 Cytometry Part A 69A:59–65 (2005)

Single-Cell Analysis of siRNA-Mediated Gene Silencing Using Multiparameter Flow Cytometry

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Received 10 November 2005; Accepted 5 December 2005

Background: Use of synthetic short interfering RNAs (siRNAs) to study gene function has been limited by an inability to selectively analyze subsets of cells in complex populations, low and variable transfection efficiencies, and semiquantitative assays for measuring protein down-regulation. Intracellular flow cytometry can overcome these limitations by analyzing populations at the single-cell level in a high-throughput and quantitative fashion. Individual cells displaying a knockdown phenotype can be selectively interrogated for functional responses using multiparameter analysis.
Methods: Lck-specific siRNA was delivered into Jurkat T cells or peripheral blood mononuclear cells (PBMCs) to suppress endogenous Lck expression. Transfected cells were fluorescently stained for intracellular Lck and analyzed using multiparameter flow cytometry. The Lck^{lo} Jurkat subpopulation was selectively analyzed for CD69 up-regulation and phospho-states of signaling proteins following T-cell receptor (TCR) stimulation. Surface expression levels of CD4 and CD8 on transfected $CD3^{+}$ gated PBMCs were correlated with intracellular Lck levels.
Results: A subpopulation of Jurkat cells with reduced levels of Lck was clearly resolved from cells with wildtype levels of Lck. Both CD69 up-regulation and ZAP70 phosphorylation were suppressed in Lck^{lo} cells when compared with those in Lck^{hi} cells upon TCR stimulation. Knockdown of intracellular Lck in primary T lymphocytes reduced surface expression of CD4 in a dose-dependent manner.
Conclusions: Multiparameter flow cytometry is a powerful technique for the quantitative analysis of siRNA-mediated protein knockdown in complex hard-to-transfect cell populations. © 2006 International Society for Analytical Cytology

Key terms: RNA interference; siRNA; multiparameter; flow cytometry; signal transduction; T lymphocytes; Lck; Jurkat

RNA interference (RNAi) has emerged as a convenient and effective tool for loss-of-function studies (1). A common strategy to detect siRNA-mediated gene silencing has been semiquantitative or quantitative RT-PCR. A disadvantage of this approach is that down-regulation at the mRNA transcript level may not extend to the protein level. This discrepancy is especially apparent for proteins with a low turnover rate (e.g. structural proteins) (1,2). To directly measure protein down-regulation, Western immunoblotting is frequently used, although it is quantitative only within a relatively narrow dynamic range (2,3). Importantly, both approaches require lysis of the sample into a mixed pool of proteins or mRNAs. This step essentially limits these assays to the analysis of relatively homogeneous populations of cells (e.g. cell lines or purified primary cells). This limitation is especially pertinent to the analysis of synthetic siRNA-transfected cell populations because it is difficult to achieve high transfection efficiencies consistently for some cell types even with extensive optimization (e.g. resting primary T lymphocytes) (1,4). Consequently, if the transfection efficiency is low and/or variable, these techniques cannot reliably monitor the degree of mRNA or protein down-regulation in the transfected subpopulation of cells. For instance, a population in which 50% of the cells are transfected with a siRNA that results in 90% protein reduction per cell would look essentially the same as a population with 90% transfected cells and 50% protein reduction per cell. Both populations have an *overall* protein reduction of 45% but the functional behavior of each population as a whole can be vastly different. It would be impossible to discriminate between these two populations using Western immunoblotting. As RNAi becomes increasingly popular as the

Contract grant sponsor: Dana Foundation, Northern California Chapter of the Arthritis Foundation; Contract grant sponsor: NIH; Contract grant numbers: DK61934, AI50854, AI50865, AR49328; Contract grant sponsor: NHLBI Proteomics; Contract grant numbers: N01-HV-28183.
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Published online 17 January 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20209

tool of choice for loss-of-function studies, alternative approaches are required to overcome the limitations imposed by these "bulk lysis" techniques.

Here we demonstrate the ability of multiparameter flow cytometry to selectively analyze cells that have experienced siRNA-mediated protein knockdown in a background of cells with wildtype protein expression levels. Since flow cytometry operates at the single-cell level, the effectiveness of siRNA-induced knockdown can be assessed independently from variabilities in transfection efficiency. Furthermore, the quantitative nature of flow cytometry permits the accurate measurement of protein knockdown over a wide dynamic range. Our strategy is to employ one parameter to measure the intracellular level of the siRNA target protein, and the remaining parameters to probe for functional responses (e.g. up-regulation or down-regulation of surface markers or intracellular protein phosphorylation). In addition, with the use of fluorescently labeled antibodies specific for cell surface markers, different subsets of cells in a heterogeneous population can be selectively studied for their response to protein down-regulation without prior cell purification steps. This is an important application because cell purification procedures, especially the ones that use antibodies for positive selection, can often lead to changes in the functional state of the cells (5).

Flow cytometry has previously been used to monitor siRNA-mediated suppression of cell surface proteins (4,6,7). However, to the best of our knowledge, only one of those studies attempted multiparameter analysis to correlate knockdown with functional changes (6). Importantly, the use of flow cytometry to assay intracellular protein knockdown has not yet been widely reported. Ohba *et al*. employed flow cytometry to monitor intracellular Bcr-Abl and c-Abl protein down-regulation but multiparameter analysis was not performed (8). In this report, we demonstrate the many unique advantages of multiparameter flow cytometry over traditional methods for detecting siRNA-mediated intracellular protein knockdown and studying its functional consequences.

As our model system, we chose to study Lck, an intracellular tyrosine kinase that is abundantly expressed in T cells (9). Lck is a well-characterized signaling protein and the functional consequences of its deficiency in T cells have been studied extensively in Lck-deficient cell lines and knockout animals (9). These qualities make Lck an ideal target of investigation for our study.

MATERIALS AND METHODS

Cell Culture and Primary Cell Isolation

Jurkat T cells (clone E6-1, ATCC) were cultured in complete RPMI (RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate and antibiotics). Human PBMCs were obtained by Ficoll-Paque density centrifugation (Amerhsam Pharmacia, Uppsala, Sweden) of whole blood from healthy donors and preactivated for 4 days prior to siRNA transfection by coincubation with anti-CD3 (clone UCHT1) and anti-CD28 (clone 9.3) coated magnetic beads (Dynabeads M-450) (Dynal Biotech, Oslo, Norway) at a cell to bead ratio of 1:3 in complete RPMI. Immediately before transfection by electroporation, magnetic beads were removed from activated cells on a magnetic particle concentrator (Dynal Biotech).

Transfection of siRNA With Electroporation

Jurkat cells in logarithmic growth or preactivated PBMCs were washed in PBS, resuspended in serum-free RPMI supplemented with 25 mM Hepes at a concentration of 10^7 cells/ml, and added to a 0.4 cm-gap gene pulser cuvette (0.25 ml/cuvette) (Biorad, Hercules, CA). An Lck- or non-specific pool of four individual siRNAs (SMARTpool siRNA reagent) (Upstate, Waltham, MA) was added to the cells at a final concentration of 1 μM. The cells were electroporated once at 280 V and 500 μF using a Gene Pulser (Biorad), rested at room temperature for 10 min, and cultured in prewarmed RPMI supplemented with 15% FCS and 25 mM Hepes in a 12-well tissue culture plate. For PBMCs, recombinant human interleukin-2 (Peprotech, Rocky Hill, NJ) was added to the culture medium at 1ug/ml to maintain viability of T lymphocytes. Cells were analyzed 48 h after transfection in all experiments except for the time course experiment shown in Figure 2.

The Lck sequences targeted by the siRNAs are as follows: siRNA #1 – 5′ GAACTGCCATTATCCCATA 3′; siRNA #2 – 5′ GAGAGGTGGTGAAACATTA 3′; siRNA #3 – 5′ GGGCCAAGTTTCCCATTAA 3′; siRNA #4 – 5′ GCACGCTGCTCATCCGAAA 3′. No significant sequence homology with other human genes was found through BLAST analysis for any of the four target sequences.

A Cy3 labeled siRNA with no significant sequence similarity to human genes (Ambion, Austin, TX) was used to estimate the efficiency of siRNA delivery under a variety of electroporation conditions. Fluorescence microscopy was used to determine the fraction of cells that were Cy3 positive.

Antibodies

Rabbit polyclonal antibody against Lck was obtained from Cell Signaling Technologies (CST; Beverly, MA), used at a dilution of 1:100, and detected using a PE-conjugated donkey anti-rabbit IgG antibody (1:200 dilution; Jackson ImmunoResearch, West Grove, PA). The following cell surface antibodies were obtained from eBioscience and used at manufacturer's suggested dilution: FITC-conjugated anti-CD4 (RPA-T4), FITC-conjugated anti-CD8a (RPA-T8), FITC-conjugated anti-CD69 (FN50), and APC-conjugated anti-CD3 (UCHT1). Alexa-Fluor 488 conjugated monoclonal antibodies specific for phospho-ZAP70 (Tyr319) and phospho-p38 MAPK (Thr180/Tyr182) were obtained from BD Pharmingen (San Diego, CA) and used at 50 ng per sample. Specificity of the phospho-antibodies were validated by probing full-length immunoblots.

Multiparameter Flow Cytometry and Cell Staining

Transfected cells (5×10^5–1×10^6 cells) were washed, resuspended in phosphate buffered saline (PBS) and fixed in 2% formaldehyde for 10 min at 37°C. For samples requiring cell surface marker staining, cells were washed twice in PBS to remove the formaldehyde, resuspended in 100 μl of FACS staining buffer (PBS with 2% FCS and 0.1% NaN_3), stained with the appropriate fluorophore-conjugated antibodies at 4°C for 20 min, and washed again to remove unbound antibodies. For samples not requiring surface staining, cells were simply washed twice in PBS. Cells were then permeabilized with 90% methanol for 30 min on ice. After washing the cells extensively with FACS buffer, they were probed with the appropriate primary and secondary antibodies for intracellular staining. In all experiments, the cells were stained with each antibody for 30 min at room temperature in a final volume of 100 μl and washed twice with FACS buffer before proceeding. Stained cells were analyzed on either a FACscan or LSR machine with a minimum of 10,000 events collected. The threshold for distinguishing high and low subpopulations in all experiments was placed at the level at which 98–99% of control untransfected cells stained with anti-Lck antibodies fell above the threshold. Cells with fluorescent intensities below the threshold were considered to be in the low subpopulation. A control untransfected stain was performed for every experiment to determine the threshold level. More advanced statistical approaches were used to determine the actual percentage of cells in each subset as described later.

Statistical Analysis

To determine the percentage of Lck^{lo} cells in Figure 2, we used the Overton cumulative subtraction algorithm in FlowJo (Ver 6.33) (TreeStar, Ashland, OR) (10). Each Lck-specific siRNA sample was compared against a non-specific control siRNA sample treated for the same duration. To estimate the level of Lck expression in the Lck^{lo} subpopulation, the following Eq. (1) was used:

$$MFI_{\text{Lcklo}} = [MFI_{\text{Lck siRNA}} - [(1 - F_{\text{Lcklo}}) \times (MFI_{\text{NS siRNA}})]]/F_{\text{Lcklo}} \quad (1)$$

where MFI_{Lcklo} is the mean fluorescent intensity (MFI) of the Lck^{lo} subpopulation in question, $MFI_{\text{Lck siRNA}}$ is the MFI of the entire Lck siRNA transfected population, F_{Lcklo} is the fraction of Lck^{lo} cells as determined using the Overton algorithm, and $MFI_{\text{NS siRNA}}$ is the MFI of the entire non-specific siRNA transfected population. All MFI values used to calculate MFI_{Lcklo} were first subtracted from the MFI of a corresponding sample stained with nonspecific rabbit gamma globulin.

To calculate the percentage of cells that were $CD69^+$ in Figure 3, we used the Overton algorithm to compare the phytohemagglutinin (PHA) stimulated subpopulation to an unstimulated Jurkat population.

Statistical difference between two populations was calculated using the Probability Binning comparison algorithm (11). A $T(X)$ value of greater than 4 indicates that the probability of two populations being the same (is less than 1 percent). We consider two populations to be significantly different when the $T(X)$ value is > 4.

Western Blotting

Western blotting was performed as previously described (12). The immunoblot in Figure 1 was probed with the Lck antibody from CST at 1:2,000 dilution for 2 h at room temperature. Each lane was loaded with twenty micrograms of total protein.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as previously described (13). Staining conditions were equivalent to those described for flow staining.

RESULTS AND DISCUSSION

Single-cell Monitoring of Intracellular Protein Knockdown

To test the feasibility of using intracellular flow cytometry to detect siRNA-induced knockdown in single cells, we transfected Jurkat T lymphocytes with a Lck-specific or non-specific control pool of four siRNAs using electroporation. The electroporation conditions were predetermined to deliver siRNA into approximately 50% of the cells, as determined using Cy3 labeled siRNA (see Materials and Methods, data not shown). Following electroporation, the cells were fixed, permeabilized, and stained with a polyclonal Lck-specific antibody. A distinct subpopulation with reduced levels of endogenous Lck was clearly resolved using flow cytometry in the Lck siRNA sample but not in the control sample (Fig. 1A). To confirm specificity of the knockdown, a full length immunoblot, containing three independent Lck siRNA transfected samples and two non-specific control samples, was probed with the same Lck antibody used for flow cytometry (Fig. 1B). Only a single band at the predicted molecular weight for Lck (57 kDa) was detected in each lane and there was a clear decrease in band intensity in Lck siRNA treated samples. Although the immunoblot confirmed specificity of the knockdown, it provided no additional information regarding the fraction of the cells that were Lck^{lo} and the magnitude of knockdown in these cells, unlike the case with flow cytometry. The fraction of Lck^{lo} cells at 48 h post-transfection varied from 32 to 68% (mean ± S.D. = (50 ± 12)%) in ten independent experiments under the same transfection conditions. This variance reinforces the importance of selective subpopulation analysis, not achievable using RT-PCR or Western immunoblotting. To verify that approximately 50% of the cells were in fact Lck^{lo}, we stained Lck siRNA transfected cells for Lck expression and visualized them under fluorescence microscopy (Fig. 1C). In accordance with what was observed using flow cytometry, the images revealed that roughly half the cells in the field were weakly stained for Lck and the rest of the

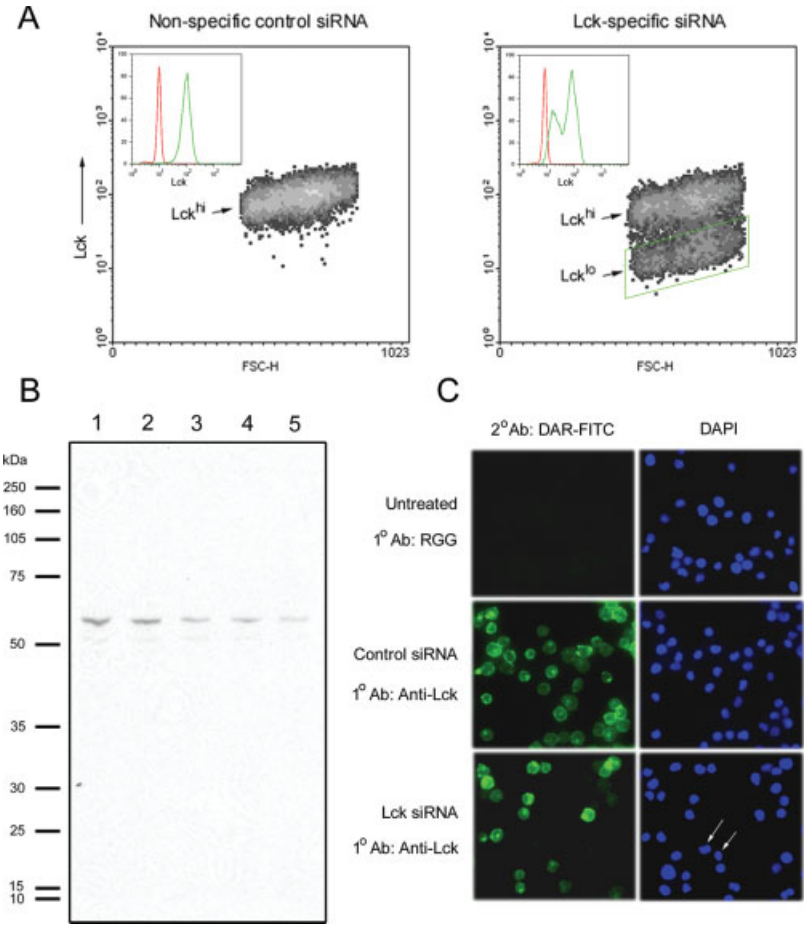

FIG. 1. Detection of siRNA-mediated protein knockdown with flow cytometry, Western blotting, and immunofluorescence microscopy. (A) Density plot of Jurkat T cells 48 h post-transfection with either a non-specific control or Lck-specific pool of siRNA. Forward scatter (FSC-H) is on the X-axis and intracellular Lck staining on the Y-axis. A representative gate circumscribing the Lck^{lo} subpopulation is shown. Histograms of the corresponding cell populations for Lck are shown in the insets (green histograms). Red histogram plots represent background staining with purified rabbit gamma globulin. These plots are representative of ten independent experiments. (B) Western immunoblot of two nonspecific control siRNA samples (lanes 1 and 2) and three Lck-specific siRNA samples (lanes 3–5). Equal amount of protein was loaded in each lane. Samples were prepared from Jurkat cells 48 h post-transfection. The blot was probed with the same Lck antibody used for flow cytometry. (C) Immunofluorescence images of Jurkat cells transfected with control siRNA or Lck siRNA. Transfected cells were fixed, permeabilized, and stained with the same Lck antibody used in flow cytometry. A FITC-conjugated donkey anti-rabbit (DAR) antibody was used for secondary detection. Cells were also stained with DAPI to reveal the location of nuclei. White arrows point to two cells with diminished Lck staining. Top-left image represents background staining with nonspecific rabbit gamma globulin (RGG).

cells were stained with the same intensity as control siRNA treated cells.

To characterize the dynamics of siRNA-mediated effects, we measured time-dependent changes in both the fraction of Lck^{lo} cells and the magnitude of knockdown in this subpopulation over six days (Fig. 2). Such an analysis with RT-PCR or Western blotting would be impossible. We found that the fraction of Lck^{lo} cells peaked around 1–2 days post-transfection and gradually decreased over the next 4 days. Within the Lck^{lo} subpopulation, the magnitude of knockdown was greatest 2 days after transfection. At this time point, Lck^{lo} cells expressed less than 10% of wildtype Lck levels. Interestingly, Lck^{lo} cells abruptly re-expressed a large fraction of their Lck as they transitioned from day 3 to 4. Because siRNA-induced Lck knockdown peaked around 48 h post-transfection, all subsequent experiments were analyzed at this time point.

Multiparameter Analysis of siRNA Transfected Cells

The ability to resolve individual cells with a knockdown phenotype using flow cytometry permits the selective interrogation of those cells for functional responses and direct comparison with wildtype cells in the same experimental sample. To demonstrate this methodology, we transfected Jurkat T cells with Lck-specific siRNA, and 48 h later, stimulated them with phytohemagglutinin

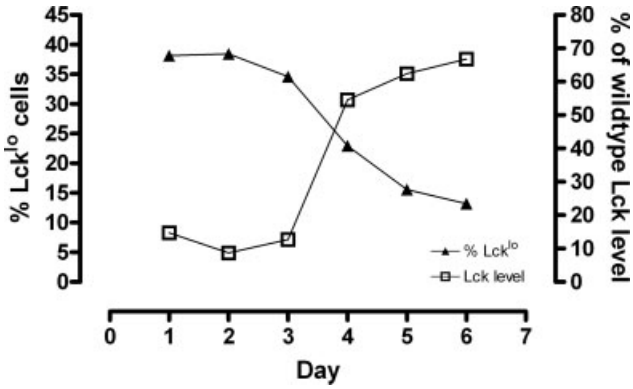

FIG. 2. Kinetics of siRNA-mediated effects over 6 days in Lck siRNA transfected Jurkat T cells. At the indicated time points, an aliquot of the transfected population was stained for intracellular Lck expression. The fraction of cells that were Lck^{lo} (left Y-axis) was determined using the Overton cumulative subtraction algorithm (see Materials and Methods for details). The level of Lck expression in the Lck^{lo} subpopulation relative to control siRNA transfected "wildtype" cells (right Y-axis) was estimated using Eq. (1) in Materials and Methods. Results shown are representative of three independent experiments.

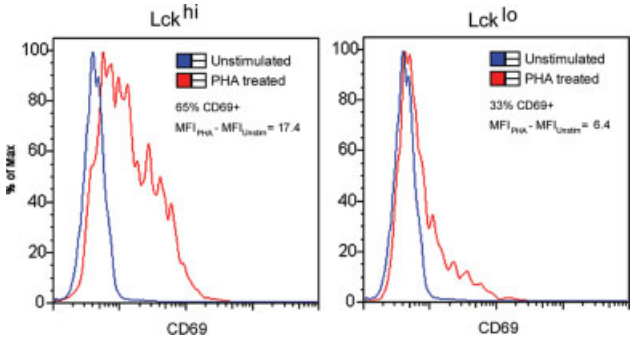

FIG. 3. Simultaneous detection of siRNA-mediated protein down-regulation and functional responses to stimulation using multiparameter flow cytometry. Jurkat T cells were transfected with Lck siRNA and 48 h later, stimulated with PHA-L at 5 μg/ml for 16 h in complete RPMI. Cells were then dually stained for CD69 up-regulation and Lck expression. Red histograms represent CD69 expression on either Lck^{hi} (left panel) or Lck^{lo} (right panel) gated subpopulations. Blue histogram represents basal CD69 staining on unstimulated Jurkat cells. MFI denotes mean fluorescent intensity. This experiment is representative of three independent experiments.

(PHA) to induce expression of CD69, an early T-cell activation marker (14). Since Lck (a Src-family kinase) is an early component of the T-cell receptor signaling pathway that plays an important role in the phosphorylation and activation of ZAP70 (15), its down-regulation should hamper CD69 induction. Accordingly, the fraction of cells that were $CD69^{+}$ and magnitude of CD69 induction (i.e. $MFI_{PHA} - MFI_{Unstim}$) were suppressed in Lck^{lo} gated cells relative to Lck^{hi} gated cells (Fig. 3). However, the Lck^{lo} cells were still responsive to stimulation as evidenced by a small but significant induction in CD69 compared with unstimulated Jurkat T cells ($P < 0.01$). This small shift could be attributed to residual Lck kinase activity (Lck^{lo} cells expressed ~10% of wildtype Lck levels; data not shown) or compensatory activity of another Src family kinase, Fyn, in T cells (16). The ability to directly compare the high and low subpopulations in the same sample is a potential benefit as it minimizes variability that is introduced when two populations of cells have to be physically separated in different vessels.

Multiparameter Phosphorylation-state Flow Cytometric Analysis of siRNA Transfected Cells

A recent advancement in the study of signaling events has been the use of phospho-specific antibodies for intracellular flow-cytometric analysis (5,17–21). By combining phosphorylation-state flow cytometry and siRNA gene silencing technologies, it should be possible to characterize downstream substrates and signaling pathways of siRNA-targeted signaling proteins. To demonstrate this application, we probed for the phosphorylation state of ZAP70 and p38 MAPK in unstimulated and anti-CD3 activated Jurkat T cells that were pretransfected with Lck siRNA 48 h earlier. ZAP70 is a well-characterized Lck substrate following TCR activation (15). In contrast, the mechanism of p38 MAPK activation and phosphorylation in T cells is not well defined. A recent study suggests that phosphorylation of p38 MAPK on the activation Thr180-X-Tyr182 motif is dependent on its autophosphorylation activity, which in turn is induced by Tyr323 phosphorylation (22). In the same study, Lck and ZAP70 were shown to be necessary for Tyr323 phosphorylation. In our experiments, CD3 crosslinking induced phosphorylation of ZAP70 and p38 MAPK in both Lck^{hi} and Lck^{lo} gated subpopulations as evidenced by significant shifts ($T(X) = > 4$) in phospho-staining (Figs. 4A and 4B, panels 1 and 2). Although downstream signaling pathways appeared to be intact in Lck^{lo} cells, a direct comparison between Lck^{hi} and Lck^{lo} gated cells revealed that the level of phospho-ZAP70 was significantly lower in Lck^{lo} cells under both unstimulated and anti-CD3 stimulated conditions (Fig. 4A, panels 3 and 4, and Table 1). Interestingly, this difference in phospho-staining was not observed for p38 MAPK under either condition (Fig. 4B, panels 3 and 4, and Table 1). Our findings suggest that Lck^{lo} cells are sufficient to induce some degree of ZAP70 phosphorylation but their basal level of phospho-ZAP70 (MFI_{Lckhi} = 14.4 vs.

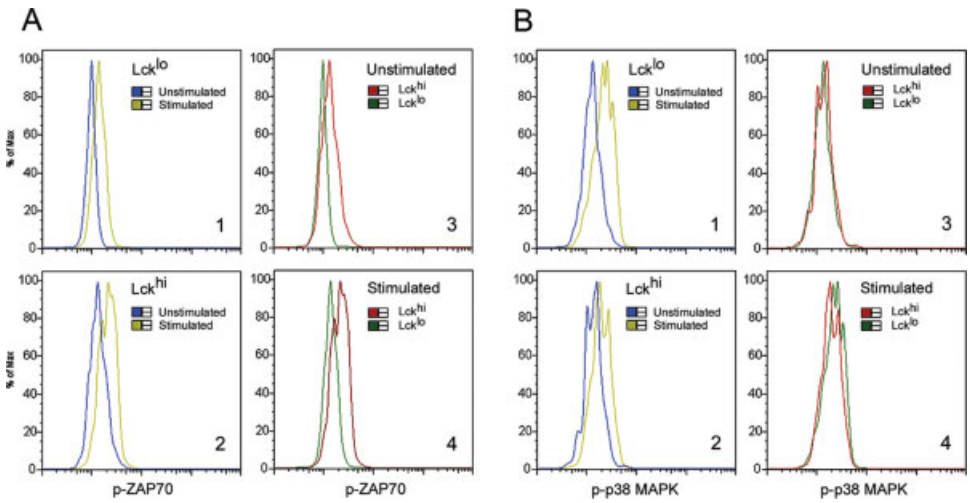

FIG. 4. Phosphorylation states of signaling proteins in unstimulated and anti-CD3 stimulated Jurkat cells that were pre-transfected with Lck siRNA. Forty eight hours after siRNA transfection, Jurkat cells were either left untreated or stimulated with an anti-CD3 antibody (10 μg/ml of HIT3a for 5 min at 37°C). After the stimulation, cells were immediately stained for Lck expression and phospho-ZAP70 (A) or phospho-p38 MAPK (B) levels. The Lck^{hi} and Lck^{lo} subpopulations were gated and their levels of phosphorylation of the indicated protein are shown as overlapping histogram plots. This experiment is representative of two independent experiments. See text for details.

Table 1
Quantitative and Statistical Analysis of Phospho-ZAP70 and Phospho-p38 MAPK Levels in Lck^{hi} and Lck^{lo} Subpopulations

Condition	Lck status	p-ZAP70 MFI	p-ZAP70 *T*(*X*)	p-p38 MAPK MFI	p-p38 MAPK *T*(*X*)
Unstimulated	High	14.4	34.1	14.7	0
	Low	9.7		13.9	
CD3 stimulated	High	22.2	37.8	21.0	0
	Low	14.3		23.9	

MFI_{Lcklo} = 9.7) and capacity to induce ZAP70 phosphorylation (ΔMFI_{Lckhi} = 7.8 vs. ΔMFI_{Lcklo} = 4.6) are reduced. We do not at this point understand why p38 MAPK phosphorylation was not equivalently affected in Lck^{lo} cells given the recent data on the role of Lck and ZAP70 in p38 MAPK activation. We surmise that since at least three intermediate steps are positioned between Lck and p38 MAPK (Lck → ZAP70 → Tyr323 phosphorylation → autophosphorylation on Thr180-X-Tyr182), any difference in activity originating from Lck becomes "masked" as signals amplify down the pathway. We believe that this phenomenon may explain why the magnitude of effect on phospho-ZAP70 was not as great as one might expect given a roughly 90% decrease in endogenous Lck expression. This experiment highlights a potential limitation of siRNA technology: the level of knockdown may not be sufficient to fully reveal the function of the siRNA target protein. Despite this limitation, our experiment shows that it is possible to apply multiparameter flow cytometry to selectively analyze siRNA-transfected cells for signaling defects.

Subset Analysis of Complex Populations Transfected With siRNA

Multiparameter flow cytometry offers the ability to analyze specific cell subsets in a complex population using cell surface marker antibodies without prior cell sorting. This ability is particularly important for the analysis of rare primary cell populations, which represents a challenge for siRNA studies using current techniques. While it is possible to purify the subset of interest before analysis, the sort itself may significantly alter the functional state of cells. Therefore, it is highly desirable to analyze cellular phenotypes with a minimal amount of manipulations. Here we demonstrate the applicability of our technique to analyze a complex primary cell population, human peripheral blood mononuclear cells (PBMCs). PBMCs isolated by Ficoll-Paque density centrifugation of whole blood from healthy donors were activated *in vitro* with anti-CD3 and anti-CD28 coated magnetic beads. After 4 days in culture, the cells were separated from the beads and transfected with Lck-specific siRNA through electroporation. Two days after transfection, the cells were stained with antibodies to CD3, and CD4, or CD8, followed by intracellular Lck staining. During our analysis, we found that surface expression of CD4 on CD3$^+$ cells was reduced in a dose-dependent manner with Lck down-regulation (Fig. 5A, panel 1). Interestingly, CD8 expression appeared to be much less affected by Lck suppression (Fig. 5B, panel 1), although both CD4 and CD8 coreceptors are known to associate with Lck (23,24). To obtain quantitative information, we divided CD4$^+$ and CD8$^+$ T cells (CD3$^+$ gated) into four additional subsets (A through D) based on vary-

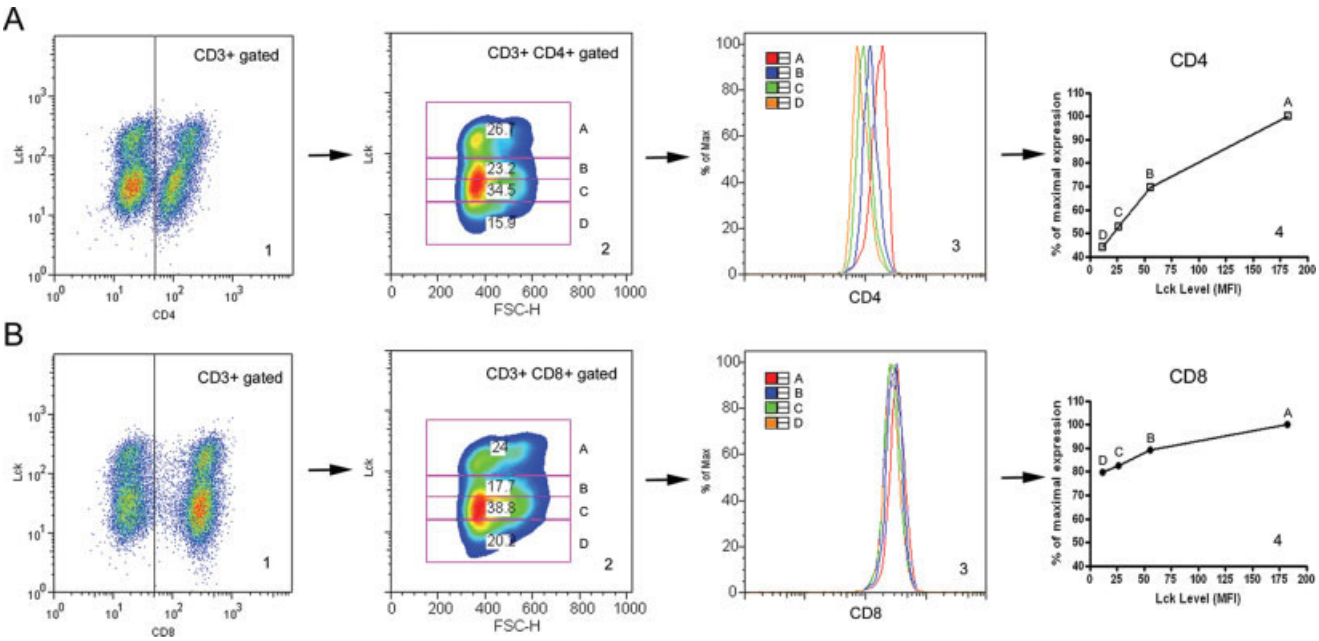

FIG. 5. Surface CD4 and CD8 expression on CD3$^+$ PMBCs in correlation with intracellular Lck levels. PBMCs isolated from healthy volunteers were activated with anti-CD3/CD28 coated beads for 4 days, transfected with Lck siRNAs, and cultured in the presence of recombinant IL-2 for 48 h. The resulting population was stained with antibodies to CD3, CD4 (A), or CD8 (B), and intracellular Lck. See text for experimental details. This experiment is representative of two independent experiments.

ing levels of Lck expression (Figs. 5A and 5B, panel 2). By displaying the level of CD4 expression of each subset as overlapping histograms, we observed a significant downward shift in CD4 expression with decreasing levels of Lck (Fig. 5A, panel 3). Surface expression of CD4 on subset D was only 43% of that on subset A (Fig. 5A, panel 4). Although there was a similar downward trend for CD8, the relative magnitude of reduction was much less than that seen with CD4 (Fig. 5B, panel 3 and 4). Our findings are consistent with previous reports showing that phorbol ester-induced dissociation of Lck from the cytoplasmic domain of CD4 leads to the internalization of CD4 through clathrin-coated pits (25,26). Our current study shows that forced suppression of endogenous Lck expression is sufficient to cause CD4 down-regulation in primary T lymphocytes, and to lesser extent, CD8 down-regulation. From an application perspective, this experiment illustrates the potential to perform "dose-dependency" experiments by taking advantage of the range in protein knockdown that is seen with siRNA-transfected populations.

This report highlights the potential power and versatility of multiparameter flow cytometry in siRNA studies of heterogeneous populations that cannot be achieved using conventional techniques. A broader range of cell types including rare primary populations such as stem cells, regulatory T cells, and dendritic cells should now be applicable to siRNA analysis. With the increasing popularity of siRNA for biological studies, multiparameter flow cytometry has the potential to make this tool even more useful for a range of biological systems. However, this technique has its limitations. Currently, many commercially available antibodies are not suitable for intracellular flow cytometry applications for reasons that have not been systematically studied. Epitope accessibility and conformation are likely to be important contributing factors and staining conditions need to optimize for each antibody. Importantly, antigen cross-reactivity potentially limits the suitability of many antibodies for flow staining. Furthermore, flow cytometers are not as sensitive as traditional Western immunoblotting, which may pose a problem for the detection of low abundance targets. With continuing technological advancements and antibody developments, these issues will likely be resolved.

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