Stable Gene Silencing in Human Monocytic Cell Lines Using Lentiviral-delivered Small Interference RNA

SILENCING OF THE p110α ISOFORM OF PHOSPHOINOSITIDE 3-KINASE REVEALS DIFFERENTIAL REGULATION OF ADHERENCE INDUCED BY 1α,25-DIHYDROXYCHOLECALCIFEROL AND BACTERIAL LIPOPOLYSACCHARIDE

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Studying mononuclear phagocyte cell biology through genetic manipulation by non-viral transfection methods has been challenging due to the dual problems of low transfection efficiency and the difficulty in obtaining stable transfection. To overcome this problem, we developed a system for mediating RNA interference in monocytic cells. The p110α isoform of phosphoinositide 3-kinase (PI3Ks) was silenced using a lentiviral vector expressing short hairpin RNA (shRNA). This resulted in the generation of stable THP-1 and U937 monocytic cell lines deficient in p110α. Notably, p110α was silenced without affecting levels of either of the other class I PI3K catalytic subunits p110β and p110δ, or the p85α regulatory subunit. The role of p110α in mediating cell adherence was examined. Monocyte adherence induced in response to either lipopolysaccharide (LPS) or 1α,25-dihydroxycholecalciferol (D3) was blocked by the PI3K inhibitor LY294002. However, although adherence induced in response to D3 was sensitive to silencing of p110α, LPS-induced adherence was not. Expression of the monocyte differentiation marker CD11b was also induced by D3 in a PI3K-dependent manner and gene silencing using shRNA showed that p110α was also required for this effect. Taken together, these findings demonstrate that LPS and D3 use distinct isoforms of class I PI3K to induce functional responses and that lentiviral-mediated delivery of shRNA is a powerful approach to study monocyte biology.

Cells of the mononuclear phagocyte series respond to a wide range of diverse stimuli and show complex cell regulation. From the perspectives of cell biology, understanding disease causation, and developing novel therapeutics, there continues to be a great deal of interest in understanding how the responses of these cells are regulated. However, study of monocyte/macrophage biology through genetic manipulation by non-viral transfection methods has been challenging (reviewed in Ref. 1). Methods involving cationic lipid and liposome-mediated delivery of DNA or physical methods such as electroporation result in low transfection efficiency in monocytic cells, loss of viability, and the difficulty of obtaining stable transfection (2, 3). An approach that has been met with greater success in monocytic cell lines is viral-mediated transduction. Although not all viruses can transduce monocytic cells efficiently, lentiviruses have been shown to do so at >90% efficiency (4–6).

RNA interference (RNAi)1 is a sequence-specific post-transcriptional gene silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) into target cells (7). RNAi is a natural regulatory mechanism that occurs in many organisms, including plants, Caenorhabditis elegans, Drosophila, and mammalian cells (reviewed in Ref. 7). The RNAi pathway begins by processing dsRNA into short (30 bp) dsRNA duplexes called small interference RNA (siRNA) by a host RNAse Dicer. The siRNA then becomes incorporated into a multicomponent nuclease complex called the RNA-induced silencing complex (RISC). RISC then uses the siRNA sequence as a guide to recognize cognate mRNAs for degradation.

Delivery of siRNAs into mammalian cells by transfection of siRNA or DNA vectors expressing short hairpin RNA (shRNA) has been shown to mediate RNAi successfully (8–11). Transfection of siRNA is transient lasting only for a week or so (12), although DNA-based vectors may last longer with drug selection (10). In contrast, viral vectors have also been used to deliver siRNA successfully, and these methods tend to provide more stable gene silencing (13–15). Here we report that human monocytic cell lines can be effectively transduced using a lentiviral vector to stably silence an endogenous lipid kinase.

The phosphoinositide 3-kinases (PI3Ks) constitute a family of at least eight different lipid kinases that phosphorylate the

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1 The abbreviations used are: RNAi, RNA interference; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; D3, 1α,25-dihydroxycholecalciferol; PtdIns, phosphatidylinositol; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; CR3, complement receptor 3; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; VDR, vitamin D receptor; VSV, vesicular stomatitis virus; dsRNA, double strand RNA; siRNA, small interference RNA; HEK, human embryonic kidney; CMV, cytomegalovirus; GFP, green fluorescent protein; LTR, long terminal repeat; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay; MFI, mean fluorescence intensity; ANOVA, analysis of variance; RPE, R-phycocerythrin.
Fig. 1. Construction of a lentiviral vector for transduction of shRNA into target cells. A, to produce the recombinant lentiviral vectors, the packaging cell line HEK 293T was co-transfected by the vector plasmid (pHR-U6-shRNA), helper plasmid (pCMVΔR8.2), and envelope plasmid (pMD.G). The general strategy in the production of lentiviral vector-delivered siRNA is to segregate the trans-acting sequences that encode for viral proteins from the cis-acting sequences (regions recognized by viral proteins) involved in the transfer of vector sequences encoding the shRNA.
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hydroxyl group of the inositol ring of phosphoinositides at the 3' position. The phosphoinositide (PI) metabolites produced as a result are known to be involved in regulating a multitude of cellular events such as mitogenic responses, differentiation, apoptosis, cytoskeletal organization, membrane traffic along the exocytic and endocytic pathways (reviewed in Ref. 16), and various other aspects of monocyte function (17–19). A considerable amount of research has led to the conclusion that this diversity of cellular control is differentially mediated by distinct PI3K isoforms (20–22). In vitro, class I PI3Ks isoforms phosphorylate phosphatidylinositol (PI), PI 4-phosphate, and PI 4,5-bisphosphate. Class I PI3K is further phosphorylate phosphatidylinositol (PtdIns), PtdIns 4-phosphate, and PtdIns 4,5-bisphosphate. Class I PI3K is further phosphorylated by class I PI3Ks. PI3K is known to be activated by cell surface receptors. Mammalian class I PI3Ks are heterodimers consisting of a regulatory subunit (p85α, p85β, p55, or other splice variants) and a p110 (α, β, or δ isoforms) catalytic subunit (23–25). Through their Src-homology 2 domain-containing p85 subunits, class I PI3K are recruited to and are activated by either cell surface receptors with intrinsic protein-tyrosine kinase activity or receptors coupled to Src-like protein-tyrosine kinases.

1α,25-Dihydroxycholecalciferol (D_3) is a biologically active form of vitamin D and plays an important role in numerous cellular and physiological processes such as calcium homeostasis and regulates cells of the hematopoietic system (reviewed in Refs. 26–28). For example, D_3 induces maturation markers such as CD11b and CD14 in monocytic cell lines such as THP-1, U-937, and HL-60 (18, 29–31). In previous work from this laboratory, D_3 was observed to activate PI3K, and PI3K activity was shown to be required for the induction of CD11b and CD14 expression by D_3 (18). D_3 also induces adherence in cells of the human promonocytic cell line THP-1 (29, 32, 33), although it is not known whether this involves PI3K. Bacterial lipopolysaccharide (LPS) is also known to enhance adherence of leukocytes in vitro (34, 35), and it also activates PI3K in monocytic cells (17, 36). We and others have previously shown that LPS induces adherence in THP-1 cells (37–40) and that this is PI3K-dependent (37). However, the roles of individual PI3K isoforms in this phenotype have not been defined.

To provide a basis for studying the roles of specific PI3K isoforms in regulating the function of cells of the mononuclear phagocyte series, the objective of the present study was to identify stable monocytic cell lines that express shRNA targeting a specific PI3K isoform. Using this approach, we examined the role of the p110α isoform of class I PI3K in mediating responses to LPS and D_3.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—RPMI 1640, Dulbecco's modified Eagle's medium, Hanks' balanced salt solution, penicillin/streptomycin, and 1 μM HEPES solution were from Stem Cell Technologies (Vancouver, BC). Phenylmethylsulfonyl fluoride, LPS from Escherichia coli (O111:B4), Polybrene, and poly-L-lysine were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). LY294002, 1α,25-dihydroxycholecalciferol, and antibodies to human p110α were from Calbiochem (San Diego, CA).

An antibody to human p110α (clone 19) was from BD Biosciences (Mississauga, Ontario). Antibodies to human p110β and actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-p85-N-SH3 antibody was from Upstate Biotechnology (Lake Placid, NY). RPE-conjugated anti-CD11b antibody and RPE-conjugated isotype-matched control antibody were from Caltag Laboratories (San Francisco, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies were from Caltag Laboratories (San Francisco, CA).

Cell Lines—The promonocytic cell lines THP-1 and U-937 (ATCC, Rockville, MD) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Burlington, Ontario), 2 mm t-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cultures were maintained without exceeding 0.5 × 10^6 cells/ml. 293T human embryonic kidney (HEK) cells, were also from ATCC and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mm t-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml), and 20 μM HEPES.

Constructs—The U6 promoter vectors pSHAG-1 and pSHAG-PF1 were kind gifts from Dr. G. J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). pSHAG-1 contains the αtR/L2 transposition elements that are compatible with Gateway Cloning Technology (Invitrogen Canada Inc.). Antisense to p110α mRNA (GenBank NM_006218) was targeted to two nucleotide segments: 5'-ATATACAT-TCTGATCCTCTCTGTGC-3' (nucleotide positions 1171–1198, referred to as α3) and 5'-CAAGACCATATCAAGTTAATCTGGG-3' (nucleotide positions 8–35, referred to as α1). The hairpin-containing sequence was created as described previously (14). The hairpin sequences p110α1 and p110α3 listed in Table I were synthesized by Qiagen Inc. (Valencia, CA). All of the sequences contained a HindIII site in the hairpin region, a site that is not present in the native pSHAG-1 vector, and BamHI and BseRI ends to enable directional cloning. The oligonucleotides were annealed and then ligated into pSHAG-1 via the BamHI/ BseRI site. DH5α E. coli (Invitrogen) were transformed, and clones were screened by HindIII digestion. pSHAG-PF1 contains a U6-driven sequence that generates a hairpin RNA that targets GL3 firefly luciferase at nucleotide positions 1619–1647 (GenBank U47296) (9). Construction of the lentiviral transducing plasmid, pHR-CMV-EGFP, packaging vector pCMVΔR8.2, and VSV envelope vector pMD.G have been described elsewhere (41, 42). Purified pSHAG-1, pSHAG-p110α1, and pSHAG-p110α3 served as entry clones. The lentiviral transducing vector pHR-CMV-EGFP was modified by inserting the Gateway vector conversion cassette (Invitrogen) in the ClaI site, which is located downstream of 5'-LTR, but upstream of the CMV promoter. The resulting pHR-Gate vector served as a destination vector, because it contained αR1/2 sites. The various entry clones were transposed to the pHR-CMV-ΔR8.2 Gateway LR Cloning Enzyme Mix by homologous recombination. The entry clones were then isolated, and the plasmids (pHR-U6, pHR-p110α1, and pHR-p110α3) were purified. All plasmid purifications were carried out using Qiagen Endofree Plasmid kits.

Lentivirus Packaging—The packaging cell line 293T HEK (5 × 10^6) was plated on poly-L-lysine-coated 100-mm tissue culture plates (Corning) and transfected the following day. 10 μg of the packaging vector pHR (pHR-U6, pHR-p110α1, or pHR-p110α3), 7.5 μg of the packaging vector pCMVΔR8.2, and 2.5 μg of the VSV envelope vector pMD.G were co-transfected by LipofectAMINE 2000 (Invitrogen), according to the manufacturer's instructions. The medium was changed the next day, and cells were cultured for another 24 h. Conditioned medium was then collected and cleaned of debris by low speed centrifugation (2,500 g for 5 min) through a pure solvent mixture filter and stored at 70 °C. This collection was repeated daily for three more days, and media from the four days were pooled and ultracentrifuged at 100,000 × g for 4 °C for 2 h. The pellet was resuspended in 500 μl of medium (overnight on a nutator (reviewed in Ref. 44). The vector plasmid contained a U6 promoter-driven shRNA coding sequence, followed by a CMV-driven reporter, enhanced green fluorescent protein (EGFP). The shRNA nucleotide sequence shown is not specific and is only intended to illustrate a generic shRNA. These elements were flanked by long terminal repeats (LTRs) and also contained cis-acting sequences that allowed the vector RNA to be packaged and, subsequently, to be reverse transcribed and integrated in the target cell. The packaging sequence (Ψ) was only present in the vector plasmid and not in the other two plasmids. Following transfection, the plasmids pCMVΔR8.2 and pMD.G were transcribed by CMV promoters, and they provided the viral structural proteins in trans. These included viral integrase, protease, reverse transcriptase, capsid matrix proteins, and vesicular stomatitis virus G protein. Together, these proteins act to trans-complement the vector by assembling the progeny viral particles, which are then limited to a single round of infection. Vector proteins were produced as well, so transfected cells were GFP-positive. Conditioned medium was then harvested, concentrated by ultracentrifugation, and stored at −70 °C. B transduction of target cells was done at a multiplicity of infection of 10:1. Virions attach at the cell surface via VSV G proteins, fuse with the cell membrane, and release the viral core. Reverse transcription and uncoating of the viral core occurs in the cytoplasm, which then integrates into the target cell genome. Following integration, the U6 and CMV promoters transcribe their respective genes, and this results in shRNA production and mRNA for the GFP reporter. The shRNA and mRNAs are exported to the cytoplasm. GFP is then translated, and the shRNA is processed by Dicer and then incorporated into the RNA-induced silencing complex (RISC) (reviewed in Ref. 14). RISC then targets and degrades cognate mRNAs.
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| Construct | 5′-Antisense sequence (28 nt) | Hairpin loop (8 nt) | Sense sequence (28 nt) | Termination (6 T–3′) |
|-----------|-------------------------------|---------------------|------------------------|----------------------|
| p110s1    | CCCCCAGGGTTCGGCCGAGGGGCTGC   | AAAAAATTGCCCCAGGGTTCGGGCCTCCGAGATCTTCAGCACTGACTATATGATATAT | | |
| p110s3    | CCCCCAGGGTTCGGCCGAGGGGCTGC   | CCAACTTACACATGAGAGCTTCACGTACGAGAAGATCGTGCAGGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA |
genome, thus generating a stable cell line (Fig. 1B). Transduced cells expressed GFP and shRNA (GFP only in HR-U6 vector). Six days post-transduction, cells were analyzed for GFP expression using flow cytometry (Fig. 2A). The lentiviral vectors were able to consistently transduce >90% of the target cells.

Stable and Specific Silencing of Class IA PI3K p110α Isoform—shRNA specific to PI3K p110α mRNA was used to induce RNA silencing by a mechanism involving the RISC (Fig. 1B). Transduced cells were expanded and examined by Western blotting. Fig. 2 (B and C) shows that transduction of THP-1 and U-937 with the HR-p110α3 viral vectors resulted in nearly complete elimination of PI3K p110α isoform expression. In contrast, transduction of cells with either lentiviral vector HR-p110α1 expressing the α1 shRNA sequence, U6-promoter control virus, or mock transduction did not affect p110α protein levels. This result was specific in that levels of other Class IA PI3K catalytic subunits p110β and p110δ, or the p85α regulatory subunits were not affected. The stability of p110α silencing was confirmed by Western blotting of cells stored in liquid nitrogen for at least 8 months, and cells that had been in continuous culture for more than 6 weeks.

Stable and Specific Silencing of Luciferase Activity in HEK 293T Cells—To verify that the hairpin construct from HR-p110α3 does not interfere with the transcription of reporter plasmids, we transduced HEK 293T cells with either HR-p110α3 or HR-FII, which produces a shRNA targeting GL3 firefly luciferase. HEK 293T cells were chosen, because they are much more receptive to transfection than are monocytic cell lines. Western blot analysis demonstrated that the level of

![Fig. 2. Transduction of monocytic cell lines by lentiviral vectors is efficient and generates stable cell lines deficient in p110α. A, flow cytometry analysis of transduced (solid histogram) or mock transduced cells (clear histogram). 10,000 cells were analyzed, and the GFP fluorescence intensity was measured on the FL1 channel. Approximately 97% of the THP-1 and U-937 cells were GFP-positive. The mean fluorescence intensity (MFI) for mock-infected cells was 8.5 for THP-1 and 6.6 for U-937. Transduced cells had MFI values of 38.3 for THP-1 and 132.3 for U-937. B and C, Western blot analyses of class IA PI3K p110 catalytic subunit isoforms (α, β, and δ), and p85 regulatory subunit in THP-1 cells and U-937 cells. Actin was used as protein loading control.](image-url)
p110α in HEK 293T cells transduced with HR-p110α3 viruses was similar to that observed in similarly treated THP-1 and U-937 cells, and as expected HR-Ff1 virus had no effect on p110α expression (data not shown). Transduced cells were then co-transfected with firefly luciferase (pGL3-SV40) and Renilla luciferase pRL-TK reporter plasmids. Forty-eight hours after transfection, cells were lysed and analyzed for luciferase activity using Promega’s Dual Luciferase Assay. The activities are reported as firefly luciferase (Pp-Luc)/Renilla luciferase (Rr-Luc). HR-Ff1-transduced cells gave a Pp-Luc/Rr-Luc ratio of <20% of mock or HR-p110α3-transduced cells (p < 0.01, post-ANOVA Tukey test). Cells transduced with shRNA targeting p110α had similar ratios as non-transduced cells (p > 0.05, post-ANOVA Tukey test). One-way ANOVA for all three cell lines p = 0.0027. Error bars indicate S.D., n = 3.

DISCUSSION

A major obstacle in studying monocyte cell biology has been the resistance of these cells to genetic manipulation, particularly when using non-viral methods. In this report, we describe a strategy for stable gene silencing in monocytic cells. Using a VSV-pseudotyped lentiviral vector, monocytic cell lines that stably expressed shRNAs targeting an endogenous gene were generated resulting in silencing of the p110α isoform of PI3K. Historically, lentiviral vectors have been shown to be superior to non-viral methods such as cationic lipid-mediated delivery of DNA vectors to monocytic cells because of their much higher transduction efficiency and longer period of transgene expression. Although the mechanism underlying the resistance of monocytic cells to DNA transfection is not known precisely, it has been proposed that much of the exogenous DNA enters the cell via endocytosis resulting in degradation of the DNA by abundant lysosomal nucleases (1). VSV-pseudotyped lentiviral vectors obviate this problem, because the viral core containing the genetic elements of interest is delivered directly into the cytosol after the viral envelope fuses with the plasma membrane of the target cell (44). Another physical approach for transfection that has not been very successful in monocytic cells is electroporation. Poor success here has been related to low viability of cells after electroporation, typically below 10–22% survival (2, 3).

Among the various viral-based approaches, lentiviral-based vectors seem to be the most promising for transduction of monocytic cells. Onco-retroviruses are similar to lentiviruses,
but the latter have a more complex genome and, consequently, a more complex replication cycle (reviewed in Ref. 44). One advantage of lentiviral vectors over onco-retroviral vectors lies in their ability to transduce both proliferating and non-proliferating cells, such as liver, muscle, retina, and neurons (45–47). This has been attributed to the presence of nuclear localization signal sequences present in lentiviral gene products (48), which are absent from onco-retroviral vectors. Interestingly, onco-retroviruses transduce mononuclear cell lines THP-1, U-937, and HL-60 at lower efficiencies (1–31%) (5, 6), even though these are proliferating cells. Furthermore, compared with onco-retroviruses, lentiviral vectors are also much less susceptible to transcriptional silencing of the viral transgene, an event that may result from methylation of foreign DNA in the vicinity of the promoter, as well as by integration of the viral elements into condensed chromatin regions (1, 49). Taken together, all of the above differences make lentiviruses potentially superior vectors for the delivery of siRNAs into mononuclear cells.

Other viruses such as adenoviruses and adeno-associated viruses have also been used to transduce mononuclear cells. Adenoviruses do not integrate into the host genome and as a result are not useful for long term expression of the exogenous sequences (1). Adeno-associated viruses, in contrast, do integrate into the host genome and have been used successfully in transducing primary human monocytes and dendritic cells (50, 51), although their efficacy in transducing human mononuclear cell lines has been low (<1%) (52). By combining the ability of lentiviral vectors to stably transduce mononuclear cell lines at a high efficiency and the potential for siRNA to mediate RNA interference, we have shown that stable gene silencing in human mononuclear cell lines is achievable (Fig. 2). Transduced cells can be propagated under normal conditions without drug selection. The silenced phenotype was stable during 6–8 weeks of continuous culture, and transduced cells could be used after long periods of storage in liquid nitrogen.

Despite recent progress in understanding the functions of PI3K family members in various cell types, study of distinct
functions of individual PI3K isoform in monocyes has been difficult due to their resistance to genetic manipulation. The PI3K inhibitors that are currently available, including LY294002 (53), wortmannin (54), and 3-methyladenine (19), are not useful in assigning function to specific isoforms, because at effective concentrations they inhibit virtually all classes of the PI3K family except for class II PI3K C2α (55). One way to obviate this problem has been to rescue PI3K inhibitor-induced phenotypes by delivering either class I PI3K or class III products such as PI 3,4,5-trisphosphate and PI 3-phosphate, respectively, by lipid carriers into cells (19). However, this approach cannot give information on the role of distinct class I PI3K isoforms. Another non-genetic approach to study the function of specific PI3K enzymes has been microinjection of inhibitory antibodies. This has proven to be useful for examining the roles of PI3K isoforms when combined with imaging studies of single cells, including the murine macrophage cell line J774 (56). However, not every type of cell can be subjected to this technique (57), and, due to the limited number of cells that can be studied, biochemical characterization of proteins is challenging. In addition, this method is transient in nature and cannot generate a stable phenotype for a prolonged period of time.

Genetic approaches to assigning function to individual class I PI3K p110 isoforms have been limited as well since gene knockouts of p110α or p110β in mice were found to be embryonically lethal (58, 59). Consequently, it has not been possible to determine with precision the roles of these isoforms in immune cells. More recently, instead of gene knockouts, mutant p110β mice were created and have been a useful alternative (60). Thus far, however, no phenotypes in monocytic cells from either knockout or mutant p110β animals have been reported (60–62).

Due to the close and complex relationships between class IA regulatory and catalytic subunits (63–65), an ideal strategy in studying the functions of specific PI3K would be to reduce the expression of individual isoforms without disturbing the molecular balance of the regulatory and catalytic subunits. This has not always been achievable. For example, p110α embryonic knockout cells had increased p85α expression (58), and the massive accumulation of p85α monomers observed was suggested to exert a dominant negative effect on the remaining class IA p110 isoforms by binding non-productively to receptors (64, 66). Conversely, in cells from p85α or p85β knockout mice, expression of p110 isoforms α, β, and δ was either reduced or normal depending on the cells studied (67–70). In this context, lentiviral-mediated RNA silencing of p110 isoforms appears to be a superior approach, because we were able to specifically reduce p110α expression while not affecting levels of either other class IA p110 isoforms or p85α (Fig. 2, B and C). Taken together, the results shown indicate that lentiviral-delivered siRNA is an efficient method for gene silencing in monocytic cells. Furthermore, by virtue of the specificity offered by RNAi, studies of individual isoforms from protein families can be done with relative ease.

In contrast to lentiviral vector HR-p110α3, transduction of the HR-p110α1 vector did not reduce cellular levels of p110α (Fig. 2, B and C). Although HR-p110α1 was originally designed as a candidate shRNA to mediate RNAi, the sequence did not bring about the desired result. Nevertheless, these cells served as useful controls for nonspecific effects of transduction and shRNA expression. There are several possible explanations for why this candidate siRNA might not have been effective. It has been suggested that target mRNA regions where hydrogen bonds form in secondary and tertiary structures can impede silencing (71, 72). Another possible explanation for the lack of effect of the HR-p110α1 vector may be that this construct targeted a region close to the AUG start codon. It has been suggested that these regions may be richer in sequences that bind regulatory proteins and this may limit the ability of the RISC complex to access the RNA target sequence (7, 73). Nevertheless, this is not an absolute restriction, because it has been shown that targeting sequences close to the start codon may successfully induce RNA silencing (71).

Vitamin D₃ and LPS are both known to induce adherence in monocytic cells (29, 32, 33, 37) and to activate PI3K (17, 18, 36). Through the use of in vitro kinase assays, we and others have previously shown that both LPS and D₃ activate PI3K in human monocytes and macrophages (17, 18, 36). Because in these studies the basic approach used was an anti-p85 antibody to immunoprecipitate the kinase, the results led to the conclusion that class IA PI3Ks are activated in response to LPS or D₃, although the involvement of other PI3K family members could not be ruled out. Because p110α is a more robust PI3K than either p110β or p110δ (60, 74, 75), we hypothesized that p110α might be the dominant class IA PI3K in mediating LPS and D₃ signaling. Based on this assumption, we predicted that by silencing PI3K p110α in THP-1 cells, a phenotype of diminished adherence induced by either LPS, D₃, or both would be observed. As shown in Fig. 4 (A and
although adherence in response to both LPS and D3 was sensitive to PI3K inhibitor LY294002, LPS-induced adherence was resistant to silencing of p110α, whereas D3-induced adherence was not. This was examined over a range of LPS concentrations to control for the possibility that LPS might only utilize p110α at lower concentrations. Thus, the findings suggest that differential utilization of p110α reflects qualitative differences between LPS and D3. These results lead to the somewhat surprising and interesting conclusion that these two agonists use signaling pathways in monocytes that activate distinct isoforms of class Iα PI3Ks at least for some functional responses. It would appear most likely that LPS-induced adherence is mediated by p110α or p110δ, because LPS is known to activate class Iα PI3K (17, 36), and LPS-induced adherence was inhibited by LY294002 (Fig. 4A). At this point, we cannot rule out the possibility that LPS activates p110α for other signaling pathways not related to adherence.

Prior studies from this laboratory showed that in THP-1 cells treated with D3, the vitamin D receptor (VDR) associated with the p85 subunit of PI3K in a ligand-dependent manner (18). In addition, within 20 min of exposure to D3, a corresponding rise in PI3K activity was observed when PI3K assays were performed on either anti-p85 or anti-VDR immunoprecipitates, and PI3K activation was linked to changes in gene expression after 24 h (18). The findings in the latter and the present reports are consistent with a model in which class Iα PI3K is activated through a steroid receptor. This model differs from the conventional paradigm in which class Iα PI3K activation occurs downstream of transmembrane receptors such as growth factor receptors, immunoreceptors (reviewed in Ref. 76), and toll-like receptor 2 (77). However, recent progress in D3 signaling research has resulted in the detection of a putative membrane-bound receptor (VDRmem) that, based upon differences in binding properties, appears to be distinct from the nuclear VDR (VDRnuc) (reviewed in Ref. 78). For example, selective binding of synthetic D3 analogs to VDRmem and not VDRnuc has shown that the former mediates non-genomic signaling effects of D3 but not delayed classic genomic responses (79). Taken together, these findings suggest the interesting possibility that class Iα PI3K may be activated by both VDRmem and VDRnuc, such that PI3K may regulate both rapid, non-genomic signaling as well as at least some delayed genomic effects of D3. Clearly in this model there would be ample opportunity for activation of PI3K through VDRmem to influence cellular responses to D3 brought about through the classic VDRnuc. Further studies will be required to identify whether VDRmem complexes with and activates PI3K p85/p110α.

The β2 integrin receptor CR3 (CD11b/CD18, αMβ2), is a marker of monocyte differentiation and can mediate adherence, phagocytosis, and leukocyte transmigration (reviewed in Ref. 80). CD11b is the α subunit of CR3 and associates non-covalently with its partner CD18. Expression of CR3 is restricted to myeloid cells, and its level of expression depends on the state of differentiation with mature neutrophils and macrophages having the highest levels (81, 82). THP-1 cells are known to express relatively low levels of CR3 in the basal state (83, 84), and D3 is known to augment expression further (18, 29, 30). We have previously reported that CR3 induction by D3 in both THP-1 cells and human monocytes is sensitive to PI3K inhibitors (18). Using THP-1 cells made deficient in p110α by RNAi, we observed a significant reduction (59 to 63%) in D3-induced CD11b expression compared with several negative control cell populations (Fig. 5). Adherence of monocytes to plastic induced by PMA has been shown to be dependent on CR3 (85). Therefore, the defect in D3-induced adherence we observed in p110α-deficient THP-1 cells (Fig. 4B) may be partially due to the attenuation of CD11b expression. However, this seems unlikely to be the entire explanation, because unstimulated THP-1 cells do express basal levels of CD11b and are not adherent, hence a role for other adhesion proteins cannot be excluded in this model system.

In conclusion, we have demonstrated the ability of VSV-pseudotyped lentiviral vectors to stably silence the PI3K p110α isoform in the myeloid cell lines THP-1 and U-937. Using transduced THP-1 cells deficient in PI3K p110α, D3-induced, but not LPS-induced adherence was shown to be dependent on p110α. D3-induced up-regulation of CD11b was also found to be dependent on p110α. The ability of lentiviral vectors to transduce both dividing and non-dividing cells and stable expression of the transgene make this a versatile strategy for gene silencing based on RNAi. Moreover, the finding that lentiviral-transduced cells expressing shRNA can be further manipulated by transfection with reporter plasmids, for instance luciferase, significantly expands the utility of this approach. One important application of this technique may be in gene therapy research, such as silencing genes in myeloid leukemia cells or other difficult to transfect cells to better understand their biology and to identify potential therapeutic targets.
