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Authors

Vora, Ashni A

Mondala, Phoebe K

Costello, Caitlin

et al.

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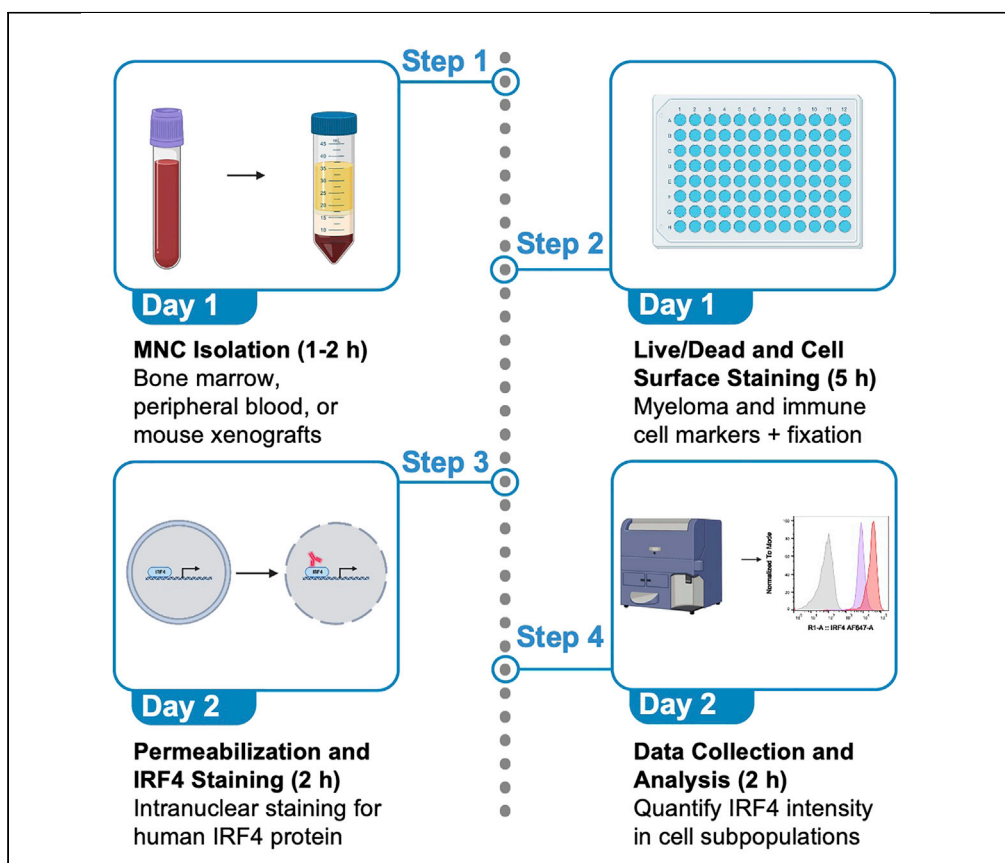
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Protocol

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Ashni A. Vora,
Phoebe K. Mondala,
Caitlin Costello, A.
Robert MacLeod,
Leslie A. Crews

lcrews@ucsd.edu

Highlights

Protocol for the quantification of human IRF4 levels by multi-parameter flow cytometry

Facilitates sensitive detection of IRF4 protein in myeloma cells and normal bone marrow

High-risk myeloma is typified by a population of CD38-high, IRF4-enriched cells

Interferon regulatory factor 4 (IRF4) is a transcription factor that regulates normal and malignant immune cell development and is implicated in multiple myeloma pathogenesis. This protocol describes the use of combined cell surface and intranuclear staining with fluorescent antibodies to measure IRF4 protein expression within myeloma and normal immune cells. IRF4 protein quantification may provide a valuable prognostic tool to predict disease severity and sensitivity to IRF4-targeted therapies. This flow-cytometry-based procedure could also be rapidly translated into a clinically compatible assay.

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Protocol

Sensitive intranuclear flow cytometric quantification of IRF4 protein in multiple myeloma and normal human hematopoietic cells

Ashni A. Vora,¹ Phoebe K. Mondala,¹ Caitlin Costello,^{2,3} A. Robert MacLeod,⁴ and Leslie A. Crews^{1,3,5,6,*}

¹Division of Regenerative Medicine, Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

²Division of Blood and Marrow Transplantation, Department of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

³Moore's Cancer Center at University of California, San Diego, La Jolla, CA 92093, USA

⁴Ionis Pharmaceuticals, Carlsbad, CA 92008, USA

⁵Lead contact

⁶Technical contact

*Correspondence: lcrews@ucsd.edu

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SUMMARY

Interferon regulatory factor 4 (IRF4) is a transcription factor that regulates normal and malignant immune cell development and is implicated in multiple myeloma pathogenesis. This protocol describes the use of combined cell surface and intranuclear staining with fluorescent antibodies to measure IRF4 protein expression within myeloma and normal immune cells. IRF4 protein quantification may provide a valuable prognostic tool to predict disease severity and sensitivity to IRF4-targeted therapies. This flow-cytometry-based procedure could also be rapidly translated into a clinically compatible assay.

For complete details on the use and execution of this protocol, please refer to Mondala et al. (2021).

BEFORE YOU BEGIN

Background

The protocol below describes the specific steps to perform combined cell surface immunolabeling with intranuclear staining and quantification of human IRF4 protein expression in primary human bone marrow mononuclear cells (BM-MNCs) or peripheral blood mononuclear cells (PBMCs) from multiple myeloma (MM) patients or healthy controls. In MM patients, abundant IRF4 expression is associated with high-risk disease (Mondala et al., 2021; Shaffer et al., 2008) and lower overall survival rates (Heintzel et al., 2008; Lopez-Girona et al., 2011). In addition, expression of other interferon-responsive and pro-inflammatory genes has been associated with cancer stem cell (CSC) generation in MM and a variety of hematological malignancies (Crews et al., 2016; Crews et al., 2015; Jiang et al., 2013; Jiang et al., 2017; Jiang et al., 2021; Lazzari et al., 2017). Thus, sensitive detection of transcription factor expression levels of IRF4 and other IRFs may have clinical relevance as prognostic tools in MM (Agnarelli et al., 2018; Heintzel et al., 2008) and other blood cancers. In previous MM immunohistochemistry studies, protein levels of IRF4 were shown to be higher in International Staging System (ISS) Stage III MM compared with Stage I and II samples (Bai et al., 2017), however a more quantitative multi-parameter detection method such as flow cytometry would be preferred to enable future clinical use of IRF4 detection assays.

This protocol is optimized for detecting IRF4 protein expression within malignant myeloma cells and other immune cell populations in a 96-well plate-based format using a two-day protocol. We have



also successfully applied this protocol, or streamlined variations of it (with fewer cell surface antibodies included), for use in tissues from patient-derived xenograft (PDX) models of MM (Lazzari et al., 2017) and in human myeloma cell lines (Mondala et al., 2021). In addition, this protocol can optionally be completed within a single day, which is preferred when optimizing additional cell surface antibodies to allow for direct comparison of fixed versus unfixed conditions.

Mononuclear cell isolation

⌚ Timing: 1–2 h

1. Prepare staining media (STM) wash buffer, to be used for MNC isolation as well as cell surface staining wash steps and incubations.
 - a. Supplement 500 mL of HBSS (without calcium and magnesium) with 2% fetal bovine serum (FBS) and 2 mM EDTA.
 - b. Store on ice or at 4°C (sterile buffer can be stored at 4°C for up to 4 weeks).
2. Freshly isolate the MNC fraction from a human bone marrow or peripheral blood sample using standard Ficoll density gradient centrifugation protocols, or carefully thaw a vial of frozen MNCs according to standard cell thawing procedures.
 - a. Standard density gradient centrifugation protocols using Ficoll-Paque™ can be found at: https://us.vwr.com/assetsvc/asset/en_US/id/16286835/contents. Application-specific protocols including isolation of plasma pre-Ficoll are also available through the Human Immune Monitoring Center at Stanford University (<https://iti.stanford.edu/himc/protocols.html>).
 - b. For more rapid processing, SepMate isolation tubes (Stem Cell Technologies) can also be used.
3. After isolation or thaw, count MNCs using a live/dead stain (Trypan blue or similar) and keep the cells in STM on ice.

Buffer dilutions and solution preparation

⌚ Timing: 30 min

4. Prepare a 1× fixative solution by diluting the 4× True-Nuclear Fix Concentrate (contains paraformaldehyde) provided in the BioLegend True-Nuclear kit.
 - a. Dilute Fix Concentrate at 1:4 in True-Nuclear Fix Diluent (1 part fixative to 3 parts diluent).
 - b. Prepare a sufficient total volume of fixative solution to allow 200 µL per well for all experimental samples and controls, plus an excess of at least 10% (200 µL × # of samples × 1.1).
5. Prepare a 1× permeabilization solution by diluting the 10× Perm Buffer (in the BioLegend kit) at 1:10 in purified water (Ultrapure sterile water is preferred).
 - a. Prepare a sufficient total volume of permeabilization solution to allow 1.3 mL per well for all experimental samples and controls, plus an excess of at least 10% (1.3 mL × # of samples × 1.1).
 - b. Refer to the True-Nuclear kit technical data sheet for supplemental guidance on buffer preparation and use (<https://www.biolegend.com/en-us/products/true-nuclear-transcription-factor-buffer-set-10859>).
6. Prepare Near-IR live/dead dye stock solution according to the manufacturer's guidelines.

Note: The diluted True-Nuclear Fix and Perm buffers should be made fresh on the day of use and should be used within 24 h (diluted buffers can be stored on ice or at 4°C until used in staining).

△ CRITICAL: Ensure that the diluted fixative solution is kept in the dark at 4°C until it is ready to use in the fixation step. Maintain all buffers and your experimental samples on ice or at 4°C until fixation (with the exception of the live/dead dye incubation step, which is performed at 20°C–25°C).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD38 PE-Cy7 (clone HB7) (product tested with this protocol, for research use only, GMP)	BD Biosciences	Cat# 335790; RRID: AB_399969
CD38 PE-Cy7 (clone HB7) (alternative product approved for in vitro diagnostic use)	BD Biosciences	Cat# 335825; RRID: AB_2868688
CD19 PE (clone HIB19)	BioLegend	Cat# 302208; RRID: AB_314238
IRF4 AF647 (clone IRF4.3E4)	BioLegend	Cat# 646408; RRID:AB_2564048
CD3 AF488 (clone HIT3a)	BioLegend	Cat# 300320; RRID:AB_493691
CD14 BV605 (clone M5E2)	BioLegend	Cat# 301834; RRID:AB_2563798
CD138 VioBlue (clone 44F9)	Miltenyi	Cat# 130-119-843; RRID: AB_2751882
Biological samples		
MM patient samples	Dr. Caitlin Costello (UC San Diego Moores Cancer Center)	N/A
PCL patient samples	Dr. Caitlin Costello and Dr. Mark Minden (University of Toronto)	N/A
Chemicals, peptides, and recombinant proteins		
Human FcR Block	Miltenyi	Cat# 130-059-901
Mouse FcR Block (optional)	BD Biosciences	Cat# 553141; RRID:AB_394656
Critical commercial assays		
True-Nuclear Transcription Factor Buffer Kit	BioLegend	Cat# 424401
Live/Dead Fixable Near-IR Dead Cell Stain Kit	Thermo Fisher Scientific	Cat# L10119
Deposited data		
Whole Transcriptome Sequencing of Human Tumor Cells and Hematopoietic Stem and Progenitor Cells During Aging and Bone Marrow Disorders	dbGaP	dbGaP: phs002291.v1.p1
Experimental models: Cell lines		
Human: H929	ATCC	Cat# CRL-9068; RRID:CVCL_1600
Human: RPMI-8226	ATCC	Cat# CCL-155; RRID:CVCL_0014
Software and algorithms		
FlowJo	BD Biosciences	https://www.flowjo.com/

Materials and equipment

Materials

- Primary normal and MM patient samples (fresh blood or bone marrow, or cryopreserved MNC fractions)
- Human myeloma cell lines as positive controls of MM cell surface antigens and IRF4 immunoreactivity
- Validated antibodies against MM and immune cell surface markers (an optimized panel is presented here, and alternative antibodies can be found in our previous study ([Mondala et al., 2021](#)))

Equipment

- Biosafety cabinet
- Tissue culture or clinical centrifuge with adapters suitable for holding 96-well plates
- 96-well deep-well round or U-bottom plates (500–1000 μ L volume recommended, with lids if available)
- Multichannel pipettor (300 μ L capacity recommended)
- FACS tubes
- Flow cytometer equipped with the appropriate lasers for the fluorophores used
- FlowJo data analysis software

Staining reagent dilution chart

Reagent	Method step(s)	Stock concentration (μg/mL)	Dilution (final concentration)	Antibody volume per well (μL)	Total volume per well (μL)
Near-IR fixable live/dead stain	9–11	not available	1:1000	0.1	100
Anti-human FcR block	13–15	not available	1:25	1.2	30
CD3 AF488	16–19	lot-specific	1:20 (lot-specific)	3	30
CD19 PE		lot-specific	1:20 (lot-specific)	3	
CD14 BV605		lot-specific	1:20 (lot-specific)	3	
CD138 VioBlue		not available	1:20	3	
CD38 PE-Cy7		25	1:100 (0.25 μg/mL)	0.6	
IRF4 AF647	29–31	500	1:100 (5 μg/mL)	1	100

Create a master antibody mixture prior to performing surface staining of cells by preparing a solution containing all cell surface antibodies (CD3, CD19, CD14, CD138, and CD38) diluted in STM in a volume sufficient to provide 30 μL of master mix per sample. Note that the surface antibodies are added as one master mix (30 μL per sample) onto cells that already contain 30 μL of diluted human FcR block in STM (60 μL total volume for cell surface antibody staining). For antibodies with lot-specific stock concentrations (e.g., 100 tests per vial), the recommended antibody dilution should remain the same across different lots. However, some further optimization may be required with different lots or with older antibody stocks. See the detailed flow cytometry guide by Cossarizza, Chang, Radbruch, et al. for more suggestions on antibody optimization in Section III.2 of (Cossarizza et al., 2019). All dyes and antibodies, and dilutions of each, should be kept on ice or at 4°C and protected from light while in use. Dilutions should be prepared on the day of use, and any leftover discarded if not used within 24 h.

⚠ CRITICAL: All antibodies and live/dead stain are light-sensitive. Limit exposure to light while preparing these dilutions by reducing ambient light (turn off overhead light in biosafety cabinet) and storing mixes in a dark container, or covered with foil.

Alternatives: For increased consistency and inter-assay reproducibility, a standardized cell type may be used for control samples, such as a large stock batch of cryopreserved, aliquoted PBMCs, or BioLegend's Veri-Cells lyophilized controls (Cat# 425004). For any non-MM samples to be used as controls, be sure to spike in human myeloma cell lines to ensure detection of myeloma cell surface antigens and IRF4-positive cells.

Optional: Include anti-mouse FcR block when using xenograft tissue samples.

STEP-BY-STEP METHOD DETAILS

Preparing the staining plate and controls

⌚ Timing: 1–2 h

This step describes the method to immunolabel primary cells in preparation for flow cytometry using antibodies and dyes. Here we provide optimized antibody dilutions for up to 10⁶ primary MNCs based on the instrumentation we have worked with. However, it is important to titrate and optimize antibody concentrations for each instrument. In addition, any alternative fluorophores and antibodies used for cell surface staining should be tested for compatibility with this fixation and permeabilization protocol. Data shown in this protocol were generated using a BD X-20 Fortessa analytical flow cytometer. The antibody dilutions used likely provide a good starting point and are within similar ranges of the dilutions recommended by the manufacturers.

1. In a standard FACS tube, prepare cells for all control samples (unstained, single-antibody stained, and antibody-deleted). Controls can be prepared using a mixture of MNCs from all conditions, or

PBMCs from healthy donors can be used in control samples after spiking in human myeloma cell lines known to express CD138, CD38, and other myeloma antigens (e.g., H929 or RPMI-8226 cells). **Troubleshooting 1**

- a. Wash cells in 2–3 mL of STM.
- b. Based on the total number of fluorophores in the panel, prepare a single-stained compensation control sample and a Fluorescence minus one (FMO) antibody-deleted sample for each fluorophore (e.g., 7 single-stain and 7 FMO samples, plus one unstained control and one full stain = 16 controls). We recommend starting with approximately 2–4 million cells resuspended in 2 mL of total STM, which should be divided amongst the control wells (e.g., 3×10^6 normal PBMCs plus 1×10^6 spiked in H929 cells). Mix cells well by pipetting.
- c. Aliquot a target quantity of 1×10^5 – 2.5×10^5 total cells per control well in a volume of 100 μ L per well.

Alternatives: As an alternative to using normal PBMCs and cell lines as controls, compensation beads (e.g., ThermoFisher UltraComp eBeads Plus Cat #01-2222-42) can be used for single-color compensation controls. Use one drop of compensation beads for each single stain control and add corresponding antibody to each control. Detailed instructions can be found in the [“Product Sheet: UltraComp eBeads Compensation Beads and UltraComp eBeads Plus Compensation Beads.”](#)

2. Dispense cells for control samples into the appropriate number of wells required for each of the above stains within the first two columns (1 and 2) of a 96-well deep-well round (U)-bottom plate.

Alternatives: Standard FACS tubes can be used in place of a 96-well plate for the rest of the protocol (e.g., polystyrene tubes, Corning Cat# 352003). The incubation volumes outlined for staining in a plate will also be compatible with tube-based staining and are designed to be low-volume to help conserve antibody reagent use. However, if higher incubation volumes are preferred for tube-based staining, antibody incubation steps can be scaled up to a final volume of 100 μ L (in which case all antibody volumes and total incubation volumes should be increased $\times 1.67$). If performing staining in FACS tubes, all wash steps may also be increased in volume (2–3 mL buffer per wash), and additional reagent volumes will also be needed for fixation and permeabilization steps.

3. Plate at least 1×10^5 cells (but no more than 1×10^6) per well for each experimental sample, with roughly equivalent numbers of cells per sample. **Troubleshooting 2**
4. Centrifuge the plate for 5 min, $300 \times g$ at 4°C. Visually verify the presence of a cell pellet in the bottom of each well after centrifugation.
5. Discard the supernatant by holding the plate firmly in one hand and then rapidly (but carefully) dump off the supernatant by inverting the plate while using a single quick plunging motion to discard the waste over a bucket containing 10% bleach. When the plate is turned back over, a small residual volume of buffer should remain in each well.

Alternatives: If FACS tubes are used instead of a microwell plate, use an aspirating pipette to remove the supernatant individually from each tube. Be sure to avoid touching the cell pellet while aspirating.

6. Gently vortex the plate, with the plate lid on, to loosen cell pellets into residual wash buffer.

△ CRITICAL: All subsequent centrifugation steps should include a visual verification of the cell pellets in the bottom of the plate, rapid discarding of the supernatant, and gentle vortexing of the plate to loosen the cell pellets. When dumping off the supernatant, do not hold the plate in an inverted position for more than a brief moment needed to rapidly dump off the supernatant. For subsequent steps, these detailed instructions will be abbreviated, but should still be followed.

Optional: In order to ensure the reproducibility and accuracy of the quantitative data obtained from samples analyzed using this protocol, we recommend including technical replicates of each sample. However, due to the inherent variabilities in working with primary material, sometimes this is not possible. We have previously observed a high degree of reproducibility between technical duplicate samples using this protocol and anticipate that a single well may be considered representative of an individual sample.

Note: With regard to designing the plate layout for your experiment, if there is sufficient room on the plate, we recommend leaving an empty column between controls and each column containing experimental samples (e.g., single-stains in column 1, FMOs in column 3, and experimental samples in columns 5, 7, and so on). This helps prevent potential cross-contamination between wells and reduces potential errors that could occur during plating and antibody addition steps. The use of individual FACS tubes for staining is another option to help eliminate any concern about potential cross-contamination between samples. Note that this may increase the time required for staining and washing the samples if >10 samples are being stained at one time.

Staining with live/dead dye

⌚ Timing: 1 h

This step stains the cells with a fixable Near-IR dye to discriminate live/dead cells.

7. Add 300 μ L of PBS (not STM) to each well using a multichannel pipettor, pipetting up and down to resuspend and wash.
8. Centrifuge the plate for 5 min, 300 \times g at 4°C. Discard supernatant and gently vortex the plate to dislodge cell pellets.
9. Dilute Near-IR live/dead dye 1:1000 in PBS to a volume of 100 μ L per sample for all samples, plus an excess of at least 10% (100 μ L \times # of samples \times 1.1).
 - a. Diluted dye can be transferred to a small reservoir to facilitate multichannel application of the solution to the wells.
10. Add diluted Near-IR dye to all the appropriate samples (do not add to unstained well, FMO Near-IR well, or any single-stain wells, other than for Near-IR single-stain).
 - a. Pipette up and down 2–3 times to thoroughly resuspend cells.
11. Incubate plate(s) in the dark for 30 min at 20°C–25°C.
12. Add 300 μ L of STM and centrifuge plate for 5 min, 300 \times g at 4°C. Discard supernatant and gently vortex the plate to dislodge cell pellets.

△ CRITICAL: Ensure that you wash the cells with PBS before staining with Near-IR live/dead stain, as the presence of FBS can interfere with the dye activity. Refer to the Near-IR live/dead kit technical data sheet for supplemental guidance on reagent preparation and use.

Staining for cell surface antigens

⌚ Timing: 2 h

This step immunolabels the cells for surface antigens to distinguish between myeloma and normal immune cell populations.

13. Dilute Human FcR block 1:25 in STM, making enough for 30 μ L per sample plus an excess of at least 10%.
 - a. All samples receive blocking solution.

14. Add 30 μ L of block solution to each sample. A small reservoir can be used to facilitate multi-channel pipetting.
 - a. Pipette up and down 2–3 times to thoroughly resuspend cells.
15. Incubate plate(s) for 20 min in the dark at 4°C.
16. During the blocking incubation step, prepare antibody master mix as outlined in the Staining Reagent Dilution Chart. **Troubleshooting 3**
 - a. Prepare a 2 \times concentrated master mix of surface-stain antibodies, diluting them appropriately in STM, making up enough volume to add 30 μ L to each experimental sample plus an excess of at least 10% (30 μ L \times # of samples \times 1.1).
17. Add master mix of surface-stain antibodies to experimental samples receiving the full stain (add 30 μ L per sample).
 - a. Pipette up and down 2–3 times to thoroughly resuspend cells.

Note: Do not remove blocking solution before adding staining mix.

△ CRITICAL: Ensure that single-stain and FMO wells do not receive the cell surface antibody master mix. Also, the IRF4 antibody does not get added into the cell surface antibody master mix, or to any single-stain or FMO controls at this stage, as it will be added after fixation.

18. For single-stain and FMO controls, add antibodies individually at the appropriate dilutions along with STM to bring the total volume of each well to 60 μ L.
 - a. When performing cell surface staining in combination with intranuclear staining, there will be some wells that receive only STM during the surface staining step (e.g., IRF4 single stain), and all FMOs will not receive IRF4 antibody until after fixation/permeabilization.
19. Incubate plate(s) for 45 min in the dark at 4°C.
20. Add 300 μ L of STM to each well and centrifuge for 5 min, 300 \times g at 4°C. Discard supernatant and gently vortex plate to dislodge pellet.
21. Repeat step 20 for a total of two washes.

Cell fixation

⌚ **Timing:** 1 h

This step fixes the cells to facilitate subsequent permeabilization and intranuclear immunolabeling.

22. After the last wash of the cell surface staining protocol, discard supernatant and gently vortex the plate to dissociate the cell pellets.
23. Add 200 μ L of 1 \times True-Nuclear fixative solution to each well. Gently pipette up and down to ensure all cells are resuspended.
24. Incubate at 20°C–25°C in the dark for 50 min (no longer than 60 min).

△ CRITICAL: Extended exposure to paraformaldehyde (in the True-Nuclear fixative solution) can destabilize tandem dyes such as PE-Cy7. Do not incubate for longer than 60 min and be sure to store samples in a fixative-free buffer if the protocol is to be paused for 12–18 h (see below).

Note: After fixation, sometimes the cell pellets are more difficult to visually identify in the bottom of the plate after centrifugation. Ensure the centrifuge has completed the run at the appropriate speed before discarding the supernatants after this point.

25. Centrifuge plate at 300 \times g for 5 min at 20°C–25°C. Discard supernatant and gently vortex the plate to dislodge the cell pellets.

26. Proceed immediately to Permeabilization and Intranuclear Staining Procedure, or pause the assay and store plate for 12–18 h (see pause point).

▮▮ Pause point: (optional) Resuspend cells in 200 μ L STM or CytoLast buffer (BioLegend) and store at 4°C in the dark for 12–18 h (no more than 24 h). Wrap sides of plate tightly with parafilm to prevent evaporation, and cover plate in foil to protect from light. To resume the protocol after a 12–18-h pause, centrifuge plate at 300 \times *g* for 5 min at 20°C–25°C, discard supernatant, and gently vortex the plate to dislodge pellets before proceeding with permeabilization and intranuclear staining.

Permeabilization and intranuclear staining

⌚ Timing: 2 h

This procedure permeabilizes the cell and nuclear membranes and immunolabels the cells using an antibody specific for human IRF4. The subsequent staining steps can be carried out at 20°C–25°C. Refer to the manufacturer’s guidelines for additional instructions on carrying out this staining in 96-well plates (<https://www.biolegend.com/protocols/true-nuclear-transcription-factor-staining-protocol-for-96-well-u-bottom-plate/4246/>) versus 5 mL FACS tubes (<https://www.biolegend.com/protocols/true-nuclear-transcription-factor-staining-protocol-for-5ml-tubes/4267/>).

27. Add 300 μ L of 1 \times True-Nuclear Perm Buffer to each well.
28. Centrifuge plate at 300 \times *g* for 5 min at 20°C–25°C. Discard supernatant and gently vortex the plate to dislodge the cell pellets.
29. Repeat steps 27 and 28 one more time for a total of two washes in Perm Buffer. Meanwhile, dilute IRF4 antibody in True-Nuclear Perm Buffer (antibody dilution of 1:100), preparing enough volume for 100 μ L per sample plus an excess of at least 10% (100 μ L \times # of samples \times 1.1). The diluted antibody can be stored on ice, protected from light, until use.
 - a. Be sure to include FMOs and single stain wells in the dilution calculation.
30. Add diluted IRF4 antibody to all experimental samples receiving the full stain (add 100 μ L per sample).
 - a. Also add diluted IRF4 antibody to the IRF4 single stain sample and all FMO samples, except IRF4 FMO.
 - b. Add 100 μ L of Perm Buffer alone to all other control samples (unstained, other single-stained samples, and the IRF4 FMO well).
31. Incubate in the dark at 20°C–25°C for 45 min.
32. Add 300 μ L of 1 \times True-Nuclear Perm Buffer to each well.
33. Centrifuge plate at 300 \times *g* for 5 min at 20°C–25°C, discard supernatant and gently vortex plate to dislodge pellet.
34. Repeat steps 32 and 33 one more time for a total of two washes in Perm Buffer.
35. Resuspend cells in 200–400 μ L of STM and acquire samples on a flow cytometer equipped with the appropriate laser configuration for the fluorophores utilized. **Troubleshooting 4**
 - a. Once staining is complete, samples should be kept on ice or at 4°C, in a dark container or covered with foil to protect from light.

Optional: If your flow cytometer is equipped with a 96-well plate adapter, prior to acquisition, transfer all samples to a standard 96-well U-bottom plate. If your flow cytometer is not equipped with a 96-well plate adapter, then samples can be transferred to microtiter tubes (multi-channel-compatible) or standard FACS tubes for acquisition.

Data acquisition and analysis

⌚ Timing: 2 h

This step processes all samples through a flow cytometer, through which different cell populations can be identified based on their cell surface receptor expression profiles and intranuclear IRF4 protein levels.

36. Ensure the flow cytometer being used includes the following lasers:
 - a. 405 nm Violet
 - b. 488 nm Blue
 - c. 561 nm Yellow Green
 - d. 633 nm Red

Note: For our optimization we used a BD X-20 Fortessa analyzer instrument. We have also tested this panel on a BD FACS Aria and obtained similar results. The following steps are a general guide for adjusting the instrument and acquisition settings in the BD FACS Diva program, but standard operating procedures should be used specific to your flow cytometry instrument.

37. Initiate and prime the flow cytometer for acquisition.
38. Use the unstained control sample to set forward scatter and side scatter voltages.
39. Use single-stained controls to set voltages of other fluorophore channels and collect data for compensation. **Troubleshooting 5**
 - a. Compensation can be performed later on during analysis in FlowJo software.

Alternatives: Compensation may also be performed through FACS DIVA software while running samples on the flow cytometer. Use standard operating procedures for your flow cytometer.

40. Use FMO samples to collect data for negative population gating.
41. Collect data from all control and experimental samples after ensuring that all the voltages are set appropriately. We recommend collecting a minimum of 20,000–50,000 cells per experimental sample (10,000 cells is typically sufficient for each compensation control and FMO).
42. Export data (FCS 3.0 format recommended) to analyze using FlowJo software.

Note: If samples are noticeably clumpy or likely to have a high proportion of dead cells, they can be filtered through a cell strainer (35 μm mesh) after staining is complete and prior to acquisition. This will be required for analysis of primary samples on some flow cytometers that are prone to clogging.

EXPECTED OUTCOMES

This protocol facilitates sensitive intranuclear flow cytometric quantification of IRF4 protein expression in primary MM and normal human hematopoietic cell types. Successful completion and analysis of primary samples will provide quantitative data on the frequency of malignant myeloma cells along with CD19⁺ B cells, CD3⁺ T cells, and CD14⁺ monocyte/myeloid populations. In addition, the intensity of IRF4 protein expression is quantified within these fractions and reported as median fluorescence intensity values (MFI). CD38-high (CD38⁺⁺) cells have been previously reported to represent the malignant plasma cell population (Leo et al., 1992), and we have recently shown that this population harbors high average IRF4 protein levels in primary high-risk MM samples compared with healthy bone marrow controls (Mondala et al., 2021). Therefore, here we utilize the CD38⁺⁺ population for quantification of IRF4 intensity in normal and malignant plasma cell populations. Figure 1 presents a suggested gating strategy for MM (Figure 1A) versus normal (Figure 1B) MNC samples using FlowJo software, along with representative immune cell populations and IRF4 MFI histograms.

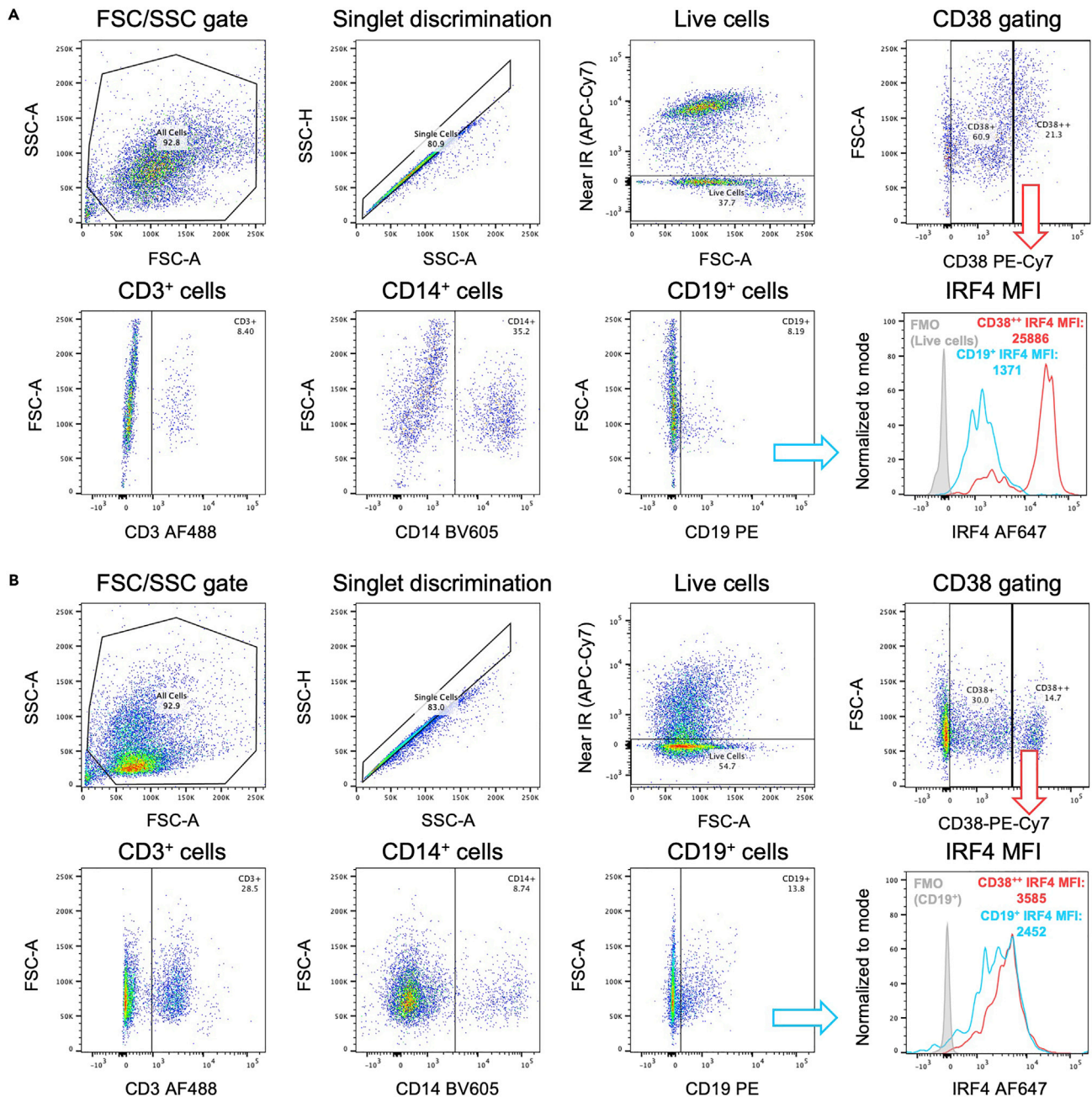


Figure 1. Gating strategy for MNC samples showing representative immune cell populations and IRF4 MFI histograms

Panels show representative FACS plots and gating strategy for discrimination of single, live cells, and subpopulations of CD38, CD3, CD14, and CD19 populations.

(A and B) Immune cell populations are shown along with IRF4 MFI histograms and values within the CD38⁺⁺ and CD19⁺ fractions in high-risk MM (MM9) PBMCs (A) compared with a normal bone marrow MNC control (B). Negative controls that did not receive the IRF4 antibody (fluorescence minus one, FMO) are shown in the histograms in gray for comparison.

With regard to the expected frequency of different cell populations detected, the immune cell compartments, and in particular the frequency of CD38⁺⁺ cells, are expected to vary substantially between different donors, between peripheral blood and bone marrow sources, and between normal and MM samples as well as treatment status. However, we detected a CD38⁺⁺ population that represented >1% of the live cell population in all normal and MM patient samples tested, with the

Table 1. Summary of clinical characteristics of primary patient samples

Sample	Age	Sex	Diagnosis	Therapy	Light chain	Cytogenetics
MM9	73	M	PCL	untreated	Kappa	N/A
MM14	83	M	ND-MM	Velcade	Lambda	CCND1/IGH gene fusion, MYC rearrangement, t(11;14), complex karyotype
MM17	58	M	R/R MM	Daratumumab/ Pomalidomide/ Dexamethasone, Neupogen	Kappa	complex karyotype, including +1q
MM20	42	M	R/R MM	previously on Revlimid, PV-POM-PACE	Kappa	N/A
MM21	54	F	R/R MM	Dexamethasone, previously on Velcade, Thalidomide, and Revlimid	Kappa	trisomy 7, trisomy 9
MM22	43	M	PCL	HyperCVAD, Dexamethasone, Bortezomib	N/A	N/A
MM23	52	F	R/R MM	N/A	Kappa	N/A
MM24	56	M	ND-MM	Dexamethasone	Kappa	complex karyotype, hyperdiploid with 8q24 translocation
MM25	56	M	PCL	untreated	Kappa	N/A
MM26	49	F	S-MM	untreated	Lambda	monosomy 13 (82%); IGH/MAF gene fusion (76%); gain of chromosome 5 (7%)
MM27	81	F	S-MM	untreated	Kappa	normal karyotype; FISH: +4p, +5, +7, +11q, +17
MM28	70	M	S-MM	untreated	Kappa	gain: 5, 7/7q, 11q; monosomy 13
NBM1	65	N/A	N/A	N/A	N/A	N/A
NBM2	53	N/A	N/A	N/A	N/A	N/A
NBM3	61	N/A	N/A	N/A	N/A	N/A

All primary patient samples were collected according to local institutional review board (IRB) guidelines under approved protocols and patient consent. PCL=plasma cell leukemia, ND=newly-diagnosed, MM=multiple myeloma, R/R=relapsed/refractory, S=smoldering, NBM=normal bone marrow, N/A=not available or not applicable

exception of one MM patient sample that was known to be on daratumumab treatment (anti-CD38 monoclonal antibody therapy) at the time of sample collection (Table 1), and had no detectable CD38-high or CD38-low populations (Figure 2A). Notably, even within a CD38-positive population in normal samples, IRF4 MFI was consistently low, while the IRF4 MFI within CD38⁺⁺ cells of MM samples was on average high and relatively variable (Mondala et al., 2021). Positive subpopulations of all other immune cell compartments are expected to be detected in most cases in both normal and MM samples.

Within human myeloma cell lines, IRF4 protein was also abundant but variable in expression, with strikingly high levels detected in H929 cells, and more moderate levels in other cell lines such as RPMI-8226 (Figure 2B), as predicted by analysis of whole transcriptome RNA-sequencing datasets from human myeloma cell lines (Keats et al., 2007; Mondala et al., 2021). Of the cell surface markers, it is also expected that only the myeloma cell surface markers will be positive on human myeloma cell lines (CD38 and CD138), with some known variability across cell lines (Muccio et al., 2016).

To demonstrate the utility of sensitive intranuclear flow cytometric quantification of IRF4 protein expression to detect differences in IRF4 levels, we also plotted IRF4 fluorescence intensity data from H929 myeloma cells treated with a lead antisense oligonucleotide (ASO) agent that inhibits human IRF4 (ION251) (Mondala et al., 2021). Intranuclear IRF4 immunolabeling and flow cytometric analysis showed that compared with a non-targeting control ASO, IRF4-targeted ASOs reduced IRF4 protein expression by over 50% (Figure 2C), which was sufficient to induce cytotoxicity in human myeloma cells after 5 days in culture (Mondala et al., 2021).

As noted above, this protocol can also be used to quantify IRF4 intranuclear protein expression in human cells expanded *in vivo* in cell line xenograft or PDX models (Lazzari et al., 2017). We

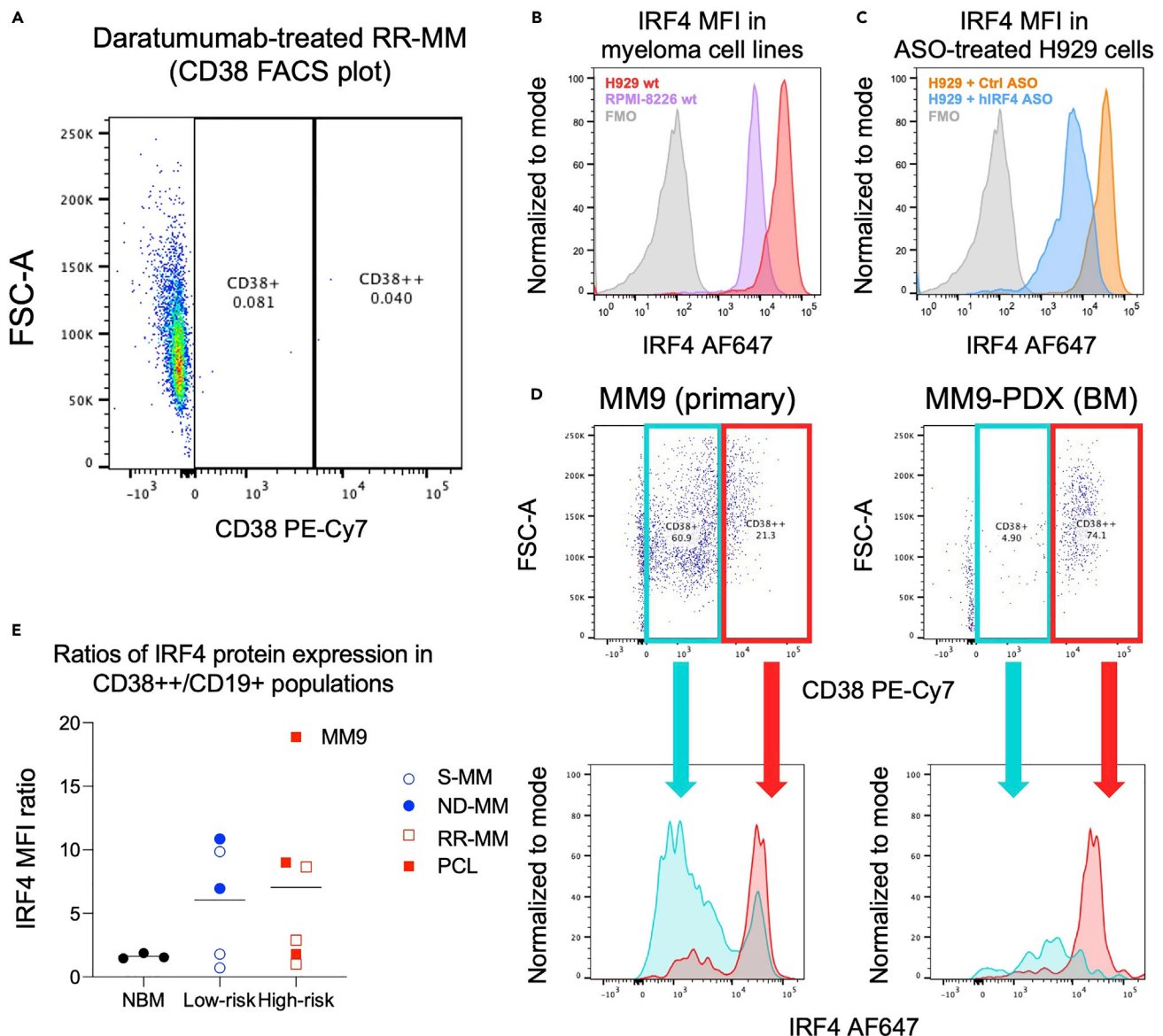


Figure 2. Characterization and quantification of CD38 frequency and IRF4 protein expression patterns in human myeloma cells

(A) Representative FACS plot showing CD38 cell surface expression in a primary MM patient sample (MM17) with relapsed/refractory (RR) disease and on daratumumab therapy at the time of sample collection. No CD38-positive population was detectable in this sample.

(B and C) Representative FACS histogram data showing IRF4 fluorescence intensity and calculated MFI values for wild-type (wt) human myeloma cell lines (H929 and RPMI-8226 cells, B) and in H929 cells treated for 3 days with antisense oligonucleotides (ASO, 2 μ M) against human (h) IRF4 compared with a negative control (Ctrl ASO) (C). Negative control cells that did not receive the IRF4 antibody (fluorescence minus one, FMO) are shown in the histograms in gray for comparison.

(D) Relative IRF4 expression levels are shown as fluorescence intensity histograms for CD38-low and CD38-high subsets of primary MM9 versus MM9-PDX bone marrow samples. For data showing tissues from experiments involving mice, all animal studies were performed in accordance with UCSD and NIH-equivalent ethical guidelines and were approved by the university institutional animal care and use committee (IACUC).

(E) Individual intra-sample IRF4 MFI ratios calculated in MM cells (CD38⁺⁺) relative to CD19⁺ B cells. S-MM = smoldering MM, ND-MM = newly diagnosed MM, PCL = plasma cell leukemia.

previously reported that the CD38⁺⁺ population exclusively harbors an IRF4-enriched population capable of regenerating disease in immunocompromised mice (Mondala et al., 2021). Figure 2D presents an extended analysis showing IRF4 MFI histograms within CD38-high and CD38-low fractions of a primary high-risk MM sample (MM9) and the bone marrow of a PDX mouse engrafted with

MM9 serially-transplanted cells. A characteristic population of FSC-high, CD38-high cells are also predominantly high in IRF4 protein expression, and this population is maintained *in vivo* after serial transplantation (Figure 2D) (Mondala et al., 2021). As anticipated, within PDX-derived hematopoietic tissues, non-myeloma, normal immune cell markers were considered undetectable (<1% of the total live cell population).

QUANTIFICATION AND STATISTICAL ANALYSIS

A combination of flow cytometry analysis software (FlowJo), Microsoft Excel, and graphing tools (e.g., GraphPad Prism) were used for data processing to quantify the frequency of malignant myeloma cells and other immune cell types in primary samples, along with the intensity of IRF4 protein expression within these immune subpopulations. For this purpose, tabular data is exported from FlowJo software for the following parameters: 1) the frequency of live, CD38⁺⁺, or CD19⁺, or CD3⁺, or CD14⁺ cells, and 2) the IRF4 median fluorescence intensity (MFI) statistic within live, CD38⁺⁺, or CD19⁺, or CD3⁺, or CD14⁺ cells. Raw values are imported into Microsoft Excel for further analysis of average frequencies of each immune cell subpopulation and average IRF4 MFI within these fractions.

Criteria for data inclusion/exclusion in our analyses involved consideration of potential disease-modifying therapeutic treatments that MM patients may have been undergoing at the time of sample collection. For example, daratumumab is a CD38-targeted monoclonal antibody therapy that could interfere with the interpretation of quantitative data in a CD38-positive population. In our dataset, one relapsed/refractory patient sample was excluded from analysis because it came from a patient that was on daratumumab at the time of sample collection, and we detected <1% CD38-positive cells in this patient sample (Figure 2A).

To further refine our analysis strategy to facilitate inter-assay comparisons in primary MM and normal samples (Table 1) and extend the clinical relevance of this procedure, we developed a novel intra-sample normalization strategy, in which we compare the IRF4 MFI within the CD38⁺⁺ population (plasma cells) versus the CD19⁺ B cell fraction (Table 2 and Figure 2E). By quantifying the relative IRF4 intensity in MM cells as a ratio compared with an endogenous immune cell compartment, this provides a reference point that may help standardize future clinical evaluation of IRF4 expression to stratify patients for treatment with IRF4-targeting agents (e.g., ION251, [clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04398485) registry number NCT04398485), or to assess clinical responses to this or other IRF4-modulating therapies (e.g., lenalidomide) (Agnarelli et al., 2018; Lopez-Girona et al., 2011).

Table 2 presents the quantification of IRF4 protein levels, calculated as a ratio using the MFI of the IRF4-AF647 antibody within CD38⁺⁺ myeloma cells relative to CD19⁺ B cells ((IRF4 MFI in CD38⁺⁺)/(IRF4 MFI in CD19⁺)). The relative IRF4 MFI ratios calculated in MM samples was on average higher in untreated and high-risk samples compared with normal controls, and thus may represent a valuable prognostic marker to predict a patient's risk for developing progressive disease.

LIMITATIONS

While CD138 (also known as syndecan-1) is the traditional plasma cell marker used to identify malignant myeloma cell populations in clinical and research settings, it is frequently rendered

Table 2. Average intra-sample IRF4 MFI ratios in MM cells relative to CD19⁺ B cells

Disease stage	Ratio of IRF4 MFI CD38 ⁺⁺ /CD19 ⁺	Sample size (n)	Standard deviation
NBM	1.63	3	0.22
S-MM	4.12	3	5.00
ND-MM	8.91	2	2.75
PCL	9.90	3	8.57
RR-MM	4.19	3	3.99

undetectable in cryopreserved samples, or in samples processed after a delay (Frigyesi et al., 2014). When there are pre-analytical variations in processing, CD38 represents a useful alternative malignant plasma cell marker for flow cytometric analysis of MM samples, due to its compatibility with analysis post-cryopreservation (Mondala et al., 2021) and maintenance during serial transplantation in PDX models (Lazzari et al., 2017). In addition, CD38-high (CD38⁺⁺) cells represent the malignant plasma cell population (Leo et al., 1992) which is enriched for IRF4 protein expression in high-risk MM compared with healthy bone marrow controls (Mondala et al., 2021). Therefore, our analysis strategy focuses on the CD38 population for quantification of IRF4 protein expression, however CD138 is also included for reference, and additional plasma cell markers such as CD319 and CD269 (Frigyesi et al., 2014) could provide other stable alternative markers for potential future addition to this or similar flow cytometry panels.

Another minor limitation of this protocol is that it is currently only compatible with flow cytometric analysis, and no sorting protocols have yet been established to isolate IRF4-positive cells for downstream functional or RNA analyses due to the necessary fixation and permeabilization steps. In addition, as new immunotherapy-based strategies continue to emerge for the treatment of MM (Costello, 2017; Costello and Mikhael, 2018; Siegel et al., 2020), flow cytometry studies that rely on cell surface marker analysis of specific subpopulations of MM cells may become more challenging to interpret. We have noted one example of a situation in which it was not possible to analyze the IRF4 MFI within a CD38-positive population due to a lack of CD38-immunoreactive cells, which is likely related to that patient's treatment status of active anti-CD38 monoclonal antibody therapy. However, with some minor modifications, the protocol presented here could potentially be adapted to serve as a cell surface antigen-agnostic method to quantify disease-associated IRF4-enriched populations on the basis of IRF4 expression in total live cells. This could enable rapid translation of IRF4 flow cytometry-based clinical quantification as a companion diagnostic to accompany novel IRF4-modulating therapies in development for MM and other lymphoid neoplasms.

TROUBLESHOOTING

Problem 1

Low levels of myeloma cell surface antigens and/or IRF4 immunoreactivity in control samples.

Potential solution

Spike in human myeloma cells known to express high levels of CD38 and IRF4 protein (e.g., H929 cells). We recommend spiking in these cells to control samples at a ratio of 1:2 to 1:4 versus control MNCs. Compensation beads can also be used in lieu of cells with sufficient expression of IRF4 protein or specific surface antigens as mentioned in the above protocol.

Problem 2

Clumping of PBMCs from primary samples or xenograft tissue.

Potential solution

Prior to staining and/or acquisition on the flow cytometer, samples may be filtered through blue-cap FACS tubes (Corning Cat# 352235) with a 35 μ m cell-strainer top to ensure a single-cell solution. To prevent aggregated cells from clogging the flow cytometer, 1% Accutase (StemCell Technologies Cat# 07922) may also be added to STM followed by thorough resuspension of the samples before data acquisition.

Problem 3

Low levels of measured CD138⁺ cell frequency in experimental and/or control samples.

Potential solution

As noted in the Limitations section above, the CD138 antigen is susceptible to rapid loss of detection with delayed sample processing or following cryopreservation. Therefore, in order to accurately

compensate for all cell surface markers, control samples should include spiked-in human myeloma cells known to express high levels of CD138 and CD38 proteins (e.g., H929 cells), or compensation beads. If alternative cell surface markers such as CD319 (*SLAMF7*) are used, U266 cells also offer a positive cell line harboring high CD319 expression.

Problem 4

High background noise due to excessive dead cells can occur when using previously cryopreserved samples.

Potential solution

Prior to cell surface immunostaining, perform a dead cell removal procedure (Miltenyi kit Cat #130-090-101) on samples with high percentages of dead cells, and filter samples through a mesh strainer prior to data acquisition on the flow cytometer. Alternatively (or if a dead cell removal kit is not available), a DNA digestion procedure using DNase I can be performed prior to cell surface immunostaining, as described by Stevens et al. for flow cytometry analyses of previously-cryopreserved primary samples (Stevens et al., 2021).

Problem 5

Changes in fluorescence spillover due to destabilization of the PE-Cy7-conjugated antibody (anti-CD38).

Potential solution

PE-Cy7 is a tandem fluorochrome that combines PE with a cyanine dye. PE-Cy7 is particularly sensitive to photo-induced degradation, resulting in loss of fluorescent signal and changes in fluorescence spillover. Extreme caution must be taken to avoid light exposure and prolonged exposure to paraformaldehyde fixative. Ensure that fixed cells are transferred to a paraformaldehyde-free buffer for storage as recommended at the optional Pause Point or analyzed within 4 h of fixation in paraformaldehyde. If concerns about spillover remain, the single-stained PE-Cy7 control sample can be evaluated for signal in the PE or other channels, and a new antibody could also be tested in case the original stock antibody has become degraded.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Leslie A. Crews (lcrews@ucsd.edu).

Materials availability

The study did not generate new unique biological reagents. All reagents used are commercially available and the catalog numbers for each have been provided in the [key resources table](#).

Data and code availability

Some of the primary MM samples included in the analyses shown here have also been subjected to whole transcriptome RNA-sequencing. The RNA-sequencing datasets are available at dbGaP (Study Accession, dbGaP: phs002291.v1.p1) (Mondala et al., 2021).

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AUTHOR CONTRIBUTIONS

L.A.C. conceived of the protocol; A.A.V. and L.A.C. tested and optimized the procedure; and A.A.V., P.K.M., and L.A.C. designed and/or performed experiments and analyzed data. C.C. provided primary patient samples, and A.R.M. provided ASO reagents. L.A.C. and A.A.V. wrote the manuscript with feedback from all authors.

DECLARATION OF INTERESTS

A.R.M. is an employee of Ionis Pharmaceuticals. All other authors declare no competing interests.

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