Optimizing a panel for flow cytometry to determine ASA's mechanism of inhibition on the mTOR pathway.

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INTRODUCTION

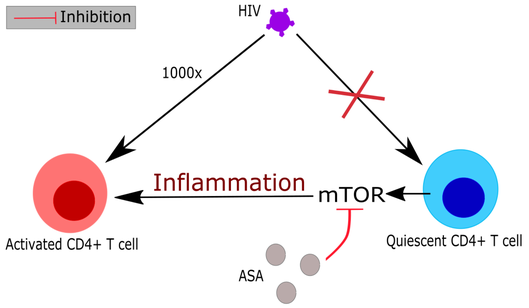


Figure 1. Graphic representation of immune quiescence. HIV replicates 1000 times more effectively in active CD4+ T cells than quiescent CD4+ T cells. mTOR induces inflammation and is involved in the activation quiescent cells. Acetylsalicylic acid (ASA) can inhibit mTOR, a potential method of inducing a quiescent phenotype.

- A quiescent phenotype is observed naturally in HIV-exposed seronegative (HESN) commercial sex workers in Nairobi, Kenya, who have lower levels of CD4+ T cell activation but can otherwise elicit a normal immune response.
- Previously, we found that found that low dose acetylsalicylic acid (ASA, brand name Aspirin) (81 mg/day) reduced HIV target cells in the female genital tract. This revealed ASA's potential in preventing HIV.

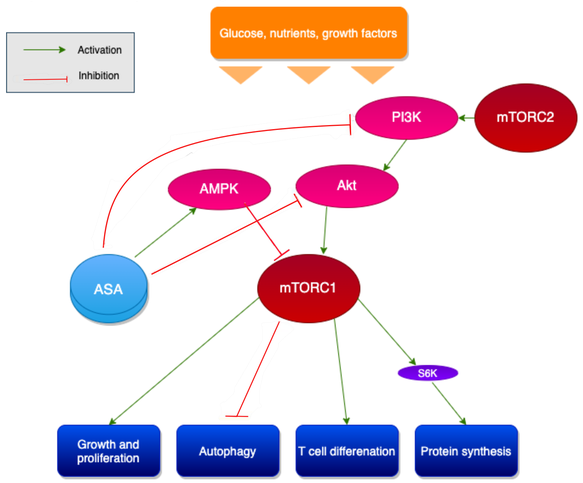


Figure 2. Graphic summary of ASA's inhibitory effect on the mTOR pathway. Orange – mTOR pathway stimuli, pink – upstream kinase, red – mTOR complex, light blue – ASA, purple – downstream effector protein, dark blue – cell functions regulated by mTOR.

- Here, we demonstrate that we have optimized a panel for flow cytometry that will allow us to determine ASA's effect on the mTOR pathway in CD4+ T cells.

OBJECTIVES & WORKFLOW

A Antibody Titration
- Reduce background noise from non-specific staining

B Plate Staining Titration
- Reduce background noise from non-specific staining

C CD3CD28 Stimulation Titration
- Replace CD3/CD28 beads with pCD3[a] and sCD28[b] to improve efficiency
- Control ratio of pCD3[a] to pCD28[b]

D Volt Titration
- Determine ideal voltage before running experimental samples (data not shown)

Figure 3. Workflow of panel optimization including objectives of each step. [a] Plate-bound CD3. [b] Soluble CD28.

METHODS

1. PBMC[a] stimulated with CD3/CD28 Dynabeads or pCD3[b] and sCD28[c] for 72 hours
2. PMBC[a] were stained with intracellular and extracellular antibodies, and live/dead stain
3. PMBC[a] were fixed and permeabilized
4. Data collected on the LSRFortessa cytometer
5. Data analyzed using FlowJo, graphs prepared in Excel

Figure 4. Overview of methods used to prepare cells for flow cytometry, data collection, and analysis. [a] Peripheral blood mononuclear cells. [b] Plate-bound CD3. [c] Soluble CD28.

RESULTS

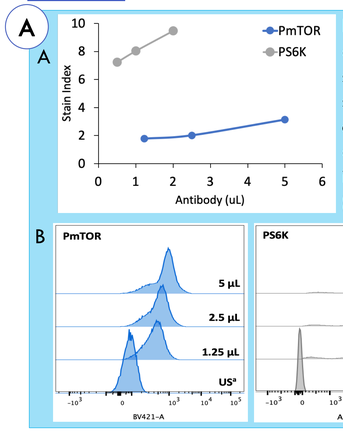


Figure 5. Determining optimal antibody volumes for intracellular staining. Cells were single-stained with different concentrations of PmTOR or PS6K antibody. (A) Results from intracellular antibody titration. (B) Spread of intracellular marker expression. [a] Unstained.

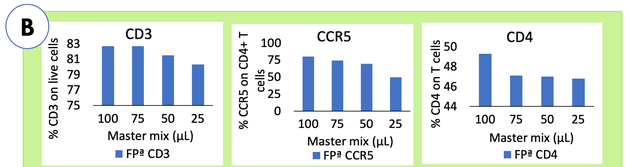


Figure 6. Titration curves from extracellular plate staining titration. Master mix containing extracellular antibodies was titrated at different volumes. [a] Frequency of parent.

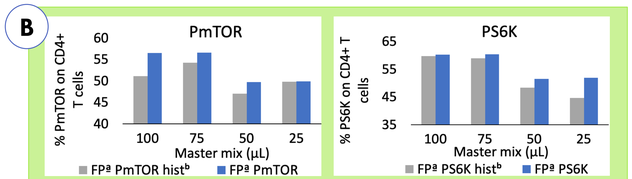
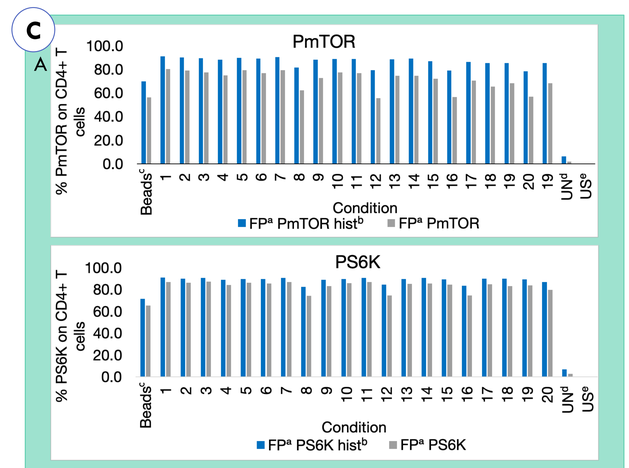


Figure 7. Titration curves from intracellular plate staining titration. Master mix containing intracellular antibodies was titrated at different volumes. [a] Frequency of parent. [b] histogram.



B

Condition	pCD3[f] µg	sCD28[g] µg	pCD3/sCD28
Beads[c]	N/A	N/A	N/A
1	0.2	0.025	8
2	0.1	0.025	4
3	0.067	0.025	2.68
4	0.05	0.025	2
5	0.2	0.02	10
6	0.1	0.02	5
7	0.067	0.02	3.35
8	0.05	0.02	2.5
9	0.2	0.013	15.385
10	0.1	0.013	7.692
11	0.067	0.013	5.154
12	0.05	0.013	3.846
13	0.2	0.01	20
14	0.1	0.01	10
15	0.067	0.01	6.7
16	0.05	0.01	5
17	0.2	0.005	40
18	0.1	0.005	20
19	0.067	0.005	13.4
20	0.05	0.005	10
UN[d]	0	0	0
US[e]	0	0	0

Figure 8. Third CD3CD28 stimulation titration. CD3/CD28 Dynabeads were run beside different ratios of pCD3 and sCD8. (A) Titration curves. (B) Ratios of pCD3/pCD28 tested corresponding to conditions in the titration curves. [a] Frequency of parent. [b] histogram. [c] CD3/CD28 Dynabeads. [d]Unstimulated. [e]Unstained. [f]Plate-bound CD3. [g]Soluble CD28.

DISCUSSION

- 5 uL of PmTOR and 2 uL of PS6K were selected based off stain indices (Figure 5).
- 50 uL of extracellular master mix (Figure 6) and 75 uL of intracellular master mix (Figure 7) were selected based off titration curves.
- Condition 16 was selected for the low range and Condition 6 was selected for the high range pCD3 and sCD28 stimulation (Figure 8).
- Ideal voltages on the cytometer were selected to use during our study (data not shown).

CONCLUSION

- Optimization ensures the reliability and reproducibility of the experiment and reduces day to day variation during experimentation.
- We have optimized a panel for flow cytometry to determine ASA's mechanism of inhibition on the mTOR pathway in CD4+ T cells.

SIGNIFICANCE

- Determining ASA's mechanism of inhibition on the mTOR pathway will provide more insight into how ASA induces a quiescent phenotype in the female genital tract.

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- Participants