STAR Protocols

Protocol

Generation of effector Vγ9Vδ2 T cells and evaluation of their response to phosphoantigen-loaded cells

Chia-Hung Christine Hsiao, Andrew J. Wiemer

christine.hsiao@uconn. edu (C.-H.C.H.)
andrew.wiemer@uconn. edu (A.J.W.)

Highlights

Protocol to define activity of phosphoantigens for Vγ9Vδ2 T cell activation

Detailed steps for Vγ9Vδ2 T cell activation, purification, and effector functions

C-HMBPP and zoledronate are described as positive controls

Vγ9Vδ2 T cells are non-canonical T cells that use their T cell receptor to detect phosphoantigens bound to the internal domain of the HMBPP receptor (butyrophilin 3/2A1 complex). This protocol describes the expansion and purification of human effector Vγ9Vδ2 T cells from human buffy coat and describes how to assess their activation by antigen-containing target cells. While specifically focused on cytokine production, this protocol can be readily adapted to evaluate other effector functions of activated Vγ9Vδ2 T cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Generation of effector Vγ9Vδ2 T cells and evaluation of their response to phosphoantigen-loaded cells

Chia-Hung Christine Hsiao[1,3,*] and Andrew J. Wiemer[1,2,4,*]

[1]Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269, USA
[2]Institute for Systems Genomics, University of Connecticut, Storrs, CT 06269, USA
[3]Technical contact
[4]Lead contact
*Correspondence: christine.hsiao@uconn.edu (C.-H.C.H.), andrew.wiemer@uconn.edu (A.J.W.)
https://doi.org/10.1016/j.xpro.2022.101422

SUMMARY

Vγ9Vδ2 T cells are non-canonical T cells that use their T cell receptor to detect phosphoantigens bound to the internal domain of the HMBPP receptor (butyrophilin 3/2A1 complex). This protocol describes the expansion and purification of human effector Vγ9Vδ2 T cells from human buffy coat and describes how to assess their activation by antigen-containing target cells. While specifically focused on cytokine production, this protocol can be readily adapted to evaluate other effector functions of activated Vγ9Vδ2 T cells.
For complete details on the use and execution of this protocol, please refer to Hsiao et al. (2022) and Hsiao and Wiemer (2018).

BEFORE YOU BEGIN

The protocol described below lists the steps for using human Vγ9Vδ2 T cells to detect antigens loaded into K562 leukemia cells (Kilcollins et al., 2016; Hsiao and Wiemer, 2018; Hsiao et al., 2022). The Vγ9Vδ2 T cells are first expanded from buffy coat peripheral blood mononuclear cells (PBMCs). These effector cells are then co-cultured with K562 cells that are loaded with phosphoantigens and the cytokine response of the Vγ9Vδ2 T cells is quantified by enzyme-linked immunosorbent assay (ELISA).

Once the PBMCs are obtained, they can be used fresh or can be frozen for future use. It is well documented that freezing PBMCs can impact certain characteristics of the cells (Costantini et al., 2003; Anderson et al., 2019; Ticha et al., 2021). Our preference is to aliquot and freeze the purified PBMCs. As the cells will be stimulated, cultured for two weeks, and purified before use, dead cells caused by the freezing process ultimately will be removed prior to the functional assay.

Because the Vγ9Vδ2 T cell receptor recognizes the HMBPP receptor, the most important attribute of the phosphoantigen-containing cells is expression of the receptor, which is a complex of butyrophilin (BTN) 3A1 and 2A1 (Karunakaran et al., 2020; Rigau et al., 2020; Vyborova et al., 2020; Cano et al., 2021; Hsiao et al., 2022). Because the phosphoantigen binding site on BTN3A1 is intracellular rather than extracellular (Hsiao et al., 2014; Sandstrom et al., 2014; Rhodes et al., 2015; Wang and Morita, 2015; Peigne et al., 2017), we refer to the cells as antigen containing cells. The phosphoantigen response is not MHC restricted (Morita et al., 1995), so it is not necessary to match MHC haplotypes.

In the PBMCs, there are monocytes that can function as the phosphoantigen-containing cells (Roelofs et al., 2009; Tomogane et al., 2022). In the co-cultures, most human cells lines that we have evaluated can function in this assay. Our earlier studies used Daudi cells, but these cells are not ideal for

loading with test compounds due to their high expression of the endogenous phosphoantigen IPP (Gober et al., 2003). K562 cells stimulate a robust response from the Vγ9Vδ2 T cells with low background from endogenous phosphoantigens and are relatively easy to genetically manipulate.

Vγ9Vδ2 T cells are also capable of self-activation, which can occur both with and without cell-to-cell contact (Laplagne et al., 2021). We observed this when attempting to re-stimulate effector Vγ9Vδ2 T cells with a second dose of phosphoantigen, which resulted in strong self-activation leading to autolysis. An important aspect of our protocol is the removal of the phosphoantigen after loading the target cells and before exposure to the T cells which prevents self-activation and clarifies results. For compounds such as HMBPP which are highly potent but slow to enter cells, significant washing is required (Hsiao and Wiemer, 2018).

This protocol uses HMBPP as the phosphoantigen for expansion of the effector Vγ9Vδ2 T cells from PBMCs. HMBPP is recommended here because it is the most potent natural phosphoantigen, providing physiologically relevant Vγ9Vδ2 T cell stimulation with little compound-mediated cell toxicity. We then describe the cytokine response of effector Vγ9Vδ2 T cells during co-culture with K562 cells that are loaded with phosphoantigens. Here, we recommend using either C-HMBPP or zoledronate as a positive control. C-HMBPP is a direct phosphoantigen (binds to BTN3A1) that is more metabolically stable than HMBPP and provides more consistent results in pulse experiments. Zoledronate is an indirect phosphoantigen which is also metabolically stable and less expensive. HMBPP, C-HMBPP, and zoledronate are available from commercial vendors (Wiemer, 2020).

Institutional permissions

All experiments conform to the relevant regulatory standards. Our blood was purchased from a vendor that follows American Association of Blood Banks guidelines using an IRB-approved consent form for the sale of blood products for research. Readers that intend to draw blood from donors themselves will need to acquire permissions from their IRB.

Thaw and culture K562 target cells

⊙ Timing: 15 min for thaw, 1 week to log growth

1. Thaw K562 cells quickly by hand or in water bath.
2. Wash once with K562 cell media to remove cryoprotectant.
    a. Dilute thawed cells to 10 mL in K562 cell media in 15 mL conical tube.
    b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
    c. Resuspend in 10 mL of fresh K562 cell media.
    d. Transfer to vented T75 flask.
    e. Dilute to 20 mL with fresh K562 cell media.
3. Grow cells in humidified incubator at 37°C and 5% CO_2.
4. Replenish media every 1–2 days to maintain cells between 5×10^4 and 5×10^5 cells/mL.
    a. Can remove 80%–90% of cells and media every 2 days and replace with fresh media.
    b. Cells should be growing in log phase prior to starting experiments.
    c. Results are more consistent if cells are split the day prior to experimental setup.

Isolate human PBMCs

⊙ Timing: 4 h per blood donor

5. Obtain 30 mL of human buffy coat.
    a. 30 mL of buffy coat has the PBMC equivalent of ~300 mL of whole blood.

⚠ CRITICAL: Donors can vary in their response to phosphoantigens. Plan to prep 3 donors initially to account for donor variability and identify phosphoantigen responsive donors. Prep 1 donor per day until familiar with protocol.

6. Remove red blood cells by centrifugation.
   a. Pour equal amounts of buffy coat into two 50 mL centrifuge tubes.
   b. Dilute each tube to 50 mL with 1× PBS and mix well by gentle inversion.
   c. Prepare twenty 50 mL centrifuge tubes.
   d. Aliquot 15 mL of lymphocyte separation solution into each centrifuge tube.
   e. Very slowly add an equal volume (∼ 5 mL) of diluted buffy coat on top of the solution.
      i. Avoid mixing. Layering is required.
      ii. Set pipettor to the lowest speed.
      iii. The tube can be slightly tilted prior to adding the diluted buffy coat to the side of the tube to avoid a sudden large amount of liquid on top of lymphocyte separation solution.
      iv. If the diluted buffy coat gets fully mixed with the separation media rather than layered on top, the preparation is ruined. Some minor mixing at the interface is normal.
   f. Centrifuge at 1500 rpm (swinging bucket) for 30 min at room temperature (20°C–25°C).
      i. Maximum acceleration can be used, no brake or minimum brake should be applied.
   g. Remove top layer (clear) and white layer (cells) together, leaving the darker red layer (RBCs).
   h. Save the top clear layer and white layer cells (these are the PBMCs) and discard RBCs.
      i. With buffy coat preparations, the clear top layer may be a small volume and difficult to remove. It is fine to keep it with the PBMC fraction and remove by washing.
7. Wash cells to remove separation media.
   a. Combine white cells from every 5 tubes into 3 tubes.
      i. This will give 12 total tubes about half full.
   b. Top off the tubes to a final volume of 50 mL with 1× PBS (takes about 30 mL/tube) and mix by inversion.
      i. It is important to have at least 50% PBS so the white blood cells pellet properly. Err on the side of too much PBS.
      ii. Can spin the first tubes while preparing the others to save time if desired.
   c. Centrifuge 10 min at 600 rcf at room temperature (20°C–25°C).
   d. Aspirate 80% of supernatant (ex 35 mL from 50 mL tube, leave 15 mL on pellet).
      i. Some cloudiness is expected at this step, these are likely platelets and can be removed, though they could be PBMCs if the cells were too dense and did not pellet properly.
   e. Resuspend cell pellet by gentle pipetting, and combine tubes to give 6 tubes.
   f. Top off the tubes to 50 mL with T cell media and mix by inversion.
   g. Centrifuge 10 min at 600 rcf at room temperature (20°C–25°C).
   h. Aspirate 100% of supernatant.
   i. Resuspend cell pellet in 10 mL T cell media by gentle pipetting, and combine tubes to give 2 tubes. Top off the tubes to 50 mL with T cell media.
   j. Should have two tubes with 100 mL total volume at this point.
      i. If cells are heavy divide the 2 tubes into 4.
   k. Reserve 10 μL for counting.
   l. Centrifuge 10 min at 600 rcf at room temperature (20°C–25°C).
      i. If the supernatant is cloudy after the last spin, spin another 5 min to make sure all cells are pelleted.
      ii. Should have a large cell pellet (about 200–400 μL pellet volume) visible at this step.
8. Count and resuspend the cells in freezing media.
   a. Dilute 10 μL of cells (reserved from step 7k) with 190 μL of trypan blue (1:20 dilution).
   b. Count using hemocytometer.
   c. Calculate total cell number per mL.
      i. Remember to multiply by volume (100 mL) and dilution factor (20×).
   d. Resuspend at 30 million/mL (or desired concentration) in freezing media.

9. Freeze cells.
   a. Add 500 μL into 500 μL cryotubes.
      i. Label tubes ahead of time if desired.
      ii. Always fill cryotubes to the recommended volume to reduce explosion risk upon thawing.
   b. Place in Styrofoam containers.
      i. We use leftover Styrofoam racks from standard 15 mL centrifuge tubes and enclose the cryotubes in between two Styrofoam racks (face the openings to each other to fit the tubes).
   c. Wrap with foil.
   d. Place in −80°C freezer overnight.
   e. After 24 h, move frozen tubes to the vapor phase of liquid nitrogen or as recommended by supplier.
   f. Each tube will have 15 million PBMCs.
   g. Typical yield: (100 tubes) (1.5×10^7 cells/tube) = 1.5×10^9 cells.

⧗⧗ **Pause point:** It is not clear how long cells can be stored effectively in liquid nitrogen. In our hands, the viability does decrease over the course of several years.

⚠ CRITICAL: Freezing in liquid nitrogen creates a temperature hazard and an explosion hazard. Be sure to wear proper personal protective equipment including hand and eye protection.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Unpurified human buffy coat (25–50 mL) from healthy males and females aged 18–65.	Research Blood Components	Item#002
Chemicals, peptides, and recombinant proteins		
Human IL-2 IS, premium grade 50 μg	Miltenyi Biotec	Cat#130-097-745
HMBPP (HDMAPP ammonium salt)	Cayman Chemical	Cat#13580
C-HMBPP (C-HDMAPP)	Cayman Chemical	Cat#13151
Zoledronate (zoledronic acid hydrate)	Cayman Chemical	Cat#14984
2-mercaptoethanol	Fisher	CAS#60-24-2
Sodium bicarbonate	Fisher	CAS#144-55-8
Dimethyl sulfoxide (DMSO)	Fisher	CAS#67-68-5
HEPES	Fisher	Cat#BP410-500
Bovine serum albumin (BSA)	Fisher	Cat#BP1600-100
Ethylenediaminetetraacetic acid (EDTA)	Fisher	CAS# 60-00-4
PE-Anti-CD3 clone UCHT1	BioLegend	Cat# 300408
FITC-Anti-TCRγ/δ clone 5A6.E91	Fisher	Cat#ENTCR2061
Critical commercial assays		
TCRγ/δ+T Cell Iso Kit, human	Miltenyi Biotec	Cat#130-092-892
MS columns	Miltenyi Biotec	Cat#130-042-201
ELISA MAX™ Standard Set Human IFN-γ	BioLegend	Cat#430101
Experimental models: Cell lines		
K562 cells	MilliporeSigma	Cat#89121407-1VL
Software and algorithms		
GraphPad Prism 6	GraphPad	www.graphpad.com/
Other		
Lymphocyte Separation Media	Corning	Cat#MT25072CV
RPMI-1640 media	Cytiva	Cat#SH30011.02
Fetal bovine serum (FBS)	Corning	Cat#MT35010CV
Penicillin-Streptomycin	Lonza	Cat#BW09-757F
Nonessential Amino Acid Solution	Corning	Cat#MT25025CI
Sodium pyruvate 100 mM	Corning	Cat#MT25000CI
Plate Sealer	Thermo Scientific	Cat#3501

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nunc MaxiSorp ELISA Plates, Uncoated	BioLegend	Cat#423501
Strip tubes	DOT Scientific	Cat#403-8PCR
500 μL cryovials	Nunc	Cat#12570216
Flat bottom 96 well plate	DOT Scientific	Cat#667196
Multichannel Pipettor 8 × 100 μL	Eppendorf	Cat#3125000036
Multichannel Pipettor 8 × 300 μL	Eppendorf	Cat# 3125000052
Centrifuge	Eppendorf	Model#5810R
Centrifuge	Eppendorf	Model#5702
Micro-centrifuge	Eppendorf	Model#5418
Mini-centrifuge	Fisher	Cat#05-090-100
Tissue culture incubator	New Brunswick	Model#170S
Tissue culture hood	Thermo Fisher Scientific	Model#1300A2
Microscope	Olympus	Model#CKX41
Miltenyi magnet	Miltenyi Biotec	Cat#130-042-102
Miltenyi multistand	Miltenyi Biotec	Cat#130-042-303
−80°C freezer	New Brunswick	Model#U410
Liquid nitrogen tank	Thermo Scientific	Cat#CY509108
Plate reader	PerkinElmer	Model#Victor X5
Spray bottle	Thermo Scientific	Cat#24011000
Rocker	Benchmark Scientific	Model#BR1000

Alternatives: Other density gradient centrifugation methods, such as lymphoprep, can be used in lieu of Lymphocyte Separation Media for isolation of the PBMCs. Although we do not expect a difference in final yields of cells with these materials, we have not compared them directly.

MATERIALS AND EQUIPMENT

RPMI-1640 media

Reagent	Final concentration	Amount
RPMI-1640 media	1×	1 bottle powder
Water	n/a	5 L
Sodium Bicarbonate	1.5 g/L	7.5 g
Total	n/a	5 L

Mix well to dissolve the bicarbonate, filter sterilize, keep sterile, store at 4°C for up to 1 year. Our recommended media does not arrive with any sodium bicarbonate, but if using a different supplier, make sure the final concentration is correct.

K562 cell media

Reagent	Final concentration	Amount
RPMI-1640 media	1×	750 mL
Heat inactivated FBS	10%	75 mL
Penn/Strep (200×)	1×	3.75 mL
Total	n/a	∼830 mL

Heat inactivate the FBS by incubation at 56°C for 45 min.
Filter sterilize, keep sterile, store at 4°C for up to 3 months.

T cell media

Reagent	Final concentration	Amount
RPMI-1640 media	1×	750 mL
2-mercaptoethanol	50 μM	3 μL
Heat inactivated FBS	10%	75 mL

(Continued on next page)

Continued

Reagent	Final concentration	Amount
Penn/Strep (200×)	1×	3.75 mL
HEPES (1 M)	10 mM	7.5 mL
Pyruvate (100×)	1×	7.5 mL
NEAA (100×)	1×	7.5 mL
Total	n/a	∼850 mL

Filter sterilize, keep sterile, store at 4°C for up to 3 months.

⚠ CRITICAL: 2-mercaptoethanol can be toxic if inhaled, add to media in fume hood (not biosafety cabinet). DMSO can be absorbed through skin, wear protective gloves.

Freezing media

Reagent	Final concentration	Amount
T cell media	n/a	80 mL
Heat inactivated FBS	n/a	10 mL
DMSO	10%	10 mL
Total	n/a	100 mL

Filter sterilize, keep sterile, store at 4°C for up to 3 months.

Concentrated IL-2

Reagent	Final concentration	Amount
Human IL-2 IS, premium grade	200 µg/mL	50 µg
Sterile water	n/a	250 µL
Total	200 µg/mL	250 µL

Divide to 50 µL aliquots, keep sterile, store at −80°C, expiration date per supplier.

IL-2 aliquots

Reagent	Final concentration	Amount
200 µg/mL IL-2	20 µg/mL	50 µL
Sterile 0.1% BSA in PBS	n/a	450 µL
Total	20 µg/mL	500 µL

Aliquot 25 µL/1.5 mL tube, keep sterile, store at −80°C, expiration date per supplier.

Working IL-2

Reagent	Final concentration	Amount
20 µg/mL IL-2 aliquot	0.5 µg/mL	25 µL
T cell media	n/a	1 mL
Total	0.5 µg/mL	1 mL

Keep sterile, store at 4°C, for up to 3 weeks, add to cells at 1:100 to get 5 ng/mL final concentration.

MACS Buffer

Reagent	Final concentration	Amount
BSA	0.5%	0.5 g
100 mM EDTA	2 mM	2 mL
PBS, pH 7.2	n/a	98 mL
Total	n/a	100 mL

Filter sterilize, keep sterile, store at 4°C, for up to 6 months.

STEP-BY-STEP METHOD DETAILS
Expansion of effector Vγ9Vδ2 T cells from PBMCs

⏱ Timing: 12–14 days

This section will describe the use of HMBPP to stimulate the PBMCs leading to activation and growth of Vγ9Vδ2 T cells.

1. Thaw PBMCs quickly by hand or in water bath.
   a. Generally we thaw 2–4 tubes (3–6 × 10^7 PBMCs) per expansion.

   ⚠ CRITICAL: When thawing PBMCs from liquid nitrogen, the general rule of thumb is to thaw cells quickly. Thawing PBMCs too slowly may result in cell aggregation/cell clump formation, which may impact the number of recovered cells. We have found it acceptable to proceed in the occasional presence of minor clumps which generally resolve after overnight incubation.

2. Wash once with media to remove cryoprotectant.
   a. Dilute thawed cells to 10 mL in T cell media in 15 mL conical tube.
   b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
   c. Resuspend in 10 mL fresh T cell media.
   d. Transfer to vented T75 flask.

   Alternatives: Cell culture dishes or 6 well plates can also be used to culture PBMCs. However, vented T75 flasks are easier to handle and have lower chance of microbial contamination.

3. Count cells and then dilute cells to a concentration of 2–4 × 10^6 / mL.
   a. As a relatively small population, the Vγ9Vδ2 T cells will clearly expand better at a relatively higher PBMC density compared to similar approaches for other T cell types. Lower densities will be detrimental to expansion, while higher densities may be tolerable.
4. Add IL-2 to a final concentration of 5 ng/mL.
   a. Use a 1:100 dilution from working IL-2 stock.
5. Add HMBPP to a final concentration of 100 nM.

   Alternatives: In lieu of HMBPP, some groups have reported using bisphosphonate drugs such as zoledronate to expand the Vγ9Vδ2 T cells from PBMCs (Roelofs et al., 2009; Zumwalde et al., 2016; Nada et al., 2017). This is a lower cost alternative of indirectly activating BTN3A1 that likely produces similar Vγ9Vδ2 effector T cells. However, only a narrow range of bisphosphonate concentrations can be used due to higher compound toxicity relative to HMBPP.

6. Grow cells in humidified incubator at 37°C and 5% CO_2.
7. After 3 days, wash to remove HMBPP.
   a. Transfer to a 15 mL or 50 mL conical tube depending on culture volume.
   b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
   c. Aspirate media using vacuum suction.
   d. Add 10 mL of fresh T cell media. No need to disturb pellet.
   e. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
   f. Resuspend cell pellet in initial volume of fresh T cell media.
   g. Add fresh IL-2 to 5 ng/mL.
   h. Transfer to a new vented T75 flask.
   i. All of the cells are transferred.

j. At this stage, counting is not recommended.

k. Visual inspection can be done, but it is not very informative. The Vγ9Vδ2 T cell population is small at this time point, and some death of other cell types is occurring.

8. At day 6 and 9 post stimulation, add fresh IL-2 to 5 ng/mL.

   a. Unlike αβ T cells, it is not usually necessary to add additional volume of media. It is not necessary to change or add media because the number of viable and proliferating Vγ9Vδ2 T cells is small (typically 1%–5% of T cell population) relative to the volume used, and the other cell types are not actively proliferating or are being killed by the Vγ9Vδ2 T cells. We have not found that media components other than IL-2 limit Vγ9Vδ2 T cell proliferation at this stage.

9. On day 12, cells are ready for purification.

> ⚠ CRITICAL: It is helpful to inspect cells at days 6 and 9. At these days, HMBPP-stimulated cells should have formed defined colonies of 10–1000 cells, whereas unstimulated cells (if used as a negative control, not always necessary) will only have some small colonies. This varies by donor. The colonies are usually visible by eye upon gentle shaking and look like healthy clumps. They can also be observed under a microscope. Prior to day 6, the total cell number may go down likely as a result of cytotoxicity caused by the Vγ9Vδ2 T cells against the other PBMCs.

> ⏸ Pause point: While we typically purify cells on day 12, it has successfully worked between days 10 and 17, giving some flexibility to the purification and subsequent functional assays. For most consistent results, keep the day constant.

Purification of effector Vγ9Vδ2 T cells

⏱ Timing: 1 h

This section will describe how to purify the expanded Vγ9Vδ2 T cells from any other PBMCs still remaining in the flask, such as resting αβ T cells. This section generally follows the recommendations of Miltenyi Biotec with some modifications. For example, our typical centrifugation protocol of 3 min at 600 rcf works fine instead of the recommended 10-min spins, saving prep time.

10. Collect all HMBPP expanded cells.

    a. Transfer cells to a 15 mL tube.

    b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).

    c. Aspirate media using vacuum suction.

11. Resuspend in 80 μL of cold MACS buffer.

    a. Should produce a very dense-looking cell suspension.

    b. The kit claims up to 1×10^7 PBMCs can be used in one prep. Since this is negative selection, the non- Vγ9Vδ2 T cells will be removed by binding to antibodies. Typically, the expanded Vγ9Vδ2 T cells have killed many of the other cells, so there are not that many cells left to remove with the cocktail.

12. Add 20 μL biotin-antibody cocktail from TCR γ/δ+ T Cell Iso Kit, human.

    a. Mix by gentle flicking.

13. Incubate for 15 min at 4°C.

    a. Usually we just put the tube into the refrigerator.

14. Wash cells.

    a. Add 900 μL of cold MACS buffer.

    b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).

15. Resuspend in 80 μL MACS buffer.

16. Add 20 μL magnetic beads.

17. Incubate again for 15 min at 4°C.

    a. Usually we just put the tube into the refrigerator.

18. Wash cells.
    a. Add 900 μL of MACS buffer.
    b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
19. Resuspend in 500 μL MACS buffer.
20. Equilibrate the magnetic column with 500 μL of cold MACS buffer.
21. Pass the 500 μL of cells through the magnetic column.
22. Elute 2 additional times with 500 μL cold MACS buffer.
23. Pellet the cells.
    a. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
24. Resuspend in 10 mL T cell media.
25. Count cells.
    a. Typical yield should be 3–30 million effector cells depending on donor and strength of expansion.
    b. If desired, assess for purity by flow cytometry staining with CD3 and γδ TCR antibodies. These cells should be >95% pure.

⏸ Pause point: After purification, the cells can be used immediately or maintained in the incubator. Typically, we will purify the cells on day 12 and use for experiments on days 13–15. Longer times can be used provided cells are maintained in fresh media and IL-2.

⏸ Pause point: Alternatively, the cells can be frozen again in liquid nitrogen following the freezing procedure above for PBMCs. Typically, storage of 3×10^6 effector cells per 500 μL cryotube will be sufficient for 2 ELISA plates upon thawing.

Phosphoantigen loading of K562 target cells

⏱ Timing: 2 h, variable

This section will describe how to load the target cells with phosphoantigen in preparation for co-culture. We will describe the process for a 5-concentration 10-fold dose response of C-HMBPP from 1×10^{-4} to 1×10^{-8} M and of zoledronate from 1×10^{-3} to 1×10^{-7} M in duplicate, with empty wells remaining in one 96-well plate for additional test compounds and unstimulated controls. We typically use one 96-well plate for up to 6 test compounds at 1 time point or 2 test compounds at 3 time points in addition to the positive controls. C-HMBPP and zoledronate are commercially available direct and indirect phosphoantigens, respectively, which will make excellent positive controls in this assay. We recommend against using HMBPP as a positive control in pulse experiments-because of its low cellular stability it does not perform well in pulse experiments such as this.

26. Dilute C-HMBPP and zoledronate (and test compounds if desired).
    a. Both compounds are originally dissolved in water. Serial dilutions are made using T cell media for ease of mixing with cells.
    b. In a strip of 200 μL tubes, prepare 10-fold serial dilutions of either compound, or both, as desired.
    c. Concentrations in the tubes should be 10× the final concentration, ranging from 1×10^{-3} to 1×10^{-7} M for C-HMBPP and 1×10^{-2} to 1×10^{-6} M for zoledronate.
    d. Volume should be at least 20 μL per tube.
    e. Compounds will be diluted an additional 10-fold when mixed with cells in later step to achieve the desired final concentrations.

Note: Some compounds with weak activity or low stability produce curves that fail to reach maximum stimulation. Therefore, a positive control such as zoledronate or C-HMBPP should be included in each experiment to allow definition of the maximal effect when plotting the curve.

Note: Zoledronate is a highly potent bisphosphonate. Other bisphosphonate inhibitors of farnesyl diphosphate synthase may be substituted with similar results, where the potency will correlate to the degree of enzyme inhibition.

27. Count K562 cells.
    a. A minimum of 4000 cells / well of a 96 well plate or 3.84×10^5 cells / 96 well plate are needed for this protocol.
    b. We recommend preparing 1.2×10^6 cells / plate to allow a bit extra, make calculations easier, and enable use of multichannel pipettors.
    c. This protocol will prepare enough cells for 48 conditions of test compounds at various doses and times in duplicate (one 96 well plate).
    d. 12 wells will be used for the C-HMBPP dose response and 12 for the zoledronate dose response including negative controls (solvent only) for each.
    e. Additional wells should be reserved for unstimulated controls (e.g., media and cells only) and standards for ELISA (see section below).
28. Collect K562 cells.
    a. Transfer 1.2×10^6 cells to a 15 mL tube.
    b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
    c. Aspirate media by vacuum suction.
29. Resuspend K562 cells in T cell media to 2×10^5 cells/mL.
    a. Add 6 mL of T cell media and resuspend by gentle pipetting to reach 2×10^5 cells/mL (1.2×10^6 cells in 6 mL).
    b. T cell media is used here instead of K562 cell media because these cells will ultimately be mixed with T cells. T cell media contains extra additives that are necessary for T cells and not K562 cells, but will not harm the K562 cells.
30. Aliquot 120 μL of cells to sterile 200 μL strip tubes.
    a. For each dose response, we will need 6 tubes of cells.
31. Using a multichannel pipettor, add 13.3 μL test compound or solvent control.
    a. Typically, we make a 10× series of test compounds in media and add 13.3 μL to each tube for a final concentration of 1× compound in 133 μL of cells.
32. Mix gently.
33. Incubate for 1 h or desired time at 37°C.

> ⚠ CRITICAL: Compounds may be incubated longer or shorter than 1 h, which depends on the expected uptake rate and potency of the specific test compound. In our hands, most of the compounds we have tested displayed time-dependent effect at exposure time with K562 cells between 15 min to 240 min. A pilot experiment at multiple time points is helpful in establishing the optimum time frame for each compound.

Note: T cell media contains HEPES buffer which allows tubes to be closed during incubation of K562 cells with test compounds (no gas exchange required).

34. During incubation, proceed to count and plate T cells as described in the next section.
35. After incubation, wash treated K562 cells 3–5× with 120 μL of T cell media.
    a. Centrifuge strip tubes for 20 s at max speed with tabletop mini centrifuge (ours is fixed at 2000 rcf).
    b. Aspirate ∼95% of media. A small cell pellet should be visible. Do not disturb the cell pellet.
    c. Using multichannel pipettor, add 120 μL of T cell media.
    d. Repeat steps 35a–35c a total of 3–5×.

Note: Some phosphoantigens, such as HMBPP, are highly potent but poorly stable. With these compounds, a high wash number is essential to remove any impact of residual

compound on the system. We have found a minimum of 5 washes is necessary to lower extra-cellular HMBPP or C-HMBPP levels to a non-impactful level (Nguyen et al., 2017).

36. Resuspend in 120 μL of T cell media using multichannel pipettor and proceed to co-culture.

Co-culture of loaded target cells with effector Vγ9Vδ2 T cells

⏲ Timing: 1 h prep, 20 h incubation

This section will describe how to mix the loaded target cells with the effector T cells for co-culture with a final volume of 200 μL per well.

37. Label a 96 well plate.
38. Count expanded Vγ9Vδ2 T cells.
39. Collect 1.2×10^6 T cells per plate.
    a. Transfer cells to a 15 mL tube.
    b. Centrifuge 3 min at 600 rcf at room temperature (20°C–25°C).
    c. Aspirate media using vacuum suction.
40. Resuspend in T cell media to 1×10^5 cells / mL.
    a. Add 12 mL of T cell media to reach a cell concentration of 1×10^5 cells / mL (1.2×10^6 cells in 12 mL).
    b. Resuspend by gentle pipetting.
41. Add 60 μL of fresh T cell media without cells to each well of a 96 well plate using a multichannel pipettor.
    a. For a media control, add 200 μL of media in duplicate wells.
    b. For co-culture conditions, 60 μL of media is first added before adding T cells and K562 cells to achieve the final volume of 200 μL (see below).
42. Distribute 120 μL of T cells to each well using a multichannel pipettor.
    a. This results in 12,000 T cells per well.
    b. We routinely distribute 80 μL of media and 120 μL of T cells to duplicate wells as a T cell only control. This is a control for the presence of T cells in a total of 200 μL solution.
    c. If loaded K562 cells are not ready to be added onto the plate, keep the plate at 37°C until ready.
43. Add 20 μL of loaded K562 cells (from step 36) to each well using a multichannel pipettor.
    a. This results in 4,000 K562 cells per well.
    b. Final effector: target cell ratio is 3:1 in final volume of 200 μL.
    c. We routinely distribute 180 μL of media and 20 μL of loaded K562 cells to duplicate wells as a K562 cells only control.
44. Incubate the cells for 20 h at 37°C and 5% CO_2.

ELISA

⏲ Timing: Overnight coating, then 5 h

This section will describe how to use ELISA to quantify the amount of interferon γ produced by the T cells. This section generally follows the recommendations of BioLegend with some modifications as noted.

45. Coat ELISA plates one day prior to the ELISA experiments, usually the same day that the co-culture is established, so that the plate can coat while the cells are incubating overnight.
46. Dilute capture antibody 1:200 in 1× coating buffer (available with kit).
    a. Though the protocol suggests making 12 mL per plate, 10 mL is sufficient providing pipetting is accurate.

Note: The BioLegend ELISA kit provides some extra reagents and can easily be stretched to use for 6 plates if all the recommended volumes are cut by 1/6.

47. Using a multichannel pipettor, coat ELISA plate with 100 μL / well of capture antibody in buffer overnight. Coat additional 16 wells for standard curve and 6 wells for controls (media only, T cells only, and K562 cells only).
    a. It is necessary to use a high protein binding plate that is designed for ELISA. A regular 96-well plate will not work.
    b. Seal with plate sealer and place in refrigerator.
48. Peel off the plate sealer the next day, use force to shake out the liquid into the sink in one quick motion.
49. Wash 4 times with Wash Buffer (PBS + 0.05% Tween 20) in spray bottle, filling up each well to a consistent level, then shaking into the sink.

Note: Attempting to wash ELISA plate wells with a multichannel pipettor or a robot is quite time consuming. We find that the spray bottle method works fastest without loss of quality. It can be made even faster by cutting the tip of the nozzle off the spray bottle. Do be careful to apply a similar volume and flow rate to the wash steps for each well.

50. After last wash, pat to dryness on a layer of paper towels, with a firm upside-down pat against a hard surface, to remove all residual liquid.
51. Dilute assay diluent (blocking buffer) to 1× with PBS.
    a. Preparing 40 mL per plate should be sufficient for the rest of the steps.
52. Block plates with 200 μL of assay diluent. Seal the plate and incubate for 1 h at room temperature on a rocker.
53. Wash 4 times and dry as above (steps 49 and 50).
54. Prepare eight 2-fold serial dilutions of the top standard from kit. We generally make the top concentration 4-fold higher than what is described in the BioLegend protocol (from 2000 pg/mL to 15.6 pg/mL), recognizing that at the highest concentration this will lose linearity.

Note: Due to potential for high interferon γ concentration, we routinely use higher concentrations of standards than recommended just in case we have underestimated the cytokine level. Because the assay loses linearity at high standard concentrations, we either drop the non-linear points from analysis or perform non-linear regression if necessary.

55. Transfer 25 μL of media (the samples) from the assay plate to ELISA plate (avoid touching the bottom of the wells).

Note: It is critical that the ELISA absorbance readings fall within the range of the standard curve and these cells should produce copious amounts of interferon γ. Therefore, we often reduce the sample volume. Because our sample volume is smaller than the standard curve volume, we adjust the final concentration to account for the difference.

56. Transfer 100 μL / well of each standard to ELISA plate.
57. Incubate sealed plate for 2 h at room temperature with gentle shaking.
58. Wash 4 times and dry as above (steps 49 and 50).
59. Dilute detection antibody 1:200 in 1× assay diluent, preparing 10 mL total per plate.
60. Add 100 μL / well of detection antibody.
61. Incubate sealed plate for 1 h at room temperature with gentle shaking.
62. Wash 4 times and dry as above (steps 49 and 50).
63. Dilute avidin-HRP 1:1000 in 10 mL of 1× assay diluent.
64. Add 100 μL / well of avidin-HRP.
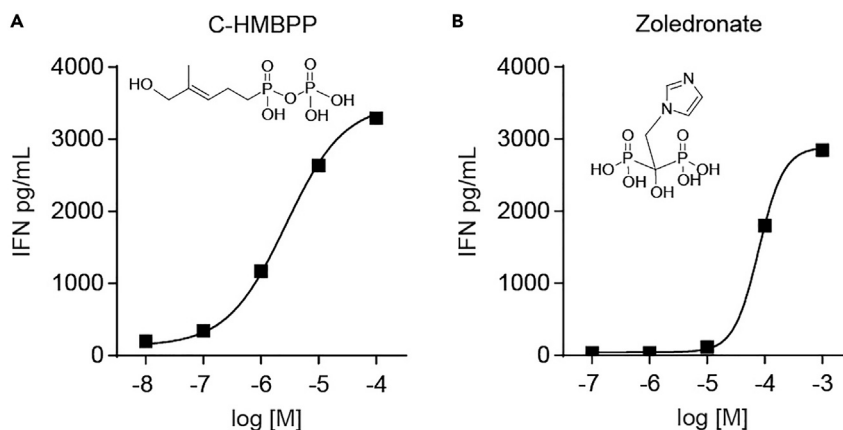65. Incubate sealed plate for 30 min at room temperature with gentle shaking.

Figure 1. Dose response curves obtained with C-HMBPP and zoledronate
Data adapted from Hsiao and Wiemer (2018) with permission.

66. Wash 5 times and dry as above (steps 49 and 50).
67. Mix TMB A and TMB B equally to form active TMB substrate solution, 10 mL total per plate.
68. Add 100 μL / well of detection reagent (TMB substrate solution).
69. Incubate 7–10 min in dark. Positive wells should turn blue in color.
70. Add 100 μL / well of stop solution when ready. Positive wells should turn color from blue to yellow.
    a. It is very important to add the stop solution, as the yellow color is detected by the plate reader.
    b. Addition of stop solution can be delayed if color is slow to develop.
71. Read absorbance at 450 nm and 550 nm (background).
    a. Manufacturer indicated to read background absorbance at 570 nm. However, due to the filter availability, we read background absorbance at 550 nm.

EXPECTED OUTCOMES

We use a wide range of doses because it is important to have at least one data point for each compound that reaches the maximum response and one data point that shows the minimum response (Figure 1). Typically, the minimum response seen in the negative controls (T cells only) is around 40–50 pg/mL of interferon γ. The response to untreated K526 cells mixed with T cells is usually between 50–200 pg/mL. The maximum response achieved at the higher doses of C-HMBPP and zoledronate in our hands averages between 3000–4000 pg/mL of interferon γ. Due to donor variability, the final concentration of interferon γ for the individual donors tends to be highly variable with maximal responses ranging from 1000–6000 pg/mL. However, the EC_{50} values of the test compounds are less variable between donors. Our reported EC_{50} for C-HMBPP at 1 h of loading time is 1.2 μM with a 95% confidence interval of 0.9–1.6 μM. For zoledronate, our reported EC_{50} is 79 μM with a 95% confidence interval of 73–85 μM (Hsiao and Wiemer, 2018). Similarly, when evaluating mutations of recombinant BTN3A1 or BTN2A1, the donor variability is high but the mutation variability is reasonable. When exploring BTN2A1 point mutations, we expressed the activity of the point mutant relative to the wild-type protein at a concentration of the test compound that produced the maximal effect (Hsiao et al., 2022). This approach was stringent enough to produce P values below 0.001 when assessed by one-way ANOVA.

QUANTIFICATION AND STATISTICAL ANALYSIS

Typically, the data generated from the ELISA are analyzed using Microsoft Excel and tested for statistical significance using GraphPad Prism.

To analyze the raw data, we first subtract the absorbance values at 550 nm from the absorbance values at 450 nm. We then plot a standard curve of the standards versus the adjusted absorbance and use the standard curve to calculate a slope. The experimental values are multiplied by the slope to calculate raw cytokine masses. They are divided by the volume, taking account the dilution factors, to calculate a concentration for each well. The duplicate wells are averaged together to produce the final concentration for that sample.

The final concentrations are transferred into GraphPad Prism for statistical analysis. Dose response data are analyzed using a log model while single concentration experiments are analyzed by ANOVA. In the log model, we use the "log(agonist) vs. response – variable slope (four parameters)" method, where the bottom and top parameters can be constrained based on the negative and positive controls. Concentrations of C-HMBPP at 1×10^{-4} M and zoledronate at 1×10^{-3} M generally produce a maximal response in this assay.

LIMITATIONS

This protocol does contain some limitations. First, not all donors are equally responsive to phosphoantigens. The results should be viewed as the activity of the compound in phosphoantigen responsive donors. Second, some phosphoantigens, such as HMBPP, have low stability. Low stability phosphoantigens do not work well for pulsing into the phosphoantigen containing cell and may fail to achieve maximal response.

TROUBLESHOOTING
Problem 1
Low yield of PBMCs following purification from buffy coat (before you begin, step 8).

Potential solution
While some donor variability is expected, low yield of PBMCs usually results from insufficient centrifugation during the wash steps. Be sure that the cells are not too dense when washing and have been appropriately diluted with PBS or media. If the pellet is smaller than expected at any step, extend the spin time.

Problem 2
Low viability/no growth of expanded Vγ9Vδ2 T cells in new donor (step 9).

Potential solution
The donor may be non-responsive. It is best to compare it to an established donor, and if the donor is a non-responder then try a different donor.

Problem 3
Low viability/no growth of expanded Vγ9Vδ2 T cells in a known responder. Cells have formed initial small colonies but then lost viability (step 9).

Potential solution
The media or one of its components may be bad. In our experience, usually this is the non-essential amino acids or the pyruvate, which have the shortest half-lives. It could also result from the FBS not being completely heat inactivated. It is better to remake the media using fresh ingredients than to try to troubleshoot a specific ingredient. The shelf life of the amino acids and pyruvate can be extended by storage of the stock solutions at −20°C.

Problem 4
Low viability/no growth of expanded Vγ9Vδ2 T cells in a known responder. Cells did not respond at all (step 9).

Potential solution

The HMBPP or IL-2 has gone bad or was not added. Even with proper storage at −80°C, the HMBPP should not be stored for longer than 2 years. We have observed some instability of the diphosphate. It is best to record the date it has been dissolved. Similarly, the IL-2 can lose activity over time even with storage at −80°C. Furthermore, while recombinant human IL-2 can stimulate mouse cells, mouse IL-2 cannot stimulate human cells well (Mosmann et al., 1987). Be sure to use human IL-2.

Problem 5

Cells expanded with HMBPP treatment, but magnetic bead isolation failed (step 25).

Potential solution

Unfortunately, the isolation kit is known to have a short shelf life and the company does not sell a smaller sized kit, making this a particularly costly part of the protocol and a potential area for future improvement. We have used the kit up to six months past the expiration date with success, but after that it does stop working. We have not yet attempted to aliquot and freeze the kit. We have tried positive selection kit from another manufacturer without success. Our current approach is to expand more cells than we need while the kit is fresh, freezing the extras in liquid nitrogen for future use when the kit has failed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrew Wiemer (andrew.wiemer@uconn.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze dataset/code.

AUTHOR CONTRIBUTIONS

Conceptualization - C.H. and A.W.; Formal Analysis - C.H. and A.W.; Funding acquisition - A.W.; Investigation - C.H. and A.W.; Project administration - A.W.; Resources - A.W.; Validation - C.H. and A.W.; Visualization - C.H. and A.W.; Writing – C.H. and A.W.; Writing – review & editing - C.H. and A.W.

DECLARATION OF INTERESTS

A.J.W. is a co-founder of Terpenoid Therapeutics. The current work did not involve the company. The other author has nothing to declare.

REFERENCES

Anderson, J., Toh, Z.Q., Reitsma, A., Do, L.A.H., Nathanielsz, J., and Licciardi, P.V. (2019). Effect of peripheral blood mononuclear cell cryopreservation on innate and adaptive immune responses. J. Immunol. Methods *465*, 61–66. https://doi.org/10.1016/j.jim.2018.11.006.

Cano, C.E., Pasero, C., De Gassart, A., Kerneur, C., Gabriac, M., Fullana, M., Granarolo, E., Hoet, R., Scotet, E., Rafia, C., et al. (2021). BTN2A1, an immune checkpoint targeting Vγ9Vδ2 T cell cytotoxicity against malignant cells. Cell Rep. *36*, 109359. https://doi.org/10.1016/j.celrep.2021.109359.

Costantini, A., Mancini, S., Giuliodoro, S., Butini, L., Regnery, C.M., Silvestri, G., and Montroni, M. (2003). Effects of cryopreservation on lymphocyte immunophenotype and function. J. Immunol. Methods *278*, 145–155. https://doi.org/10.1016/s0022-1759(03)00202-3.

Gober, H.J., Kistowska, M., Angman, L., Jeno, P., Mori, L., and De Libero, G. (2003). Human T cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J. Exp. Med. *197*, 163–168. https://doi.org/10.1084/jem.20021500.

Hsiao, C.C., Nguyen, K., Jin, Y., Vinogradova, O., and Wiemer, A.J. (2022). Ligand-induced interactions between butyrophilin 2A1 and 3A1 internal domains in the HMBPP receptor complex. Cell Chem. Biol. https://doi.org/10.1016/j.chembiol.2022.01.004.

Hsiao, C.C., and Wiemer, A.J. (2018). A power law function describes the time- and dose-dependency of Vγ9Vδ2 T cell activation by phosphoantigens. Biochem. Pharmacol. *158*, 298–304. https://doi.org/10.1016/j.bcp.2018.10.035.

Hsiao, C.H., Lin, X., Barney, R.J., Shippy, R.R., Li, J., Vinogradova, O., Wiemer, D.F., and Wiemer, A.J. (2014). Synthesis of a phosphoantigen prodrug that potently activates Vγ9Vδ2 T-lymphocytes. Chem. Biol. *21*, 945–954. https://doi.org/10.1016/j.chembiol.2014.06.006.

Karunakaran, M.M., Willcox, C.R., Salim, M., Paletta, D., Fichtner, A.S., Noll, A., Starick, L., Nohren, A., Begley, C.R., Berwick, K.A., et al. (2020). Butyrophilin-2A1 directly binds germline-encoded regions of the Vγ9Vδ2 TCR and is essential for phosphoantigen sensing. Immunity *52*, 487–498.e6. https://doi.org/10.1016/j.immuni.2020.02.014.

Kilcollins, A.M., Li, J., Hsiao, C.H., and Wiemer, A.J. (2016). HMBPP analog prodrugs bypass energy-dependent uptake to promote efficient BTN3A1-mediated malignant cell lysis by Vγ9Vδ2 T lymphocyte effectors. J. Immunol. *197*, 419–428. https://doi.org/10.4049/jimmunol.1501833.

Laplagne, C., Ligat, L., Foote, J., Lopez, F., Fournie, J.J., Laurent, C., Valitutti, S., and Poupot, M. (2021). Self-activation of Vγ9Vδ2 T cells by exogenous phosphoantigens involves TCR and butyrophilins. Cell Mol. Immunol. *18*, 1861–1870. https://doi.org/10.1038/s41423-021-00720-w.

Morita, C.T., Beckman, E.M., Bukowski, J.F., Tanaka, Y., Band, H., Bloom, B.R., Golan, D.E., and Brenner, M.B. (1995). Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. Immunity *3*, 495–507. https://doi.org/10.1016/1074-7613(95)90178-7.

Mosmann, T.R., Yokota, T., Kastelein, R., Zurawski, S.M., Arai, N., and Takebe, Y. (1987). Species-specificity of T cell stimulating activities of IL 2 and BSF-1 (IL 4): comparison of normal and recombinant, mouse and human IL 2 and BSF-1 (IL 4). J. Immunol. *138*, 1813–1816.

Nada, M.H., Wang, H., Workalemahu, G., Tanaka, Y., and Morita, C.T. (2017). Enhancing adoptive cancer immunotherapy with Vγ2Vδ2 T cells through pulse zoledronate stimulation. J. Immunother. Cancer *5*, 9. https://doi.org/10.1186/s40425-017-0209-6.

Nguyen, K., Li, J., Puthenveetil, R., Lin, X., Poe, M.M., Hsiao, C.C., Vinogradova, O., and Wiemer, A.J. (2017). The butyrophilin 3A1 intracellular domain undergoes a conformational change involving the juxtamembrane region. FASEB J. *31*, 4697–4706. https://doi.org/10.1096/fj.201601370rr.

Peigne, C.M., Leger, A., Gesnel, M.C., Konczak, F., Olive, D., Bonneville, M., Breathnach, R., and Scotet, E. (2017). The juxtamembrane domain of butyrophilin BTN3A1 controls phosphoantigen-mediated activation of human Vγ9Vδ2 T cells. J. Immunol. *198*, 4228–4234. https://doi.org/10.4049/jimmunol.1601910.

Rhodes, D.A., Chen, H.C., Price, A.J., Keeble, A.H., Davey, M.S., James, L.C., Eberl, M., and Trowsdale, J. (2015). Activation of human γδ T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin. J. Immunol. *194*, 2390–2398. https://doi.org/10.4049/jimmunol.1401064.

Rigau, M., Ostrouska, S., Fulford, T.S., Johnson, D.N., Woods, K., Ruan, Z., McWilliam, H.E.G., Hudson, C., Tutuka, C., Wheatley, A.K., et al. (2020). Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells. Science *367*, eaay5516. https://doi.org/10.1126/science.aay5516.

Roelofs, A.J., Jauhiainen, M., Monkkonen, H., Rogers, M.J., Monkkonen, J., and Thompson, K. (2009). Peripheral blood monocytes are responsible for γδ T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br. J. Haematol. *144*, 245–250. https://doi.org/10.1111/j.1365-2141.2008.07435.x.

Sandstrom, A., Peigne, C.M., Leger, A., Crooks, J.E., Konczak, F., Gesnel, M.C., Breathnach, R., Bonneville, M., Scotet, E., and Adams, E.J. (2014). The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2 T cells. Immunity *40*, 490–500. https://doi.org/10.1016/j.immuni.2014.03.003.

Ticha, O., Moos, L., and Bekeredjian-Ding, I. (2021). Effects of long-term cryopreservation of PBMC on recovery of B cell subpopulations. J. Immunol. Methods *495*, 113081. https://doi.org/10.1016/j.jim.2021.113081.

Tomogane, M., Omura, M., Sano, Y., Shimizu, D., Toda, Y., Hosogi, S., Kimura, S., and Ashihara, E. (2022). Expression level of BTN3A1 on the surface of CD14+ monocytes is a potential predictor of γδ T cell expansion efficiency. Biochem. Biophys. Res. Commun. *588*, 47–54. https://doi.org/10.1016/j.bbrc.2021.12.060.

Vyborova, A., Beringer, D.X., Fasci, D., Karaiskaki, F., van Diest, E., Kramer, L., de Haas, A., Sanders, J., Janssen, A., Straetemans, T., et al. (2020). γ9δ2T cell diversity and the receptor interface with tumor cells. J. Clin. Invest. *130*, 4637–4651. https://doi.org/10.1172/JCI132489.

Wang, H., and Morita, C.T. (2015). Sensor function for butyrophilin 3A1 in prenyl pyrophosphate stimulation of human Vγ2Vδ2 T cells. J. Immunol. *195*, 4583–4594. https://doi.org/10.4049/jimmunol.1500314.

Wiemer, A.J. (2020). Structure-activity relationships of butyrophilin 3 ligands. ChemMedChem *15*, 1030–1039. https://doi.org/10.1002/cmdc.202000198.

Zumwalde, N.A., Haag, J.D., Sharma, D., Mirrielees, J.A., Wilke, L.G., Gould, M.N., and Gumperz, J.E. (2016). Analysis of immune cells from human mammary ductal epithelial organoids reveals Vδ2+ T cells that efficiently target breast carcinoma cells in the presence of bisphosphonate. Cancer Prev. Res. *9*, 305–316. https://doi.org/10.1158/1940-6207.capr-15-0370-t.