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Use of transfected *Drosophila* S2 cells to study NK cell activation

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Abstract

Determining the contribution of individual receptors to natural killer (NK) cell function is complicated by the multiplicity of activating and inhibitory NK cell receptors. Mammalian target cells typically express a variety of ligands for NK cell receptors. Engagement of NK cell receptors by antibodies may not mimic activation by natural ligands. To define requirements for activation and dissect the contribution of receptors to NK cell function, we have generated *Drosophila* Schneider line 2 (S2) cell transfectants expressing ligands for NK cell receptors. The evolutionary distance between *Drosophila* and mammals greatly reduces the potential of recognition of insect cell molecules by mammalian NK cells. Here, we present methods for maintenance and transfection of S2 cells, as well as protocols for their use in NK cell assays.

Keywords

Drosophila; S2; insect cells; NK cells; activation; inhibition; transfection

1. Introduction

The activation of Natural Killer (NK) cells is controlled by combinations of activating and inhibitory receptors expressed by NK cells. Integration of the signals downstream from a wide variety of receptors determines whether or not the NK cells respond (1–4). Higher expression of activating ligands on target cells induces activation of NK cells, whereas higher expression of inhibitory ligands prevents it. Human NK cells express a wide variety of activating receptors, which respond to a wide variety of ligands. The Natural Cytotoxicity Receptors (NCRs) NKp46, NKp44, and NKp30, and receptors NKG2D, 2B4 (CD244), CD2, LFA-1 (CD11a/CD18), and DNAM-1 (CD226) are some of the receptors known to deliver activating signals to NK cells. Engagement of combinations of these receptors can activate NK cell functions such as adhesion, cytotoxicity, and cytokine secretion (5–7). NK cells are also potent mediators of antibody-dependent cellular cytotoxicity (ADCC), through the low affinity Fc_γ RIII (CD16). Inhibitory receptors are expressed on NK cells in a clonally distributed manner, where individual NK cells may express single or multiple inhibitory receptors in an essentially random manner. Inhibitory receptors in humans include members of the killer cell Ig-like receptor (KIR) family, which recognize alleles of HLA Class I, members of the CD94/NKG2 family that recognize HLA-E, and several other inhibitory receptors that bind to non-MHC ligands (4, 8).

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The response of NK cells to signals from individual receptors, or combinations of receptors, is often studied by masking the receptors with antibodies in order to block a response to a given target cell (9–13), or by crosslinking the receptors on the surface of NK cells with antibodies in order to provoke a response in the absence of target cells (7, 14). Such experiments can be very informative; however, antibodies typically have much higher affinities for receptors than the natural ligands do, and may fail to stimulate those receptors in the physiological way. This is particularly true for receptors such as integrins, that undergo conformational changes upon ligand binding (15, 16), which may not occur following antibody crosslinking.

Cell lines that are susceptible or resistant to NK cell cytotoxicity are also used to probe NK cell responses. Ectopic expression of ligands in cell lines has revealed much about the contributions of both activating and inhibitory receptors to the regulation of NK cells (9, 13, 17, 18). Interpretation of such experiments can be complicated by the expression of many, often uncharacterized, ligands on target cell lines. For example, the EBV transformed B cell line 721.221 expresses ICAM-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50) (ligands for the integrin LFA-1), CD48 (ligand for 2B4), and B7 (CD80, a ligand of CD28) (19). The erythroid cell line K562 expresses ULBP2 and MICA/B (ligands for NKG2D) (20, 21) along with ICAM-1 and ICAM-2 (22). Additionally, the Fc γ R⁺ mouse cell line P815, which is often used to stimulate NK cells with antibodies via redirected lysis (10, 11), expresses the mouse ICAM-1, which binds to human LFA-1 (23). In addition, P815 cells are more sensitive to lysis by NK cells that express high levels of NKp46, suggesting that they may express ligands for this NCR (24). Ligands for the NCRs have not been fully characterized, and the pattern of expression on many NK target cell lines is unknown.

These uncertainties created a need for a target cell line in which expression of ligands is more easily characterized and controlled. A cell line derived from an evolutionarily distant species, which would also be easy to transfect and maintain, would be a useful surrogate target cell. Schneider line 2 (S2) cells fulfill these requirements. The S2 cell line was derived from late-stage embryos of *Drosophila melanogaster* (25). The cells grow in a loose monolayer. S2 cells are easily transfected. They can be adapted to growth in serum free medium, suitable for the purification of recombinant secreted proteins. S2 cells have been used to produce and purify proteins in sufficient quantities for structural and biochemical studies (26). Expression of exogenous proteins is often accomplished by transfection with the pRmHa3 plasmid, which uses the *Drosophila* metallothionein gene promoter for inducible expression, allowing high expression even for proteins that prove deleterious to the growth of S2 cells (27).

Transfected S2 cells have been used previously to investigate the minimal requirements for T cell activation and co-stimulation. S2 cells expressing peptide-loaded MHC class-I, either alone or in combination with ICAM-1 and B7, were used as antigen presenting cells (APCs) for the stimulation of naïve T cells expressing the 2C transgenic TCR (28, 29). The results demonstrated that signaling through the TCR alone is not sufficient to activate naïve CD8⁺ T cells, and that B7 and ICAM-1 could provide the required co-stimulation for activation. Optimal co-stimulation occurred with both B7 and ICAM-1 expressed on the same MHC class-I⁺ S2 cell. Besides confirming the two-signal hypothesis for T cell activation, these data also demonstrated that co-engagement of the TCR with a co-stimulatory receptor is sufficient for T cell activation.

The use of S2 cells as APCs for T cells indicated that this *Drosophila* cell line could be used to reconstitute a sensitive target cell for NK cells. We have successfully used S2 cells to investigate the response of primary human NK cells to stimulation and inhibition through individual receptors (5, 30, 31). A notable advantage of this system is that resting NK cells,

freshly isolated from human blood, can be used directly in functional assays with S2 cell transfectants, without further manipulation. In most assays examining degranulation or cytokine secretion, untransfected S2 cells induce minimal or no response from either primary NK cells or NK cell lines. Expression of individual ligands by stable transfection is sufficient to induce responses such as adhesion (5) and granule polarization (30, 31), whereas multiple ligands may be required to induce other responses such as degranulation (Y. Bryceson, unpublished). Transmembrane proteins expressed in S2 cells are glycosylated, which may be important for recognition by some NK cell receptors. S2 cells are easily transfected, either transiently or stably, and can be substituted for mammalian target cells in many NK cell assays with little or no modification. We will present here methods for stable transfection of S2 cells, methods for isolation of cells expressing the transfected protein, as well as specific protocols for the use of S2 cells in assays for NK cell function.

2. Materials

2.1 Care and Culture of S2 Cells

2.1.1 Standard Culture

1. S2 culture medium: Schneider's *Drosophila* Medium (Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan UT; complement inactivated by heating at 56°C for 30 minutes).
2. 75 cm² tissue culture flasks (Costar, Corning, Lowell MA).
3. Freezing Medium: Fresh S2 cell culture medium mixed with an equal volume of conditioned medium (the medium in which S2 cells have been grown) supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis MO). Alternatively, freezing medium can be composed of 90% FBS supplemented with 10% DMSO.
4. Reagent for inducible expression: Expression from metallothionein promoter-based plasmids is induced by the addition of cupric sulfate (CuSO₄, Sigma Aldrich) to the cell culture to a final concentration of 1 mM. A 100 mM CuSO₄ stock solution is made in deionized water, and then sterilized by filtration through a 0.45 µm filter.

2.1.2 Adaptation of S2 Cells to Culture in Serum Free Conditions

1. Bio Whittaker Insect-Xpress medium (Lonza, Basel, Switzerland).
2. Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 0.2% BSA (see Note 1)
3. Fetal Bovine Serum (Hyclone).
4. 75 cm² tissue culture flasks (Costar, Corning).
5. 125 ml Disposable Erlenmeyer Flask with 0.2 micron PTFE Vented Closure (Bellco Glass Inc, Vineland, NJ).
6. Orbital Shaker capable of handling 125 ml flasks, at 27°C.

¹In functional assays involving S2 cells adapted to serum free conditions, medium supplemented with 0.2% BSA must be used in place of serum-containing medium. Addition of serum to adapted cells will allow the recovery of cholesterol-containing microdomains.

2.2 Transfection of S2 Cells

2.2.1 Calcium Phosphate Transfection

1. HEPES buffered saline (HBS) for calcium phosphate transfection: 50 mM HEPES, 1.5 mM Na₂HPO₄, and 280 mM NaCl, in water, adjusted to pH 7.1. Sterilized by filtration through a 0.45 µm filter.
2. CaCl₂ for calcium phosphate transfection: 2 M CaCl₂ (Sigma Aldrich) in water, sterilized by filtration through a 0.45 µm filter.
3. 6-well tissue culture plates (Costar, Corning).
4. pAc5.1-V5-His: plasmid for constitutive expression in S2 cells using the *Drosophila* Actin 5 promoter (Requires a licensing agreement with Invitrogen).
5. pRmHa3: plasmid for inducible expression in S2 cells using the metallothionein promoter (available to not-for-profit labs from the *Drosophila* Genome Research Center, Bloomington IN).
6. Plasmids for selection of transfectants, which include pNeoFly (27), pCoBlast (Invitrogen) and pCoHygro (Invitrogen).
7. Agents for selection of stable transfectants: Geneticin (G418 sulfate, Invitrogen), Puromycin dihydrochloride (Sigma Aldrich), Hygromycin B (Roche Diagnostics GmbH, Basel Switzerland), Blasticidin S HCl (Invitrogen).
8. 6 ml polypropylene, round bottom tubes, sterile (Falcon, BD Biosciences, San Jose CA).
9. 15 ml polystyrene conical screw cap tubes (Sarstedt, Newton NC).

2.2.2 Transfection with Cellfectin

1. Schneider's *Drosophila* Medium (Invitrogen) ± 10% FBS.
2. Cellfectin Transfection Reagent (Invitrogen).
3. Expression and drug resistance plasmids and agents for selection of stable transfectants (see Section 2.2.1).
4. 6-well tissue culture plates (Costar, Corning).
5. 6 ml polypropylene, round bottom tubes, sterile (Falcon, BD Biosciences).

2.2.3 Enrichment of Expressing Cells

1. Antibodies for the transfected proteins. For Fluorescence Activated Cell Sorting (FACS), antibodies directly coupled to fluorescent labels may be used when available. Alternatively, unlabeled primary antibodies can be stained with fluorescently labeled secondary antibodies. For enrichment of cells expressing a given protein using magnetic beads coated with goat anti-mouse antibodies, unlabeled primary antibodies are recommended.
2. Buffer for FACS sort: PBS supplemented with 2% heat inactivated FBS and 2 mM EDTA.
3. FACS tubes: 5 ml polystyrene tubes, 12 × 75 mm (Falcon, BD Biosciences).
4. Dynabeads Goat anti-mouse IgG and selection magnet (Invitrogen).
5. 14 ml Polypropylene Round-Bottom Tubes (Falcon, BD Biosciences).
6. Flat bottomed 96-well plates (Costar, Corning).

2.3 S2 Cells as Targets in NK Cell Assays

2.3.1 CD107a Degranulation Assay

1. Anti-CD56 mAb (clone NCAM 16.2) PE (BD Bioscience).
2. Anti-CD107a mAb (clone H4A3) FITC (BD Bioscience).

2.3.2 Conjugate Assay

1. PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich).
2. CellTracker Green CMFDA (Invitrogen).
3. Hank's Buffered Saline Solution (Invitrogen) supplemented with 5% FBS.
4. IMDM + 10% FBS
5. FACS tubes: 5 ml polystyrene tubes, 12 × 75 mm (BD Biosciences).
6. 0.5% Paraformaldehyde (16% solution from Electron Microscopy Sciences, Hatfield PA, diluted in phosphate buffered saline (PBS)).

2.3.3 Perforin Polarization Assay

15 ml polystyrene conical screw-cap tubes (Sarstedt).

Hank's Buffered Saline Solution (HBSS) (Invitrogen) supplemented with 3% FBS.

Poly-D-Lysine coated 2-well Culture Slides (BD BioCoat, BD Biosciences).

4% Paraformaldehyde (16% solution from Electron Microscopy Sciences diluted in PBS).

Normal Donkey Serum (Jackson ImmunoResearch Laboratories, West Grove PA) reconstituted in 10 ml water.

Triton X-100 (Sigma Aldrich).

Permeabilization Buffer: 10% Normal Donkey Serum and 0.5% Triton X-100 in PBS.

Staining Buffer: 3% Normal Donkey Serum and 0.5% Triton X-100 in PBS.

CellTracker Green CMFDA (Invitrogen).

Anti-perforin antibody, clone G9 (Thermo Scientific, Rockford IL).

Alexa Fluor 568 conjugated goat anti-mouse secondary antibody (Invitrogen).

Prolong Gold antifade reagent (Invitrogen).

2.3.4 Flow Cytometry Based Cytotoxicity Assay

PKH67 Green Fluorescent Cell Linker Kit (Sigma Aldrich).

15 ml polystyrene conical screw cap tubes (Sarstedt).

Iscove's Modified Dulbecco's Medium with L-Glutamine and 25 mM HEPES (Invitrogen).

Propidium Iodide solution, 1 mg/ml (Sigma Aldrich).

FACS tubes: 5 ml polystyrene tubes, 12 × 75 mm (BD Biosciences)

3. Methods

3.1 Care and Culture of S2 Cells

3.1.1 Standard Culture

1. S2 cells grow at or slightly above room temperature. Additional carbon dioxide (CO₂) is not required. Incubators, without CO₂ injection, can be used to maintain an optimal temperature of ~27°C. Alternatively, S2 cells will grow reasonably well in a drawer, or any location at room temperature that is protected from light.
2. S2 cells should be thawed at room temperature. Since S2 cells undergo a heat shock at 37°C, thawing the cells in a warm water bath will reduce viability.
3. Cell cultures should be maintained between 1×10^6 cells/ml and 2.5×10^6 cells/ml in Schneider Cell medium supplemented with 10% FBS. Cells grow best when diluted no more than 1:3 at each passage. Maintenance of a healthy culture of growing cells requires a 1:2 passage every other day (*see* Note 2).
4. Freezing of S2 cells is most successful when large numbers of cells from a healthy culture are frozen, such as 20 to 30 million cells per vial. S2 cells can be frozen in a variety of freezing media. We have used mainly 45% fresh culture medium combined with 45% conditioned S2 cell medium (the medium in which the cells have been grown) supplemented with 10% DMSO. Alternatively, a simple mixture of 90% FBS supplemented with 10% DMSO has been used.
5. Proteins can be expressed by transfection with either a constitutive or inducible promoter-containing plasmid. Plasmid pAc5.1 (Invitrogen) drives expression with a *Drosophila* Actin 5 promoter, which results in constitutive expression. Plasmid pRmHa3 uses a metallothionein promoter, the activity of which is induced with 1 mM CuSO₄. 48 hours of induction with 1 mM CuSO₄ is sufficient to yield maximal expression of transfected proteins. A fraction of the cell culture is reserved, without induction, for continued culture. Constitutive expression is more convenient, as the transfected proteins are always expressed and variability introduced by induction is avoided. Expression from a constitutive promoter is often more uniform over time than induced expression, which often yields heterogeneous expression levels. Constitutive expression can also be beneficial in transient transfections, where cells already dealing with the stress of the transfection may be adversely affected even further by the induction in cupric sulfate. Inducible expression is advantageous when a transfected protein is toxic to the S2 cells. Induced expression is also often used for large scale protein production in S2 cells, where constitutive expression may slow the expansion of the cultures. The cells can be expanded without expressing the protein, and expression can be induced in 24–48 hours.
6. Co-expression of multiple proteins in transfected S2 cells can be achieved either by co-transfection of all the required plasmids at once or by sequential transfections (*see* Notes 3 and 4).

²S2 cells in a healthy culture are semi-adherent. When passaged into new tissue culture plates or flasks, most of the cells will adhere to the plastic fairly firmly. The cells can be detached from off the plastic by repeated washes with medium using a pipette. After several passages in the same flask, the cells will not adhere as much, although they will proliferate and remain trypan blue negative. When in suspension, healthy S2 cells will grow individually, with minimal clustering or clumping. Expression of transfected proteins can be greatly affected by the health of the cells. Cells in overgrown cultures will often express lower levels of protein, whether expression is driven from a constitutive or an inducible promoter. S2 cultures in poor health can often be rescued by seeding cells into fresh medium at 1×10^6 cells/ml and allowing the cells to grow to 2.5×10^6 cells/ml. Once the cells are proliferating at a normal rate, protein expression will often recover to previous levels.

7. Expression of MHC class I molecules in S2 cells requires co-transfection with β_2 -microglobulin. Furthermore, exogenous peptides have to be loaded onto MHC class I because insect cells do not have the endogenous peptide loading machinery (e.g. TAP transporter, chaperones) (32). Peptide loading is very efficient because “empty” class I- β_2 -microglobulin dimers are readily expressed at the surface of S2 cells at 27°C. Appropriate peptides are added to the S2 cell culture at a final concentration of 1 μ M one day prior to the assay with NK cells.
8. Using S2 cells in NK cell assays is often a matter of simply replacing the conventional target cells in the assay with S2 cells. However, there are two important considerations. First, insect cells undergo heat shock and will eventually die at 37°C. Second, S2 cells exposed to human serum that has not been heat-inactivated are killed in less than 1 hour. Each of those issues can be addressed as follows. The viability of S2 cells following incubation at 37°C is not significantly reduced for at least 3 hours. The assays described here using S2 cells at 37°C do not exceed 3 hours. S2 cells grow happily at 27°C in heat-inactivated bovine serum. However, our primary human NK cells are usually cultured in human serum, which is not routinely heat-inactivated. To avoid exposure of S2 cells to human serum, the simplest approach is to wash and resuspend NK cells in medium containing FBS prior to the assays with S2 cells. Although, in our hands, primary human NK cells survive and proliferate best in medium containing 10% human serum, they remain functional in medium containing FBS in short term assays.

3.1.2 Adaptation of S2 Cells to Culture in Serum Free Conditions—S2 cells used for production of proteins are often cultured in serum-free conditions in order to simplify the purification of the protein of interest. Serum-free culture of S2 cells has an additional advantage, of interest when using S2 cells as target cells in NK cell assays. *Drosophila* does not synthesize sterols. Its sole source of cholesterol is from the diet. Culture of S2 cells in serum-free medium is therefore a method to deplete the cells of cholesterol without using compounds such as β -methylcyclodextrin. Therefore, S2 cells grown in serum-free conditions may be a useful tool to investigate the role of cholesterol-dependent membrane microdomains on the behavior of ligands of NK cell receptors. A previous report indicated that the inclusion of MICA in lipid rafts on target cells is necessary for the NKG2D-dependent response of NK cells (33).

1. S2 cells to be adapted should be thawed from frozen stocks and cultured in 75 cm² flasks in Insect-Xpress medium supplemented with 5% FBS. Cells should be expanded until a healthy growing culture at 2×10^6 cells/ml is obtained.
2. Pellet 150×10^6 cells at 135 g. Resuspend in 30 ml Insect-Xpress + 5% FBS and transfer the cell suspension to a 125 ml disposable Erlenmeyer Flask.
3. Expand the cells by culturing on a shaker at 140 RPM at 27°C for 2–3 days.
4. Pellet 150×10^6 cells at 135g. Resuspend in 30 ml Insect-Xpress + 2.5% FBS and transfer the cell suspension to a 125 ml disposable Erlenmeyer Flask.
5. Again, expand the cells by culturing on a shaker at 140 RPM at 27°C for 2–3 days.

³We have successfully co-transfected S2 cells with six different proteins simultaneously. It is likely that an even greater number of proteins could be simultaneously transfected.

⁴Any given protein is usually expressed at a similar level in independently transfected S2 cells. For instance, expression of ICAM-1 is almost always high, whereas other molecules have proven more difficult to express. In the latter case, it is preferable to isolate S2 cells expressing a decent level of the troublesome protein after a first round of transfection, and to “super-transfect” plasmids for other proteins in a second round of transfection using a different drug resistance plasmid. For example, S2 cells expressing CD48 were isolated first, prior to sequential transfections for expression of additional proteins.

6. Perform three more cycles of pelleting 150×10^6 cells, resuspending in Insect-Xpress medium, and expanding the cells in shaking culture for 2–3 days, using medium that contains 1% FBS, then 0.5% FBS, and finally no serum.
7. Cells should be cultured in serum free medium for a further 2 weeks before use in an assay (*see Note 1*).

3.2 Transfection of S2 Cells

For most NK cell assays, it is preferable to use S2 cells that express ligands at a fairly uniform level. To this end, stable transfectants of S2 cells are usually generated, from which the desired expressing cells can be enriched or cloned, when necessary. Two protocols for the generation of stable S2 cell lines are presented here. In some cases, transient transfections of S2 cells can be used. Transient expression is advantageous to test expression from new plasmids, for preliminary functional assays (where appropriate), and for the rapid screening of a very large number of transfectants. However, there is less control over protein expression levels in transient transfections, and there will usually be a sizeable fraction of the S2 cells that do not express the transfected proteins (*see Note 5*).

3.2.1 Calcium Phosphate Transfection

1. For transfection, S2 cells should be growing logarithmically, at a density around 2×10^6 /ml, with high viability. Cells can be counted on a hemacytometer with trypan blue staining.
2. The day before transfection, spin down 3×10^6 cells per transfection in a 15 ml conical tube at 215g. Resuspend the cells at 1×10^6 cells per ml, and plate 3 ml (3×10^6 cells) in a well of a 6-well plate. Incubate overnight at 27°C.
3. Set up the transfection mix. In one 6 ml tube, aliquot 300 μ l of HBS. In a second 6 ml tube, mix 36 μ l of 2 M CaCl_2 with 19 μ g of sterile DNA and sterile water to a total volume of 300 μ l. This mixture should contain 18 μ g of the plasmid expressing the protein of interest and 1 μ g of the desired selection plasmid (*see Notes 6 and 7*). Vortex the tube containing the HBS at a moderate setting. While vortexing, slowly add the CaCl_2 /DNA mixture dropwise to the HBS. This process should take approximately one minute. Incubate the mixture at room temperature for 30 minutes.
4. Add the DNA mixture to the S2 cells that had been plated the day before. The mixture should be added to the medium on the cells in a dropwise manner. Gently

⁵Transient expression of proteins can be achieved by transfection with either calcium phosphate or Cellfectin method. Proteins will be expressed almost immediately following the addition of the transfection mixtures, with maximal expression occurring at 48–72 hours. Either protocol should be followed to Step 5 (Section 3.2.1 for calcium phosphate transfection or Section 3.2.2 for Cellfectin transfection), where the transfection mixtures are removed by washing, and the cells are cultured for 48 hours. If induction is required, 1 μ M CuSO_4 should be added 24 hours after the washes, and induction should proceed for a further 24–48 hours.

⁶There are a number of plasmids using different promoters that can be used to obtain expression of exogenous proteins in *Drosophila* cells. We have used pRmHa3 and pAc5.1 plasmids successfully, as described in step 5 of section 3.1.1. Our cDNA constructs for expression in S2 cells have contained the entire coding sequence of the protein of interest, including the signal sequence. Where possible, 5'- and 3'-untranslated regions of the mRNA were included in the expression constructs. It has not been necessary to alter the cDNA sequences in order to get good expression of the various human and mouse proteins in S2 cells.

⁷The plasmids used for expression of selectable drug resistance must have promoters for constitutive expression. Commercial plasmids exist for selection of S2 cells with blasticidin or hygromycin (Invitrogen), as well as plasmids made by individual researchers for selection with puromycin and neomycin. It is not necessary to use expression vectors that carry both a selectable marker and the coding region of the protein of interest. It is much more convenient to co-transfect plasmids containing the selectable marker together with the plasmids for protein expression. S2 cells are transfected by the calcium phosphate method with 19 μ g of DNA, of which 18 μ g is the expression plasmid(s) and 1 μ g is the selection plasmid. In cases where expression of multiple exogenous proteins is desired, the plasmids are mixed in equal amounts to a total of 18 μ g of DNA (for example, 6 μ g each of 3 different plasmids).

swirl the plate in order to mix the medium and DNA. Incubate the cells overnight with the transfection mixture at 27°C.

5. Pipet the cells into a 15 ml conical tube. Wash the well with 5 ml of S2 medium in order to recover adherent cells. Pellet the cells at 215g, and wash the cells once with 10 ml of medium. Plate the cells into a fresh well in a 6-well plate in 3 ml medium, and incubate for 48 hours at 27°C.
6. Add the appropriate selection (see Note 8). Incubate the cells for up to 2 weeks, until the resistant cells expand through the selection. The drug resistant cells should be assayed for protein expression by flow cytometry.

3.2.2 Cellfectin Transfection

1. The day before the transfection, plate 1×10^6 cells into a well of a 6-well plate in 3 ml of Schneider's *Drosophila* medium + 10% FBS. Culture the cells overnight.
2. Prepare the transfection mixture.
 - a. In a 6 ml polypropylene tube, prepare a mixture consisting of 0.5 µg of the drug resistance plasmid and 5 µg of each protein expression plasmid in 100 µl of serum-free Schneider's *Drosophila* medium (see Notes 6 and 7). The DNA should be sterile.
 - b. In a second tube, add 10 µl Cellfectin Reagent to 90 µl serum-free Schneider's *Drosophila* medium.
 - c. Add the Cellfectin-containing medium to the tube containing the DNA mixture. Mix gently.
 - d. Incubate the combined mixture at room temperature for 15 minutes.
 - e. Add 800 µl serum-free Schneider's *Drosophila* medium to the mixture.
3. Gently aspirate the medium from the S2 cells. Carefully wash the cells once with 2 ml serum-free Schneider's *Drosophila* medium. Aspirate the wash medium.
4. Add the Cellfectin/DNA mixture to the cells. Incubate the cells for 18 hours at 27°C.
5. Replace the DNA-containing medium with Schneider's *Drosophila* medium + 10% FBS. Incubate for 48 hours at 27°C.
6. At this point, the cells can be analyzed by flow cytometry for the expression of proteins from constitutively expressing plasmids, and protein expression from inducible plasmids can be induced with 1 mM CuSO₄ and examined after another 48 hours.
7. To select stable transfectants, the cells from step 5 should be harvested and collected by centrifugation, and resuspended in 3 ml Schneider's *Drosophila* medium + 10% FBS containing the required selection reagent (see Note 8). Cells can be re-plated into the same well of the 6-well plate in which the transfection was

⁸The appropriate drug concentration for killing of untransfected S2 cells and selection of resistant S2 cells must be determined experimentally by serial dilutions. We have had success selecting resistant S2 cells with 1 mg/ml G418, 6 µg/ml puromycin, and 300 µg/ml hygromycin. Puromycin and hygromycin kill untransfected cells within 7 days. After their addition, the transfected S2 cell culture can be left alone until resistant cells grow, which usually occurs in 2 weeks. As G418 kills cells much more slowly, it is recommended to monitor live cell numbers. If the number reaches 3×10^6 cells/ml the cells should be removed from the medium by centrifugation and re-plated at 1×10^6 cells/ml in fresh medium containing G418. Protein expression may not be detectable for 2 weeks in G418 selection.

performed. The cells should be incubated at 27°C, and the medium exchanged for fresh growth medium every 7 days, until stably transfected cells begin to expand.

3.2.3 Enrichment of Expressing Cells—Cells that have been selected through drug resistance often contain only a subpopulation of cells that express the protein(s) of interest. Although this amount of expression is sometimes sufficient for use in certain assays, it is usually preferable to obtain a more uniform profile of expression.

1. Expressing cells can be enriched with magnetic beads. We have had success with Dynabeads from Invitrogen, simply following the manufacturer's protocol. This type of enrichment works best with relatively high levels of expression. The S2 cells must be stained with a saturating amount of primary antibody, and must be kept cold (preferably on ice) for the duration of the procedure. S2 cells are phagocytic. If the S2 cells are allowed to phagocytose magnetic beads at room temperature, the enrichment for expression will be contaminated with non-expressing cells.
2. Expressing cells can be isolated by FACS. Again, it is critical to obtain the brightest antibody staining possible to ensure the removal of non-expressing cells. When expression of a protein is marginal, it is recommended that only the brightest cells are selected.
3. After enrichment of expressing cells by magnetic beads or FACS, it is necessary to regularly monitor expression of the transfected proteins. We have found that these enriched populations tend to lose expression during culture, presumable due to selective expansion of cells with lower expression. More stable, uniform expression can be obtained by deriving clones from the resistant populations, immediately following transfection or after enrichment by beads or sorting. To clone S2 cell transfectants, plate cells in medium with selection in 96-well plates at decreasing cell concentrations beginning at 10 cells per well. Threefold dilutions of the cells down to a concentration of 0.3 cell per well can be plated. Proliferating clones will usually be visible within two weeks. Clones should be expanded into larger volumes of medium until sufficient cells are available to screen by flow cytometry. Multiple clones, often with varying amounts of protein expression, should be selected for experimentation (see Note 9).

3.3 S2 Cells as Targets in NK Cell Assays

The usefulness of the S2 cells relies on the ability to use them in a variety of assays investigating NK cell function. We have successfully performed a variety of flow cytometry- and microscopy-based assays, using the S2 cells as target cells to investigate responses of human NK cells to a variety of ligands for NK cell receptors. We have used S2 cells to monitor NK cell degranulation as measured by the surface expression of lysosome-associated membrane protein 1 (LAMP-1, also called CD107a) (31), binding of NK cells to target cells in a conjugation assay (5), clustering of activating and inhibitory receptors at NK-S2 cell synapses (34, 35), signaling induced in NK cells by contact with S2 cells (36), and the intracellular polarization of perforin-containing granules to the site of contact with target cells (31). We have also used S2 cells as target cells in a modified cytotoxicity assay. Of particular note, we have generated, through Anaspec (San Jose, CA), a rabbit polyclonal

⁹Selection of transfected clones is a time consuming process, usually requiring an additional month after resistant cells have been obtained. To accelerate the process by several weeks, one can isolate expressing cells by FACS or by enrichment with antibodies coupled to magnetic beads. However, cell sorting will usually result in a broader range of expression than cell cloning, and will often result in the loss or a reduction of protein over time. Therefore, it is necessary to regularly monitor expression in a sorted population and to repeat cell sorting when necessary.

antisera against an S2 cell membrane preparation. Addition of this serum to S2 cells, provides a potent, physiological stimulus to human NK cells for ADCC through CD16 (31). Untransfected S2 cells provide negative controls for each of these assays.

3.3.1 CD107a Assay—The detection of NK cell degranulation through monitoring of the surface expression of CD107a has rapidly become the method of choice. This assay works well with S2 target cells. For a thorough description of the CD107a assay, please refer to the chapter in this issue by Bryceson *et al.*, as well as our previous work (31). Due to a relatively high auto-fluorescence of S2 cells it is important to obtain a bright staining of NK cells with the CD56 antibody in order to obtain a stringent gate for NK cells. This may require a two-step staining protocol, using a secondary antibody against the CD56 antibody. Note that the E:T ratio used with S2 cells is 1:5 instead of the 1:2 used for other target cells.

3.3.2 Conjugation Assay

1. This assay has been adapted from Burshtyn *et al.* (37), which is also described for use with mammalian target cells in a chapter in this issue. If necessary, induce expression in S2 cells transfected with a metallothionein-based plasmid with 1 mM CuSO₄ 48 hours before the conjugation assay. If the S2 cells express MHC molecules, those molecules will need to be loaded with a compatible peptide. Peptides should be added to the cells one day before the assay to a final concentration of 1 μM. Expression of ligands on the S2 cells should always be monitored by flow cytometry on the day of the assay (*see Note 10*).
2. Each combination of NK cell and target cell should be assayed for conjugation at a series of time points. We routinely perform 0, 5, 10, 20 and 40 minute time points. With S2 cell targets, each point in the assay will include 1×10^5 NK cells and 4×10^5 target cells, for a final E:T ratio of 1:4. In the following steps, be sure to label sufficient cells for the assay.
3. Label the S2 cells with the red PKH26 dye, according to the manufacturer's protocol, summarized here (*see Note 11*).
 - a. Pellet the required number of S2 cells by centrifugation at 300g.
 - b. Count the cells. Use about 5×10^6 for labeling (scale up as necessary); put 5×10^6 cells into a new tube and bring them up to 10 ml total volume with serum free medium. Pellet the cells again.
 - c. While the cells are in the centrifuge, prepare the Fluorescent dye. For labeling, you need 100 μl of "2× dye" for every 10^6 cells. 2× dye for 5×10^6 cells is 588 μl of Diluent C (supplied with the labeling kit) + 12 μl of PKH26.
 - d. Aspirate the medium from the cells. Resuspend the target cells in 100 μl of Diluent C for every 10^6 cells. Add the 2× dye to the cells, 100 μl for each 1×10^6 cells.

¹⁰Expression of proteins on transfected S2 cells should always be verified on the day of an assay. Expression level is dependent on the condition of the cells and can vary widely.

¹¹Alternatively, the S2 cells can be labeled with Celltracker dyes (Celltracker Green as a replacement for PKH67 and Celltracker Orange for PKH26). The cells should be incubated, in their standard medium at 10×10^6 cells/ml, with 4 μg/ml of the Celltracker reagent for 30 minutes at room temperature. The cells should be washed twice with their standard medium, and then allowed to rest in 10 ml of medium in a 15 ml tube at room temperature for 30 minutes. Celltracker dyes are more convenient to use than the lipophilic PKH dyes. Additionally, the S2 cells occasionally display reduced viability after labeling with the PKH reagents. However, S2 cells labeled with the PKH dyes often display more uniform fluorescence than cells labeled with Celltracker, simplifying the FACS analysis. The appropriate label for a given application should be determined experimentally.

- e. Incubate 5 minutes at room temperature. Add an equal volume of serum to the cells and incubate 1 minute at room temperature.
 - f. Wash the cells twice with Schneider cell medium + 10% FBS. After the second wash, resuspend the cells in 5 ml of medium + 10% FBS. Incubate the cells at 27°C for 2 hours.
4. Label the NK cells with Celltracker Green. The cells should be incubated, in their standard medium at a concentration of 10×10^6 cells/ml, with 1 µg/ml of the Celltracker Green for 30 minutes. The cells should be washed twice with their standard medium, and then allowed to rest in 10 ml of medium in a 15 ml tube for 30 minutes at 37°C.
 5. Wash both the NK and S2 cells twice with HBSS + 5% FBS. Resuspend the cells in HBSS with 5% FBS, and count them. Pellet the cells, and resuspend the NK cells at a concentration of 1×10^6 cells/ml and the S2 cells at a concentration of 4×10^6 cells/ml using ice cold HBSS with 5% FBS.
 6. To a series of FACS tubes (one tube for each time point), add 1×10^5 NK cells and 4×10^5 of the appropriate S2 cells (100 µl each), for an E:T ratio of 1:4. Vortex the cells quickly to mix.
 7. Pellet the cells, at 4°C, for 3 minutes at 20g.
 8. Incubate the tubes in a 37°C water bath for the desired lengths of time. When each time point is reached, vortex the appropriate tubes and then fix the cells by adding 1 ml of 0.5% paraformaldehyde.
 9. Acquire samples on a flow cytometer, using stained NK and S2 cells alone as compensation controls. Conjugate pairs are represented by green and red double-stained events. Results are typically represented as the percentage of all NK cells that are in conjugates relative to the total number of NK cells.

3.3.3 Perforin Polarization Assay—NK cells respond to contact with sensitive target cells by polarizing their perforin-containing lytic granules to the site of contact with the target cell. This polarization can be detected through fluorescent microscopy. Certain S2 cell transfectants have been shown to induce polarization of granules in human NK cells (30, 31).

1. NK cells and S2 cells will ultimately be mixed at a 1:1 E:T ratio, with 1×10^6 of each cell type per sample. The required number of each transfected S2 cell should be labeled, in standard medium at a concentration of 10×10^6 cells/ml, with 1 µg/ml of Celltracker Green for 30 minutes. The cells should be washed twice in standard medium, and then allowed to rest in 1 ml of medium in a 15 ml tube for 30 minutes. Wash twice with 10 ml HBSS + 3% FBS. Resuspend at 10×10^6 cells/ml.
2. Pellet the required number of NK cells. Wash twice with 10 ml HBSS + 3% FBS. Resuspend at 10×10^6 cells/ml.
3. In a 15 ml conical tube, combine 1×10^6 NK cells and 1×10^6 target cells (100 µl each). Vortex briefly to mix the cells.
4. Pellet the cells at 20g for 3 minutes.
5. Incubate in a 37°C water bath for 20 minutes.
6. Transfer the cells to poly-D-lysine coated culture slides. Resuspend the cells gently to preserve cell:cell contacts. Incubate the cells on the slides for 60 minutes at room temperature.

7. Aspirate the medium. Wash the cells gently twice with 1 ml PBS. Fix the cells by adding 1 ml 4% paraformaldehyde. Incubate for 20 minutes at room temperature.
8. Wash the fixed cells twice with PBS.
9. Permeabilize the cells with 0.5 ml of 10% normal donkey serum (NDS) and 0.5% Triton X-100 in PBS. Incubate at room temperature for 30 minutes with gentle shaking.
10. Stain the cells with 3 µg/ml anti-perforin antibody (clone G9) in 3% NDS, 0.5% Triton X-100 in PBS. Incubate for 1 hour at room temperature with gentle shaking.
11. Wash three times, for 5 minutes each, with 1 ml PBS.
12. Stain with a fluorescently labeled goat anti-mouse secondary antibody in 3% NDS and 0.5% Triton X-100 in PBS. We typically use an Alexa Fluor 568 conjugated secondary antibody at a concentration of 1 µg/ml. Incubate for 1 hour at room temperature with gentle shaking.
13. Wash three times, for 5 minutes each, with 1 ml PBS.
14. Aspirate the PBS, and allow the slides to air dry.
15. Seal the samples on slides under a coverslip, using a drop of Prolong Gold antifade reagent as a mounting medium.
16. The cells should be imaged with a fluorescent microscope. We have acquired images on a Zeiss LSM510 confocal microscope. Z-stacks are acquired and used to create 3-dimensional reconstructions of the cell contacts. Contacts between a perforin-containing NK cell and a Celltracker Green labeled target cell are identified, and the perforin is scored as polarized or not polarized by the visual appearance of the cell:cell conjugate. In most experiments, NK cells in which granules have polarized are obvious. Large numbers of cell contacts must be analyzed for rigorous analysis. In order to acquire sufficient data, acquire microscope images under relatively low magnification (*e.g.* multiple 40× fields). We typically analyze 150–200 contacts for each transfected S2 cell analyzed.

3.3.4 Cytotoxicity Assay by Flow Cytometry—One situation where S2 cells cannot be simply substituted for conventional target cells is cytotoxicity assays. Most cytotoxicity assays require that target cells be loaded with a radioactive or fluorescent vital label, like chromium-51, Europium, or calcein. Lysis of target cells is monitored by the release of the vital label into the medium. S2 cells label poorly with these reagents, and they have a high rate of spontaneous release. These factors combine to leave only a very small window between spontaneous and maximal release, making it very difficult to detect specific NK cell-mediated cytotoxicity. Killing of S2 cells is therefore best measured using a flow cytometry based assay, in which the S2 cells are labeled with a green lipophilic dye, mixed with NK cells, and then stained with propidium iodide to detect cytotoxicity.

1. If necessary, induce expression in S2 cells transfected with a metallothionein-based plasmid with 1 mM CuSO₄ 48 hours before the cytotoxicity assay. If the S2 cells express MHC molecules, those molecules will need to be loaded with a compatible peptide. Peptides should be added to the cells the day before the assay to a final concentration of 1 µM. Expression of ligands on the S2 cells should always be monitored by flow cytometry on the day of the cytotoxicity assay (*see* Note 10). Untransfected S2 cells must be included as a control in the assay (*see* Note 12).
2. Label S2 cells with the green PKH67 dye, according to the manufacturer's protocol, as described in step 3 of section 3.3.2 (*see* Note 11).

3. During the incubation, spin down the NK cells, and wash with Iscove's medium containing 10% FBS. The number of NK cells required will depend on the effector to target ratios being used. Each point requires 20,000 targets cells, and is done in duplicate. Typically, for S2 cells, the following E:T ratios are performed: 30:1, 10:1, 3:1, 1:1 and target cells alone (spontaneous release). Setup the dilutions of NK cells. Ultimately, the target cells will be resuspended at a concentration of 20,000 cells in 100 ml, and the NK cells will be set up in dilutions containing the appropriate number of NK cells in 100 ml. For example, for the 30:1 points, the NK cells will need to be at a concentration of 6×10^5 NK cells per 100 ml.
4. Prepare FACS tubes. The cytotoxicity assay will be conducted directly in the FACS tubes. Label 2 tubes for duplicates of every target/dilution combination.
5. Pipet 100 μ l of the NK cell dilutions into appropriately labeled tubes. Place these tubes in the 37°C incubator until the 2-hour incubation of the labeled target cells is complete.
6. When the 2-hour incubation of the labeled target cells is complete, bring the volume of the target cells up to 10 ml with Schneider's medium containing 10% FBS, and spin down the cells at 215g. Wash the cells twice more with 10 ml of serum-containing medium.
7. Resuspend the target cells in 4 ml of Schneider cell medium with 10% FBS. Count the cells.
8. Transfer 800,000 cells to a new tube, and bring the volume up to 4 ml, resulting in a concentration of 20,000 cells in every 100 μ l.
9. Add 100 μ l (20,000 cells) to each tube with the NK cells. Vortex each tube. Then spin the tubes at 20g for 5 minutes.
10. Put the tubes at 37°C for 3 hours.
11. Add propidium iodide to each tube to a final concentration of 50 μ g/ml, and vortex gently to mix.
12. On the flow cytometer, acquisition should be set up so that the green target cells, very bright in FL1, are gated on and the PI profile for those cells is displayed. Acquire at least 2000 gated target cells.
13. The percentage of cells that are positive for both the PKH67 dye and propidium iodide is the percentage of S2 cells lysed in each sample. Values from duplicate samples are usually averaged. The percentage specific lysis is calculated according to the formula $[100 \times (\% \text{ PI}^+ \text{ cells} - \% \text{ Spontaneous Death}) / (100 - \% \text{ Spontaneous Death})]$ where spontaneous death is determined with a sample containing only labeled S2 cells.

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¹²It is common to encounter problems with high killing of untransfected S2 cells in cytotoxicity assays. To minimize such occurrences, it is essential to seed S2 cells at optimal numbers and to allow them to proliferate for at least a week prior to the assay. However, nonspecific killing of untransfected S2 cells may also be due to constitutive release of perforin and granzymes by activated NK cells. We have not found a reliable solution to this problem. Assays for NK cell degranulation and for granule polarization in NK cells are very reliable and focus on the functions of interest (*i.e.* NK cell responses) rather than the fate of S2 cells. Nevertheless, target cell lysis may occasionally be a useful parameter of NK cell function, as it requires the combination of granule polarization and degranulation (31).

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