Intracellular Staining of PUMA in Primary PBMC Lymphocytes V.1

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Protocol status: Working
We use this protocol and it's working

DISCLAIMER

ABSTRACT

Flow cytometric assessment of Intracellular PUMA levels is useful when wanting to assess PBMC subsets but are limited by sample as with primary patient samples. Here I describe a protocol for intracellular PUMA staining validated with PUMA knockdown and induction with PUMA inducing treatments.

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MATERIALS

10X Intracellular Staining Buffer
1% Saponin, 10% BSA, 20% FBS, 0.02% Sodium Azide, PBS
Per 50 mL combine:
10 mL FBS
500 mg saponin
5 g BSA
Dissolve in 50 mL PBS
Add 100uL 10% Sodium Azide (Final concentration 3mM. Saponin can contain bacterial spores so a
preservative should be added)
Sterile filter. May have to sterile filter multiple times to remove particulates. Store at 4C.

Staining

1 Treat at least 1 million PBMCs per sample, including flow cytometry controls (unstained, single stain controls, and FMO controls)

2 Harvest cells at 20 hours and wash with PBS.

3 Stain cells with anti-CD3, anti-CD4 and anti-CD19 for 20 minutes at 4 degrees in the dark. Create unstained, single stain controls, and FMO controls.

4 After staining, wash with PBS and resuspend 1 million cells in 100uL of PBS-diluted 2% formaldehyde for 10 minutes at RT.

5 Add 1mL of PBS and spin down the cells at 800xg for 5 minutes.

6 Wash two times with 1x intracellular staining buffer (see materials).

7 Stain for intracellular PUMA in 1x intracellular staining buffer: 50ul staining volume per sample with 1:50 final dilution Puma (Cell signaling D30C10) Rabbit mAb

8 Stain at 4 degrees for 1 hour in the dark.

9 Spin down and wash two times with 1x intracellular buffer.

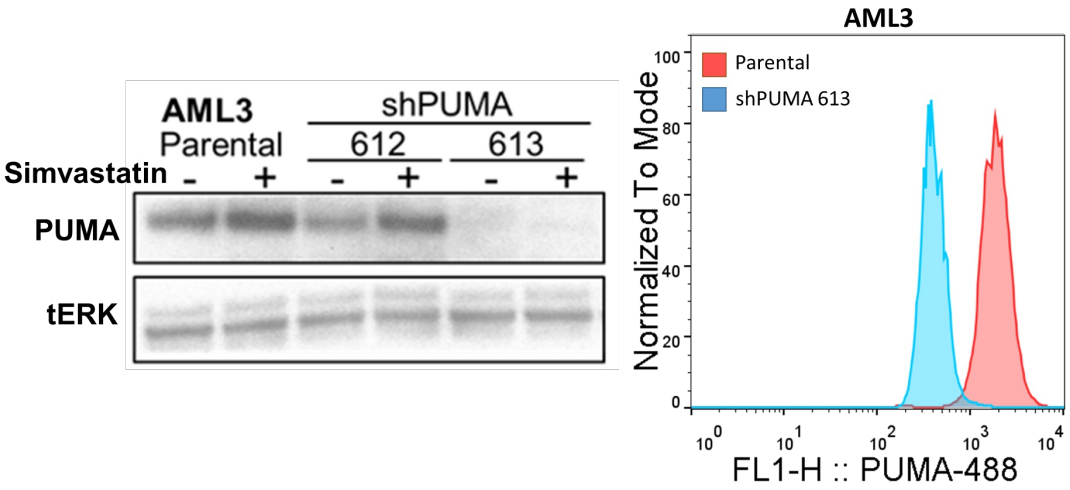
10 Prepare the secondary Invitrogen's anti-Rabbit IgG 647 #A-21244 at Final Dilution 1:500 in 1x staining buffer. Be sure to stain the PUMA FMO with the secondary antibody.

11 Stain for 1 hr in the dark at RT.

12 Wash twice with 1x intracellular buffer and once with PBS. Resuspend in 100-150uL PBS and samples are ready to run.

Validation

13 Protocol used in PUMA knockdown cell line validated by western blot.



Lower levels of PUMA in PUMA knockdown cell lines.

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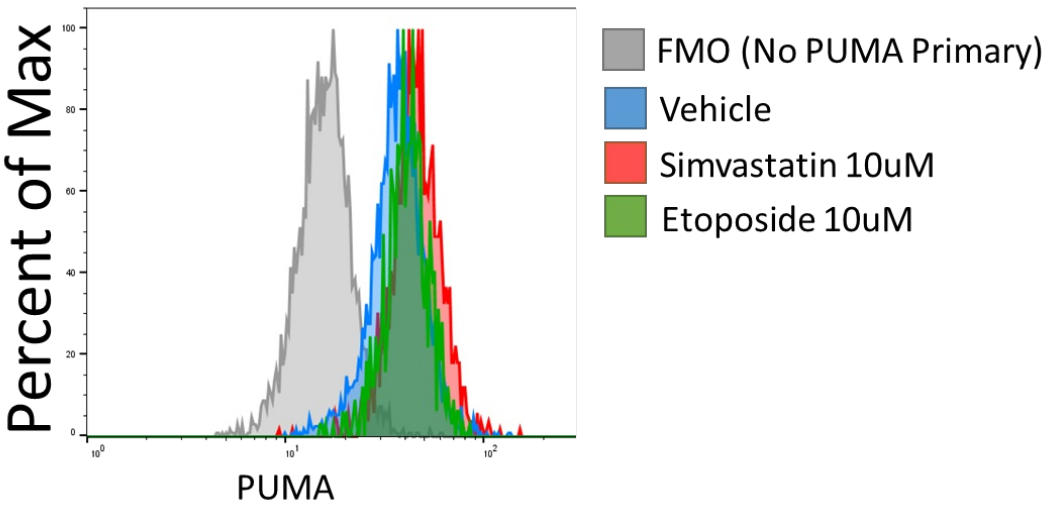

L363 normally upregulate PUMA 2 fold by western. Differences in treatment-induced PUMA upregulation by flow is smaller, closer to 1.4 fold difference after subtracting off MFI of PUMA FMO. This is suggestive of off-target binding of the PUMA antibody that cannot be corrected for with FMO. Nevertheless, this assay still can capture the increase in PUMA by simvastatin and etoposide.

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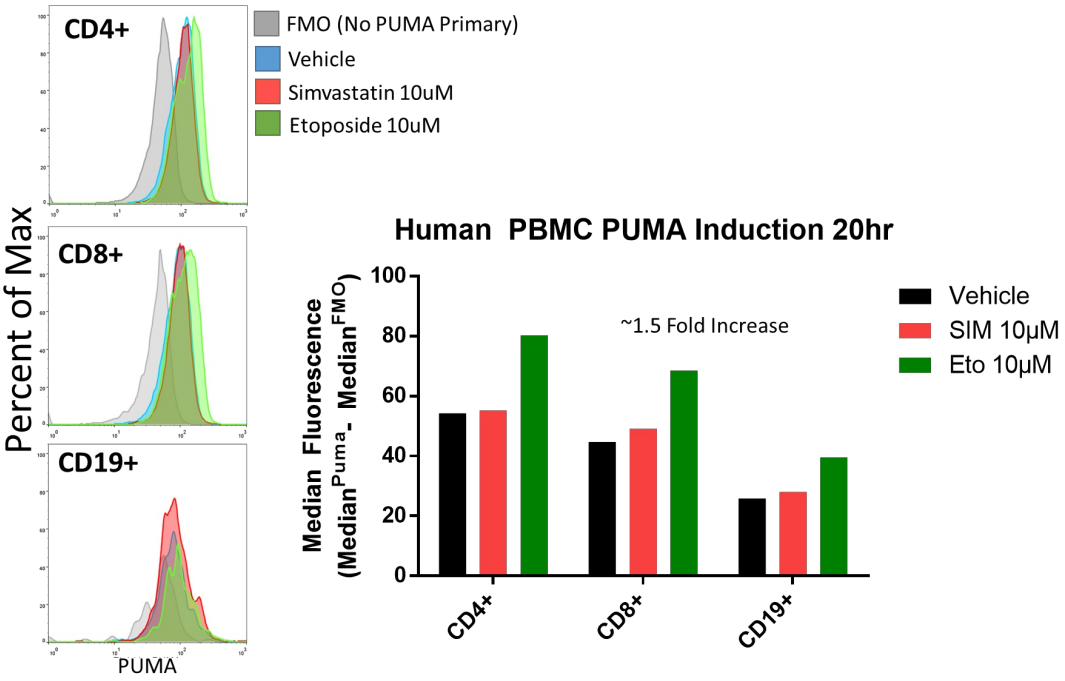

Etoposide upregulates PUMA levels in normal PBMC whereas simvastatin does not., consistent with western blot.