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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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NK cells, FcγRIIIa engagement, therapeutic antibodies, small molecules, gene expression, degranulation, cytokine release, signaling

SUMMARY:

A protocol for studying FcγRIIIa-driven events by therapeutic antibodies in human natural killer cells is described here. This artificial stimulation platform permits the interrogation of downstream effector functions such as degranulation, chemokine/cytokine production, and signaling pathways mediated by the FcγRIIIa and Fc portions of antibodies involved in binding.

ABSTRACT:

One mechanism of action for clinical efficacy by therapeutic antibodies is the promotion of immune-related functions, such as cytokine secretion and cytotoxicity, driven by FcγRIIIa (CD16) expressed on natural killer (NK) cells. These observations have led to research focusing on methods to increase Fc receptor-mediated events, which include removal of a fucose moiety found on the Fc portion of the antibody. Further studies have elucidated the mechanistic changes in signaling, cellular processes, and cytotoxic characteristics that increase ADCC activity with afucosylated antibodies. Additionally, other studies have shown the potential benefits of these antibodies in combination with small molecule inhibitors. These experiments demonstrated the molecular and cellular mechanisms underlying the benefits of using afucosylated antibodies in combination settings. Many of these observations were based on an

artificial in vitro activation assay in which the FcγRIIIa on human NK cells was activated by therapeutic antibodies. This assay provided the flexibility to study downstream effector NK cell functions, such as cytokine production and degranulation. In addition, this assay has been used to interrogate signaling pathways and identify molecules that can be modulated or used as biomarkers. Finally, other therapeutic molecules (i.e., small molecule inhibitors) have been added to the system to provide insights into the combination of these therapeutics with therapeutic antibodies, which is essential in the current clinical space. This manuscript aims to provide a technical foundation for performing this artificial human NK cell activation assay. The protocol demonstrates key steps for cell activation as well as potential downstream applications that range from functional readouts to more mechanistic observations.

INTRODUCTION:

Over the last few decades, there has been tremendous focus on developing targeted cancer therapies using antibodies. Therapeutic antibodies, such as trastuzumab and rituximab, operate through multiple mechanisms, including the prevention of dimerization of signaling molecules and mobilization of the immune system[1,2]. The latter is accomplished through antibody-dependent cellular cytotoxicity (ADCC), in which lymphocytes called natural killer (NK) cells are brought to a target cell by the antibody[1,2]. By placing the cells in proximity with each other, the NK cell is activated and can lyse a tumor/target cell through the secretion of effector molecules[3].

At the molecular level, the Fab portion of the antibody binds its cognate antigen expressed on the tumor cells, while its Fc portion engages the FcγRIIIa expressed on NK cells to bring the two cells together[1,2]. After engagement of the FcγRIIIa, signaling pathways (i.e., MAPK and PI3K pathways) drive cytoskeletal rearrangement, cytokine production, and cytotoxicity[4–9]. Thus, ADCC is an FcγRIIIa-driven event mediated by NK cells and antibodies.

Because ADCC was thought to be a mechanism of action for these therapeutic antibodies, researchers searched for methods to increase ADCC by modifying the antibody. One modification was the removal of fucose on the oligosaccharide chain attached to asparagine 297, which increases the binding affinity of the Fc portion of the antibody to the FcγRIIIa[10–12]. In animal studies, mice receiving afucosylated antibodies exhibited slower tumor growth compared to mice treated with its fucosylated counterpart[13]. More importantly, obinutuzumab (e.g., Gazyva, an approved afucosylated antibody) showed better efficacy relative to rituximab (e.g., Rituxan, its fucosylated counterpart) in patients diagnosed with chronic lymphocytic leukemia or follicular lymphoma[14,15].

Until recently, the mechanisms underlying increased ADCC via afucosylated antibodies were unknown. Combined with the fact that there are numerous research programs developing therapeutic antibodies to utilize FcγRIIIa-driven mechanisms to target cancer cells, it is imperative to develop in vitro assays that examine the molecular and cellular aspects promoted by these antibodies. This provides fundamental understanding of the mechanisms of action as

well as the potential to discover biomarkers. As such, an artificial activation assay was developed to study antibody-dependent FcγRIIIa-mediated functions in addition to signaling and cellular characteristics[8]. Through these studies, the mechanisms underlying increased efficacy of afucosylated antibodies have been elucidated, in which enhanced binding affinity increases signaling to promote cellular properties and cytotoxic characteristics[8].

The current trend in clinical trials is use of a combination of therapeutic molecules[16]. One of the most commonly mutated pathways is the PI3K pathway, which has prompted tremendous effort in developing small molecule inhibitors that target components of this pathway[17–20]. Yet, how these molecules act in combination with therapeutic antibodies is relatively unknown, especially in combinations where the inhibitor may affect molecules that require the PI3K pathway in order to function, such as those driven by therapeutic antibodies.

To this end, the in vitro assay employed for the afucosylated antibody studies has also been used to study the combination of PI3K inhibitors and therapeutic antibodies. These studies defined the molecular characteristics of PI3K inhibition on therapeutic antibody PI3K-driven events and described how afucosylated antibodies can offset this loss of signaling[9]. These findings are relevant, as they lend potential guidance for designing clinical trials. In addition, this series of experiments also led to the first described observations for kinetic regulation of the PI3K signaling pathway to modulate chemokine/cytokine transcription and production, which may serve as potential biomarkers[9].

The artificial in vitro activation assay used to define the signaling and cellular characteristics described above has been designed to study FcγRIIIa-driven events in NK cells mediated by antibodies in the absence of target cells. Without target cells in the system, all of the signaling events and functions observed can be attributed directly to the NK cells. In the presented assay, antibody is added to purified NK cells, at which point the Fc portion binds the FcγRIIIa. This is followed by crosslinking of the antibody using an anti-human κ light chain antibody to artificially stimulate the cells. Crosslinking of the antibody mimics binding of the target antigen to generate a signaling platform that elicits downstream events. Depending on the length of stimulation, researchers can assess signaling, cellular processes, cytotoxic characteristics, and effector functions[8,9]. Similarly, this assay also provides flexibility in studying these events when antibodies are combined with other molecules[9].

Together, this is an ideal in vitro assay to study therapeutic antibodies that elicit NK cell responses through their FcγRIIIa as part of the mechanism of action. This protocol describes the performance of this in vitro activation assay and provides insight into the various readouts that can be performed.

PROTOCOL:

The following protocol is in accordance with the guidelines of iQ Bioscience's human research ethics committee.

1. Isolation of PBMCs and enrichment/purification of NK cells

NOTE: Other methods for the isolation of peripheral blood mononuclear cells (PBMCs) and enrichment/purification can also be performed.

1.1. Draw 200 mL of blood under regulated conditions into a tube containing heparin.

1.2. Add 15 mL of the density gradient medium into a 50 mL tube with a porous barrier incorporated. Spin the tube at 1000 *x g* for 30 s.

1.3. Add 12.5 mL of blood into the tube followed by another 12.5 mL of PBS, and gently invert 3x. Spin the tube at 800 *x g* for 15 min at room temperature (RT) with no breaks.

1.4. Prepare a 50 mL conical tube with 40 mL of PBS for each tube with a porous barrier while the cells are spinning.

1.5. Carefully remove the tubes and inspect after centrifugation. Check for a thin, visibly white layer of PBMCs above the porous barrier, between the 20 mL and 25 mL demarcations on the 50 mL tube.

1.6. Carefully remove as much liquid as possible from the top using a pipette without disturbing the thin, white PBMC layer.

1.7. With a clean 10 mL serological pipette, gently remove the PBMC layer in addition to the clear, yellowish liquid layer down to the filter of the tube.

1.8. Expel the PBMCs and liquid into the 50 mL conical containing PBS prepared in step 1.4. Spin tubes at RT and 300 *x g* for 5 min.

1.9. Aspirate the PBS wash and add an additional 40 mL of PBS. Spin tubes at RT and 300 *x g* for 5 min.

1.10. After washing, count the cells and resuspend them in 40 µL of 2% BSA/PBS per 1 x 10^7 cells.

1.11. Proceed to the isolation of NK cells using the method of choice. Choices include manual or automated magnetic bead-based sorting[9,21]. Fluorescence-activated cell sorting can also be performed[22].

1.12. Remove a small aliquot of cells to determine the purity of NK cells by flow cytometry. Gating strategy includes the following: gate on live cells based on forward vs. side scatter profile, then assess the purity based on NK markers (e.g., CD3, CD56) in the specific live population. Typical purity is >95%.

1.13. After isolation is completed, spin cells at RT and 300 *x g* for 5 min to pellet and aspirate.

1.14. Resuspend the cell pellet to 1 x 10^7 cells/mL in RPMI 1640 with nutrients, 10% heat-inactivated FBS, 1 mM sodium pyruvate, 55 mM 2-ME, and 10 mM HEPES (pH = 7.2).

2. Antibody-mediated activation of NK cells via FcγRIIIa

2.1. Set a refrigerated microcentrifuge to 4 °C.

2.2. Dispense 1 x 10^6 (100 µL of) resuspended NK cells into 1.5 mL tube(s) and place on ice.

NOTE: Cells can be dispensed into a 96 well U-bottom plate if there are numerous samples.

2.3. Prepare 1 µL of 100 µg/mL rituximab (or antibody of interest) per sample and add 1 µL to the cells for a final concentration of 1 µg/mL antibody.

NOTE: Other molecules can be added to the cells at this point or earlier for the assessment of combination effects. Here, ritixumab was used for stimulation. In prior studies, small molecule inhibitors have been added to stimulations[9].

2.4. Incubate the sample on ice for 30 min. During incubation, prepare 50 µL of 50 µg/mL anti-human κ light chain antibody per sample in media and warm to 37 °C on a heat block or in a water bath.

2.5. After incubation of cells on ice, add 1 mL of ice-cold media and spin at 135 *x g* for 5 min at 4 °C. Wash again with 1 mL of ice cold-media.

2.6. Aspirate the supernatant after the last wash and add 50 µL of the anti-human κ light chain mixture prepared in step 2.5.

2.7. Immediately incubate samples for the end-user determined timepoints at 37 °C on a heat block or in a water bath.

2.8. Stop stimulation by adding 1 mL of ice-cold media and immediately spin samples in a refrigerated centrifuge at 135 *x g* for 5 min. Wash once more with 1 mL of ice-cold media.

3. Downstream applications and readouts

3.1. Interrogation of signaling molecules in FcγRIIIa-stimulated NK cells by western blot analysis

NOTE: Different protein separation and membrane transfer apparatuses may be used.

3.1.1 After the last washing with ice-cold media (step 2.8), lyse cells with 20 µL of RIPA buffer containing phosphatase and protease inhibitors mix for 30 min on ice.

3.1.2 Spin tubes at 2100 *x g* for 15 min at 4 °C. Transfer the lysate to a clean 1.5 mL tube and add the reagents required for protein separation.

3.1.3 Separate proteins on SDS-PAGE gel and transfer onto a nitrocellulose or PVDF membrane following standard protocols.

3.1.4 Block and probe the membrane according to the manufacturer's datasheet for the primary detection antibody (here, pAKT, pPRAS 40, and pERK1/2 were used).

3.1.5 Add HRP conjugated secondary antibody and chemiluminescence reagent per the manufacturer's recommendations on the PVDF membrane. Use X-ray film or detection instrument to visualize (here, pAKT was detected at 60 kDa, pPRAS40 at 40 kDa, and pERK1/2 at 42 kDa and 44 kDa).

3.2. Isolation of mRNA and preparation for gene expression analysis of FcγRIIIa-stimulated NK cells (**Table of Materials**)

3.2.1 After the timepoint for stimulation is reached (step 2.7), place the tube on ice and immediately spin in a refrigerated centrifuge at 135 x *g* for 5 min (similar to step 2.8).

3.2.2 Remove the supernatant and wash 2x with 1 mL of ice-cold PBS.

3.2.3 Lyse cells using guanidium isothiocyanate RNA extraction (**Table of Materials**). Use 1 µg of mRNA to perform reverse transcription using random hexamers with a commercial kit (**Table of Materials**).

NOTE: Any method of RNA extraction or cDNA synthesis can be used[9,23,24].

3.2.4 Freeze cDNA at -20 ˚C until gene expression analysis.

3.3. Cytoskeletal rearrangement assessment in FcγRIIIa-stimulated NK cells

3.3.1 After the last washing with ice-cold media (step 2.8), resuspend the cell pellet with 50 µL of 3.7% paraformaldehyde for 10 min at RT.

3.3.2 Add 1 mL of PBS and spin at 135 *x g* for 5 min at RT. Repeat this washing once more.

3.3.3 Add 100 µL of 0.1% Triton X-100/PBS for 5 min to permeabilize cells, and spin cells at 135 *x g* for 5 min at RT to pellet.

3.3.4 Add 100 µL of 5 units/mL AF488-labeled phalloidin diluted in 1% BSA/PBS and incubate for 20 min at RT.

3.3.5 Add 1 mL of PBS and spin at 135 *x g* for 5 min at RT to wash. Resuspend the cell pellet in the desired volume for flow cytometric analysis.

3.4. Assessing degranulation of FcγRIIIa-stimulated NK cells using CD107a surface staining

3.4.1 Incubate the cells with the antibody of interest on ice as described in steps 2.1–2.4. During incubation, prepare 50 µL of antibody mixture per sample. Prepare the mixture in cell media with a final concentration of 50 µg/mL anti-human κ light chain antibody and 1 µg/mL fluorochrome-labeled CD107a.

3.4.2 After incubation of the cells on ice, add 1 mL of ice-cold media. Spin at 135 *x g* for 5 min at 4 °C, then wash again with 1 mL of ice cold-media.

3.4.3 Aspirate the supernatant after the last washing, then add 50 µL of the anti-human κ light chain mixture and incubate at 37 °C for the desired timepoints.

3.4.4 At the desired timepoints, add 1 mL of PBS and spin at 135 *x g* for 5 min at RT. Aspirate supernatant and add 100 µL of 4% paraformaldehyde. Incubate at RT for 10 min.

3.4.5 Add 1 mL of PBS and spin at 135 *x g* for 5 min at RT to wash. Resuspend the cell pellet in the desired volume for flow cytometric analysis.

3.5. Assessment of chemokine/cytokine production from FcγRIIIa-stimulated NK cells

NOTE: Different methods and/or kits may be used for assessment of chemokine and cytokine production.

3.5.1 After the timepoint for stimulation is reached (step 2.7), immediately place the tube on ice and spin in a refrigerated centrifuge at 135 x g for 5 min at 4 °C.

3.5.2 Transfer the supernatant into a clean vessel. Freeze the supernatant until chemokine or cytokine assessment using the desired assay.

NOTE: Cell pellets can now be washed with ice-cold PBS and processed for signaling, gene expression, and cytoskeletal rearrangement studies.

REPRESENTATIVE RESULTS:

It is essential that NK cell purity is high, because the Fc portion of antibodies can bind the FcγRIIIa expressed on other cell types, such as monocytes. With high purity, the observations made can be attributed directly to FcγRIIIa-driven events in NK cells. Here, NK cells had greater than 90% purity based on CD56 and CD3 stains (**Figure 1**). In addition, the viability was >95%. Care should be used when using isolations with lower viability. To ensure events were driven by the FcγRIIIa, western blots for phospho-AKT (pAKT), phospho-PRAS40 (pPRAS40), and phospho-

ERK1/2 (pERK1/2) were performed, in which NK cells were activated for 1–5 min. As shown, an accumulation of these molecules was observed (**Figure 2**). Similarly, activated NK cells expressed MIP-1α, MIP-1β, IFN-γ, and TNF-α, as shown by gene expression analysis (**Figure 3**).

Additionally, cytoskeletal rearrangement was observed in activated cells (**Figure 4**). A percentage of NK cells stimulated for 4 h expressed CD107a on the cell surface (**Figure 5**). Additionally, IFN-γ, TNF-α, MIP-1α, MIP-1β, and RANTES were detected in the supernatant after 3 h of stimulation (**Figure 6**). These readouts are expected based on published studies, as activated NK cells will have these phosphorylated proteins, as well as the gene expression and production of the mentioned chemokines and cytokines[8,9]. In all experiments, stimulation conditions should include an anti-human κ light chain only control and antibody without anti-human κ light crosslinking control. These NK cells should not show any phospho-signaling, degranulation, chemokine/cytokine production, or chemokine/cytokine gene expression.

FIGURE LEGENDS:

Figure 1: Representative flow profiles of NK cell purity after isolation from PBMCs. PBMCs were isolated from blood of a healthy donor, followed by enrichment of NK cells using a negative selection method for human NK cell isolation (**Table of Materials**). Cells were stained with CD56 and CD3 before and after enrichment to determine purity. Representative dot plots of CD56 vs. CD3 before and after isolation.

Figure 2: FcγRIIIa-activated NK cells exhibit phosphorylated signaling molecules. Cells were stimulated with rituximab for 2 min, and cell lysates were made according to the protocol. Lysates were separated on a 4%–12% gel, followed by transfer onto a PVDF membrane. The membrane was probed with antibodies against pAKT, pPRAS40, pERK1/2, and actin.

Figure 3: FcγRIIIa-activated NK cells express chemokine and cytokine genes. NK cells were stimulated with rituximab for 0 h, 0.5 h, and 1 h. mRNA was collected, reverse-transcribed, and subjected to qPCR analysis for MIP-1α, MIP-1β, RANTES, IFN-γ, and TNF-α (**Table of Materials**). (**A**) Relative expression of MIP-1α, MIP-1β, and RANTES at each timepoint. (**B**) Relative expression of IFN-γ and TNF-α at each timepoint. Values were normalized to the actin gene.

Figure 4: FcγRIIIa-activated NK cells exhibit cytoskeletal rearrangement. NK cells were stimulated with rituximab for 0 min, 5 min, 15 min, and 30 min, followed by assessment for cytoskeletal rearrangement by phalloidin staining and flow cytometry. The ratio of MFI at experimental timepoint to the MFI at time 0 of NK cells stimulated with rituximab (circle, solid line) or secondary antibody alone (square, dotted line). Bars represent the SD of four replicates. Asterisks represent statistical significance based on a two-tailed unpaired Student's t-test (*$p < 0.05$).

Figure 5: FcγRIIIa-activated NK cells express CD107a. NK cells were stimulated with rituximab for 4 h followed by assessment for degranulation by CD107a and flow cytometry. (**A**)

Percentage of NK cells expressing CD107a after stimulation with rituximab (white bars) or anti-human κ light chain antibody (gray bars) for 0 h and 4 h. (**B**) CD107a MFI of NK cells stimulated with rituximab (white bars) or anti-human κ light chain antibody (gray bars) after 0 h and 4 h of treatment.

Figure 6: FcγRIIIa-activated NK cells secrete chemokines and cytokines. NK cells were stimulated for 0, 0.5, 1, 3, and 6 hr with rituximab. Supernatant was collected to measure release of MIP-1α, MIP-1β, RANTES, IFN-γ, and TNF-α by a flow- and bead-based cytokine assessment method (**Table of Materials**). (A) MIP-1α, MIP-1β, and RANTES production at each timepoint. (**B**) IFN-γ and TNF-α production at each timepoint.

DISCUSSION:

This protocol describes methods for studying FcγRIIIa-driven events in NK cells mediated by antibodies. These techniques permit the evaluation of potential mechanisms of action of therapeutic antibodies, which is suggested to be ADCC[1,2]. Specifically, these methods provide flexibility in studying underlying molecular signaling pathways and cellular processes that are responsible for ADCC. They also allow observation of other effector functions, such as chemokine and cytokine production. In addition, these methods allow the identification of potential biomarkers and molecules that may be targeted to modulate ADCC.

The basis for this protocol is the artificial stimulation of NK cells through the FcγRIIIa with antibodies in the absence of target cells. Antibody-bound target cells typically serve to promote the crosslinking of Fc receptors to form a platform that drives signaling and downstream effects. Instead, crosslinking is accomplished using an anti-human κ light chain antibody in this assay, bypassing the need for target cells to stimulate the NK cells. Without target cells, the results and observations can be attributed directly to the NK cells, assuming that the purification process is successful.

Importantly, using anti-human κ light chain antibody to crosslink the antibody does not interfere with the binding affinity of the Fc portion for the FcγRIIIa, an interaction that dictates the strength of response[10–13]. Indeed, studies have shown that afucosylated antibodies increase ADCC due to their increased affinity for the FcγRIIIa[10–13]. Subsequent studies showed that this increased affinity is unaffected by the anti-human κ light chain secondary antibody substitute and can be used to study the basis for increased ADCC[8]. To ensure that the NK cell is stimulated through crosslinking of the antibody, two negative controls should be included: 1) therapeutic antibody only without the secondary anti-human κ light chain antibody, and 2) the secondary anti-human κ light chain antibody only. In both cases, no signaling or effector function should be generated.

This method also provides flexibility for studying the effects of therapeutic antibodies on small molecule inhibitors. The inhibitor can be added before crosslinking with the secondary antibody so that the inhibitor has time to engage its target. However, studies should be performed to

393 determine the optimal time of inhibitor pretreatment; thus, the inhibitor has a maximal effect
394 on stimulation. With that said, researchers may also choose to study the effects of an inhibitor
395 after stimulation. In this case, the inhibitor may be added after crosslinking to study how it
396 influences signals and processes that are already generated. Together, the method described
397 here provides maximal flexibility in studying combinatorial effects of different small molecule
398 inhibitors with therapeutic antibodies.
399
400 As mentioned, above, a variety of readouts can be performed after stimulation. Western
401 blotting can be performed to study signaling using SDS-PAGE and membrane transfer systems
402 from various vendors. Similarly, gene expression can also be assessed using various RNA
403 extraction methods, reverse transcription reagents, and gene expression instruments. Finally,
404 staining for intracellular or extracellular protein can also be performed, in which samples can be
405 analyzed using different flow cytometers. For intracellular cytokine and CD107a staining (step
406 3.4, which can be assessed simultaneously), monensin and/or brefeldin A should be added to
407 maximize signals. We have used different platforms for each experimental goal and still
408 observed similar results. Therefore, the method can be complemented with various reagents,
409 platforms, and instruments, depending on the study.
410
411 The crosslinking time for stimulation will depend on the goal of the study. If signaling studies
412 are desired, typical crosslinking stimulation time is between 2 min and 10 min. pAKT, pPRAS40,
413 and pERK1/2 accumulation peaks at 2 min and disappears after 10 min[8,9]. For functional studies
414 (i.e., those involving chemokine/cytokine production), cells must be stimulated for at least 30
415 min, depending on the analyte[9]. Gene expression analysis also typically requires 30 min of
416 stimulation[9]. Caution should be exercised when using RANTES gene expression as a readout, as
417 RANTES mRNA production is independent of transcriptional activation since it is already stored
418 in cells for prompt translation and release of protein upon stimulation[25]. Degranulation, in
419 contrast, requires at least 3 h of stimulation. Despite these general observations, researchers
420 should perform kinetic studies to determine the optimal stimulation time for the particular
421 molecules of interest.
422
423 Similarly, researchers should titrate the antibody of interest to determine the optimal
424 concentration, because antibodies with different specificities bind to FcγRIIIa with different
425 affinities, even if they are of the same isotype. For example, rituximab and trastuzumab are
426 both an IgG1 isotype, but trastuzumab binds more strongly to the valine polymorphism of
427 FcγRIIIa than rituximab[26,27]. This difference in affinity may lead to functional differences, such as
428 degranulation, as observed in published studies[8].
429
430 Determining the optimal concentration is also important because of the low affinity the Fc
431 portion of the antibody has for the FcγRIIIa. This may result in washing off the antibody, since
432 the protocol includes washing steps after binding of the antibody to the Fc receptor. This may
433 then lead to a lack of sensitivity in the assays, as suggested by the low percentage of CD107a
434 positive cells after stimulation (**Figure 5**). However, determination of the optimal concentration
435 should provide confidence that results are not due to a lack of sensitivity. In addition, cells are
436 clearly activated in the biochemical and functional assays that use bulk cell as opposed to single

cell readouts (**Figure 2, Figure 3, Figure 6**).

The protocol is also limited since it does not entirely mimic what occurs physiologically. The secondary anti-human K antibody used is to imitate the crosslinking generated by target antigen expressed on cells. Here, a saturating amount of secondary antibody is added to generate the maximum response. However, distinct target cells will express different levels of antigen, which will affect crosslinking and response. Currently, this platform is not optimized to mimic the effects of different antigen expression levels.

Another factor to consider when performing these experiments is donor-to-donor variability due to different genetic backgrounds and immunological histories among individuals. Therefore, care must be taken when comparing NK cell responses from different donors across the same assays. Similarly, only general conclusions should be made when different donors are used.

Altogether, the described method is a simple and flexible stimulation platform to study antibody driven FcγRIIIa-mediated events in NK cells. It has been used to better understand the basis for the increased ADCC and efficacy observed with afucosylated antibodies[8]. This method was also employed in a study combining therapeutic antibodies and PI3K small molecule inhibitors[9]. Additionally, a previously unknown mechanism for chemokine and cytokine production regulated by pS6 was identified[9]. Therefore, future studies using this artificial signaling platform can further elucidate mechanisms of regulation for effector functions driven by the FcγRIIIa. It may also potentially identify new molecules important for these mechanisms as well as new roles for known molecules.

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DISCLOSURES:
All authors are or have been employees or consultants for iQ Biosciences.

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Figure 1

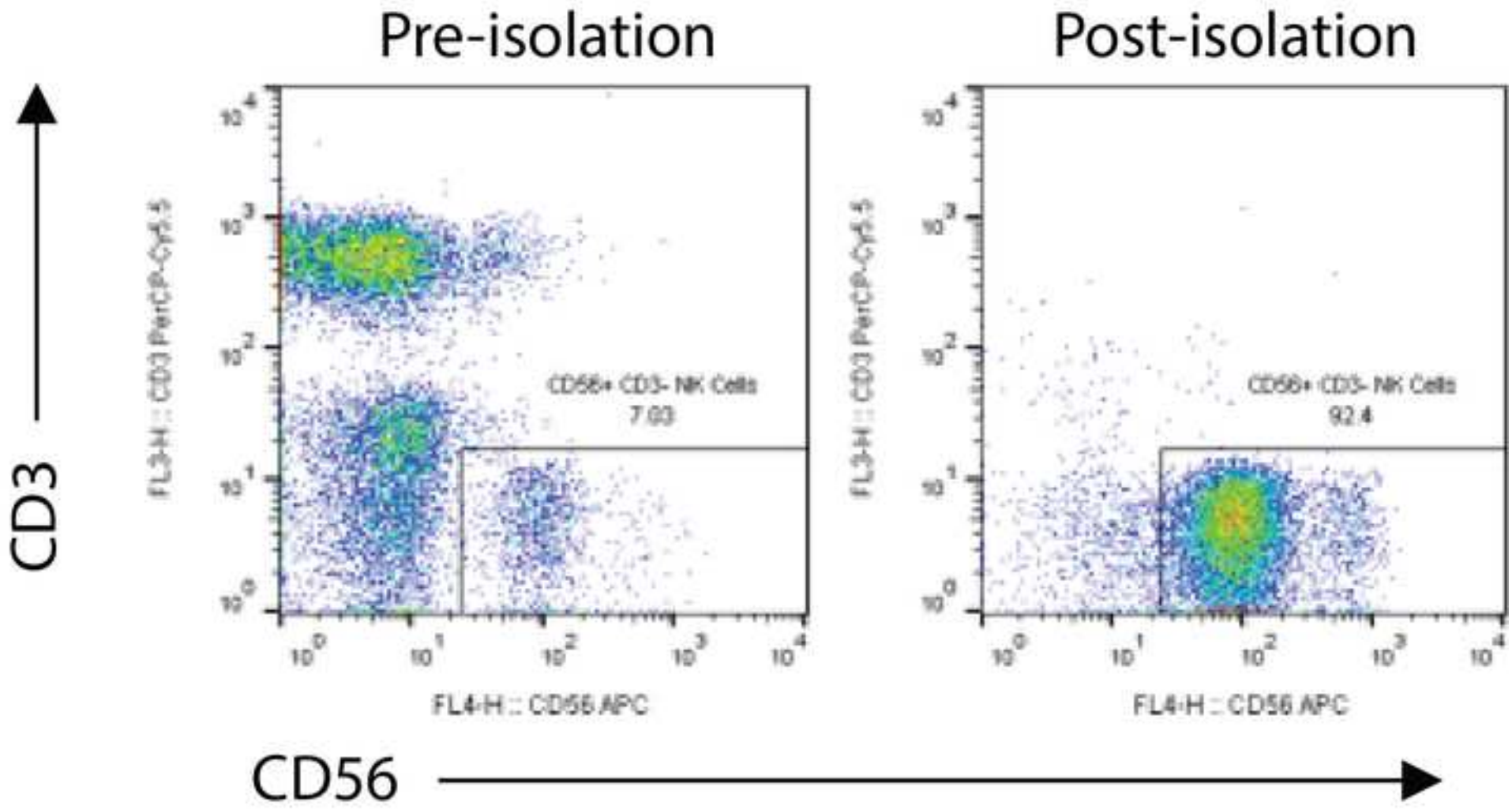


Figure 2

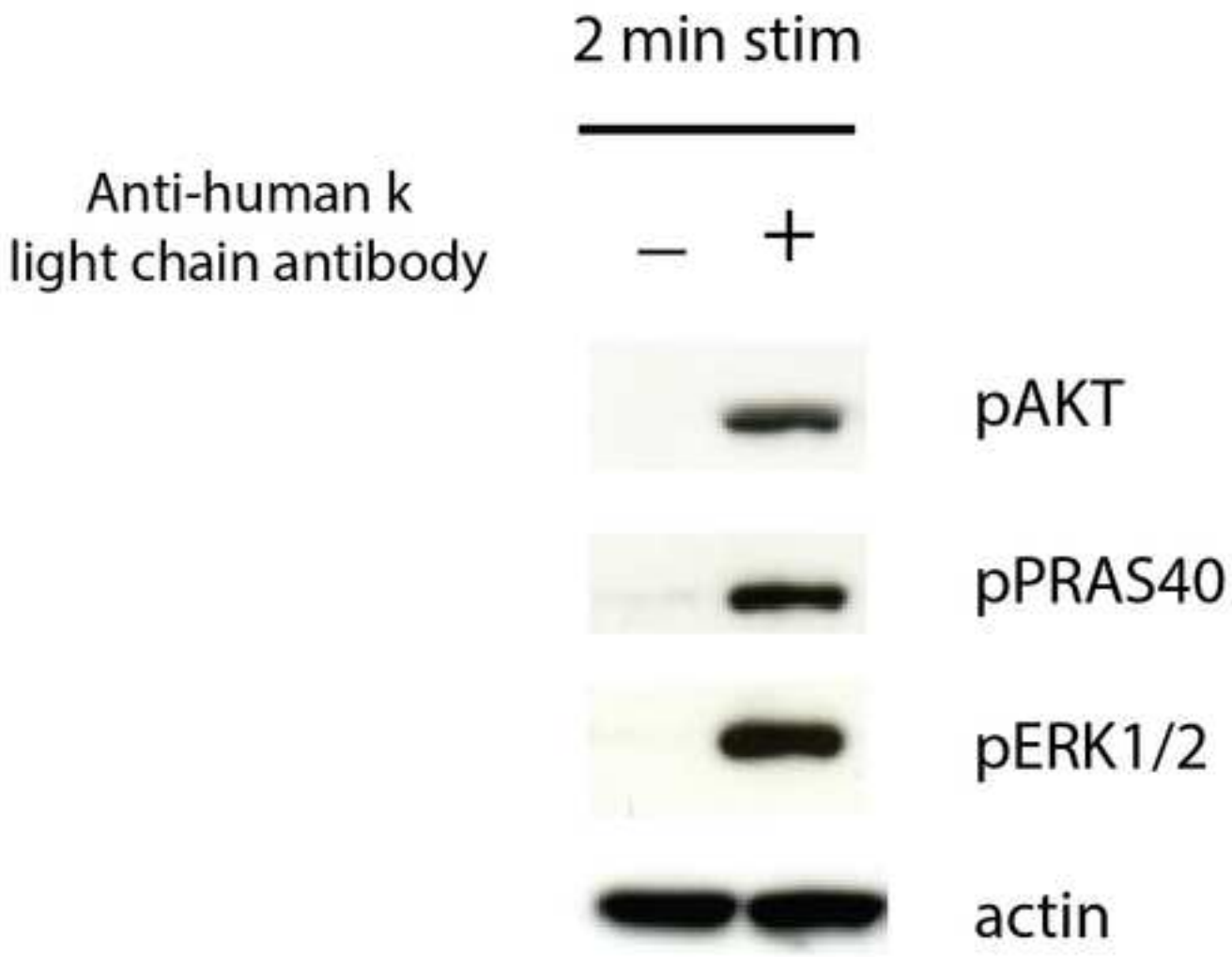
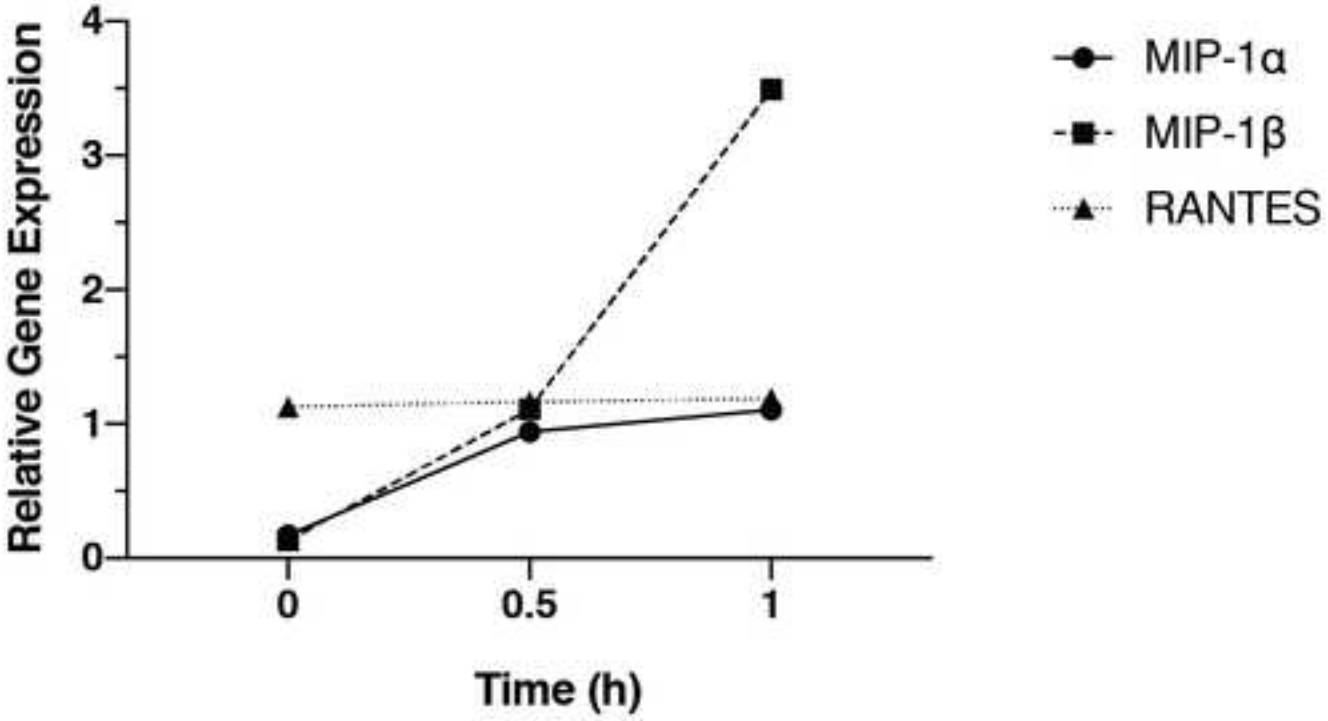


Figure 3

A.



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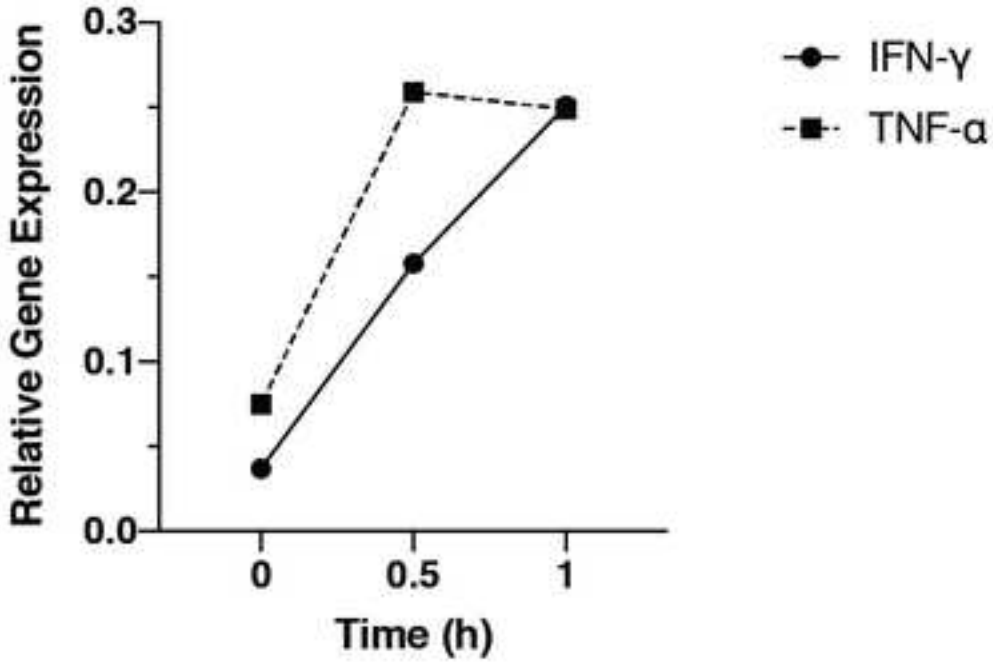


Figure 4

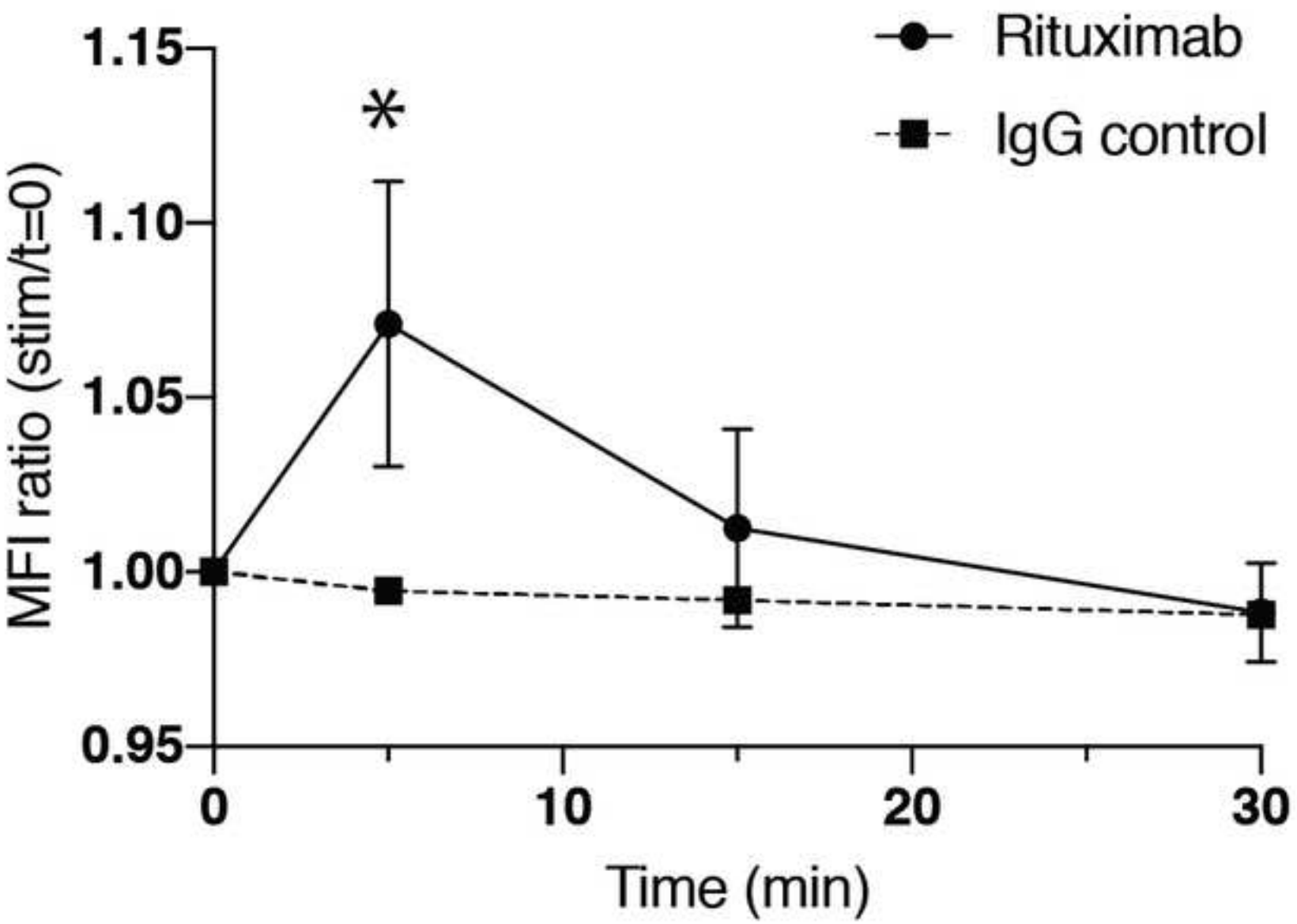


Figure 5

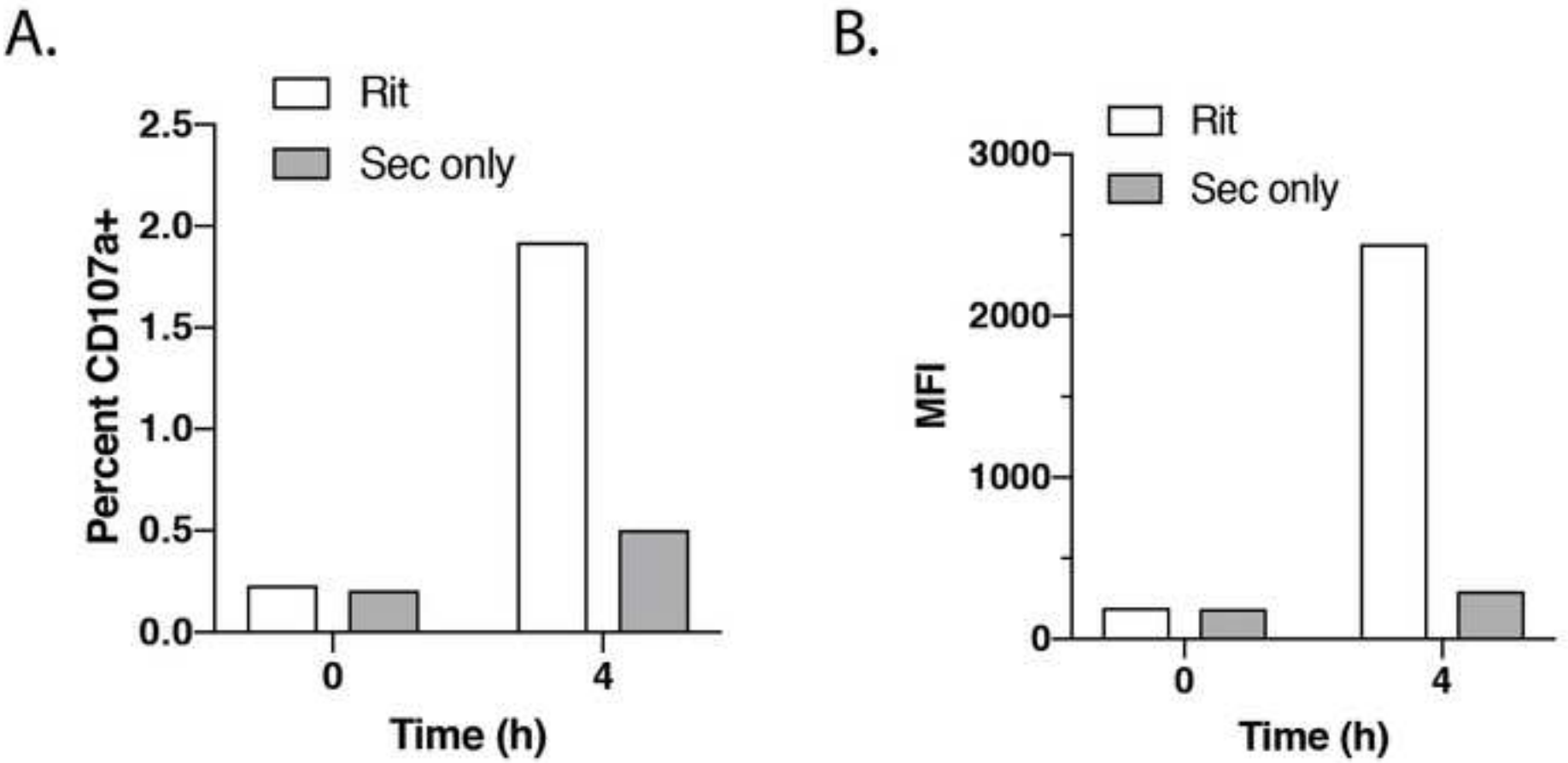
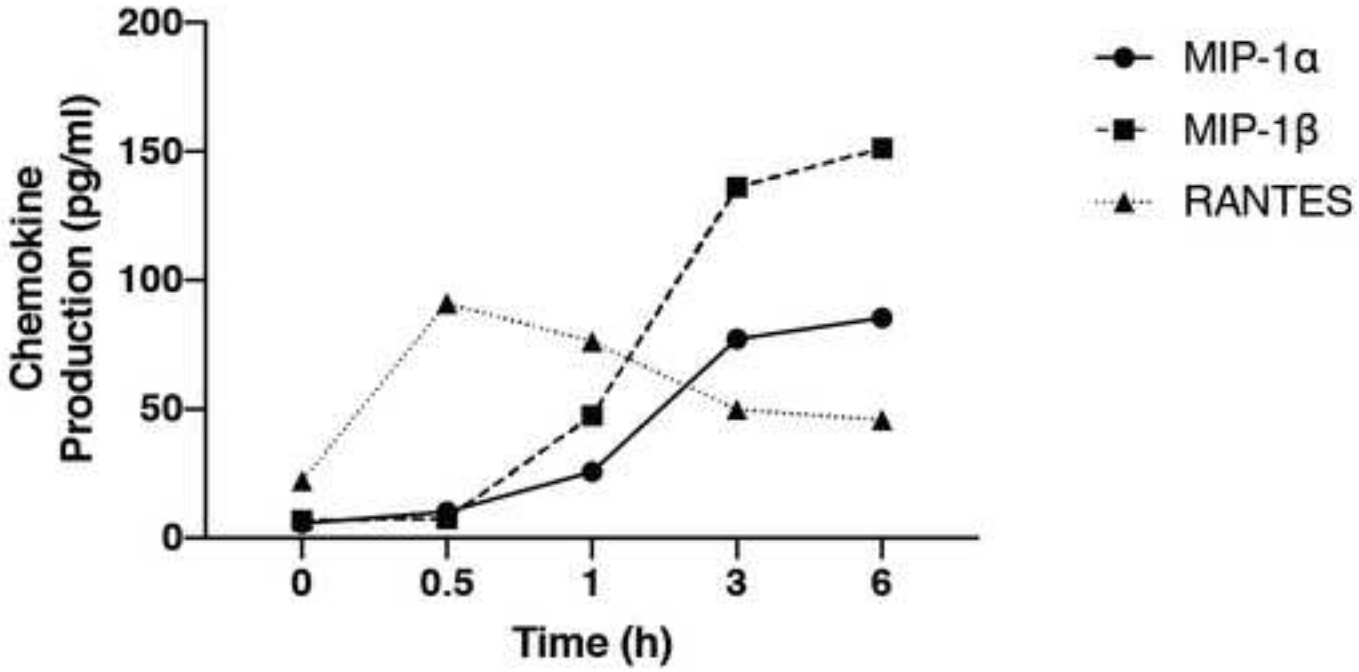


Figure 6

A.



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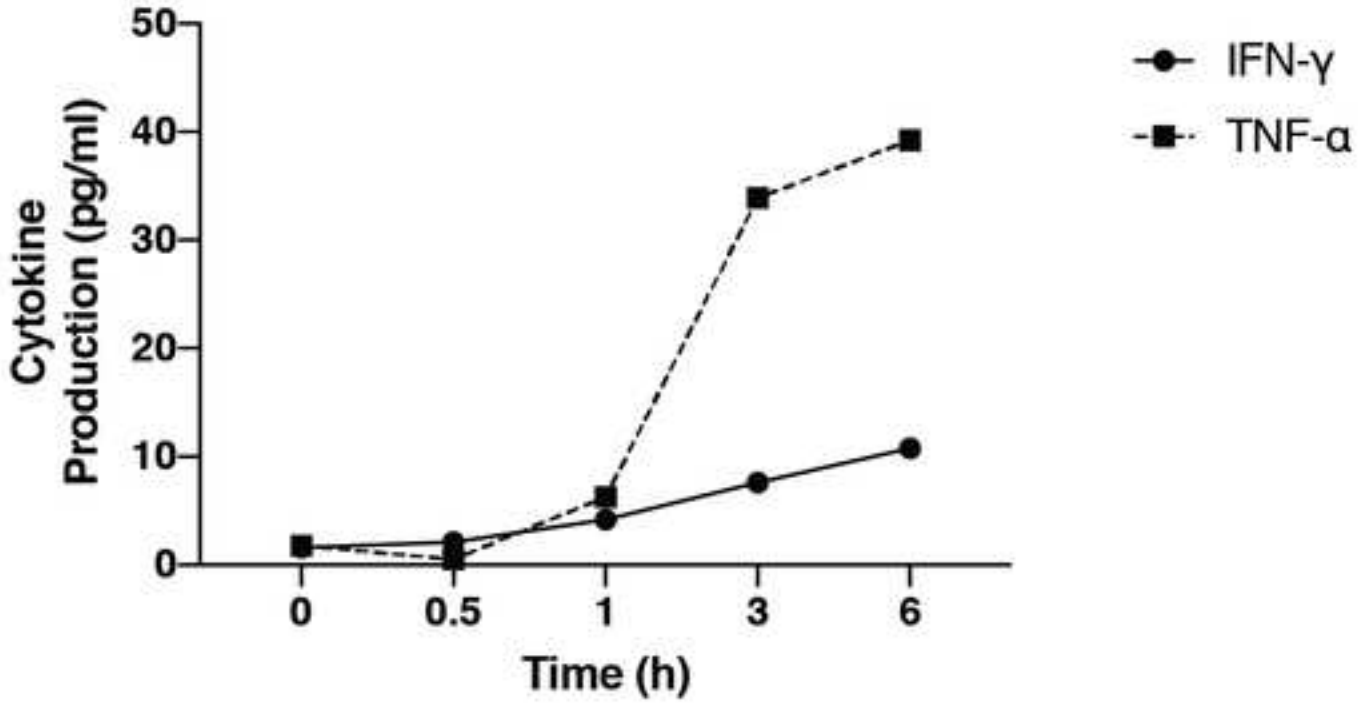

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
16% paraformaldehyde	Thermo Fisher Scientific	50-980-487	
Alexa Fluor 488 phalloidin	Thermo Fisher Scientific	A12379	
anti-mouse HRP antibody	Cell Signaling Technologies	7076	
AutoMACS instrument	Miltenyi		NK cell isolation method; another isolation instrument may be used
B-mercaptoethanol	Thermo Fisher Scientific	21985023	
Bovine Serum Albumin Fraction V, fatty acid free	Millipore Sigma	10775835001	
CD107a APC antibody	Biolegend	328620	
Cytokine 30-Plex Human Panel	Thermo Fisher Scientific	LHC6003M	chemokine/cytokine method; another chemokine/cytokine analysis method may be used
FBS	Hyclone	SH30071.01	
Goat anti-human κ light chain antibody	Millipore Sigma	AP502	
Halt Protease and Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific	78440	
HEPES	Thermo Fisher Scientific	15630080	
High Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368814	cDNA transcription method; used according to manufacturer's protocol
IFN-ɣ primers	Thermo Fisher Scientific	Hs00989291_m1	
Leukosep tube (50 ml conical with porous barrier)	Greiner	227290	
Lymphoprep (density gradient medium)	Stemcell	7851	
MIP-1α primers	Thermo Fisher Scientific	Hs00234142_m1	
MIP-1β primers	Thermo Fisher Scientific	Hs99999148_m1	

NK cell isolation kit	Miltenyi	130-092-657	NK cell isolation kit; another isolation kit may be used
NuPAGE 4-12% Bis-Tris Protein Gels	Thermo Fisher Scientific		another protein separation system may be used
pAKT (S473) antibody	Cell Signaling Technologies	4060	
pERK1/2 antibody	Cell Signaling Technologies	4370	
pPRAS40 antibody	Cell Signaling Technologies	13175	
PVDF membrane	Thermo Fisher Scientific		nitrocellulose may be used
RANTES primers	Thermo Fisher Scientific	Hs00982282_m1	
RIPA buffer	Millipore Sigma	R0278	
RPMI w/glutamax	Thermo Fisher Scientific	61870	
Sodium pyruvate	Thermo Fisher Scientific	11360070	
TNF-α primers	Thermo Fisher Scientific	Hs00174128_m1	
Triton X-100	Thermo Fisher Scientific	85111	
TRIzol	Thermo Fisher Scientific	15596018	RNA isolation method; used according to manufacturer's protocol
Xcell Blot II Transfer Module	Thermo Fisher Scientific		another protein separation system may be used
Xcell SureLock Protein Gel Electrophoresis Chamber System	Thermo Fisher Scientific		another protein separation system may be used
β-actin HRP antibody	Abcam	ab6721	
β-actin primers	Thermo Fisher Scientific	Hs00982282_m1	

Dear Editor,

We thank the editors for their helpful comments and respond to each of the items in our resubmission. Point-by-point responses (in normal font) to Editor's comments (in italics) are provided and identified in the revised manuscript by blue highlights.

Editorial comments:

1. *The editor has formatted the manuscript to match the journal's style. Please retain.*

We have not changed the formatting in the manuscript.

2. *Please address minor specific comments marked in the manuscript.*

We have addressed all the minor specific comments marked in the manuscript. They are highlighted in blue.

3. *Once done please ensure that the highlighted section is no more than 2.75 pages. Some of the shorter steps can be combined.*

We have combined the shorter steps, and the (yellow) highlighted section is just at 2.75 pages.

We hope the revisions are sufficient for acceptance. If not, we are happy to make further revisions to make it suitable for publication.

Regards,
Scot Liu