

# Identification of Cell-surface Proteins from Highly Purified Subpopulations of T-cells Produced by Fluorescence-activated Cell-sorting from Human Blood

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## Objective

The goal of this work is to identify new cell surface markers from highly purified, sorted subpopulations of cells. Phenotypically distinct populations of cells can be produced by fluorescence-activated cell sorting (FACS) and the global protein differences between the populations can be investigated by the techniques of proteomics, including nanoflow LC/MS<sup>2</sup>. In our initial studies, we have examined the combined use of FACS to produce highly purified populations of CD4+ and CD8+ T-cells with long-gradient nanoflow LC/MS<sup>2</sup> to analyze the peptides released from those cell surface proteins that are susceptible to cleavage by trypsin.

## Introduction

A goal of proteomics is the ability to detect all proteins in a particular biological subsystem, such as a cell type or cellular subfraction. Fluorescence-activated cell sorting (FACS) is a powerful tool for isolation and characterization of purified cell populations. FACS uses cell surface markers, DNA content, biochemistry, or morphology (light scatter) to sort a specific cell subpopulation from a cell suspension.

Methods coupling liquid chromatography (LC) with tandem mass spectrometry (MS/MS) have been shown to enable the rapid identification of many proteins without a requirement for prior purification. Using this technology multiple peptide fragments are separated, analyzed and linked to their parent proteins using genomic databases. An advantage of the LC/MS/MS analysis is that even low abundance proteins can often be detected.

The objective of this study was to combine the power of the LC/MS/MS with FACS to enable the identification of unique cell surface proteins on specific subsets of human T-cells. To achieve this objective it was first necessary to prepare sufficient quantities of purified CD8+ and CD4+ cells for analysis and to develop and evaluate a method to selectively release peptides and proteins from the cell surface.

Analysis of cellular proteomes using these methods typically requires at least several million cells per sample. An advantage of the FACS technology for this study was that millions of cells can be purified in a short time, and the procedure has little or no effect on cell viability, which is important in minimizing the number of intracellular proteins in the preparations.

## Materials and Methods

**Labeling and Sorting of T-Cells.** Whole blood (60ml) drawn from healthy donors was treated with a combination of fluorescein-labeled anti-CD4 and phycoerythrin-labeled anti-CD8 antibodies (BD Biosciences, San Jose, CA). The blood samples containing the labeled T-cells were then centrifuged over Ficoll-Paque PLUS (Pharmacia, Uppsala, Sweden) in order to separate the lymphocytes from red blood cells and granulocytes. The harvested cells were washed twice with phosphate buffered saline, pH 7 (PBS) containing 0.5% Pluronic F-68 (Sigma, St Louis, MO). The CD4+ and CD8+ subsets of lymphocytes were sorted by single drop deposition using a BD FACSVantage SE (Figure 2). We used multiparameter analysis, including the forward and side scatter properties of cells and surface fluorescence, to gate the CD4 and CD8 T-cell subpopulations for sorting. Sort purity was confirmed by analyzing portions containing 10<sup>4</sup> of the sorted cells on a BD FACScan.

**Selective Digestion of Lymphocyte Cell Surface Proteins.** Isolated cells (1-50 x 10<sup>6</sup>) were washed with Hank's balanced salt solution (HBSS) to remove extracellular protein. Cells were treated with reducing agent tris(2-carboxyethyl)phosphine (TCEP), 10 mM in HBSS, for 20 minutes. After removing the TCEP, the cells were resuspended in 20 µg/mL trypsin (sequencing grade modified) in PBS and incubated at room temperature for 15-30 min. The cells were centrifuged, saving the supernatant for further digestion (overnight at 37°C). Validation studies were performed to evaluate the efficiency of trypsin cleavage for a variety of cell surface markers using fluorescent antibodies to CD proteins (Figure 4). Membrane permeability and cell viability post-treatment, were evaluated by trypan blue dye exclusion and by lack of propidium iodide uptake.

**Analysis of peptide mixtures and data analysis.** Peptide mixtures were analyzed by LC/MS/MS on a Surveyor HPLC (ThermoFinnigan, San Jose, CA) equipped for nanoflow operation, coupled to an LCQ-Decca ion-trap mass spectrometer (ThermoFinnigan). Prior to analysis, the digested samples were reduced by treatment with 10 mM TCEP for 5 min at 90°C, loaded into 96-well microtiter plates and maintained at 4°C in the autosampler. Sample volumes of approximately 100 microliters were pre-concentrated onto a poly(styrene)divinylbenzene trap and then eluted from the trap onto a reversed-phase packed-tip column containing C18-BioBasic (75 µm i.d. x 10 cm) at a flow rate of 100 nL/min. The gradient conditions were 0% - 40% B (B = 0.1% formic acid in acetonitrile, A = 0.1% formic acid in water) over 6 hours. Data were collected using dynamic exclusion, with three MS/MS scans for every full MS scan (Figure 5). Database searching was performed using TurboSequest against a human protein database, allowing for up to two missed cleavages per peptide. TurboSequest results were summarized using a newly devised scoring algorithm that uses correlation values produced by Sequest to assign statistical significance to the matches obtained.

## Flowchart for Sample Preparation and Analysis



Fig. 2) Principles of fluorescence-activated cell-sorting

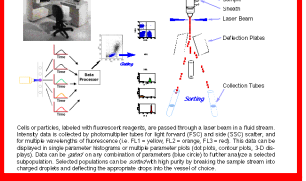


Fig. 3) Typical data from sort of CD4+ and CD8+ T-cells



Fig. 4) Efficiency of cell-surface protein-release by trypsin



## Results

The results of triplicate LC/MS analyses of both CD4+ (12-15 x 10<sup>6</sup> cells) and CD8+ (5 x 10<sup>6</sup> cells) sorted-cell-sample digests revealed that the samples were highly complex and consisted of large numbers of peptides. Examples depicting the base-peak chromatogram for each type of sample are presented in Figure 5 for (a) CD4+ and (b) CD8+ cells. Results of the database searches revealed that, despite the presence of many intracellular proteins, several integral-membrane and membrane-associated proteins could be identified in the samples. A total of 367 proteins were identified in the CD8+ cells and out of these, 44 were known to be membrane-bound and 14 had CD designations.

Fig. 5a) Base peak ion chromatogram of peptides released by trypsin from the surface of CD4+ cell

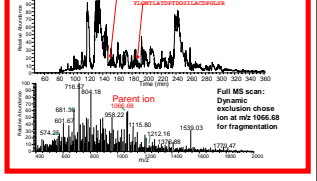


Fig. 5c) CD11A protein identified on the surface of CD4+ cell. MS/MS spectra of two identified peptides.

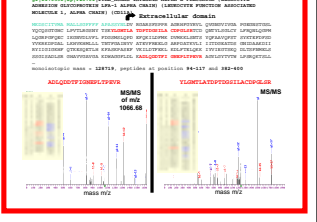


Fig. 5b) Base peak ion chromatogram of peptides released by trypsin from the surface of CD8+ cell

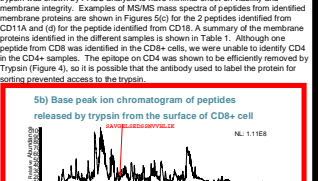


Fig. 5d) CD18 protein identified on the surface of CD8+ cells. MS/MS spectra of peptide matching CD18.

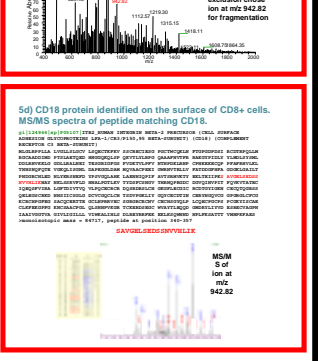


Table 1) Proteins Identified in CD4+ Cells

Protein Name	Accession Number	CD Designation
CD4	P01721	CD4
CD8	P01722	CD8
CD11A	P01723	CD11A
CD18	P01724	CD18
CD28	P01725	CD28
CD29	P01726	CD29
CD30	P01727	CD30
CD31	P01728	CD31
CD32	P01729	CD32
CD33	P01730	CD33
CD34	P01731	CD34
CD35	P01732	CD35
CD36	P01733	CD36
CD37	P01734	CD37
CD38	P01735	CD38
CD39	P01736	CD39
CD40	P01737	CD40
CD41	P01738	CD41
CD42	P01739	CD42
CD43	P01740	CD43
CD44	P01741	CD44
CD45	P01742	CD45
CD46	P01743	CD46
CD47	P01744	CD47
CD48	P01745	CD48
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CD60	P01757	CD60
CD61	P01758	CD61
CD62	P01759	CD62
CD63	P01760	CD63
CD64	P01761	CD64
CD65	P01762	CD65
CD66	P01763	CD66
CD67	P01764	CD67
CD68	P01765	CD68
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CD193	P01890	CD193
CD194	P01891	CD194
CD195	P01892	CD195
CD196	P01893	CD196
CD197	P01894	CD197
CD198	P01895	CD198
CD199	P01896	CD199
CD200	P01897	CD200

Table 1) Proteins Identified in CD8+ Cells

Protein Name	Accession Number	CD Designation
CD8	P01722	CD8
CD11A	P01723	CD11A
CD18	P01724	CD18
CD28	P01725	CD28
CD29	P01726	CD29
CD30	P01727	CD30
CD31	P01728	CD31
CD32	P01729	CD32
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CD71	P01768	CD71
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CD74	P01771	CD74
CD75	P01772	CD75
CD76	P01773	CD76</