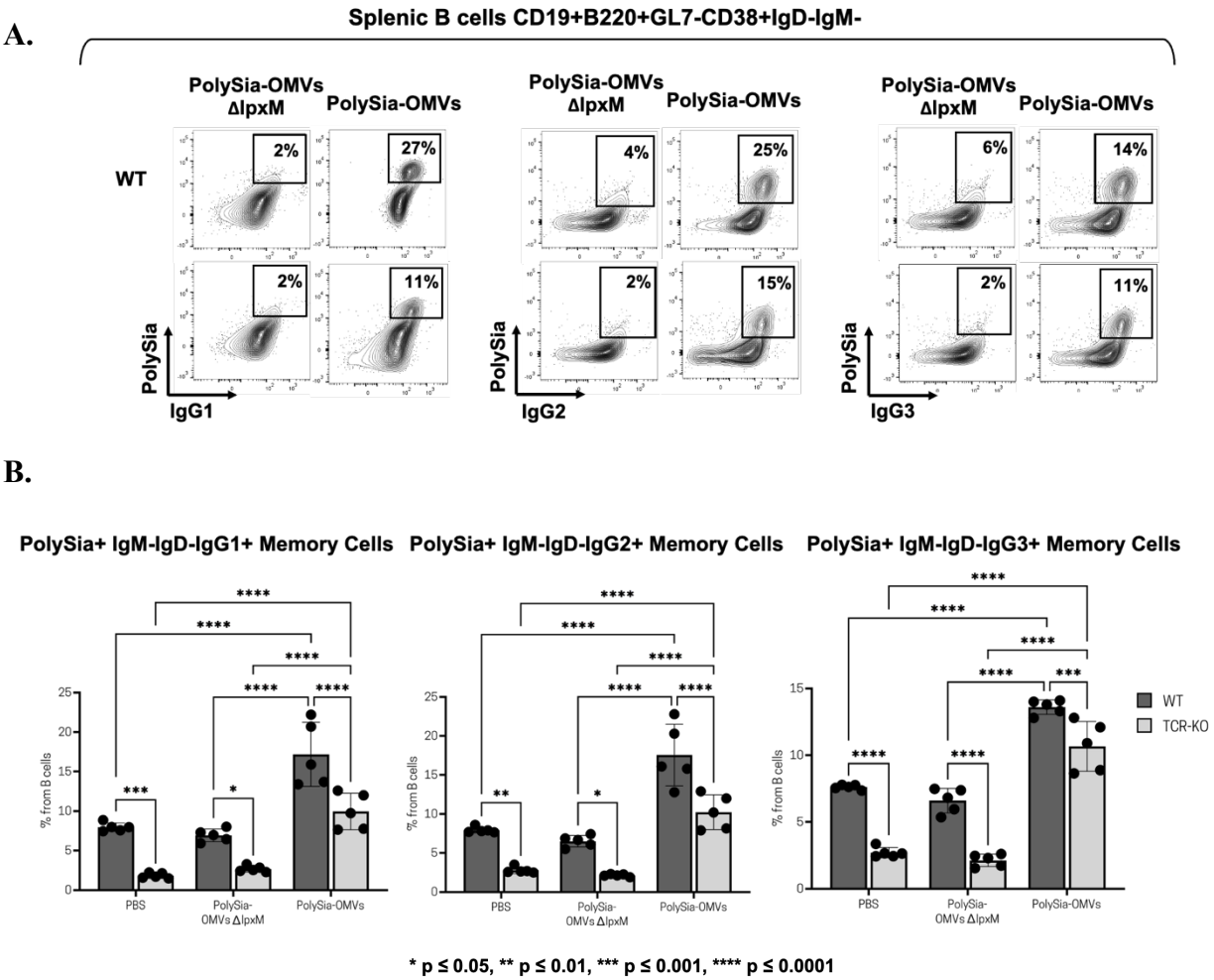

Figure 5. Flow cytometry analysis of splenic class-switched memory B cells. Splenic B cells were analyzed for memory B cell markers GL7- and CD38+, as well as expression of antibody isotypes IgG1, IgG2, and IgG3. ***(A)*** Representative contour plots of IgG1, IgG2, or IgG3 memory B cells from one mouse of each group. Square gates represent the population of memory B cells that express an IgG subtype and bind to polySia. ***(B)*** *P*ercentage of total memory B cells that recognize polySia and are class-switched from the spleen of five mice per group. Bars represent the statistical mean, error bars the standard deviation, and circles the value for each mouse within the group. Statistical significance was determined for p values < 0.05 using the Turkey-Kramer post hoc honest significant difference (HSD).

Peritoneal cavity B2 cells drive a T-dependent immune response to polySia

The peritoneal cavity is a fluid-filled region housing the abdominal organs, as well as a unique collection of leukocytes involved in tissue repair and homeostasis [10]. Peritoneal cavity B cells were defined by expression of CD19 and B220 on their surface (Fig. 6A). For mice immunized with hexa-acylated polySia-OMVs, WT and TCR-KO mice peritoneal cavity B cells were similarly activated, and had significantly greater activation compared to the controls and mice immunized with penta-acylated polySia-OMVs. For mice immunized with penta-acylated polySia-OMVs, WT mice had greater peritoneal cavity B cell activation than TCR-KO mice (Fig. 6B). There was no significant B1 cell activation with respect to the controls in the peritoneal cavity for WT and TCR-KO mice immunized with penta-acylated polySia-OMVs, while both mice groups displayed significant B1 cell activation when immunized with hexa-acylated polySia-OMVs (Fig 6C). A peritoneal cavity B2 cell population was observed to have significant activation in WT mice immunized with penta-acylated polySia-OMVs in comparison to TCR-KO and control mice (Fig. 6D); a trend that resembles what was seen in the IgG blood titers. Since B2 cells typically operate in a T cell-dependent manner [17], this result indicates that this population of peritoneal cavity B2 cells might be the primary source of antibodies in the T-dependent response to penta-acylated polySia OMVs.

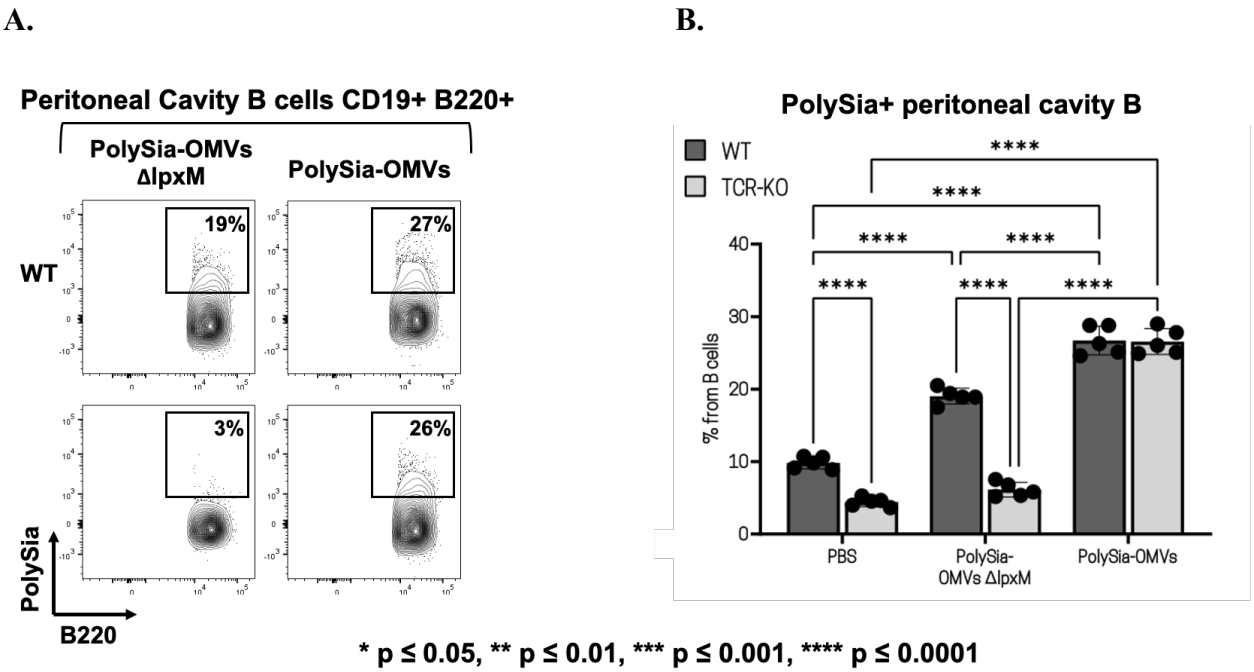

Figure 6. Flow cytometry analysis for peritoneal cavity B cells. ***(A)*** Representative contour plots of total B cells from the peritoneal cavity of one mouse from each group. Square gates represent the population of B cells that recognize polySia. ***(B)*** Percentage of total B cells that recognize polySia from the spleen of five mice per group. ***(C)*** B cells that recognize polySia are further characterized by their phenotype as B1 or ***(D)*** B2 cells. Bars represent the statistical mean, error bars the standard deviation, and circles the value for each mouse within the group. Statistical significance was determined for p values < 0.05 using the Turkey-Kramer post hoc honest significant difference (HSD).

Class-switched polySia-specific B cells were also discovered in the peritoneal cavity (Fig. 7). There was no significant activation of polySia-specific, class-switched B cells in the peritoneal cavities of TCR-KO mice independent of the structure of lipid A in the OMVs, suggesting that there is a primarily T-dependent response to polySia driven by B2 cells. In particular, there was a significantly increased presence of polySia-specific B cells expressing IgG2 in WT mice immunized with penta-acylated polySia-OMVs, which may account for previously reported IgG titers in these mice. This elevation is of interest because IgG2 is of particular importance in the adaptive immune response to bacterial capsular polysaccharide antigens [22].

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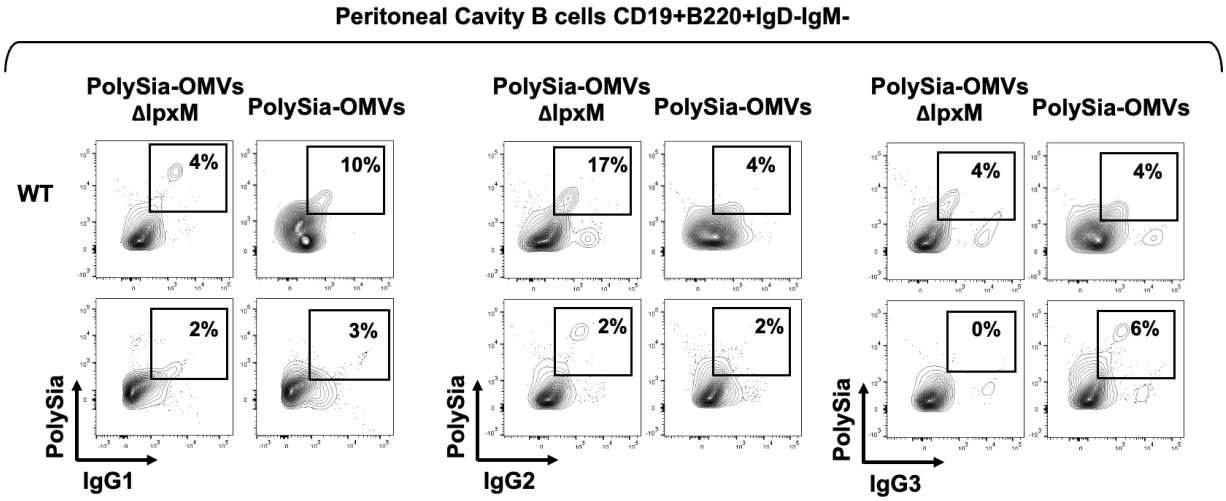

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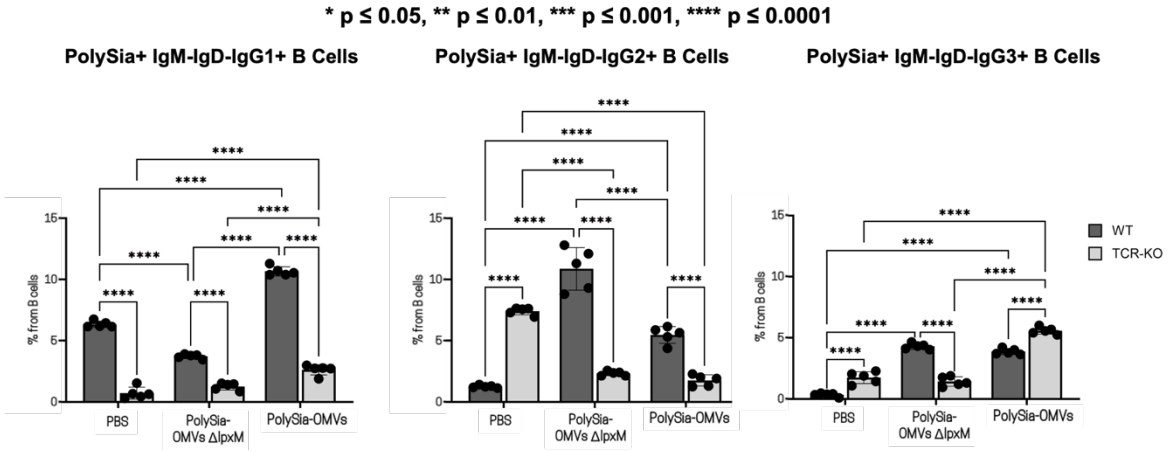

Figure 7. Flow cytometry analysis of peritoneal cavity class-switched B cells. Peritoneal cavity B cell populations of WT and TCR-KO mice were analyzed for expression of B cell markers CD19 and B220, as well as their binding to polySia and isotypes IgG1, IgG2, and IgG3. ***(A)*** Representative contour plots of IgG1, IgG2, or IgG3 memory B cells from one mouse of each group. Square gates represent the population of memory B cells that express an IgG subtype and bind to polySia. ***(B)*** Percentage of total memory B cells that recognize polySia and are class-switched from the peritoneal cavity of five mice per group. Bars represent the statistical mean, error bars the standard deviation, and circles the value for each mouse within the group. Statistical significance was determined for p values < 0.05 using the Turkey-Kramer post hoc honest significant difference (HSD).

DISCUSSION

In this study, we investigated the immune response of WT and T cell-deficient mice to polySia-OMVs with either a penta- or hexa-acylated lipid A structure. The results demonstrate that immunization with both penta- and hexa-acylated polySia-OMVs produces glycan-specific IgGs through different immune mechanisms. Penta-acylated polySia-OMVs induced a T-dependent response, primarily driven by B2 cells that produce IgG2 in the peritoneal cavity, while hexa-acylated polySia-OMVs induced a T-independent response, primarily driven by B1 cells in both the spleen and peritoneal cavity. Penta-acylated polySia-OMVs did not result in significant activation of glycan-specific memory B cells, while the immune response to hexa-acylated polySia-OMVs was characterized by expansion of a class-switched memory B cell population in the spleen.

Since B-1 cells are predominantly abundant in the peritoneal cavity [8], from where they can expand and produce antibodies within the innate immune response, it is interesting to observe a population of splenic B-1 cells contributing to a T-independent adaptive immune response after immunization with hexa-acylated polySia-OMVs. It is possible that since hexa-acylated polySia-OMVs generate a stronger innate immune response due to the high affinity of native lipid A with

murine TLR4, that this innate inflammatory signaling contributes to splenic B1 cell activation and to the migration of non-resident B1 cells to the spleen for class-switching. In fact, Ha et al. [4] demonstrated that the engagement of TLR4 in B1 cells with bacterial molecules can release B1 cells from the peritoneum and drive their migration to the spleen where they can contribute to the adaptive immune response. This accumulation of IgG producing B1 cells has also been observed in the onset of systemic lupus erythematosus (SLE), an autoimmune response characterized by the presence of abundant autoreactive antibodies. Enghard et al. [3] showed that a population of autoreactive B1a cells from the peritoneal cavity, and not splenic B1b cells, accumulates in the spleen during the onset of SLE, where they class-switch into IgG producing cells. Given that polySia is a self-antigen in mice [13], it is possible that autoreactive B1a cells specific to polySia persist in the peritoneum, but only migrate and class-switch in the spleen in the presence of inflammatory signals.

B-2 cells also make up a relatively small proportion of the peritoneal cavity B cell population, and the results indicate a strong dependence on T cells for their activation after immunization with penta-acylated polySia-OMVs. Moreover, splenic B2 cells, which are traditionally engaged in T-dependent immune responses, seem to not contribute to the response to polySia. Since the peritoneal cavity does not have germinal centers where B cells can engage with T cells for activation [20], it is likely that these T-dependent B-2 cells are not activated in a conventional manner. One possibility is that a population of circulating B2 cells is driven to milky spots in the omentum by recognition by β7 of MAd-CAM1, from which they can circulate in and out to the peritoneal cavity [1]. Although milky spots do not work as traditional secondary lymphoid tissues, recent studies have shown that B2 cells in milky spots undergo similar reactions as those

in lymph nodes, including T cell activation and isotype switching [10, 18]. However, it is not clear what causes the potential upregulation of β7 in these B2 cells, or what drives the activation to occur in the omentum rather than the spleen, especially given that the immunization was done subcutaneously and not intraperitoneally.

Overall, the results of this study show that comparable levels of anti-glycan IgGs in blood after immunization with glycOMVs can be reached through more than one mechanism. It also demonstrates that, in the immune response to polySia, the strength of the innate immune response drives different populations of B cells for activation and class-switching in distinct tissues. The migration and activation of B1 cells in the spleen after immunization with hexa-acylated OMVs in a T-cell independent manner supports the findings that this population of B cells have extensive roles in the immune response to glycans beyond the production of natural antibodies. Their absence in the immune response to polySia after immunization with penta-acylated OMVs indicates that the idiotype of these B1 cells is likely autoreactive, and that high levels of pro-inflammatory signals are required to break tolerance in the peritoneum. In addition, although the activation of B2 cells in response to T-dependent antigens in milky spots has been previously observed, it has only been reported after either intraperitoneal immunization of a T-dependent antigen, or after removal of other lymphoid tissues that support T-dependent activations (e.g. spleen). Our study shows a T-dependent activation of B2 cells in the peritoneum without the presence of a T-dependent antigen or intraperitoneal immunization, and without the alteration of other lymph nodes. Thus, there must be other unknown signals that drive B2 cells for activation and class-switching in the omentum rather than the spleen, and that could be overshadowed or non-existent during high levels of inflammation.

MATERIALS AND METHODS

Antibodies

Antibodies against GL7 (GL7), CD38 (90), IgM (RMM-1), and IgD (11-26c.2a) were purchased from Biolegend. Antibodies against IgG1 (MOPC-21), IgG2a/b (R2-40), and IgG3 (R2-38) were purchased from BD Biosciences. Antibodies against TCRa/b (H57-597), CD45R (B220 - RA3-6B2), CD11b (M1/70), CD23 (B3B4), CD5 (53-7.3), and biotin were purchased from ThermoFisher Scientific.

Preparation of OMVs

To prepare OMVs for immunization, *E. coli* cells of strain JC8031 or JC8033 containing the plasmids pNeuD and pLCBS were grown in 200 mL of Luria-Bertani (LB) medium at 37°C with shaking at 250 rpm. The culture density (OD_{600nm}) was allowed to reach 0.3, at which time glycan expression was induced by adding 0.2% arabinose and 0.1M IPTG. Cells were then harvested after an additional incubation for 6 hours at 37°C with shaking at 250 rpm. The cultures were centrifuged at 8,000 rpm for 15 minutes at 4°C to separate OMVs from the *E. coli* cells. Cell debris was removed by flowing the supernatant through a 0.2 μm sterile filter into an autoclaved bottle. The supernatant was further centrifuged at 28,000 rpm for 3 hours at 4°C for final isolation of OMVs. Collected OMVs were resuspended in 600 μL of 1X PBS, filtered through a Millex-HV syringe filter unit (0.45μm), and finally stored in 150 μL aliquots at -20°C. OMV concentration was measured through total protein assay using the QuantiPro BCA assay kit (Sigma-Aldrich, USA).

Mouse Immunizations

All animal studies were approved by the Institutional Animal Care and Use Committee at Cornell University under the protocol 2012-0132. To measure the T-dependence of the immune response to glycOMVs, we used mice that are genetically deficient in T-cells. These mice have a knockout for the gene TCRa, resulting in depletion of all T cells except gamma T cells. We used 60 adult female mice, with 30 having the TCRa knockout and the other 30 being wild type C57BL/6 mice.

Each of these two groups were further divided into subgroups based on the immunization strategy, resulting in 10 groups as described below, with each group having 6 mice. Five groups were C57BL/6, and the other 5 groups TCRa -/-. In addition to varying the presence or absence of glycosylation on the OMVs, we also varied the structure of lipid A on the OMVs to be either penta- or hexa-acylated. Each group was immunized using a 27G syringe, subcutaneously and over the shoulders with either 100 µL Phosphate Buffer Saline (PBS), 100 µL containing 10 µg of penta-acylated OMVs, 100 µL containing 10 µg of hexa-acylated OMVs, 100 µL containing 10 µg of penta-acylated OMVs displaying polysialic acid, or 100 µL containing 10 µg ofhexa-acylated OMVs displaying polysialic acid. Each mouse was boosted with a similar dose 28 and 42 days after the initial immunization.

Blood Draws

Blood was collected from each mouse from the mandibular sinus immediately before and 14 days after the first immunization, and immediately before and 7 days after each boost. After every blood draw procedure, the blood was allowed to coagulate and was centrifuged at 5,000

rpm for 10 minutes to separate red blood cells from blood sera. The trial was stopped 56 days after the first immunization, when mice were euthanized by CO_2 and splenectomies, B and T cell isolations, and blood draws from cardiac punctures were conducted.

Lymphocyte Collection and Cell Sorting

Immediately after euthanasia, lymphocytes from the peritoneal cavity were isolated following the protocol described by Ray and Dittel [19]. In brief, a 27G needle was used to inject 5 mL of cold 1X PBS with 3% fetal bovine serum (FBS) into the peritoneal cavity. The peritoneum was then massaged to release lymphocytes and a 25G needle was used to collect the PBS solution. The solution was centrifuged at 400 rcf for 8 minutes. Cells were resuspended in PBS for cell labeling. Samples did not contain any visible blood or fat contaminations.

Single-cell suspensions were prepared from the spleens through mechanical disruption and passage through a 100 μm cell strainer. Cells were centrifuged at 400 rcf and 4°C for 10 minutes. The cells were resuspended in RBC lysing buffer and incubated at room temperature for 5 minutes. Lysed RBC were removed by centrifugation at 400 rcf and 4°C for 10 minutes. Cells were resuspended in PBS for cell labeling.

The EasySep™ Mouse Pan-B Isolation Kit (StemCell, USA) was used for B cell enrichment following the instructions from the manufacturer. Cells were stained on ice for 30 minutes with the appropriate combinations of fluorophore-conjugated antibodies and polySia in 5% BSA in PBS. After five washes with 5% BSA in PBS, stained cells were recorded on a BD

FACSMelody™ (BD Biosciences, USA) and analyzed using the FlowJo software (Tree Star, USA). In all cases, doublets were excluded.

Blood Sera Enzyme-Linked Immunosorbent Assay

At the end of the trial, the blood sera were then used to analyze the presence of IgG, IgM, IgA and IgE specific to polysialic acid via indirect ELISA. Serum antibody titers were determined by measuring the lowest serial dilution that produced a signal at least three standard deviations above the background.

Statistical Analysis

Statistical significance of differences between groups was determined using Turkey-Kramer post hoc honest significant difference (HSD), student's *t* test (unpaired), or two-way ANOVA. A p value <0.05 was considered to be statistically significant for all tests. Data was plotted using GraphPad Prism 4.0.

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APPENDIX

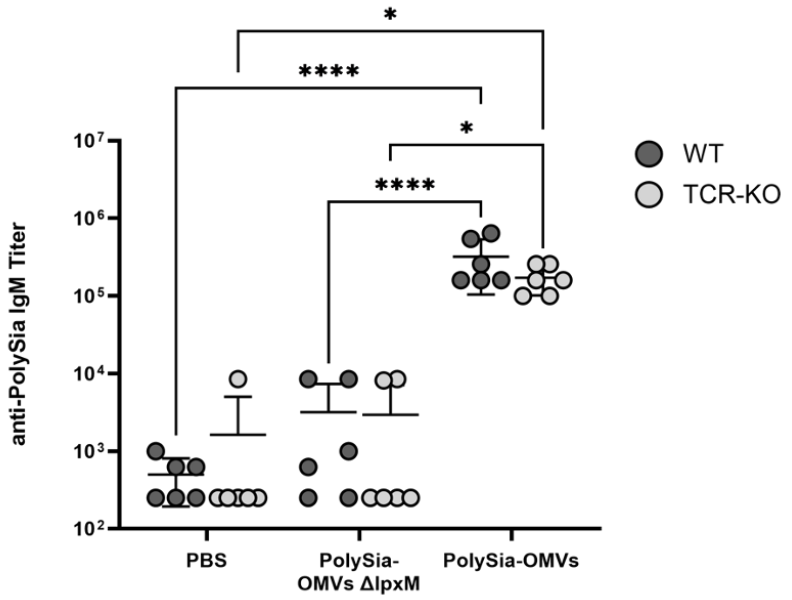

Figure S1. For WT mice immunized with hexa-acylated polySia-OMVs, a significantly greater IgM response was mounted when compared to penta-acylated polySia-OMVs. There was no significant difference between WT and TCR-KO for either immunization group. Asterisks are used to indicate statistical significance in an unpaired t-test, with four asterisks (****) indicating statistical significance of $p < 0.0001$. WT and TCR-KO mice immunized with hexa-acylated polySia-OMVs had similar IgM titers at the end of the trial, which could indicate that these antibodies arose from B-1 cells which often activate in a T-independent manner [17]. Higher IgM titers for mice immunized with hexa-acylated polySia-OMVs indicate a stronger innate immune response compared to mice immunized with penta-acylated polySia-OMVs.