Spring 4-27-2018

Factors Involved In Suppression of Human Vγ9Vδ2 T-Lymphocytes And Impact of Checkpoint Blockades On The Effector Functions of Vγ9Vδ2 T-Lymphocytes

MinJi Choi
minji.choi@uconn.edu

Andrew Wiemer
School of Pharmacy, andrew.wiemer@uconn.edu

Andrea Hubbard
School of Pharmacy, andrea.hubbard@uconn.edu

Brian Aneskievich
School of Pharmacy, brian.aneskievich@uconn.edu

UNIVERSITY SCHOLAR PROJECT

HONORS THESIS

FACTORS INVOLVED IN SUPPRESSION OF HUMAN Vγ9Vδ2 T-LYMPHOCYTES AND IMPACT OF CHECKPOINT BLOCKADES ON THE EFFECTOR FUNCTIONS OF Vγ9Vδ2 T-LYMPHOCYTES

By:

MinJi Choi

University of Connecticut

School of Pharmacy

2018

Chair and Major Project Advisor: Dr. Andrew Wiemer, Department of Pharmaceutical Sciences

Advisor: Dr. Andrea Hubbard, Department of Pharmaceutical Sciences

Advisor: Dr. Brian Aneskievich, Department of Pharmaceutical Sciences

Honors Advisor: Dr. Brian Aneskievich, Department of Pharmaceutical Sciences

Abstract

Chronic antigenic stimulation leads to T cell exhaustion, which drastically dampens T cell effector functions. Upon exhaustion, T cells progressively lose their proliferative capacity, cytokine production, and cytotoxic effector functions against tumors. T cell exhaustion involves programmed cell death-1 (PD-1), which is an inhibitory co-stimulation receptor. Limited scope of studies has been done regarding impact of PD-1 expression on the effector functions of γδ T cells. In this study, we investigated expression of PD-1 and its impact on effector functions of human γδ T cells. When γδ T cells were stimulated with phosphoantigens (HMBPP and POM_2-C-HMBP) and analyzed using flow cytometry, PD-1 expression peaked on day 5 and showed gradual decline thereafter. Treatment of γδ T cells with IL-2 without phosphoantigen stimulation can induce steady and continuous increase in PD-1 expression throughout the course of experiment. These results suggest that PD-1 may be expressed on highly primed γδ T cells. Upon concurrent dose response and time course experiments over 5-day period, we found that 10 μM POM_2-C-HMBP treatment induced the maximum PD-1 expression on day 5. To demonstrate effect of anti-PD-1 monoclonal antibody on effector function of γδ T cells against tumor cells (K562), we quantified γδ T cells' IFN-γ release by using ELISA and measured the absorbance. Contrary to our initial hypothesis, addition of anti-PD-1 monoclonal antibody did not appear to improve the effector function of γδ T cells, as difference in IFN-γ release among different treatment groups was not statistically significant. However, we found that increase in concentration of phosphoantigens increased effector functions of γδ T cells as demonstrated through increasing IFN-γ release.

Introduction

Detection of and response to antigens is a central function of the immune system. T cells are key to these processes – their T cell receptors directly interact with antigens and/or their accessory molecules, and subsequent T cell receptor signaling initiates an immune response to the antigen. There are two types of T cell receptor dimers encoded in the human genome, the αβ (alpha beta) and the γδ (gamma delta) T cell receptors. αβ T cells are recognized as the predominant circulating lymphocytes, whereas γδ (Vγ9Vδ2) T cells compose a minor fraction of circulating lymphocytes found in human peripheral blood. Unlike αβ T cells that contribute to adaptive immune response via antigen-specific effector functions and memory phases, γδ T cells feature combined roles in the immune system [1]. γδ T cells participate in tumor surveillance and bridge two core features of the immune system – conventional innate and adaptive immune responses – with their unique T-cell repertoire.

Recognition of antigens by γδ T cells is TCR-dependent but HLA-independent; antigen detection through γδ T cell receptors is not restricted to antigenic peptides that are complexed to HLA [2]. This allows interplay of γδ T cells between innate and adaptive immune response and broadens effector functions of γδ T cells in tumor surveillance. HLA-independent antigen recognition is represented by a specific subset of γδ T cell receptors including Vγ9Vδ2, which is the most prominent subset of γδ T cells human adults. As a pattern recognition receptor, Vγ9Vδ2 TCR allows Vγ9Vδ2 T cells to participate in innate-like immune response; Vγ9Vδ2 TCR facilitates recognition of diphosphate antigens derived from various pathogenic microbes, parasites, and tumors through detection of 'molecular patterns' inherent in them. The most potent γδ T cell activators include hydroxymethylbutenyl diphosphate (HMBPP) and isopentenyl diphosphate (IPP), which are intermediates of cellular isoprenoid biosynthesis [3-4].

Accumulation of IPP is detected in dysregulated self-cells and certain tumors, as they may upregulate the mevalonate biosynthetic pathway. Accumulation of intracellular IPP in the tumors enables recognition by and activation of γδ T cells [5-6]. Upon activation, γδ T cells mount wide arrays of effector functions. They can lyse infected or transformed cells through release of cytotoxic perforin and granzymes and activate death-inducing receptor pathways, such as FAS and TNF-related apoptosis-inducing ligand receptors [7-8]. Therefore, in addition to mediating innate arm of immune response, γδ T cells are primed for their roles in adaptive arm of immunity. γδ T cells are highly effective at killing tumor cells and protecting the host via IFN-γ. IFN-γ inhibits angiogenesis and promotes HLA-1 expression by tumor cells, which subsequently enhances CD8+ effector cell responses against tumor [9]. Various interactions between γδ T cells and other effector T cells that mediate innate and adaptive immune responses carry significant therapeutic implications in utilizing γδ T cells in cancer immunotherapy.

One of the drawbacks that underlie cancer immunotherapies includes concept of T cell exhaustion. Exhausted T cells refer to dysfunctional T cells upon chronic antigen stimulation that are not able to further mount effector immune response. Upon exhaustion, T cells progressively lose their proliferative capacity, cytokine production, and subsequently, cytotoxic functions against tumors. T cell exhaustion involves expression of costimulatory markers, or ‘immune checkpoints’, such as PD-1 and BTLA. The elevated expression of immune checkpoints on T cells from cancer patients is often considered markers of exhausted T cells, which cannot mount effective anti-tumor activities [10].

It is particularly important to understand that effector functions of γδ T cells are vastly affected by immune checkpoints, as checkpoint blockades are gaining heavy clinical use in anticancer immunotherapy. Immune checkpoints refer to series of inhibitory pathways wired to T cell responses as part of baseline homeostatic mechanism to maintain peripheral self-

tolerance. Under normal physiological conditions, checkpoints are critical in preventing over-activation of immune system and in protecting subsequent tissue damaging [11]. However, tumors up-regulate immune checkpoint protein ligands as means of immune escape, which gives rise to poorly immunogenic tumor that can readily progress. PD-1 and BTLA are two prominent checkpoint receptor proteins upregulated in γδ T cells following γδ T cell activation [12].

Programmed cell death-1 (PD-1) and its interaction with PDL-1/2 delivers inhibitory signal to TCR signal transduction, which under normal physiology would lead to phosphorylation of ZAP70. PDL-1/2 is often expressed on the surface of tumor cells, as they utilize suppressive signals mediated by various checkpoints as one of survival mechanisms. Phosphorylation of ZAP70 leads to downstream signal cascade that promotes T cell proliferation and cytokine release. Once phosphorylation of ZAP70 is halted upon PD-1 and PDL-1/2 interaction, downstream events that lead to T cell effector functions are subsequently inhibited [13]. PD-1 expression on Vγ9Vδ2 T cells increases in the presence of phosphoantigens and under subverted local tumor microenvironment. Furthermore, it is known that PD-1 activation upon its interaction with ligand PD-L1/2 attenuates effector functions of Vγ9Vδ2 T cells against infectious diseases and tumor [14-15]. However, when PD-1 mediated suppression of TCR signal cascade is blocked by using anti-PD-1 monoclonal antibodies, effector functions of highly primed γδ T cells are enhanced [16]. Therefore, blocking suppressive checkpoint signals by using monoclonal antibodies reverses γδ T cell anergy and exhaustion in tumor environment and promotes continuous survival and proliferation of γδ T cells; by facilitating γδ T cell tumor infiltration and anti-tumor cytokine productions (i.e., IFN-γ), anti-PD-1 monoclonal antibodies targeting γδ T cells will increase chance of tumor rejection and host protection.

Along with PD-1, B and T lymphocyte attenuator (BTLA) is checkpoint receptor protein expressed on γδ T cells that interacts with herpesvirus entry mediator (HVEM). HVEM is often expressed on surface of various tumor cells. BTLA plays important role in limiting T cell activation to maintain baseline homeostasis and promotes maintenance of dendritic cells and memory T cells [17]. Upon activation, BTLA signal inhibits release of IL-17 and TNF by specific subset of γδ T cells. And the susceptibility of γδ T cells to inhibitory signaling of BTLA implies therapeutic utilization of monoclonal antibodies targeting BTLA to competitively inhibit BTLA-HVEM interaction [18].

Use of monoclonal antibodies targeting checkpoint inhibitor proteins has accomplished considerable success in treating solid tumors and hematological malignancies. As a marker of highly activated or exhausted T cells, PD-1 has been shown to result in anti-tumor responses in patients with renal cell carcinoma, melanoma, colorectal cancer, and non-small-cell lung cancer [19-20]. Monoclonal antibodies targeting BTLA+ CD8+ effector T cells has resulted in positive clinical response, suggesting that functional differences may exist between BTLA+ and BTLA- T cells, which ultimately result in differential anti-cancer therapeutic potency in patients [21].

Despite the fact that γδ T cells bridge innate and adaptive immune responses that can possibly broaden cancer therapeutic approach, the majority of checkpoint blockade studies have been exclusively focused on αβ T cells. Furthermore, current literature suggests that Vγ9Vδ2 T cell cancer immunotherapies demonstrate critical drawback in rapid exhaustion. As previously mentioned, Vγ9Vδ2 T cell exhaustion within the tumor microenvironment will attenuate Vγ9Vδ2 T cell's proliferative and effector capacities. However, Vγ9Vδ2 T cell exhaustion can be reversed upon inhibition of suppressive signals mediated by various checkpoint receptor proteins and their interaction with ligands [22]. Therefore, it is important to characterize checkpoints that suppress and attenuate highly functional effector Vγ9Vδ2 T

cells. Understanding of the impact of immune checkpoint interactions on Vγ9Vδ2 T cell signaling and effector functions will contribute to broadened understanding of essential factors that are required for long term survival, proliferation, and sustained activation of Vγ9Vδ2 T cells. This understanding will aid in resolving current drawbacks related to Vγ9Vδ2 T cells in the context of immunotherapies and further suggest implication of Vγ9Vδ2 T cells as promising therapeutic agents in treating cancer.

Materials/Methods

Vγ9Vδ2 T Cell Culture

Human peripheral blood mononuclear cells (hPBMCs) were isolated from blood that was obtained from Research Blood Components (Boston, MA). Cells were frozen in freezing media (10% DMSO, 20% fetal bovine serum [FBS], 70% RPMI media) in liquid nitrogen until needed for experiments. hPBMCs were re-suspended in fresh T cell media (RPMI-1640, 10% heat-inactivated FBS, 1x4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pyruvate, nonessential amino acids, β-mercaptoethanol [BME]) [23]. Cells were added to 12-well plates according to respective conditions (control, HMBPP, and POM_2-C-HMBP). Cells were stimulated with phosphoantigens hydroxymethylbutenyl diphosphate (HMBPP) or POM_2-C-HMBP for 3 days and washed. 15 μL of human IL-2 (5 ng/mL) was supplemented every 3 days. Cells were cultured for another 10 days after phosphoantigen was washed for flow cytometry analysis. Experiments were performed using cells from at least 3 different donors.

POM_2-C-HMBP compound

POM_2-C-HMBP (Figure 1) was synthesized by Dr. David F Wiemer at the University of Iowa and provided to us.

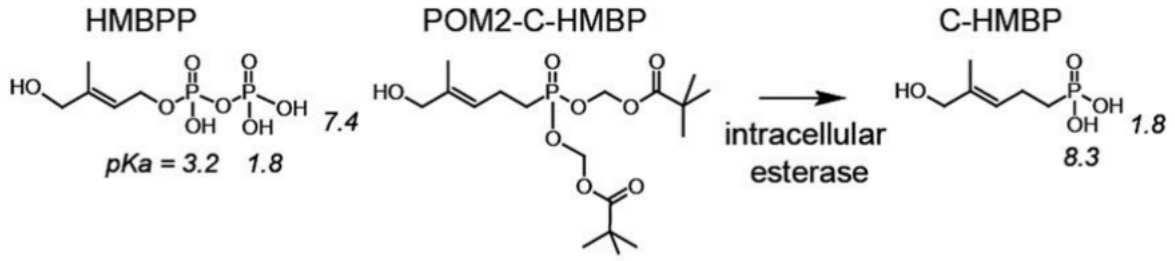

Figure 1. Chemical structures of HMBPP and POM_2-C-HMBP used in this study. The pKa of each acidic hydrogen atom (as determined by Marvin software) is given in italics [24]. Vγ9Vδ2 T cells detect phosphoantigens. Certain malignant tumors and infected cells contain express high levels of HMBPP or IPP, which will activate Vγ9Vδ2 T cells to mount

immune responses. HMBPP is a compound considered to be the most potent naturally Vγ9Vδ2 T cells activator.

Vγ9Vδ2 T Dose Response

Cells were cultured the same way throughout the experiments. 0.1 μM, 1 μM, and 10 μM of HMBPP and POM_2-C-HMBP were prepared and used to treat hPBMC donors. 15 μL of human IL-2 (5 ng/mL) was supplemented every 3 days. Phosphoantigens were washed 3 days after initial stimulation, and cells were cultured for additional 3 days for subsequent flow cytometry analysis. Experiments were performed using cells from at least 3 different donors.

Vγ9Vδ2 T Cell Flow Cytometry

Time course flow cytometry was performed on day 0-5, 7, 9, and 12 using 10 nM phosphoantigen concentration and on day 0-5 for dose-response experiment. Each sample was washed and resuspended in 200 μL of fluorescence-activated cell sorting buffer (2% BSA in PBS). Gamma delta T cell receptor (TCR) was labeled using FITC-anti-human pan gamma delta TCR monoclonal antibody (Thermo Fisher Scientific, Rockford, IL, 5A6.E9) Cells were co-stained with APC-anti-human CD279/PD-1 (BioLegend, San Diego, CA, EH12.2H7). Samples were washed twice and fixed in 16% paraformaldehyde before flow cytometry analysis.

Vγ9Vδ2 T Dose Response and Time Course

Cells were cultured the same way throughout the experiments, and 0.1 μM, 1 μM, and 10 μM of HMBPP and POM_2-C-HMBP were prepared and used to treat hPBMC donors. 15 μL of human IL-2 (5 ng/mL) was supplemented every 3 days. Phosphoantigens were washed 3 days after initial stimulation for all conditions, and cells were stained for

subsequent flow cytometry analysis from day 1 to day 5, according to different conditions. Experiments were performed using cells from at least 3 different donors.

Vγ9Vδ2 T Cell Negative Selection and K562 Pre-loading

hPBMCs were stimulated with 10 μM of HMBPP and were treated with IL-2. Cells were expanded until day 5, as day 5 was found to be the day of maximum PD-1 expression from the previous dose response experiment. Phosphoantigens were washed on day 3 for 3 times, and hPBMCs were resuspended in T cell media with IL-2 treatment.

In order to ensure that we examine the effector function of Vγ9Vδ2 T cells among hPBMCs, we used negative selection method to purify Vγ9Vδ2 T cells. Negative selection allows isolation of Vγ9Vδ2 T cells by magnetically labeling non-Vγ9Vδ2 T cells and retaining them on a MACS Column, while purified T cells pass through the column. During K562 pre-load and incubation period, T cell negative selection was done by using Miltenyi Biotec Human TCRγ/δ+ Cell Isolation Kit.

Final concentration of 200,000 K562 cells/mL was counted and used for pre-loading K562 target cells. K562 cells were re-suspended in 200 μL of T cell media and were treated with 0.1 μM, 1 μM, and 10 μM of HMBPP and POM_2-C-HMBP in 1mL tube according to conditions. K562 cells pre-loaded with phosphoantigens were incubated at 37 degrees for 2 hours, washed 3 times afterwards, and re-suspended in 200 μL of T cell media. Experiments were performed using cells from at least 3 different donors.

Vγ9Vδ2 T Cell and K562 Co-incubation and IFN-γ ELISA

A Final concentration of 200,000 cells/mL for pre-loaded K562 cells and 100,000 cells/mL for purified T cells were counted and mixed in a 96 well plate for ELISA. 20 μL of pre-loaded K562 cells and 120 μL of T cells were mixed for co-incubation, and LEAF Purified anti-human PD-1 monoclonal antibody (BioLegend, San Diego, CA, EH12.2H7)

was added to the desired wells. Wells were brought up to final volume of 200 μL with T cell media and incubated for 24 hours at 37 degrees. Supernatant on 96-well plate was used for ELISA experiment using BioLegend Human IFN-γ ELISA Max Deluxe Set, and ELISA was performed as duplicate. Experiments were performed using cells from at least 3 different donors.

Results

Stimulation of PBMCs with phosphoantigens increases populations of Vγ9Vδ2 T cells

We first wanted to confirm that phosphoantigens expand Vγ9Vδ2 T cell population over time, so we stimulated hPBMCs with 100 nM of HMBPP and analyzed % of γδ TCR expression using flow cytometry (Figure 1). hPBMCs were stimulated with 100 nM of HMBPP for 3 days, and cells were treated with IL-2 every 3 days for total of 12 days. Subsequently, cells were stained with γδ TCR and were analyzed by flow cytometry from day 1 to day 12. Upon stimulation with 100 nM HMBPP, Vγ9Vδ2 T cell population gradually increased until day 12 (55.1%) compared to day 1 (10.97%).

Stimulation of Vγ9Vδ2 T cells with phosphoantigens increases PD-1 expression

After determining that the phosphoantigens expanded Vγ9Vδ2 T cell population, the next goal was to examine effect of the phosphoantigen stimulation on Vγ9Vδ2 T cell PD-1 expression. To investigate this, we stimulated hPBMCs with 100 nM of HMBPP and POM_2-C-HMBP for 3 days with addition of IL-2 every 3 days (Figure 2-3). Negative control Vγ9Vδ2 T cells were only treated with IL-2 without phosphoantigen stimulation (Figure 4). Cells were co-stained with antibodies towards the γδ TCR and/or PD-1 and were analyzed by flow cytometry from day 0 to day 12. Upon stimulation, 14% of POM_2-C-HMBP and HMBPP treated Vγ9Vδ2 T cells expressed PD-1 as early as day 1 (24 hours after stimulation), and 15% of unstimulated control expressed PD-1 on day 1. PD-1 expression peaked on day 5 in both POM_2-C-HMBP and HMBPP treated Vγ9Vδ2 T cells (23% and 30%, respectively), and the level of PD-1 expression gradually declined thereafter, as proportion of Vγ9Vδ2 T cells increased; nevertheless, PD-1 expression on unstimulated Vγ9Vδ2 control continued to increase until day 12 without showing decline, as 37% of unstimulated Vγ9Vδ2 T cells expressed PD-1 on day 12 compared to 16% on day 1.

Stimulation of Vγ9Vδ2 T cells with 10 μM of POM_2-C-HMBP for 5 days induces maximum PD-1 expression

Previous time course experiment demonstrated that PD-1 expression peaks on day 5 with phosphoantigen treatments. Prior to investigating the effect of anti-PD-1 monoclonal antibodies on the effector function of Vγ9Vδ2 T cells, our goal was to determine the optimal phosphoantigen concentration that induces maximum Vγ9Vδ2 T cell PD-1 expression. To examine this, we concurrently performed dose response and time course experiments over 5-day period (Figure 5).

We stimulated hPBMCs with 0.1 μM, 1 μM, and 10 μM of HMBPP and POM_2-C-HMBP for 3 days with addition of IL-2 every 3 days. Negative control Vγ9Vδ2 T cells were only treated with IL-2 without phosphoantigen stimulation. Cells were co-stained with antibodies towards the γδ TCR and/or PD-1 and were analyzed by flow cytometry from day 1 to day 5. As expected, maximum PD-1 expression was seen on day 5 in all conditions except condition treated with 0.1 μM HMBPP (23.3%). Maximum PD-1 expression was seen in condition treated with 10 μM POM_2-C-HMBP, as 41.55% of 10 μM POM_2-C-HMBP treated cells expressed PD-1 at day 5. As noted in previous dose response experiment, PD-1 expression on unstimulated Vγ9Vδ2 control continued to increase and peaked on day 5, as 19.3% of unstimulated Vγ9Vδ2 T cells expressed PD-1 on day 5 compared to 6.675% of unstimulated Vγ9Vδ2 T cells on day 1.

Higher concentration of phosphoantigens increase release of IFN-γ from Vγ9Vδ2 T cells upon co-incubation with K562, while addition of PD-1 mAb decreases release of IFN-γ.

Previous research conducted by Koca showed that K562 blasts are multipotential, hematopoietic malignant cells that spontaneously differentiate into progenitors of the erythrocyte, granulocyte, and monocytic series [25]. DM Benson performed flow cytometry

and verified that K562 cells express PD-L1, which would down-regulate effector functions of T cells in tumor microenvironment [26].

Once we determined that maximum PD-1 expression was seen on day 5 and that 10 μM POM_2-C-HMBP induced the highest expression among other treatments, we next wanted to determine the effect of anti-PD-1 monoclonal antibody on effector function of Vγ9Vδ2 T cells against tumor cells (K562). To examine this, we quantified Vγ9Vδ2 T cells' IFN-γ release by using ELISA. PBMCs were stimulated for 3 days with phosphoantigens and on day 5 were purified using T-cell negative selection. Purified T cells were subsequently co-incubated with K562 cells that express PD-L1/2. Release of IFN-γ and K562 expression of PD-L1/2 were determined by ELISA and flow cytometry, respectively (Figure 6).

We observed that Vγ9Vδ2 T cells treated with higher concentration of phosphoantigen led to higher absorbance reading, as cells released higher amount of IFN-γ. In negative control samples where cells were only treated with IL-2 and anti-PD-1 monoclonal antibodies, mean absorbance reading was 108, 113, 100, and 125 for cells treated with HMBPP, HMBPP and anti-PD-1, POM_2-C-HMBP, and POM_2-C-HMBP and anti-PD-1, respectively. Compared to negative control, at 10 μM concentration, mean absorbance readings were 298, 271, 252, and 274 for cells treated with HMBPP, HMBPP and anti-PD-1, POM_2-C-HMBP, and POM_2-C-HMBP and anti-PD-1, respectively.

Discussion

PD-1 expression peaks in Vγ9Vδ2 T cells on day 5 following stimulation with phosphoantigens

Although the function of checkpoint inhibitors and PD-1 in αβ T cells has been extensively studied, little is known about roles of PD-1 in Vγ9Vδ2 T cells and their possible impacts on therapeutic utilization. A previous study demonstrated that human Vγ9Vδ2 T cells expressed PD-1 upon stimulation with phosphoantigens (HMBPP) with maximum expression on day 3, and gradual decline thereafter [14].

In this study, we found that HMBPP and POM_2-C-HMBP stimulations at subsequently lower concentration compared to previously used concentration (10 μM) [14] can induce Vγ9Vδ2 T cells to express PD-1, which reached maximum levels within 5 days. PD-1 expression showed steady decline thereafter. We also revealed that treatment of Vγ9Vδ2 T cells with IL-2 alone can cause Vγ9Vδ2 T cells to express PD-1 with steady increase until day 12 without decline in expression. Vγ9Vδ2 T cells require IL-2 for clonal expansion, and IL-2 treatment was continued throughout the course of experiment. Therefore, it is possible that PD-1 was continuously expressed on highly primed Vγ9Vδ2 T cells without decline, as a cellular mechanism to avoid over-activation of Vγ9Vδ2 T cells, in which it would lead to potential auto-immunity in physiologic function.

There does not appear to be difference between HMBPP and POM_2-C-HMBP in terms of maximum effect on PD-1 expression

We conducted the flow cytometry to determine percentage of PD-1 expression in Vγ9Vδ2 T cells using two different types of phosphoantigens, HMBPP and POM_2-C-HMBP, at the same concentration of 100 nM (Figure 2-3). On day 12, 17% of Vγ9Vδ2 T cells treated with HMBPP 100 nM expressed PD-1, and 18% of Vγ9Vδ2 T cells treated with POM_2-C-HMBP 100 nM. This could indicate that Vγ9Vδ2 T cells respond to phosphoantigens with

shared structural moiety in a similar way in terms of surface checkpoint protein expression. Possible limitation to these experiments with phosphoantigens is that the molecules share similar structural moiety, and we did not test multiple phosphoantigens that may prime Vγ9Vδ2 T cells to vastly different extent. Also, underlying mechanism by which the phosphoantigens induce PD-1 expression in Vγ9Vδ2 T cells is not entirely known, which

There does not appear to be dose dependency in PD-1 expression on Vγ9Vδ2 T cells in time course experiment

We conducted concurrent dose response and time course experiment to determine percentage of PD-1 expression in Vγ9Vδ2 at different concentrations of HMBPP over the course of 5 days. Although result from ELISA experiment showed statistically significant dose dependency in IFN-γ release, PD-1 expression did not differ significantly among different treatments. There is a limitation in the design of our study, as the doses that we chose for this experiment might not have been in the right range to observed a clear dose response effect. Future experiment with doses of each compound on the lower range may clarify the relationship between dose of phosphoantigens and PD-1 expression in Vγ9Vδ2 T cells.

Blocking PD-1 with anti-PD-1 monoclonal antibody does not appear to increase IFN-γ production by Vγ9Vδ2 T cells

IFN-γ release was more closely related to the concentrations of treatment rather than to the addition of PD-1 antibodies, as IFN-γ release increased and peaked with 1 μM of HMBPP (304.33) and POM_2-C-HMBP (216). Our initial hypothesis was that adding anti-PD-1 monoclonal antibody would increase IFN-γ release by counteracting the known role of checkpoint protein PD-1. However, it is difficult to determine why the effector function slightly decreased with the addition of anti-PD-1 monoclonal antibody, although difference was not statistically significant.

A major limitation to this experiment is that our experimental model may not perfectly represent tumor microenvironment, in which immune effector cells' anti-tumor functions are downregulated largely due to complex tumor-derived signals [27]. There are multiple signaling pathways involved in down-regulation of anti-tumor functions of these immune effector cells, and our negative selection purification method may have eliminated potentially significant immune cell interactions that could affect PD-1 and PD-L1/2 activities among human PBMCs. Another limitation could be that the experiment was done using 3 different donors, which may show variability in response to treatments with phosphoantigens and anti-PD-1 monoclonal antibody.

Future directions and significance

Through the methods and experiments described so far, we have been able to confirm that both HMBPP and POM_2-C-HMBP induce PD-1 expression in Vγ9Vδ2 T cells. There does not seem to be dose-dependency in PD-1 expression, as there was no statistically significant difference in percentage of PD-1 expression induced by both types of phosphoantigens. However, higher concentration of phosphoantigens led to higher release of IFN-γ from Vγ9Vδ2 T cells, as indicated by an ELISA experiment. This indicates that PD-1is expressed in activated Vγ9Vδ2 T cells primed by structurally similar types of phosphoantigens, and effector function of Vγ9Vδ2 T cells depends on the activation. We have investigated the potential synergistic effect of anti-PD-1 monoclonal antibodies on the effector function of Vγ9Vδ2 T cells, but so far have been unsuccessful in determining its clear role. In fact, results indicated decrease in IFN-γ release from Vγ9Vδ2 T cells treated with anti-PD-1 monoclonal antibody, but differences are not statistically significant. Recent study done by Zumwalde demonstrated that Vγ9Vδ2 T cells transferred from human PBMCs exhibit potent antitumor effector functions even in the absence of checkpoint inhibitor

treatment [28], which might suggest different roles of PD-1 in Vγ9Vδ2 T cells compared to those in αβ T cells and thus warrants further investigation in exact roles and mechanisms of PD-1 in Vγ9Vδ2 T cells.

Further investigation into the exact roles and mechanism of PD-1 in Vγ9Vδ2 T cells may help to identify different pathways that should be utilized for Vγ9Vδ2 T cells as potential adjuvant or immunotherapeutic agent. Once we can identify the exact role and mechanism by which PD-1 plays in Vγ9Vδ2 T cells and clarify the difference in roles of PD-1 in different types of T cells, we can try to utilize Vγ9Vδ2 T cells by targeting unique mechanisms by which PD-1 works in Vγ9Vδ2 T cells. Future studies into tumor cell lines that extensively express PD-L1/2 and are resistant to current immunotherapeutic agents may also be beneficial for development of future therapeutic modalities. For example, checkpoint inhibitors have been extensively utilized in the field of oncology, yet there have not been studies done to examine specific types of tumors that are resistant to immunotherapy and the mechanisms they escape the targeted therapeutic agents. Therefore, the research conducted thus far is the beginning of work that could enhance novel immunotherapeutics for oncology, and potentially other auto-immune disorders as well.

Figures

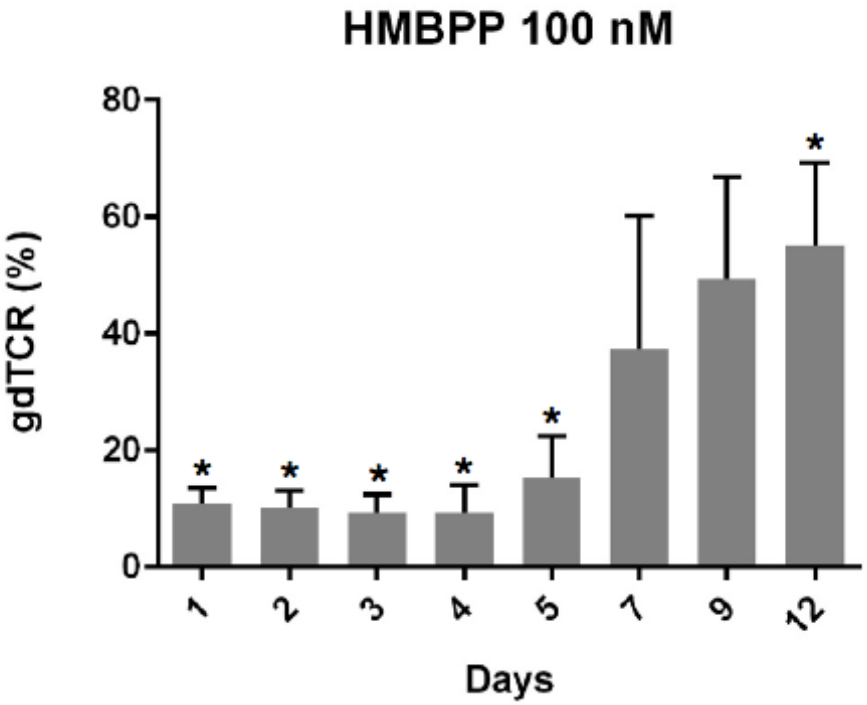

Figure 1. Expression of the γδ TCR following 100 nM HMBPP stimulation. PBMCs were stimulated for 3 days with 100 nM HMBPP. The percentage of cells expressing the γδ TCR was determined by flow cytometry. Bars represent mean values and error bars represent standard deviations of three independent experiments. *$p < 0.05$ by one way ANOVA. Bars are compared to the different days (column).

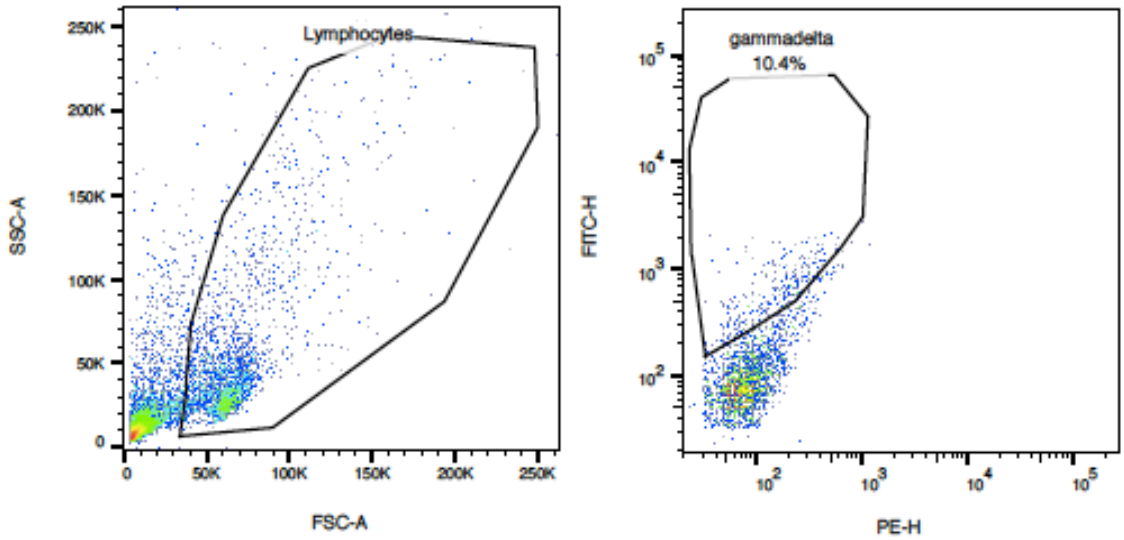

Figure 1-A. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 1. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 10.4% of T cells out of entire donor 11 PBMCs expressed γδ TCR on day 1 following 100 nM HMBPP stimulation.

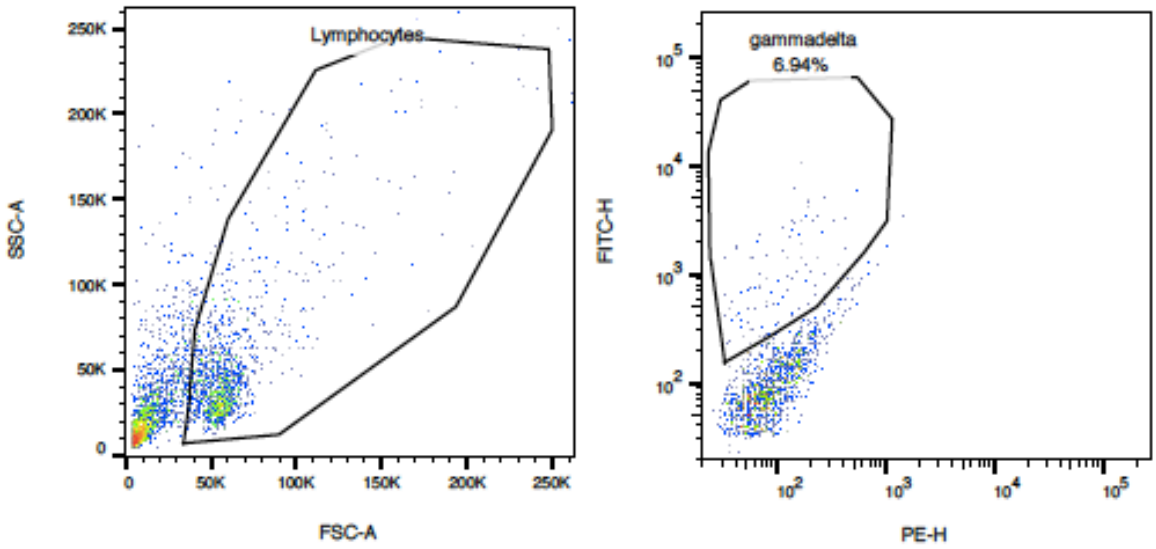

Figure 1-B. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 5. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 6.94% of T cells out of entire donor 11 PBMCs expressed γδ TCR on day 5 following 100 nM HMBPP stimulation.

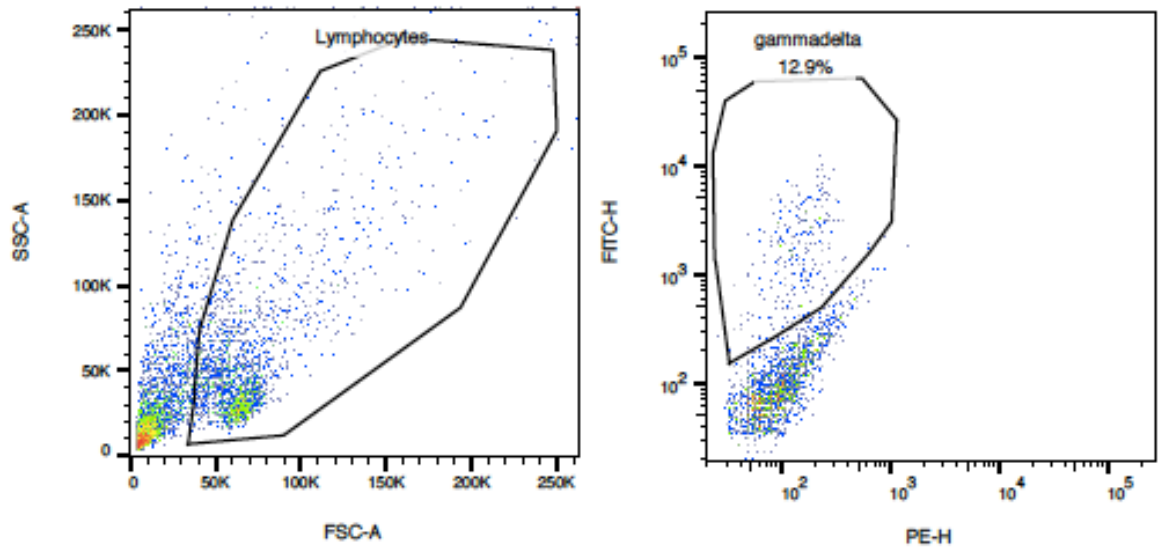

Figure 1-C. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 7. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 12.9% of T cells out of entire donor 11 PBMCs expressed γδ TCR on day 7 following 100 nM HMBPP stimulation.

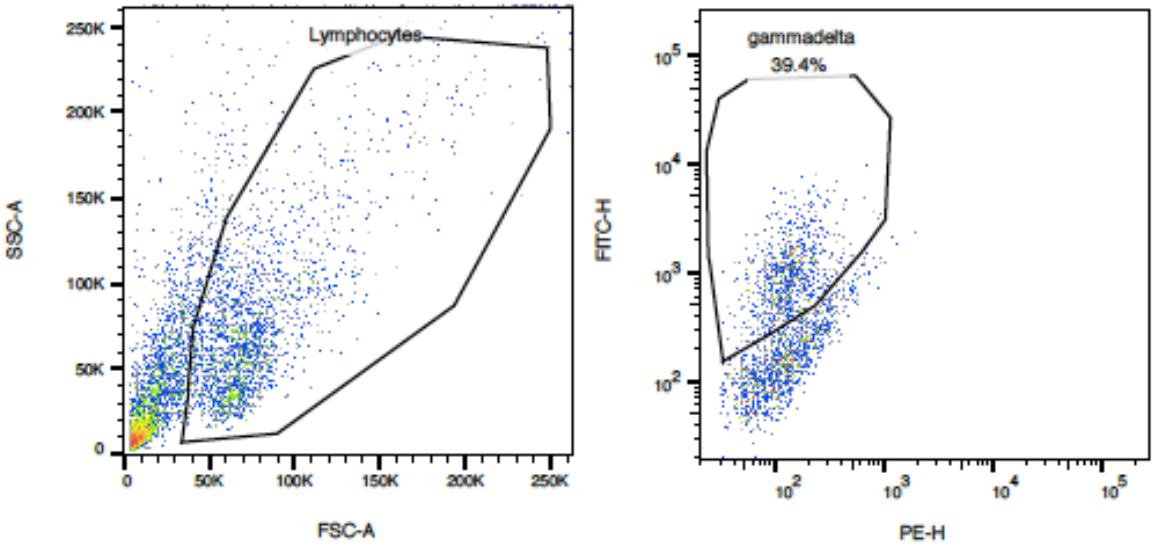

Figure 1-D. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 12. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 39.4% of T cells out of entire donor 11 PBMCs expressed γδ TCR on day 12 following 100 nM HMBPP stimulation.

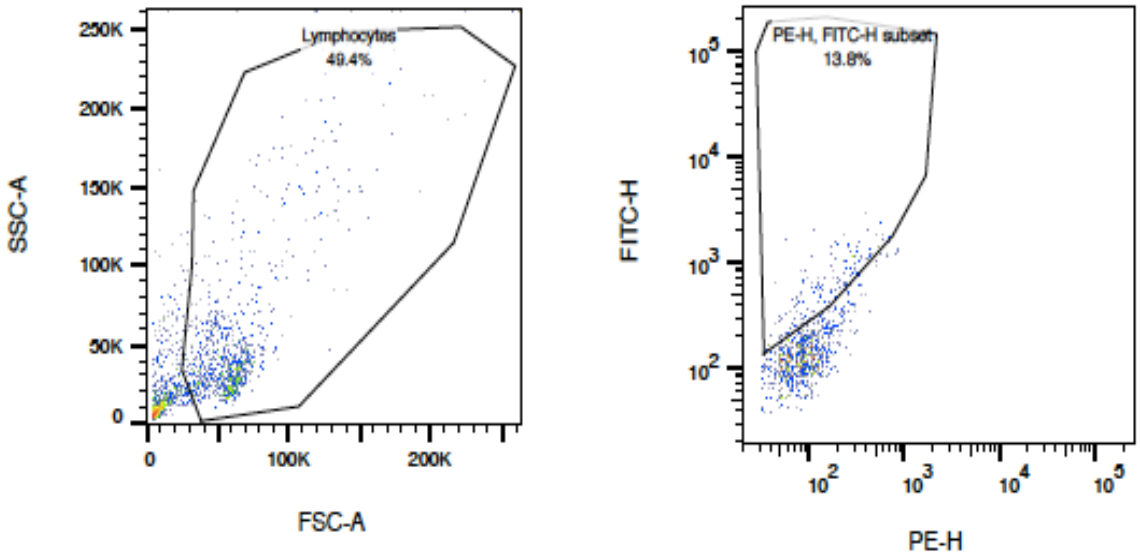

Figure 1-E. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 1. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 13.9% of T cells out of entire donor 12 PBMCs expressed γδ TCR on day 1 following 100 nM HMBPP stimulation.

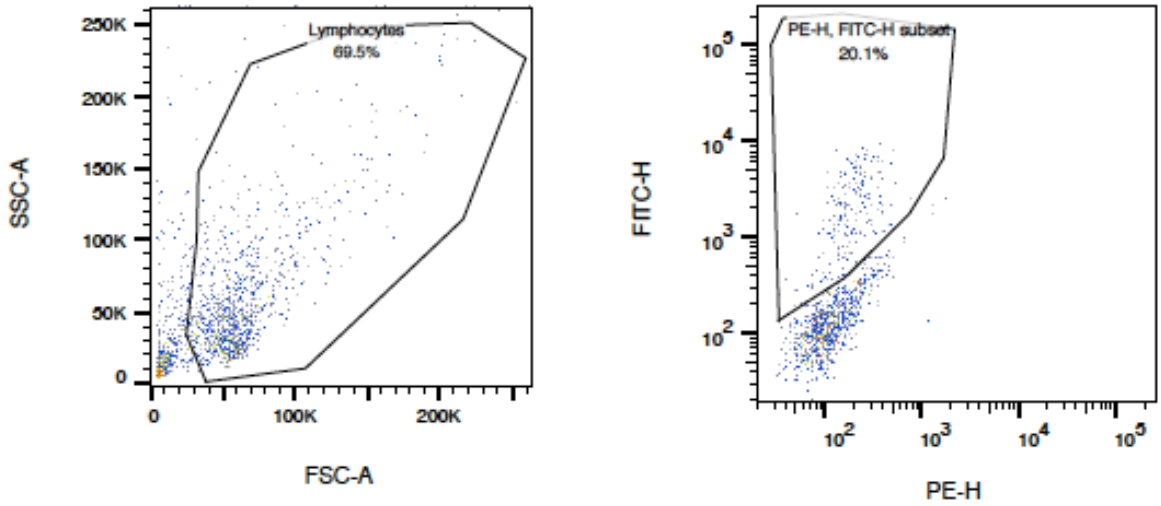

Figure 1-F. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 5. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 20.1% of T cells out of entire donor 12 PBMCs expressed γδ TCR on day 5 following 100 nM HMBPP stimulation.

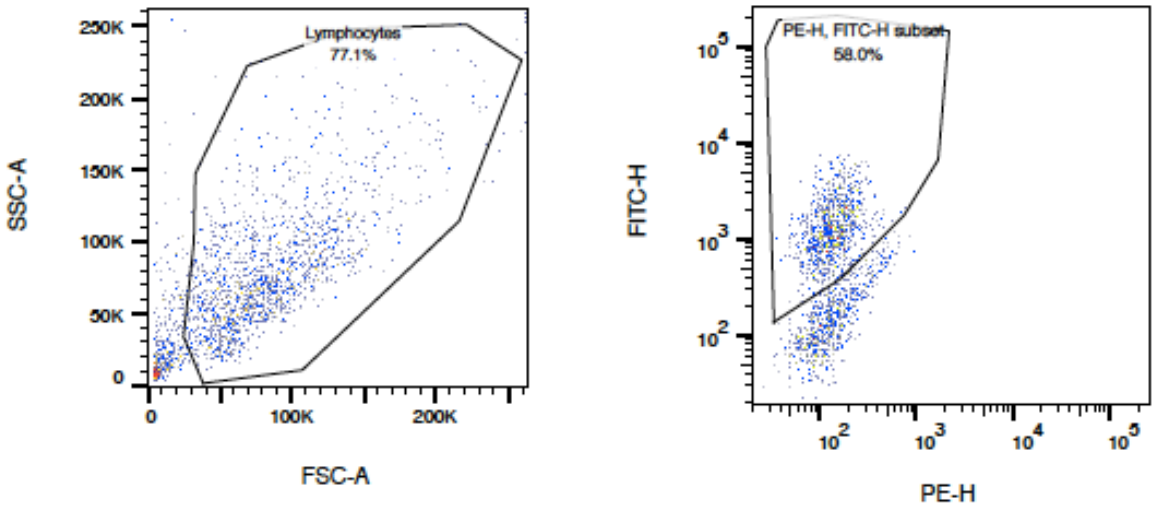

Figure 1-G. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 7. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 58.0% of T cells out of entire donor 12 PBMCs expressed γδ TCR on day 7 following 100 nM HMBPP stimulation.

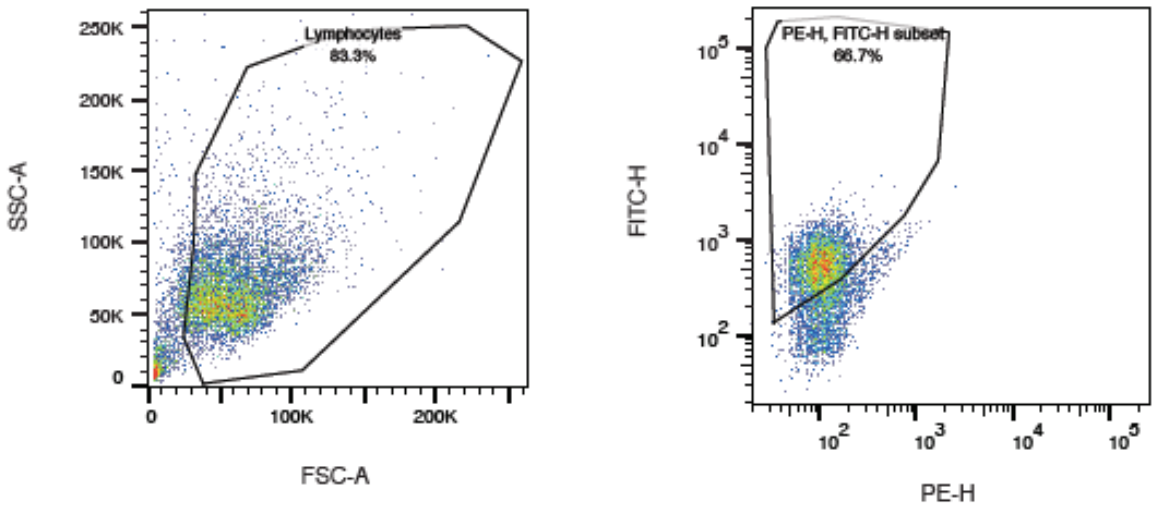

Figure 1-H. Expression of the γδ TCR following 100 nM HMBPP stimulation on day 12. PBMCs were stimulated for 3 days with 100 nM HMBPP. Cells were stained with γδ TCR on days 1-7, 9, and 12 and were analyzed by flow cytometry from day 1 to day 12. Panel on the right indicates that 66.7% of T cells out of entire donor 12 PBMCs expressed γδ TCR on day 12 following 100 nM HMBPP stimulation.

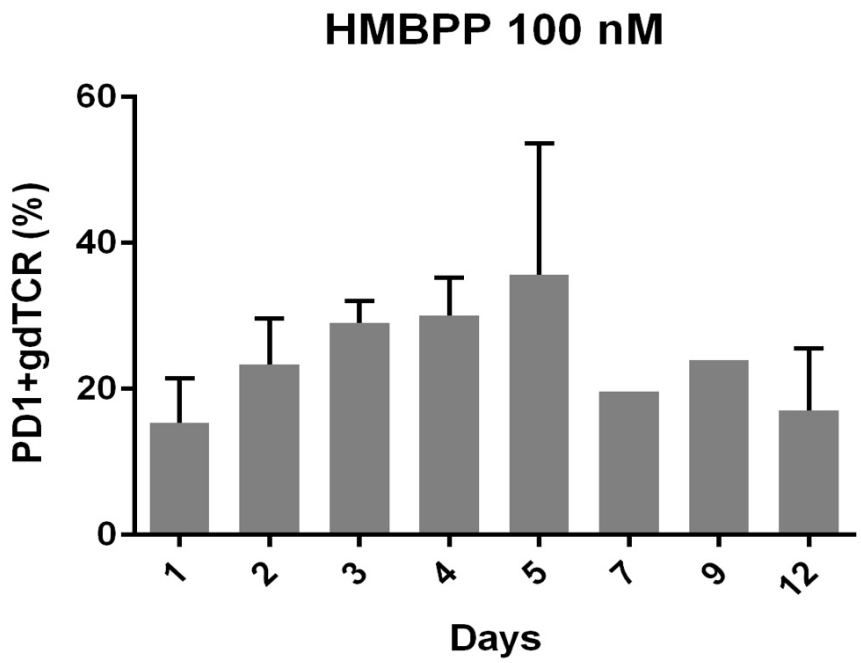

Figure 2. Expression of the γδ TCR and PD-1 following 100 nM HMBPP stimulation. PBMCs were stimulated for 3 days with 100 nM HMBPP. The percentage of cells expressing the γδ TCR and PD-1 was determined by flow cytometry over 12 days. Bars represent mean values and error bars represent standard deviations of three independent experiments. *$p < 0.05$ by one way ANOVA.

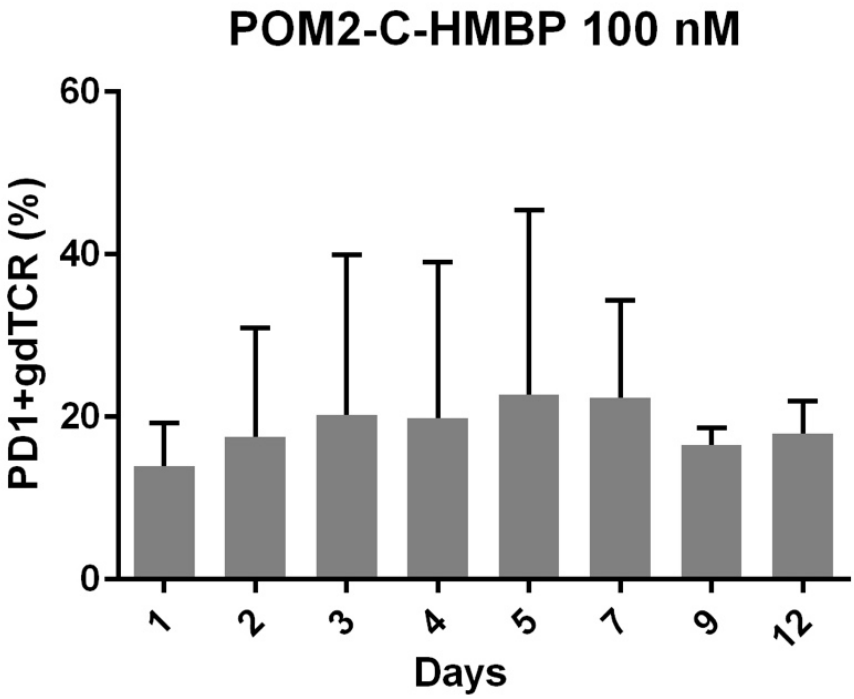

Figure 3. Expression of the γδ TCR and PD-1 following 100 nM POM_2-C-HMBP (054) stimulation. PBMCs were stimulated for 3 days with 100 nM POM_2-C-HMBP (054). The percentage of cells expressing the γδ TCR and PD-1 was determined by flow cytometry over 12 days. Bars represent mean values and error bars represent standard deviations of three independent experiments. *$p < 0.05$ by one way ANOVA.

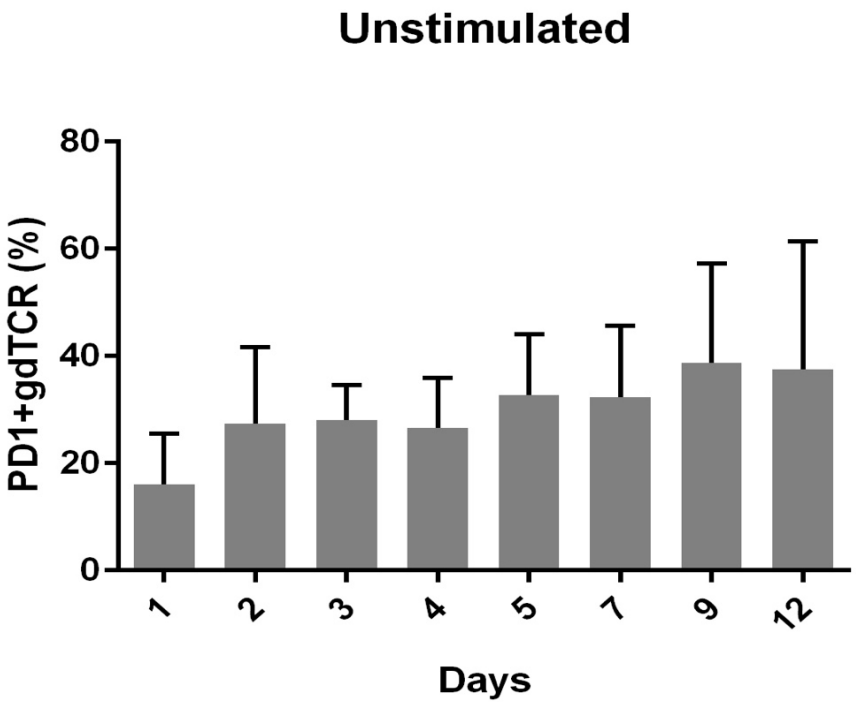

Figure 4. Expression of the γδ TCR and PD-1 in unstimulated PBMCs. Control PBMCs were left unstimulated throughout the entire course of experiment. The percentage of cells expressing the γδ TCR and PD-1 was determined by flow cytometry over 12 days. Bars represent mean values and error bars represent standard deviations of three independent experiments. *$p < 0.05$ by one way ANOVA.

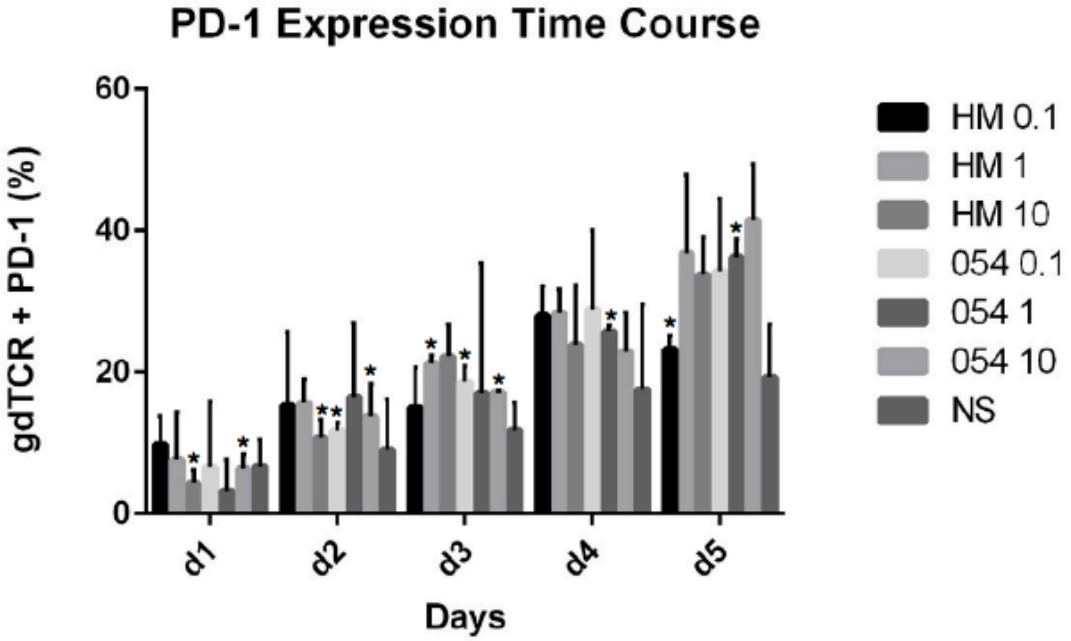

Figure 5. Dose response of γδ TCR and PD-1 expression. PBMCs were stimulated for 3 days with 0.1 μM, 1 μM, and 10 μM of HMBPP and POM_2-C-HMBP (054) for respective conditions. The percentage of cells expressing the γδ TCR and PD-1 was determined by flow cytometry over 5 days. Bars represent mean values and error bars represent standard deviations of three independent experiments. *$p < 0.05$ by one way ANOVA. Bars are compared to the different concentrations (row).

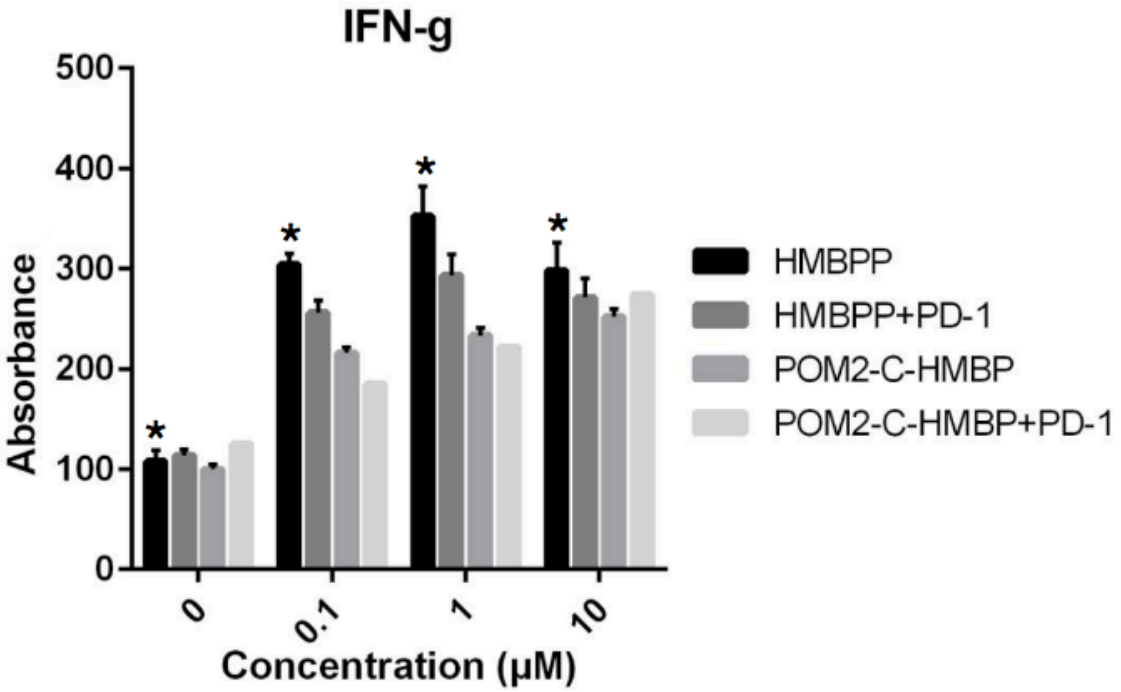

Figure 6. Secretion of IFN-γ following addition of PD-1 blocking monoclonal antibody. PBMCs were stimulated for 3 days with phosphoantigens and on day 5 were co-incubated with K562 cells that express PD-L1/2. Release of IFN-γ and K562 expression of PD-L1/2 were determined by ELISA and flow cytometry, respectively. *$p < 0.05$ by one way ANOVA. Bars are compared to the different concentrations (row).

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