ADVL0317 study

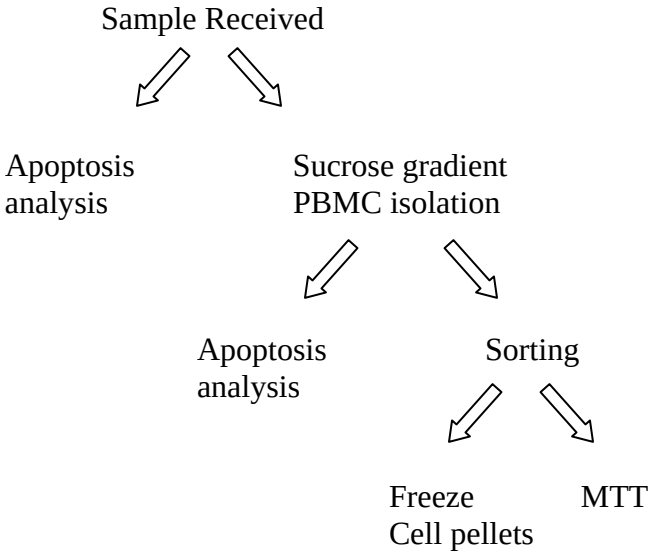
(PS-341/bortezomib in patients with leukemia)

I. Overview of study:

We are interested in determining the amount of apoptosis in leukemic samples before and after drug treatment, which we determine by **flow cytometry** (analysis of annexin/7AAD). We also **freeze cell pellets** (for protein and DNA work) and (if cells are available) perform an in vitro **cytotoxicity assay** using MTT (Kathy Scorsone will do this part).

If the percent blasts is high in the marrow sample (≥90%), there is no need to **FACS sort** the sample. However, if the % blasts is <90% in the bone marrow, Chris will sort out the lymphoblasts (and normal lymphocytes if there are enough) for us to use for cell freezing and (if there are enough) MTT analysis. We do the flow cytometry on samples before PBMC isolation and after PBMC isolation by sucrose gradient. Cells analyzed by flow cytometry before PBMC isolation are processed using PharmLyse, which is similar to FACS-lyse.

Overview diagram:



II. Sample preparation

A. the night before: set up positive control for Annexin flow cytometry assay:
   a. Count ALL cell line and add $1x10^6$ cells into 2 ml RPMI + 10% BGS in 6-well assay plate.
   b. Add 2 ul 1 mg/ml actinomycin D (final concentration 1 ug/ml).
   c. Mix by rocking plate.
   d. Incubate overnight at 37 C, 5% CO_2.

B. contacting folks: We will either receive either a single peripheral blood sample (if the WBC count is high) or both peripheral blood and bone marrow. Once we know a marrow is coming, please contact Mike Cubbage/Chris for cell sorting @4-4252 and contact Tartiyana for FACS at @4-4791 (usually a one-hour slot). Samples arrive by FedEx between 10-11 am. Book slots for 1pm for flow sorting and in the afternoon for Tartiyana (FAX analysis) on the same day. The samples will arrive pretreatment, on day 1 (no sorting), day 8 (sorting) and day 18 (sorting), so slots can be booked ahead once the treatment start date is known.

- Put the Lymphoprep (for cell separation) or Histopaque (for sorting) at room temperature on the morning of sample arrival.
- When the sample is received
    1. Log into logbook, ask Terzah for sample # (COG#) if not on sheets that come with sample. Record sample type, diagnosis and shipment condition
    2. log in volume of each sample.
- Take an aliquot of 100ul of marrow in an Eppendorf tube to April Durrett (4-4666, 11th floor) for WBC count. The WBC count tells how to dilute sample for sucrose gradient
- Take an aliquot of 200ul marrow in an Eppendorf tube to Mike (4-4252, 10th floor) for blast %. This % helps to decide if the sample needs to be sorted.
- ***Before PBMC isolation** : remove 500,000 cells (~500ul if 1000 WBC/ul, or 100 ul if 5000 WBC/ul) peripheral blood and 2,000,000 cells bone marrow for apoptosis analysis. Place in 3 ml FACs tubes and set aside for PharmLyse and staining. (see below)

Isolation of PBMC by sucrose gradient:

- Label 50 ml polypropylene tubes. In a 50ml polypropylene tube add 15ml of Lymphoprep solution (if sample needs to be sorted use Histopaque instead of Lymphoprep). If total WBC in the peripheral blood is >4000 cells/ul, dilute to 4X10^{6}cells/ml in HBSS medium. Also dilute the bone marrow sample in HBSS medium to a cell count of 4x106 cells/ml (based on WBC count from April)
- Carefully layer diluted blood and marrow samples over an equal volume of sucrose solution. Try not to mix the layers.
- Centrifuge the tubes for 30 min at 1200rpm (300xg). After centrifugation the sample will layer:
    - bottom layer: RBC's
    - Next layer: (slightly turbid) lymphoprep or Histopaque
    - Third layer: *thin layer of cells (PBMC)*
    - top layer: media/plasma layer.
    - Note: The cells are at the *interface* between lymphoprep and plasma.
- Remove the interface with a pipettman (p1000) into a clean, labeled 50 ml tube. Dilute the PBMC to 10ml with 1XPBS and mix gently. Take an aliquot of 90ul and add 10ul of tryphan blue (1:1). Count the cells using hemocytometer.
- Add 30ml 1XPBS with 2%FBS (=FACS wash buffer) to cells and spin the cells for 5-7 min @1000rpm. Aspirate supernatant.

- Remove aliquot of cells for FACS analysis (see below) and process the rest for sorting (if needed):

If sorting needed:
----- **Step 1.** Sorting (do first)
----- **Step 2**. Apoptosis (set aside)
----- **Step 3**. Freeze pellets (1X10^7 cells)-after sort
----- **Step 4**. MTT assay (3.7X10^6 cells)-after sort
or before sort if % blast >70%. Can skip if cell number low (dilute to 1X10^6 cells/ml and bring to Kathy@4-4028)

If no sorting needed
----- **Step 2**. Apoptosis
----- **Step 3**. Freeze pellets (1X10^7 cells per tube, can freeze up to 10 tubes if lots of cells)
----- **Step 4**. MTT assay (3.7X10^6 cells min, can use up to 2x10^7 cell if available). See above caveats.

Step 1. Cell Sorting: (done on 10th floor in Flow lab –Mike/Chris@ 4-4252)

- Before sorting: remove 5X10^5 to 1X10^6 cells/sample for apoptosis/FACS analysis (step 2) and remove 2x10^6 cells of marrow (for gating in apoptosis assay).
- Resuspend remaining cells in 750ul of 1X binding buffer. If more than 4X10^7 cells, resuspend cell to 4X10^7 cells/ml in 1X binding buffer.
- Add CD45-APC- 1ul for each 1X10^6 cells. Vortex briefly and incubate 10min in dark @room temp. If the cells are low CD45 expressers (Mike will let you know), also add CD10-FITC (1 ul/1x10^6 cells)
- If >750ul divide sample into 500ul for each 3ml tube for spinning (for eg: if 8X10^7 cell, add 2ml 1Xbinding buffer, incubate @ RT, then divide into 4 tubes (500ul each) for centrifugation).
- Wash cells once with 2 ½ ml 1XFACS wash buffer. Take an aliquot and count the cells.
- Resuspend to 4X10^7 cells/ml in RPMI1640 + 10%FBS + antibiotics in opaque plastic tubes for sorting. Place 4X10^7 cells/ml (1ml) in each tube. Take some extra clear 3ml tubes with 500 ul medium (500ul RPMI1640 + 20%FBS + antibiotics) to FACS lab to place the sorted cells into after sorting.
- After sorting plan the experiments accordingly – (cell count usually on side of tubes)
  -if 1X10^7 cells or less –freeze as cell pellets only: go to step3 (i.e., Freeze pellets for protein lysates) below
  -if >1.4X10^7 cells freeze cell pellets (step 3) and dilute cells for MTT assay (step 4) below.

Step 2. FACS analysis(done on 7th floor in FACS lab-Tartiyana@4-4791)

For each sample tube 1-3X10^5 cells are needed for staining. ALWAYS run one control (like Jurkat, JM1 etc) AND gates with the patient samples:

Positive control: Terzah will prepare control (cell line cells treated with 1ng/ul of Actinomycin D O/N). Will have 300,000 treated cells in incubator in cell culture room.

Gates: You can skimp on the number of cells needed for gates if the patient marrow cell count is low (100,000/gate x 3 = 300,000 cells). Stain each of these cell populations with only one antibody. (see FACs worksheet)

It is good idea to **prepare the FACS worksheet** before starting the exp. Fill in the sheet and make a copy for Tartiyana to submit along with samples.

PharmLyse Procedure:

Use for samples set aside prior to sucrose gradient. This aliquot will have a lot of RBC, which we can remove using the PharmLyse reagent. Dilute 1 ml Pharmlyse concentrate up to 10 ml in ddH20.

- Add sample (up to 200 ul) to 2 ml PharmLyse reagent
  - If lysing more than 200 ul, scale up amount of Pharm lyse 1:100 i.e. 5 ml of PharmLyse for 500 ul of blood or marrow.
- Vortex sample and incubate in the dark for 15 minutes
  - If lysing volume is more than 2 ml, it helps to rotate the tube or vortex it every 2-3 minutes to help the lysing process
- Centrifuge at 100 rmp (200xg) for 5 minutes
- Remove the supernatant (will be red) and wash the pellet with FACs wash buffer (fill tube)
- Count the cells after lysis and washing and dilute to $1x10^6$/ml for FACs analysis. (use 100-500 ul for each sample).
  - Freeze remaining cells as a cell pellet (Step 3) if lots of extra (more than $5x10^6$ cells)

Based on results from Flow lab, cells will be stained with either CD45-APC alone (if bright CD45) or with CD45-APC and CD10-FITC (if dull CD45)

- Spin the cells ($3X10^5$-$5X10^5$ for each sample) in a 3ml FACS tube for 5min @100rpm at 4^0C. Aspirate supernatant.
- Wash pellet once with 2.5ml FACS wash buffer and resuspend in 500ul 1X binding buffer
- Stain the cells with CD45-APC (and CD10-FITC if needed) in appropriate tubes (see FACS template sheet)
- Incubate in dark for 10min
- Wash once in cold FACS buffer (2 ½ ml) and resuspend cells in 500ul 1X binding buffer
- Add 2ul Annexin-FITC to appropriate tubes
- Add 0.5ul 7AAD to appropriate tubes
- Incubate 10min in dark and wash once in cold FACS buffer
- Resuspend in 500ul FACS wash buffer and bring to Tartiyana (in box to keep dark) along with completed FACS template sheet.

Step 3. Freeze pellets for protein lysates: (stored at -80^0C)

- Freeze all cells remaining after completing Apoptosis or MTT (if done)

- Wash cells in 10ml cold 1XPBS twice
- Resuspend cells to $1X10^7$cells/ml
- Label the tubes with sample name/no, type of sample (either blood or marrow), cell no and date prepped. For example:
    - Top of tube: COG #____, blood, pretreatment
    - Side of tube: number of cells frozen, date prepped
- Aliquot 1ml into Eppendorf tubes and spin in cold microcentrifuge. (1000 rpm for 7 min) Aspirate the supernatant.
- Freeze cell pellets at -80^0C and note in the lab notebook and logbook where tubes have been stored.

Step4. MTT assay:

- Resuspend $3.7X10^6$ cells to $1X10^7$ cells ($2X10^7$ cells if have extra) to $1X10^6$ cells/ml in RPMI1640 + 20%FBS + pen-strep & gentamycin .
- Bring cells to Kathy Scorsone (Extension 4-4219) (or Terzah) for plating in MTT assay. NOTE: leave resuspended cells 37^0C incubator in tissue culture flask until use. If in 50ml conical tube, loosen 50ml conical cap so cells can breathe

Instructions for Kathy:

- Dilute +control cells (Jurkat) to $0.75X10^5$/ml
- Plate primary cells using robot (if $1x10^7$ cells) or just three rows by hand if only $4x10^6$ cells (columns 3-11 only)
- On Day 2 add drug PS341 (31ul stock@1.3mM + 4ml of 1XPBS- 1:4 dilution)

#	Drug dilutions	Working stock (nM)	Final conc. (nM)
(1)	31ul stock into 4 ml 1XPBS	10,000	1000
(2)	1ml of (1) into 3 ml 1XPBS	2500	250
(3)	1ml of (2) into 3 ml 1XPBS	620	62
(4)	1ml of (3) into 3 ml 1XPBS	150	15
(5)	1ml of (4) into 3 ml 1XPBS	40	4
(6)	1ml of (5) into 3 ml 1XPBS	10	1
(7)	1ml of (6) into 3 ml 1XPBS	2.5	0.25
(8)	1ml of (7) into 3 ml 1XPBS	0.6	0.06

- Incubate the plates for 48 hrs
- On last day harvest the cells after adding MTT.

FACS Template sheet:

Gate controls	**CD45-APC**	**CD10-FITC (if needed)**	**Annexin-PE**	**7AAD**
1. Marrow	+	-	-	-
2 Marrow	-	+	-	-
3. Marrow	-	-	+	-
4. Marrow	-	-	-	+
5. + Control (Jurkat +Actinomyin)	+	+	+	+
6a. Peripheral Blood Before sucrose gradient	+	+	+	+
7a. Peripheral blood After sucrose gradient	+	+	+	+
8a. Marrow Before sucrose gradient	+	+	+	+
9a. Marrow After sucrose gradient	+	+	+	+
6b . Pre.txt (Day 1)— Before sucrose gradient	+	+	+	+
7b. Pre-tx (day 1)-- After sucrose gradient	+	+	+	+
8b . 6 hrs txt (Day 1) Before sucrose gradient	+	+	+	+
9b. 6 hours txt (Day1) After sucrose gradient	+	+	+	+
10. 12 hrs txt Day 1) Before sucrose gradient	+	+	+	+
11. 12 hrs txt (Day 1) After sucrose gradient	+	+	+	+
12. 24 hrs txt (Day 1) Before sucrose gradient	+	+	+	+
13. 24 hrs txt (Day 1) After sucrose gradient	+	+	+	+