RNAi in human monocyte-derived dendritic cells using shRNA vectors

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Method Article

Keywords: Human monocyte-derived dendritic cells, shRNA, RNAi, Vpx

Posted Date: December 14th, 2010

DOI: https://doi.org/10.1038/protex.2010.208

Abstract

Human monocyte-derived dendritic cells \(MDDC) constitute a widely used experimental model to study human dendritic cells. However, loss-of-function experiments in MDDC have been limited by the poor efficiency of traditional RNAi techniques. We have developed a RNAi method in MDDC using shRNA lentiviral vectors. Importantly, this method of MDDC transduction does not appear to interfere with the process of DC differentiation. Furthermore, this method does not provoke MDDC maturation. This process is highly efficient and >90% transduction is routinely obtained. This protocol is focused on the use of shRNAs in the widely available LKO.1 backbone, with puroR or GFP as a marker. It is adaptable to other lentiviral shRNA vectors, to gain-of-function approaches \(overexpression) and for differentiation into macrophages instead of dendritic cells.

Introduction

Human monocyte-derived dendritic cells \(MDDC) constitute a widely-used experimental model to study human dendritic cells. However, loss-of-function experiments in MDDC have been limited by the poor efficiency of traditional RNAi techniques. In our hands, lipid-based transfection of siRNA is largely ineffective. Fluorescently-labelled siRNA can be detected after lipofection of MDDC by microscopy or cytometry, but these siRNAs appear located in intracellular vesicles, and not in the cytoplasm, which is consistent with the highly endocytic nature of MDDC. Another technique, electroporation, can be effective with some siRNAs, but is extremely toxic by nature and leads to a large proportion of cell death. This can be a highly confounding factor in studying MDDC function, since MDDC can respond to the presence of apoptotic cells, among other factors. We have developed a RNAi method in MDDC using shRNA lentiviral vectors. These vectors are typically derived from HIV-1. Due to a post-entry restriction to HIV-1 infection in MDDC, transduction of MDDC with HIV-1-derived vectors is highly inefficient. This restriction can be overcome by the Vpx accessory protein found in other lentiviruses such as SIVmac. SIVmac particles can be used as helper particles to facilitate MDDC transduction by alleviating the restriction to HIV-1 \[1]. We have used this property to transduce MDDC with shRNA vectors in a highly efficient manner. Importantly, this method of MDDC transduction does not appear to interfere with the process of DC differentiation, as shown by the normal expression of DC markers \(DC-SIGN, CD1a, HLA-DR) and the down-regulation of CD14 \(see Figure). Furthermore, this method does not provoke MDDC maturation \[2]. As we show in the accompanying paper, HIV-1 induces MDDC maturation only after integration and de novo expression of viral proteins. In brief, fresh monocytes are transduced with shRNA-encoding lentiviral particles and SIVmac virus-like particles. The SIVmac particles carry the Vpx protein but are devoid of genomic RNA. After 6 days of differentiation, MDDC expressing the shRNA are obtained. This process is highly efficient and >90% transduction is routinely obtained with non-concentrated, non-purified viral supernatants. In such cases, antibiotic selection or cell sorting are usually not required and the total cell population can be used as is for further experiments. We have used this protocol successfully to inhibit the expression of DC-SIGN, Cyclophilin A and IRF-3 at the RNA level, the protein level and at the functional level. This protocol is focused on the use of shRNAs in the widely available LKO.1 backbone, with puroR or GFP as a

marker. It is adaptable to other lentiviral shRNA vectors, to gain-of-function approaches \(overexpression) and for differentiation into macrophages instead of dendritic cells.

Reagents

- A source of adult human blood - Plasmid pCMV-VSVG \(Addgene) - Plasmid pCMV-ΔR8.91 \(Addgene) - Plasmid pLKO.1puro \(Addgene) - A lentiviral vector expressing GFP such as pLKO.1gfp \[2], pTRIP \[3], etc. - Plasmid pSIV3+ \[1] - Invitrogen PureLink HiPure plasmid kit - FicollPAQUE - Miltenyi human CD14+ microbeads - DC culture media: RPMI + 10% heat-inactivated fetal bovine serum + PenStrep + 2 mM L-Glutamine + 100 µM ß-mercaptoethanol - recombinant human IL-4 \(eBioscience) - recombinant human GM-CSF \(eBioscience) - 293T cells \(ATCC) - 293T culture media: DMEM : + 10% heat-inactivated fetal bovine serum + PenStrep + L-Glutamine - TransIT-293 \(Mirus) - Polybrene \(hexadimethrine bromide; Sigma)

Equipment

- Magnetic cell separator - Flow cytometer

Procedure

Research involving human blood is subject to specific regulations. Ensure compliance with regulations at your host institution, including reviewing by your Institutional Review Board. Research involving HIV-derived vectors is also subject to specific regulations. Check with your host institution the level of biosafety required. _Timing is based on days of MDDC differentiation_ **DAY –3** 1. Plate 293T cells at $0.5x10^6$/well in 6-well plates **DAY –2** 2. Transfection with TransIT-293 following manufacturer's protocol. Per well, use 8 µL TransIT-293 + 3 µg total DNA. DNA is made with Invitrogen HiPure kits. Ratios for transduction are: _Helper particles:_ - 0.4 µg pCMV-VSVG - 2.6 µg pSIV3+ _Transduction particles:_ - 0.4 µg pCMV-VSVG - 1 µg pCMV-ΔR8.91 - 1.6 µg pLKO.1puro shRNA vector or a GFP-encoding lenviviral vector \(pLKO.1gfp, pTRIP, etc) **DAY –1** 3. Change media \(3 ml per well of 293T culture media) **DAY 0** 4. Isolate mononuclear cells from adult or cord blood on a FicollPAQUE gradient 5. Use Miltenyi human CD14+ beads and procedure to isolate $CD14^+$ monocytes and check their purity 6. Plate $5x10^6$ monocytes in 5 ml MDDC culture media in a 10 cm plate with recombinant human GM-CSF at 10 ng/ml and recombinant human IL-4 at 50 ng/ml 7. Add 1 µg/ml polybrene 8. Filter culture supernatants from transfected 293T cells with 0.45 µM yellow filters \(Corning) 9. Add 2.5 ml of helper particle supernatant and 2.5 ml of shRNA particle supernatant to the monocytes **DAY 3** 10. Add an additional 2.5 ml of fresh MDDC culture media containing GM-CSF and IL-4 **DAY 5** 11. Add an additional 2.5 ml of fresh MDDC culture media containing GM-CSF and IL-4 **DAY 6** 12. Analyze. Check transduction efficiency by cytometry when a lentiviral vector encoding GFP was used. Check knockdown efficiency by real-time PCR and western blotting.

Troubleshooting

More details about lentiviral shRNA vectors and particle production can be found on the Addgene website: http://www.addgene.org/pgvec1?f=c&cmd=showcol&colid=170&page=2 http://www.addgene.org/pgvec1?f=c&cmd=showcol&colid=170&page=3 MDDC are highly sensitive cells and will get activated by traces of contaminants, too much cell death or harsh manipulation. General precautions: - Prepare plasmid DNA with a low-endotoxin procedure - Test different batches of fetal bovine serum - Use filter tips at all times

Anticipated Results

20-80% of the original number of monocytes should be recovered as MDDC after 6 days. Variation may be attributed to donor-to-donor differences, to quality of the blood source and the efficiency of monocyte purification. >90% of the recovered MDDC should appear transduced at day 6 \(see Figure).

References

\[1] Goujon, C. et al., With a little help from a friend: increasing HIV transduction of monocyte-derived dendritic cells with virion-like particles of SIV\(MAC). Gene therapy 13, 991-994 \(2006). \[2] Manel, N. et al., A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells. Nature, in press. \[3] Zennou, V. et al., HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 101, 173–185 \(2000).

Acknowledgements

We thank Mickaël Ménager and Brandon Hogstad for critical reading of this protocol.

Figures

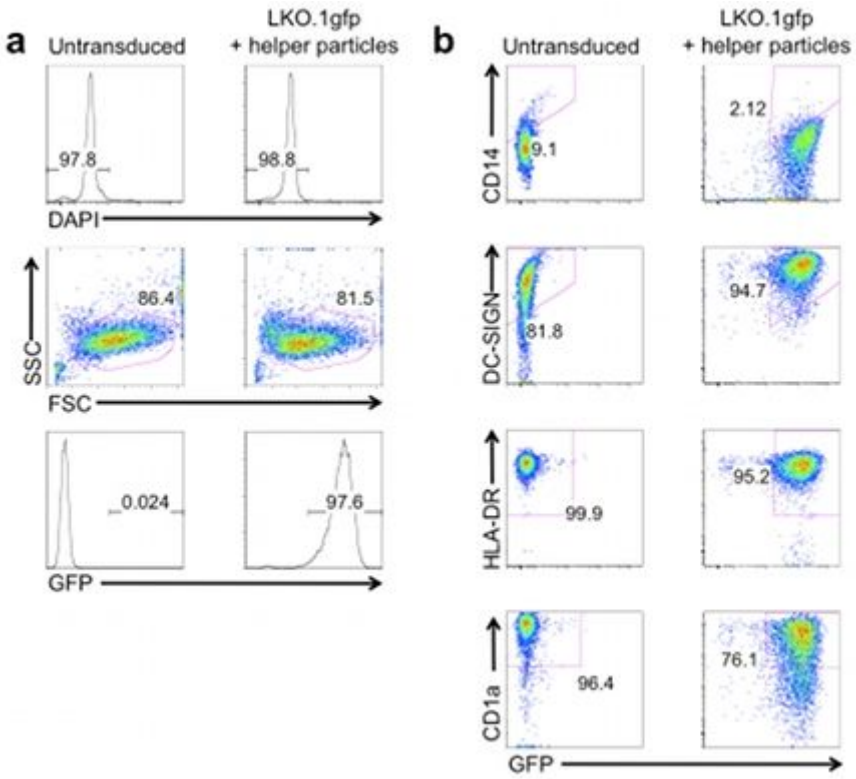

Figure 1

Figure Differentiation of untransduced MDDC and MDDC transduced with the shRNA vector Differentiation of untransduced MDDC and MDDC transduced with the shRNA vector LKO.1gfp encoding GFP in combination with helper particles. MDDC were labeled at day 6 with DAPI and antibodies against CD14, DC-SIGN, HLA-DR and CD1a (a) Gating strategy and expression of GFP, from top to bottom. (b) Expression of GFP vs CD14, DC-SIGN, HLA-DR and CD1a.