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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

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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.

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.

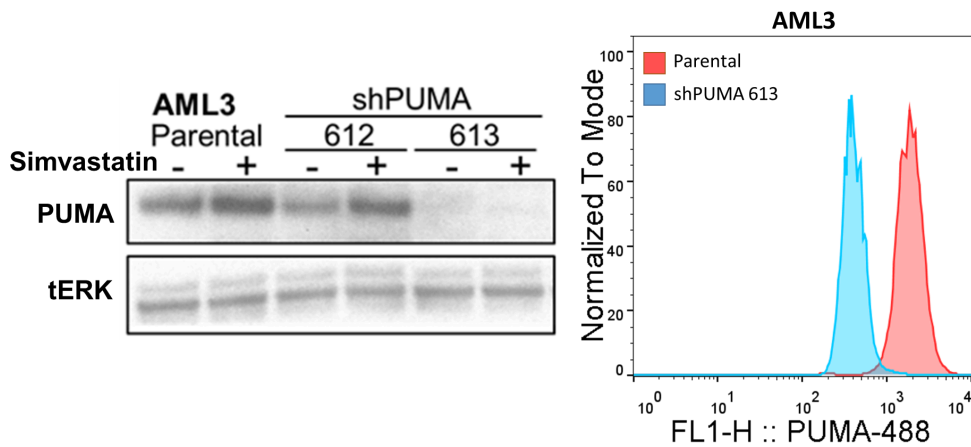
Troubleshooting

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.

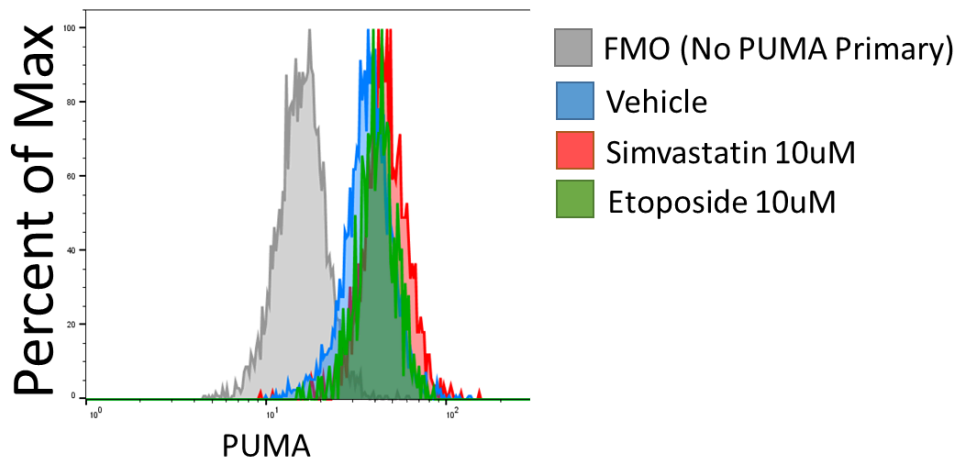
Validation

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



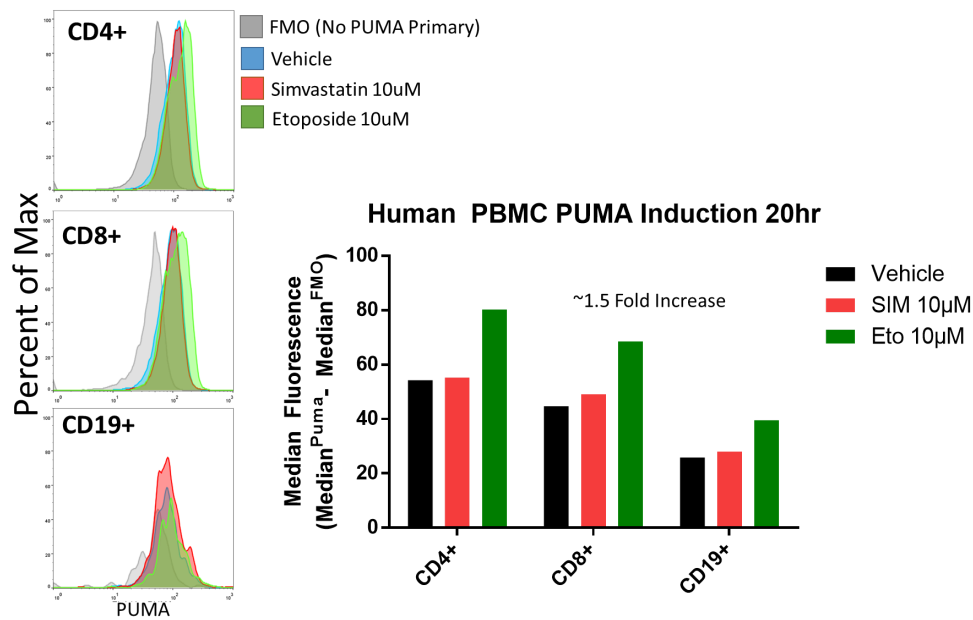
Lower levels of PUMA in PUMA knockdown cell lines.

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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.