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TITLE:

A Plate-Based Cytotoxicity Assay for the Assessment of Rat Placental Natural Killer Cell Cytolytic Function

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KEYWORDS:

cytotoxicity, natural killer cell, isolation, lactate dehydrogenase, immunology, inflammation, hypertension

SUMMARY:

Here, we provide a detailed methodology to isolate and assess cytotoxic function of natural killer cells from placentas by a colorimetric plate assay. The reduced uterine perfusion pressure rat model of placental ischemia was chosen to demonstrate the antibody-mediated isolation and assessment of the cytotoxic function of natural killer cells.

ABSTRACT:

It is well known that decidual natural killer (NK) cells play a critical role in establishment and maintenance of normal pregnancy. Recent studies have demonstrated an altered population of circulating and decidual NK cells in women who suffer from adverse pregnancy complications such as recurrent miscarriage and preeclampsia. Studies from our group have shown that hypertension in pregnancy is associated with an increased population of activated NK cells in the placenta based on the expression of surface activation markers. This manuscript provides a detailed protocol to assess the cytotoxic function of NK cells isolated from placentas in a preeclampsia-like animal model of surgically induced placental ischemia. The following steps are described in detail: generation of single cell suspension, NK cell isolation, ex-vivo stimulation, effector:target cell co-culture, and the cytotoxicity assay.

INTRODUCTION:

Preeclampsia is a hypertensive disorder of pregnancy characterized by fetal growth restriction, end organ damage and chronic immune activation. Chronic immune activation in women with

preeclampsia leads to increased circulating and placental inflammatory cytokines, an imbalance in CD4+ T Cells populations, and an increased population of activated Natural Killer (NK) cells[1]. Studies recently published by our lab demonstrate a role for NK cells in causing some of the pathophysiology associated with preeclampsia in the Reduced Uterine Perfusion Pressure (RUPP) rat model of preeclampsia. Using flow cytometry to measure surface expression of activation markers on NK cells, an increased population of activated NK cells in the circulation and placentas of RUPP rats compared to normal pregnant (NP) rats was observed[2].

To confirm the flow cytometry observations, functional studies to assess the cytotoxic activity of NK cells isolated from the placentas of NP and RUPP rats were performed. There are several methods available for the assessment of cytotoxic function of cytotoxic $CD8^+$ T cells and NK cells. The gold standard for functional cytotoxic analysis is the chromium release assay[3]. Other developed protocols utilized include flow cytometry[4], image cytometry[5], calcein release[6], and most recently bioluminescence[7]. This video will provide a detailed protocol on using the well-established lactate dehydrogenase (LDH) release assay to measure cytotoxic function of NK cells using a commercially available LDH cytotoxicity assay kit.

PROTOCOL:

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. The care and handling of the animals were in accord with the National Institutes of Health guidelines for ethical animal treatment.

1. Lymphocyte cell isolation from placentas

1.1. Remove one placenta from the rat uterus (gestation day 19) and place in 10 mL of ice-cold PBS[8].

1.2. Place one placenta on a 100 µm filter and sit in a Petri dish containing 13.5 mL of RPMI and 1.5 mL of FBS (total volume is 15 mL). Use the flat side of a syringe plunger to push the placenta through the filter into the Petri dish.

1.3. Prepare 3 sets of 15 mL conical tubes for each tissue. Add 3 mL of density gradient medium (see **Table of Materials**) to each tube, then carefully overlay 5 mL of homogenized placenta into each tube.

1.4. Centrifuge for 25 min at 100 x *g* at room temperature (RT) with **no brake.** Collect the **thin white buffy layer** with a transfer pipette.

NOTE: After centrifugation, 3 layers are visible, the red RPMI at the top, the white buffy layer in the middle, and the clear density gradient medium layer at the bottom. Use a transfer pipette to pull up the white buffy layer from the tube. Combine buffy layer from all tubes of the same placenta into 1 tube.

1.5. Add 10 mL of RPMI to combined buffy layers. Centrifuge for 5 min, 300 x *g*, at 4 °C and discard supernatant.

2. Isolation of natural killer cells

2.1. Resuspend cell pellet in 50 µL of ice-cold PBS

2.2. Add biotin-labeled CD3 antibody to pelleted cells according to the manufacturer's protocol and mix well with a pipette. Place the tube in a tube rotator and incubate 20 min at 4 °C.

2.3. Add 1 mL of RPMI, centrifuge for 10 min at 400 x *g* and 4 °C, and discard supernatant.

2.4. Resuspend pellet in 1 mL of RPMI and combine with 150 µL of magnetic beads in a 1.5 mL microcentrifuge tube. Place the microcentrifuge tube in a tube rotator and rotate while incubating for 30 min at 4 °C.

NOTE: Pull out release buffer at this time and allow to reach RT in the biosafety cabinet.

2.5. Place the tubes in the magnet for 1 min. Collect supernatant and save CD3- cell population in a 15 mL tube on ice.

NOTE: This is the CD3- population of cells.

2.6. Remove the tube from the magnet and add 1 mL of RPMI. Mix cells and beads 5 times with a pipette.

2.7. Repeat step 2.5.

2.8. Centrifuge the CD 3- population of cells for 10 min at 400 x *g* and 4 °C and discard supernatant. Resuspend the cell pellet in 50 µL of ice-cold PBS

2.9. Add biotin-labeled CD161a antibody to $CD3^-$ cells according to the manufacturer's protocol and mix well. Place tube in a tube rotator and incubate for 20 min at 4 °C.

2.10. Add 1 mL of RPMI, centrifuge for 10 min at 400 x *g* and 4 °C, and discard supernatant. Resuspend pellet in 1 mL of RPMI and combine with 150 µL of magnetic beads in a 1.5 mL microcentrifuge tube.

2.11. Place microcentrifuge tube in a tube rotator and rotate while incubating for 30 min at 4 °C.

2.12. Place the tubes in a magnet for 1 min. Collect supernatant and discard $CD3^-$/$CD161a^-$ cells.

2.13. Remove tube from magnet and add 1 mL of RPMI. Mix cells and beads 5 times with a pipette.

2.14. Repeat step 2.12.

2.15. Remove microcentrifuge tubes from the magnet and add 1 mL of RT Release buffer. Place tube on tube rotator and rotate while incubating for 15 min at RT.

2.16. Place tubes in magnet for 1 min. Collect supernatant in a new 15 mL conical tube on ice.

NOTE: This is the CD3-/CD161a+ population of NK cells.

2.17. Remove tube from magnet and add 1 mL of RT RPMI. Mix cells and beads 5 times with pipette.

2.18. Repeat step 2.16, placing supernatant in the same tube. Mix well and take a 20 µL sample to count cells

NOTE: Keep tube on ice.

2.19. Centrifuge CD3-/CD161a+ cells for 10 min, 400 x *g* at 4 °C, then remove supernatant. Resuspend the cells in RPMI (10% FBS, 1% Pen/Strep, 2 ng/ mL IL-2) and seed at a concentration of 3 x 10^5 cells/well in a 6-well plate in 2.5 mL of NK Cell Activation Media. Incubate cells for 48 h at 37 °C, 5% CO_2 in a humidified incubator.

3. Cytotoxicity assay: retrieving NK Cells or YAC1 from culture or passing cells

NOTE: All steps must be conducted under the hood. All cell tubes must be kept on ice at all times.

3.1. Use a glass serological pipet to collect YAC1 cells and media from flask, place in a 50 mL tube on ice, and mix well. Take 20 µL to count cells.

3.2. Spin the YAC1 cells for 10 min at 300 x *g* and 4 °C. Count cells while these are spinning.

3.3. Add trypsin/EDTA to each well in a NK cell 6-well plate. Tap the plate and place in incubator.

3.4. After cells have incubated with trypsin/EDTA for ~5 min at 37 °C, scrape the plate/flask with a sterile plate scraper. Add 1 mL of NK Cell Media to each well.

3.5. Collect cells and media with serological pipet and collect in a 15 mL centrifuge tube. Take 20 µL to count cells.

3.6. Spin the NK cells for 10 min, 400 x *g* at 4 °C. Count the sample of cells from step 3.5 during this centrifugation.

NOTE: View the culture plates under a microscope before discarding them to make sure there are no more cells adhered to the bottom of the wells. Since the experiment uses NK cells and YAC1 cells, be sure to count each one **separately**.

3.7. Count cells and resuspend at the concentrations determined in optimization trials to test for the appropriate number of target:effector ratio. This will be achieved by re-suspending the pellet in their corresponding media to make the following cell concentrations: YAC1 at 4 x 10^5 cells/mL and NK cells at 2 x 10^7 cells/mL.

4. Cytotoxicity assay: assay protocol

4.1. Use a round bottom, culture treated 96-well plate to set up the plate as suggested in **Table 1**. This table shows the experimental controls and 3 sets of NK experimental columns. This can be expanded to a total of 10 NK experimental columns in a 96-well plate.

4.2. Centrifuge the assay plate at 250 x *g* for 4 min to be certain that the effector and target cells are in contact. Incubate the 96-well plate for 5 hours in a humidified chamber incubator at 37 °C, 5% CO_2 to achieve ample contact between target and effector cells and target cell lysis by effector cells.

NOTE: The protocol can be paused here.

4.3. 45 min prior to harvesting supernatants, add 10 µL of 10x Lysis Solution to the Target Cell Maximum LDH Release wells (Wells 1E, 1F, 1G, and 1H) and place the plate back in the humidified chamber.

4.4. After the incubation time is completed, centrifuge the plate at 250 x *g* for 4 min. Using a multichannel pipettor, transfer 50 µL aliquots from all wells to a fresh 96-well flat-bottom assay plate.

NOTE: Do not touch the bottom of the wells so that cells are not transferred into the fresh assay plate. Do not use a cultured treated plate as the fresh assay plate.

4.5. Make Assay Reagent.

4.5.1. After Assay Buffer has reached room temperature, add 12 mL of Assay Buffer to one Substrate Mix bottle. Invert and shake gently until completely dissolved. 1 bottle is enough for two 96-well plates.

NOTE: Assay Buffer should be protected from light while being thawed. Mix immediately prior to use.

4.6. Add 50 µL of Assay Reagent to all wells in the assay plate from step 4.6. Cover the plate with foil to protect it from light and incubate for 30 min at room temperature.

NOTE: Newly made Assay Reagent should be stored in a freezer. Reagent is good for approximately 8 weeks.

4.7. Add 50 µL of Stop Solution to each well. Read plate within 1 hour after adding Stop Solution and record the absorbance at 490 nm. Representative data is shown in **Table 2**.

5. Calculation of results

5.1. Calculate the average absorbance value from the Culture Medium Background wells and subtract from all absorbance values for Experimental, Target Cell Spontaneous LDH Release and Effector Cell Spontaneous LDH Release wells.

5.2. Calculate the average absorbance values for the Volume Correction Control wells and subtract from the absorbance values acquired for the Target Cell Maximum LDH Release Control wells.

5.3. Use the corrected values from Steps 5.1 and 5.22 in the following formula to calculate percent cytotoxicity for each effector:target well.

$$\%\ Cytotoxicity = \frac{Experimental - Effector\ Spontaneous - Target\ Spontaneous}{Target\ Maximum - Target\ Spontaneous} \times 100$$

RESULTS

Placental NK cells obtained from NP and RUPP rats were incubated for 5 hours with target cells in their respective medias at a ratio of 50:1 (NK:target). Absorbance was recorded at 490 nm and the raw data is shown in **Table 2**. The average absorbance of the Culture Medium Background and the Volume Correction control wells were calculated. These averages were subtracted from the appropriate wells indicated in the manufacturer's protocol and are represented in **Table 3**. The corrected values were then used to obtain the% cytotoxicity using the protocol provided by the manufacturer (**Table 4**).

FIGURE AND TABLE LEGENDS:

Table 1. Assay Plate Layout. TCS = Target cell Spontaneous LDH Release (50 µL target cells + 50 µL target cell medium); TCM = Target Cell Maximum LDH Release (50 µL target cells + 50µL target cell medium); VCC = Volume Correction Control (50 µL target cell medium + 50 µL NK Cell medium); CMB = Culture Medium Background Control (50 µL target cell medium + 50 µL NK Cell medium); ECSR = Effector Cell Spontaneous Release (50 µL NK cells + 50 µL NK cells medium); EW = Experimental wells (50 µL target cells + 50 µL NK cells).

Table 2. Raw absorbance data of an assay plate containing the experimental controls in columns 1 and 2 and experimental wells to measure the cytotoxicity of placental NK cells from NP and RUPP rats against YAC1 target cells.

Table 3. Corrected Absorbance. Absorbance of wells after subtraction of average absorbance of control wells according to the manufacturer's protocol. Column 1, Wells 1-4, are TCS absorbances values – the average CMB absorbance value. Column 1, Wells 5-8, are the TCM absorbance values – the average VCC absorbance value.

Table 4. Cytotoxicity Calculations. Average percent cytotoxicity of replicate samples of placental NK cells isolated from NP and RUPP rats.

DISCUSSION:

There are a number of important key notes to consider for optimal results. The sterility of the cells utilized is very important. After collection of the placenta, it is important that preparation and isolation of the NK cells are performed under sterile conditions in a biosafety cabinet. Furthermore, because all cells release LDH upon cellular damage, care should be taken to obtain a high viability of NK cells after isolation and during the co-culture process. Too much spontaneous LDH release from the NK cells can often result in negative, unusable data

There are some aspects of this protocol that will be specific to the user and their needs. For example, there are various methods for isolation of mononuclear cells from placenta in addition to the methods in this article, such as mechanical and enzymatic disaggregation. Additionally, it is up the user to determine the optimal target cells number and effector:target ratio that will be suitable for their experiment. In our hands, 10,000 target cells and an effector:target ratio of 50:1 was determined to be optimal for our experiments. These numbers may change depending on the target cells chosen and on the tissue from which the NK cells are isolated. Other investigators have utilized this method to assess NK function of cells isolated from liver and spleen[9]. Therefore, this method can be employed in various areas of research, not just in studies of preeclampsia, as we have chosen to use it.

The gold standard for cytotoxicity assessment is the chromium release assay[5]. While this method is reliable and reproducible, the most obvious limitation is the use of radioactive exposure risk to personnel and radioactive disposal. Other, non-radioactive cytotoxicity assays such as the flow cytometry based and calcien release assays are reported to have comparable results to CRA[6,10,11]. However, flow-cytometry-based assays require additional specialized training of personnel in flow cytometry to run the assay. The LDH release assay doesn't require any specialized training, as this test only requires simple pipetting to perform the assay. Additionally, while the LDH release assay can be utilized using standard spectroscopy or fluorescent detection, the calcien release and flow-cytometry-based methods require equipment capable of detecting fluorescence. Limitations of the LDH release assay include an underestimation of cell death due to incomplete release of LDH from damaged cells.

In this assay, the measurement of LDH released into the media occurs by an enzymatic reaction in which iodonitrotetrazolium (a tetrazolium salt) is converted into formazan (red in color). The absorbance is measured by standard spectroscopy and the amount of damaged cells in the culture is proportional to the intensity of the red color. This assay allows for accurate determination of damaged or injured cells in a sample. Finally, this colorimetric assay can also be

used with other cell types to assay cytotoxicity occurring via cell-mediated as well as chemical-mediated mechanisms.

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DISCLOSURES:

No conflicts of interest, financial or otherwise, are declared by the authors.

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Table 1

1	2	3	4	5
TCS	VCC	ECSR-1	ECSR-2	ECSR-3
TCS	VCC	ECSR-1	ECSR-2	ECSR-3
TCS	VCC	ECSR-1	ECSR-2	ECSR-3
TCS	VCC	ECSR-1	ECSR-2	ECSR-3
TCM	CMB	EW-1	EW-2	EW-3
TCM	CMB	EW-1	EW-2	EW-3
TCM	CMB	EW-1	EW-2	EW-3
TCM	CMB	EW-1	EW-2	EW-3

Table 2

1	2	3	4	5
		NP	RUPP	NP
0.615	0.484	0.473	0.463	0.471
0.586	0.484	0.458	0.474	0.469
0.61	0.486	0.464	0.459	0.469
0.578	0.495	0.478	0.458	0.482
0.605	0.55	0.524	0.526	0.543
0.576	0.504	0.519	0.502	0.528
0.565	0.512	0.515	0.513	0.531
0.563	0.581	0.526	0.508	0.519

Table 3

1	2	3	4	5
	NP	RUPP	NP	
0.12775	-0.01425	-0.02425	-0.01625	
0.09875	-0.02925	-0.01325	-0.01825	
0.12275	-0.02325	-0.02825	-0.01825	
0.09075	-0.00925	-0.02925	-0.00525	
0.06825	0.03675	0.03875	0.05575	
0.03925	0.03175	0.01475	0.04075	
0.02825	0.02775	0.02575	0.04375	
0.02625	0.03875	0.02075	0.03175	

Table 4

	NP	RUPP	NP
	128.9916	108.8235	93.69748
	63.44538	118.9076	66.80672
	75.92593	72.75132	64.28571
	66.27907	63.17829	83.33333
Average % Cytotoxicity	**83.66049**	**90.91518**	**77.03081**

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL Eppendorf tube	Fisher	5408129	
100 µL Filter	Fisher	22363549	Nylon Mesh
15 mL conical tube	Fisher	0553859A	
3 mL syringe	Fisher	14823436	
50 mL conical tube	Fisher	7203510	
6-well cell culture plate	Corning	720083	
96-well Tissue Culture Plate	CELLTREAT	229190	Sterile, Round Bottom
AOPI	Nexcelom	CS201065ML	
Cell scraper	Fisher	8100241	
Cellometer Disposable Counting Chambers	Nexcelom	CHT4-SD100	
Cellometer Vision Image Cytometer	Nexcelom	N/A	
Cytotox 96 Non-Radioactive Cytotoxicity Assay Kit	Promega	G1780	
Dynabeads Flowcomp Flexi Kit	Invitrogen	11061D	
DynaMag-2 Magnet	Invitrogen	12321D	
EDTA	Sigma Aldrich	EDS-100G	
FBS	Atlanta Biologicals	S11150H	
Flow Cytometry Tube	Corning	352008	
Lymphoprep	Fisher	NC0460539	Density gradient medium; 4 x 250 mL
PBS	Fisher	SH3025801	10 x 500 mL
Penicillin/Streptomycin	Gibco	15140122	
Petri dishes	Fisher	9720500	Without Pad
Purified Mouse anti-Rat CD161a	BD Biosciences	555006	
Purified Mouse anti-Rat CD3	BD Biosciences	554829	
Recombinant Rat IL-2	R&D Systems	502-RL	
RPMI	Gibco	11875135	1640 Medium
T25 flask	Corning	430639	
Trypsin	ThermoFisher	15090046	
YAC-1 cell	ATCC	TIB-160	

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RESPONSE TO THE REVIEWERS

We would like to thank the Reviewers for their valuable and constructive comments, which we have considered carefully. Please find a point-by-point response to the issues raised.

Editorial Comments:

1. 1.1: Please provide more details or a reference for placenta isolation here (if the latter, please do not highlight for filming).

The authors have provided a reference for removal of the placenta from the uterus. See lines 70-71.

2. 4/5: While this is a commercial kit, please provide a little more detail about the procedure here, in particular as it appears that the results largely come from these sections. Also, calculations are fine to have in the written protocol; they just shouldn't be highlighted for filming.

The authors have provided the details in these sections and also removed the highlights from the calculations section (section 5) so that it will not be filmed. See lines 209-268

3. Figure 1: This would probably be better as a Table. I've provided a placeholder Figure (which will be disregarded when this is published) so that you can remove this Figure.

The authors have uploaded previous figure 1 as a table. It is now Table 2.

4. Figure 1/Table 2: Please describe columns 1 and 2.

Columns 1 and 2 in these tables are now described in the new Table 1. These are the required control wells that are described in the manufacturer's protocol. We have edited the legend for the new Table 1 (previously table 4) so that it described what is contained in columns 1 and 2 of the new Tables 2 and 3 (previously Figure 1/Table 2). See lines 289-295. Column 2 has been removed from Table 2.

5. Table 4 is not cited in the text. It is also a little confusing-how do the numbers at top correspond to the columns below? What are 7-12, more experimental wells?

Table 4 has now been cited in the text. It is now Table 1. See lines 211-212.

The numbers at the top have now been removed. The numbers were showing the columns in a 96-well plate.

See Table 1

Columns 7-12 were more experimental wells, but have been removed. We have added a note in the text stating that the number of experimental well columns can be expanded to 10 when using a 96-well plate. The table of the plate assay layout now only includes the columns used in this particular experiment.

See lines 212-215 and Table 1