Published in final edited form as:

Methods Mol Biol. 2019 ; 1943: 323–331. doi:10.1007/978-1-4939-9092-4_21.

Evaluating the regulation of cytokine levels after siRNA treatment in antigen-specific target cell populations via intracellular staining

Rima Kandil^a, Daniel Feldmann^b, Yuran Xie^b, and Olivia M. Merkel^{a,b,*}

^aDepartment of Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universität München, Germany

^bDepartment of Pharmaceutical Sciences, Wayne State University, Detroit, Michigan, USA

Summary/Abstract

RNA interference (RNAi) offers a promising base for therapeutic knockdown of clinically relevant genes. Local delivery routes as well as targeted delivery to specific cell populations have been shown to circumvent several hurdles of successful siRNA delivery in vivo. To evaluate and quantify the treatment effect in a precise way, next to measuring the downregulation on gene and protein levels, it is equally essential to investigate the influence on down-stream factors such as generated cytokines. Here, we describe an expressive method to specifically isolate the desired target cells and determine their levels of intracellular cytokines by flow cytometry using the example of murine lungs after pulmonary in vivo transfection with siRNA.

Therefore, the lungs of treated mice are harvested and processed into single cell suspensions, in which CD4 positive T cells are marked by antibody-coupled magnetic beads and isolated via magnetic separation. These purified target cells are then fixed and permeabilized, making their intracellular interleukins accessible for staining with fluorescently labeled antibodies. Thus, the cytokine levels and hence the precise influence of the siRNA treatment on intracellular conditions can be measured.

Keywords

siRNA delivery; knockdown; lung; inflammation; GATA-3; OVA challenged mice; flow cytometry; cytokines; T cell isolation; intracellular staining

1 Introduction

Small interfering RNAs (siRNA) offer the theoretic potential to silence the expression of any chosen gene with a known sequence (1). Over the last two decades, the regulatory mechanism of RNA interference (RNAi) has aroused great interest for therapeutic purposes and found its way into several human clinical trials (2). Despite all progress, however, there

*Corresponding Author: Prof. Dr. Olivia Merkel, Department Pharmazie, Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, Haus B, 81377 München, Germany, TEL: +49-89-2180-77025, FAX: +49-89-2180-77020, Olivia.Merkel@cup.uni-muenchen.de.

are still hurdles that impede translation into the clinical routine. Since siRNA, due to its poor pharmacokinetics, was recognized at an early stage to be extremely problematic for systemic administration, a majority of the recent studies focuses on local delivery routes (3).

Pulmonary application, as such a way, not only circumvents several of the barriers that need to be overcome by nucleic acids and protects the sensitive payload from degradation, e.g. by serum nucleases, but obviously also enables reductions of doses, and therefore, side effects (4). Furthermore, the therapeutics are instantly available at the target region where they are supposed to bring about their effect (5).

For immune-related diseases, this target is oftentimes displayed by the cellular contingent of the immune system. Activated T cells, as the most important cellular mediators in immune responses, are anticipated to be targeted and treated in several related studies (6). One of the numerous diseases, in whose pathophysiology T cells play a crucial role, is asthma, a chronic inflammatory disease of the airways characterized by infiltration of immune cells including T helper 2 cells (T_H2 cells), a type of activated T cells (ATC), in the lung (7). These CD4⁺ T cells orchestrate various of the cytokine-based inflammatory cascades by secretion of interleukins such as IL-13, which in turn are produced upon activation of the key transcription factor GATA-3 (8). There are attempts to capture the cells and their receptors directly (9, 10) as well as indirect methods that aim to modulate the cytokine environment (11). Beyond targeting these single cytokines in particular, for example with respective antibodies (12), the down-regulation of GATA-3 is a promising approach to early-on undermine pathologic pathways (13) and was recently even proven successful in a human phase II clinical trial employing DNazymes (14).

To further characterize and optimize similar approaches in vivo, suitable animal models and appropriate read-out parameters to quantify the treatment success are imperative. A well-established and extensively studied method to examine inflammatory and immune responses is the Ovalbumin (OVA)-sensitization mouse model (14) which is well suited, but by far not limited, for the use in asthma research (15). In these experimentally challenged animals, it is possible to check the distinct influence of siRNA treatment on enhanced cytokine levels in specific T cell subsets. To achieve this, an essential step is to isolate the desired cell type, which can be accomplished by antibody-based magnetic cell separation as a straightforward technique (16). In order to access intracellular cytokines, the membrane of the obtained cells then has to be permeabilized, which is commonly approached with organic solvents or detergents such as saponin (17). The respective cytokines are now attainable for fluorescently-labeled antibodies and can easily be stained and detected by flow cytometry as described in this chapter.

2 Materials

2.1 CD4⁺ T cell isolation

1. Optional. 0.7 % NaCl solution for cell counting.
2. Isolation buffer: phosphate-buffered saline (PBS), pH 7.2, 0.5 % bovine serum albumin (BSA), 2 mM EDTA, filtrated and degassed.

3. CD4⁺ T cell Isolation Kit, mouse (Miltenyi Biotec, Bergisch Gladbach, Germany)
Components:
CD4⁺ T cell biotin-antibody cocktail, mouse
Anti-biotin microbeads
4. LS Separation Columns (Miltenyi Biotec, Bergisch Gladbach, Germany).
5. MidiMACS Separator and appropriate rack (Miltenyi Biotec, Bergisch Gladbach, Germany).
6. Centrifuge cups, 1.5 ml.

2.2 Cell Fixation and Permeabilization

1. Paraformaldehyde: 1% v/v in PBS buffer, freshly prepared.
2. Wash buffer: PBS supplemented with 2 mM EDTA and 0.5% v/v fetal calf serum.
3. Saponin buffer: 0.3% w/v saponin (Carl Roth, Karlsruhe, Germany) in PBS buffer.
4. FACS tubes.

2.3 Antibodies

1. Rat anti-mouse CD16/CD32 (Fc-Block; BD Biosciences, Heidelberg, Germany), 1:100 dilution in 0.3% saponin buffer.
2. Anti-mouse CD4-PE, clone REA604 (Miltenyi Biotec, Bergisch Gladbach, Germany).
3. Isotype control: Rat anti-mouse IgG1-PE, clone M1-14D12 (eBioscience, Frankfurt, Germany).
4. Rat anti-mouse IL-13-PE, clone eBio13A (eBioscience, Frankfurt, Germany), 1:10 diluted in 0.3% saponin buffer.
5. Rat anti-mouse IL-17A-eFluor 450®, clone eBio17B7 (eBioscience, Frankfurt, Germany), 1:10 diluted in 0.3% saponin buffer.
6. Attune NxT acoustic focusing cytometer (Life technologies, Carlsbad, USA) or similar flow cytometer equipped with the following lasers and filter settings:
PE: excitation: 488 nm, emission filter: 574/26; eFluor 450®: excitation: 405 nm, emission filter: 440/50.

3 Methods

For in vivo transfection, harvesting of the lungs and preparation of single cell suspensions, please refer to Chapter 18. With these single cell suspension, obtained from lungs or other desired organs, respectively, proceed with the following steps.

3.1 Isolation of CD4⁺ T cells (see Note 1)

1. Dilute the obtained single cell suspension in 10 ml of a 0.7 % NaCl solution and count the cells using a cell counting device, such as the Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, California, USA) (see Note 2).
2. Centrifuge the cell suspension for 10 min at 350 g.
3. Discard the supernatant and resuspend the cell pellet in 40 µl of isolation buffer per 10⁷ total cells (see Note 3).
4. Optional: Take an aliquot of the original untreated cell population aside for later comparison.
5. Add 10 µl of biotin-antibody cocktail per 10⁷ total cells.
6. Mix well and incubate for 5 minutes in the refrigerator (2-8 °C).
7. Add 30 µl of isolation buffer and 20 µl of anti-biotin microbeads per 10⁷ total cells.
8. Mix well and incubate for 10 minutes in the refrigerator (2-8 °C).
9. For cell separation, place the LS column in the magnetic field of a MidiMACS Separator.
10. Rinse the column with 3 ml of isolation buffer (see Note 4).
11. Apply the cell suspension onto the column and collect the flow-through containing the unlabeled, enriched CD4⁺ T cells (see Note 5).
12. Wash the column with 3 ml of isolation buffer, collect the flow-through and combine with the effluent from step 10 (see Note 6).
13. Optional: To obtain the labeled non-CD4⁺ cells, remove the column from the separator, place it on a collection tube and add 5 ml of isolation buffer. Immediately flush out the remaining cells by firmly pushing the plunger into the column, collecting the flow-through containing the non-CD4⁺ cells.

3.2 Validation of successful isolation

1. To determine the fraction of CD4⁺ T cells in the enriched sample, count all cells and transfer triplicates of about 300.000 cells each of the original untreated cell sample, the CD4⁺ fraction and the non-CD4⁺ fraction into FACS tubes. (see Note 7)
2. Prepare additional samples as blank and isotype controls.
3. Centrifuge the aliquots of 300.000 cells per tube for 10 min at 350 g.
4. Discard the supernatant and resuspend the cell pellets in 35 µl PBS buffer.
5. Add 10 µl of 1:10 diluted Fc-block and 5 µl of the antibody against CD4 (or the appropriate isotype control), vortex and incubate for 10 min at 4 °C in the dark.
6. Centrifuge the stained cells for 5 min at 350 g.

7. Discard the supernatant, wash with 500 μ l PBS buffer three times, and centrifuge the cells a fourth time for 5 min at 350 g.
8. Discard the supernatant and resuspend the cell pellet in 400 μ l PBS buffer.
9. Adjust the laser power at the flow cytometer using the isotype controls and unstained samples.
10. Gate the cells to exclude debris and cell clumps. Count at least 30.000 events.
11. Determine the fraction of CD4⁺ cells in the PE channel as shown in Figure 1. (see Note 8)

3.3 Quantification of intracellular cytokine levels

1. Centrifuge the isolated CD4⁺ T cells for 10 min at 350 g.
2. Discard the supernatant and resuspend the pellet in 4 ml fresh 1% paraformaldehyde solution and incubate for 15 min on ice.
3. Centrifuge the fixed cells for 10 min at 350 g.
4. Discard the supernatant and resuspend in 5 ml wash buffer.
5. Centrifuge cells for 10 min at 350 g.
6. Discard the supernatant and resuspend the pellet in 4 ml wash buffer.
7. Count the cells and transfer about 300.000 cells per sample into FACS tubes.
8. Prepare additional samples as blank and isotype controls.
9. Centrifuge the aliquots for 10 min at 350 g.
10. Discard the supernatant, add 100 μ l 0.3% saponin buffer per sample for permeabilization, mix and incubate for 15 min at 4 °C.
11. Centrifuge FACS tubes for 5 min at 350 g.
12. Discard the supernatant, add 10 μ l diluted Fc-block, add 10 μ l of the appropriate dilutions of the antibodies against IL-13 and IL-17A (or isotype controls), vortex and incubate for 25 min at 4 °C in the dark.
13. Centrifuge the stained cells for 5 min at 350 g.
14. Discard the supernatant, wash with 100 μ l 0.3% saponin buffer twice, and centrifuge the cells a third time for 5 min at 350 g.
15. Discard the supernatant, wash with 100 μ l wash buffer once, centrifuge the cells again for 5 min at 350 g, and resuspend them in 200 μ l wash buffer.
16. Adjust the laser power at the flow cytometer using the isotype controls and unstained samples.
17. Gate the cells to exclude debris and cell clumps. Count at least 30.000 events.

18. Determine the mean fluorescence in the PE channel (representing IL-13) as well as in the eFluor 450® channel (representing IL-17A) as shown in Figures 2 and 3.

4 Notes

1. Throughout the whole isolation process, it is advised to work fast, always keep the cells cold and use pre-cooled solutions (2-8 °C). Ideally, also pre-cool the columns in the fridge, so that they have a similar temperature as the isolation buffer.
2. Alternatively use a haemocytometer counting chamber.
3. For cell numbers lower than 10^7 , use the same volumes as indicated. For higher cell numbers, scale up all reagent volumes accordingly.
4. Always wait until the column reservoir is completely empty, before proceeding with the next step.
5. A volume of not less than 500 µl is required for successful separation, smaller volumes should be filled up with buffer at this point.
6. If some of the T cells are desired to be kept in culture for further experiments, it is strongly recommended to stimulate them, e.g. with antibodies against CD3e (Hamster anti-mouse CD3e, clone 145-2C11 (BD Biosciences, Heidelberg, Germany)) and CD28 (Hamster anti-mouse CD28, clone 37.51 RUO (BD Biosciences, Heidelberg, Germany)). Therefore, coat the appropriate number of wells of a 96-well plate with 1 µl of CD3 antibody diluted in 200 µl of PBS buffer per well. Incubate overnight at 4 °C or, alternatively, at 37 °C for 3 h. Wash three times with rising volumes of PBS buffer and dispense $1-2 \times 10^6$ cells per well in 100 µl RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, USA). Add 0.2 µl of CD28 antibody diluted in 100 µl of RPMI-1640 medium per well and incubate the plate at a humidified atmosphere with 5 % CO₂ at 37 °C.
7. To check the cell viability after isolation, the samples can, additionally, be incubated with dead cell stains, such as DAPI (Biolegend, San Diego, USA) and distinguished by flow cytometry.
8. A purity value of at least 90 % is expectable.

Acknowledgement

This work was supported by the ERC Starting Grant ERC-2014-StG – 637830 “Novel Asthma Therapy”. The authors are grateful to Ayse Kilic for expert support.

References

1. Mocellin S, Provenzano M. RNA interference: learning gene knock-down from cell physiology. *J Transl Med.* 2004; 2(1):39. [PubMed: 15555080]

2. Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. *Advanced drug delivery reviews*. 2007; 59(2–3):75–86. [PubMed: 17449137]
3. Merkel OM, Rubinstein I, Kissel T. siRNA delivery to the lung: what's new? *Adv Drug Deliv Rev*. 2014; 75:112–28. [PubMed: 24907426]
4. Paranjpe M, Muller-Goymann CC. Nanoparticle-mediated pulmonary drug delivery: a review. *Int J Mol Sci*. 2014; 15(4):5852–73. [PubMed: 24717409]
5. Lam JK, Liang W, Chan HK. Pulmonary delivery of therapeutic siRNA. *Advanced drug delivery reviews*. 2012; 64(1):1–15. [PubMed: 21356260]
6. Kim NH, Nadithe V, Elsayed M, Merkel OM. Tracking and treating activated T cells. *J Drug Deliv Sci Technol*. 2013; 23(1):17–21. [PubMed: 24660025]
7. Jeffery PK. Pathology of asthma. *Br Med Bull*. 1992; 48(1):23–39. [PubMed: 1617392]
8. Ray A, Cohn L. Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *The Journal of clinical investigation*. 1999; 104(8):985–93. [PubMed: 10525032]
9. Gwinn WM, Damsker JM, Falahati R, Okwumabua I, Kelly-Welch A, Keegan AD, et al. Novel approach to inhibit asthma-mediated lung inflammation using anti-CD147 intervention. *J Immunol*. 2006; 177(7):4870–9. [PubMed: 16982929]
10. Biedermann T, Schwarzler C, Lametschwandtner G, Thoma G, Carballido-Perrig N, Kund J, et al. Targeting CLA/E-selectin interactions prevents CCR4-mediated recruitment of human Th2 memory cells to human skin in vivo. *Eur J Immunol*. 2002; 32(11):3171–80. [PubMed: 12555662]
11. O'Reilly S, Hugle T, van Laar JM. T cells in systemic sclerosis: a reappraisal. *Rheumatology (Oxford)*. 2012; 51(9):1540–9. [PubMed: 22577083]
12. Pelaia G, Vatrella A, Maselli R. The potential of biologics for the treatment of asthma. *Nat Rev Drug Discov*. 2012; 11(12):958–72. [PubMed: 23197041]
13. Sel S, Wegmann M, Dicke T, Sel S, Henke W, Yildirim AO, et al. Effective prevention and therapy of experimental allergic asthma using a GATA-3-specific DNase. *J Allergy Clin Immunol*. 2008; 121(4):910–6 e5. [PubMed: 18325571]
14. Krug N, Hohlfeld JM, Kirsten AM, Kornmann O, Beeh KM, Kappeler D, et al. Allergen-induced asthmatic responses modified by a GATA3-specific DNase. *N Engl J Med*. 2015; 372(21):1987–95. [PubMed: 25981191]
15. Xie Y, Kim NH, Nadithe V, Schalk D, Thakur A, Kilic A, et al. Targeted delivery of siRNA to activated T cells via transferrin-polyethylenimine (Tf-PEI) as a potential therapy of asthma. *J Control Release*. 2016; 229:120–9. [PubMed: 27001893]
16. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. *Cytometry*. 1990; 11(2):231–8. [PubMed: 1690625]
17. Jamur MC, Oliver C. Permeabilization of cell membranes. *Methods Mol Biol*. 2010; 588:63–6. [PubMed: 20012820]

Figure 1.

Dot and histogram plots obtained after measuring the mean fluorescence intensity (MFI) in the PE channel after staining with an anti-CD4-PE antibody. To exclude debris, cells are gated in the FSC-SSC dot plots on the left. CD4⁺ cells are gated in the respective middle and right plot. From top to bottom the following samples can be seen: original cells before separation, isolated CD4⁺ fraction, non-CD4⁺ fraction.

Figure 2.

For illustration, an untreated blank sample and a stained sample of CD4⁺ T cells are shown after measuring the MFI in the PE channel. The respective peak and dot cloud of the positive stained sample distinctly shift to higher fluorescence values, indicating high levels of the respective cytokine as well as a high number of positive cells.

Figure 3. Mean fluorescence values from the respective channels can be depicted in bar graphs. Here, the cytokine levels after pulmonary treatment of OVA challenged mice with GATA-3 siRNA compared to a scrambled control sequence (siNegCon) are shown after assessment via intracellular staining of the isolated CD4⁺ T cells from the lungs.