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Title: Assessment of Human Natural Killer Cell Events Driven by FcγRIIIa Engagement in the Presence of Therapeutic Antibodies

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **no**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **no**

3. **Filming location:** Will the filming need to take place in multiple locations? **no**

Introduction

1. Introductory Interview Statements

Videographer: Interviewee headshots are required. Take a headshot for each interviewee.

Authors: While filming the interview portion, our videographer will also photograph you for the **JoVE Dedicated Author Webpage**. Please look at this **example**. For questions about the author profile pages and pictures, please contact **author.liaison@jove.com**.

Authors: Please memorize the interview statements prior to your filming day.

1.1. **Annalise Petriello:** This method can used to dissect the molecular and cellular mechanism of FcγRIIIa-driven events, without the need for target cells that express the antigen **[1]**.

1.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

Ethics Title Card

1.2. Procedures involving human subjects have been approved by the Institutional Review Board (IRB) at iQ Biosciences.

Protocol

Please use this draft script to help you prepare for filming day.
- Filming should take no more than 10 minutes per step. If a step will take more than 10 minutes, prepare the product from that step in advance.

2. Antibody-Mediated Activation of NK Cells

2.1. Set a refrigerated microcentrifuge to 4 degrees Celsius **[1]** or place a microcentrifuge into a refrigerated space.

2.1.1. Talent sets microcentrifuge to 4 degrees Celsius. **NOTE: Not filmed.**

Talent walks into a cold room (4 degrees Celsius) with a microcentrifuge. *Videographer: This is one of the most important steps for viewers to see.* **NOTE: Use as 2.1.1.**

2.1.2. Added shot: Talent places the microcentrifuge on a surface in the cold room and sets it up.

2.2. Dispense 100 microliters of resuspended natural killer cells into 1.5-milliliter tubes or a 96-well U-bottom plate, and place the cells on ice **[1-TXT]**.

2.2.1. Talent dispenses cells into tubes and places tubes on ice. **TEXT: 100 microliters = 1 x 10^6 cells**

2.3. Prepare rituximab, or other antibody of interest, at a concentration of 100 micrograms per milliliter **[1]**. Add 1 microliter to each tube of cells, for a final concentration of 1 microgram per milliliter **[2]**, and incubate the cells on ice for 30 minutes **[3]**.

2.3.1. Talent prepares solution of antibody.
2.3.2. Talent adds small amount of antibody solution to tubes.
2.3.3. Talent places cells on ice and starts timer. *Videographer, obtain multiple reusable takes. This can be reused for 6.1.1.* **NOTE: Used again in 6.1.1.**

2.4. During incubation, prepare a solution of anti-human κ *(pronounce kappa)* light chain antibody in media, at a concentration of 50 micrograms per milliliter. The amount of antibody solution needed will be 50 microliters per cell sample **[1]**. Warm the antibody solution to 37 degrees Celsius on a heat block or in a water bath **[2]**.

2.4.1. Talent prepares antibody solution.
2.4.2. Talent places antibody solution on a heat block. *Videographer: This is one of the most important steps for viewers to see.*

2.5. When incubation of the cells is complete, add 1 milliliter of ice-cold media to each tube **[1]** and centrifuge the cells at 135 times *g* and 4 degrees Celsius for 5 minutes **[2]**.

2.5.1. Talent adds ice-cold media to tubes.
2.5.2. Talent centrifuges cells

2.6. Wash the cells again with 1 milliliter of ice-cold media **[1]**. Aspirate the supernatant after the last wash, and add 50 microliters of the anti-human κ light chain antibody solution to activate the cells **[2]**.

2.6.1. Talent washes cells again with ice-cold media (remove media and then add media).
2.6.2. Talent removes supernatant and adds a small amount of the antibody solution.

2.7. Immediately place cell samples at 37 degrees Celsius on a heat block or in a water bath, and incubate them until the desired time-points for analysis or other procedures **[1]**.

2.7.1. Talent places cell samples in water bath. *Videographer: This is one of the most important steps for viewers to see.*

2.8. **Annalise Petriello**: The best way to handle multiple samples is to use a floating rack in a 37-degree water bath, which can be taken out and placed on ice to stop all reactions immediately **[1]**.

2.8.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

2.9. At the desired time-point for Western Blot analysis or assessment of cytoskeletal rearrangement, stop activation by adding 1 milliliter of ice-cold media **[1]** and immediately spin samples in a refrigerated centrifuge at 135 times *g* for 5 minutes **[2]**. Wash once more with 1 milliliter of ice-cold media **[3]**.

2.9.1. Add 1 milliliter of ice-cold media. *Videographer: This is one of the most important steps for viewers to see.*
2.9.2. Spin samples in refrigerated centrifuge
2.9.3. Wash samples once more with 1 milliliter of ice-cold media.

3. Interrogation of Signaling Molecules by Western Blot Analysis

3.1. Lyse the cells by adding 20 microliters of RIPA buffer containing phosphatase and protease inhibitors mix **[1]**. Place the tubes on ice for 30 minutes **[2]**. Then centrifuge tubes at 2100 times *g* for 15 minutes at 4 degrees Celsius **[3]**.

3.1.1. Talent adds buffer to each tube of cells.
3.1.2. Talent places tubes on ice

3.1.3. Talent places tube in centrifuge.

3.2. Transfer the lysate to a clean 1.5-milliliter tube and add the reagents required for protein separation **[1]**. Then, proceed with protein separation and Western blot analysis as per the investigator's preferred procedure **[2]**.

3.2.1. Talent transfers the lysate to a clean tube and adds reagents.
3.2.2. Talent begins adding samples to an SDS-PAGE gel. **NOTE: Not filmed.**

3.3. After running the gel, transfer the proteins onto a nitrocellulose or PVDF membrane following standard protocols **[1]**. Continue with Western blot analysis as per the investigator's preferred procedure **[2]**.

3.3.1. Talent transfers the proteins from the gel onto a membrane. **NOTE: Not filmed.**
3.3.2. Talent working with Western blot apparatus. **NOTE: Not filmed.**

4. Isolation of mRNA and Preparation for Chemokine/Cytokine Assessment

4.1. To stop activation, remove the cell samples from the water bath and place them on ice **[1]**. Immediately spin in a refrigerated centrifuge at 135 times g for 5 minutes **[2]**.

4.1.1. Talent removes cell samples from water bath and places them on ice.
4.1.2. Talent places cell samples in refrigerated centrifuge.

4.2. Transfer the supernatant to a clean vessel, and freeze it for later assessment of chemokine or cytokine production**.** Wash the pellet twice with 1 milliliter of ice-cold PBS **[1]** **[2]**.

4.2.1. Talent transfers supernatant to a clean vessel and places it in freezer. *Video editor: show 4.2.1 and 4.2.2 as a multipanel.*
4.2.2. Talent begins washing pellet with ice-cold PBS.

4.3. Extract RNA and produce cDNA using the investigator's preferred protocol **[2]**.

4.3.1. Talent adds extraction mix to tubes.
4.3.2. Talent begins process of producing cDNA. **NOTE: Not filmed.**

4.4. Freeze the cDNA at negative 20 degrees Celsius until gene expression analysis **[1]**.

4.4.1. Talent places tubes in freezer.

5. Cytoskeletal Rearrangement Assessment

5.1. Resuspend the cell pellet with 50 microliters of 3.7 percent paraformaldehyde **[1]**. After 10 minutes at room temperature, add 1 milliliter of PBS and spin at 135 times *g* for 5 minutes at room temperature **[2]**. Repeat this washing once more **[3]**.

5.1.1. Talent resuspends cell pellet in paraformaldehyde.
5.1.2. Talent adds PBS and places tubes in centrifuge.
5.1.3. Talent begins to repeat wash procedure.

5.2. Add 100 microliters of 0.1 percent Triton X-100–PBS to permeabilize cells **[1]**. After 5 minutes, pellet the cells by centrifugation at 135 times *g* and room temperature for 5 minutes **[2]**. Then, add 100 microliters of phalloidin and incubate for 20 minutes at room temperature **[3-TXT]**.

5.2.1. Add solution to permeabilize cells.
5.2.2. Place tubes in centrifuge.
5.2.3. Add phalloidin to tubes. **TEXT: 5 units/mL AF488-labeled phalloidin diluted in 1% BSA/PBS**

5.3. To wash the cells, add 1 milliliter of PBS and spin the tubes at 135 times *g* for 5 minutes at room temperature **[1]**. Resuspend the cell pellet in the desired volume for flow cytometric analysis **[2]**.

5.3.1. Talent adds PBS and places tubes in centrifuge.
5.3.2. Talent removes supernatant and resuspends pellet.

6. Assessing Degranulation Using CD107a Surface Staining

6.1. While the cells are incubating on ice with rituximab or other antibody of interest **[1]**, prepare 50 microliters of antibody mixture per sample **[2-TXT]**.

6.1.1. Cells incubating on ice. *Videographer/video editor, please reuse footage from 2.3.3.* **NOTE: Use 2.3.3.**
6.1.2. Talent prepares antibody mixture. **TEXT: Mixture: 50 µg/mL anti-human κ light chain antibody; 1 µg/mL fluorochrome-labeled CD107a**

6.2. After the final wash and aspiration of the cells, add 50 microliters of the antibody-CD107a mixture **[1]**. Then, incubate at 37 degrees Celsius **[2]**.

6.2.1. Talent adds small amount of antibody-CD107a mixture.
6.2.2. Talent places cells in incubator.

6.3. At the desired timepoints, add 1 milliliter of PBS to the cells, and spin at 135 times *g* for 5 minutes at room temperature **[1]**. Aspirate the supernatant and add 100 microliters of 4 percent paraformaldehyde **[2]**. Incubate at room temperature for 10 minutes **[3]**.

6.3.1. Talent adds 1 milliliter of PBS to the tube and places tube in centrifuge.
6.3.2. Talent removes supernatant and adds paraformaldehyde.
6.3.3. Talent places cells in incubator.

6.4. Add 1 milliliter of PBS and spin at 135 times *g* for 5 minutes at room temperature to wash **[1]**. Resuspend the cell pellet in the desired volume for flow cytometric analysis **[2]**.

6.4.1. Talent adds PBS to tubes, and places tubes in centrifuge.

6.4.2. Talent removes supernatant and resuspends cell pellet.

Results

7. Results: Downstream Applications of NK Cell Activation Assay

7.1. Natural killer cells were isolated from PBMCs and stained with CD56 and CD3. The cells had a greater than 90 percent purity, and viability of greater than 95 percent **[1-TXT]**.

7.1.1. LAB MEDIA: Figure 1. **TEXT: NK: Natural Killer**

7.2. Cells lysates were separated on an SDS-PAGE gel. After transfer onto a PVDF membrane, western blots for pAKT, pPRAS40, and pERK1/2 were performed. An accumulation of these molecules indicates that events were driven by the FcγRIIIa *(pronounce F-C-gamma-R-3-A)* **[1]**.

7.2.1. LAB MEDIA: Figure 2.

7.3. Natural killer cells were stimulated with rituximab. mRNA was collected, reverse-transcribed, and subjected to qPCR analysis **[1]**. Activated natural killer cells expressed MIP-1α *(pronounce M-i-P-1-alpha)*, MIP-1β *(pronounce M-i-P-1-beta)*, RANTES *(pronounce ran-tees)* **[2]**, IFN-γ, *(pronounce i-F-N-gamma)*, and TNF-α *(T-N-F-alpha)* **[3]**.

7.3.1. LAB MEDIA: Figure 3.
7.3.2. LAB MEDIA: Figure 3. *Video editor, emphasize Figure 3A*.
7.3.3. LAB MEDIA: Figure 3. *Video editor, emphasize Figure 3B.*

7.4. After stimulation with rituximab for 0 to 30 minutes, natural killer cells underwent phalloidin staining and flow cytometry. Cytoskeletal rearrangement was observed in activated cells **[1]**.

7.4.1. LAB MEDIA: Figure 4.

7.5. Natural killer cells were assessed for degranulation by CD107a and flow cytometry. Rituximab increased the percentage of cells expressing CD107a **[1]**.

7.5.1. LAB MEDIA: Figure 5.

7.6. Natural killer cells were stimulated for 0, 0.5, 1, 3, and 6 hours with rituximab. Supernatant was collected and analyzed by a flow- and bead-based cytokine assessment method. As expected based on previously published studies **[1]**, the chemokines MIP-1α, MIP-1β, and RANTES **[2]** and the cytokines IFN-γ and TNF-α **[3]** were detected in the supernatant **[4]**.

7.6.1. LAB MEDIA: Figure 6.

7.6.2. LAB MEDIA: Figure 6. *Video editor please emphasize Figure 6A*.

7.6.3. LAB MEDIA: Figure 6. *Video editor please emphasize Figure 6B.*

7.6.4. LAB MEDIA: Figure 6.

Conclusion

8. Conclusion Interview Statements

8.1. **Annalise Petriello:** A small subset of stimulated CD8 T cells express FcγRIIIa, and this protocol may help with the identification of biomarkers for therapeutics that mediate their effect through FcγRIIIa-driven events **[1].**

8.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.